Universidade de Aveiro Departamento de Química
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Sónia Marília de Almeida e Castro

Efeito de tratamentos de pressão e temperatura na actividade e estabilidade da pectina metilesterase e na textura de pimento (*Capsicum annuum*)

Effect of pressure and temperature treatments on pectin methylesterase activity and stability and texture of bell pepper (*Capsicum annuum*) fruit

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dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Bioquímica, realizada sob a orientação científica do Professor Doutor Jorge Saraiva, do Departamento de Química da Universidade de Aveiro

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Esta dissertação é dedicada aos meus Pais e ao Álvaro.

o júri

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palavras-chave

Capsicum annuum, (in)activação, pectina metilesterase, textura, tratamentos de temperatura e combinados de temperatura e pressão.

resumo

De entre os diversos métodos de conservação de alimentos, o processamento térmico é o mais comum. No entanto, a alta pressão tem ganho interesse já que inactiva microorganismos e várias enzimas relacionadas com a qualidade alimentar, enquanto que outros atributos (cor, aroma, vitaminas) são mantidos. Do mesmo modo, a aplicação de pressão a baixas temperaturas (abaixo de 0^{o}C) tem tido um interesse crescente na área do processamento por congelação, já que este provoca danos irreversíveis na textura de diversos frutos e vegetais.

Industrialmente, os pimentos são congelados, após branqueamento térmico, para serem consumidos crus em saladas. Considerada como um importante parâmetro de qualidade no pimento, a textura é muitas vezes afectada por alterações que ocorrem durante o processamento, onde se incluem as modificações ao nível da pectina, devido a reacções químicas e/ou à acção de enzimas endógenas, tal como a pectina metilesterase (PME).

Tratamentos de pressão de 100 e 200 MPa foram estudados como possíveis substitutos para o branqueamento térmico de pimentos. Os resultados revelaram que os pimentos tratados por pressão apresentam de um modo geral melhor qualidade em diversos parâmetros, nomeadamente ao nível da proteína solúvel, do conteúdo em ácido ascórbico e da textura (firmeza). Os pimentos tratados por pressão apresentam também uma textura melhor após congelação/descongelação que os pimentos branqueados termicamente.

Como se verificou que apenas os pimentos verdes apresentavam actividade de PME (nos pimentos vermelhos não se detectou actividade de PME), usaram-se os pimentos verdes para estudar as características desta enzima sob diversas condições de pressão e temperatura. A enzima foi assim purificada através de um passo único por cromatografia de afinidade e caracterizada em termos bioquímicos. A enzima apresentou 2 bandas por SDS-PAGE, com 33 e 37 kDa, e um pH óptimo de 7,5 e uma temperatura óptima a pH neutro entre 52,5 e 55^{o}C.

A inactivação isotérmica da enzima PME purificada seguiu uma cinética de inactivação que pode ser descrita através de um modelo de inactivação fraccional (55-57^{o}C, pH 7,5) e de um modelo bifásico (58-70^{o}C, pH 7,5; 62-76^{o}C, pH 5,6). A pH ácido, a enzima PME purificada apresentou uma estabilidade térmica superior. No extracto não purificado, a pH 5.6, a inactivação isotérmica foi igualmente descrita por um modelo bifásico, entre 62^{o}C e 76^{o}C. Estes resultados de inactivação térmica da enzima PME purificada e no extracto não purificado indiciam a presença de várias isoformas com diferente estabilidade térmica. A enzima nestes dois sistemas apresentou ainda um comportamento relativamente estável aos dois pHs face a diversos tratamentos de pressão-temperatura.

Quando se submeteu os pimentos, em pedaços e puré, a tratamentos de pressão a temperaturas moderadas (25°C, 40°C), verificou-se um aumento da actividade da PME, o que poderá estar relacionado com um aumento da extractibilidade da PME, provavelmente devido alterações na estrutura da parede celular. Em puré, a enzima revelou-se mais estável do que em pedaços às condições de temperatura e pressão estudadas.

A estabilidade da enzima purificada a diversos tratamentos de pressão (0,1-800MPa) e temperatura (10-64°C) foi também estudada em tampão a pH 5.6. Com os dados cinéticos obtidos para a fracção lábil da enzima, construiu-se um diagrama P-T de inactivação da enzima, onde se verificou um efeito antagonista na inactivação a $P \leq 300$MPa e $T > 54$°C. A actividade da enzima purificada foi igualmente estudada sob pressão e temperatura (0,1-600MPa e 18-65°C), na presença de pectina a pH 5,6. Observou-se um óptimo de actividade a condições de pressão e temperatura moderadas (200MPa, 55°C). Adicionalmente, utilizou-se um modelo polinomial do 3º grau (derivado de um modelo termodinâmico) para descrever quer a dependência da constante cinética de inactivação da fracção lábil da enzima PME, quer a dependência da taxa de produção de metanol pela enzima, nas diferentes condições de pressão e temperatura estudadas.

Por último, estudou-se o efeito de tratamentos de pressão e temperatura, isoladamente e em combinação, na textura do pimento. Entre 75°C e 95°C, a cinética de degradação da textura do pimento verde obedeceu a um modelo de conversão fraccional. A 90°C, a degradação da textura do pimento foi substancialmente retardada, quando o pimento foi submetido a pré-tratamentos moderados de temperatura (55°C, 60min) ou de pressão (200MPa, 25°C, 15min). A combinação destes tratamentos com imersão em cálcio ($CaCl_2$, 0,50% (w/v)) retardou ainda mais a degradação de textura.

A congelação desviada por pressão ("high pressure shift freezing" - HPSF) não alterou a firmeza dos pimentos pré-tratados ($P > 0.05$), enquanto que a congelação convencional à pressão atmosférica diminuiu significativamente ($P < 0.05$) a firmeza de pimentos quando submetidos aos mesmos pré-tratamentos. Dos pimentos congelados por congelação desviada por alta pressão, os pré-tratados por alta pressão apresentam uma firmeza substancialmente melhor, que os pré-tratados por temperatura, após conservação a –18°C durante 2,5 meses. Estes resultados mostram claramente a vantagem dos pré-tratamentos para melhorar a textura do pimento verde, e da congelação desviada por pressão, para manter a textura do pimento verde durante a congelação e a conservação a –18°C, comparada com a congelação à pressão atmosférica.

Os tratamentos de pressão e temperatura aplicados não foram suficientes para causar a inactivação completa da enzima PME, ao pH natural dos pimentos, enquanto que os tratamentos moderados, economicamente mais vantajosos, parecem ter um efeito favorável na activação da enzima e, por conseguinte, na melhoria da textura. A congelação com recurso a pressão permite ainda obter pimentos pré-processados com melhor textura face à congelação convencional à pressão atmosférica. Os resultados deste trabalho ilustram claramente o potencial dos tratamentos de pressão e de pressão combinados com temperatura, para in(activar) a PME de pimento, adicionando um valor acrescentado à alta pressão como tecnologia de (pre-)processamento, na melhoria da qualidade do pimento verde processado, no que diz respeito às suas propriedades texturais.

keywords

Capsicum annuum, (in)activation, pectin methylesterase, texture, temperature and combined temperature-pressure treatments.

abstract

Among several food preservation methods, thermal processing is the most commonly used. However, high pressure technology is gaining interest since it inactivates vegetative microorganisms and some food quality related enzymes, while quality attributes (colour, flavour, vitamins) are maintained. Moreover, the application of pressure at sub-zero temperatures is also a technology that is becoming of interest regarding the freezing processing, since freezing causes irreversible texture losses in fruits and vegetables.

Industrially, peppers are frozen after thermal blanching, in order to be consumed raw in salads. Considered as one of the most important quality attributes in peppers, texture is mostly affected by changes that occur during thermal treatments, like changes in pectins, caused by chemical conversions and/or by the action of endogenous enzymes, such as pectin methylesterase (PME).

Pressure treatments of 100 and 200MPa were studied as a possible alternative to thermal blanching of peppers. The results revealed that pressurized peppers presented, in general, better quality in several parameters, namely soluble protein, ascorbic acid content and texture (firmness). Peppers treated by pressure also presented better texture after freezing/thawing than thermally blanched peppers.

Since PME activity was only detected in green peppers (PME was not found red peppers), green peppers were used to study the characteristics of PME upon several pressure and temperature conditions. Pepper PME from green bell pepper was purified through a single-step affinity chromatography and biochemically characterized. The enzyme presented two bands on SDS-PAGE, with 33 and 37 kDa, an optimum pH of 7.5 for PME activity, and its optimum temperature at neutral pH was between 52.5 and 55.0^{o}C.

Isothermal inactivation of purified pepper PME could be described by a fractional conversion model (55-57^{o}C, pH 7.5) and a biphasic model (58-70^{o}C, pH 7.5; 62-76^{o}C, pH 5.6). At acidic pH, purified enzyme presented a higher thermal stability. In the crude extract (pH 5.6), the isothermal inactivation was also described by a biphasic model, between 62^{o}C and 76^{o}C. These results indicate the presence of several isoforms, with different thermal stabilities. The enzyme in these two systems still showed a rather stable behaviour towards several pressure-temperature treatments at both pHs.

When pepper pieces and puree were submitted to pressure and mild temperatures (25^{o}C, 40^{o}C), an increase in PME activity was observed, which might be related with an increase in PME extractability, probably due to changes in the cell wall structure. The enzyme in pepper puree samples revealed to be very pressure stable.

The stability of purified pepper PME to pressure (0.1-800MPa) and temperature (10 to 64°C) was also studied in buffer at pH 5.6. With kinetic data obtained for the labile fraction of the enzyme, a pressure-temperature inactivation diagram was constructed, where an antagonistic effect was observed in the inactivation at $P \leq 300MPa$ and $T > 54°C$. Purified pepper PME activity was also considered under combined high-pressure/temperature treatments (18-65°C, 0.1-600MPa) in the presence of pectin at pH 5.6. An optimum in the activity at mild pressure-temperature conditions (200MPa, 55°C) was observed. Moreover, a third-degree polynomial model (derived from a thermodynamic model) was successfully applied to describe the temperature/pressure dependence of the inactivation rate constants of the labile pepper PME fraction as well as the heat-pressure dependence of the initial rates of purified pepper PME-catalyzed methanol formation.

Finally, the effect of several pressure and temperature treatments, separately and combined, on pepper texture was also evaluated. Between 75°C to 95°C, the texture degradation kinetics of green pepper could be accurately described by a fractional conversion model. At 90°C, texture degradation of pepper was substantially retarded when pepper was submitted to pre-treatments of temperature (55°C, 60min) and pressure (200MPa, 25°C, 15min). The combination of these treatments with calcium ($CaCl_2$, 0.50% (w/v)) immersion retarded even more the texture degradation.

High pressure shift freezing (HPSF) did not change the firmness of pre-treated peppers ($P > 0.05$), while conventional freezing at atmospheric pressure decreased ($P < 0.05$) the firmness of peppers submitted to the same pre-treatments. From the peppers frozen by HPSF, those pre-treated by high pressure showed a firmness substantially better than the ones thermally treated, after freezing storage at -18°C during 2.5 months. These results clearly showed the advantage of pre-treatments to improve green pepper texture, and of HPSF to maintain the texture of green pepper during freezing and storage at -18°C, when compared to freezing at atmospheric pressure.

The applied pressure/temperature treatments revealed to be insufficient to cause fully PME inactivation at the natural pH of peppers, while mild treatments, economically more advantageous, seemed to have beneficial effect in the activation of PME and, as a consequence, in the improvement of texture. HPSF also considerably improves the texture of pre-processed peppers, when compared to conventional freezing at atmospheric pressure. The results of this work clearly illustrate the potential of pressure and pressure combined with temperature treatments to (in)activate PME, giving to high pressure an add-value as a (pre-)processing technology, in the improvement of the quality of processed green bell pepper, regarding its textural properties.

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LIST OF SYMBOLS

E_a	Activation energy [$kJ.mol^{-1}$]
V_a	Activation volume [$cm^3.mol^{-1}$]
$\Delta\kappa$	Compressibility factor [$cm^6.J^{-1}.mol^{-1}$]
D	Decimal reduction time [min]
D_{ref}	Reference decimal reduction time [min]
ΔS	Entropy change [$J.mol^{-1}.K^{-1}$]
ΔS_0	Standard entropy change [$J.mol^{-1}.K^{-1}$]
A	Enzyme activity [mL 0.01N NaOH/min; U]
K	Equilibrium constant for the process
A_∞	Final fraction enzyme activity [mL 0.01N NaOH/min; U]
ΔG	Free energy change [$J.mol^{-1}$]
ΔC_P	Heat capacity [$J.mol^{-1}.K^{-1}$]
k	Inactivation rate constant [min^{-1}]
k_L	Inactivation rate constant of the labile fraction [min^{-1}]
k_S	Inactivation rate constant of the stable fraction [min^{-1}]
A_0	Initial enzyme activity [mL 0.01N NaOH/min; U]
A_L	Enzyme activity of the labile fraction [mL 0.01N NaOH/min; U]
A_S	Enzyme activity of the stable fraction [mL 0.01N NaOH/min; U]
m	Number of observations

P	Pressure [MPa]
n	Reaction order
k_0, k_{ref}	Reference inactivation rate constant [min^{-1}]
P_0, P_{ref}	Reference pressure [MPa]
T_0, T_{ref}	Reference temperature [K]
R^2	Squared correlation factor
ΔG_0	Standard free energy change [$J.mol^{-1}$]
T	Temperature [K]
$\Delta\zeta$	Thermal expansibility [$cm^3.mol^{-1}.K^{-1}$]
t	Treatment time [min]
R_P	Universal gas constant [8.314 $cm^3.MPa.mol^{-1}.K^{-1}$]
R, R_T	Universal gas constant [8.314 $J.mol^{-1}.K^{-1}$]
ΔV	Volume change [$cm^3.mol^{-1}$]
ΔV_0	Standard volume change [$cm^3.mol^{-1}$]
z_P	*z*-value [MPa]
z_T	*z*-value [ºC]

LIST OF SUB/SUPERSCRIPT

∞	After prolonged treatment time
L	Labile
S	Stable
0, ref	standard, reference
P	Pressure
T	Temperature
≠	Transition state

LIST OF ABBREVIATIONS

EC	Enzyme classification number (Nomenclature Committee of the International Union of Biochemistry and Molecular Biology)
EU	European Union
HGA	Homogalacturonan
HMM	High molar mass
HP	High pressure
HPSF	High-pressure-shift freezing
HPAF	High-pressure-assisted freezing

HPAT	High-pressure-assisted thawing
HPST	High-pressure-shift thawing
pI	Isoelectric point
k-value	Inactivation rate constant value
LOX	Lipoxygenase enzyme
MM	Molar Mass
PME	Pectin methylesterase enzyme
POD	Peroxidase enzyme
PG	Polygalacturonase enzyme
PGA	Polygalacturonic acid
PPO	Polyphenol oxidase enzyme
RG-I	Rhamnogalacturonan I
RG-II	Rhamnogalacturonan II
SHU	Scoville Heat Units
TL-PME	Thermolabile fraction of PME
TS-PME	Thermostable fraction of PME
UK	United Kingdom
USA	United States of America
z-value	Temperature change necessary to obtain a 10-fold change of the D-value [ºC]

General Introduction

Since man has discovered fire, his ability to change the properties of food has enlarged dramatically. With the use of fire, he was able to cook his food. By doing so and probably without knowing, he released nutrients and energy which were not available for him in uncooked food. More readily observable were the changes of taste of food and its texture. As man became more knowledgeable about his world, his needs regarding food and nutrition became more extensive. Later, the food preservation problem arose, since it had to be transported over long distances and kept during long periods. In 1785, Nicolas Appert invented a conservation process based on enclosing food in jars and immersing in boiling water for a period of time. The sterilisation principles were not yet known but the method seemed to work. The spoilage of food by yeasts and their inactivation by heat was later explained by Louis Pasteur in 1864. Since then, one of the main objectives of Food Engineering has been to develop ways to preserve and extend the shelf-life of food. And nowadays, more than ever, is gaining a particular interest because consumer is looking for products with an extended shelf-life and simultaneously with a high quality level. In recent years, an increasing demand for nutritious, fresh-like food products with a high organoleptic quality and an acceptable shelf-life can be noted.

Currently, thermal processing (e.g., pasteurisation, sterilisation) is the most frequently used method for food preservation, an effective method for endogenous enzymes and microorganisms inactivation but, at the same time, leading to a decrease in organoleptical quality of the final product. Apart from thermal processing, alternative technologies are being studied, in order to minimize the side effects of the heat treatment. Among these technologies, high pressure processing is gathering an up-coming interest, even though its application in the food area is not new since it was first reported for treated milk by Hite (1899).

High pressure processing allows the inactivation of vegetative microorganisms at pressures around 400 to 600MPa, including bacteria, yeast and moulds (e.g., Smelt, 1998; Mañas and Pagán, 2005), as well as (in)activation of several food quality-related enzymes, such as polyphenoloxidase, peroxidase, pectin methylesterase and polygalacturonase (e.g., Seyderhelm *et al.*, 1996; Hendrickx *et al.*, 1998), while maintaining the initial quality attributes, as opposed to thermal processing. Increasing the pressure to 800MPa, most

inactivation reactions are strongly accelerated and in combination with temperatures higher than 80°C, even bacterial spores are irreversibly inactivated. Due to the extreme pressure resistance of the bacterial spores and of some food related enzymes, high pressure technology must be combined with temperature.

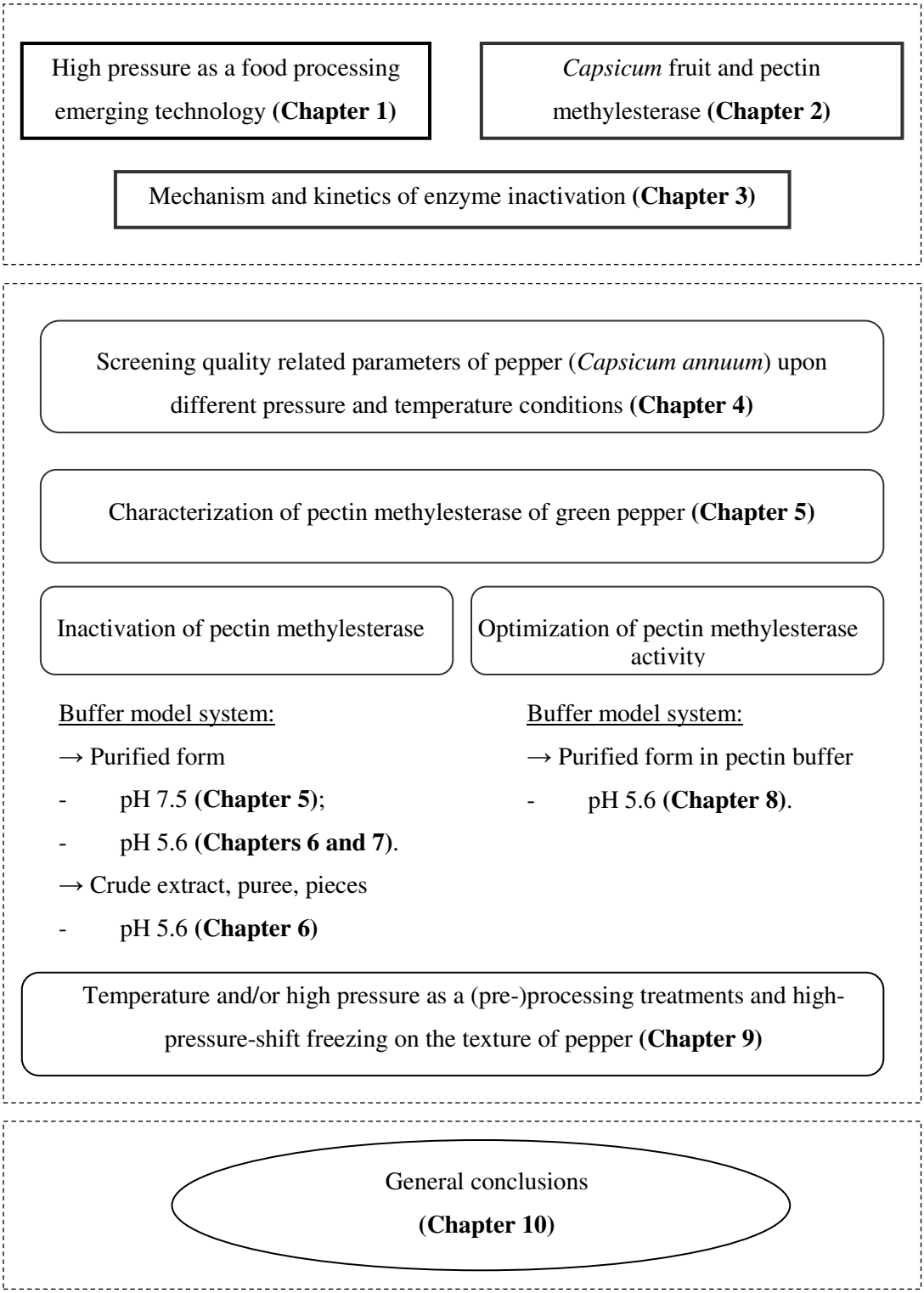
Besides microorganisms and enzymes inactivation, high pressure still presents other advantages when compared to heat treatment, including the retention of flavour and colour, as well as nutritive components, leading to a better preservation of the raw material qualities (Hayashi, 1989; Eshtiaghi *et al.*, 1994; Van den Broeck *et al.*, 1998). High pressure can also be effective to produce food with unique textural properties and to provide partially prepared foods (Hayashi, 1989). An additional benefit is the influence of pressure on enzymes and its reactions. Enzymes might be affected in different ways (Cheftel, 1992): (i) pressurization at room temperature may bring reversible or non-reversible, partial or complete, enzyme inactivation, depending on the enzyme and the process conditions; (ii) an enzymatic reaction may be enhanced or inhibited by pressure, depending on the negative or positive value of the reaction (or activation) volume (Van den Broeck *et al.*, 2000a,b; Verlent *et al.*, 2004a,b); (iii) a macromolecular substrate (protein, starch granules) may become more sensitive to enzymatic depolymerization or modification once it has been unfolded or gelatinized by pressure; (iv) intracellular enzymes may be released in the extracellular fluids or the cell cytoplasm, provided the cell membrane or membranes of intracellular organelles are altered by pressure. This might facilitate enzyme-substrate contact, which can provide a potential for development of new texture products.

Like heating, freezing has long been established as an excellent method for preserving food products, with the potential of high retention of food quality over long storage periods, as food quality decay is slowed. Bacterial growth and many chemical and/or enzyme reactions are unable to occur. At the same time, ice crystals formation adversely affects the texture of many foods, especially in fruits and vegetables which have higher water content. Since water expands when frozen, cell walls in food are often ruptured. Nevertheless, if the food is quickly frozen less damage to the structure of the product is done. Recently, high pressure processes in which phase transitions (water-ice equilibrium) take place, such as high pressure shift freezing, have attracted the attention of many food researchers (e.g.,

Cheftel *et al.*, 2000; Otero *et al.*, 2002). In high pressure shift freezing, the small ice crystals formation is instantaneous and homogenous throughout the whole volume of the product, because of the high degree of supercooling reached on pressure release. The food product frozen by this process is less texture damaged than by the traditional freezing techniques (Fuchigami *et al.*, 1996, 1997; Koch *et al.*, 1996; Otero *et al.*, 1998, 2000).

Nevertheless, there is "no beauty without the beast"… High pressure technology has currently a stressing disadvantage associated to the high costs of both equipment and process, contributing to the elevated cost of purchasing high pressure-treated food products in the market. Large scale industrial implementation of high pressure technology is, however, progressing. There is a wide variety of high pressurized food products spread all over the world, as it will be further pointed out in this work. The scientific basis that assesses quantitatively the impact of high pressure processes on both food safety (for "regulatory approval") and quality (for "consumer' acceptance") is also becoming available. Quantitative data on the effects of pressure and temperature on the safety and quality aspects of food are indispensable for design and evaluation of optimal high pressure processes, i.e. processes resulting in the maximum quality retention within the constraints of the required technologies should deliver, apart from the promised quality improvement, an equivalent, or preferably, enhanced level of safety. Therefore, food technologists ought to join efforts with scientists in order to solve equipment and process problems, as well as satisfying the consumers' demands.

Peppers are most appreciated for their quality attributes, and texture in particular when they are consumed raw in salads. The production of frozen peppers intended to be consumed raw includes a thermal blanching process, prior to freezing, which causes some undesirable effects on nutritional and texture attributes of peppers. For these reasons, peppers were used as a case study in this work, to evaluate: (i) the possibility of pressure treatments to substitute the thermal blanching, with lower damaging effects on quality attributes; (ii) the use of thermal and pressure pre-treatments to promote the catalytic action of the enzyme pectin methylesterase, to improve the texture of peppers prior to processing and (iii) the use of high pressure shift freezing, instead of the conventional atmospheric freezing, to better keep the texture of frozen peppers. A schematic presentation of the research strategy used in this study is shown in **Scheme 1**.



Scheme 1 Schematic presentation of the research strategy.

An overview of high pressure technology, combined with temperature, its effect on several quality parameters as well as its application in the food industry is discussed in detail in **Chapter 1**. **Chapter 2** describes the general characteristics of peppers (*Capsicum*

annuum), widely appreciated in the every cuisine, including the Portuguese, and with an increasing consumer's acceptance. A review on pectin methylesterase and its related substrate, pectin, is also presented. **Chapter 3** discusses both mechanism of thermal and high pressure inactivation of enzymes and outlines enzyme inactivation models.

A preliminary study related with the impact of pressure and/or temperature treatments on several quality attributes of green and red peppers, such as enzymes, vitamin C and texture, was done in **Chapter 4**. Afterwards, the experimental work was converged onto the biochemical characterization of pectin methylesterase from green bell pepper as well as on its pressure-temperature (in)activation kinetic. The reason why this enzyme was focused on, was because its importance in the context of vegetable food commodities. It causes cloud loss in juices and modifies texture of fruit and vegetable-based products. On one hand, PME should be inactivated to avoid cloud destabilization of juices, on the other hand, modulation of texture is more complex: uncontrolled activity of PME can lead to texture loss and hence its inactivation should be considered, whereas a controlled activity can enhance texture. In **Chapter 5**, green pepper PME was extracted, purified by affinity chromatography column and biochemically characterized. Process stability of purified green pepper PME at the optimum pH, at 7.5, is also discussed. **Chapter 6** focused on process stability of green pepper PME in model systems (pure form and crude extract), pepper puree and intact pepper tissue at the natural pepper pH, at 5.6. Inactivation kinetics of green pepper PME by combined high-pressure and temperature treatments (pH 5.6) is described in **Chapter 7**. Different primary and secondary models were applied to model the inactivation kinetics. In **Chapter 8**, the identification of pressure and temperature combinations for optimal pepper PME activity in a pectin model system (pH 5.6) was done. **Chapter 9** focuses on the effect of different thermal and thermal/high pressure pre-treatments on green pepper texture. This chapter also includes results related with high pressure technology as a freezing technology in comparison with more conventional freezing methods ("slow" and "rapid" cryogenic freezing), and their effect upon storage at different temperatures. Finally, the **general conclusions**, in **Chapter 10**, together with indications of future work, were drawn from the previous chapters.

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CHAPTER ONE High pressure as a food processing emerging technology

1.1 Introduction

There is a food market tendency for more convenient, fresher, less heavily processed (e.g., processed with less heat), more natural and healthier products. This can be achieved with minimal processing methods that preserve food, but also retain to a greater extent their nutritional quality and sensory characteristics, by reducing the reliance on heat as the main preservation action. Very short shelf-life products require preservation methods that will prolong their shelf-life and improve their quality. Traditionally, fermented foods have many of the mentioned characteristics; irradiation, often called cold pasteurisation, and chilling, together with controlled or modified atmosphere, are two of the more widely adopted minimal processing methods. However, only irradiation can destroy microorganisms, while the other two rely on microbial growth inhibition to preserve food. But even though traditional techniques continue to be important, there is much interest in new and alternative techniques, the so-called non- or low-thermal techniques that may be applied commercially.

Currently, non- or low-thermal food processing techniques are regarded with special interest by the food industry. Among non-thermal technologies, high pressure processing, also described as high hydrostatic pressure, or ultra high pressure processing, has been recently introduced with success in some food sectors. In this chapter, it will be given an overview about high pressure technology, its effects on different aspects relevant for food processing and preservation, and a review of the current status of high pressure technology with respect to its application in the food industry.

1.2 High pressure: a new technology?

Fruit and vegetable derived products are traditionally produced by thermal processing, sometimes even combined with chemical preservatives, which inhibits microbial spoilage and reduces enzymatic activity, present in unprocessed food. Although ensuring safety and shelf-life of fruit and vegetable products, thermal pasteurisation dramatically affects many quality factors of fresh fruits, particularly texture, colour, aroma and vitamins content. Besides, the

"magical" slogan of present day, food market is not only safety combined with shelf-life, but also with freshness. Therefore, several alternative or novel food processing technologies, with/without the use of heat, are being explored in order to provide safe, fresh-tasting, and nutritive foods. Several new minimal processing technologies are presented in **Table 1.1**. Minimal processing of foods covers a wide range of technologies and methods, for preserving foods during their transport from the production site to the consumer and can be applied in various stages of the food production chain, in storage of raw materials, processing, packaging and distribution. During minimal processing, changes of fresh-like quality attributes of the foods vary as little as possible (minimally), while the processing gives the food product sufficient shelf-life for the transport from processing to consumers.

Table 1.1 Minimal processing methods (adapted from Ohlsson, 1994).

Process	**Mechanism**
New thermal processing methods	
Ohmic heating High frequency heating Microwave heating	Optimized heating regime reduces levels of undesired microorganisms while minimizing thermally induced quality losses
Sous-vide technology	Mild and controlled heating and cooling
Non- or low-thermal processing methods	
High pressure technology	(In)activation of enzymes and destruction of microorganisms under high pressure
Irradiation	Ability of microorganisms to reproduce eliminated
High electric field pulses	Microbial cell rupture due to uneven distribution of electrical charge across cell

There has also been an increasing interest in developing other combinations of existing and novel methods to achieve mild preservation, including microbial inactivation (Ohlsson, 1994). This methodology of combining preservation treatments is not new in principle of action. Many of the traditional preservation methods are based on such combinations, in order to obtain enough protection against microbiological growth. Modern hurdle technology studies

the interactions of the various preservation steps on microbiological development and quality changes during storage.

High pressure processing seems a very promising technique for the food industry, as it offers numerous opportunities for developing new stable foods with an extended shelf-life, with high nutritional value and excellent organoleptic characteristics – minimally processed but safe for consumers, as already mentioned. And even though often referred as a new technology, the use of high pressure in food processing is an adjustment of a technology that is commonly employed in other industrial processes (ceramics, diamonds, super-alloys, and sheet metal forming). Moreover, even the idea of using high pressure in food processing is not new. The first report of high pressure being used as a food preservation method was by Hite (1899). He reported that milk "kept sweeter for longer" after a pressure treatment of *ca* 600MPa (1 hour) at room temperature. During the next 15 years, Hite and his co-workers examined a variety of foods and reported that while pressure could be used to extend the shelf-life of fruits (bananas, pears, peaches, plums, elderberries), it was less successful with vegetables (Hite *et al.*, 1914). It seemed that fruit and fruit juices responded well to high pressure because "yeasts and other organisms having most to do with decomposition are very susceptible to pressure", while vegetables preservation was related to the presence of spore-forming bacteria that could survive and grow in the low acid environment. Therefore, biological effects of high pressure, like inactivation of microorganisms, are known for decades. However, food preserved by high pressure only became a commercial reality in the last 15 years. The commercial interest of high pressure in the food industry has occurred first in Japan, in the early 1990s, and 6 years later in Europe and the United States (**Table 1.2**).

As it can be seen in **Table 1.2**, a number of food products are currently available mainly in United States and Japan, including fruit juices, guacamole, sauces, oysters and packaged cured ham. However, in Europe the method is more or less unknown for the consumer and food manufacturers face novel food regulation impediments due to the European Union (EU) legislation. EU, on the other hand, in the last ten years funded a number of research projects on high pressure treatment of food, including how to deal with consumer attitudes to high pressure processed food (Butz *et al.*, 2003a). Those who perceived the greatest personal

advantage from the technology tended to include a higher proportion of young educated people. According to Butz *et al.* (2003a), without personal experience and based largely on information provided on a statement card, high pressure processing was acceptable to the majority of consumers interviewed in France and Germany, and with some reservations in the United Kingdom (UK). The majority of the potential buyers were conditional buyers. For both British and Germans, it was most important that the high pressure treated products were not more expensive than the conventional products and that there will be a health benefit from their consumption. Contrary to them, the French consumers were more prepared to pay a bit more for the products, to whom quality and shelf-life increase was clearly important.

Table 1.2 Commercial high-pressure processed products marketed (Avure Technologies, 2004).

Type	Products	Distribution
Ready-to-eat meat products	Ham, carved chicken breast, beef	Spain, United States
Seafood	Ceviche (raw fish and/or shellfish in a citrus marinade) salad, oysters, mussels, abalone, sea food salads, dried and salted cod	Australia, Italy, USA
Fruits and vegetables	Guacamole, avocado halves and pulp, apple sauce, several fruit blends, chopped onions	United States
Beverages	Carrot and apple fruit purees, strawberry juices, orange and apple juice, lemonade and apple cider	France, Italy, Portugal, United States, UK

The initial emphasis of high pressure processing was, in fact, directed towards food preservation with the goal of extending product shelf-life with minimum impact on product quality. Subsequently, the great potential of high pressure for physical modification of structure and function of food and food constituents, as well as the possibility for new processes developments has also been recognised. Threfore, the application of high pressure processing is rapidly becoming the most well known emerging food processing technology as an alternative to heat treatments based on its ability to produce value-added and fresher foods (Palou *et al.*, 2002).

High pressure processing subjects liquid and solid foods, with or without packaging, to pressures between 100 and 800MPa. The International System (SI) unit of pressure is Pascal (Pa) or Newton *per* square meter ($N.m^{-2}$). A number of different measures of pressure are used throughout the scientific world. Some units of pressure are listed in **Table 1.3**. Because the pressure level of high pressure processing is much higher than several Pascal, the standard prefix mega-(10^6) is often multiplied to the Pascal, yielding mega-Pascal (MPa). Thus, a pressure of 101MPa is equivalent to 1.01kbar, 997atm or $14.6\times10^3 lb.in^{-2}$ (psi). These pressures are higher than those naturally occurring on the Earth surface, but routinely used in pressure industrial processes. To put into perspective, two 5000kg elephants balancing on a $1cm^2$ surface will produce a pressure force of 1000MPa.

Table 1.3 Pressure units in use.

Pa	atm	bar	Kg-f.cm^{-2}	psi (lb.in^{-2})
0.1013×10^6	0.9998	1.013	1.033	14.69

Note: The difference between the atm, bar and kg-f.cm^{-2} units is usually ignored if precise numerical values are not too important.

In the introduction section of this chapter, high pressure technology was described as a non- or low-thermal food processing technique. However, during pressure, temperature can be applied (from below 0ºC to above 100ºC, up to 115ºC). High pressure processing when combined with mild- to high-temperature seems to have an effect similar to heat-pasteurisation and -sterilisation, respectively. It is also possible to freeze a food sample under pressure, to enhance ice nucleation by fast pressure release (as it will be further discussed in section 1.6.), to keep a sample at sub-zero temperatures without ice crystal formation, to reach the glassy state of water by fast cooling under pressure, or to thaw a frozen sample under pressure below 0°C.

The commercial exposure time to pressure can range from millisecond pulse to over 20min. However, keeping the sample under pressure for extended periods of time does not require any additional energy (Cheftel and Culioli, 1997). The work of compression during high pressure treatment will uniformly increase the temperature of foods through adiabatic heating. The

magnitude of the temperature rise is determined by the initial temperature of the product and its composition (Balasubramanian and Balasubramaniam, 2003). According to Ting *et al.* (2002), water has *ca* 3ºC increase *per* 100MPa. However, if the food contains, for example, a significant amount of fat, such as chicken or beef fat, the temperature rise can be larger, 4.5ºC and 6.3ºC *per* 100MPa, respectively. The adiabatic heating is reversible upon pressure release.

1.3 High pressure principles

There are two principles that describe the effect of high pressure. The first principle, Le Chatelier's principle, states that any phenomenon (phase transition, chemical reactivity and reaction or change in molecular configuration) accompanied by a volume change is influenced by pressure. In general, the effect of pressure on a physicochemical process at equilibrium is governed by the volume change of the process (ΔV, $cm^3.mol^{-1}$). The relationship between ΔV and K, the equilibrium constant for a certain process, derives from equation 1.1 and 1.2 into equation 1.3:

$$\left(\frac{\partial \ln K}{\partial P}\right)_T = -\frac{1}{RT}\frac{\partial\left(\Delta G^0\right)}{\partial P} \tag{1.1}$$

$$\frac{\partial\left(\Delta G^0\right)}{\partial P} = \Delta V \tag{1.2}$$

$$\left(\frac{\partial \ln K}{\partial P}\right)_T = -\frac{\Delta V}{RT} \tag{1.3}$$

where ΔG^0 is the standard Gibbs free energy ($J.mol^{-1}$), R the universal gas constant (8.314 $cm^3.MPa.mol^{-1}.K^{-1}$); T the absolute temperature (K); P the pressure applied (MPa) and K, the equilibrium constant. The subscript T means that the process occurs at constant temperature. Thus, the application of pressure will cause the equilibrium to shift in favour of the state with the lowest overall volume. The magnitude and sign of ΔV for changes in the protein structure, for example, will depend on the specific molecular interactions (Mozhaev *et al.*, 1994). The processes associated with volume decrease are favoured by pressure increases, whereas

processes related with volume increase are inhibited by increments on pressure. **Figure 1.1** shows the volume changes undergone by a bottle of orange juice upon pressurization.

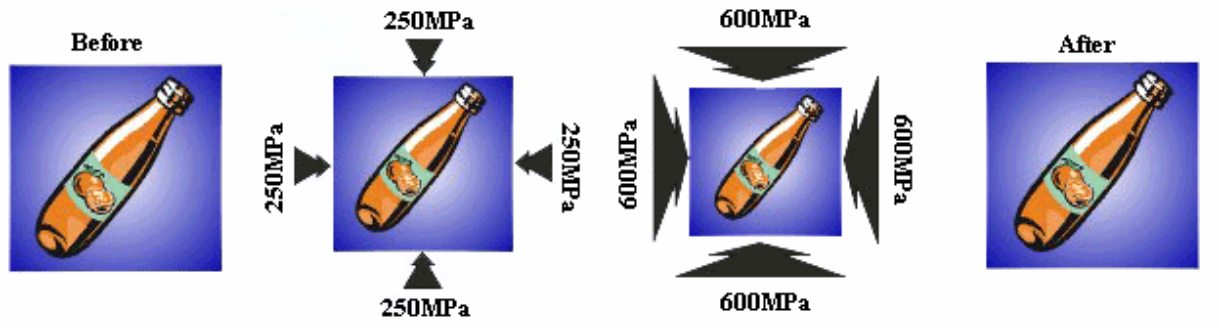

Figure 1.1 A schematic representation of an orange juice bottle under two different pressure levels, 250 and 600MPa (adapted from www.avure.com).

One of the key problems related to heat treatments is the difficulty to achieve uniform distribution of temperature within the product without the risk of over-processing. In order to reach the desirable value of temperature in the centre of the product, long treatment times are required due to slow heat penetration to the core of the product and, as a consequence, less quality of the final product is achieved. High pressure processing overcomes this problem through the second principle, known as the isostatic principle, which states that pressure is instantaneously and uniformly transmitted, independent of the size and geometry of the food or food packaging (Knorr, 1996). This also causes scale-up, from laboratory to industrial level, to be easily carried out, since the process is volume independent and pressure gradients inside the sample do not exist.

Nevertheless, the effect of pressure is similar to the heat effect on biological material and food. The difference is that, at room temperature, high pressure basically keeps covalent bonds intact as the liquid water is compressed, an unique characteristic of high pressure processing (Hayashi, 1989; Heremans, 1992). According to Silva and Weber (1993), the volume decrease accompanying protein denaturation appears to be due to the formation or rupture of non-covalent bonds (i.e., changes in the conformational volume) and from rearrangements of solvent molecules (i.e., changes in solvation volume). So, as opposed to high temperatures, which lead mostly to irreversible protein denaturation and enzyme inactivation, high pressure

often causes reversible effects on proteins/enzymes at low pressure range (<200MPa). Irreversible effects caused by higher pressure levels, including protein denaturation and enzyme inactivation, due to chemical modification or unfolding of single chain proteins occur above 300MPa (Masson, 1992). Quality and function retention and improvement is also made possible by limiting processing effects on non-covalent bonds (Weber and Drickamer, 1983; Hayashi, 1989; Cheftel, 1991; Heremans, 1992; Kunugi, 1993; Balny *et al.*, 1997; Knorr, 1999).

Moreover, the application of high pressure on food processing in combination with mild- or low temperature (Knorr, 1999), or even sub-zero temperatures such as pressure freezing/thawing, non-frozen storage and formation of ice polymorphs (Kalichevsky *et al.*, 1995), can lead to enhancement of quality of food products, with high energy efficiency and practically without waste generation.

1.4 High pressure equipment

Compared to today's high pressure processing equipment, the prototype system utilized in the 1890s by Hite was very primitive. Nowadays, with advances in computational analysis and new materials, high capacity pressure systems can be manufacture to allow reliable high pressure treatment of food products at even higher pressures than those used by Hite (Hoover, 1993).

In a high pressure process, the food product to be treated is placed in a pressure vessel capable of sustaining the required pressure. Therefore, the main key components in high pressure technology, besides the pressure vessel, are the pressure generating pumps or pressure intensifiers and the pressure transmission fluid. Currently, there are a number of different pressure transmission media being used, including water, mixtures of water and small proportions of water soluble oils, which act simultaneously as a lubricant and as an anticorrosion agent, like castor oil and glycol mixtures. Nevertheless, water is still considered

to be the ideal medium for food industry applications in order to avoid any potential contamination of the product.

As far as the type of industrial high pressure treatment is concerned, it can be applied as batch or as semi-continuous process (**Figure 1.2**). The selection of equipment depends on the kind of food product to be processed. Solid food products or food with large solid particles can only be treated in a batch mode. Liquids, slurries or other pumpable products have the additional option of semi-continuous production (Ting and Marshall, 2002).

Figure 1.2 A batch high pressure equipment (left), with a commercial size vessel of 215-liter capacity from Avure Technologies (Kent, WA, United States); a semi-continuous high pressure equipment (right) (http://grad.fst.ohio-state.edu/hpp/HPPoverview.htm).

Currently, most high pressure machines in industrial plants used for food processing work in a batch process, whereby the product is placed in a high pressure chamber and the vessel is closed, filled with pressure transmitting medium and pressurized either by pumping pressure transmission medium into the vessel as it can be seen in **Figure 1.3**, or by reducing the volume of the pressure chamber, using a piston. Once the desired pressure is achieved, the pump or piston is stopped, the valves are closed and the pressure is maintained without any further energy input. After the required time has elapsed, the system is depressurized, the vessel opened and the product unloaded (Ting and Marshall, 2002). When product package is necessary after treatment, additional systems, such as an aseptic filling station, are then

required (Ting and Marshall, 2002). As it can be seen in **Figures 1.1** and **1.3**, and further in **Figure 1.6**, the food product can be packaged in a plastic bottle or in a pouch, as long as it is a flexible container.

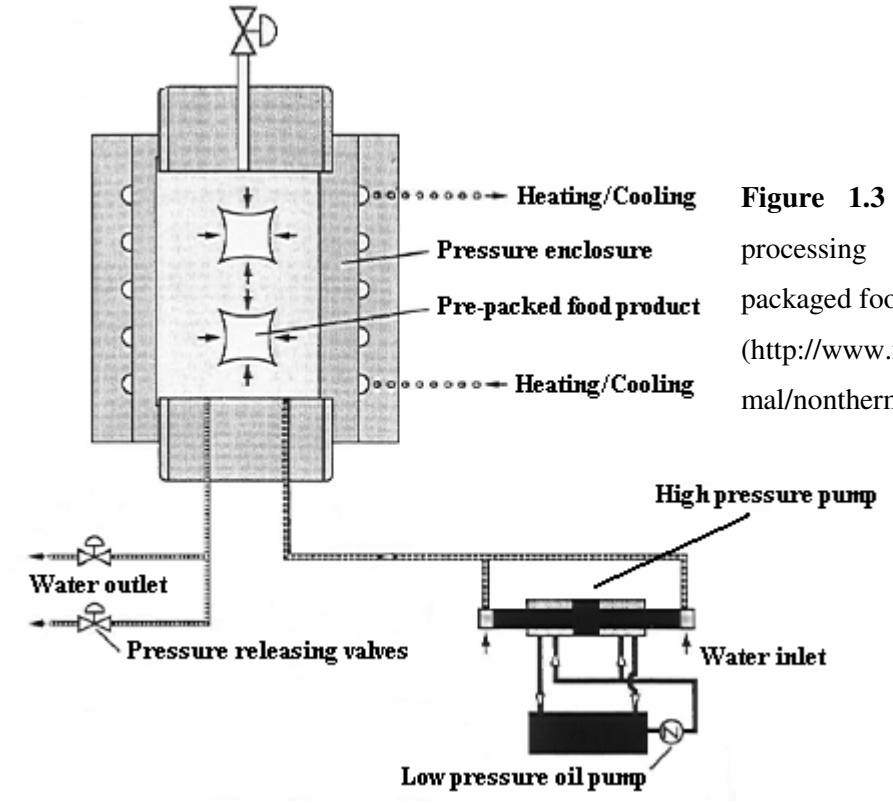

Figure 1.3 A typical high pressure processing system for treating pre-packaged foods (http://www.fao.org/ag/ags/agsi/Nonthermal/nonthermal_1.htm).

Some semi-continuous systems for treating liquids use a pressure vessel with a free piston to compress liquid foods (**Figure 1.4**). A low-pressure food pump is used to fill the pressure vessel and, as the vessel is filled, the free piston is displaced. Once filled, the inlet port is closed and high-pressure process water is introduced behind the free piston to compress the liquid food. After an appropriate holding time, releasing the pressure on the high-pressure process water decompresses the system. The treated liquid is discharged from the pressure vessel to a sterile holding tank through a discharge port. A low-pressure water pump is used to move the free piston towards the discharge port. The treated liquid food can be filled aseptically into pre-sterilized containers.

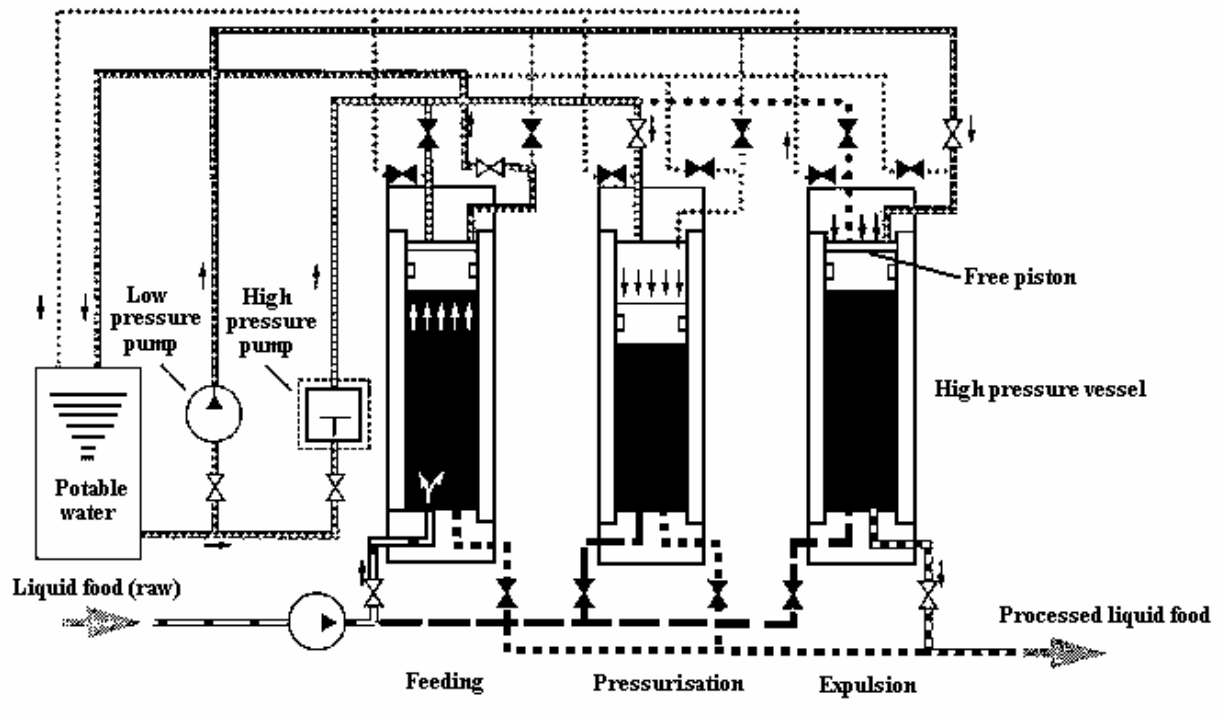

Figure 1.4 A multivessel arrangement for semi-continuous high-pressure processing of a liquid food (http://www.fao.org/ag/ags/agsi/Nonthermal/nonthermal_1.htm).

Both batch and semi-continuous equipments can still be adapted to pulsed operation by programming a series of treatment cycles of short duration prior to discharging the treated food, which can improve the high pressure processing of food products. Even though both types of equipment are available on an industrial scale, batch process is more widely used for commercial food production than semi-continuous, since different food can be processed without cross-contamination concerns, with no need for clean-up between runs, and without the risk of large quantities of foods becoming contaminated in case of equipment malfunction.

The commercial available high pressure equipments for food industry have pressure vessels from 35L up to 687L of volume capacity and working at 600 and 310MPa, respectively. The volume capacity vessel of 687L, for example, is often used in the shellfish industry, using high pressure as a processing tool for oysters, clams and lobsters. However, the higher the volume of the vessel, the lower the pressure level allowed due to operation constraints. Pre-stressed wire-wound vessels, as well as for top and bottom seals, are needed for safe, durable and

reliable operation for larger volumes at higher pressures, which will increase the equipment costs. Equipment and processing costs are typically estimated to be less than 0.08€ *per* kg of the food processed (Meyer *et al.*, 2000). Even though a relatively low pressure level is usually applied in the industry due to volume operation constraints, in the research field, high pressure equipments can allow more stressing limits in terms of both pressure (up to 1400MPa) and temperature (up to 140ºC), since smaller vessel equipments are used (**Figure 1.5**).

Figure 1.5 High pressure units. (A) 8-vessels of 8mL working at P_{max} 1000MPa and -30ºC<T<100ºC, with glycol-oil mixture (TR-15, Resato) as pressure medium; (B) single vessel (590mL volume, internal diameter 50mm, height 300mm) (SO. 5-7422-0, Engineered Pressure Systems International EPSInt, Belgium), working at P_{max} 600MPa and -30ºC<T<70ºC, with mixture of propylene glycol (60% Dowcal N, Switzerland) as pressure medium. Both working systems are from Laboratory of Food Technology (Katholieke Universiteit Leuven, Belgium).

One of the first and most classical as well as successful examples of industrial high pressure processing is pressurized avocado. AvoMex Inc.'s is a company that has been using this technology for a decade to produce fresh-tasting guacamole (**Figure 1.6**), a traditional Mexican sauce, before branching into other products such as salsa, juices and ready-to-eat meals.

Figure 1.6 AvoMex commercializes guacamole product since 1997.

Guacamole is made of avocado puree, dried onions, salt, among other ingredients, and is a quality sensitive product, which can suffer a noteworthy impact upon thermal processing,

particularly in flavour and colour. On the contrary, high pressure treatments do not significantly affect the sensory acceptability and colour of guacamole when compared to the non-processed product (Palou *et al.*, 2000). Nowadays, the AvoMex Company operates three plants with about 10 batch and semi-continuous high pressure units (Sabinas, Mexico), with almost 1 million lbs. of avocados alone processed each week. High pressure treated avocado is now in USA national distribution mainly due to an unsatisfied consumer demand for guacamole microbiologically safe, chemical additive-free, fresh-like tasting and convenient to use.

Therefore, due to recent equipment advances, successful commercialisation of high pressure products and a consumer demand for minimally processed, high quality and safe foods have lead to substantial research interest in high pressure technology. This rising interest allowed a more detailed study of the effect of high pressure on the food constituents and their consequence on food quality parameters, which in turn, will lead to the optimisation of process parameters and, at the same time, the production of food with better quality. Nevertheless, high pressure technology applied to food industry is still considered only for high-added value products or products that are impossible to obtain by other commercially available technologies. An increase in taste, flavour, texture and nutritional value of the processed products would be appreciated by consumers who are willing to pay a premium price for the increased quality level of the food (Donsí *et al.*, 1996; Butz *et al.*, 2003a).

1.5 Effect of high pressure in quality related parameters

The main effects of pressure related to food systems comprise the inactivation of microorganisms; the modification of biopolymers, including enzyme (in)activation, protein denaturation and gel formation; the susceptibility to enzyme action as well as quality retention, such as stability of colour, flavour or palatability; and the functionality of the product as exemplified by density changes, freezing and melting temperature or textural attributes (Knorr *et al.*, 1992; Knorr, 1993). Pressure can also lead to gelatinization of starch and inactivation of microorganisms under ambient temperature and produces unique texture products (Hayashi

and Hayashida, 1989; Balny and Masson, 1993). According to Hayashi (1989), reactions like Maillard, off-flavour formation and vitamin destruction do not occur during pressurization.

High pressure processing can potentially modify the functional proprierties of food constituents (e.g., proteins) and even increase the yield of certain food products, a very important economic issue for food manufacturers. Mor-Mur and Yuste (2003) reported that the weight loss was significantly higher in heat-treated sausages than in high pressure treated samples. Therefore, high pressure can allow producers to create new markets not possible with the conventional technologies and such benefits are only now being exploited. According to Ting and Marshall (2002), consumers are generally willing to pay more for greater perceived value.

1.5.1 Microorganisms

The microbial population present in fruits and vegetables are the main agents responsible for food spoilage and food poisoning, and therefore food preservation procedures are targeted towards them. Most of the microorganisms present in fresh vegetables are saprophytes, such as *coryniforms,* lactic acid bacteria, spore-formers, coliforms*, micrococci*, and *pseudomonas*, derived from soil, air, and water contamination. Due to the raw fruits acidity, the primary spoilage organisms are fungi, predominantly moulds and yeasts, such as *Saccharomyces cerevisiae*, *Aspergillus niger, Penicillum spp, Byssochlamys fulva*, *B. nivea*, *Colletotrichum gloeosporioides*, and bacteria such as *Clostridium pasteurianum*, *C. perfringes*, and *Lactobacillus spp*. Psychrotrophic bacteria are able to grow in vegetable products, like *Erwina carotovora*, *Pseudomonas fluorescens*, *P. auriginosa*, *P. luteola*, *Bacillus species*, *Cytophaga jhonsonae*, *Xantomonas campestri* and *Vibrio fluvialis* (Alzamora *et al.*, 2000).

Even though fresh-cut fruits and vegetables are generally rinsed with chlorine solution (50-100ppm), the wash step does not eliminate all microorganisms (Torriani and Massa, 1994). According to Babic *et al.* (1996), fresh-cut spinach washed with 50ppm chlorine solution contained mesophilic aerobic bacteria, psychrotrophic bacteria, *Pseudomonadaceae*,

Enterobacteriaceae, *Vibrionaceae*, coliforms, *Micrococcaceae* and yeasts, and they were found inside of broken cells or cells adjacent to broken tissue. Besides, during the washing step, solutes and pigmented compounds are removed from the cut surfaces, resulting in a direct reduction of the physicochemical characteristics as well as in an increase of the tissue deterioration (Toivonen and Stan, 2004). Microorganisms, such as *Listeria monocytogenes*, *Salmonella*, *Escherichia coli* O157:H7 and other pathogens of concern were also found in fresh-cut vegetables, even after packaging (Pao *et al.*, 1997; Alzamora *et al.*, 2000).

Food preservation methods currently used by the food industry rely on the inhibition of microbial growth and/or on microbial inactivation. Thermal treatment, one of the most widely used procedures for microbial inactivation in foods, causes unwanted side-effects in the sensory, nutritional and functional properties of food. This limitation, together with increasing consumer demand for fresh-like foods, has promoted the development of alternative methods for microbial inactivation, among which high pressure is attracting much interest. Commercialized high pressure treated fresh-cut fruit refrigerated salads have revealed to be microbiologically safe and, at the same time, convenient and labour saving (Garrett *et al.*, 2003; Parish *et al.*, 2003). Another successful example is the oysters shucking process. Previously regarded as a laborious and costly since it was a hand–made, pressurized oysters turned the shucking process much easier and the original flavour profile of oysters is maintened and, at the same time, their refrigerated shelf-life was extended (**Figure 1.7**).

Figure 1.7 The dangerous and difficult hand-shucking process and the commercially available high pressurized oysters.

Optimum shucking pressure for releasing the adductor muscle from the shell range between 250-310 MPa (time dependent) and causes minimum changes to sensory qualities as well as provide unique value-added products for the industry (He *et al.*, 2002). Besides, high pressure processing eliminates the high safety risk to production workers

and reduces the microbial risks to consumers when raw oysters are eaten, due to the inactivation of several microorganisms, such as several *Vibrio* strains (Berlin *et al.*, 1999). As a consequence, the geographical area where they can be marked increases.

Initial investigations on mechanism of high pressure inactivation of microorganisms appear to be related with the cytoplasmatic membrane (Cheftel, 1995; Smelt, 1998). High pressure seems to damage bacterial membranes, namely the molecular organization of the lipid-peptide complex, by disrupting the phosphatidic acid bilayer membrane structure, and consequently affecting the transport phenomena involved in nutrient uptake and disposal of cell waste. Intracellular fluid compounds have been found in the cell suspending fluid after high pressure treatment demonstrating that leaks occur while cells are held under pressure (Shimada *et al.*, 1993; Perrier-Cornet *et al.*, 1999). High pressure orders phospholipids bilayers, causing the fatty acyl-chains to pack together more tightly, and reducing membrane fluidity (Kato and Hayashi, 1999; Mentré and Hui Bon Hoa, 2001). Membranes with a higher relative proportion of unsaturated fatty acids can compensate this effect and maintain the membrane in a functional liquid crystalline state. Besides, these disordered regions are barely accessible to water molecules, leading to an increase in the baroresistance. Other structures inside the cell have also been proposed as potential key targets for inactivation by high pressure, since many organelles such as the nucleous, mitochondria, endoplasmatic reticulum, Golgi apparatus and lysosomes or vacuoles are all enveloped by membranes (Kato and Hayashi, 1999). Pressure treated *E. coli* may maintain a physically intact cytoplasmatic membrane upon decompression even in dead cells (Mañas and Mackey, 2004). High pressure is then multi-target in nature, with cellular membrane as the key target, but in some cases additional damaging events such as extensive solute loss during pressurization, protein coagulation, key enzyme inactivation and ribosome conformational changes, together with impaired recovered mechanisms, seem also to kill bacteria (Mañas and Pagán, 2005).

However, high pressure treatment related with microorganism inactivation should not be taken lightly, since it may not always completely inactivate microorganisms, but rather injure only a proportion of the population. Survival curves have shown pronounced “tails”, concave upwards in shape (Humpheson *et al.*, 1998; Benito *et al.*, 1999; Smelt *et al.*, 2002). This

upward concavity has been explained on a biphasic behaviour basis that reflects two first order inactivation curves for two distinct microbial subpopulations, each one homogeneous in resistance (Humpheson *et al.*, 1998), or a continuous distribution of resistance within the microbial population (Smelt *et al.*, 2002). Recovery of the injured cells will depend on the conditions after treatment and this has implications for microbiological counts (Patterson *et al.*, 1995).

In most cases, vegetative bacterial cells are inactivated at relatively low pressure levels, around 400-600MPa, while bacterial endospores can be extremely pressure resistance, up to 1000MPa. Still, significant variations between spores from different species and also between different strains were observed (Patterson *et al.*, 1995; Smelt, 1998; Benito *et al.*, 1999). Moreover, low pressures (<200MPa) can trigger spore germination (Gould and Sale, 1970), which might suggest that spores can be killed in two stages: a first one at low pressure treatment to trigger the germination process, followed by a second treatment, at higher pressures, to kill the germinated spores (Heinz and Knorr, 2001).

Up-to-date, high pressure technology has been almost limited to pasteurisation processes in the food industry, due to the presence of quite high pressure resistant microorganisms. Even though the effect of temperature together with high pressure inactivation is considered complex, it is known that high pressure treatments when combined with mild temperature can lead to higher lethal effects (Patterson and Kilpatrick, 1998; Alpas *et al.*, 2000), which is attributed to a higher degree of damage on proteins (Sonoike *et al.*, 1992). The latter authors obtained complete inactivation of *L. casei* Y1T9018 and *E. coli* JCM1649 under various combinations of temperature and pressure (0-60ºC, 0.1-400MPa). Therefore, combination of pressure with higher temperatures can also be an approach to overcome pressure resistance of bacterial spores (Cheftel, 1995; Heinz and Knorr, 2001), in order to produce shelf-stable low-acid food products such as vegetables, milk, or soups. High pressure sterilisation is possible by starting high pressure treatments at elevated temperatures (e.g., 60-90ºC), and using the adiabatic heating upon compression for rapid heating to higher temperatures in a pulsed manner, resulting in destruction of spores and commercial products stable at ambient conditions (Meyer *et al.*, 2000; Matser *et al.*, 2004). Hayakawa *et al.* (1994) developed a

method of sterilizing *Bacillus stearothermophilus* spores through 6-cycles of oscillatory pressurization (600MPa, 70°C), while inactivation was not complete within 60min under continuous pressurization. Spores from *C. sporogenes* PA 3679 (ATCC 7953) in beef and carrot broth were also inactivated after being treated at 800MPa and 80-90ºC (Maggi *et al.*, 1996). This approach has been considered for commercial production of shelf-stable foods and is the subject of a number of patents designed to achieve the commercial sterilisation of food which has a pH greater than 4.5. One such patent describes a two or more pressure cycles (>530MPa) and elevated temperature (>70ºC) to reach this goal (Meyer, 2000).

Yeasts are an important group of spoilage microorganisms, especially in acidic foods, but none considered as a vital food pathogen. Shimada *et al.* (1993) investigated the yeast cell ultrastructure and cytoskeleton after pressurization and noted that above 200MPa the cell wall was damaged and the subcellular structure was altered, especially the nucleous and mitochondria. Later, Perrier-Cornet *et al.* (1999) distinguished two yeast populations after high-pressure treatment (250MPa, 15min): pressure inactivated cells during pressure holding time with an irreversible volume decrease (about 35% of the initial volume) and viable cells, which were less affected, related with the previous abovementioned biphasic behaviour. By application of multiple-pulsed pressure treatments to pineapple juice, Aleman *et al.* (1996) observed an increase in the inactivation rate of yeasts. Pressure-induced cell permeabilization could be the cause of yeast inactivation under pressure.

However, toxic mould growth is already a safety concern in foods. Butz *et al.* (1996) examined responses of the heat-resistant moulds, such as *Byssochlamys nivea*, *B. fulva*, *Eurotium* (*Aspergillus fischeri*), *Eupenicillium* sp. and *Paecilomyces* sp. to high pressure (300-800MPa) in combination with different treatment temperatures (10-70°C). As expected, all the vegetative forms were inactivated by exposure to 300MPa (25°C) within a few minutes, but ascospores required treatment at higher pressures (300-600MPa, 10-60ºC), except for ascospores from *B. nivea* (>600MPa, >60ºC).

Successful implementation of a novel technology for food preservation relies on the progress in the field of mechanisms of inactivation. The resistance of microorganisms to pressure varies

considerably depending on the pressure range applied, temperature and treatment duration, and type of microorganism, as already mentioned. In general, the pressure resistance increases from vegetative cells < yeasts < ascospores < bacterial spores (Patterson *et al.*, 2005). The nature of food is also important, as it may contain substances which might protect the microorganism from pressure damage. To design the appropriate conditions (pressure range, processing temperature, initial temperature of sample, holding time and packaging type) for high pressure processing, it is essential to know precise tolerance levels of different microbial species regarding pressure as well as the mechanisms by which the tolerance level can be minimised. Inappropriate use of the high pressure processing parameters may adversely affect the outcome of this novel technology.

1.5.2 Enzymes

Enzymes occur naturally in biological systems and can, together with processing, affect functional properties of foods in many ways. Endogenous enzymes can have beneficial or detrimental effects on foods. Some enzymes are positively utilized during food processing for recovery of by-products, for developing new products, for achieving higher rates and levels of extraction, or for improving food quality in terms of flavour and texture. Food spoilage can be caused by enzymes naturally present in food or produced by certain microorganisms. Some key enzymes in fruit and vegetable processing include polyphenol oxidase (PPO), responsible for enzymatic browning and the concomitant quality deterioration, peroxidase (POD) which gives rise to unfavourable flavours, lipoxygenase (LOX) which induces changes in flavour, colour and nutricional value, and pectin methylesterase (PME), which is responsible for cloud destabilization and consistency changes. Therefore, in most food processing steps, control of the enzymatic activity is required, either to promote beneficial effects of the enzymatic activity during processing or to eliminate/retard the enzymatic reaction in case of detrimental enzymatic action.

1.5.2.1 Polyphenol oxidase (PPO, EC 1.14.18.1)

A number of researchers have carried out investigations into the effect of high pressure on PPO (EC 1.14.18.1), a group of copper proteins that catalyze the oxidation of phenols to their quinone derivatives, which can then spontaneously polymerize. Their action can give rise to enzymatic browning in many damaged fruits and vegetables (Gomes and Ledward, 1996), often undesirable, and responsible for inferiour sensory attributes and losses in nutrient quality. Therefore, PPO inactivation is highly desirable. However, PPO is found to be very pressure resistant, since the pressure level required to inactivate this enzyme surpasses the one needed to inactivate microbial vegetative cells. Besides, different sources show different pressure-temperature behaviours. Mushroom, potato, and avocado PPO are very pressure stable, since treatments at 800-900MPa are needed to reduced enzyme activity at room temperature (Eshtiaghi *et al.*, 1994; Gomes and Ledward, 1996; Weemaes *et al.*, 1997, 1998b), whereas plum PPO is even more stable, since it is not inactivated at room temperature by pressures of up to 900MPa (Weemaes *et al.*, 1998c). Acidification treatments were found to significantly reduce the pressure stability of both avocado and mushroom PPO (Weemaes *et al.*, 1997, 1998a). Grape, strawberry, apricot and apple PPO seem to be more pressure sensitive. Apricot, strawberry, and grape PPO could be inactivated by pressures exceeding 100, 400 and 600MPa, respectively (Jolibert *et al.*, 1994; Amati *et al.*, 1996; Weemaes *et al.*, 1998c). Eshtiaghi and Knorr (1993) showed that potato PPO was inactivated when pressurised at 900MPa and 45°C for 30min, whereas at ambient temperature this treatment only inactivated the enzyme at low pH. Gomes and Ledward (1996) found that activity of commercial PPO extracted from mushrooms decreased steadily on treatment between 100 and 800MPa, for 1-20min in phosphate buffer (pH 6.5) and complete inactivation only occurred at 800MPa for at least 5min. Victoria grape PPO has also been described as a pressure resistance enzyme (Rapeanu *et al.*, 2005). Previously, Weemaes *et al.* (1998c) suggested that both pH modification and addition of anti-browning agents (benzoic acid, EDTA, 4-hexylresorcinol, glutathione and sodium chloride) could enlarge the pressure process efficacy. Besides the abovementioned factors, thermal treatments prior to pressure application on vegetables minimises enzymatic and oxidative reactions (Hoover, 1997). Palou *et al.* (1999) observed

residual PPO activity (<5%) in banana puree when a 7min blanch treatment was followed by high pressure treatment at 689MPa for 10min. Combined temperature and pressure treatments also seemed to act synergistically on PPO from avocado, Victoria grape and strawberry, with the exception in the high temperature-low pressure region, where an antagonistic effect was observed (Weemaes *et al.*, 1998a; Rapeanu *et al.*, 2005; Dalmadi *et al.*, 2006).

In addition to high pressure inactivation of PPO, low pressure levels have been reported to induce enzyme activation, from apple (Jolibert *et al.*, 1994; Anese *et al.*, 1995), mushroom (Gomes and Ledward, 1996), onion (Butz *et al.*, 1994), pear (Asaka and Hayashi, 1991), and strawberry (Cano *et al.*, 1997). For example, PPO from mushroom and pear showed an increase in the enzyme activity after pressurizing at 400MPa for 10min (Asaka and Hayashi, 1991; Gomes and Ledward, 1996); which could be ascribed to conformational changes or to a conversion of a latent enzyme form to an active form by release from the membrane (Asaka *et al.*, 1994; Gomes and Ledward, 1996). In tissues, apparent activation of PPO may take place as a result of membrane alterations and decompartmentation of the enzyme and its substrate (Butz *et al.*, 1994; Joliber *et al.*, 1994).

1.5.2.2 Peroxidase (POD, E.C. 1.11.1.7)

PODs (E.C. 1.11.1.7) are implicated in a number of higher plant processes and their catalytic reaction is involved in the oxidation of a large number of aromatic structures at the expense of H_2O_2. Besides being the most heat stable vegetable enzyme, POD is, at least in some cases, extremely pressure resistant. In high pressured treated green beans, POD was 88% inactivated after a treatment at 900MPa for 10min at room temperature (Quaglia *et al.*, 1996). Quaglia *et al.* (1996) ascribed that an increase in temperature enhanced POD green beans inactivation at 600MPa, while no significant differences were detected when pressure level increased up to 700MPa. An increase in the pressurization time also seemed to have no significant effect. However, less intense pressure-temperature conditions (300-400MPa) were satisfactory to inactivate POD from strawberry puree and orange juice at room temperature (Cano *et al.*, 1997).

1.5.2.3 Lipoxygenase (LOX, EC 1.13.11.12)

LOX (EC 1.13.11.12) constitute a group of enzymes that catalyzes lipid oxidation and pigment bleaching found ubiquitously in plants. Direct and indirect LOX catalyzed reactions in food systems significantly contribute to changes in colour, flavour, texture and nutritional properties, which can be desirable and undesirable (Richardson and Hyslop, 1985). As opposite to thermal inactivation, pressure inactivation is much less reported either on a quantitative basis (Ludikhuyze *et al.*, 1998a,b; Indrawati *et al.*, 1999b, 2001) or on a qualitative basis (Heinisch *et al.*, 1995; Seyderhelm *et al.*, 1996). In literature, threshold pressure for inactivation in a narrow range between 400-600MPa have been reported (Heinisch *et al.*, 1995; Ludikhuyze *et al.*, 1998a; Indrawati *et al.*, 1999b; Tangwonchai *et al.*, 1999). Soybean LOX is more pressure stable around room temperature and temperature changes enhanced the pressure inactivation effect (Ludikhuyze *et al.*, 1998a,b). LOX from green beans and peas has been successfully inactivated by high pressure at low/sub-zero temperatures (Indrawati *et al.*, 1999b, 2001). Similarly to PPO from other sources, an antagonistic effect between high temperature and low pressure was noted for pea LOX. For soybean and green bean LOX, an antagonistic effect between low temperature (<30ºC) and high pressure (>500MPa) has been observed (Ludikhuyze *et al.*, 1998b; Indrawati *et al.*, 1999b). Therefore, in what LOX inactivation is concerned, pressure blanching can be proposed as an alternative to the conventional thermal blanching treatment and, in some cases, a single pressure treatment at sub-zero temperatures could replace the sequential process of conventional blanching and freezing.

Since the work presented in this thesis is mainly devoted to pectin methylesterase (PME), its biochemical characterization as well as the effect of thermal, pressure and combined thermal/pressure treatments on the PME activity and stability will be discussed in detail in Chapter 2.

1.5.3 Texture

Much research has been done on plant cell walls in relation to texture (e.g., Van Buren, 1979; Brett and Waldron, 1996; Harker *et al.*, 1997; Ng *et al.*, 1998; Toole *et al.*, 2000; Parker *et al.*, 2000), considering the mechanical properties of the tissues. The texture of fruits and vegetables is highly dependent on the chemical and physical properties of the cell walls. In general, pressures up to 350MPa can be applied to plant systems without any major effect on the overall texture and structure (Knorr, 1995). Several studies revealed that pressure treatment of fruits and vegetables can cause both firming and softening (Basak and Ramaswamy, 1998), the effects being dependent on pressure level and pressurization time. At room temperature and low pressure (100MPa), instantaneous pressure softening was caused by compression of cellular structures without disruption, while at higher pressures (>200MPa) severe texture loss can be gradually recovered and some products become even more firm than than their fresh counterparts. This effect might be attributed to the action of PME that is only partially inactivated by pressure. Simultaneous disruption of cell structures allows interaction of the enzyme with the pectin substrate. Hence, de-esterified cell wall pectin can cross-link with divalent ions, mainly Ca^{2+}, leading to compactness of the cell structure. It has also been suggested that the firming effect obtained with mild heat treatments alone or even combined with calcium chloride treatments (Hoogzand and Doesburg, 1961; Hsu *et al.*, 1964; Stolle-Smits *et al.*, 1998; Vu *et al.*, 2003) may be attributed to the action of heat-activated PME and/or to increased Ca^{2+} diffusion into the tissue.

High-pressure pre-treatments alone or combined with temperature seem to have a similar effect, or even a greater effect, when compared to thermal pre-treatments, regarding softening in several plant tissues induced by the process conditions. Shimada *et al.* (1990) examined the textural changes of several fruits and vegetables after pressure treatment at 500MPa (15min), at room temperature. They found that texture of soybeans and rice did not change, while taproot vegetables, potatoes, pears and persimmons became softer. When Sila *et al.* (2004) high-pressure pre-treated carrot (200-500MPa, 60°C, 15min) a more pronounced texture improvement was observed when compared with thermal pre-treatment at 20°C and 40°C. Firmness of pressurised carrots (700MPa, 45min) was found to be the same as raw carrots and

higher than cooked carrots (3 and 30min) (Fuchigami *et al.*, 1996). Krebbers *et al.* (2002a) concluded that both one-pulse high pressure (500MPa, 20ºC, 60s) and two-pulsed high pressure treatments (1000MPa, 75ºC, 80s, with 30s at 0.1MPa interval) of green beans have potential to substitute conventional (90ºC, 4min) preservation techniques, such as blanching, pasteurisation and sterilisation, with improved textural properties.

1.5.4 Flavour

When food products are thermally processed, flavour is another quality parameter that is affected. However, high pressure processing, as previously mentioned, limits its major impact to non-covalent bonds. Consequently, food keeps, besides colour, taste, and nutritional value (e.g. vitamins), and the natural flavour of the untreated material (Hayashi, 1989). For most fruit juices, the potencial of high pressure mainly arises from the fact that fresh flavour can be maintained by high pressure. In most cases, trained sensory panels are unable to differenciate between fresh and pressurized juice made from the same raw material, e.g. from orange (Ogawa *et al.*, 1990). When carried out at room or even refrigerated temperatures, the application of high pressure does not induce any formation of cooked flavours. Raw fish or meat, fresh fruit purees or juices, and various flavour extracts can be processed without any alteration of their raw or fresh taste and flavour characteristics (Shimada *et al.*, 1990). Flavour of soybeans and rice was maintained after pressurizing at 500MPa for 15min at room temperature (Shimada *et al.*, 1990). In some occasions, the taste of pressurized meat has been reported to be sweeter than that of the control meat (Cheftel and Culioli, 1997). Also, high pressure processing (300MPa, 30min, 25ºC and 40ºC) changed the fresh onions odour towards that of braised or fried onions (Butz *et al.*, 1994).

Even when higher temperatures are used during high pressure processing flavour retention is improved. Processed herbs, which are food components that largely contribute to the smell and taste of products, are a good example to illustrate the negligible effect of high pressure sterilisation on flavour, as demonstrated for basil when pressure sterilisation was compared to

conventional heat sterilisation (Krebbers *et al.*, 2002b). However, the texture was clearly more similar to heat treated basil than to fresh basil.

1.5.5 Colour

Colour is regarded as a major food quality attribute in determining product acceptability, and the degree of importance seems to be dependent upon a particular food system under consideration. Another disadvantage of thermal food processing is the modification of pigments, altering the original colour of food. With regard to green colour, a common change in processed green plants is the loss of chlorophyll. The conversion of brilliant green into olive brown due to the formation of pheophytins, metabolites of chlorophyll without the magnesium atom, is considered as an index of the severity of heat processing. Little is known about the influence of pressure in such degradation reactions. Tauscher (1998) reported that there were no effects found in high pressure treated alcoholic model solutions, while dramatic changes in aqueous model systems were observed. But Van Loey *et al.* (1998) noted that both chlorophyll *a* and *b* presented extreme pressure stability at room temperature and significant reductions were only noted for temperatures exceeding 50ºC. Also, in pressure treated minced broccoli at 600MPa and 75ºC, chlorophylls demonstrated to be remarkably stable (Butz *et al.*, 2002). Contrary to conventional heat processing, high pressure sterilisation results in a good retention of the colour even though its effects on colour can be strongly product dependent. Some chlorophyll containing green products are affected in a similar way as in retort processing, e.g. green beans, while other products show less changes in colour, e.g. spinach (Matser *et al.*, 2004).

Carotenoids, which occur in many fruits and vegetables, are potentially antioxidants and may be decomposed by oxidation due to their conjugated double bonds. Thermal processing also may decompose β-carotene. However, Butz *et al.* (2002) observed that for high pressure treated (600MPa, 60min, 25ºC) and thermal treated (95ºC, 60min) tomato homogenate, no changes were observed in lycopene and β-carotene contents, regarding the control sample.

Stability of pigments can be explained by matrix effects, as within tissues pigments are often compartimentalised and thus more protected from adverse influences. The accessibility/extractability of different carotenoids in samples that were high pressure treated also seemed to be dependent on the matrix as well as on the effect of pressure on the matrix. While pressurised tomato pulp and carrot homogenate (600MPa, 25ºC, 10min) reduce the accessibility of the solvent to carotene and therefore reduced its extractability, even though total concentration of carotenoids was unaffected (Butz *et al.*, 2003b), fresh orange juice high pressure treated at 350MPa produced a significant increase (20-43%) in the carotenoid content due to a better extraction of α- and β-carotenes (de Begoña *et al.*, 2002). The colour of tomato puree has been shown to improve and the lycopene content was higher following high pressure processing (700MPa, 90ºC, 30s) compared with a 40% loss after conventional sterilisation (Krebbers *et al.*, 2003). Also, Rovere *et al.* (2000) evaluated the colour of meat containing-tomato sauce after high pressure sterilisation at 900MPa and 110ºC, and concluded that, as opposite to conventional retorting sterilisation, high pressure sterilisation did not affect the colour.

1.5.6 Other quality parameters

Vitamins are extremely important components of our diet and therefore its retention during processing is regarded as significant markers of overall nutrient recovery. Many thermal treatments of food result in losses of vitamins whereas high pressure seems to have a different effect. Pressures between 100 to 600MPa used to preserve a range of citrus juices, including Satsuma mandarin juice, Valencia orange juice and lemon juice, caused no changes in vitamin C content (Ogawa *et al.*, 1990). Horie *et al.* (1991) reported that strawberry jam prepared by high pressure treatment retained 95% of its initial vitamin C content and was preferred by a taste panel over a heat-processed jam. Quaglia *et al.* (1996) found that pressurization resulted in a higher retention of ascorbic acid (82%, 900MPa), when compared to water blanching of peas (12%) even though the results could be improved if the samples were packaged without water. Orange juice pressurized up to 500MPa (1min) showed no substantial modification in

the content of vitamin C (Donsí *et al.*, 1996). However, vitamin C is unstable when high temperature (65 to 85ºC) and high pressure conditions (850MPa) are applied (Van den Broeck *et al.*, 1998), with an observed synergistic effect of both factors. However, it should be pointed out that changes in vitamin content are usually noticed in model systems, indicating that the food matrixes, so far studied, seem to exert a protective effect (Butz *et al.*, 2003b). Under realistic production conditions, water soluble vitamins, including vitamin C, appear to be not or only little affected by pressure treatment (Van den Broeck *et al.*, 1998; Butz *et al.*, 2003b). Like water soluble vitamins, fat soluble vitamins seemed to be unaffected or only slightly influenced by pressure treatment (Fernandez-Garcia *et al.*, 2001; Sanchez-Moreno *et al.*, 2003).

Fruits and vegetables contain potential constituents, with beneficial functional properties, like antimutagens, e.g., flavonoids and coumarins. The antimutagenicity of pressurised tomato pulp (600MPa, 25ºC, 5min) was comparable to untreated samples, while heat treated samples completely lost their antimutagenicity capacity (Butz *et al.*, 2002). Further analysis in different products (oranges, apples, peaches, mixed citrus juices, carrots, tomatoes, strawberries and raspberries) led to the conclusion that, in most cases, high pressure (600MPa, 25ºC and 44ºC; 800MPa, 44ºC) did not induce loss of beneficial substances in fruits and vegetable matrices (Butz *et al.*, 2003b).

Contradictory reports exist regarding the oxidation of fats in foodstuffs caused by high pressure treatments. Such changes are often not clearly distinguished from changes occurring during storage (Angsupanich and Ledward, 1998). Residual enzymatic activities, fatty acid spectrum, water content, pH-values, degree of oxidation before pressure treatment, pro- and antioxidants all have a decisive influence on the pressure-induced changes in lipids and the progress of oxidation during storage. Structural changes in the cell membrane up to destruction of the cellular agglomerate and decompartmentation also have an effect on the oxidation of lipids. High pressure treated meat and fish can result in lipid oxidation increase, probably due to the release of free metal ions into the tissue. Oxidation, if not controlled, can negatively affect the colour and flavour of such products (Cheah and Ledward, 1996, 1997; Angsupanich and Ledward, 1998). According to Cheah and Ledward (1996), the rate of lipid

oxidation in minced pressurized pork at 800MPa for 20min prior to storage at 4ºC was similar to that induced by heat treated samples (80ºC, 15min), and higher than the one observed for the controls. It appeared that there was a critical pressure region between 300-400MPa for inducing marked changes in lipid oxidation in minced pork samples, since pressures lower than 300MPa had little effect on lipid oxidation. However, when lipid oxidation of pressurized (300-500MPa, 30min, 20ºC) or cooked (90°C, 15min) minced chicken breast was evaluated, in general, pressurized samples presented less oxidation compounds than cooked samples (Beltran *et al.*, 2003).

1.6 High pressure freezing

Freezing has long been established as an excellent method for preserving food products, with the potential of high retention of food quality over long storage periods. Generally, freezing preserves taste, texture and nutritional value of foods better than any other preservation method; as a result, ever-increasing quantities of food are being frozen throughout the world. In traditional freezing processes (such as plate contact or air blast), when food comes into contact with the refrigerating medium, ice nucleation occurs in the region next to the refrigerated border and is controlled by the magnitude of supercooling reached in this zone. Therefore, the main potential disadvantage of freezing foods is the risk of damage caused by formation of ice crystals. Controlling ice crystal formation inside food is very important and the quality of food products depends on how fast ice crystal formation occurs. It is well known that "slow freezing" induces the formation of large extracellular ice crystals that may cause mechanical damage on cell structure in tissue based food products (e.g., fruit, vegetables, meat), as well as inducing protein denaturation, while "rapid freezing" enhances nucleation and the formation of smaller crystals (Cheftel *et al.*, 2000). However, very rapid freezing (e.g., using liquid nitrogen) may also cause macroscopic cracks due to non-homogeneous volume expansion. After thawing, excessive drip and texture softening is observed. Detrimental reactions are also enhanced by solute concentration effects and closer enzyme-substrate interactions.

Besides how fast ice crystal formation occurs, the type of ice formed during freezing of foods is another factor that affects the quality of the product. The density of the type of ice that is formed under pressure is higher than that of water and the ice does not expand in volume during phase transition, which reduces tissue damage. Water has many physical and chemical properties that are significantly affected by pressure (Otero *et al.*, 2002). Pressure opposes reactions associated with volume increase, such as freezing of water at atmospheric pressure, which constitutes the basis of a new field of high pressure food applications, such as high-pressure freezing, thawing and storage of food at temperatures in the low-temperature range, below 0°C without freezing. Therefore, and during the past decade, high-pressure freezing has been envisaged as a promising freezing method by the food industry, due mainly to its potentiality for improving the kinetics of the process and the characteristics of the ice crystals thus formed (Cheftel *et al.*, 2000). The different processes in the high pressure low temperature domain are schemastically shown in **Figure 1.8**. Freezing under high pressure can be processed as either high-pressure-shift (HPSF) or high-pressure-assisted freezing (HPAF). While HPSF is achieved by depressurizing the sample in the liquid region under high pressure processing, HPAF is basically the normal freezing process occurring under a constant pressure. Thawing processes under high pressure can be carried out as high-pressure-assisted (HPAT) or high-pressure-shift thawing (HPST). A frozen product can so be forced to the liquid region in the phase diagram by applying high pressure, allowing faster thawing. Different classifications and terminology can been found in literature for these processes (Urrutia Benet *et al.*, 2004).

Recently, the effect of pressure on the water–ice equilibrium, such as HPSF, has attracted the attention of many food technologists and engineers, since this process permits particularly an increase in the ice-nucleation rate. HPSF is a unique technique used to produce small and uniform ice crystals, which minimizes damage in frozen foods. Ice I is the only ice modification susceptible to occur in HPSF, because is the only one with a negative slope in the phase transition line. The principle of HPSF involves reducing the temperature of a food sample, held in a high-pressure vessel whose temperature is regulated at sub-zero level, below 0ºC under pressure.

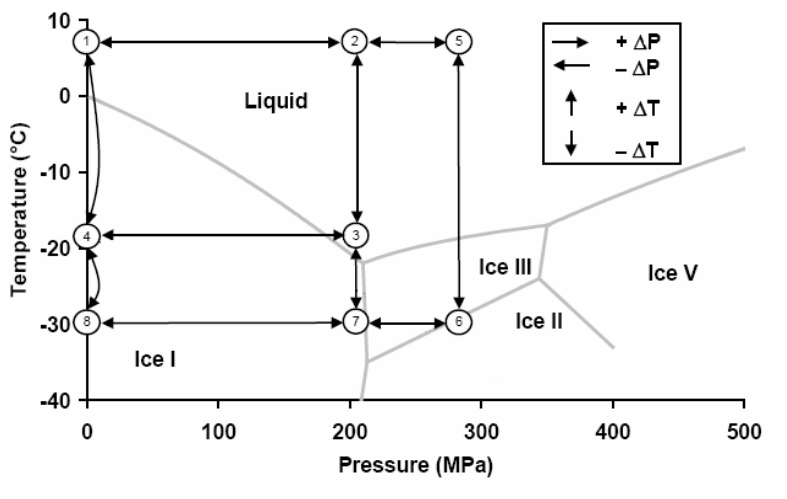

Figure 1.8 The phase diagram of water, with possible theoretical processing pathways. 1-4-8: Freezing at atmospheric pressure; 1-2-3-7-8: Pressure-assisted[1] freezing (HPAF); 1-2-5-6-7-8: Pressure-assisted freezing to ice III (HPAF III); 1-2-3-4-8: Pressure-shift[2] freezing (HPSF); 8-4-1: Thawing at atmospheric pressure; 8-7-3-2-1: Pressure-assisted thawing (HPAT); 8-7-6-5-2-1: Pressure-assisted thawing from ice III (HPAT III); 8-4-3-2-1: Pressure-induced[3] thawing (HPIT) (adapted from Urrutia-Benet *et al.*, 2005).

The food sample, previously equilibrated at a temperature above the initial freezing temperature of the food (e.g., room temperature), is placed in the high pressure equipment, the vessel is closed and the pressure is then increased up to a level which freezing is not possible (Knorr *et al.*, 1998). During this first step, the water in the food system remains in the liquid phase. Holding pressure and temperature at these conditions for the required time causes a drop in the sample temperature until the regulated temperature of the media is reached. At ≈-22°C and pressure up to 207MPa (the triple point liquid/ice I/ice III), water can still remain in the liquid state as is explained by the fact that pressure opposes the volume increase associated with the formation of ice I (Cheftel, 1992). After reaching the desired pressure-temperature coordinates at which the sample water remains liquid, pressure is released and subsequently, the temperature decreases due to the adiabatic expansion process. After this first negative slope, the path shows that the sample temperature increases: an inflection point

[1] Phase transition at constant pressure;
[2] Phase transition due to pressure change;
[3] Phase transition initiated with pressure change and continued at constant pressure.

shows the start of nucleation of ice I, i.e., the area of nucleation of ice I is first reached. When the difference between the sample temperature and that corresponding to the current pressure becomes excessively large (supercooling), a sudden temperature rise is observed when nucleation of ice I occurs. Crystallisation is induced simultaneously in the entire supercooled sample by fast pressure release, with the intention of obtaining small ice crystals, uniformly distributed in the sample, and, as a consequence, the process time as well as the quality of the frozen material is improved. Each degree K of supercooling causes about 10-fold increase in the ice nucleation rate (Kalichevsky *et al.*, 1995). To enable complete freezing of the sample, additional cooling of the sample is needed because of the large heat of the ice formation. **Figure 1.9** shows in detail the schematic theoretical path (A) and experimental paths (B) for HPSF.

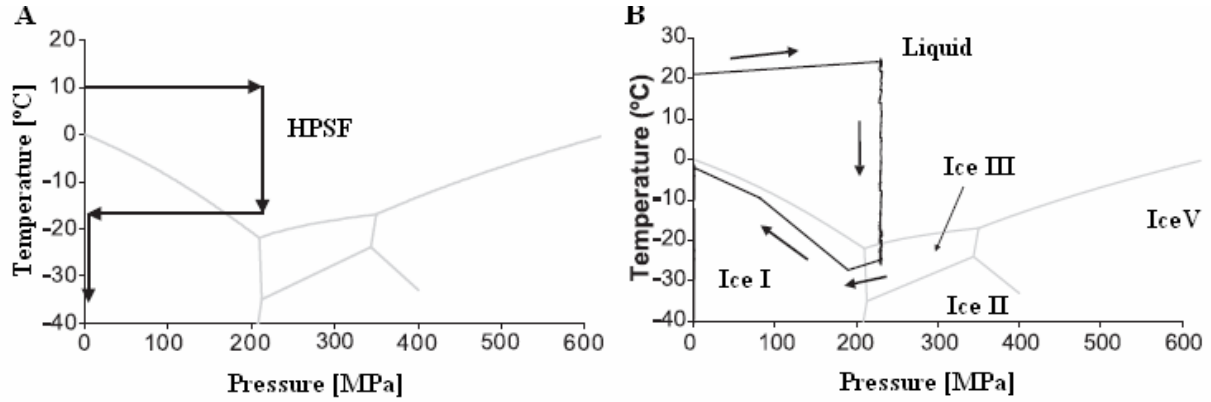

Figure 1.9 Schematic theoretical path (A) and experimental path (B) for HPSF (adapted from Urrutia-Benet *et al.*, 2004).

The temperature gradient in the food sample before and after the pressure release has been reported to be the reason of the phase transition time reduction (Urrutia-Benet *et al.*, 2005). It should be mentioned that at the point at which the pressure release has already crossed in the experimental path the phase transition line of ice III, the sample is still in an unfrozen state, due to the existence of a liquid metastable phase at these conditions of pressure and temperature (Schlüter *et al.*, 2004). The existence of a liquid metastable phase in the domain of ice I can be used to increase the real temperature gradient before pressure release (**Figure 1.10**).

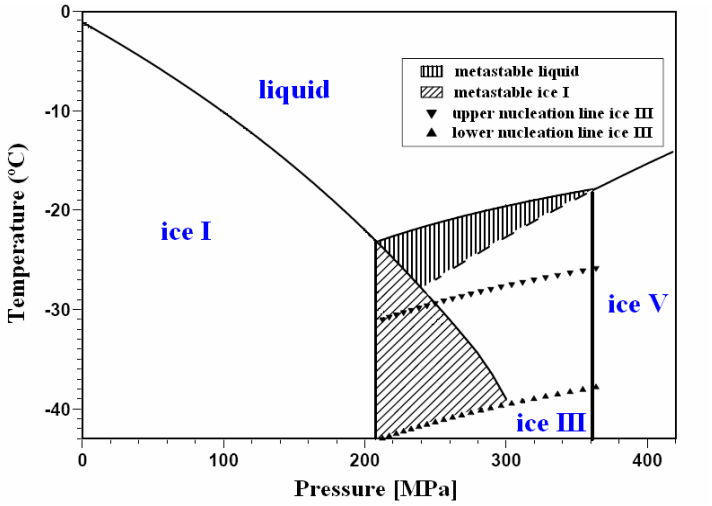

Figure 1.10 Modified phase diagram for potato, where the limits of the metastable phases can be seen (adapted from Schlüter *et al.*, 2004).

The range of ice III nucleation is defined between the upper and the lower nucleation line (Schlüter *et al.*, 2004). The area of metastable liquid indicates a region where no crystallisation was observed. Before the determination and explanation of the metastable phases (Schlüter *et al.*, 2004), it was believed that -20ºC and 210MPa (**Figure 1.8**, point 3) was the lowest temperature and highest pressure possible before pressure release for HPSF processes. However, there is the "conventional" (and most reported in the literature) HPSF process and the "enhanced" one, both shown in **Figure 1.11**, where the plateau time is minimized and consequently the textural damage to food products is diminuished. The effect of sub-zero temperature-high pressure processing on protein denaturation, enzyme activity and microorganisms inactivation has already been reported.

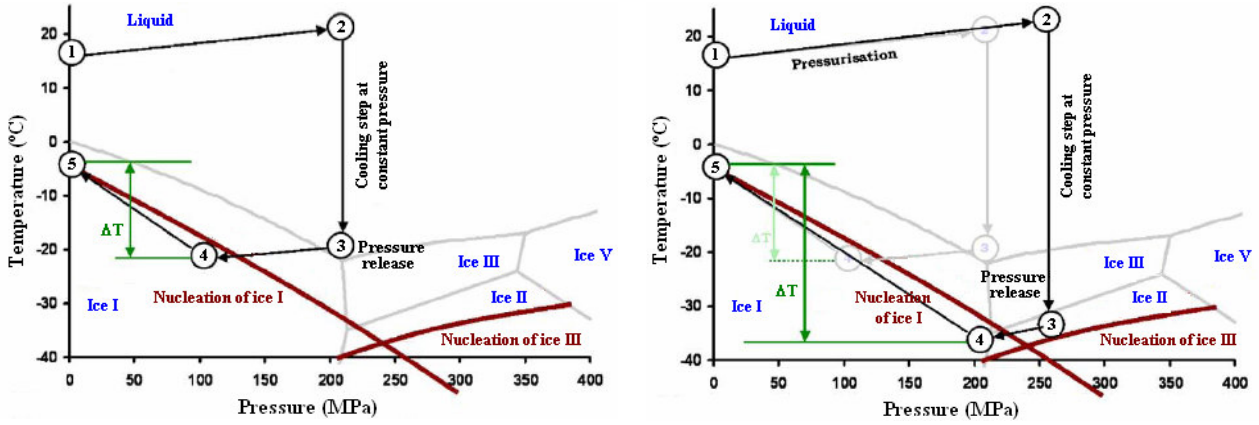

Figure 1.11 The "conventional" HPSF process (left) and the "enhanced" HPSF process (right) (adapted from Urrutia-Benet *et al.*, 2005).

When carboxypeptidase Y was treated at pressures higher than 300MPa and temperatures lower than -5ºC, it underwent an irreversible inactivation, while for pressures below 200MPa, cold inactivation process appeared to be reversible (Kinsho *et al.*, 2002). Indrawati *et al.* (1998) also provided data regarding several enzyme solutions frozen either by fast pressure release (200 to 400MPa, -22 to -10ºC), or under constant pressure (350MPa and 400MPa, both at -22ºC). α-amylase from *Bacillus subtilis* and horseradish POD were slightly and reversibly inactivated, while LOX was irreversibly inactivated (60% at 200MPa). Even after long periods of time under pressure (80-135min) only a slight, reversible inactivation of mushroom PPO (around 15% at 200MPa and -15ºC) while PME was not or only slightly inactivated. In agreement with Indrawati *et al.* (1998) results, Urrutia *et al.* (2005) found a slight PPO inactivation after freezing potatoes at 260MPa and -27ºC. Both results should be compared carefully, given that the activity examined in this work is measured directly from a potato tissue and not from extracts or enzyme solutions, where no influence of cell damage plays a role on the measured activity. However, the results found by Préstamo *et al.* (2005) may appear to be in contradiction. HPSF treatments of potato cubes (210MPa at -20ºC, 180MPa at -16ºC, 150MPa at -12ºC or 120MPa at -8ºC) were not enough to inactivate the PPO, but a considerable decrease in PPO activity at 210 MPa and -20ºC was described in comparison to control: only 30% of the PPO activity remained after HPSF at 210MPa and -20ºC. While Urrutia *et al.* (2005) analysed the PPO activity after the HPSF and storage at -20ºC for one week, Préstamo *et al.* (2005) analysed the PPO activity, after HPSF and pressure expansion, and soaking in liquid nitrogen for 15 min, followed by storage at -80ºC.

Hashizume *et al.* (1995) observed that the inactivation impact on *Saccharomyces cerevisiae* in 0.85%NaCl at 180MPa reached 3 log cycles in about 7min at -20ºC and in 20min at -10ºC. Pressurization at 190MPa and -20ºC was as effective as treatment at 320MPa and 20ºC to inactivate *S. cerevisiae*. Increasing NaCl concentration offered some protection against pressure inactivation at -20ºC while an increase in sucrose concentration had the opposite effect. Hayakawa *et al.* (1998) submitted several microorganisms suspensions (*S. cerevisiae*, *Zygosaccharomyces rouxii*, *Aspergillus oryzae*, *A. niger*, *Lactobacillus brevis*, *E. coli IFO*

3972, *Staphylococcus aureus*) to freezing at -20ºC and pressure (140-180MPa), and reported inactivation ratios of ≥5 log cycles for all, except *S. aureus* (~4 log cycles).

Further studies of enzymes and microorganisms inactivation at high pressure and sub-zero temperatures in various media and food are required to elucidate the possible specific effects of HPSF. But as far as research results allow to conclude, it does not seem that high pressure at sub-zero temperatures could be used as a substitute for thermal blanching plus atmospheric pressure freezing of vegetables with respect to enzyme inactivation, except perhaps with respect to LOX. If effective, for microorganisms it could have practical applications with respect to microbial load of frozen foods. Key quality related parameters (e.g., colour, texture, microstructure) have already been evaluated at a pilot scale level and showed to have better responses for HPSF than for atmospheric freezing (Urrutia-Benet *et al.*, 2005).

1.6.1 Potential of high pressure freezing in food applications

As a result of the small ice crystals that are formed during the pressure release at sub-zero temperatures, HPSF seemed to have advantageous effects on texture of plant based food products, compared to other freezing techniques (Fuchigami *et al.*, 1996, 1997; Koch *et al.*, 1996; Otero *et al.*, 1998, 2000). Examination of plant tissue materials carried out for fruits and vegetables indicated better texture and microstructure of the tissue after HPSF. When peach and mango were submitted to HPSF (200MPa, -20ºC), their original tissue structure was maintained to a great extent, since problems associated with thermal gradients are minimized, HPSF prevents quality losses by avoiding freeze-cracking or large ice crystals formation (Otero *et al.*, 2000). Tofu frozen by fast pressure release from 200MPa and -18ºC displayed, after thawing, less exudation and a texture near to the unfrozen tofu samples, and clearly better than that obtained for air blast frozen tofu in air at -20ºC and atmospheric pressure (Kanda *et al.*, 1992). Both texture and histological structure of frozen carrots were improved by freezing at 200, 340, 400MPa at -20ºC (Fuchigami *et al.*, 1996). Moreover, the structure of carrot slices was not altered after freezing by fast pressure release (200MPa, -20ºC or 100MPa, -10ºC) in

contrast to still air freezing at 0.1MPa and -30ºC (Fuchigami *et al.*, 1997). HPSF was also investigated for potato cubes (Koch *et al.*, 1996) or whole eggplants (Otero *et al.*, 1998). In both cases, freezing by fast pressure release enhanced quality, as compared to air-blast freezing, in terms of drip loss and texture. Pressure (240 and 280MPa) treated potato samples frozen at -20ºC maintained the texture characteristics, while the atmospheric treated samples showed a decrease in the texture, in both cases with respect to the corresponding fresh samples (Urrutia-Benet *et al.*, 2005). Even for leafy vegetables, where freezing at atmospheric pressure has a detrimental effect on texture, HPSF seemed to have advantages. Even though pressure frozen Chinese cabbage samples became flexible, thus losing the crispness of raw samples, HPSF (200-400MPa, -20ºC) proved to be more effective in improving both texture and histological structure than freezing at -30°C and atmospheric pressure (Fuchigami *et al.*, 1998).

1.7 Conclusions

The application of any new technology presents significant challenges to food technologists and food researchers. High pressure processing offers the food industry a technology that can achieve the food safety level of heat pasteurisation while meeting consumer demand for fresher-tasting minimally-processed foods. In addition, other favourable organoleptic, nutritional and rheological properties of high pressure processed foods have been demonstrated following high pressure, in comparison to heat processing. The retention of colour and aroma and the preservation of nutritive components, are enormous benefits to both the food processing industry and consumers. From a food processing/engineering perspective, key advantages of high pressure applications to food systems are the independence of size and geometry of the sample during processing, possibilities for low temperature treatment and the availability of a waste-free, environmentally-friendly technology. High pressure treatment of food within the range of 200 to 800MPa is a novel process in food technology employed to eliminate microorganisms, to selectively affect the activity of food enzymes, to improve food texture, and to change functional food properties. The application of high pressure is

especially promising to achieve preservation of minimally processed foods, as the treatment does not compromise the sensorial quality of food to the same extent as do thermal treatments with a comparable bactericidal effect. It is advantageous to achieve food preservation by mild-pressure treatment in order to minimize quality deterioration and to reduce both equipment and energy costs. Aiming the production of high-quality, firm processed fruit and vegetable products, there is a call for optimization of the conventional heat technologies as well as their combination with the new emerging technologies, like high pressure (e.g., combined thermal/high pressure treatments, high pressure freezing and thawing).

High pressure is an environmentally-friendly, industrially-tested technology that offers a natural alternative for the processing of a wide range of different food products. It is a technology with many obvious advantages. However, the range of commercially-available high pressure-processed products is relatively small at the present but there are opportunities for further development and the production of a wide range of high pressure-treated products. At the moment, the main drawbacks of high pressure treatment are the implied costs (equipment, product and process know-how) as well as the impossibility to extend the semi-continous processing for all types of food products. But as high pressure food processing develops further, and as the technology matures and producers gain experience, lower equipment and operation cost can be anticipated, so that both economics and safety of processed products can be obtained.

CHAPTER TWO *Capsicum* fruit and Pectin methylesterase

2.1 Introduction

During processing of food products, quality attributes and nutritional value are inevitably affected. Texture is one of the key sensorial parameters associated with *Capsicum* fruits. It is well known the relation between texture and pectic substances, since textural changes on fruit and vegetable tissues are accompanied by significant changes in the characteristics of these polysaccharides. Taking into account this consideration, to improve texture or reduce softening, the respective pectic degrading enzymes should be considered as the target for suppression or control. Hence, it is clear that the enzyme pectin methylesterase (PME) is of major importance in food processing and the investigation of its behaviour in response to physical factors (pressure, temperature, time) is essential in order to enhance or inhibit its activity as desired.

In this chapter, the main quality attributes of *Capsicum* fruits are discussed as well as the present technologies associated with different products obtained from them. The biochemical properties of the enzyme PME are also discussed, so as to understand its mode of action in fruit and vegetable tissues, and to recognize its relation with softening and textural changes during processing. Other closely related topics with this enzyme like assays to measure PME activity as well as the biochemistry of pectic substances, and the substrate of this cell wall degrading enzyme are also mentioned.

2.2 The fruit *Capsicum*

Capsicum fruits are historically associated with the journey of Christopher Columbus to the Americas (Heiser, 1976). The plant encountered by Columbus, whose fruit mimicked the pungency of the black pepper (*Piper nigrum* L.), was called red pepper as its pods were red. However, the plant was not the black pepper, but an unknown plant later classified as *Capsicum*. While he was given the credit for introducing this fruit in Europe and, subsequently, to Africa and Asia, it has been known since the beginning of civilization in the Western Hemisphere, and probably evolved from Bolivia or Peru (**Figure 2.1**), and has been a

part of the human diet since about 7500 B.C. (MacNeish, 1964). Heiser (1976) stated that apparently Native Americans were growing *Capsicum* plants between 5200 and 3400 BC, which places them among the oldest cultivated crops of the Americas.

Figure 2.1 *Capsicum* fruit:
- Places of origin
- Paces of crop growing.

The botanical classification of *Capsicum* members showed at least two major species, e.g., *C. annuum* and *C. frutescens* (Bailey and Bailey, 1976). The varieties that belong to the first group are classified as mild-flavoured sweet-peppers, large and less hot, or even with the lack of this characteristic property, while the other species include smaller and hotter fruits. More recently, the genus *Capsicum* was considered to have approximately 22 wild species and 5 domesticated ones, i.e., *C. annuum*, *C. baccatum*, *C. chinense*, *C. frutescens*, and *C. pubescens* (Bosland, 1996) (**Figure 2.2**).

Figure 2.2 Several varieties of *Capsicum* fruit.

Despite their peculiar differences, most of the cultivars commercially cultivated in the world belong to the species *C. annuum* (Salunkhe and Desay, 1984). Several trivial names are used interchangeably for plants from the *Capsicum* genus, like pepper, chili, chile, chilli, aji, paprika, and *Capsicum*. *Capsicum* researchers use chile, pepper, or aji, as colloquial speech terms, whilst the term *Capsicum* is reserved for taxonomic discussion. Also, trivial names like sweet chillis, bell peppers, paprika and cayenne are considered to belong to the *C. annuum*; while hot chili, jalapeños and tabasco peppers are generally considered to belong to *C. frutescens*. Bell pepper generally refers to non-pungent blocky chile types. Also most sweet peppers are bell-shaped,

and therefore the name bell pepper is very common. But sweet peppers come in a range of shapes from round to oblong, to tapered, with a smooth and shiny skin, in a wide range of colors. Additional confusion is present within species designation, because *C. annuum* is sometimes called *C. frutescens* in the scientific literature (Heiser and Smith, 1953).

2.2.1 General characteristics

The most appreciated characteristics in fruits and vegetables, and in *Capsicum* without exception, are colour, flavour, texture, shape, and absence of external defects, characteristics that are affected by chemical and biochemical changes that occur during pre- and post-harvesting conditions. After pepper was brought to Portugal and Spain, following the discovery of America, growers have selected many pepper cultivars for the properties and characteristics that made them so popular or most profitable. The result is a great number of very different cultivares showing a wide range of morphological and organoleptic characteristics. In **Table 2.1** it can be seen an illustrative composition of two varieties of peppers, in different maturity stages.

Table 2.1 Biochemical composition in two varieties of peppers (*Capsicum annuum*) (per 100g of fresh weight) (adapted from Guil-Guerrero *et al.*, 2006).

Variety		Moisture (g)	Crude protein (g)	Available carbohydrate (g)	Lipids (g)	Neutral detergent fiber (g)	Ash (g)	E (kcal)
Lamuyo	Green	94.7±1.3	0.70±0.06	1.83±0.24	0.19±0.06	1.04±0.11	1.02±0.09	11.3±0.9
	Yellow	92.8±1.2	0.96±0.02	2.63±0.51	0.50±0.04	1.09±0.11	1.34±0.10	18.2±1.6
	Red	92.5±1.0	0.81±0.02	2.78±0.32	0.60±0.06	1.22±0.10	1.42±0.14	19.0±1.1
Italian	Green	93.7±1.7	0.71±0.01	2.11±0.24	0.38±0.02	1.43±0.09	1.21±0.12	14.2±0.8
	Red	89.4±1.4	1.20±0.10	4.82±0.44	0.53±0.04	1.55±1.14	2.07±0.16	27.6±2.0

Breeding programs on *Capsicum* varieties focused on disease resistance, crop yield, nutritional value, e.g. vitamin C, and carotenoids levels (Govindarajan, 1985; Minguez-Mosquera *et al.*, 1994; Levy *et al.*, 1995; Pérez-Gálvez *et al.*, 1999; Chen *et al.*, 2003), and different pungency level. For example, *Capsicum annuum* variety Califonia was considered exceptional for human nutrition due to its high vitamin C content (348-370mg.100g fresh weight^{-1}) (Guil-Guerrero *et al.*, 2006). But geneticists and biochemists are not the only ones concerned, since food processors and technologists are also interested in developing new and quality appealing products to the consumers. Minguez-Mosquera *et al.* (1994) verified that *Capsicum annuum* varieties *Bola* (sweet) and *Agridulce* (spicy, highly coloured) presented certain drawbacks as crops. Therefore, breeding trials were carried out between 1990 and 1992 to obtain varieties from *Agridulce* that were nonpungent, and had a high colouring capacity, namely Jaranda and Jariza. Besides, both varieties after dehydration demonstrated to yield a dry fruit with carotenoid concentration similar to that the initial one (Minguez-Mosquera *et al.*, 2000).

2.2.1.1 Colour

Colour changes occur during ripening, transport, and storage of fresh *Capsicum* fruits as well as in several pepper-based proccessed products. Most *Capsicum* fruits are dark green when immature, and red if allowed to ripen (**Figure 2.3**).

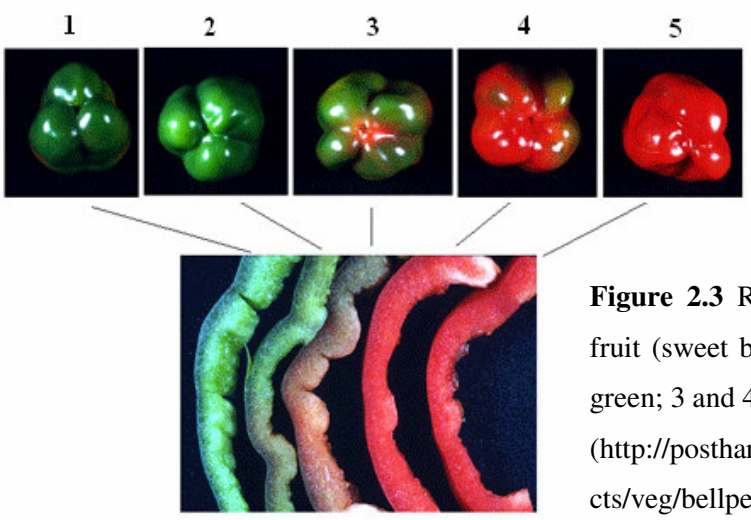

Figure 2.3 Ripening stages colours of *Capsicum* fruit (sweet bell pepper): 1 - dark green; 2 - light green; 3 and 4 – turning red; and 5 – red. (http://postharvest.ucdavis.edu/Produce/ProduceFacts/veg/bellpepper_graphics.shtml).

Green peppers are often harvested before they are ripen and are usually less expensive because they can withstand transport and tend to last long. But when allowed to ripen, peppers can turn from green to yellow, orange, red, purple or even black (Simmone *et al.*, 1997). New cultivars offer both mature and immature peppers in red, yellow, orange, purple, brown or even white colours (**Figure 2.4**). During the ripening process of *Capsicum* fruits, changes in colour result from the degradation of chlorophyll and *de novo* synthesis of carotenoid pigments (Minguez-Mosquera *et al.*, 1994). Some carotenoids are exclusive to this genus, like capsanthin and capsorubin (**Figure 2.5**), and their synthesyis is influenced by temperature and illumination to which the fruit is exposed (Kanner *et al.*, 1977; Minguez-Mosquera and Hornero-Mendez, 1994).

Figure 2.4 Several colours of *Capsicum* fruit (bell pepper).

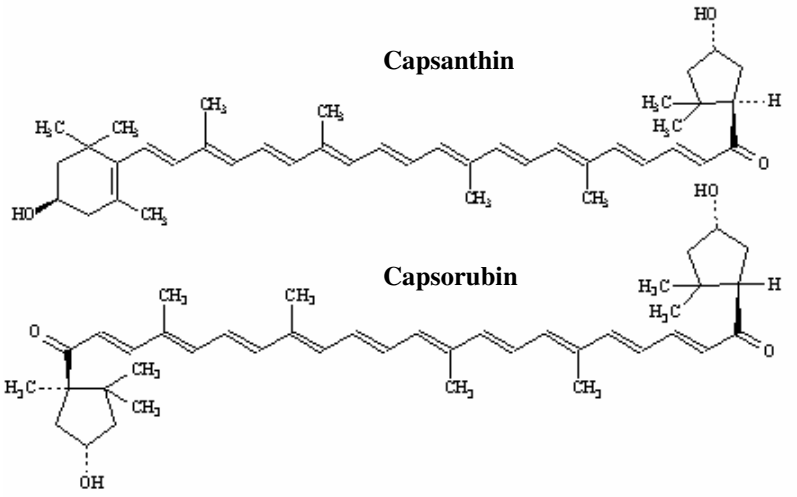

Figure 2.5 Chemical structures of capsanthin and capsorubin carotenoids, only found in *Capsicum* fruits.

The intense red colour of *C. annuum* fruits has been used since ancient times as a source of pigments to add or change the color of foodstuffs, making them more attractive and acceptable for the consumer. Pepper used as food colorant has traditionally been in the form of paprika (ground powder), although today oleoresins are widely used. The mechanism of chlorophyll disappearance is complex and still not fully understood, due to its rapidity and colourless catabolites formation (Moser and Matile, 1997). But it seems that the chlorophyll degradation

pathway consists of three main steps involving different enzymes, namely chlorophyllase, Mg-dechelatase and pheophorbide *a* oxygenase (Vicentini *et al.*, 1995). For example, in varieties containing a chlorophyll-retaining gene, the total chlorophyll content suddenly increases at the onset of ripening, principally due to a sharp increase of chlorophyll *b*. From this moment onward, the content of chlorophyll, and chlorophyll *a* in particular, decreases, but not to an extent that it completely disappears (Ferrer and Costa, 1991; Hornero-Mendez and Minguez-Mosquera, 2002), giving the fruit a characteristic chocolate colour.

In the ripen stage of this type of varieties where it seems that there is a coexistence of chlorophyll and a high level of chlorophyllase activity, which suggests that the chlorophyll catabolism may be affected in several ways, e.g., by the presence of other chlorophyll-degrading enzymes, by restricted chlorophyll and chlorophyllase contact or even by delayed degeneration of chloroplast structures (Hornero-Mendez and Minguez-Mosquera, 2002). Several studies have focused on the improvement of retention of β-carotene, α-carotene and β-cryptoxanthin, well known antioxidants, during processing and storage (e.g., Minguez-Mosquera and Hornero-Mendez, 1994; Daood *et al.*, 1996; Howard and Hernandez-Brenes, 1998; Markus *et al.*, 1999). In general, the compounds responsible for the attractive colours in vegetables are highly heat-sensitive and therefore lost during the heat treatments. Besides the conversion of chlorophylls into pheophytins caused by heating, which leads to a change from bright green to olive-brown (Ihl *et al.*, 1998), there is an appreciable loss of carotenoids as well as a large increase in content of browning compounds (Ramakrishnan and Francis, 1973).

2.2.1.2 Pungency

Pungency is another important quality attribute of the so-called hot peppers (fruits of several *Capsicum* species) that is very appreciated in many foods. It depends on the production of a certain type of alkaloid compounds, the capsaicinoids, only found in this genus (Hoffman *et al.*, 1983; Govindarajan *et al.*, 1987). Most of the capsaicinoids are pungent, but a group of non-pungent capsaicinoid-like substances, named capsinoids, has also been reported (Kobata

et al., 1998). The capsaicinoids are produced in glands on the placenta, the white "ribs" that run down the middle and along the sides of the fruit (Fujikawe *et al.*, 1982). And because the seeds are in close contact with the ribs, they are also often hot.

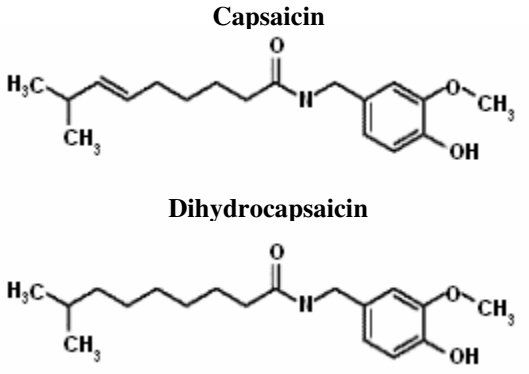

Figure 2.6 Chemical structures of capsaicin and dihydrocapsaicin, the most abundant capsaicinoids found in *Capsicum* fruits.

Capsaicin or (E)-N-(4-hydroxy-3-methoxybenzyl)-8-methylnon-6-enamide), the most ubiquitous capsaicinoid found in *Capsicum* fruits, is together with dihydrocapsaicin (**Figure 2.6**), the second most abundant, and responsible for 80-90% of the *Capsicum* fruits pungency compounds (Iwai *et al.*, 1979; Govindarajan, 1986), while in the remaining fraction, minor capsaicinoids are considered.

Capsaicin is an incredibly powerful and stable alkaloid seemingly unaffected by cold or heat, which retains its original potency despite time, cooking, or freezing. The pungency, caused by the direct effect of capsaicin and its analogues on the pain receptors in the mouth and throat, is often expressed based on the Scoville Heat Units (SHU) (**Figure 2.7**), according to its concentration in capsaicin (Scoville, 1912).

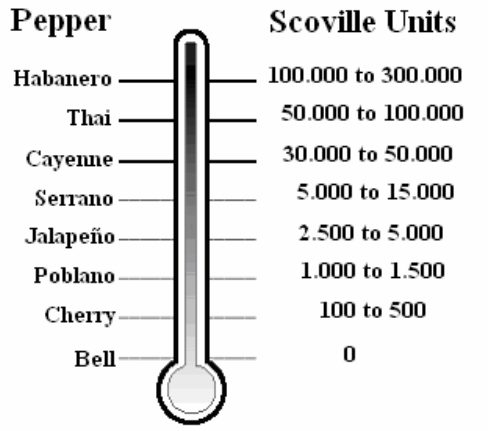

Figure 2.7 The Scoville scale, where several varieties of *Capsicum* are classified according to the Scoville Heat Units (SHU).

Pure capsaicin rates over 16.000.000 SHU, while Habañero is among the most pungent varieties with 200.000 SHU (Bosland, 1992). The amount of total capsaicinoids varies greatly but the proportions of capsaicin and dihydrocapsaicin range from 77 to 90% in the hot fruits of the species *C. annuum* and from 89 to 98% in those from *C. frutescens* (Govindarajan *et al.*, 1987).

The content of capsaicinoids is affected by the genetic make-up of the cultivar, weather conditions, growing conditions, and fruit ripening stage (e.g., Contreras-Padilla and Yahia, 1998; Estrada *et al.*, 2000). As previously mentioned, plant breeders can selectively develop cultivars with varying degrees of pungency. Also, growers can somewhat control pungency by the amount of stress to which they subject their plants. According to Lindsay and Bosland (1995), the pungency level is increased with any stress to which pepper plant might be submitted, such as environmental stresses. Capsaicin and dihydrocapsaicin have been shown to decrease in fruits after cellular disruption and such decrease is apparently due to temperature-dependent oxidation (Kirschbaum-Titze *et al.*, 2002). Probably, pepper cells have some enzyme or enzymes that are able to oxidize capsaicinoids. It seems that there is some evidence pointing to peroxidase (EC.1.11.1.7) as the enzyme directly related to capsaicinoid metabolism by oxidation (Bernal *et al.*, 1993a,b; Díaz *et al.*, 2004), as it will further mentioned in section 2.4.2.

2.2.1.3 Flavour

Luning *et al.* (1994b) studied the flavour attributes of two different varieties of *Capsicum* fruits, at three maturation stages, by a sensorial panel. Green maturation stages were characterized by attributes like grassy, cucumber and green bell pepper aroma whereas the ripe stages had a distinct red bell pepper aroma. Likewise, Chitwood *et al.* (1983) observed that "green" sensory attributes, such as grassy, fresh green bean and garbanzo green bean aroma, discriminated different cultivars. During ripening of bell peppers, the volatiles associated with the "green" aroma notes disappear (Luning *et al.*, 1994c).

2.2.1.4 Texture

Colour has a great impact on appearance consideration, but quality loss is also observed with changes in texture, another important quality criterion for many fruit and vegetables products.

While genetic background is the major contributor to the texture of a plant food, other factors, such as morphology, cell wall-middle lamella structure, cell turgor, water content and biochemical components, all affect texture (Harker *et al.*, 1997). Texture changes of fruits and vegetables take place during ripening, storage and processing, as a consequence of changes in cell-wall polysaccharides, mainly pectin. Pectin can be degraded by enzymatic and non-enzymatic ways. The non-enzymatic way, β-elimination, is a chemical reaction that takes places at elevated temperatures and alkaline conditions (Keijbets and Pilnik, 1974), while the enzymatic reactions are catalysed by a combined action of pectic enzymes, mainly PME and polygalacturonase (PG), a subject that will be further discussed in section 2.5 of this chapter. The final degradation products are short and de-esterified pectin chains that cause an increase of pectin solubility, loosening of cell wall and softening of tissues (Van Buren, 1979).

Texture of *Capsicum* fruits, and in particular their crispiness, is an important quality attribute to consumers. The firmness (hardness) of processed peppers has been evaluated by different methods, such as the puncture force, for cooked sweet peppers, rehydrated dried bell peppers and heat treated Jalapeño pepper (Chang *et al.*, 1995; Domínguez *et al.*, 2001; Heredia-Leon *et al.*, 2004; Villarreal-Alba *et al.*, 2004), the extrusion force to evaluate frozen jalapeño (Quintero-Ramos *et al.*, 1998), the compression to shear a sample of Jalapeño pepper rings (Gu *et al.*, 1999), and cutting with a single blade, to evaluate effects of packaging and storage of green bell pepper (Senesi *et al.*, 2000).

The possibility of using milder process conditions for preservation of tissue integrity and the improvement of quality has also been explored (Heredia-Leon *et al.*, 2004). Pre-heating *Capsicum* tissues between 50ºC and 60ºC before canning can reduce canning-induced softening in several plant tissues (Wu and Chan, 1990; Lee and Howard, 1999; Domínguez *et al.*, 2001; Villarreal-Alba *et al.*, 2004). This firming effect may be attributed to the action of heat-activated PME and/or to increased Ca^{2+} diffusion into the tissue. High pressure treatments alone or combined with temperature when applied to several fruits and vegetables seem to have a similar effect when compared to thermal treatments (Basak and Ramaswamy, 1998).

2.2.1.5 Nutritional and safety parameters

Peppers are an important source of nutrients in human diet, and an excellent source of vitamins (A, C, and E) as well as neutral and acidic phenolic compounds, known as important antioxidants in a variety of plant defence responses. Levels of these compounds can vary due to genotype and maturity and are influenced by growing conditions and losses after processing (Mejia *et al.*, 1988; Howard *et al.*, 1994, 2000; Lee *et al.*, 1995; Daood *et al.*, 1996; Simmone *et al.*, 1997; Osuna-Garcia *et al.*, 1998; Howard *et al.*, 2000).

As far as vitamin C is concerned, there have been some contradictory results regarding its content during ripening. In general, vitamin C content tends to increase from green to red maturation stage (Rhaman *et al.*, 1978; Howard *et al.*, 1994; Osuna-Garcia *et al.*, 1998; Guil-Gerrero *et al.*, 2006). Luning (1995) reported that the content in vitamin C increases during the first developmental stages (±80-180mg/100g fresh weight) of *Capsicum* fruit, and slowly decreases in the red maturation stage (±200mg/100g fresh weight), while Yahia *et al.* (2001) observed a dramatic loss of vitamin C in bell pepper fruit much earlier than the full colour intensity stage, attributed to a sudden increase in the activity of a vitamin C related enzyme, the ascorbic acid oxidase. Also, the use of different methodology, different cultivars and subjective determination of fruit developmental stages could support the different results obtained for vitamin C content in bell peppers. Vitamin C content showed a wide variation among the varieties and stages of fruit development (Khadi *et al.*, 1987; Guil-Gerrero *et al.*, 2006). Simone *et al.* (1997) also concluded that it was impractical to group cultivars based on their ascorbic acid content because the level seemed to change with colour stages and cultivars.

During ripening, tocopherol, often referred as vitamin E, is present in the seeds as γ-tocopherol as well in the pericarp as α-tocopherol and they both increase during ripening (Biacs *et al.*, 1992; Osuna-Garcia *et al.*, 1998). Xanthophylls, also found in *Capsicum* fruits, have been shown to be effective free radical scavengers (Matsufuji *et al.*, 1998) and may be important for the prevention of age related macular degeneration and cataracts (Seddon *et al.*, 1994). Their

composition and concentration can vary due to differences in genetics and degree of ripening (Markus *et al.*, 1999).

Sweetness and sourness of *Capsicum* fruits seemed to be related with both sugar and organic acids contents. According to Luning *et al.* (1994a), sweetness appeared to be typical of ripen stages and closely associated with glucose, fructose, total sugar, and dry matter. However, differences in sweetness in the same stage of maturation could be also related with the composition of organic acids. Turning and ripen stages seemed to be associated with a sourness taste and correlated with the amount of organic acids, especially citric acid and to a lesser extent ascorbic acid, which increase markedly during ripening (Luning *et al.*, 1994a).

According to González-Aguilar (2002), the most common decay microorganisms found in *Capsicum* fruits are *Botrytis*, *Alternaria*, and soft rots of fungal and bacterial origin. Field sanitation and prevention of wounds on the fruit help to reduce its incidence. Still, hot water dips at 53-55°C (4min), can effectively control *Botrytis* rot without causing fruit injury. And since the presence of *Alternaria* black rot, especially on the stem end, is a symptom of chilling injury, the best way to control *Alternaria* is to store the *Capsicum* fruits at 7°C. Bacterial soft rot, caused by several bacteria which attack damaged tissue, can occur on washed or hydrocooled peppers, where water sanitation was inadequate. *Capsicum* fruits are also affected by many of the diseases caused by virus, insects, and nematode pests that affect tomato.

2.3 Technology associated with *Capsicum* fruits

Capsicum fruits are considered to be a perishable product, like any other fruit and vegetable, and therefore not suitable for long term storage (Senesi *et al.*, 2000). These fruits should be received at 7ºC and stored between 7-10ºC, temperatures at which chilling injury and ripening are minimal (Barth *et al.*, 2002). Operations involved in minimal processing (e.g., washing, coring, slicing or cutting) of horticultural products disrupt the plant tissues and the products become more perishable than the intact ones (Watada *et al.*, 1996). Consequently, fresh-cut products (salad or salsa) require very special attention because of the magnitude of enzymatic

and respiratory factors as well as microbiological concerns that will have an impact on safety. For example, fresh-cut bell peppers should be stored between 0-5ºC in order to maintain visual quality and compositional characteristics (Barth *et al.*, 2002).

Due to their distinct colours, pungency and flavour, the different varieties of *Capsicum* fruit have a wide range of food application (Govindarajan, 1986; Govindarajan *et al.*, 1987). Besides fresh-cut products, *Capsicum* fruits can be consumed as processed product (canned, frozen, pickled, fermented, and dehydrated), as immature (green) or mature fruit (red, yellow, and white), and as a spice (ground powder). As a consequence of processing, changes in several quality parameters can occur. Changes regarding pungency (Govindarajan, 1985), colour pigments (Ramakrishnan and Francis, 1973; Minguez-Mosquera and Hornero-Mendez, 1994; Minguez-Mosquera *et al.*, 1994, 2000; Ihl *et al.*, 1998; Sgroppo *et al.*, 2001), flavour (Luning *et al.*, 1995a) and texture (Chang *et al.*, 1995; Gu *et al.*, 1999; Senesi *et al.*, 2000; Domínguez *et al.*, 2001; Villarreal-Alba *et al.*, 2004) have already been reported.

Drying, one of the oldest and still widely practised methods of preservation, is used to conserve perishable fruits, to reduce storage volume and weight and to decrease transportation costs (Govindarajan, 1985). Dried peppers can be stored in moisture- and vapor-proof packaging in good conditions for several months. Together with drying, grinding or solvent extraction might be associated in order to produce either paprika or oleoresins, both considered as natural carotenoids concentrates and generally used as a food colorant. Changes in colour of paprika are greatly dependent on the *Capsicum* fruit used (Minguez-Mosquera and Hornero-Mendez, 1994), whereas in oleoresins, the carotenoids content depends on the conditions used (Jarén-Galán and Minguez-Mosquera, 1999). Besides colour, quantitative and qualitative composition of volatile compounds of bell peppers can also change dramatically upon drying (Luning *et al.*, 1995a). Pepper pastes or purees are such products capable of retain colour and flavour in a semi-solid form, with similar characteristics of the fresh ones and are very convenient to use. Red pepper paste is one of the major semi-solid sauces in Korea (*kochujang*) and most appreciated in the Mexican food, produced from fermentation of mixed ingredients.

The increase in the consumption of processed pepper demands high quality parameters, with texture as one of the most important attributes. Low temperature blanching represents an important technology to achieve this. For example, pre-blanching of peppers at a moderate temperature for a suitable period of time and then blanching in boiling water will result in higher firmness, compared to peppers directly blanched (Wu and Chang, 1990). Blanched diced peppers at 65ºC for 49min showed a 64% increase in the puncture force over the control, after being dried and rehydrated (Domínguez *et al.*, 2001). Also, by combining extended blanching with calcium addition, it is possible to modify the texture of *Capsicum annuum* fruits in a controlled way (Saldana and Meyer, 1981; Gu *et al.*, 1999; Lee and Howard, 1999; Domínguez *et al.*, 2001). Calcium firms plant tissues by cross-linking with polysaccharide molecules. And by increasing the cohesive properties of the cell wall middle lamella complex may prevent the leaching of phytochemicals out of pepper fruit during pasteurisation and storage, resulting in greater antioxidant activity retention (Lee and Howard, 1999).

Peppers are also frequently frozen as cubes or small sticks, with or without a previous blanching treatment, contrary to blanching requirement of most low-acid vegetables. Matthews and Hall (1978) concluded that unblanched peppers were superior in flavour, texture, and appearance when compared to the blanched (100ºC, 2.5min) frozen peppers. However, when halved frozen peppers were previously blanched in $CaCl_2$ solutions at moderate temperatures for a period of time, with an holding time before blanching again at higher temperatures, followed by freezing, the optimum conditions for texture quality were found within the range of 63-66ºC for 11-14min, in 0.17-0.22M $CaCl_2$ followed by an holding time range of 56-66min (Márquez-Meléndez *et al.*, 2001). Nevertheless, freeze/thaw cycle operations can result in severe changes in the food product, and peppers are no exception, such weakening of the cell wall and some degree of cell separation due to destruction of the cytoplasmatic membrane structure, and loss of turgor. As a consequence, these changes will produce a severe effect on the texture of frozen products (Préstamo *et al.*, 1998). Frozen peppers can also be used in cooked dishes where colour and flavour are of more importance than texture, and those quality attributes can also be improved by adequate blanching as a

result of removal of gases. The demand of the fast food industry for frozen red pepper cubes or slices considerably increased in recent years.

Capsicum fruits can still be pickled and given the same treatment as cucumber salt stock (Salunkhe and Desay, 1984). Commercially mixed vegetable pickles are usually made from vegetables temporarily preserved by fermentation and stored in brines of 7-15% of salt content. The low temperature used in pickling helps to preserve the texture of the peppers.

There is also the possibility to make pepper juice since it is considered an excellent addition to any vegetable juice due to its high level of vitamin C. However, some consumers might find the flavour of green peppers a little bitter for the taste when used in juicer recipes, and take yellow and red peppers instead, since they are sweeter and melt into the other fruit flavours to give a subtle but specific taste. Noteworthy is the medicinal application, dating back to Mayas, of *Capsicum* fruits related with capsaicinoids. The pharmaceutical industry uses capsaicin as a counter-irritant balm for external application, a relieve pain used in post-operative pain for mastectomy patients and for amputees suffering from phantom limb pain (Carmichael, 1991).

2.4 Enzymes in the fruit *Capsicum*

2.4.1 Cell wall carbohydrate degrading enzymes

The softening that occurs in any fruit and vegetable tissue is primarily due to a change in cell-wall carbohydrate metabolism, resulting in a net decrease in certain structural components (Labavitch, 1981; Bartley and Knee, 1982). The changes in cell-wall carbohydrate composition result from the action of hydrolytic enzymes produced in the plant tissue, namely PME and PG, both considered to be implicated, due to an increase in their activities with ripening and with the changes in the cell wall pectin content (Fisher and Bennett, 1991).

2.4.1.1 Pectin methylesterase and polygalacturonase

Contradictory information has been published regarding PME and PG with respect to their activity during the *Capsicum* fruit development. According to Fisher and Bennet (1991), the activity of these two enzymes often increase as ripening continues. However, during the ripening of *Capsicum* fruits, it seemed to be an overall decrease in PME activity (Jen and Robinson, 1984; Sethu *et al.*, 1996; Prabha *et al.*, 1998).

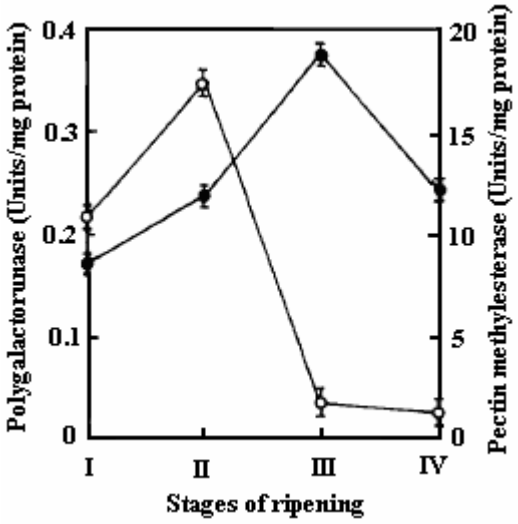

Figure 2.8 Activity of PME (o) and PG (•) during different stages of ripening (I, dark green; II, light green; III, turning; and IV, red) of *Capsicum annuum* (adapted from Sethu *et al.*, 1996).

As it can be seen in **Figure 2.8**, in the early stages of ripening (stages I and II), there was an initial increase up to a maximal of PME activity, followed by a decrease (Jen and Robinson, 1984; Sethu *et al.*, 1996). In contrast, PG activity increased to a maximal level at turning stage (III), however, pepper PG activity in sweet bell-type cultivars was found to be lower by a factor of 116–164, than that of the tomato fruit enzyme (Jen and Robinson, 1984). According to Sethu *et al.* (1996), the texture of the pepper fruits declined concomitantly with the increase in PG activity, but did not correlate with PME activity, since this enzyme decreased as the softening increased. The overall texture loss of the fruits at room temperature could be prevented if they were stored at 8ºC.

Other studies, e.g., that of Gross *et al.* (1986) did not detect PG activity in any stage of fruit development of hot pepper. Unlike the fruits of most bell and hot peppers that lose their texture gradually during ripening, ripe fruits of wild peppers are characterized by rapid

deterioration of the tissue and by their deciduous nature (Bosland and Votava, 2000). It seems that firm-fruited domesticated pepper varieties lack expression of endo-PG (Rao and Paran, 2003). The rapid texture deterioration (termed soft flesh) and the easy separation of the pod from the calyx were eliminated from most of the cultivated germplasm during domestication, because mature fruits can fall on the ground and they have a very limited shelf-life.

Since the experimental work in this thesis is devoted to PME from *Capsicum annuum* fruit, characteristics and function of this enzyme will be further described in detail in section 2.5.

2.4.2 Other enzymes

In addition to PME and PG, a variety of glycanases and glycosidases have been assigned roles in fruit cell-wall metabolism (Fisher and Bennett, 1991). The behaviour of glycanases, namely cellulase, xylanase, mannanase, glucanase and galactanase, and glycosidases, like α-D-mannosidase and β-D-galactosidase, were monitored during the ripening of *Capsicum* fruits (Sethu and Prabha, 1997). The activities of cellulase and xylanase increased up to a maximum in the early stages of ripening, followed by a decrease as ripening proceeded; while mannanase, glucanase and galactanase were present constantly in a significant amount at all stages. As glycosidases are concerned, α-D-mannosidase and β-D-galactosidase increased in the early stages, and thereafter remained constant. β-hexoaminidase, another glycosidase that may have an important function in fruit ripening through deglycosylation and generation of free *N*-glycans (Priem *et al.*, 1993), was found in *Capsicum* fruits. This enzyme activity seemed to have a first increase during fruit development followed by a further increase during ripening (Jagadeesh and Prabha, 2002).

Two major peroxidase isoenzyme groups can be distinguished in *Capsicum* by their individual isoelectric point (pI). The first major group is composed of peroxidase isoenzymes of acidic pI, and the second group corresponds to peroxidase isoenzymes of basic isoelectric point (Bernal *et al.*, 1993c). According to Díaz *et al.* (2004), within the group of basic peroxidase isoenzymes, two subgroups may also be defined based in their pI (low/high). Previosly, the

localization of *Capsicum* POD was mainly in the placenta and the outermost epidermal cell layers (Bernal *et al.*, 1993a, 1994). Recent studies, have located peroxidase isoenzymes of acidic pI bound to cell walls and in the cell-wall free space (Díaz *et al.*, 2004). The first subgroup of basic peroxidase isoenzymes (low pI), is exclusively located in cell walls, while the second subgroup (high pI) is located both in the cell walls and the vacuoles.

PODs have also been considered to play an important role in the metabolism of alkaloids. Capsaicinoids in hot peppers, like other plant alkaloids, are accumulated in the fruit during the early stages of maturation and later undergo a rapid turnover and degradation (Iway *et al.*, 1979; Pomar *et al.*, 1997). Through in *vitro* studies, Bernal *et al.* (1993b,c) suggested that POD is involved on the degradation of capsaicinoids and on the oxidation of capsaicin and dihydrocapsaicin. This co-localization of peroxidase and capsaicin in vacuoles and free space of the placenta in over-ripened fruits is in agreement with the possible role of POD in capsaicinoid turnover in pepper fruits. Later, Contreras-Padilla and Yahia (1998) demonstrated that there is an inverse relationship between the evolution of capsaicinoids and POD activity during development stages, maturation and senescence of two hot varieties of *Capsicum* fruits. A basic POD of high pI appeared to be directly related to capsaicinoid metabolism since capsaicin, dihydrocapsaicin and their phenolic precursors were easily oxidized by this enzyme (Bernal *et al.*, 1993b,c, 1995). Pomar *et al.* (1997) demonstrated that capsaicin oxidation was also possible by an acidic POD found in *Capsicum* fruits.

Pinski *et al.* (1971) determined LOX activity in several fruit and vegetable tissues and found that in red bell peppers it was very low as compared to other vegetables, such as eggplant, which also belong to the Solanaceae family. Later, some evidences for the presence of LOX in red pepper seeds were given by Daoods and Biacs (1986). Minguez-Mosquera *et al.* (1993) reported that LOX activity decreased during ripening of some *Capsicum annuum* varieties, but suggested that seasonal influences levelled down this phenomenon.

The relation between some lipid derived volatile compounds in bell peppers, formed upon tissue disruption, and LOX activity was suggested by Wu and Liou (1986). And more recently, Luning *et al.* (1994a,c) suggested that the composition and odour characteristics of

C_6 aldehydes and alcohols, may be partially responsible for distinct aroma differences between green and red peppers, and that their pattern during ripening, might be related with LOX enzymatic reaction (Luning *et al.*, 1995b). Besides differences in flavour that might result from the action of LOX, this enzyme seems to be involved in colour changes during ripening. *In vitro* studies revealed that the products formed during the enzymatic reaction catalyzed by pepper LOX have a strong destructive action on the carotenoids (Jarén-Galán and Mínguez-Mosquera, 1999).

2.5 Pectin methylesterase (PME, E.C. 3.1.1.11)

As previously mentioned, PME catalyses the de-esterification of a polygalacturonic polymer, leading to the formation of polygalacturonic acid, which can cross-link with Ca^{2+} ions to form a precipitate of calcium pectate (Walkinshaw and Arnot, 1981; Powell *et al.*, 1982; Alonso *et al.*, 1995). As a consequence, the action of PME can cause cloud loss of fruit and vegetable juices during processing and storage (Laratta *et al.*, 1995a; Rombouts *et al.*, 1992). On the other hand, PME activity can also contribute to (1) the enhancement of texture of fruit and vegetable based products (Alonso *et al.*, 1995; Fuchigami *et al.*, 1995); (2) the effective increase of the extracting yield of juices by conventional methods (3) and the promotion of water removal from tissues, e.g. during drying (Manabe, 1982). Therefore to make use of the advantages that PME action can bring to food processing and to avoid its detrimental effects, knowledge about the enzyme and its substrate (pectin) is required.

2.5.1 Physiological role *in vivo*

PME has been found in all plant tissues and in some plant cell wall-degrading microorganisms or insects (Rexová-Benková and Markovič, 1976) and has been implicated in a number of processes including cell growth (Moustacas *et al.*, 1991), fruit ripening (Gaffe *et al.*, 1994; Tieman and Handa, 1994; Steele *et al.*, 1997), abscission and senescence (Liners and van

Cutsem, 1992), pathogenesis (Collmer and Keen, 1986), cambial cell differentiation (Guglielmino *et al.*, 1997) and seed germination (Ren and Kermode, 2000). A close correlation between PME activity and the level of methanol in fruit tissues, from both wild-type tomato and a PME antisense mutant was observed, indicating that PME is on the primary biosynthetic pathway for methanol production in tomato juice (Frenkel *et al.*, 1998). Methanol oxidation to CO_2 could result in the incorporation of methanol carbon into metabolites via Calvin-Benson cycle, and in this way PMEs action could play a role, albeit indirect, in the photosynthetic metabolism on the plant (Micheli, 2001).

2.5.2 Localization in the plant tissue

Goldberg *et al.* (1992) found that PMEs are mainly present in the middle lamella and in the cell junctions, which are parts of the cell very rich in acidic pectins, while esterified pectins seemed to be distributed throughout the cell walls (Goldberg *et al.*, 1986). The different localization of PMEs and their substrate might explain why plant cells can contain high levels of esterified pectins, together with enough PME that could easily de-esterify them completely. In young cell walls, active PMEs coexist with highly methylated pectins. Most PMEs are tightly bound to cell walls, and this probably unable them to de-esterify the methylated pectins, unless a favorable ionic environment permits them to move to other parts of the cell wall. In mung bean, where several PME isoforms were detected (Bordenave and Goldberg, 1993), the fixation strength varies greatly from one isoform to another, and therefore, weakly bound isoforms might be the most active *in vivo*. Hence, plant tissue might regulate PME activity through control of ionic concentrations inside the cell wall (Bordenave, 1996).

2.5.3 Structure of pectin methylesterase

Several primary structures of plant PMEs have already been published in detail (Jenkins *et al.*, 2001; Johansson *et al.*, 2002; D'Avino *et al.*, 2003). According to Johansson *et al.* (2002),

carrot, potato and tomato leaf PMEs consist of 319 amino-acid residues, while for tomato fruit PME, D'Avino *et al.* (2003) obtained 317 residues and Markovic and Jörvall (1986) showed the protein chain to be 305 residues long. The amino-acid sequence differences might be attributed to varietal differences or several isozymes, as well as misinterpretations of peptide overlaps in the direct protein analysis and difficulties in identification of peptides used in protein sequencing (Ray *et al.*, 1988). However, for these different PMEs, the sequence similarity is high and the residues at the active site are conserved, with two aspartic acids, an arginine, two glutamines and most of the aromatic residues lining in the active site (**Figure 2.9**). The amino-acid residue Arg225 is hydrogen bonded with Asp157, which is suitably positioned for nucleophilic attack on the carbon, while Asp136 would act as an acid/base during catalysis. The glutamine residues, Gln113 and Gln135, could form an anion hole for stabilization of the negatively charged tetrahedral intermediate.

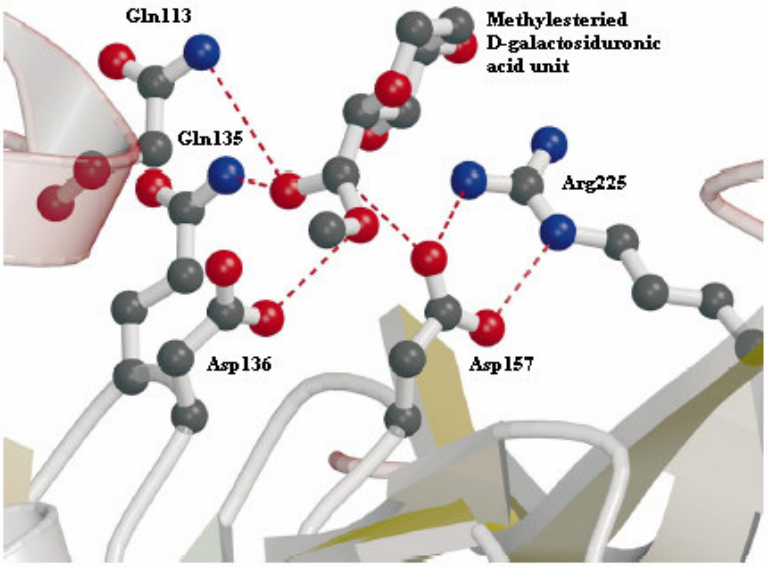

Figure 2.9 The active site of carrot PME, together with the substrate, a methyl-esterified D-galacturonic acid unit (Johansson *et al.*, 2002).

Another similarity between PMEs from different sources is the secondary structure as reported by Jenkins *et al.* (2001), Johansson *et al.* (2002) and D'Avino *et al.* (2003). PME belongs to the family of the so-called parallel β-helix proteins. The β-prism organization of carrot PME has been described as a three parallel β-sheets and with eight regular β-helix turns, with

strands in all three β-sheets (**Figure 2.10**). The relation between the secondary structure of an enzyme and its thermal and pressure stability will be discussed further in Chapter 3.

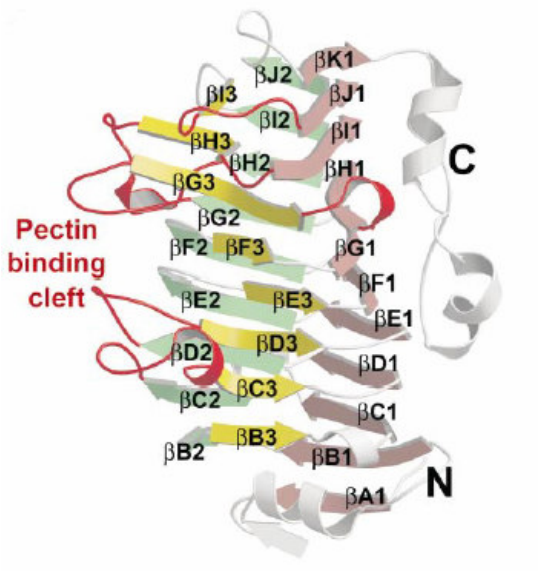

Figure 2.10 The structure of carrot PME, with a β-helix structure consisting of three parallel β-sheets organised in a prism like fashion. The sheets PB1, PB2, PB3 are colored brown, green and yellow, respectively. Strands are numbered from βA1 to βK1 in sheet PB1, βB2 to βJ2 in sheet PB2, and βB3 to βI3 in sheet PB3 (Johansson *et al.*, 2002).

2.5.4 Pectin methylesterase properties

2.5.4.1 Biochemical properties

The existence of multiple PME isoforms has been reported in tomato (Pressey and Avants, 1972), orange (Versteeg *et al.*, 1978), banana (Brady, 1976; Ly-Nguyen *et al.*, 2002a), lemon (Evans and MacHale, 1978), apple (Castaldo *et al.*, 1989), kiwi (Giovane *et al.*, 1990), grapefruit (Seymour *et al.*, 1991b; Guiavarc'h *et al.* 2005), papaya (Lourenco and Catutani, 1984; Lim and Chung, 1993), flax and mung bean (Gaffé *et al.*, 1992; Bordenave and Goldberg, 1993), green bean (Laats *et al.*, 1997), cherry (Alonso *et al.*, 1996), and persimmon (Alonso *et al.*, 1997a). Usually, these isoforms differ in their isoelectric point (pI) and/or their molar mass (MM) and even substrate specificity (Bordenave, 1996). Their relative proportions may vary greatly, according to the development stage as well as the source organ considered. For example, in mung bean (*Vigna radiata*), the relative amounts of the four major PME isoforms vary along the hypocotyl (Bordenave and Goldberg, 1993). A certain PME isoform can be specific of one organ and/or expressed in a precise moment of the development stage. For instance, one of the tomato isoforms is specifically expressed in the mature fruit

(Harriman *et al.*, 1991). Different isoforms can still have different action patterns on polygalacturonic polymer and the action pattern can also be influenced by the extent of methyl esterification of the substrate and pH (Catoire *et al.*, 1998; Denès *et al.*, 2000b). The biochemical properties of some purified plant PMEs are summarized in **Table 2.2**. The MM of plant PMEs range from 21 to 57kDa and most of them have their pI between 7 and 11, although some acidic PMEs have already been reported (Lin *et al.*, 1989; Bordenave and Goldberg, 1993; Alonso *et al.*, 1996).

Table 2.2 Biochemical characteristics of purified or partially purified plant PMEs.

Source	MM (kDa)	pI	Optimal pH	Reference
Apple	55	-	6.5-7.5	Castaldo *et al.* (1989)
	28	-	-	
Apple	36	>9	7.5	Denès *et al.* (2000a)
	34.5	>9		
Banana	30	8.8-8.9	6.0-7.0	Brady (1976)
	30	9.3-9.4	6.0-7.0	
Banana	38	8.9	6.0-8.0	Ly-Nguyen *et al.* (2002a)
	45	8.2		
		7.0		
		5.8		
		4.7		
		4.5		
		4.0		
Carrot	25	>8.66	7.4	Alonso *et al.* (2003)
Carrot	34	>9.0	6.5-8.5	Ly-Nguyen *et al.* (2002b)
Cherry	27.2	>8.66	7.0	Alonso *et al.* (1996)
	55.9	7.05	5.2-8.0	
	55.9	6.36	<5.2	
	55.9	5.24	5.8	
Cucumber	28.1	-	6-7	Guiavarc'h *et al.* (2003)
	35.1	-	6-7	
Grapefruit	36	>10	7.0	Seymour *et al.* (1991b)
	53.5	>10	7.8	
Grapefruit	31.5	-	7.0	Guiavarc'h *et al.* (2005)
	23.7	-		
Green bean pod	46.0	8.4-9.8	9.5-9.0	Laats *et al.* (1997)
	30.0	10.5->11.5	-	
Green bean seed	44	9.8	-	Laats *et al.* (1997)
	28.5	10.5->11.5	7-9	

(Continue on the next page)

Table 2.2 Biochemical characteristics of purified or partially purified plant PMEs (cont.).

Source	MM (kDa)	pI	Optimal pH	Reference
Jelly fig achenes	38	3.5	6.5-7.5	Lin *et al.* (1989)
Kiwi	57	7.3	-	Giovane *et al.* (1990)
Lemon				Macdonald *et al.* (1993)
- Peel	35	>11	5.9	
- endocarp	33	8.8	5.9	
Orange Valencia	36	8.38	7.0	Hou *et al.* (1997)
	36	8.67	7.0	
	36	8.95	7.0	
	36	9.82	7.0	
	53	8.67	7.0-8.5	
	53	9.18	7.0-8.5	
Orange Navel	36.2	10.05	7.6	Versteeg *et al.* (1978)
	36.2	>11	8.0	
	54.0	10.2	8.0	
Papaya	53	-	8.0	Lourenco and Catutani (1984)
Papaya	28	>9		Lim and Chung (1993)
	27	>9		
Peach	36.3	-	8.0	Javeri and Wicker (1991)
	33.9	-	8.0	
Persimmon	51	8.4	7.4	Alonso *et al.* (1997a)
	30	6.9	7.8	
Soursop	24.1	-	7.0–8.0	Arbaisah *et al.* (1997)
	29.1	-		
Strawberry	33.5	>9.0	6.0-8.0	Ly-Nguyen *et al.* (2002c)
	43			
Tomato	35.5	-		Pressey and Avants (1972)
	27	-		
	23.7	-		
	24.3	-		
Tomato	23	-		Tucker *et al.* (1982)
Tomato	23.8	9.3		Pressey and Woods (1992)
	24.2	8.9		
Tomato	35	>9.3	5-10	Giovane *et al.* (1994)
	35	>9.3	8.5-10	
	35	>9.3	8.5-10	

Similar to other cell wall proteins, PMEs can be glycosylated (Giovane *et al.*, 1990; Seymour *et al.*, 1991b; Rillo *et al.*, 1992) and their isoforms can still differ on the degree and nature of glycosilation as reported by Giovane *et al.* (1990) for kiwi fruit. However, Ray *et al.* (1988)

and Glover and Brady (1994) found low or even zero level of glycosylation for tomato and peach PMEs, respectively.

2.5.4.2 Enzymatic properties

PMEs are highly specific for methylesters of D-galacturonans, since the de-esterification of ethyl or propyl groups as well as methyl alginates are not catalyzed by this enzyme. Due to the need of a free carboxyl group in the pectin chain, very high methylated pectin can inhibit PME activity (Evans and MacHale, 1978; Seymour *et al.*, 1991b). PME hydrolyzes the ester bond of esterified galacturonic units, producing negatively charged polymer (pectic acid) and methanol (**Figure 2.11**).

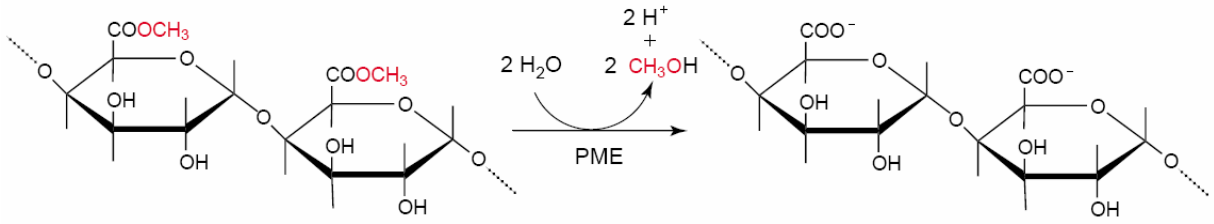

Figure 2.11 De-methylesterification of pectin by pectin methylesterase.

Johansson *et al.* (2002) have suggested a mechanism of action of PME where the amino-acid residue Asp157, hydrogen-bonded with both side chain oxygens to Arg225, acts as a nucleophilic group for the carboxymethyl carbonyl carbon of pectin substrate (**Figure 2.9**). The glutamine side chains 113 and 135 would then stabilize the negatively charged intermediate, through an anion hole. The amino-acid residue Asp136 may act, in the first cleavage step where methanol leaves, as an acid; and in the next step, as a base, by extracting an hydrogen from an incoming pectin molecule to cleave the covalent bond between the pectin substrate and the amino-acid residue Asp157 to restore the active site. Jenkins *et al.* (2001) suggested another mechanism where the aspartate active site would be responsible for the de-protonation of a catalytic water molecule.

Broadly, PMEs are thought to have two types of action patterns on the polygalacturonic polymer: single (or block-wise) and multiple-chain (or non-block-wise, random) mechanism (Micheli, 2001), as shown in **Figure 2.12**. It appears that most of the plant PMEs de-esterify pectins in a block-wise fashion (Micheli, 2001).

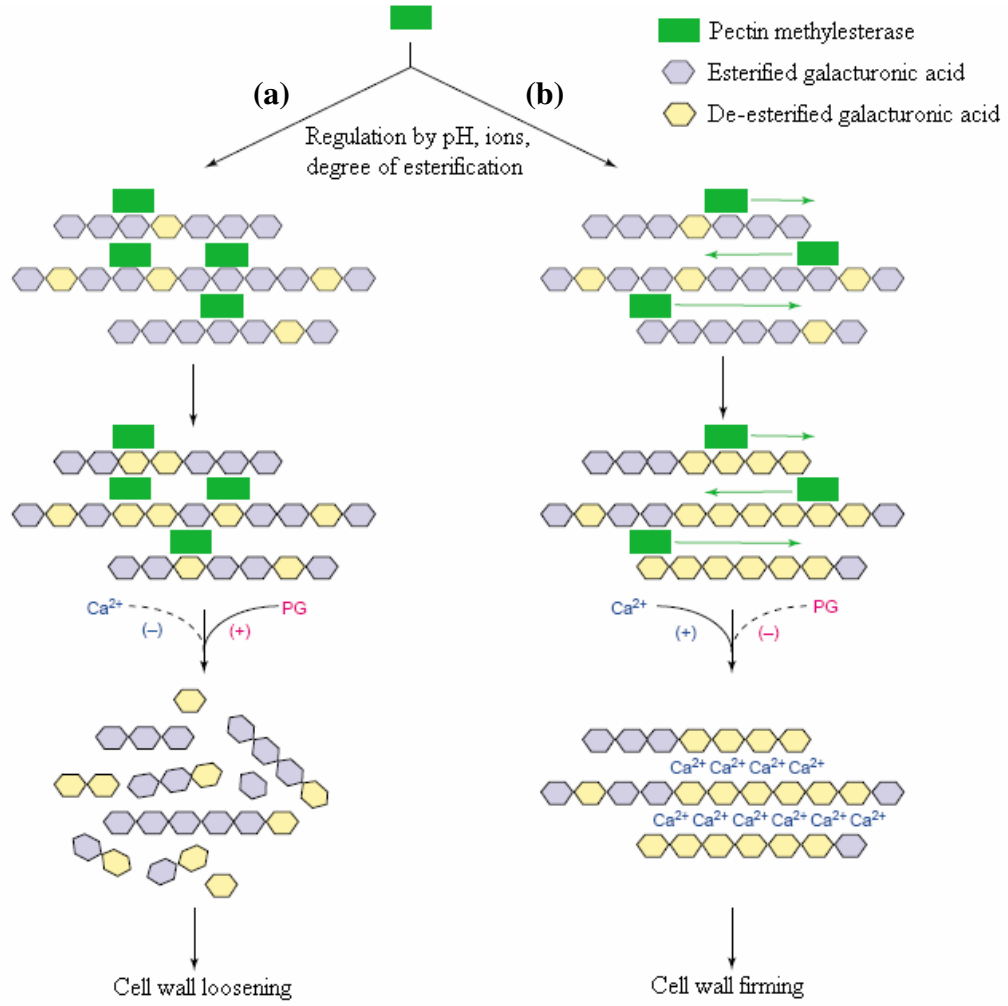

Figure 2.12 Schematic representation of modes of action of PMEs. Depending on the medium properties and origin, PMEs can act randomly (a) promoting the action of pH-dependent cell wall hydrolases such as PG, or can act block-wise (b) giving rise to blocks of free carboxyl groups that interact with bivalent ions, like Ca^{2+} (adapted from Micheli, 2001).

In contrast to plant PMEs, the action of fungal PMEs is regarded as random - multiple chain mechanism. However, fungal PME from *Trichoderma reesei* and *Erwinia chrysanthemi* PME

de-esterifies pectin in blocks (Markovic and Kohn, 1984; Christensen *et al.*, 2001). More recently, the work from Willats *et al.* (2001a) suggested that some plant PMEs may also have a non-block-wise action pattern, and additionally that PMEs could have the potential to modify or fine-tune matrix properties, at a local level, in specific regions of the cell wall in response to functional requirements. The consequences of these two types of de-esterification are very different. In blockwise mechanism, de-esterification favours pectin gelification in the presence of calcium, where the action of PG is limited, contributing to the cell wall stiffening (Micheli, 2001), whereas randomly mechanism, de-esterification favours PG and other enzymes, e.g. pectin lyase, and therefore pectin de-polymerization.

The rate of the enzymatic reaction catalyzed by plant PMEs seems to be influenced by several extrinsic (temperature, pressure) and intrinsic factors (pH, cations). As far as pH optimum is concerned, plants PMEs usually present their maximal activity from 6.0 to 8.0 (**Table 2.2**), probably due to the interactions between the positively charged enzyme and the free carboxyl groups of galacturonic residues, which resulted from the enzymatic reaction. At basic pHs, above the p*I*s, where PMEs are neutral or negatively charged, it has been proposed that the enzyme can release the substrate due to electrostatic repulsion between the negatively charged enzyme and the free carboxylate groups (Nari *et al.*, 1991).

Above pH 7.0 and in a wide range of temperatures, pectins can undergo spontaneous de-esterification which will in turn enhance PME activity, since it needs free carboxyl groups to act. Another reaction that can also occur at neutral and alkaline pH during heating is β-elimination. In β-elimination reaction, the glycosidic bonds are slipt (Keijbets and Pilnik, 1974), with the formation of a double bond between the C_5 and C_4 of the recent formed non-reducing end group of a D-galacturonic acid residue (**Figure 2.13**).

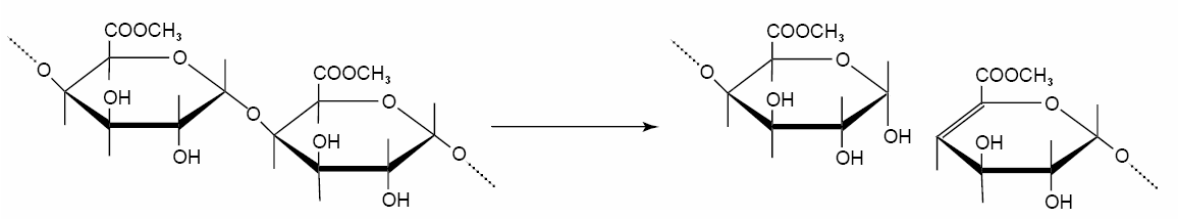

Figura 2.13 Reaction of β-elimination of pectin.

Several authors have reported that metal ions are important for PMEs activity in both plant cell-wall extension and in food processing (Rexová-Benková and Markovic, 1976; Moustacas *et al.*, 1991; Nari *et al.*, 1991; Bordenave, 1996). The fixed negative charges in the plant cell wall generate an electrostatic potential difference between the outside and inside of the plant cell. During cell wall extension, pectins are incorporated in the cell wall in the methylated state, and as a consequence the fixed charge density declines and the cell wall potential decreases. Moustacas *et al.* (1991) suggested that the electrostatic potential difference decrease will activate PME and restore the initial electrostatic potential difference value. This process is amplified by the attraction of metal ions to the polyanionic cell-wall matrix. PME is not directly activated by metal ions (cations), but they tend to release enzyme molecules that were initially bound to blocks of carboxy groups, by binding to carboxylate groups of pectin. The increase of metal-ion concentration also results in the activation of wall-loosening enzymes (Nari *et al.*, 1991). Plant PME activity increases with the metal ions concentration up to an optimum level, above which activity usually decreases. The apparent "inhibition" of PME by high salt concentrations may be considered as a protection against an excessive increase of the electrostatic potential. This inhibition may be explained by the fact that PME needs some carboxyl groups adjacent to the ester bond to be cleaved, in order to allow the reaction to proceed and, if these are blocked by metal ions, the reaction cannot occur. According to Leiting and Wicker (1997), mono- and divalent cations at the same ionic strength activate PME differently. With NaCl, the optimal concentration for activation of PME activity is *ca* 0.2M (Lee and MacMillan, 1968; Brady, 1976; Lourenco and Catutani, 1984; Lin *et al.*, 1989). Among the divalent cations, Ca^{2+} is one of the most important ions. At low concentrations (5-25mM), Ca^{2+} strongly enhances PME activity; whereas at higher concentrations, possibly due to pectate gel formation, the reaction rate is decreased (Leiting and Wicker, 1997). The nature of the anion might also influence this process. Salt addition allows a shift of the optimum pH towards acidic values (Lin *et al.*, 1989; Moustacas *et al.*, 1991).

2.5.5 Pectin, the pectin methylesterase substrate

Primary wall and middle lamella are the most important parts in the cell wall of edible plants. The basic structure of the primary cell wall consists of cellulose fibrils and a matrix composed by other polysaccharides (pectin, hemicellulose) together with lower amounts of structural glycoproteins (e.g., extensins), ionically and covalently bound minerals (e.g. calcium and boron), enzymes and water (**Figure 2.14**).

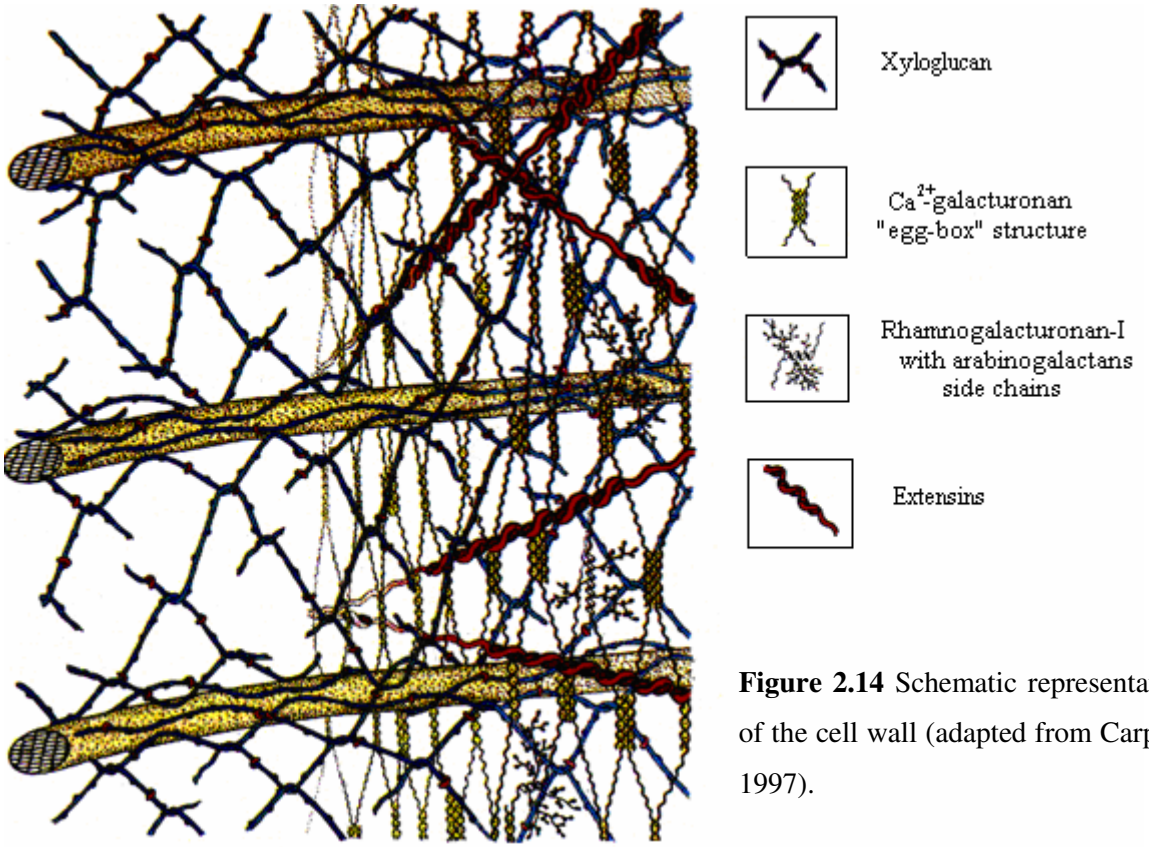

Figure 2.14 Schematic representation of the cell wall (adapted from Carpita, 1997).

The surface of the cellulose microfibrils are coated with xyloglucan polymers, which are attached to each other with hydrogen bonds. A second network, next to the cellulose/xyloglucan matrix, is the pectin network, mainly composed of galacturonans. The pectic network fills the spaces between the cellulose/xyloglucan network and determines the pore size of the cell wall (Carpita, 1997). About 1/3 of the dry amount of the primary cell wall consists of pectic substances and they can constitute an even greater proportion of the dry

amount of the middle lamella (Van Buren, 1979; Willats *et al.*, 2001b). Middle lamella (0.1-0.2μm) is an extension of the primary wall without cellulose fibrils and is the outermost part of the plant cell wall. And finally, a third network co-existing in the cell wall is a network of structural proteins, where extensins, a family of hydroxyproline rich glycoproteins, constitute the major group.

Pectins consist of highly carboxyl-methylated linear homogalacturonan regions ("smooth" regions), which alternate with "hairy" regions consisting of a rhamnose-galacturonic backbone with arabinose side chains (Willats *et al.*, 2001b) (**Figure 2.15**). Several domains can be classified in pectin polymers, such as homogalacturonan (HGA), rhamnogalacturonan I and II (RG-I and RG-II, respectively) (Vincken *et al.*, 2003).

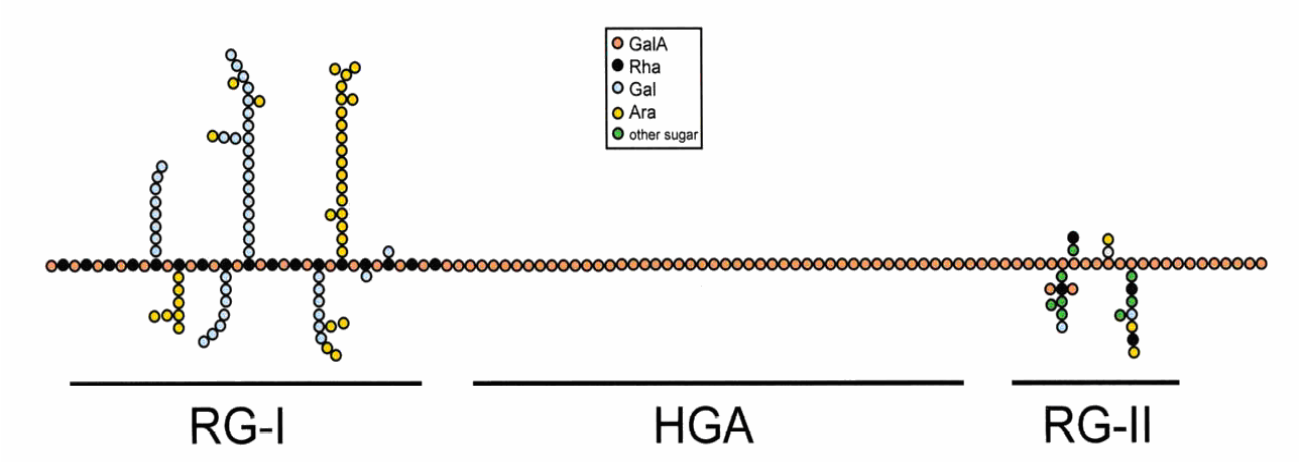

Figure 2.15 Simplified schematic diagram to show some of the features of the three major polysaccharide domains of pectin: homogalacturonan (HGA), rhamnogalacturonan-I (RGA-I) and rhamnogalacturonan-II (RGA-II). Both RGA-I and RGA-II are thought to be covalently attached to HGA, but at the present there is no evidence for a direct linkage between RGA-I and RGA-II (adapted from Willats *et al.*, 2001b).

It is thought that the three regions of pectins can be covalently linked to form a pectic network throughout the primary cell wall matrix and middle lamellae (Willats *et al.*, 2001b). This network has considerable potential for modulation of its structure by the action of several cell

wall-based enzymes (Morra *et al.*, 2004), including PME (**Figure 2.16**). HGA may, depending on the plant source, also be partially *O*-acetylated at C_3 or C_2 (Ishii, 1997).

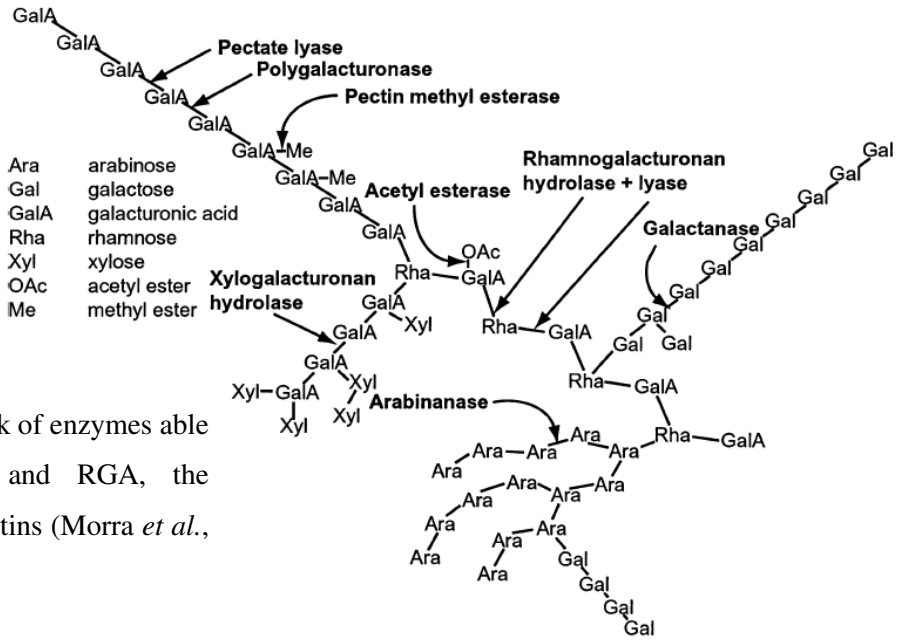

Figure 2.16 Sites of attack of enzymes able to modify the HGA and RGA, the structural elements of pectins (Morra *et al.*, 2004).

2.6 Plant pectin methylesterases in food processing

Fruit and vegetable juices are generally commercialized both as clear beverages and cloudy drinks. The turbidity of fruit and vegetable juices is due to a fine suspension of particulates with a size range from 0.4-5.0μm. These particulates are a mixture of cell wall fragments and oil droplets, with which quality factors such aroma, taste and colour, are related. Since PME occurs naturally in fruit and vegetables, electrostatically bound to the cell walls, it is impossible to avoid enzyme contamination in the juices (Rombouts *et al.*, 1992). In fresh and pasteurized cloudy juices, PME catalyzes the de-esterification of pectin, leading to low-methoxyl pectin formation, which is capable of Ca^{2+} cross-links (insoluble calcium flocculation). In order to avoid cloud loss, it is advisable to reduce PME activity to less than 10^{-4} U/mL of sample (Rothschild *et al.*, 1975; De Sio *et al.*, 1995; Laratta *et al.*, 1995b). The critical degree of esterification at which pectin becomes susceptible to cation precipitation was determined by Baker (1979) as 14-21%, while Ben-Shalom *et al.* (1985) quantified the value

as 36%. Above this value, flocculation might occur due to factors other than PME or calcium ions. If the levels of pectin are also very high, as in concentrates, gelification can occur. Pectins and pectates are known to form gelatinous masses under suitable conditions and, in general, two types of gels can be distinguished: gels of polysaccharides, acids and pectins and pectate gels. The first is formed when the concentration of polysaccharides is higher than 65%, at low pH and in presence of highly esterified pectins, while the second gel, is formed between low esterified pectins and bivalent cations. In the latter case, tha action of PME plays an importante role.

It has already been stated that texture loss of several plant-based food products, including peppers (Lee and Howard, 1999; Domínguez *et al.*, 2001; Villarreal-Alba *et al.*, 2004) can be reduced after blanching at mild temperatures due to the action of heat-activated PME. But both effects, activation and inactivation of pectinases, can be beneficial in processing fruit and vegetable based products. The combined action of PME and PG can lead to drastic textural changes of fruits and vegetables, since the action of PME makes pectin more susceptible for further degradation by PG, and therefore care should be taken. Enzyme inactivation studies have shown that, under suitable high-pressure/temperature conditions, PG can be inactivated, whereas PME can remain active (Crelier *et al.*, 2001; Fachin, 2003; Verlent *et al.*, 2004a,b). Commercial tomato PME was found to be activated under low pressure treatments at mild temperature (~300MPa, 60-65°C) (Van den Broeck *et al.*, 2000b). Therefore, controlled activity of PME with selective PG inactivation can result in some benefits regarding texture improvement of several processed fruits and vegetables (Alonso *et al.*, 1997b). The knowledge of these enzymes characteristics and especially PME, and how it is affected by processing is essential.

2.7 Conclusions

Peppers are becoming more popular in recent years because of their distinct colours, intense taste and unique flavour. They can be consumed fresh or processed, as immature (green) or as

mature fruit (yellow, orange, red), as a spice or as a vegetable. The texture, and, in particular, the crispness of peppers, is an important quality attribute to consumers. It is known that vegetable texture is closely related to the pectic substances and to activities of pectolytic enzymes. Prominent among the enzymes implicated in the softening of fruits and vegetables during ripening are PME and PG due to their relation with the cell-wall pectic content and nature. The action of PME can bring beneficial and detrimental effects on fruit and vegetable-based food products. These effects are due to PME catalyzed reaction yielding acidic pectin, and the subsequent cross-linking between the acidic pectin and Ca^{2+} ions to form calcium pectate or the subsequent action of PG. The knowledge of the characteristics and structure of PME, as well as its mode of action are important tools to the minimization of detrimental effects and maximization of beneficial ones. A number of technological approaches including thermal treatments, combined high pressure and mild temperature treatments (including sub-zero temperatures, as in high pressure shift freezing) have been lately studied to address the problem of cloud loss of fruit and vegetable juices during processing and storage or to enhance the firmness of fruit and vegetable-based food products. A similar approach was done in this thesis for the case of sweet bell peppers. And since PME can exist in multi-isoforms showing different processing stability towards temperature and pressure, the properties of PME isoforms should be taken into account when designing and optimizing pressure-temperature processes.

CHAPTER THREE Mechanisms and kinetics of enzyme inactivation

3.1 Introduction

Enzyme inactivation in food is generally performed by thermal processing, an effective procedure to inactivate both enzymes and microorganisms. Unfortunately, there are negative side effects of thermal processing regarding nutritive and sensory quality. High pressure is being investigated as an alternative or complementary technology (e.g., with mild-temperature) for food processing and preservation, since the original properties of fresh food are better kept, while enzymes and vegetative microorganisms are inactivated. In this chapter, mechanisms and kinetics of enzyme inactivation, and pectin methylesterase (PME) in more detail, under thermal, pressure and combined pressure-temperature treatments, will be discussed.

3.2 Proteins and enzymes

Proteins have delicate and balanced structures, maintained by interactions within the protein chain and with the surrounding solvent (Jaenicke, 1991). Changes in the external factors, such as pressure and temperature, can disturb the delicate balance of intramolecular and solvent-protein interactions and originate, partial or even complete, unfolding/denaturation of the polypeptide chain. And since enzymes are a special class of proteins, in which biological activity arises from an active site and due to a particular three-dimensional conformation of the molecule, the effect of both temperature and pressure on enzymes is usually approached using the basic knowledge about proteins (Richardson and Hyslop, 1985).

It should also be mentioned that the term denaturation in literature is often used as a synonym for inactivation, although this use is not always correct. Denaturation implies a conformational change of a biological macromolecule which entails a reversible or an irreversible loss of its ability to perform a certain biological function. Along with denaturation (conformational change), interactions between macromolecules or modification of functional groups, together with or even without conformational changes, can be responsible for protein inactivation. Unless specially indicated that no changes have occurred in the primary structure of a protein

or in the degree of its aggregation, the term inactivation should be used referring loss of biological activity (Mozhaev and Martinek, 1982). And in general, the term enzyme inactivation is used indicating loss of enzymatic activity.

3.3 Mechanism of enzyme inactivation

3.3.1 The effect of temperature

The structure of proteins is dynamic and, as such, is constantly changing regarding the surrounding medium, i.e., pH, temperature, pressure and salts (Balny and Mason, 1993; Philips *et al.*, 1994). The covalent bonds (peptide and disulphide bonds) and non-covalent bonds (hydrogen bonds, electrostatic interactions, hydrophobic interactions and van der Waals forces) determine the structure and specific conformation of proteins (Balny and Mason, 1993; Philips *et al.*, 1994). Under normal conditions, the native, catalytically active structure of an enzyme is maintained by a fragile balance of different non-covalent forces. The active site of an enzyme, mainly composed of amino acid residues with apolar side-chains, consists both of a binding site and a place where chemical reactions occur (Richardson and Hyslop, 1985). The volume of the native protein structure depends on three factors: (i) the volume of the constitutive atoms, (ii) the volume of the internal cavities, and (iii) the solvation volume (Masson, 1992).

In thermal inactivation, both non-covalent and covalent bonds are affected. The optimum temperature for enzyme activity, in most of the cases, is around 30-40ºC, where the catalytic activity accelerates specific biological reactions with an extraordinary catalytic power, and above 45ºC, the enzyme begins to denature (Richardson and Hyslop, 1985). With the exception of hydrophobic interactions, all of the abovementioned non-covalent forces diminish upon an increase in temperature and the protein molecule unfolds (Klibanov, 1983). As the temperature increases, the hydrogen bonds de-stabilize and, as a consequence, there will be a disturbance on the secondary structure (Casal *et al.*, 1988; Fabian *et al.*, 1993). Upon unfolding, the hydrophobic regions of the protein become exposed to the solution, which is

thermodynamically unfavourable. In order to reduce the free energy of the system, the unfolded molecules interact with each other in the hydrophobic spots, leading to protein aggregation (Mozhaev and Martinek, 1982; Klibanov, 1983). The aggregation can be reduced if the pH medium is far way from the isoelectric point of the protein, since the electrostatic repulsion of the similar charged surface of the aggregate is large (Mozhaev and Martinek, 1982). In very dilute solutions, unfolded molecules can refold intra-molecularly to new kinetic or thermodynamically stable structures, different from the native enzyme conformation. In general, this new conformation is catalytically inactive but remains even after cooling, since a kinetic barrier prevents spontaneous refolding to the native structure (Klibanov, 1983). The effect of temperature on the tertiary structure is the result of counteracting exothermic interactions (electrostatic and van der Waals interactions, hydrogen bonds) and endothermic hydrophobic interactions. The heat stabilisation of hydrophobic interactions reaches its maximum around 60-70ºC (Richardson and Hyslop, 1985) and decreases after because of the breakdown of the water structure (Scheraga *et al.*, 1962; Brandts, 1967; Damodaran, 1996). Thermal processing of oligomeric enzymes also results in dissociation into individual subunits, since quaternary structure is due to non-covalent association of proteins units of the same interaction type as secondary and tertiary structures (Mozhaev and Martinek, 1982).

At extreme temperatures (e.g., 100ºC), changes in covalent bonds can occur, causing interchange reactions, like inter- and intramolecular thiol-disulphide, oxidation of cysteine sulfhydryl groups or tryptophan indole ring, deamination of asparagine and/or glutamine and deteriorative reactions caused by the nucleophilic character of the ε-amino group of lysine and terminal α-amino acid groups (Klibanov, 1983; Ahern and Klibanov, 1985; Mozhaev *et al.*, 1988; Volkin *et al.*, 1991).

3.3.2 The effect of pressure: inactivation, stabilization and activation

The main characteristics related with high pressure processing of food systems include microorganisms inactivation; modification of biopolymers, including enzyme inactivation and

activation, protein denaturation and gel formation; changes in susceptibility of enzyme action as well as quality retention such as stability of color and flavor; and change in product functionality as exemplified by density changes, freezing and melting temperature or textural attributes (Knorr *et al.*, 1992; Knorr, 1993).

With the exception of oxidation of the sulfhydryl groups and thiol-disulphide interchange reactions (Cheftel, 1991; Tanaka *et al.*, 1996; Funtenberger *et al.*, 1997), covalent bonds of proteins remain unaffected by pressure (Cheftel, 1991; Heremans, 1992; Kunugi, 1993; Balny *et al.*, 1997), up to 1200MPa (Weber and Drickamer, 1983). Therefore, the primary structure is only slightly affected (Cheftel, 1991; Heremans, 1992; Masson, 1992; Mozhaev *et al.*, 1994). On the other hand, changes in secondary, tertiary and quaternary structures of proteins do occur during high pressure treatments.

Responsible for maintaining the secondary structure of proteins, the hydrogen bonds are almost insensitive to pressure (Mozhaev *et al.*, 1994, 1996) and only affected at elevated pressure levels (>700MPa), which will lead to irreversible denaturation (Masson, 1992; Balny and Masson, 1993). At this level, where the secondary structure is affected, β-pleated sheets seem to be even more pressure stable than α-helical structures (Gross and Jaenicke, 1994). However, hydrogen bonds might be destroyed or strengthened depending on the working system (Van Eldik *et al.*, 1989; Gross and Jaenicke, 1994). It seems that electrostatic interactions in biomolecules are weaker at elevated pressures. Pressure induces reversible denaturation of chymotrypsin due to the dissociation of a salt bridge in the active site (Heremans and Heremans, 1989). Another example is the dissociation constants of weak acids which vary with pressure. Phosphate buffer has a high ionization volume in contrast with Tris(hydroxymethyl)-aminomethane-hydrochloric acid buffer (Tris-HCl buffer), that has nearly zero ionization volume (Kitamura and Itoh, 1987).

Opposite to the effect of pressure on the secondary structure, the well organized tertiary structure suffers significant changes above 150-200MPa (Cheftel, 1991; Balny and Masson, 1993; Barbosa-Cánovas *et al.*, 1997), probably due to an equilibrium disturbance of hydrophobic and electrostatic interactions. These structural modifications are accompanied

with large hydration changes, which are assumed to be the major source of volume decrease associated with pressure denaturation of enzymes (Gross and Jaenicke, 1994; Barbosa-Cánovas *et al.*, 1997). The rupture of electrostatic interactions is accompanied by an exposure of charged groups, having the tendency to order water molecules around them, leading to volume reduction (Heremans, 1992; Mozhaev *et al.*, 1994, 1996). On the other hand, the disruption of hydrophobic interactions exposes non-polar amino acid residues to the (aqueous) solution, around which the "loosely packed" water structure is reorganised (Gross and Jaenicke, 1994; Hayakawa *et al.*, 1996; Mozhaev *et al.*, 1996). From the above mentioned, it is clear that hydrophobic and electrostatic interactions are the main responsibles for pressure denaturation of monomeric enzymes.

The quaternary structure is markedly affected by pressure above 150-200MPa, due to its effect in hydrophobic interactions, where oligomeric enzymes dissociate into individual subunits (Balny and Masson, 1993; Barbosa-Cánovas *et al.*, 1997). However, it has already been reported that several quaternary structures are insensitive to pressure (Messens *et al.*, 1997). Higher pressure levels induce protein unfolding and re-association (aggregation) of the dissociated subunits (Ohmiya *et al.*, 1989; Silva and Weber, 1993). In **Table 3.1**, general critical pressure levels found in literature for different behaviours of protein/enzyme under high pressure treatments are presented.

Besides inactivation, pressure-induced stabilisation under high temperature conditions has also been observed for α-chymotrypsin and PPO (Mozhaev *et al.*, 1996; Weemaes *et al.*, 1998b). A possible explanation of pressure stabilization against heat inactivation regarding α-chymotrypsin lays on the opposing effect of pressure and temperature on the ability of protein functional groups to interact with water (Mozhaev *et al.*, 1996). According to Mozhaev *et al.* (1996), during the initial step of thermal inactivation, α-chymotrypsin looses a certain number of water molecules, while pressure inactivation is accompanied by large hydration changes due to the exposure of both charged and non-polar groups.

Table 3.1 Critical pressure levels for different behaviours of protein/enzyme under high pressure treatments.

PRESSURE	STRUCTURE EFFECT	REFERENCES
>150-200MPa	Quaternary structure: oligomeric enzymes dissociate into individual units.	Balny and Masson (1993); Barbosa-Cánovas *et al.* (1997)
>150-200MPa	Tertiary structure: hydrophobic and electrostatic interactions, with large hydration changes	Cheftel (1991); Balny and Masson (1993); Gross and Jaenicke (1994); Barbosa-Cánovas *et al.* (1997)
200-600MPa	Tertiary structure	Balny and Masson (1993); Gross and Jaenicke (1994); Hayakawa *et al.* (1994)
>700MPa	Secondary structure	Balny and Masson (1993)
>1200MPa	Covalent bonds	Weber and Drickamer (1983)

The antagonistic effect of pressure and temperature on PPO might be related to the pressure stabilization of hydrogen bridges due to the large amount of these bonds present on the enzyme which will stabilise the α-helical structure. Based on several studies, Boonyaratanakornkit *et al.* (2002) presented a general model for pressure-induced stabilization of proteins (**Figure 3.1**).

As previously mentioned, the active site which is responsible for the biological activity of enzymes, is brought together by the three dimensional conformation of the molecule. Therefore, enzymes submitted to pressure treatments may display inactivation or activation, depending on the volume changes associated with modifications in the tertiary and quaternary structures at the pressure level applied (Cheftel, 1992; Heremans, 1992). In this sense, pressure can affect product functionality, providing a good potential basis for development of new processes for food preservation or product modifications (new texture/taste). In the case of pressure treatment, enzyme activation can also arise from pressure induced decompartmentalization (Butz *et al.*, 1994; Jolibert *et al.*, 1994; Gomes and Ledward, 1996). In intact tissues, enzymes and substrate are often separated by compartmentalization, which

can be destroyed upon application of low pressure (Jolibert *et al.*, 1994; Butz *et al.*, 1994). Pressure-induced membrane damage and the resulting leakage of enzyme and substrate can result in enzyme-substrate contact. The enzymatic reaction resulting from this contact can, in turn, be accelerated or decelerated by pressure, depending on the reaction volume of the enzyme catalyzed reaction (Morild, 1981).

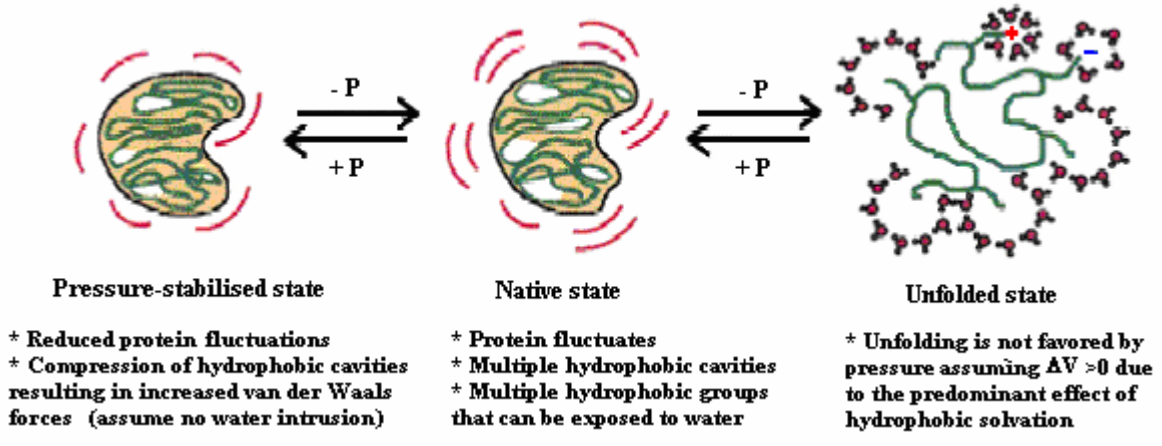

Figure 3.1 Mechanism for pressure stabilization of enzymes. Interactions between the unfolded protein and water molecules are included for the unfolded protein (adapted from Boonyaratanakornkit *et al.*, 2002).

3.4 Inactivation kinetics of enzymes

The kinetic models and the kinetic parameters estimation are necessary for design and optimization of temperature and combined temperature-pressure processing of food products. The following section is related to the different equations used in enzyme inactivation kinetics. The regression methods applied to estimate the kinetic parameters with the maximum likelihood of being correct are discussed in the further sections.

3.4.1 Primary models

3.4.1.1 n^{th} order kinetic model

In general, the decrease of concentration of the active enzyme (A) as a function of time (t) can be expressed by the n^{th} order kinetic model shown in equation 3.1:

$$\frac{dA}{dt} = -k \cdot A^{n} \tag{3.1}$$

where k is the inactivation rate constant and n the represents reaction order. Due to the linearity between concentration of the active enzyme and the enzymatic activity, A will be considered as enzyme activity. The integration of the abovementioned differential equation between the initial (A_0, time zero) and final (A, time t) conditions, for constant extrinsic conditions (e.g., temperature and pressure) and intrinsic factors, will result in the following equation (Eq. 3.2), an expression of enzymatic equation as a function of time. The inactivation rate constant (k-value) and the reaction order (n) can be estimated by non-linear regression analysis.

$$A = \left[A_0^{1-n} + (n-1)k \cdot t\right]^{\frac{1}{1-n}} \tag{3.2}$$

3.4.1.2 First-order kinetic model

In many cases, inactivation of enzymes can often be described by a first-order kinetic model (Eagerman and Rouse, 1976), where n in equation 3.1 equals 1. Therefore, under isobaric-isotherm conditions the decrease of enzymatic activity versus time can be described by equation 3.3, a simplified form of equation 3.1:

$$\ln\left(\frac{A}{A_0}\right) = -k \cdot t \tag{3.3}$$

When the natural logarithm of $\left(\frac{A}{A_0}\right)$ is plotted as a function of treatment time, the inactivation rate constant (k) can be derived by a linear regression analysis.

In food processing and for first-order reactions, the traditional approach to describe changes in microbial populations as a function of time has been the use of the survivor curve equation (Eq. 3.4), where kinetics can be express in terms of the D-value, the decimal reduction time at a certain temperature or pressure. The D-value is estimated from the reciprocal slope of the regression line of $\log\left(\frac{A}{A_0}\right)$ versus treatment time at constant temperature:

$$\log\left(\frac{A}{A_0}\right) = -\frac{t}{D} \tag{3.4}$$

Since the D-value is also defined as the time at, a given temperature and pressure, needed for 90% reduction of the initial activity (A_0), a relationship between the D-value and the more general inactivation rate constant (k-value) can be established (Eq. 3.5) by comparison of equation 3.3 and 3.4, where the D-value is inversely proportional to the k-value:

$$D = \frac{\ln(10)}{k} \tag{3.5}$$

However, in some cases deviations from first-order inactivation behaviour of enzymes has been observed. In these cases, other models should be used. The most common are presented in the next sections.

3.4.1.3 Fractional-conversion model

The fractional conversion model (Eq. 3.6), a special case of the first-order inactivation model, which takes into account a non-zero activity (A_∞-value) after a prolonged heating and/or pressurizing time. This model considers that the enzyme inactivates, following a first order decay, up to a point where the activity remains unchangeable with respect to time.

$$A = A_\infty + (A_0 - A_\infty)\exp(-k \cdot t) \tag{3.6}$$

This relation is valid in the temperature and/or pressure domain where only the labile enzyme fraction inactivates whereas the activity of the stable fraction (A_∞-value) remains unchangeable with respect to time. The inactivation rate constant (k-value) and the remaining activity after treatment (A_∞-value) can be estimated using non-linear regression analysis. It should be stressed that for experiments at constant temperature and/or pressure, the heating and/or pressurizing time should be long enough so that the remaining activity, A_∞, is no longer changing with respect to time (Van den Broeck *et al.*, 1999a,b). This non-zero activity may or may not be a function of applied temperature and pressure. This inactivation behaviour has been observed for several enzymes, e.g., broccoli mirosinase (Ludikhuyze *et al.*, 1999), orange PME (Van den Broeck *et al.*, 1999b) and avocado PPO (Weemaes *et al.*, 1998a).

3.4.1.4 Parallel or distinct isoenzyme kinetic model

In the presence of isoenzymes with different processing stability, e.g. in the case where one is more thermal (pressure) resistant than the other, in which each isoenzyme is inactivated in parallel according to a first-order reaction, a biphasic kinetic model or distinct isoenzyme kinetic model can be used (Ramesh *et al.* 1998; Indrawati *et al.*, 1999a, 2000; Van den Broeck *et al.*, 2000a). The inactivation process can be accounted as described in Eq. 3.7:

$$A = \sum_{i=1}^{n} A_i \exp(-k_i \cdot t) \tag{3.7}$$

where A is the total, experimentally measured activity; A_i is the enzyme activity of the i^{th} fraction at time zero (t = 0); and k_i is its inactivation rate constant of the i^{th} enzyme fraction.

When there are several isoenzymes present, which show different behavior towards temperature and/or pressure, and both inactivate according to a first-order kinetic model, there is a fast inactivation period followed by a decelerated decay. For constant extrinsic (e.g., pressure, temperature) and intrinsic conditions and assuming that the inactivation of both fractions is independent of each other, the inactivation can be modeled according to equation 3.8.

$$A = A_L \exp(-k_L \cdot t) + A_S \exp(-k_S \cdot t) \quad (3.8)$$

where A_L and A_S refer, respectively, to the enzymatic activity of labile and stable fractions, and k_L and k_S are the inactivation rate constants of labile and stable fractions, respectively. The inactivation rate constants (k_L- and k_S-value) and the activity of each fraction (A_L and A_S), can be derived by non-linear regression analysis.

3.4.1.5 Consecutive step kinetic model

A succession of (two) irreversible first-order steps (Eq. 3.9), e.g., an irreversible conversion of the native enzyme to an intermediate with lower specific activity and subsequent irreversible conversion of the intermediate to an inactive enzyme form (Weemaes *et al.*, 1998a; Ludikhuyze *et al.*, 1999), can be described by a consecutive step kinetic model. Activity lost in each first-order step (A_1 and A_2) and inactivation rate constants (k_1 and k_2) can be estimated by non-linear regression analysis.

$$A = \left[A_1 - A_2 \left(\frac{k_1}{k_1 - k_2} \right) \right] \exp(-k_1 \cdot t) + \left[A_2 \left(\frac{k_1}{k_1 - k_2} \right) \right] \exp(-k_2 \cdot t) \quad (3.9)$$

It should be noted that, both in parallel or isoenzyme model and consecutive model, plotting the logarithm of the residual activity versus time results in the so called biphasic decay profiles.

3.4.2 Secondary models

3.4.2.1 Temperature dependence

At constant pressure, the temperature dependence of the inactivation rate constant can be expressed in terms of the activation energy (E_a, $kJ.mol^{-1}$) and estimated using the Arrhenius model (Eq. 3.10) (Arrhenius, 1889):

$$\ln(k) = \ln(k_{ref}) + \left[\frac{E_a}{R_T}\left(\frac{1}{T_{ref}} - \frac{1}{T}\right)\right] \quad (3.10)$$

where T and T_{ref} are the absolute temperature (K) and the chosen reference temperature (K), respectively; k is the inactivation rate constant (min^{-1}); k_{ref} is the inactivation rate constant at T_{ref} (min^{-1}); R_T (8.314 $J.mol^{-1}.K^{-1}$) is the universal gas constant. When the natural logarithm is plotted as a function of the reciprocal of absolute temperature at constant pressure, the activation energy (E_a) can be derived from the slope based on the linear regression analysis.

In food processing and technology, the Thermal Death Time model is often used where the temperature dependence is usually expressed in terms of the *z*-value. The *z*-value is characterized by the temperature change necessary to obtain a 10-fold change of the D-value, and is calculated by the following equation (Eq. 3.11):

$$\log(D_T) = \log(D_{T_{ref}}) - \frac{T - T_{ref}}{z_T} \quad (3.11)$$

where D_{ref} is the D-value (min) at the chosen reference temperature (T_{ref}). When the logarithm of D is plotted as a function of the absolute temperature (T), the z_T-value (ºC) can be obtained from the slope, based on the linear regression analysis. The subscript T in the D- and in z-value indicates that the experiments were done at constant pressure.

3.4.2.2 Pressure dependence

The pressure dependence of the enzyme inactivation rate constant (k) at constant temperature can be expressed in terms of the activation volume (V_a), based in the Eyring equation (Eyring *et al.*, 1946), from which equation 3.12 can be obtained:

$$\ln(k) = \ln(k_{ref}) - \left[\frac{V_a}{R_P T}(P - P_{ref})\right] \quad (3.12)$$

where P and P_{ref} are the pressure and the chosen reference pressure (MPa), respectively; V_a is the activation volume ($cm^3.mol^{-1}$); k is the inactivation rate constant (min^{-1}); k_{ref} is the inactivation rate constant at reference pressure (min^{-1}); R_P is the universal gas constant (8.314 $cm^3.MPa.mol^{-1}.K^{-1}$); and T is the absolute temperature (K). The activation volume (V_a) can be estimated by the slope obtained by the linear regression analysis of the natural logarithm of k-value versus pressure at contant temperature. In some cases, the plot may not be linear. In these cases, the activation volume is temperature dependent ($V_a = f(T)$) and another equation is needed to estimate the pressure dependence of the inactivation rate constant (Ludikhuyze *et al.*, 1998b; Indrawati *et al.*, 2000).

In general for biological systems, V_a-values are difficult to interpret at the molecular level because of the many interactions involved (Mozhaev *et al.*, 1994). As previously mentioned, volume increasing reactions will tend to be inhibited by pressure, while reactions leading to a decrease in volume will tend to be promoted. Therefore, equation 3.12 also illustrates that pressure accelerates reactions having negative activation volumes, and the more negative the activation volume, the greater the acceleration with pressure. The activation volumes can also vary with temperature. According to Mozhaev *et al.* (1996), the activation volume of α-chymotrypsin was 2.5-fold more negative at 50ºC than at 20ºC when it was submitted to pressures from 1bar to 3kbar (~0.1-300MPa).

Analogous to the log-linear relationship between D-value and temperature, there is often a relationship between D-value and pressure, where *z*-value is defined as the pressure change necessary to obtain a 10-fold change of the D-value and calculated as expressed in equation 3.13. When the decimal logarithm of D-value is plotted as a function of pressure, the z_P-value can be obtained from the slope based on a linear regression analysis. The subscript P in D- and in z-value indicates that the experiments were done at constant temperature.

$$\log(D_P) = \log(D_{P_{ref}}) - \frac{P - P_{ref}}{z_P} \qquad (3.13)$$

3.4.2.3 Combined pressure-temperature dependence

Based on complete experimental kinetic data sets for enzyme inactivation pressure-temperature, iso-rate contour diagrams can be contructed, i.e. two-dimensional diagrams representing combinations of pressure and temperature resulting in the same inactivation rate constant. These iso-rate contour diagrams for pressure-temperature inactivation of enzymes (as well as microorganisms) are often elliptically shaped, similar to combined pressure and temperature dependence of a protein denaturation over a broad range of pressures and temperatures (**Figure 3.2**).

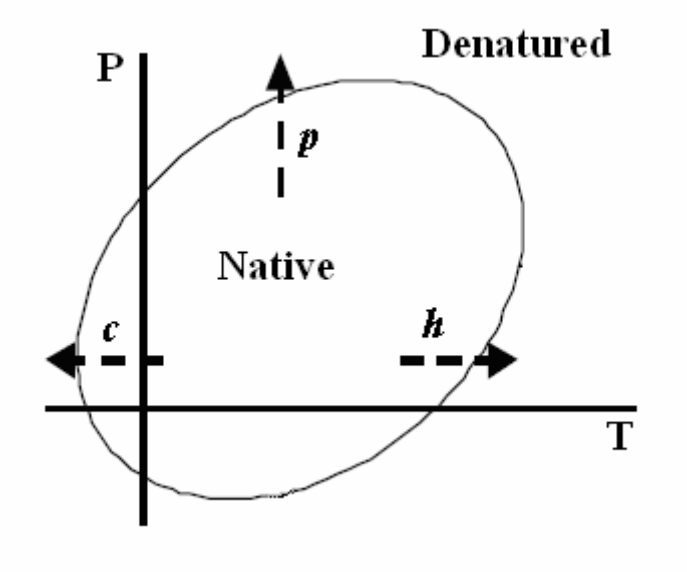

Figure 3.2 Schematic representation of the elliptic phase diagram of proteins. The arrows marked by the letters *p*, *h* and *c* show specific denaturation ways known as pressure, heat and cold denaturation (adapted from Smeller, 2002).

A number of different mathematical approaches have been used in order to describe the elliptical iso-rate contour diagram, depicting combined pressure and temperature dependence of enzyme inactivation over a broad range of pressures and temperatures. The elliptical pressure-temperature kinetic diagram for enzyme inactivation can be approached on an empirical basis, either using a (i) modified Arrhenius relation (Weemaes *et al.*, 1998b; Van den Broeck *et al.*, 2000a) or (ii) a modified Eyring equation (Ludikhuyze *et al.*, 1998b), as a starting point to build the models as shown in **Table 3.2**, or on (iii) a thermodynamic basis. The accuracy of these models can be evaluated by plotting the experimental k-values versus k-values calculated according to the model and the concomitant parameters, as it will be further illustrated in section 3.5.2.

Table 3.2 Secondary models using Arrhenius or the Eyring equations as starting point to express pressure-temperature dependence of the inactivation rate constant.

Enzyme	Model	Approach	Authors
Avocado PPO	$\ln(k) = (a + bP + cP^2 + dP^3) + \left[\frac{E_a^0 \exp(-nP)}{R_T}\left(\frac{1}{T_{ref}} - \frac{1}{T}\right)\right]$ (3.14)	Modified Arrhenius equation	Weemaes *et al.* (1998b)
Orange PME	$\ln(k) = (a + bP + cP^2 + dP^3) + \left[\frac{f - eP}{R_T}\left(\frac{1}{T_{ref}} - \frac{1}{T}\right)\right]$ (3.15)	Modified Arrhenius equation	Van den Broeck *et al.* (2000a)
Soybean LOX	$\ln(k) = (a_2T^2 + b_2T + c_2) + \left[\frac{a_1 T \exp(-b_1 T)}{R_P T}(P - P_{ref})\right]$ (3.16)	Modified Eyring equation	Ludikhuyze *et al.* (1998b)

(i) The empirical approach using the Arrhenius equation

When the temperature dependence of the inactivation rate constant can be expressed by the Arrhenius equation for the pressure range tested, an empirical approach using the Arrhenius equation as a starting point can be used. In this case, the inactivation rate constant and the activation energy are both function of pressure, [$k_{ref} = f_1(P)$] and [$E_a = f_2(P)$], respectively, at the reference temperature (Weemaes *et al.*, 1998b; Van den Broeck *et al.*, 2000b). Considering the substitution of those two terms in equation 3.10, yields equation 3.17:

$$\ln(k) = \ln(f_1(P)) + \left[\frac{f_2(P)}{R}\left(\frac{1}{T_{ref}} - \frac{1}{T}\right)\right] \quad (3.17)$$

And as an example, the mathematical model that describes the pressure-temperature dependence of the inactivation rate constants using Arrhenius equation as a starting point is illustrated for avocado PPO (Weemaes *et al.*, 1998b) in equation 3.14 (**Table 3.2**) by substitution of equations 3.18 and 3.19 in equation 3.17. It should be mentioned that in this case, the pressure dependence of k_{ref} could be described by a third-degree polynomial model.

$$\ln(k_{ref}) = (a + bP + cP^2 + dP^3) \quad (3.18)$$

$$E_a = E_a^0[\exp(-nP)] \quad (3.19)$$

The coefficients designated as a, b, c, d, n and E_a^o are model fitting parameters.

For orange PME, the mathematical model that Van den Broeck *et al.* (2000a) used to describe the pressure-temperature dependence of the inactivation rate constants using Arrhenius equation as a starting point is illustrated in equation 3.15 (**Table 3.2**). In this case, the effect of pressure on E_a was a linear function. By substitution of equations 3.18 and 3.20 in equation 3.17, equation 3.20 is obtained. The coefficients designated as e and f are model fitting parameters.

$$E_a = f - eP \quad (3.20)$$

(ii) The empirical approach using Eyring equation

The Eyring equation can be used as a starting point to model the dependence of the inactivation rate constant as a function of pressure and temperature, when it is valid over the entire temperature domain. In this case, both the inactivation rate constant and the activation volume are function of temperature [$k_{ref} = f_1(T)$ and $V_a = f_2(T)$] (Ludikhuyze *et al.*, 1998b; Indrawati *et al.*, 2000). Inserting the terms in the Eyring equation (3.12), equation 3.21 is obtained:

$$\ln(k) = \ln(f_1(T)) - \left[\frac{f_2(T)}{RT}(P - P_{ref})\right] \quad (3.21)$$

As an example, the combined pressure-temperature inactivation of soybean LOX was modeled using Eyring equation as a starting point (Ludikhuyze *et al.*, 1998b) using equation 3.16 (**Table 3.2**) which is obtained by substitution of equations 3.22 and 3.23 in equation 3.21.

$$V_a = a_1 T \exp(-b_1 T) \tag{3.22}$$

$$\ln(k_{ref}) = a_2 T^2 + b_2 T + c_2 \tag{3.23}$$

For equations 3.22 and 3.23, the coefficients designated as a_1, b_1,a_2, b_2, and c_2 are model fitting parameters.

In the present study, (iii) a thermodynamic-based kinetic model governing the behaviour of the system during pressure and temperature change was used as a general equation (Eq. 3.31) to describe the pressure-temperature inactivation of PME under investigation. This model has been often used with success to analyse a number of enzyme inactivation data towards temperature-pressure treatments and can be regarded as a generic model in the field (Hawley, 1971; Morild, 1981; Weemaes *et al.*, 1998b; Indrawati *et al.*, 2002).

(iii) Thermodynamic-based kinetic model

A thermodynamic equation governing the behaviour of a system during a pressure and a temperature change has been previously described by Hawley (1971) and later by Morild (1981). Hawley (1971) started from the assumption that there are only two distinct states of the protein (native and denatured) and the transition between them is a two-state process. Starting from this assumption, Hawley (1971) calculated the Gibbs free energy difference (ΔG) between these states:

$$\Delta G = G_{denatured} - G_{native} \tag{3.24}$$

$$d(\Delta G) = -\Delta S dT + \Delta V dP \tag{3.25}$$

As a first approximation, one can assume ΔS and ΔV to be nearly constant within limited P and T range, and use the integrated equation 3.26, obtained from equation 3.25:

$$\Delta G = \Delta G_0 - \Delta S(T - T_0) + \Delta V(P - P_0) \tag{3.26}$$

Since the entropy change (ΔS) and volume change (ΔV) vary both with pressure and temperature according to equations 3.27 and 3.28, respectively:

$$d(\Delta S) = \left(\frac{\partial \Delta S}{\partial T}\right)_P dT + \left(\frac{\partial \Delta S}{\partial P}\right)_T dP \tag{3.27}$$

$$d(\Delta V) = \left(\frac{\partial \Delta V}{\partial T}\right)_P dT + \left(\frac{\partial \Delta V}{\partial P}\right)_T dP \tag{3.28}$$

Inserting appropriately defined quantities, these equations may be re-written as:

$$d(\Delta S) = \left(\frac{\Delta C_P}{T}\right)_P dT - \Delta\zeta\, dP \tag{3.29}$$

$$d(\Delta V) = \Delta\zeta\, dT + \Delta\kappa\, dP \tag{3.30}$$

Upon integration from an arbitrarily chosen reference point T_0, P_0 to P,T, equation 3.26 can be re-written as equation 3.31 (Hawley, 1971; Morild, 1981):

$$\begin{aligned}\Delta G = \Delta G_0 + \Delta V_0(P - P_0) - \Delta S_0(T - T_0) + \frac{1}{2}\Delta\kappa(P - P_0)^2 + 2\Delta\zeta(P - P_0)(T - T_0) \\ - \Delta C_p\left[T\left(\ln\frac{T}{T_0} - 1\right) + T_0\right]\end{aligned} \tag{3.31}$$

where ΔC_p is the heat capacity change $(T\partial\Delta S/\partial T)_P$; $\Delta\zeta$ the thermal expansibility factor $((\partial\Delta V/\partial T)_P = -(\partial\Delta S/\partial P)_T)$ and $\Delta\kappa$ the compressibility factor, $(\partial\Delta V/\partial P)_T$. What Hawley (1971) obtained was a second-order curve, which turned to be elliptical in the case of proteins (**Figure 3.2**). In the frame of this theory, temperature, pressure and cold denaturation are the only three very special ways of protein denaturation (Smelt, 2002). One of the practical consequences of this phenomenon is the stabilisation of the protein against heat denaturation by low pressures (Heremans, 2002).

The above thermodynamic model (Eq. 3.31) can be converted into a kinetic model through the transition state theory of Eyring, assuming that the enzyme inactivation is accompanied by the

formation of a metastable activated state ($^{\neq}$) which exists in equilibrium with the native enzyme. This conversion is based on the substitution of equations 3.32 and 3.33 in equation 3.31, yielding equation 3.34.

$$\Delta G^{\neq} = -R_T \cdot T \cdot \ln\left(K^{\neq}\right) \tag{3.32}$$

$$K^{\neq} = \frac{k \cdot h}{r \cdot k_B \cdot T} \tag{3.33}$$

$K^{\neq}$ is the thermodynamic equilibrium constant; k is the rate constant; r is the transmission coefficient; k_B is the Boltzmann constant (1.38×10^{-23} J.K^{-1}); h is the Planck constant (6.26×10^{-34} J.s) and R_T is the universal gas constant (8.314 J.mol^{-1}.K^{-1}). Equation 3.31 represents the modified thermodynamic model that describes the inactivation rate constant as a function of pressure and temperature.

$$\begin{aligned}\ln(k) = \ln(k_0) &- \frac{\Delta V_0^{\neq}}{R_T T}(P - P_0) + \frac{\Delta S_0^{\neq}}{R_T T}(T - T_0) - \frac{\Delta \kappa^{\neq}}{2R_T T}(P - P_0)^2 \\ &+ \frac{\Delta C_p^{\neq}}{R_T T}\left\{T\left[\ln\left(\frac{T}{T_0}\right) - 1\right] + T_0\right\} - \frac{2\Delta\zeta^{\neq}}{R_T T}(P - P_0)(T - T_0)\end{aligned} \tag{3.34}$$

P_0 (MPa) and T_0 (K) are reference pressure and temperature, respectively; k (P, T) and k_0 (P_0, T_0) are inactivation rate constants (min^{-1}); $\Delta\kappa^{\neq}$ is the compressibility factor (cm^6.J^{-1}.mol^{-1}); $\Delta\zeta^{\neq}$ is the thermal expansibility factor (cm^3.mol-1.K^{-1}); $\Delta C_p^{\neq}$ is the heat capacity (J.mol^{-1}.K^{-1}); $\Delta V_0^{\neq}$ (cm^3.mol^{-1}) and $\Delta S_0^{\neq}$ (J.mol^{-1}.K^{-1}) are the volume and entropy change, respectively, between the native and denaturated states at P_0 and T_0. The model parameters can be estimated using a non-linear regression analysis, involving an iterative numerical procedure based on the minimal sum of squares.

Since the shape of pressure-temperature contour diagrams either for protein denaturation (Hawley, 1971) and for enzyme inactivation (Weemaes *et al.*, 1998b; Van den Broeck *et al.*, 2000a) are mostly elliptical, equation 3.35 can also be used to predict the dependence of the

inactivation rate constant as a function of pressure and temperature (Hashizume *et al.*, 1995; Reyns *et al.*, 2000).

$$\ln(k) = \alpha + \beta(P - P_0) + \gamma(T - T_0) + \delta(P - P_0)^2 + \varepsilon(P - P_0)(T - T_0) + \phi(T - T_0)^2 \qquad (3.35)$$

where α, β, γ, δ, ε and ϕ are model parameters. It can be noticed that equation 3.35 is very similar to equation 3.34. Smeller (2002) proposed a possible modification in the vicinity of the reference point, resulting in the following second-order approximation (Eq. 3.33):

$$T\left(\ln\left(\frac{T}{T_0}\right) - 1\right) + T_0 = \frac{(T - T_0)^2}{2T_0} \qquad (3.36)$$

Equation 3.34 can then be written as a second-degree polynomial (elliptic) equation (Eq. 3.37):

$$\begin{aligned} \ln(k) = \ln(k_0) - \frac{\Delta V_0^{\neq}}{R_T T}(P - P_0) + \frac{\Delta S_0^{\neq}}{R_T T}(T - T_0) - \frac{\Delta\kappa^{\neq}}{2R_T T}(P - P_0)^2 \\ + \frac{\Delta C_p^{\neq}}{R_T T}\frac{(T - T_0)^2}{2T_0} - \frac{2\Delta\zeta^{\neq}}{R_T T}(P - P_0)(T - T_0) \end{aligned} \qquad (3.37)$$

When applying the resulting model it is important to recognize both the power and the limitations of the model. According to Morlid (1981), this equation, in principle, describes the pressure-temperature behavior of most phenomena. One limitation of this equation is that only the difference in thermal expansion, compressibility factor, and heat capacity between denatured and native state are obtained. Also, it should be taken into account that this model (and thus the elliptical shape of the iso-rate P, T contour) results from the fact that the series expansion is truncated after the second-degree terms (Smeller, 2002). Leaving out higher degree terms implies that $\Delta\kappa$, ΔCp and $\Delta\zeta$ are independent of temperature and pressure, and if any of these show pressure or temperature dependence, higher degree terms appear not to be negligible and as a consequence the ellipse can be distorted (Yamaguchi *et al.*, 1995; Smeller and Heremans, 1997). In a recent study on PME inactivation (Ly-Nguyen *et al.*, 2003a,b), third-degree terms were required in the model to accurately describe its pressure-temperature

inactivation kinetics. Therefore, an extended analysis of ln(k) (T,P) is necessary, involving higher order terms (Eq. 3.38).

$$\ln(k) = \ln(k_0) - \frac{\Delta V_0^{\neq}}{R_T T}(P - P_0) + \frac{\Delta S_0^{\neq}}{R_T T}(T - T_0) - \frac{\Delta \kappa^{\neq}}{2R_T T}(P - P_0)^2 + \frac{\Delta C_p^{\neq}}{R_T T}\frac{(T - T_0)^2}{2T_0} - \frac{2\Delta \zeta^{\neq}}{R_T T}(P - P_0)(T - T_0) + \text{higher} - \text{order terms} \quad (3.38)$$

When the following higher-order terms up to the third-degree are introduced in the equation 3.38, equation 3.39 is obtained:

$$\frac{2\Delta \zeta_{2A}^{\neq}}{R_T T}(P - P_0)^2(T - T_0), \qquad \frac{\Delta \kappa_2^{\neq}}{2R_T T}(P - P_0)^3,$$
$$\frac{2\Delta \zeta_{2B}^{\neq}}{R_T T}(P - P_0)(T - T_0)^2, \qquad \text{and} \quad \frac{\Delta C_{2P}^{\neq}}{2R_T T T_0}(T - T_0)^3$$

The subscript "2" refers to the coefficients ($\Delta\kappa$, ΔC_p, $\Delta\zeta$) of the higher-order terms.

$$\ln(k) = \ln(k_0) - \frac{\Delta V_0^{\neq}}{R_T T}(P - P_0) + \frac{\Delta S_0^{\neq}}{R_T T}(T - T_0) - \frac{\Delta \kappa^{\neq}}{2R_T T}(P - P_0)^2 + \frac{\Delta C_p^{\neq}}{R_T T}\frac{(T - T_0)^2}{2T_0} - \frac{2\Delta \zeta^{\neq}}{R_T T}(P - P_0)(T - T_0) + \frac{2\Delta \zeta_{2A}}{R_T T}(P - P_0)^2(T - T_0) + \frac{2\Delta \zeta_{2B}}{R_T T}(P - P_0)(T - T_0)^2 + \frac{\Delta \kappa_2^{\neq}}{2R_T T}(P - P_0)^3 + \frac{\Delta C_{2P}}{2R_T T T_0}(T - T_0)^3 \quad (3.39)$$

The resulting equation (Eq. 3.39) can then be transformed into a linearized equation so that a multi-linear regression analysis can be applied (Eq. 3.40):

$$\ln(k) = a + AX_1 + BX_2 + CX_3 + DX_4 + EX_5 + FX_6 + GX_7 + HX_8 + IX_9 \quad (3.40)$$

where A, B, C, D, E, F, G, H, and I are model fitting parameters and

$$a = \ln(k_{ref}) \qquad X_1 = -\frac{(P - P_0)}{T} \qquad X_2 = \frac{(T - T_0)}{T}$$

$$X_3 = -\frac{(P - P_0)^2}{T} \qquad X_4 = \frac{(T - T_0)^2}{T} \qquad X_5 = -\frac{(P - P_0)(T - T_0)}{T}$$

$$X_6 = \frac{(P - P_0)^2 (T - T_0)}{T} \qquad X_7 = \frac{(P - P_0)(T - T_0)^2}{T} \qquad X_8 = \frac{(P - P_0)^3}{T} \qquad X_9 = \frac{(T - T_0)^3}{T}$$

By using multiple linear regression analysis, multivariate tests across the multiple dependent variables can be performed and the number of significant variables in the model selected. The "forward" selection procedure in the multiple linear regression analysis allows to start with no variables in the model and to calculate the F* statistic for each independent variable, which will reveal if the contribution of the several variables is significant to the model.

As a measure for the quality of the model fitting to the experimental data, the corrected r^2 and the model standard deviation (SD) are also calculated using the equations 3.41 and 3.42, respectively.

$$\text{Corrected } r^2 = \left[1 - \frac{(m-1)\left(1 - \frac{SSQ_{regression}}{SSQ_{total}}\right)}{(m-j)} \right] \tag{3.41}$$

$$SD = \sqrt{\frac{SSQ_{residual}}{(m-j)}} \tag{3.42}$$

Where m is the number of observations, j is the number of model parameters, SSQ is the sum of squares, and SD is the standard deviation. Besides the corrected r^2 and SD, the Mallow's coefficient (C_p) statistic was also computed; since C_p is a measure for the total sum of squared errors, where the error of incorporating all the variables in the best chosen model is taken into account and is defined as (Eq. 3.43):

$$C_p = \left(\frac{SSE_p}{s^2}\right) - (N - 2p) \tag{3.43}$$

where s^2 is the mean error sum of squares for the full model; SSEp is the error sum of squares for a model with p parameters including the intercept; N is the number of observations. The best situation occurs when C_p is close to p.

3.5 Process stability of pectin methylesterase

3.5.1 Thermal stability of pectin methylesterase

The most common procedure to inactivate PME in different food products is by thermal processing at atmospheric pressure. An overview about PME thermal inactivation kinetics is presented in **Table 3.3**. Thermal inactivation kinetics of tomato PME is often described by a first order reaction, even in different systems, e.g., water, buffer or juice. But it must be noticed that the inactivation kinetics of PME in water was described by a first-order model (Van den Broeck *et al.*, 2000b) and by a biphasic model (Crelier *et al.*, 1995). The latter behaviour was attributed to the existence of (at least) two forms of PME, being one more thermostable.

Table 3.3 Kinetic parameters for thermal inactivation of PME from different sources.

Source	System	Kinetic model	T range (ºC)	z-value (ºC)	References
Apple	Citrate-phosphate buffer (pH 4.0)	First order	45-55	9.2	Denès *et al.* (2000a)
Banana	Tris buffer (pH 7.0)	First order	65-72.5	5.89	Ly-Nguyen *et al.* (2002a)
Cucumber	Bis-Tris buffer (pH 6.7) + 1.25M NaCl Tris buffer (pH 7.5)	First order	55-65	5.66 6.57	Guiavarc'h *et al.* (2003)
Carrot	*Situ*	First order	50-95	n.d.	Tijskens *et al.* (1997)
Carrot	Tris buffer (pH 7.0)	Fractional conversion	48-60	n.d.	Ly-Nguyen *et al.* (2002b)
Grapefruit	Tris buffer (pH 7.0)	Fractional conversion	56-62ºC	n.d.	Guiavarc'h *et al.* (2005)
Orange	Juice	Biphasic	60-90	z_1=6.5 Z_2=11	Versteeg *et al.* (1980b)

(Continue on the next page)

Table 3.3 Kinetic parameters for thermal inactivation of PME from different sources.

Orange	Pulp	Biphasic	60-90	z_L=10.8 z_S=6.5	Wicker and Temelli (1998)
Orange	Water	Fractional conversion	60-67	n.d.	Van den Broeck *et al.* (1999a)
Papaya	Citric acid-phosphate buffer: - pH 4.0 - pH 7.5	First order	 55-70 60-75	 8.38 7.8	Fayyaz *et al.* (1995)
Potato	*Situ*	First order		n.d.	Tijskens *et al.* (1997)
Strawberry	Tris-HCl buffer (pH 7.5)	Fractional conversion	54-63	n.d.	Ly-Nguyen *et al.* (2002c)
Tomato	Juice	First order	73-78 73-88 78-88	11.2 15.6 27.8	De Sio *et al.* (1995)
Tomato	Juice	First order	60-75	6.3[a]	Crelier *et al.* (2001)
Tomato	Juice	First order	69.8-77.8	4.8-5.2	Anthon *et al.* (2003)
Tomato	Tris-HCl buffer (pH 7.5)	First order	75-90	23 (PME1) 15 (PME2) 24 (PME3)	Laratta *et al.* (1995a)
Tomato	Puree	Biphasic	60-75	z_L=1.7 z_S=7.5	Crelier *et al.* (1995)
Tomato	Water	Biphasic	60-75	z_L=9.8 z_S=6.2	Crelier *et al.* (1995)
Tomato	Citrate buffer (pH 4.0)	First order	66.4-74.5	5	Lopez *et al.* (1997)

Since food products derived from fruits and vegetables can contain multiple forms and different amounts of each PME form, it is reasonable to expect that these two factors will determine different temperature-time requirements for thermal pasteurisation with respect to PME inactivation. In most studies, the higher molar mass (HMM) form of PME was assigned as the most thermal stable fraction (TS-PME) (Seymour *et al.*, 1991b). However, thermally stable samples of red grapefruit (Cameron and Grohamann, 1995) and from citrus juices tissue culture cells (Cameron *et al.*, 1994) were not characterized by a HMM.

The determination of residual activity of the thermostable form of PME is often required in certain food products submitted to heat treatment. Eagerman and Rouse (1976) determined 1min as the time required for citrus juices PME inactivation at 90ºC, where a conventional log

linear model was applied to describe the heat pasteurisation of citrus juices. Versteeg *et al.* (1980a) showed that time-temperature relationship to inactivate PME in citrus juices was due to the presence of a heat stable PME isoenzyme, which represented 5% of the total activity and was characterized by a D-value at 90ºC of 23 seconds. The presence of a thermal labile fraction (TL-PME) and a TS-PME fraction in citrus juices was confirmed by Wicker and Temelli (1988). These authors reported a D-value at 90ºC of 32 seconds for TS-PME, close to the D-value (30 sec, 90ºC) obtained by Eagerman and Rouse (1976), while Rothschild *et al.* (1975) found 95% of PME inactivation in citrus juices after a heat treatment of 80ºC for 15 seconds.

PME from Navel orange at pH-value of 3.2 was found to accelerate thermal inactivation as compared to water (pH 5.2), whereas at a pH of 3.7 and 4.2 a higher thermostability was observed (Van den Broeck, 1999b). This thermal inactivation increase for citrus juices was already reported by Rothschild *et al.* (1975), Eagerman and Rouse (1976), and Nath and Ranganna (1977). Purified cucumber PME is most thermostable in the pH range of 6.0 to 7.0, but the stability rapidly decreases for pH values lower or higher (Guiavarc'h *et al.*, 2003). Variations of pH conditions may greatly influence the degree to each the enzyme can survive a heat treatment and influence the time-temperature conditions associated to inactivate the enzyme. By exposing juice to extreme pH-values (pH 2.0 and pH 12.0), the labile form could be inactivated in 5 min, but not the stable form (Sun and Wicker, 1996). These authors concluded that pH affected the stability and accessibility of hydrophobic domains to solvent for the thermolabile form of PME from marsh grapefruit. Previously, Seymour *et al.* (1991a) have already reported that TS-PME from grapefruit was characterized by a higher relative amount of hydrophobic amino acids and a higher degree of glycosylation. More recently, Hou *et al.* (1997) found that TS-PME isolated from Valencia oranges was less hydrophobic than TL-PME. These experimental observations, lead to the possibility of the hydrophobic character of PME as being a heat stability promoting factor.

Besides pH, sucrose is another factor that might influence the inactivation of PME. It seems that sucrose possesses a protecting effect on the activity of PME as it acts as a non-competitive inhibitor regarding pectin binding for the active site. It stabilizes the structure of the enzyme

and increases its thermal resistance (Van den Broeck *et al.*, 1999b; Guiavarc'h *et al.*, 2003). The thermal inactivation of PME extracted from Navel oranges slowed down in presence of 20% sucrose whereas a low concentration of sucrose (4%), naturally present in orange juice, stabilized Navel orange PME to a small extent (Van den Broeck *et al.*, 1999b). An environment of lower water activity due to sucrose may be an important factor involved in PME stabilization. The thermal stability of purified tomato PME increased 6-fold in the presence of sucrose when compared to citrate buffer (0.1M). According to Guiavarc'h *et al.* (2003), polyols with the exception of mannitol, have even a greater stabilizing effect than sugars. An increase in concentration of soluble solids in juices also increases the stability of PME (Marshall *et al.*, 1985).

3.5.2 High pressure stability of pectin methylesterase

Pressure has been referred to wield a protective effect on heat inactivation of PME. As for thermal inactivation, pressure inactivation of PME from several sources has also been described in literature. Combined pressure-temperature treatments partially inactivated orange, carrot, banana and graprefruit PME and its inactivation was modeled by fractional conversion kinetics (Van den Broeck *et al.*, 1999a; Ly-Nguyen *et al.*, 2003a,b; Guiavarc'h *et al.*, 2005). An overview of the kinetic models applied to describe pressure inactivation kinetics of PME from different sources is presented in **Table 3.4**.

For both orange PME from commercial source (dissolved in desionised water, pH 4.5) and extracted from pulp (citric acid buffer, pH 3.7), a pressure increase to 700-900MPa at room temperature was required to inactivate the labile fraction. At higher temperatures, the inactivation rate accelerated, indicating the synergistic effect of pressure and temperature (Van den Broeck *et al.*, 2000a). Increasing pressure caused an increase of the inactivation rate constant. In the high-temperature ($T \geq 57°C$) and low-pressure ($P \leq 300MPa$) region, on the contrary, a clearly antagonistic effect of pressure and temperature was observed, from contour diagrams based in kinetic data sets for enzyme pressure-temperature inactivation (**Figure 3.3**).

Increasing pressure caused a decrease of the inactivation rate constant. In **Figure 3.3**, it can also be seen that orange PME is less pressure stable in an acid medium than in water.

Table 3.4 Kinetic parameters for pressure inactivation of PME from different sources.

Source	System	Kinetic model	P/T range (MPa, ºC)	Refences
Banana	Tris buffer (pH 7.0)	Fractional conversion	0.1-900, 30-76ºC	Ly-Nguyen *et al.* (2003a)
Carrot	Tris buffer (pH 7.0)	Fractional conversion	600-700, 10ºC	Ly-Nguyen *et al.* (2002b)
Orange	Juice	First order	100-400	Basak and Ramaswamy (1996)
Orange	Water	Fractional conversion	600-900, 20-30ºC	Van den Broeck *et al.* (1999b)
Grapefruit	Tris buffer (pH 7.0)	Fractional conversion	0.1-800, 10-62ºC	Guiavarc'h *et al.* (2005)
Strawberry	Tris buffer (pH 7.0)	Fractional conversion	850-1000, 10ºC	Ly-Nguyen *et al.* (2002c)
Tomato	Puree	First order	400-800, 30-75ºC	Crelier *et al.* (1995)
Tomato	Water	First order	100-900, 40/60ºC	Van den Broeck *et al.* (2000b)
Tomato	Citrate buffer (pH 4.4)	First order	600-650, 50ºC	Fachin (2003)
Tomato	Juice	First order	550-700, 50ºC	Fachin (2003)

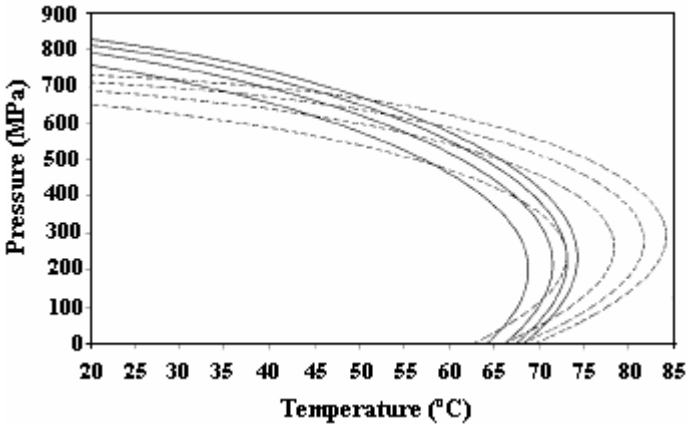

Figure 3.3 Predicted pressure-temperature kinetic diagram for the P/T inactivation of commercial orange peel PME in deionized water (full line) and extracted orange PME in buffer pH 3.7 (dashed line), based on parameters estimated by equation 3.15. The inner and outer lines represent P/T combinations for which k = 0.02 and 0.08 min^{-1}, respectively (adapted from Van den Broeck *et al.*, 2000a).

Purified thermolabile carrot PME (Ly-Nguyen *et al.*, 2003b) exhibited an antagonistic effect even at lower temperatures (<300MPa, >50ºC) when compared to orange PME, while the

same effect was observed for purified thermolabile PME from banana (≤300-400MPa, ≥64ºC) and grapefruit (<300 MPa, ≥58ºC) at higher temperatures (Ly-Nguyen *et al.*, 2003a; Guiavarc'h *et al.*, 2005). Also, Fachin *et al.* (2002) observed an antagonistic effect of pressure and temperature when they treated tomato PME both in juice and in purified form, at different combinations of pressure and temperature.

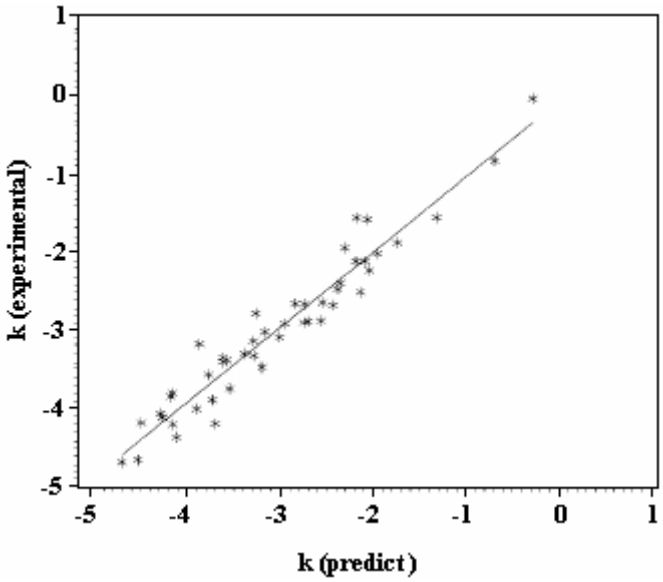

Figure 3.4 Correlation between the k values of extracted orange PME, determined from experimental work, and the k-values estimated using the model described in equation 3.15.

Using equation 3.15, the inactivation data of commercial orange peel PE in water as well as extracted orange PE in citric acid buffer pH 3.7 could be accurately fit. To analyze the quality of parameter estimation, a plot of experimental k-values versus k-values calculated using the estimated parameters was made (**Figure 3.4**). The divergence from the bisector can be seen as an indicator for the accuracy of the model and its parameters; that is, the more the calculated and estimated values mutually differ, the less successful the model is. A satisfactory correlation between these values was found.

Threshold pressures for inactivation of PME from different sources as a function of the media in which the inactivation is carried out have been reported to vary from 150 to 1200MPa. For example, the use of pressure cycles at 800MPa and 30ºC did not affect tomato PME activity until the submission of five cycles (Crelier *et al.*, 1995). Most researchers found only partial inactivation of PME during pressure treatments, and ascribed this behaviour to the existence of a pressure stable PME form, similar to the behaviour previously mentioned for thermal

inactivation – the presence of (at least) one thermostable isoenzyme. Since pressure pasteurized food products, like fruit and vegetable juices and pieces, are treated at pressures (around 500MPa) a certain amount of PME will still remain active in the processed product. Therefore, the determination of the remaining PME activity is of importance since during storage, PME will promote demethylation of pectin, which can then cross-link with Ca^{2+} ions present. In fruit and vegetable juices is very common to observe turbidity loss, usually called cloud loss, resulting in a two phase system, a sediment phase and a colourless and odourless liquid phase. Obvious decreases in quality as well as in commercial value will occour in these products.

If cloud loss in juice caused by the remaining activity of PME fraction is to be avoided, orange juice should be transported and stored under refrigerated conditions (Ogawa *et al.*, 1990; Donsí *et al.*, 1996). Ogawa *et al.* (1990, 1992) reported that even though no complete inactivation of orange PME was obtained, there was no reactivation of the enzyme under the refrigerated conditions. According to Ogawa *et al.* (1990), orange PME seemed to be protected from pressure inactivation by an increase in the soluble solids content.

3.5.3 Activity of pectin methylesterase at elevated pressure

Controlled activity of texture related enzymes, namely PME and PG, during (pre)processing can be useful to improve food products texture, i.e., tomato based products. Either thermal or combined thermal/pressure processing can induce both PME and PG activation. At atmospheric pressure, purified tomato PME is most active at pH 7.2 and 55ºC, whereas at higher pressures (100-300MPa) at the same pH, the optimal temperature shifts to higher values (60 to 65ºC) (Van den Broeck *et al.*, 2000b). At temperatures higher than 55ºC, where PME inactivation starts at ambient pressure, PME activity reaches a maximum at 100 and 200MPa; while at higher pressures, up to 300MPa, the activity decreases but is still higher than at atmospheric pressure. Verlent *et al.* (2004a) during similar experiments with tomato PME at its natural pH (around 4.4) and 8.0, concluded that at atmospheric pressure, the optimal temperature for the enzyme activity was around 35ºC and 45ºC, respectively. An enhancing

effect of pressure on the enzyme activity was observed for all the temperatures tested: a higher enzymatic activity was observed at elevated pressure than at atmospheric pressure. Other researchers also obtained an increase in PME activity at 400MPa and 45ºC (Shook *et al.*, 2001).

3.6 Process stability of polygalacturonase regarding pectin methylesterase stability conditions

It is also of interest to shortly point out the thermal and pressure stability of polygalacturonase, a pectin de-polymerizing enzyme, co-existing with PME in most fruits and vegetables. PG is present in tomato fruit in at least two forms, namely PG1 and PG2 (Pressey and Avants, 1971), which differ in size and heat stability, being PG2 named heat labile and PG1 heat stable (stable at 65ºC for 5min) (Fachin, 2003). For complete inactivation of PG in tomato juice, the sample must be treated at 93ºC for 3min (Crelier et al, 2001). Fachin *et al.* (2003) reported 14% residual activity of PG when tomato juice was treated at 70ºC during 60 minutes. This enzyme could only be completely inactivated at temperatures above 90ºC.

As for thermal inactivation, it should be mentioned that opposite to PME, PG appeared to be a pressure labile enzyme. PG in tomato juice can be completely inactivated at some rather low pressure-temperature combinations, e.g. 550MPa-20ºC (Fachin *et al.*, 2003). When tomato PG was submitted to 500MPa-60ºC (Crelier *et al.*, 1999) and 800MPa-25ºC (Shook *et al.*, 2001) total enzyme inactivation was obtained. But Tangwongchai *et al.* (2000) only obtained partial inactivation after 500MPa and 20ºC.

Verlent *et al.* (2004b) investigated the enzymatic reaction of purified tomato PG on polygalacturonic acid (PGA) as a substrate during combined high pressure/temperature treatment in a temperature range of 25ºC to 80ºC and in a pressure range of 0.1 to 500MPa at pH 4.4. The optimal temperature for PG-catalyzed hydrolysis of PGA shifted to lower values at elevated pressure when compared to atmospheric pressure (55-60ºC). At constant temperature, the enzymatic activity decreased with increasing pressure, and was more

pronounced at higher temperatures. At 500MPa, no PG activity was detected at any temperature tested. At elevated temperatures, no PG activity was observed, even at lower pressure levels.

Due to the very different stability to pressure of both PME and PG, it seems to be possible to pressure process food products, in order to obtain selective (in)activation, that is, to have PME active while PG is inactivated. In this way, de-methylation of pectins can occur but not pectin de-polymerization. The low-methoxyl pectin chains can still crosslink in the presence of divalent ions, such as Ca^{2+}. This can be used to improve texture of fruits and vegetable products. Regarding tomato-based products at pH 4.4, and comparing results of both endogenous pectin degrading enzymes, PME and PG, PME activity is almost optimal at 400MPa and 55ºC while PG shows no activity (Verlent *et al.*, 2006).

3.7 Conclusions

Although thermal treatment is efficient in enzymes and microorganisms inactivation, it can cause detrimental changes in nutritional and sensorial attributes of food (flavor, texture, color). High pressure processing can also destroy microoganisms and inactivate enzymes, but causes less damaging effects on nutritional and sensorial attributes of food. This potential has been the driven force for food related high pressure research and the development of new non-conventional food products.

Mild pressures, within the range of 100-300MPa, can induce reversible changes in proteins, including limited conformational changes of monomers and oligomers dissociation. Higher pressure levels can cause irreversible changes, such as complete denaturation, secondary structure alteration and aggregates formation. Pressure combined with (high) temperature can act both antagonistically and synergistically depending on the enzyme under study and the environmental conditions, like pH.

PME is a fairly thermostable enzyme and PME activity can still be found at relative high temperatures. Since PME is also a rather pressure stable enzyme, its inactivation requires combined temperature-pressure treatments. Besides, through controlled activation of PME and inactivation of PG, a better texture of fruits and vegetables can be obtained. Thermal, pressure and combined thermal-pressure treatments can promote PME controlled activity while avoiding PG activity, opening possibilities to enhance texture of vegetable-based products.

The work presented in this thesis is the first reported study related with the purification and characterization of PME from sweet bell peppers, as well as its thermal, pressure and combined thermal-pressure stability and activity. Kinetic data related with PME (in)activation as well as the related models were also studied to describe the effect of temperature, pressure and combined temperature-pressure on sweet bell pepper PME activity and stability.

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CHAPTER FOUR

Screening quality related parameters of pepper (*Capsicum annuum*) upon different pressure and temperature conditions

High pressure treatments as a possible alternative to the thermal blanching of bell pepper fruit (*Capsicum annuum* L.)

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ABSTRACT

The effect of pressure treatments of 100 and 200MPa and thermal blanching, in conditions carried out at industrial level, on quality of red and green bell peppers was compared. Pressure treated peppers showed a lower reduction on soluble protein and ascorbic acid contents, with red peppers presenting even an increased content of ascorbic acid, compared to the unprocessed samples, up to 20%. Peroxidase and pectin methylesterase showed a higher stability to pressure, particularly the latter enzyme, while polyphenol oxidase was inactivated to the same final level by thermal blanching and pressure treatments. The washing step with chlorine solution prior to the blanching and pressure treatments is of fundamental importance to achieve microbial conformity of processed peppers consumption. Pressure treated green and red peppers presented a better firmness before and after tunnel freezing at -30ºC and upon thawing, compared to thermally blanched peppers at 80ºC and 98ºC. Red peppers showed a higher sensitivity to lose firmness than green peppers. In general, the quality parameters of green and red peppers had a different behaviour to thermal blanching and pressure treatments. The results indicate that pressure treatments of 100 and 200 MPa have good potential to be used as alternative to the thermal blanching of red and green bell peppers, yielding peppers with, globally, better quality attributes.

Keywords: *Capsicum annuum*, heat and pressure treatments, ascorbic acid, enzymes, texture

INTRODUCTION

Fruit peppers have grown in popularity in recent years, mainly due to their occurrence in a wide variety of colours, shapes, and sizes and their characteristic flavour (Lucier and Lin, 2001). Native from the Americas, pepper is a Solanaceous fruit belonging to the same family as tomato, potato and eggplant, with most pepper varieties belonging to the *Capsicum annuum* L. species and almost all sweet peppers turn from green to yellow, orange, red, or purple as the ripening process proceeds.

Peppers are mainly used to produce dehydrated products (such as paprika), pickled peppers, and sliced or diced frozen peppers to be used in pizzas and other frozen foods or to be eaten raw, as salads. The demand for sliced and diced frozen peppers has been increasing considerably in the last years, due to consumers' willingness to eat raw, minimally processed vegetable products, as part of healthier food habits.

Prior to freezing, vegetables are subjected to thermal blanching whose main purpose is to inactivate deleterious enzymes and reduce the microbial load (Cano, 1996), since many of the quality changes that occur during distribution and storage of vegetable-based foods, are due to detrimental reactions catalyzed by enzymes, such as peroxidase, polyphenol oxidase, lipoxygenase, and pectin methylesterase (e.g., Versteeg et al., 1980; Williams et al., 1986; McEvily et al., 1992; Bahçeci et al., 2004). However, heating causes also losses of sensorial (texture, taste, flavour, and colour) and nutritional (vitamins/nutrients) quality attributes (Lund, 1977), such as reduction of ascorbic acid content (Laing et al., 1978; Lathrop and Leung, 1980; Rao et al., 1981; Howard et al., 1994). For this reason, it is desirable to keep

heat treatment conditions at a level strictly sufficient to cause inactivation of the deleterious enzymes and adequate microbial load reduction, to minimize quality losses. This is particularly relevant for frozen sliced or diced peppers intended to be eaten raw, since excessive thermal blanching causes considerable deleterious effects on texture, which is particularly important for this product due to its characteristic texture properties, namely firmness and crispness. This has been a driven force for the pepper processing food industry to seek for other processing methodologies or technologies that can substitute the conventional thermal blanching, but cause less damaging effects on fruit pepper quality.

In the last 15-20 years, high pressure processing has been increasingly explored and used to process foods, to destroy microorganisms and inactivate enzymes, with minimal deleterious effects on quality (Ogawa et al., 1990; Cheftel, 1991; Knorr, 1993; Anese et al., 1995; Seyderhelm et al., 1996; Hendrickx et al., 1998; Torres and Velazquez, 2005). A considerable number of high pressure pasteurized food products are already commercially available worldwide, including fruit juices and fruit salads (Torres and Velazquez, 2005).

The aim of this work was to evaluate the possible use of high pressure treatments to substitute the conventional thermal blanching applied to bell pepper fruit prior to freezing, to obtain a final product with better quality. To compare both processes the following parameters were quantified: soluble protein content, the activity of the enzymes peroxidase, polyphenol oxidase and pectin methylesterase; ascorbic acid content, firmness, microbial load (total aerobic mesophiles, Enterobacteraceae, total and faecal coliforms, and presence of *Escherichia coli*). These are usual parameters quantified at industrial level, to evaluate the adequacy of thermal blanching, of peppers fruits. The effect of freezing on firmness, as carried out at industrial

conditions to produce commercial frozen peppers, was also evaluated for both thermally blanched and high pressure treated peppers. This study was carried out with peppers in two different colour stages: green and red. Physicochemical characterization of the peppers used was also carried out.

MATERIALS AND METHODS

Materials. Green and red bell peppers (*Capsicum annuum* L.) were supplied by a local company. The fruits were harvested, brought to the company and immediately taken to the laboratory, where they were stored at 4±1ºC, until further use. All chemicals used in this study were of analytical grade.

Physicochemical analysis. *Dry matter* and a*sh content.* One gram of homogenised pepper fruit was dried until constant weight was obtained, first at 70ºC (about 3hours) and, subsequently, at 105ºC (about 16hours) to quantify the dry matter (adapted from AOAC, 1990). Afterwards, the dried fruit pepper residue was burnt in a muffle at 525ºC for 16hours and the residue weighted to determine the ash content (adapted from AOAC, 1990).

pH, titratable acidity, and soluble solids. Pepper juice was extracted from a 10g sample with an Ultra-Turrax (T25, IKA-Labortechnik), followed by centrifugation (10000g, 10min), at 4ºC. The supernatant was recovered for pH, titratable acidity, and soluble solids measurements. The pH was measured at 20ºC. Titratable acidity was determined by titration with 0.1N NaOH until pH 8.1 was reached and reported as g citric acid/100 g fresh weight.

Soluble solids content was determined at 20ºC with a refractometer and reported as ºBrix. All assays for the physicochemical analysis were performed in triplicate.

Protein content. The protein content (g protein/100g fresh weight) was quantified using the Folin-Lowry method (Lowry et al., 1951) and bovine serum albumin (BSA) as standard.

Ascorbic acid. Five grams of pepper fruit were homogenised with 50mL of 4% (w/v) solution of metaphosphoric acid for 15min. The mixture was filtered and diluted to 100mL and divided into several aliquots. The aliquots were then frozen in liquid nitrogen and stored at -20ºC until quantification of ascorbic acid content (AsA). All these operations were carried out with protection from light, using aluminium foil, to avoid oxidation of AsA. An aliquot of AsA extract was thawed and filtered through a 0.45µm millipore filter prior to injection onto the chromatographic column. AsA was determined based on the method described by Daood et al. (1994). The HPLC apparatus used consisted of an L-6200A pump, with a 20µL injection loop and a L-4250 UV-Vis detector, with a D-2500 Chromato-integrator. The column was a LiChrosorb 100 RP-18 column (250×4.6mm), with particle size of 5µm (Merck). The mobile phase was constituted by 0.1M phosphate and methanol (97:3), containing 0.75mM ammonium tetrabutylhydroxide at pH 2.75, and a flow rate of 1.0mL/min was used. The detection was performed at 254nm for AsA, which was first identified and further quantified by comparing retention time, absorption spectra and peak areas with those of AsA standard (Sigma).

Enzymatic activity. *Crude extract preparation*. Fifty grams of thermal and pressure treated and unprocessed pepper samples were thawed at 4ºC, homogenized with 75mL of 0.2M

sodium phosphate buffer (pH 6.5), a relatively low ionic strength buffer, and 4% (w/w) polyvinylpyrrolidone (PVP) in a Warring blender, followed by agitation during 1h at 4ºC. The homogenate was filtered through several layers of cheesecloth and then centrifuged (10000g, 20min) at 4ºC. The supernatant, further designated enzymes soluble fraction (SF) extract was collected and the precipitate was re-suspended in 30mL of 0.2M sodium phosphate buffer (pH 6.5), followed by agitation for 30min, and extraction performed again alike, to ensure that all enzymes from SF were extracted. The resulting pellet was mixed with 50mL of 0.2M sodium phosphate buffer (pH 6.5) with 1M NaCl for 2h, a high ionic strength buffer, in order to obtain the enzymes ionically-bound fraction (IF) extract. Extraction was carried out twice as described for the SF. All the extracts were divided into aliquots, frozen in liquid nitrogen, and stored at -20ºC until quantification of the enzymatic activities. All procedures to obtain the enzymatic extracts were carried out at 4ºC and the enzymatic activities were determined in triplicate. No activity was found for the enzymes studied in the second extraction of SF and IF, revealing that first extraction was complete in both cases. Protein content was quantified using the same extracts used for quantification of enzymatic activities and no protein was found for the second extraction of SF and IF, revealing that the first extraction was also complete. Total enzymatic activity for each enzyme and total protein content, was calculated as the sum of the values obtained for SF and IF fractions. The standard deviation, S_T, for the total amount of enzymatic activity for each enzyme and protein content, was calculated by $S_T = \sqrt{S_{SF}^2 + S_{IF}^2}$ (Miller and Miller, 2000), where S_{SF} and S_{IF} are, respectively, the standard deviation obtained for the SF and IF fractions. All enzymatic determinations were performed in triplicate.

Polyphenol oxidase. A spectrophotometric assay using cathecol as substrate at pH 6.5, was used to quantify polyphenol oxidase (PPO) activity, as described by Weemaes et al. (1997), using a 6405 UV/Vis JenWay spectrophometer. The reaction mixture consisted of 1000µL of substrate at 25ºC and 40µL of enzyme extract. PPO activity was determined from the slope of the linear portion of the curve, relating absorbance at 411nm with time, and was expressed as $\Delta Abs_{411}.min^{-1}.(100g\ fresh\ weight)^{-1}$.

Pectin methylesterase. Activity determination was carried out according to the method described by Hagerman and Austin (1986). In order to achieve a constant starting pH for the reaction, all the solutions (pectin, indicator dye and water) were adjusted to pH 7.5 with 2M NaOH. The enzymatic extracts were also adjusted to pH 7.5 with 0.5M NaOH. Four mL of citrus pectin solution (0.5%, w/v) were mixed with 300µL of bromothymol blue (0.01%, w/v), and distilled water and enzyme extract, up to 6mL. Decrease of absorbency was recorded at 620nm, at 25ºC and pectin methylesterase (PME) activity was expressed as $\Delta Abs_{620}.min^{-1}.(100g\ fresh\ weight)^{-1}$. The initial absorbency (around 0.28) remained constant until the enzyme was added, indicating that no reaction occurred in the absence of the enzyme.

Peroxidase. Activity was determined based on the method described by Worthington (1978). The substrate solution was composed by hydrogen peroxide (0.975mM), phenol (83.1mM) and 4-aminoantipyrine (1.19mM) in 0.1M sodium phosphate buffer (pH 7.0). To 1.450mL of daily prepared substrate solution, incubated at 25ºC, 50µL of enzymatic extract were added and the increase in absorbency recorded at 510 nm. The slope of linear portion of the curve

relating absorbance at 510 nm with to time was used to calculate the enzyme activity, expressed in $\Delta Abs_{510}.min^{-1}.(100g\ fresh\ weight)^{-1}$.

Texture measurements. The texture of the peppers was measured using a texture analyser (TA-HDplus, Stable Micro System) with a 7 mm diameter hole, and the following parameters: 5kg force load cell, 2mm diameter aluminium cylinder probe, and $2.0mm \cdot s^{-1}$ test speed. The property "firmness" (hardness), the maximum force applied to puncture the pepper tissue, was measured as an indicator of texture, which is very similar to the one performed by mastication that takes part during eating. The measurements were done on both sides of the pepper tissue that is, from the skin and the flesh sides. Rupture of the skin from the flesh side required a lower force when compared with the same action from the skin side. An average value of firmness from 5 puncture measurements (skin and flesh sides) was calculated for each experimental condition. Texture analysis of pepper pre-treated samples was carried within one hour after the pre-treatments have been applied, and the samples were kept at 4 ºC during this period. All the measurements were conducted at room temperature. The results are presented as relative firmness (%), calculated from the ratio between the firmness of the control, untreated sample and the thermally blanched or pressure treated samples.

Microbial analysis. For the microbiological study, the pepper samples were washed with a chlorine solution (5ppm), before being submitted to thermal blanching and pressure treatments. Quantification of total aerobic mesophiles, Enterobacteraceae, total and faecal coliforms, and presence of *Escherichia coli*, are the most important microorganisms quantified, to assess microbial conformity of minimally processed ready-for-use fresh vegetables and vegetable salads or raw foods of vegetable nature ready-for-use (French

legislation is an example - Arrêté du 22 mars, 1993). These same microorganisms were quantified in this work, by the most probable number (MPN) method. An amount of 10g of pepper sample was weighted under aseptic conditions, 90mL of sterile 0.1% peptone water added, and the mixture was homogenised in sterile bags using a Stomacher (Model 400, Laboratory blender) for 2.5min. All decimal dilutions were prepared from this homogenate. Counts of total aerobic mesophiles were obtained by incubation in tryptophane broth (TB) (Oxoid, Hampshire, UK) at 30ºC until 72 hours. For the quantification of Enterobacteraceae, serial dilutions were seeded in glucose broth (GB) at 37ºC for 24 hours. Both total and faecal coliforms were quantified by seeding graded dilutions of the samples in lactose broth (LB) and incubating at 30ºC and 37ºC, for total coliforms and faecal coliforms, respectively, for 24 hours. Presence of *Escherichia coli* was also investigated, by checking the capacity of faecal coliforms to produce indol, using tergitol-BCIG (5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid) by incubation at 44ºC for 24 hours.

Thermal blanching and pressure treatments. The pepper samples were cut in slices of 15×75mm and placed in a plastic bag that was heat sealed. Temperature, pressure and time duration of the thermal blanching and pressure treatments studied are shown in **Table 1**. Temperatures and time duration of the thermal blanching treatments were the same as those typically used for the industrial production of frozen pepper: for very ripened peppers, with lower firmness, the temperature of 70ºC is applied, while for ripened peppers, from different varieties with medium to high firmness, temperatures of 80ºC and 98ºC are, respectively, applied. Time duration of the treatments (1 or 2.5min) is used additionally to better adjust, at

each temperature, the applied treatment to the precise firmness stage of the peppers, with 1 min being used for lower firmness peppers and 2.5min for higher firmness peppers.

Table 1. Temperature, pressure and time conditions used for thermal blanching and pressure treatments of green and red peppers.

Sample code	Operating conditions
C	Control, unprocessed sample
BI.1	Blanching at 70ºC for 1min
BI.2	Blanching at 70ºC for 2.5min
BII.1	Blanching at 80ºC for 1min
BII.2	Blanching at 80ºC for 2.5min
BIII.1	Blanching at 98ºC for 1min
BIII.2	Blanching at 98ºC for 2.5min
PI.1	Pressurizing at 100MPa for 10min
PI.2	Pressurizing at 100MPa for 20min
PII.1	Pressurizing at 200MPa for 10min
PII.2	Pressurizing at 200MPa for 20min

The lowest blanching temperature (70ºC) is less frequently used, particularly to produce frozen peppers intended to be eaten raw, because peppers are not usually blanched at a maturation stage in which they show already appreciable loss of firmness. Nevertheless, this blanching temperature was studied to evaluate the effect of a low, less damaging, blanching temperature on pepper quality. The packaged samples were immersed in a thermostated water bath (Grant, Y28), pre-set at the adequate temperature, for 1 or 2.5min, cooled immediately in a water bath at 4ºC for 5min, and then equilibrated at room temperature for the texture measurements.

For the pressure treatments, the pepper samples were packed in a second plastic bag, which was heat sealed under vacuum. Pepper samples were then pressurized using an Autoclave Engineers (Erie, Pa., USA) isostatic press (Model IP3-23-30). Samples were placed in the cylindrical pressure chamber (i.d. 76mm, height 610mm), which contained a pressure medium consisting of water, containing 2% hydraulic fluid (Hydrolubric 142; E.F. Houghton and Co., Valley Forge, Pa., USA), pressure was build up at room temperature (18-20ºC), up to 100 or 200MPa and maintained during 10 or 20min, followed by decompression. Pressure build-up and decompression took 15s each. After decompression, texture measurements were carried out.

For determination of soluble protein content, enzymatic activities, microbiological analysis, and determination of ascorbic acid content, thermally blanched and pressure treated peppers were frozen in liquid nitrogen and stored frozen at -20ºC until used.

Effect of freezing on firmness. To carry out the freezing experiments, thermally blanched and pressure treated peppers were kept at 4ºC, transported to the company and frozen in a tunnel freezer at -30ºC, with an air-blast frozen system, following the same procedure used to freeze commercial peppers. The frozen samples were stored at -20ºC and prior to the texture measurements, the samples were thawed and equilibrated at room temperature

Data analysis. ANOVA and bilateral Tukey´s test were carried out to determine significant differences ($P < 0.05$).

RESULTS AND DISCUSSION

Physicochemical analysis. *Dry matter* and *ash content.* Moisture content is within the range (89.4-94.7%) reported in literature for pepper fruits (Guil-Gerrero et al., 2006). The moisture content of green peppers (94.5±0.4%) is significantly ($P < 0.05$) higher than that of the red peppers (92.8±0.2%) (**Table 2**), which is also in agreement with results reported by Luning et al. (1994a,b) and Roura et al. (2001). The ash content (**Table 2**) is within the range (1.02-2.07%) found by Guil-Gerrero et al. (2006) for several pepper fruit varieties.

Table 2. Physicochemical parameters (±standard deviation, n=3) of green and red bell peppers.

Physicochemical parameters	Green	Red
Moisture (%)[a]	94.5±0.4	92.8±0.2
Ash content (%)[a]	1.38±0.01	1.33±0.02
pH	4.65±0.01	4.81±0.01
Titratable acidity (g/L of citric acid)	10.0±0.5	26.1±0.9
Soluble solids (ºBrix at 20ºC)	5.27±0.01	6.01±0.01

[a] % on fresh weight basis.

pH, titratable acidity, soluble solids. The pH was lower than the values found for Dutch sweet pepper (pH 5.6) and Padrón ($5.01 < pH < 6.37$) varieties (Estrada et al., 2000; Castro et al., 2005), but in agreement with the values reported for pH of the New Mexican-type variety, (5.4 to 4.6) (Biles et al., 1993) and significantly ($P < 0.05$) higher for red pepper (**Table 2**). Titratable acidity increased ($P < 0.05$) about 2.5 fold from green to red peppers (**Table 2**), results that are similar to those reported by Luning et al. (1994b). According to these authors, this increase in titrable acidity could be caused by an increment of the concentration of

protonated organic acids. The amount of soluble solids (**Table 2**) found in this work was within the range reported by Guil-Gerrero et al. (2006) and significantly ($P < 0.05$) higher (about 14%) for red pepper.

Protein content. Total and SF protein contents were not significantly different ($P > 0.05$) for unprocessed green and red peppers, while protein content of IF was higher ($P < 0.05$) for untreated red peppers (**Figure 1**). The protein content of the IF constituted about 1% of the total amount of protein of green peppers and almost 10% for red peppers. Blanching treatments reduced the soluble protein content by about 15% to 60% in green pepper and 15% to 35% in red pepper, an effect that increased progressively with the increment of the blanching temperature, which indicates a progressively higher protein thermal denaturation. Pressure treated green peppers showed a decrease in soluble protein content of about 32 to 45%, a reduction that was lower than that obtained with blanching at 80ºC and 98ºC, and higher than that obtained with 70ºC. Pressure treated red peppers showed no variation (10min, $P > 0.05$) or an increase of the protein content (20min, $P < 0.05$) compared with the control sample, results that are clearly better than those observed for the blanching treatments. These results show that pressure treatments caused a lower reduction on protein solubility, an effect that was particularly noticeable for red peppers, indicating a higher possibility on protein bioavailability and a higher protein value of pressure treated peppers when compared to blanched peppers. Globally, the protein content of red peppers was less affected by both blanching and pressure treatments, indicating an effect of the colour stage.

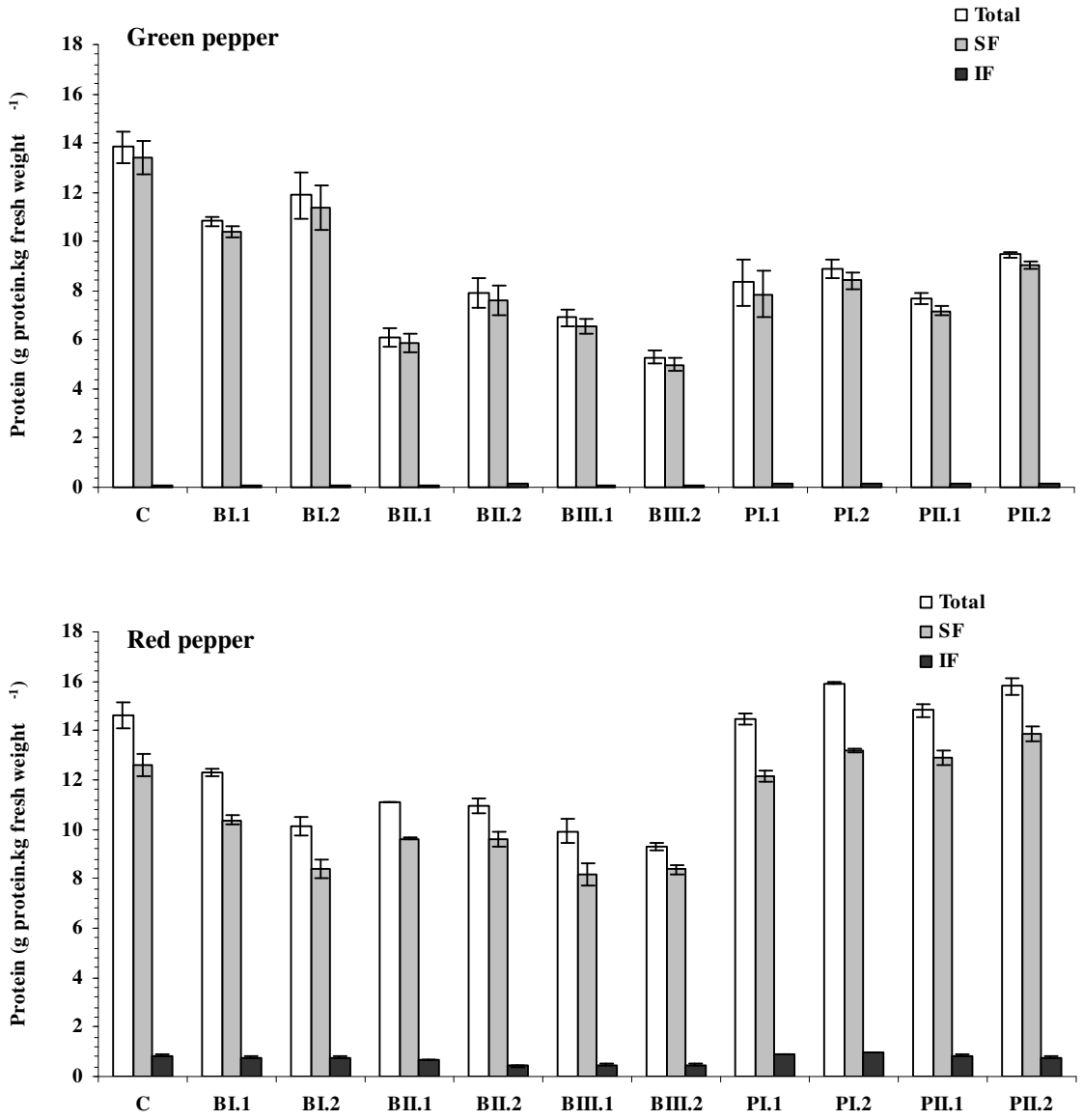

Figure 1 Protein (g protein.kg fresh weight^{-1}) of the soluble fraction (SF), ionically-bound to the cell wall fraction (IF), and total content (SF + IF), measured for unprocessed, thermally blanched, and pressure treated green and red peppers. The bars represent the standard deviation (n = 3).

Ascorbic acid. AsA content increased significantly ($P < 0.05$) from green to red peppers (**Figure 2**), a result that is in accordance with previous studies (Rhaman et al., 1978; Howard

et al., 1994; Osuna-Garcia et al., 1998; Guil-Gerrero et al., 2006). AsA content found for both green (88.5±1.5mg/100g fresh weight) and red peppers (107.4±2.3mg/100g fresh weight) are within the ranges found in other studies, for green (12-180mg/100g fresh weight - Luning, 1995; Simonne et al., 1997; Yahia et al., 2001; Castro et al., 2003) and red peppers (75 to 277mg/100g fresh weight - Lee et al., 1995; Howard et al., 1994; Simmone et al., 1997; Osuna-Garcia et al., 1998). These results confirmed peppers as a good source of AsA, with 100g exceeding the Recommended Daily Allowance (RDA) of 60 mg (Carr and Frei, 1999) by, respectively, 48% and 79%, for green and red peppers.

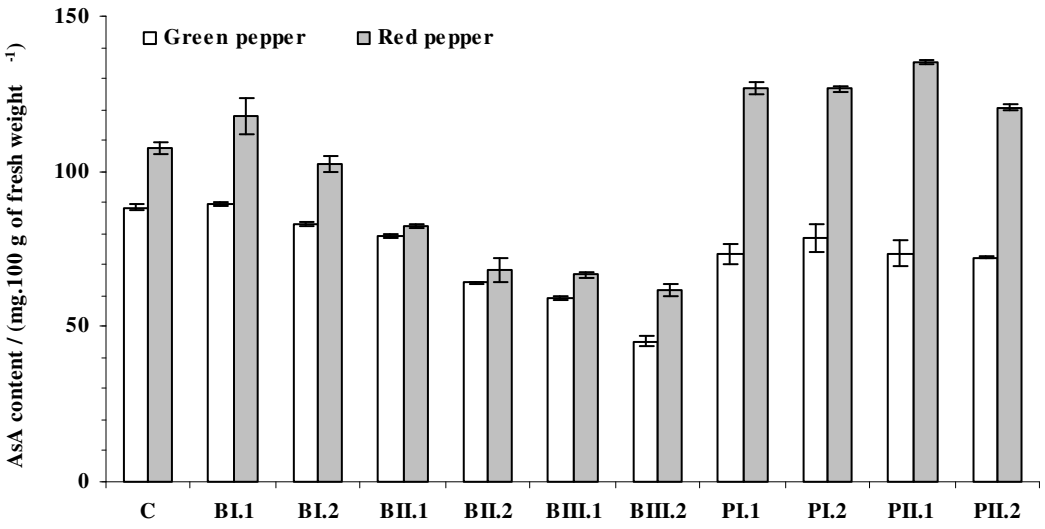

Figure 2 Effect of thermal blanching and pressure treatments, on AsA content (mg of AsA.100g fresh weight^{-1}) of green and red peppers. The bars represent the standard deviation (n = 3).

AsA content decreased progressively as blanching conditions were more severe (higher temperature and longer treatment time duration), being this effect particularly important for

treatments BII.2, BIII.1, and BIII.2, where it reached a value of about 45% and 30% for green and red peppers, respectively. Jalapeño pepper cultivar blanched prior to pasteurization lost 75% of its AsA (Howard et al., 1994), while for the same variety, Saldana and Meyer (1981) found that AsA was well retained, after blanching and pasteurization. Matthews and Hall (1978) reported a 40% loss of AsA during water blanching of green peppers. Differences in ascorbic acid retention may be attributed to differences in genetics, maturity stage, brine composition, blanching method, and pasteurisation time and temperature (Lee and Howard, 1999), that can cause different degrees of inactivation of ascorbic acid oxidase and removal of residual oxygen from vegetable tissue (Selman, 1994). For example, retention values for AsA obtained from water blanched peas could be improved if samples were packaged without water (Quaglia et al., 1996).

Pressurized green bell peppers showed a decrease of about 15-20% of AsA content (**Figure 2**), while rep peppers showed an increase of about 10-20% ($P < 0.05$). Orange juice showed no substantial modification in the composition of AsA when pressurized at 500MPa (Donsí et al., 1996; Garcia et al., 2001). Even higher pressure levels (900MPa) resulted in a higher retention of AsA (82%) of peas, when compared to water blanching (12%) (Quaglia et al., 1996). The reason for the augmentation of AsA in pressurized red peppers is unknown, but may be related to an increased extractability, that might result from the pressurization process (Sancho et al., 1999), to a higher stability to pressure compared to temperature, to other components present, e.g. oxidation inhibitors, which can inhibit the oxidation of ascorbic acid (Lee et al., 1995), or to the colour stage, since degradation of AsA also depends greatly on the food matrix (Van den Broeck et al., 1998; García et al., 2001). Globally, pressurized green bell peppers showed

a similar to higher retention of AsA for green peppers when compared to the blanching treatments of 80ºC and 98ºC, respectively, while pressurized red peppers showed a higher content, around 50-100% when compared to the same treatments. Therefore, AsA was clearly better retained in pressurized red peppers than in green peppers, indicating an effect of the colour stage, as already observed for the soluble protein content.

Enzymatic activity. *Polyphenol oxidase*. PPO activity of green peppers was found to about 50% higher than that of red peppers due to a decrease of about a half of the activity of SF extract of red peppers (**Figure 3**). SF extract represented 85% and 70% of total activity for green and red peppers, respectively. PPO activity in eggplant was also found to be mostly in the SF (Concellón et al., 2004). Also, during ripening of olive fruit, PPO activity was found to increase which could be related with the blackening process of this fruit (Saraiva et al., 2007). A gradual decrease in PPO activity during medlar fruit development was also observed, followed by an increase during the ripening period (Aydin and Kadioglu, 2001).

PPO was found to be more stable to both thermal blanching and pressure treatments in red peppers (particularly PPO of the SF), with pressure treatments having no effect on total activity, and treatments BII.2, BIII.1, and BIII.2 causing a progressive decrease on activity up to 25% (**Figure 3**). For green peppers, PPO activity showed, generally, a progressive decrease on activity (from 25% to 75%), from blanching treatment BI.1 to BIII.2, while all pressure treatments caused a decrease of activity of about 50%. Overall, PPO activity in SF extract of green and red peppers was more sensible to blanching and pressure treatments than in IF extract.

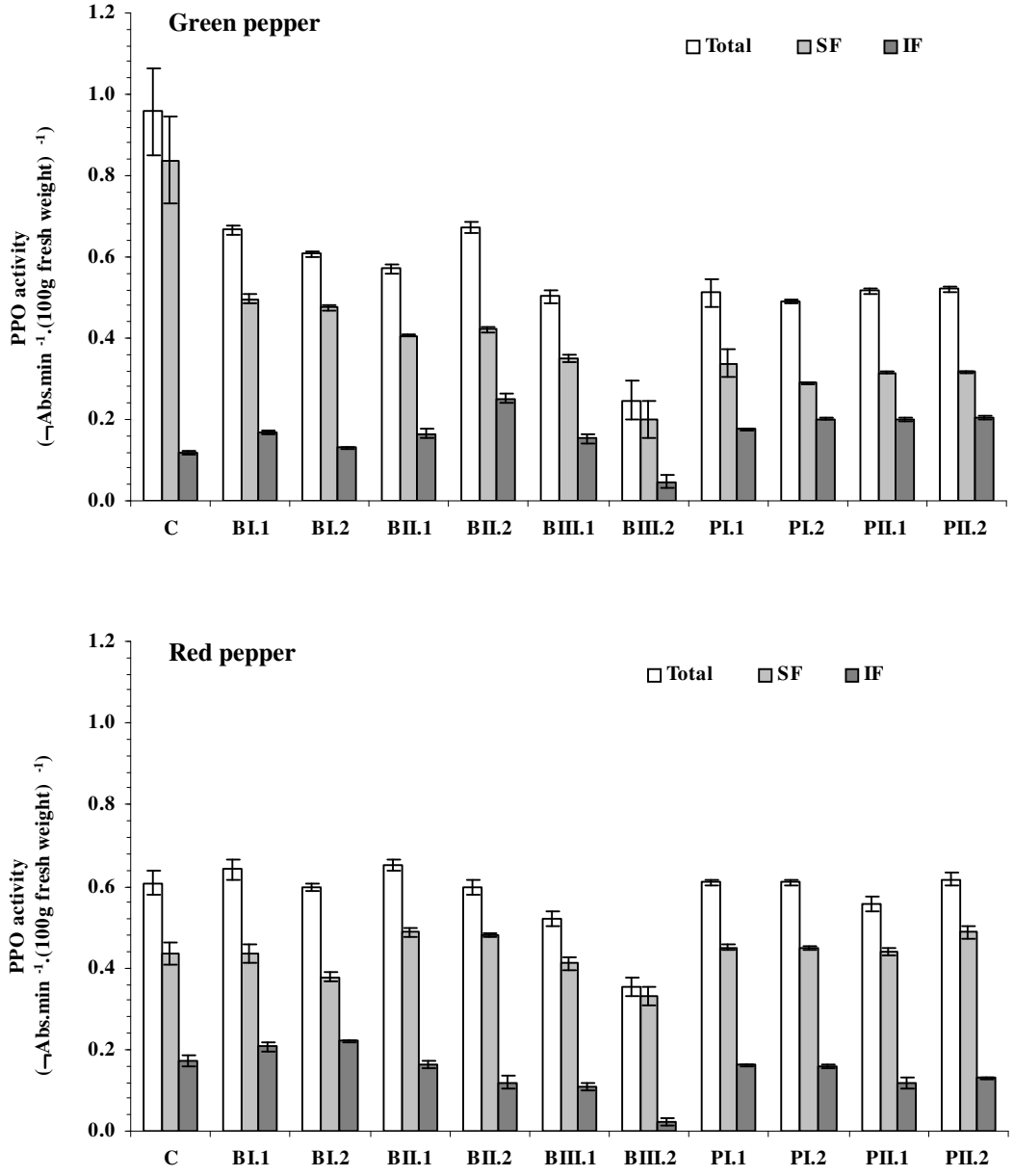

Figure 3 Effect of thermal blanching and pressure treatments on polyphenol oxidase activity of green and red peppers (SF – activity of the soluble fraction; IF – activity of the ionically-bound to the cell wall fraction; Total –activity of SF + IF). The bars represent the standard deviation (n = 3).

Globally, green and red pressure treated peppers showed a PPO activity level similar to that of thermally blanched peppers, except for the most severe blanching treatment (BIII.2) that caused a higher degree of PPO inactivation. Several authors have reported PPO from different origins as a rather baroresistant enzyme (Gomes and Ledward, 1996; Seyderhelm et al., 1996; Hendrickx et al., 1998; Weemaes et al., 1998; López-Malo et al., 1999; Palou et al., 2000), but this barostability depends from the source. While mushroom, potato, and avocado PPO are very pressure stable, since treatments at 800-900MPa, since treatments at 800-900MPa are needed to reduced enzyme activity at room temperature (Eshtiaghi *et al.*, 1994; Gomes and Ledward, 1996; Weemaes *et al.*, 1997, 1998b), apricot, strawberry, and grape PPO could be inactivated by pressures exceeding 100, 400 and 600MPa, respectively (Jolibert *et al.*, 1994; Amati *et al.*, 1996; Weemaes *et al.*, 1998). PPO of red peppers is more stable to pressure and temperature than PPO of green peppers, results that indicate also an effect of the colour stage.

Pectin methylesterase. PME activity was only detected for green peppers, even when increased amounts of enzymatic extract were used and longer reaction times were studied. PME activity was equally distributed between SF and IF extracts (**Figure 4**). Both Jen and Robinson (1984) and Sethu et al. (1996) reported a decrease in PME activity in C. *annuum* fruits during ripening, results that are in agreement with those reported in this work. It is worth mentioning that no PME activity was detected for both thermally blanched and pressure treated red peppers. As can be seen in **Figure 4**, PME activity in green peppers declined progressively, as blanching temperature and time increased, until reaching basically absence of activity for treatment BIII.2. Castro et al. (2005) concluded that green pepper PME was completely inactivated after heating at 80ºC for 5min, both in crude extract and in Tris buffer

at pH 5.6, results that are in agreement with those obtained in this work. PME of the IF extract showed some tendency to be slightly more resistant to temperature than PME from the SF extract.

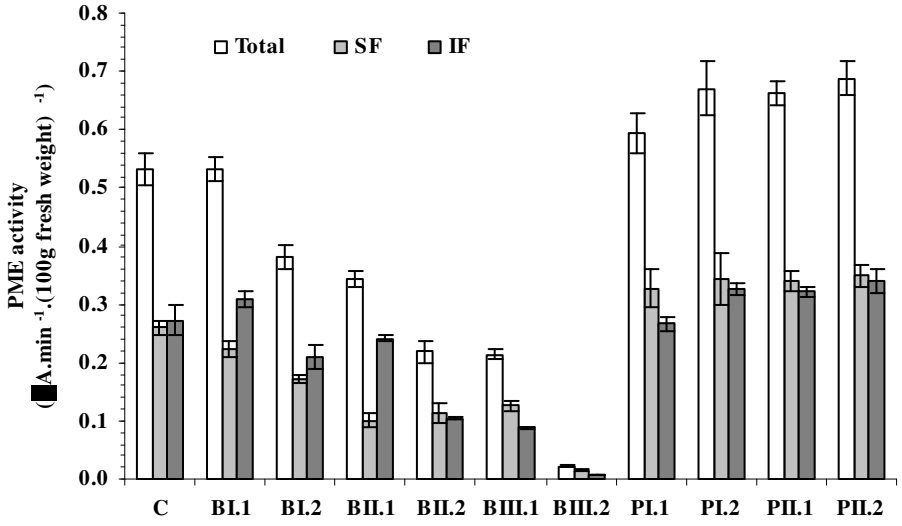

Figure 4 Effect of thermal blanching and pressure treatments on pectin methylesterase activity of green d peppers (SF – activity of the soluble fraction; IF – activity of the ionically-bound to the cell wall fraction; Total – activity of SF + IF). The bars represent the standard deviation (n = 3).

Pressure treated green peppers showed a slight increase in activity, which was due to the increment of PME activity of both SF and IF. Castro et al. (2005) also found that PME activity of green peppers pieces increased, after the pepper pieces have been pressure treated up to 500MPa at room temperature. Likewise, Shook et al. (2001) observed significant increase of PME activity, when diced tomatoes were pressurized at 400MPa and 45ºC compared non-pressurized samples. Increase (activation) of PME activity of tomato (*L. esculentum*) cell

cultures, caused by pressure treatments up to 150MPa, was ascribed by Dörnenburg and Knorr (1998) to a more effective extraction of the enzyme, due to damage of plant cell wall/membrane and changes in cell wall association state of the enzyme. Potato tubers PME showed activation caused by blanching treatments (5min) between 60-70°C, with 55ºC being the minimum temperature for the activation effect to be observed (González-Martinez et al., 2004). This blanching induced activation of PME can be attributed to the same causes indicated above for pressure induced activation and might also be the reason for the increase of PME activity found in this work for pressure treated red peppers. The absence of PME activity in red peppers, indicates again an effect of the colour stage.

Peroxidase. Peroxidase activity was found to be mainly present in SF, for both green and red peppers, and no significant ($P > 0.05$) variation was found between green and red peppers (**Figure 5**). In Habanero chile peppers (*Capsicum annuum* Jacq.) POD activity was found to decrease during the fruit development (Contreras-Padilla and Yahia, 1998). In tomato (Thomas et al., 1981) and strawberry (Civello et al., 1995), POD activity decreased with ripening. In other cases, POD activity has been found to increase with fruit ripening (Silva et al., 1990, Thomas et al., 1981; Aydin and Kadioglu, 2001). All the applied treatments reduced significantly ($P < 0.05$) POD activity, for both SF and IF extracts, particularly all the blanching treatments that, with exception of treatment BI.1 for red peppers, inactivate POD almost completely (**Figure 5**).

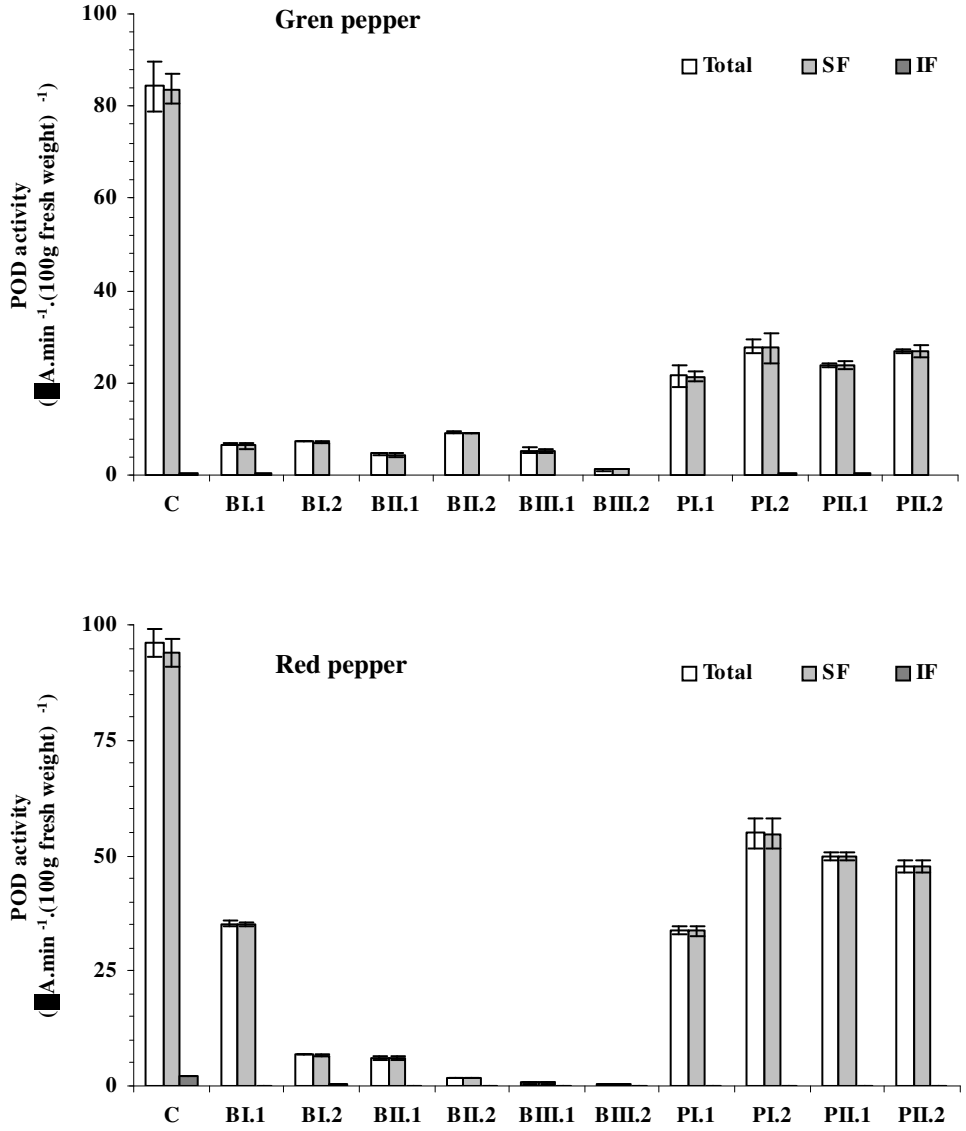

Figure 5 Effect of thermal blanching and pressure treatments on peroxidase activity of green and red peppers (SF – activity of the soluble fraction; IF – activity of the ionically-bound to the cell wall fraction; Total – activity of SF + IF). The bars represent the standard deviation (n = 3).

POD of the SF extract was more stable to the pressure treatments, particularly in red peppers, where activity reduction was about 50-70%. In general, POD is well known as a heat-stable

enzyme, and for this reason is used to evaluate the adequacy of fruits and vegetables thermal blanching (Burnette et al., 1977; Williams et al., 1986; Barrett and Theerakulkait, 1995). For instance, Bahçeci et al. (2004) found a blanching treatment at 90ºC for 3min was necessary to inactivate 90% of the activity of green bean POD. The results obtained in this work show that pepper POD has a low stability to temperature. Results found in literature indicate that POD can show a wide spectrum in what concerns stability to pressure. Pressure treated green beans at 900MPa for 10min at room temperature showed an 88% inactivation of POD and combination with temperature treatments enhanced the inactivating effect at 600MPa (Quaglia et al., 1996). Green bean POD showed 75% residual activity after being pressure treated at 500MPa for 60s at room temperature (Krebbers et al., 2002). Less intense pressure conditions (300 and 400MPa) could inactivate POD from strawberry puree and orange juice at room temperature (Cano et al., 1997). In tomato puree, an increase in POD activity was reported for pressure treatments below 350MPa at room temperature, while a significant inactivation was obtained above 350MPa (Hernández and Cano, 1998). Pepper POD shows a low stability to pressure, with POD from red peppers presents a higher stability to pressure than POD from green peppers.

Using absence of POD activity as an indicator of adequate thermal blanching is inadequate for peppers, since pepper POD shows a lower stability to temperature compared to PPO and PME.

Microbial analysis. For green and red peppers, thermal blanching caused a reduction of total aerobic mesophiles (about 2 decimal reductions – 2D reductions) and faecal coliforms (about 1D reduction), except for red peppers and blanching treatments BI.1 and BII.1 (**Figure 6**). For red peppers, thermal blanching also caused about 2D reduction for enterobacteraceae (except

for treatments BI.1 and BII.1). Generally, pressure treatments caused a slight lower reduction on microbial counts, compared to blanching treatments. This is expectable, since the pressure levels used (100 and 200 MPa) are much lower than those used (500-600 MPa) to pasteurize food products (Torres and Velazquez, 2004).

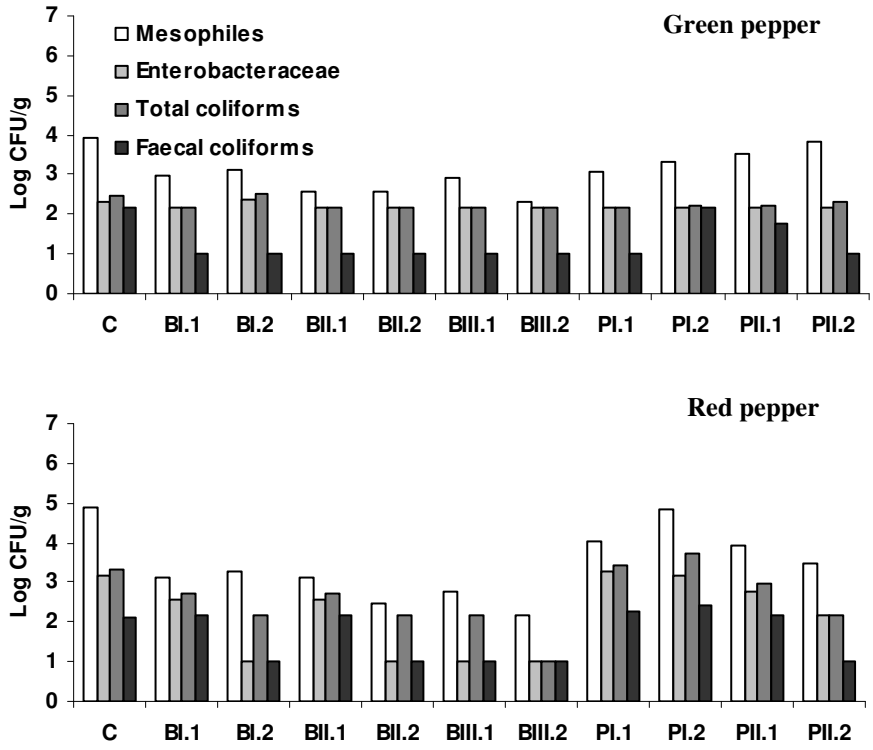

Figure 6 Effect of thermal blanching and pressure treatments on the microbial counts of total aerobic mesophiles, enterobacteraceae, and total and faecal coliforms of green and red pepper samples.

Most of the microorganisms present in fresh-cut fruits and vegetables are derived from soil, air, and water contamination. The presence of coliforms on fresh-cut produce should not be surprising since many coliforms are associated with the soil. Their presence at levels of 10^2 to

10^4 colony forming units (CFU)/g on produce is not rare, when enumeration is done on media selective for Enterobacteriaceae (Nyguyen-the and Carlin, 1994). Microorganisms, such as *Listeria monocytogenes*, *Salmonella*, *Escherichia coli* O157:H7 and other pathogens of concern were also found in washed fresh-cut vegetables, even after cutting and packaging (Pao *et al.*, 1997; Alzamora *et al.*, 2000). Beuchat (1996b) and Heard (2002) reported that aerobic mesophilic counts can range from 10^3 to 10^8 (CFU)/g, in fresh vegetable produce. French legislation (Arrêté du 22 mars, 1993) imposes maximum acceptable values for total mesophilic counts, faecal coliforms, and *E. coli*, for minimally processed ready-for-use fresh vegetables and vegetable salads, or raw foods of vegetable nature ready-for-use of, respectively, 5×10^6, 10^3, and 10^3 (all values per g). For this type of products, a maximum acceptable value of 10^4 for total coliforms is also common to be requested by legislation or buyers to the processing suppliers. As can be seen in **Figure 6**, all thermally blanched and pressurized samples showed a microbial content, for total mesophilic counts and total and faecal coliforms, below the maximum acceptable values, indicating conformity of the peppers so processed for consumption. *E. coli* counts were less than 10 CFU/g in all samples. The results depicted on **Figure 6** also point out the importance for the microbial quality of the peppers prior to the thermal blanching and pressure treatments, and so of the washing step with chlorine solution (5ppm), for the microbial quality of the final product. In fact the treatments caused at maximum 2D reductions of the microbial load of the raw peppers and microbial conformity of the processed peppers was achieved by washing with chlorine solution.

Texture. Firmness (kg-force) measured from the skin side (green, 1.12±0.13; red, 1.03±0.06) was about 3-fold higher ($P < 0.05$) than firmness measured from the flesh side (green, 0.446±0.043; red, 0.414±0.074), for both green and red peppers.

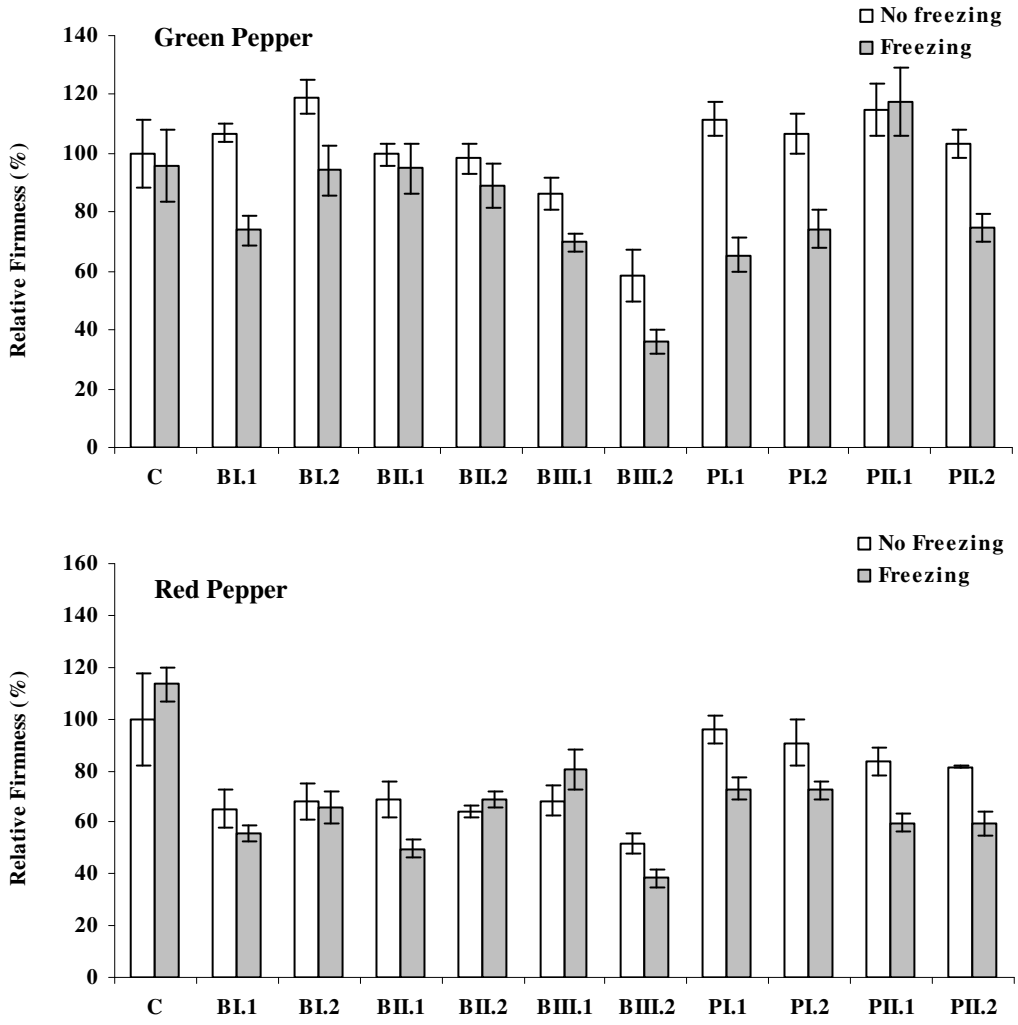

Figure 7 Effect of thermal blanching and pressure treatments and freezing on the firmness, measured from the skin side, of green and red peppers. The bars represent the standard deviation (n = 5).

Generally, the results show a tendency, for firmness of red peppers to be more sensitive to both the thermal blanching and the pressure treatments, than firmness of green peppers. This is particularly evident for thermally blanched red peppers that showed a reduction of firmness, measured from the skin side (**Figure 7**), to about half the value of the unprocessed sample. These results might be related to the absence of PME activity in red peppers. For green peppers, PME activity can cause de-methylation of pectin molecules in the middle lamella (Wu and Chang, 1990; Hoogzand and Doesburg, 1961; Hsu et al., 1965, Stolle-Smits et al., 1998; Fuchigami et al., 1995; Alvarez et al., 2001). The de-esterified pectins are consequently less susceptible to β-eliminative degradation and, therefore, more heat resistant and less soluble, which is generally thought to increase the cell-cell adhesion (Ng and Waldron, 1997; Stolle-Smits et al., 1998). This hypothesis is supported by observation of the results shown in **Figures 4**, **7**, and **8** for green peppers: decrement of firmness caused by the blanching treatments follows a pattern similar to the decrease of PME activity caused by the same treatments, while for pressure treatments that caused no reduction of PME activity, no decrease of firmness was observed. In fact, PME activity due to pressure activation (treatments higher than 100MPa) has been reported in literature as causing beneficial effects on firmness. Absence of PME activity in red peppers can be due to absence of the enzyme, or to the occurrence of a well known PME inhibitor, a glycoprotein that usually appears or increases its amount with ripening (Giovane et al., 2004). Globally, pressurized peppers showed similar to better values for firmness, compared to thermally blanched peppers.

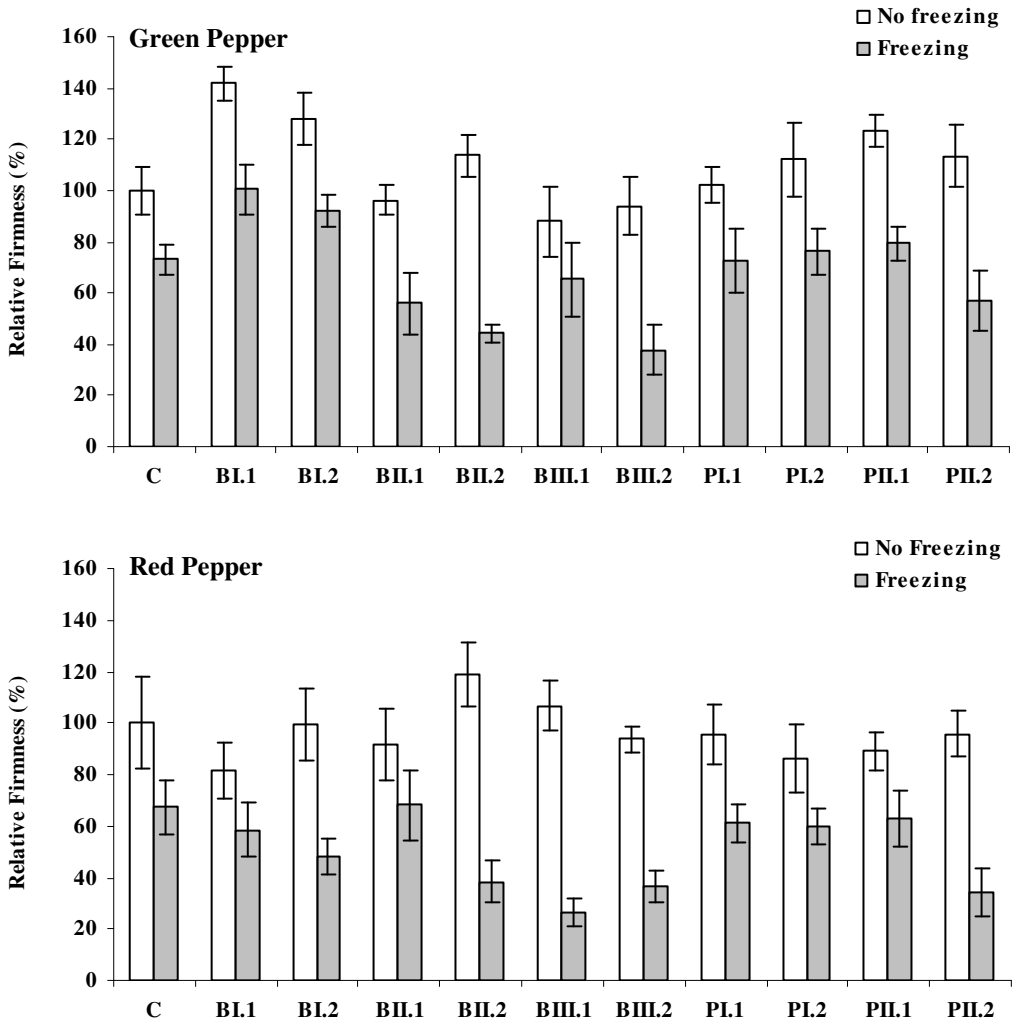

Figure 8 Effect of thermal blanching and pressure treatments and freezing on the firmness, measured from the flesh, of green and red peppers. The bars represent the standard deviation (n = 5).

Effect of freezing on firmness. A decrease of about 27% and 33% in firmness, measured from the flesh side, was observed for frozen/thawed non-treated green and red peppers, respectively, while firmness quantified from the skin side was not affected. In addition, freezing caused a higher decrease on firmness, for the processed samples, when it was

measured from the flesh side, indicating, possibly, the occurrence of more detrimental effects on pepper flesh cells. Firmness of red peppers showed also a higher sensitivity to freezing than green peppers, from both the skin and flesh sides, which might be related to the absence of PME activity in red peppers, as explained above. Thawed peppers that were pressure treated, showed a similar to better texture, compared to the thermally blanched peppers, being the latter particularly evident for the higher temperature blanched peppers. These results point out the potential beneficial effect on firmness of pressure treatments, as alternative blanching treatments to the conventional thermal blanching.

CONCLUSIONS

Pressure treatments caused a lower reduction on soluble protein and ascorbic acid contents, than thermal blanching, particularly for red peppers, that showed and increased content of ascorbic acid, compared to the unprocessed samples.

In general, green and red pressure treated peppers showed a level of residual polyphenol oxidase activity similar to that of thermally blanched peppers, except for the most severe blanching treatment (98ºC, 2.5min). Polyphenol oxidase of red peppers showed a higher stability to pressure and temperature than polyphenol oxidase of green peppers. Pectin methylesterase activity was only detected in green peppers and its activity declined progressively, as blanching temperature and time increased, while the pressure treatments caused a slight increase of its activity. Peroxidase was more stable to pressure than to temperature, particularly for red peppers and showed a lower stability to the thermal blanching

treatments, than polyphenol oxidase and pectin methylesterase, which unables the possible use of peroxidase as an indicator of adequate thermal blanching. Thermal blanching and pressure treatments caused only 1-2 decimal reductions on microbial load, pointing out the importance of the washing step with chlorine solution for the microbial quality of the final product.

Firmness was equally to better retained in pressure treated peppers than in thermally blanched peppers, before and after freezing, with red peppers showing higher sensitivity to lose firmness. Globally, the quality parameters of green and red peppers showed a different behaviour to thermal blanching and pressure treatments and pressure treatments of 100 and 200MPa constitute potential alternatives to the thermal blanching of red and green bell peppers, yielding peppers with higher soluble protein and ascorbic acid contents and better firmness.

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CHAPTER FIVE

Activity and process stability of purified green pepper (*Capsicum annuum*) pectin methylesterase

CHAPTER SIX **Process stability of *Capsicum annuum* pectin methylesterase in model systems, pepper puree and intact pepper tissue**

CHAPTER SEVEN	**Inactivation of pepper (*Capsicum annuum*) pectin methylesterase by combined high-pressure and temperature treatments**

CHAPTER EIGHT

Identification of pressure/temperature combinations for optimal pepper (*Capsicum annuum*) pectin methylesterase activity

CHAPTER NINE

Effect of temperature, pressure and calcium soaking pre-treatments and pressure shift freezing on the texture evolution of green bell peppers (*Capsicum annuum*)

CHAPTER TEN General conclusions

10.1 An overview of the present work

The study concerning the possible use of pressure treatments (100 and 200MPa) as an alternative to thermal blanching, indicate that the pressure treatments caused a lower reduction on soluble protein and ascorbic acid contents, increasing even the of ascorbic acid content of red peppers, compared to the unprocessed samples. Pressure caused an inactivation effect similar to thermal blanching on polyphenol oxidase, while peroxidase was more stable to the pressure treatments applied. Pectin methylesterase activity was only detected in green peppers, its activity declined progressively, as blanching temperature and time increased, while the pressure treatments caused a slight increase of its activity. The pressure treatments caused a lower reduction on the microbial load than the blanching treatments. Firmness was better retained in pressure treated peppers than in thermally blanched peppers, before and after freezing. Globally, it can be conclude that pressure treatments of 100 and 200MPa constitute potential alternatives to the thermal blanching of red and green bell peppers, yielding peppers with higher soluble protein and ascorbic acid contents and better firmness.

PME from green bell peppers was successfully extracted and purified by affinity chromatography column, containing a PME inhibitor extracted from kiwi. PME showed two bands on SDS-PAGE, with 33 and 37kDa, and several acidic and basic bands on the IEF with silver nitrate. The optimum pH for PME activity at 22°C was 7.5, and its optimum temperature at neutral pH was between 52.5 and 55.0°C. The purified pepper PME required the presence of 0.13M NaCl for optimum activity. Isothermal inactivation of purified pepper PME in 20mM Tris buffer (pH 7.5) could be described by a fractional conversion model for lower temperatures (55-57°C) and a biphasic model for higher temperatures (58-70°C). Isothermal inactivation of purified pepper PME in 20mM Tris buffer (pH 7.5) could be described by a fractional conversion model for lower temperatures (55-57°C) and a biphasic model for higher temperatures (58-70°C). The enzyme showed a relative stable behaviour toward high-pressure/temperature treatments at the optimum activity pH. Stability of PME towards temperature and/or pressure on PME was also studied at pH 5.6, the natural pH of peppers, and in different systems (purified form, crude extract, pepper pieces and puree). Within the temperature range studied (22–80ºC, 5min), pepper PME in both pure form and crude extract

was gradually inactivated showing a biphasic inactivation behaviour. Heated pepper samples showed a maximum of residual PME activity around 55ºC (15min). Isothermal inactivation of pepper PME in purified form and crude extract at pH 5.6 could be described by a biphasic inactivation model for the temperature range studied (62–76ºC). A stable behaviour towards high-pressure/temperature treatments (400–800 MPa/25–60ºC) was observed for crude extract and purified pepper PME. PME in pepper puree samples revealed to be very pressure stable. Mild temperatures combined with pressure treatments of pepper tissue increased PME activity, which can be caused by an increased PME extractability from pepper tissue, probably due to a disruption effect on the cell wall structure. This effect can be used to improve the structure of processed peppers, with beneficial texture changes.

Futher studies on purified green bell pepper PME (pH 5.6) related with pressure and/or temperature inactivation (at mild temperatures between, 10–64ºC, in combination with high pressure, 0.1–800MPa) where done. Inactivation of the labile fraction under mild-heat and high-pressure conditions could be accurately described by a fractional conversion model, while a biphasic model was used to estimate the inactivation rate constant of both fractions at more drastic conditions of temperature/pressure. At lower pressures ($P \leq 300$MPa) and high temperatures ($T > 54$ºC), an antagonistic effect of pressure and temperature was observed.

The study of "in situ" activity of the enzyme, during thermal and/or pressure treatment (18–65ºC, 0.1–600MPa), was performed in a model system of pectin at pH 5.6, indicated that at atmospheric pressure, the optimal temperature for activity of purified pepper PME was found within the range of 50–55ºC. Increasing pressure up to 200-300MPa, on the other hand, was efficient in stimulating the catalytic activity at elevated temperature. The activity of purified pepper PME showed a maximum at 200MPa and 55ºC.

A third-degree polynomial model (derived from a thermodynamic model) successfully described the temperature/pressure dependence of the inactivation rate constants of the labile fraction of pepper PME. Likewise, the heat–pressure dependence of the initial rates of purified pepper PME-catalyzed methanol formation could be described by a third-degree polynomial

model (derived also from a thermodynamic model) in the same way as inactivation rate constants. This methodology allowed to build iso-rate contour plots.

These results clearly illustrate the potential of combined high pressure–temperature treatments to control PME activity, through the increment of its activity (by activation or increased stability), which can be an add-value of high pressure processing of fruits and vegetables regarding textural properties.

Such possibility was investigated, by carrying out studies of firmness changes of green bell pepper under different processing conditions. Thermal texture degradation kinetics of pepper tissue between 75-95°C could be accurately described by a fractional conversion model. The firmness of pepper tissue increased when it was submitted to several heat, pressure, and combinations of heat/pressure and calcium soaking pre-treatments. Pre-heating at 55ºC during 60min and mild heat/high-pressure treatments (200MPa at 25ºC, 15min) yielded the best results, which were further improved when combined with calcium soaking. These pre-treatments significantly slowed down thermal texture degradation of pepper at 90ºC, a typical temperature used for pepper blanching prior to freezing. The above mentioned pre-treated samples showed a significant reduction in firmness when frozen by a "slow" and "rapid" cryogenic process at 0.1MPa, while the same pre-treated samples showed no changes in firmness when they were frozen by high-pressure shift freezing at 200MPa. Pressure pre-treated pepper showed a better retention of firmness upon frozen storage (-18ºC) for 2.5 months, than thermal pre-treated peppers. These results illustrate the potential of thermal, pressure, combined thermal/pressure, and calcium soaking pre-treatments to improve texture of pepper and of high pressure shift freezing also to retain texture during freezing and frozen storage.

As a general conclusion, it can be stated that the results of this work allowed to: (i) conclude that pressure treatments (100 and 200MPa) can be a viable alternative to the conventional thermal blanching of peppers; (ii) characterize PME from green peppers, biochemically and in what concerns its stability and inactivation kinetics, under thermal, pressure and combined thermal/pressure conditions; (iii) confirm the potential use of high pressure treatments, with or

without simultaneous application of temperature, to control PME activity/inactivation, in order to improve firmness of green peppers; (iv) verify that high-pressure-shift freezing permits to freeze green peppers that retain firmness at a level that is clearly better than that of peppers frozen at atmospheric pressure. The beneficial effects of high pressure processing indicated in the two last points, can be extrapolated for foods of vegetable origin in general.

10.2 Future work

Since most endogenous PMEs are pressure stable enzymes, and pepper PME is no exception, pressure inactivation of this enzyme needs very high pressures, sometimes up to 1000MPa.

- The heat stable fraction of pepper PME was assumed to be also pressure resistant. However, a purification of PME and a full characterization of the different isoenzymes are required to confirm this assumption and the biochemical molecular relation between different isoenzymes and thermal, pressure, and combined thermal/pressure biphasic inactivation kinetics.

The high pressure stability of PME makes high pressure processing not applicable from an economical and engineering point of view, when PME inactivation is the prime target, as already focused in Chapter One. In order to evercome this problem, high pressure processing needs to be combined with other techniques, such as mild temperature, and for these type of studies, iso-rate contour plots are important tools to design and optimize high pressure/thermal processes.

- During this study, experiments under isothermal-isobaric conditions were conducted with slow build-up of pressure, with the exception of real model systems. This approach is useful for academic studies but fast pressure build-up dynamic pressure processing conditions should also be studied to fill the gap between possible practical applications.

Since, PME behaviour under pressure-temperature treatments depends not only on extrinsic factors but also on intrinsic factors, such as pH and other proteins and food constituents

research needs to be done, regarding PME activity, stability and inactivation kinetics and pepper related products in real foods and in real processing conditions, like pressure pasteurisation and pressure/temperature sterilisation.

The beneficial effects on firmness, caused by thermal and pressure treatments and by high pressure shift freezing, should also be related to the postulated alterations on the methylation degree of pectins and constitution, structure, and arquitecture of cell wall.

List of publications and communications based on the Thesis work

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EFFECT OF PRESSURE AND BLANCHING TREATMENTS ON ENDOGENOUS ENZYMATIC ACTIVITY AND VITAMIN C CONTENT OF BELL PEPPER FRUIT

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The effect of high pressure and blanching treatments on green bell pepper was studied. Both processes caused changes on pectin methylesterase activity and vitamin C and protein contents. Polygalacturonase activity was not detected in any of the samples studied. There seems to be no significant difference caused by both types of processing.

Keywords: High pressure; Blanching; Bell pepper; Enzymes; Vitamin C

INTRODUCTION

Bell pepper fruits (*Capsicum annuum*) are commercially blanched prior to freezing for storage and further raw consumption. Blanching of bell pepper fruits aims to decrease contaminating flora to accepted values to ensure safe raw consumption. However, blanching causes colour, texture and flavour changes and decreases the nutritional value. In the present work, the effect of conventional blanching and high pressure treatments of green bell pepper sticks was studied. The effect of both processes was analysed for their effects on the enzymatic activities of pectin methylesterase (PME) and polygalacturonase (PG) and soluble protein and vitamin C contents. These two enzymes were chosen because they can cause important textural changes through their action on pectins.

EXPERIMENTAL PROCEDURE

Green bell peppers were purchased at a local market and thoroughly washed before each experience, to avoid floral contamination that could affect the enzyme activity measurements. About 100 g of green bell-pepper tissue were cut in small sticks (3×10 cm), vacuum packed in sealed plastic bags and subjected to the blanching and pressure treatments. The treatment conditions were: 70 °C/60 s (BI.1), 70 °C/150 s (BI.2), 80 °C/60 s (BII.1), 80 °C/150 s

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(BII.2), 100 MPa/10 min. (PI.1), 100 MPa/20 min. (PI.2), 200 MPa/10 min. (PII.1) and 200 MPa/20 min. (PII.2). After each processing, the samples were immediately frozen in liquid nitrogen and stored at −20 °C for subsequent analysis. To obtain the enzyme extracts, each sample was thawed at 4 °C and homogenised in a Blender using sodium phosphate buffer (0.2 M, pH 6.5) and polyvinylpyrrolidone (PVPP), followed by gentle agitation at 4 °C, during an hour, and centrifugation. The supernatants obtained were frozen and stored at −20 °C for enzymatic activities, vitamin C and soluble protein quantification.

For the determination of PME activity, to 4 mL of substrate solution, consisting of 0.5% (w/v) citrus pectin solution (Fluka, 76280) and 0.300 mL of bromothymol blue, was added an adequate amount (typically 300 μL) of enzyme extract and the mixture was left to react at 25 °C for up to 3 hours [1]. In order to achieve a constant starting pH, the pH was previously adjusted to 7.5 with concentrated NaOH. Absorbance readings were taken at 620 nm each half an hour to determine the activity, which was calculated as the slope of the linear portion of the curve relating absorbance and time, and expressed as $\Delta O.D._{620\,nm}$/min. The PG activity determination was carried out at 276 nm after incubating the enzyme extract (5–125 μL) with 200 μL of polygalacturonic acid 0.4% (w/v) (Sigma, P-3889), 75 μL of 200 mM of acetate buffer (pH 4.4) and desionised water, in a final volume of 400 μL, at 30 °C, for a period of time up to 48 hours. The quantification of reducing sugars was done using 2-cyanoacetamide [2]. The 2,6-dichlorophenolindophenol method was used to determine the ascorbic acid content [3]. The soluble protein content was measured using the Folin-Lowry method [4]. All determinations were carried out, at least in duplicate, and the effect of the applied treatments was statistically analysed.

RESULTS AND DISCUSSION

Soluble protein content was found to be significantly different ($P < 0.05$) for all treatments, except for sample BII.2 when compared to non-processed bell pepper (Fig. 1). The other

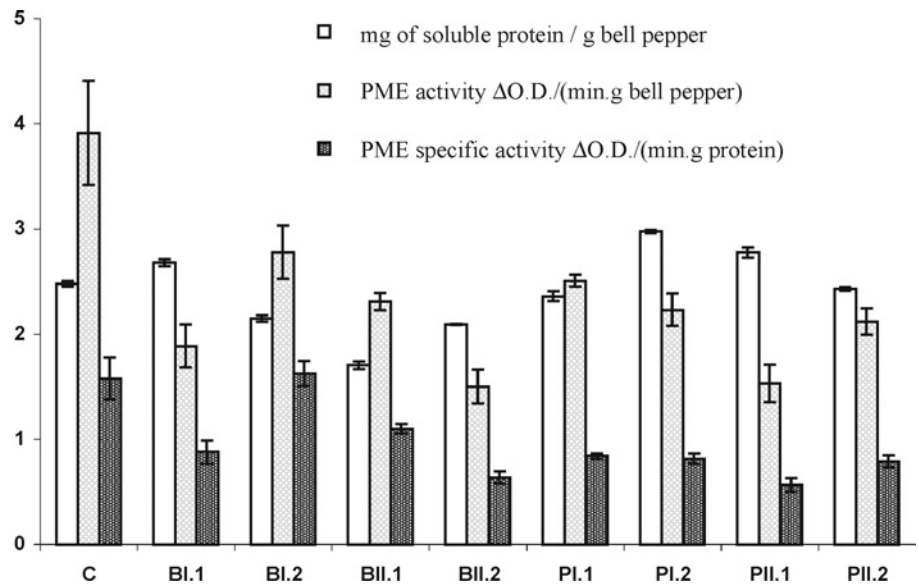

FIGURE 1 The effect of the applied treatments on soluble protein, PME activity and specific activity (C refers to non-processed sample; error bars indicate the standard deviation; the result for the PME activity are shown multiplied by 1000).

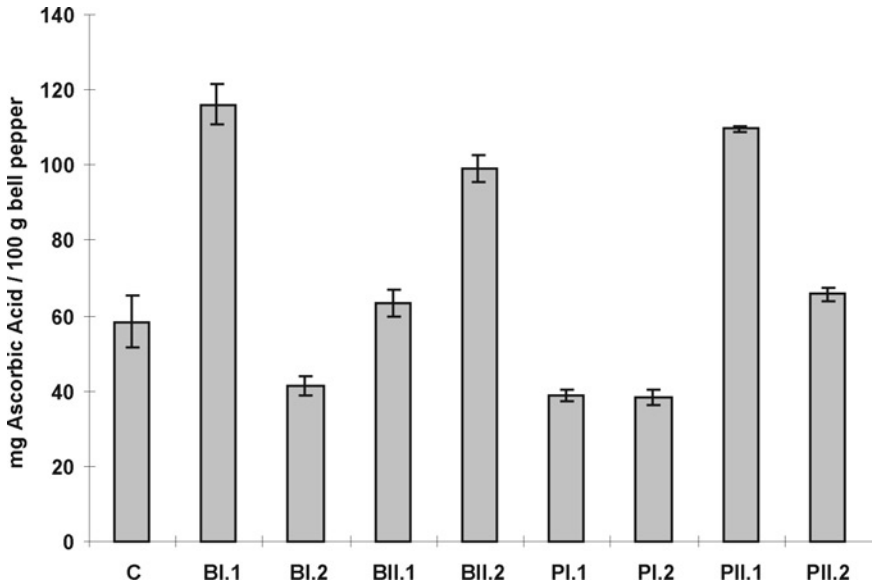

FIGURE 2 Effect of the applied treatments on ascorbic acid content of bell pepper (C refers to non-processed sample; error bars indicate the standard deviation).

blanching treatments (BI.1, BI.2, and BII.1) reduced the protein contents, ranging from 69% to 87%, while pressure-treated samples showed higher values (from 109% to 123%). All the treatments studied caused a reduction in the PME activity ($P < 0.05$), from 65% to 40% (Fig. 1). The effect of the treatments on PME specific activity followed a similar pattern. For commercial PME in Tris buffer at pH 7, it was reported that a minimum pressure of 800 MPa and 45 °C was needed for inactivation [5] while mild pressures and temperatures were able to inactivate PME in orange juice [6].

In the conditions used in this work, it was impossible to detect PG activity. Neither was PG activity detected in hot pepper, at several maturation stages, ranging from immature green to 100% red surface colour [7].

The ascorbic acid content determined in the control sample (55 mg/100 g of fresh bell pepper) is within the range (from 12 to 180 mg/100 g of fresh tissue) reported in literature [8–10]. No systematic pattern was observed for the effect of the applied treatments on ascorbic acid content (Fig. 2). The ascorbic acid content of samples BI.1, BII.2 and PII.1 increased, whereas those of samples BI.2, PI.1 and PI.2 decreased ($P < 0.05$), and the remaining ones showed no significant effect. The reason for the increase in ascorbic acid content can be related, at least partially, to the limitations of the analytical method, which is affected by other reducing substances. HPLC quantifications of ascorbic acid are underway to clarify these results. Other works have indicated a slight increase (up to 15%) or no reduction of ascorbic acid in pressure/temperature treated potato cubes [11] and orange juice [12], respectively.

CONCLUSION

Globally, it can be concluded that the pressure treatments studied caused an increase in soluble protein content, whilst blanching showed an opposite effect. Both types of processes decreased significantly the activity of PME. No detectable activity was found for PG in

all cases. No particular trend was observed for the effect of the applied treatments on the ascorbic acid content, though both types of processing showed significant variations compared to the unprocessed bell pepper. Overall, the results obtained indicate that the pressure and blanching treatments studied caused similar effects on bell pepper, which makes the former a feasible alternative to the latter. To evaluate further this possibility, it is necessary to study pressure processing efficacy to reduce bell pepper contaminating flora, to levels compared to those obtained by blanching.

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JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Activity and Process Stability of Purified Green Pepper (*Capsicum annuum*) Pectin Methylesterase

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Pectin methylesterase (PME) from green bell peppers (*Capsicum annuum*) was extracted and purified by affinity chromatography on a CNBr-Sepharose-PMEI column. A single protein peak with pectin methylesterase activity was observed. For the pepper PME, a biochemical characterization in terms of molar mass (MM), isoelectric points (p*I*), and kinetic parameters for activity and thermostability was performed. The optimum pH for PME activity at 22 °C was 7.5, and its optimum temperature at neutral pH was between 52.5 and 55.0 °C. The purified pepper PME required the presence of 0.13 M NaCl for optimum activity. Isothermal inactivation of purified pepper PME in 20 mM Tris buffer (pH 7.5) could be described by a fractional conversion model for lower temperatures (55−57 °C) and a biphasic model for higher temperatures (58−70 °C). The enzyme showed a stable behavior toward high-pressure/temperature treatments.

KEYWORDS: ***Capsicum annuum*; pepper; pectin methylesterase; purification; characterization; thermal and high-pressure stability**

INTRODUCTION

Peppers have become more popular in recent years due to their chemical composition (e.g., vitamins), and a wide variety is nowadays available on the market. Most varieties belong to the *Capsicum annuum* species, and they can be consumed fresh or processed, as immature (i.e., green) or as mature fruit (i.e., yellow, orange, red), as a spice or as a vegetable, because of their distinct colors, intense taste, and unique flavor. The texture, in particular, the crispness, of peppers is an important quality attribute to consumers. It is known that vegetable texture is closely related to the pectic substances and to activities of pectolytic enzymes (*1*). Prominent among the enzymes implicated in the softening of fruits and vegetables during ripening are polygalacturonase and pectin methylesterase (PME) due to their relationship with the cell-wall pectic content. Although peppers, like tomatoes, belong to the Solanaceae family, there is little information in the literature on the pectolytic enzymes and their relationship with biochemical cell-wall changes of bell peppers and texture (*2*−*4*). One reason such information is lacking is probably due to the anticipated low enzyme activities in peppers, as the texture degradation in peppers is a slow process (*2*). Nevertheless, PMEs play a central role in texture evolution; the control of its activity, through knowledge of its dependence on parameters such as temperature and pH, is of great practical importance in the food industry for protecting and improving the texture and firmness of several processed fruits and vegetables (*5*). Thermal processing is still one of the most frequently used methods for food preservation, and one approach to optimize the heat treatment of fruits and vegetables in order to maximize the overall quality is to develop a model considering, among other parameters, the (in)activation kinetics for relevant enzymes to predict quality changes during processing and subsequent storage. PME has been extracted and purified from many different sources and characterized in terms of biochemical properties and thermal stability (e.g., see refs *5*−*12*). In this work, PME was extracted from green peppers and purified by affinity chromatography. The purified pepper PME obtained was biochemically characterized and submitted to thermal and high-pressure inactivation.

MATERIALS AND METHODS

Materials. Green bell peppers (*C. annuum*) were purchased from a local auction (Mechelen, Belgium). Apple pectin [degree of esterification (DE) = 75%] was obtained from Fluka Chemicals Co. (Buchs, Switzerland). CNBr-Sepharose 4B resin was purchased from Sigma (St. Louis, MO). All other chemicals were of analytical grade.

Methods. *Pectin Methylesterase Purification.* PME was extracted from peppers with 0.2 M tris(hydroxymethyl)aminomethane buffer (i.e., Tris buffer) (pH 8.0) with 1 M NaCl, followed by purification by affinity chromatography on a CNBr-Sepharose 4B-PME inhibitor column and finally stored at −80 °C in 20 mM Tris buffer (pH 7.5) using the procedure as described by Ly-Nguyen et al. (*11*).

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Pectin Methylesterase Assay. PME activity was measured by continuous recording of titration of carboxyl groups released from a pectin solution using an automatic pH-stat (Metrohm, Switzerland) and 0.01 N NaOH solution. Routine assays were performed with a 3.5 mg/mL apple pectin solution (DE = 75%, 30 mL) containing 0.117 M NaCl (pH 7.0) at 22 °C. The activity unit of PME is defined as the amount of enzyme required to release 1 μmol of carboxyl groups per minute, under the aforementioned assay conditions.

Protein Determination. Protein concentration was determined using Sigma procedure TPRO-562. Bovine serum albumin (Sigma) was used as a standard.

Gel Electrophoresis. A PhastSystem (Amersham Biosciences, Uppsala, Sweden) was used for both SDS-PAGE and IEF experiments. SDS-PAGE was performed using Fast Gel homogeneous 20% and PhastGel Tris−tricine SDS buffer strips. Samples were boiled for 5 min at 100 °C in a buffer containing SDS (2.5%) and β-mercaptoethanol (5%). The molecular mass markers were phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), and lactalbumin (14.1 kDa). The IEF was performed in a Phastgel IEF medium (polyacrylamide gels) in the pH range of 3−9, using a calibration kit (Pharmacia) containing 11 proteins with isoelectric points ranging from 9.3 to 3.5. Gel staining was performed with silver nitrate according to the method of Heuskeshoven and Dernick (*13*), using the equipment from Pharmacia.

Effect of Pectin and NaCl Concentration on Pectin Methylesterase Activity. The effect of substrate concentration (apple pectin, DE = 75%, 30 mL) was determined by measuring the activity of purified PME in the presence of various substrate concentrations (0.10−7.5 mg/mL), with 0.117 M NaCl at pH 7.0. The K_m and maximum rate (V_{max}) were determined by nonlinear regression analysis using the Michaelis−Menten equation. The effect of NaCl concentration on the PME activity was studied in the range of 0−0.3 M NaCl.

Effect of pH on Pectin Methylesterase Activity. The pH dependence of the purified pepper PME activity was assayed titrimetrically at 22 °C with 0.01 N NaOH after adjustment of the pH of the reaction solution to one of the pH values tested (3.5−9.0). Corrections were made to each experiment for the spontaneous de-esterification of pectin at alkaline conditions.

Effect of Assay Temperature on Pectin Methylesterase Activity. The effect of the assay temperature on the purified pepper PME activity was tested under standard assay conditions (pH 7.0) with various temperatures. The temperature (22−70 °C) was controlled by means of a circulating water bath. Corrections were made to each experiment for the spontaneous de-esterification of pectin at elevated temperatures. The activation energy (E_a) of PME-catalyzed pectin de-esterification was calculated using the Arrhenius equation

$$k = A \exp(-E_a/RT) \quad (1)$$

where k is the rate constant, R is the gas constant (8.314 J mol^{-1} K^{-1}), A is a pre-exponential factor, and T is the temperature (K).

Thermal and Pressure Stability of Purified Pepper Pectin Methylesterase. The thermal stability of purified pepper PME was investigated at pH 7.5 within a temperature range of 22−70 °C. Thermal treatments were performed by immersing glass capillaries (Hirschmann, Germany) with the enclosed enzyme solution, in a temperature-controlled water bath, during 5 min. After the treatments, the capillaries were immediately cooled in ice water. Residual activities of PME were measured within 60 min of storage at 0 °C. Pressure treatments were conducted in a multivessel high-pressure apparatus (eight vessels of 8 mL) (Resato, Roden, The Netherlands). The pressure medium is a glycol−oil mixture (TR-15, Resato). To enclose the enzyme solution, flexible microtubes of 0.3 mL were used (Elkay, Leuven, Belgium). The microtubes were placed in the pressure vessels, already equilibrated at desired temperature (25 and 60 °C). Pressure was built slowly (100 MPa/min) to minimize temperature increases due to adiabatic heating. After pressure buildup, an equilibrium period of 2 min to allow the temperature to evolve to its desired value was taken into account. After 15 min, the pressure was released, the samples were immediately cooled in an ice−water bath, and the residual activities of PME were measured within 60 min of storage in ice water.

Thermal Inactivation Kinetics of Purified Pepper Pectin Methylesterase. Thermal inactivation of purified pepper PME was investigated within a temperature range of 55−70 °C at pH 7.5. Isothermal treatments were performed in a temperature-controlled water bath using glass capillaries (Hirschmann, Germany) to enclose the enzyme solution. After the treatments, the capillaries were immediately cooled in ice water. Residual activities of PME were measured within 60 min of storage at 0 °C.

Kinetic Data Analysis. Inactivation of enzymes can often be described by a first-order kinetic model (*14*). When there are several isozymes present, which show different behavior toward temperature and/or pressure, that is, labile and stable fractions, and both inactivating according to a first-order kinetic model (*15*−*18*), a biphasic kinetic model (eq 2) or distinct isozyme model can be used. There is a fast inactivation period followed by a decelerated decay.

$$N_L \xrightarrow{k_L} I_L \quad N_s \xrightarrow{k_S} I_S \quad A = A_L \exp(-k_L t) + A_S \exp(-k_S t) \quad (2)$$

The subscripts L and S indicate labile and stable enzyme fractions, respectively. The residual activity from the labile and stable fractions as well as the inactivation rate constants can be estimated by nonlinear regression analysis. When only the labile fraction inactivates, whereas the activity of the stable fraction does not change with respect to time, a fractional conversion kinetic model should be applied. A fractional conversion model takes into account the residual activity after prolonged thermal and/or pressure treatment (eq 3):

$$N + RF \xrightarrow{k} I + RF \quad A = A_\infty + (A_0 - A_\infty) \exp(-kt) \quad (3)$$

A_∞ is the residual activity after prolonged treatment time. The inactivation rate constant (k) and the residual activity (A_∞) are estimated by nonlinear regression analysis. It should be stressed that for experiments at constant temperature and/or pressure, the heating and/or pressurizing time should be long enough so that the remaining activity, A_∞, is no longer changing with respect to time (*19*, *20*). The temperature dependence of inactivation rate constants can be estimated using the Arrhenius model (eq 4)

$$\ln(k) = \ln(k_0) + \left[\frac{E_a}{R}\left(\frac{1}{T_0} - \frac{1}{T}\right)\right] \quad (4)$$

where T and T_0 are the absolute temperature (K) and the reference temperature (K), respectively; k_0 is the rate constant at T_0, E_a is the activation energy (kJ mol^{-1}), and R (8.314 J mol^{-1}K^{-1}) is the universal gas constant. The activation energy can be estimated by linear regression analysis on eq 4.

RESULTS AND DISCUSSION

Purification of Pepper Pectin Methylesterase by Affinity Chromatography. PME is located in the cell walls of higher plants and is ionically bound to the cell wall. Hence, a high ionic strength buffer is required for extraction from the cell walls (0.2 M Tris buffer, 1 M NaCl, pH 8.0). In **Figure 1**, the elution profile of pepper PME from the affinity column shows that inert proteins were removed with 2 mM KH_2PO_4 buffer (pH 6.0) containing 0.5 M NaCl. Purified pepper PME was then eluted with a high ionic strength and high pH buffer (50 mM Na_2CO_3, 1 M NaCl, pH 9.85), and a single peak of proteins and PME activity was obtained. Each replicate purification of the enzyme showed a single protein and activity peak. The PME activity found in the crude extract (2.5 units/g of fresh material) was in the range found in previous work on sweet bell peppers at different stages of maturity (*2*). Purified pepper PME had a maximum activity of 242.5 units/mg of protein corresponding to at least a 10.3-fold enrichment and an overall yield of at

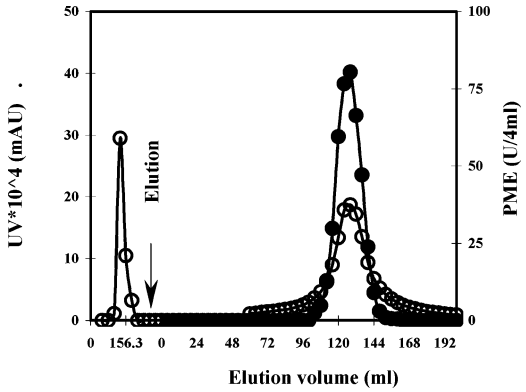

Figure 1. Elution profile of green pepper PME on a PMEI-CNBr-Sepharose 4B column: UV absorbance (measured at $\lambda = 280$ nm) (○); PME activity (●). Washing solution was 2 mM KH_2PO_4 buffer, containing 0.5 M NaCl at pH 6.0. The elution buffer was 50 mM Na_2CO_3, containing 1 M NaCl at pH 9.85.

Table 1. Extraction and Purification of Pepper Pectin Methylesterase

	activity (units)	protein (mg)	specific activity (units mg^{-1})	recovery (%)	purification factor
crude extract	1999	84.8	23.6	100	1
purified PME	417	1.72	242.5	20.9	10.3

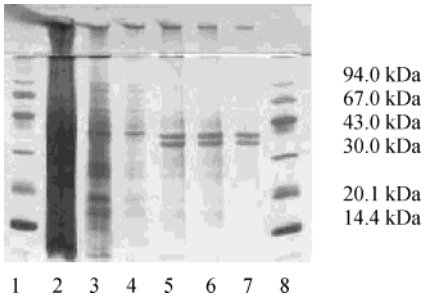

Figure 2. SDS-PAGE of green pepper PME: (lanes 1 and 8) SDS-PAGE standards; (lanes 2–4) crude green pepper extract; (lanes 5–7) pepper PME after affinity chromatography.

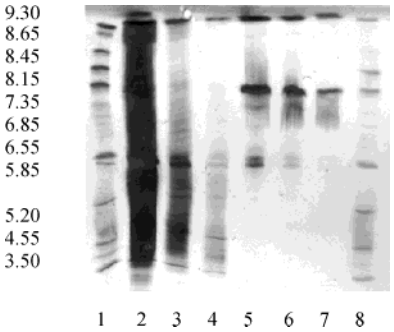

Figure 3. IEF of green pepper PME: (lanes 1 and 8) p*I* standards; (lanes 2–4) crude extract; (lanes 5–7) pepper PME after affinity chromatography.

least 20.9%, based on the total enzymatic activity of crude extract (**Table 1**).

On SDS-PAGE, the purified pepper PME produced two bands with identical intensities (**Figure 2**). After comparison with the electrophoretic mobility of the standard proteins, the bands obtained indicated molecular masses of 33 and 37 kDa, which are in the range of other plant PMEs purified from different sources (*5*, *7*−*9*, *21*, *22*). On the IEF gel, purified pepper PME showed several bands between 6.0 and 9.3 (**Figure 3**), of which two were cathodic (p*I* values of 7.9 and 7.5) and two were anodic (p*I* values of 6.3 and 6.1). There was also observed another band at a p*I* > 9.3 (around 9.6 determined by extrapolation), suggesting that there might be some other forms. Generally, the isoelectric point of plant PMEs is between 7 and 11, but some acidic forms have also been reported (*6*, *8*, *11*, *22*).

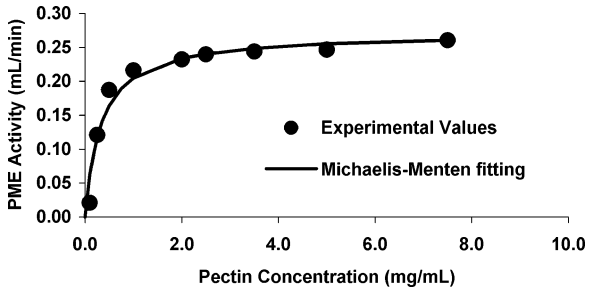

Figure 4. Activity of green pepper PME as a function of substrate concentration. Assay conditions: apple pectin (DE 75%), pH 7.5, 22 °C, 0.117 M NaCl.

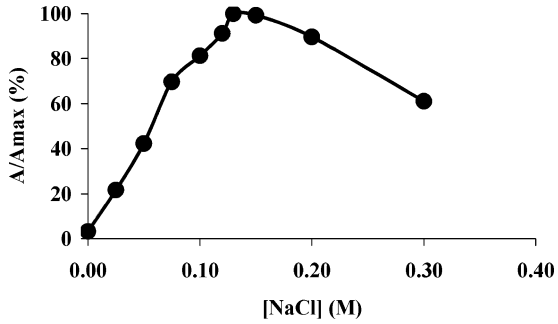

Figure 5. Activity of green pepper PME as a function of salt concentration. Assay conditions: apple pectin (DE 75%) solution 3.5 mg/mL, pH 7.5, 22 °C.

Effect of Pectin and NaCl Concentration on Pectin Methylesterase Activity. The activity of pepper PME as a function of apple pectin concentration was investigated (**Figure 4**). The kinetic parameters K_m and the maximum rate (V_{max}) were determined by nonlinear regression analysis as 0.329 mg/mL and 0.272 mL/min, respectively. For routine assays (substrate concentration of 3.5 mg/mL) the initial rate of the enzyme represented 98% of the maximum rate. Pepper PME activity, like any other plant PME, depends on the NaCl concentration in the assay (**Figure 5**). The activity increased with the salt concentration up to 0.13 M, but when the NaCl level was raised further, the activity decreased gradually. At 0.3 M NaCl, ∼60% of the activity at optimal NaCl concentration (0.13 M) was observed. According to this study, pepper PME possesed only 3% activity in the control assay (no NaCl added) in comparison to the activity at 0.13 M NaCl. Previous studies on purified apple PME reported also a maximal enzyme activity for 0.13 M NaCl at pH 7 (*10*). The effect of NaCl on plant PMEs varies considerably, and even PMEs from different varieties of the same fruit have shown different optimum NaCl concentrations for their maximum activities (*21*, *23*). The stimulatory effect of the salt on the pepper PME activity is quite high, with 13-, 25-, and 30-fold increases in the activity for 0.05, 0.1, and 0.15 M of NaCl concentrations, respectively, as compared to the control.

Effect of pH on Pectin Methylesterase Activity. The study of purified pepper PME activity as a function of pH reveals (**Figure 6**) that the activity increases rapidly from 4.5 to 6.5 (79%). The optimum pH value of 7.5 is within the optimal pH range of 7−9 of most plant PMEs from different sources (*23*).

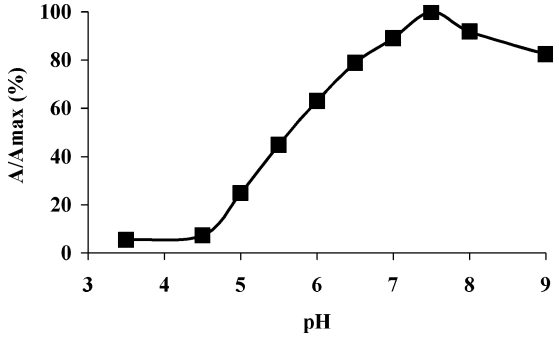

Figure 6. Activity of green pepper PME as a function of pH. Assay conditions: apple pectin (DE 75%) solution 3.5 mg/mL, 22 °C, 0.117 M NaCl.

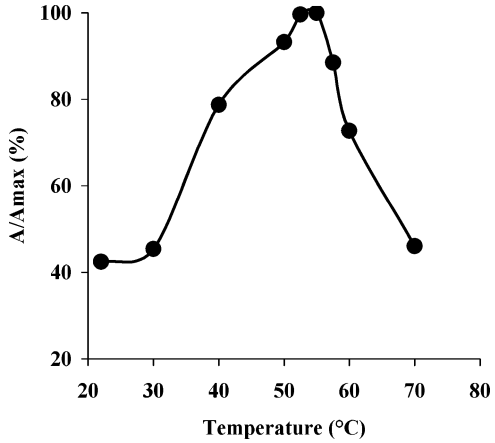

Figure 7. Activity of green pepper PME as a function of temperature. Assay conditions: apple pectin (DE 75%) solution 3.5 mg/mL, pH 7.5, 0.117 M NaCl.

Above pH 7.5, the PME activity remains relatively high over the range of alkaline pH. At pH 9.0 there is still 83% of PME activity at optimal pH.

Effect of Temperature on Pectin Methylesterase Activity. The effect of the reaction temperature on PME activity is shown in **Figure 7**. From 30 to 40 °C, there was a sudden increase in PME activity, up to 79%. Maximal PME activity was observed around 52.5−55 °C. From the slope of the Arrhenius plot (not shown) of the data in **Figure 7**, the activation energy (E_a) of pepper PME was estimated to be 22.8 kJ mol^{-1} (5445 cal mol^{-1}), from 22 to 55 °C. This value is in agreement with values of 5000, 5600, 5740, 5800, and 6200 cal mol^{-1}, which have been reported for cucumber ionically bound PME (*24*), orange PME 1 and 2 (*25*), apple PME (*26*), and potato PME (*27*), respectively.

Temperature and Pressure Stability of Purified Pepper Pectin Methylesterase. In **Figure 8**, relative residual activity is plotted as a function of inactivation temperature. Within the temperature range of inactivation, purified pepper PME was gradually inactivated. At 60 °C, 50% of PME activity was lost after 5 min of treatment, whereas at 68 °C, >90% of the PME activity was lost under the same conditions and at 70 °C purified pepper PME is completely inactivated. Two inactivation phases can be observed (**Figure 8**). The onset of the first inactivation phase is estimated at ∼50−55 °C, and the onset of the second one at ∼62 °C. Pressure stability at 25 and 60 °C of purified pepper PME was screened by pressurizing samples for 15 min in the pressure range of 400−800 MPa (**Figure 9**). At 25 °C, there is a slight decrease on the relative residual activity in the pressure range studied, whereas at 60 °C the PME is quite stable

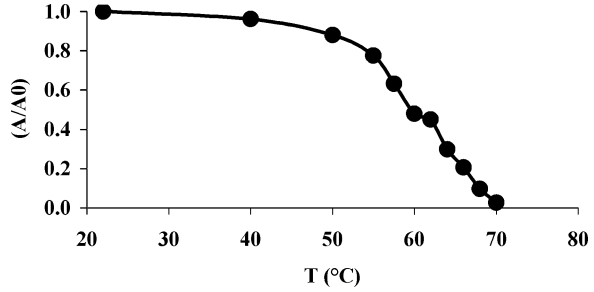

Figure 8. Thermal stability of purified green pepper PME. Residual activity was measured after 5 min of treatment at different temperatures. Assay conditions: apple pectin (DE 75%) solution 3.5 mg/mL, pH 7.5, 0.117 M NaCl.

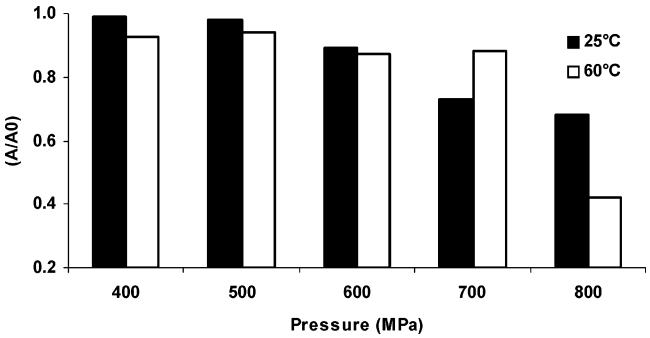

Figure 9. Pressure stability of purified green pepper PME at 25 and 60 °C. Residual activity was measured after 15 min of treatment. Assay conditions: apple pectin (DE 75%) solution 3.5 mg/mL, pH 7.5, 0.117 M NaCl.

until 700 MPa. This could be due to the effect on the heat labile form of PME at the lower temperature, whereas at the high temperature the heat labile form might already have been inactivated during pressure buildup and in the equilibration period. When the adiabatic effect in the pressure range studied is taken into consideration, the residual PME activity after the 2 min of equilibration for 25 and 60 °C is already around 90−80% and 78−42%, respectively, compared to the control at 25 °C and at atmospheric pressure (data not shown). Crelier et al. (*28*) concluded that tomato PME was stabilized against thermal denaturation at pressures above atmospheric pressure and up to 500−600 MPa. In more recent studies on tomato PME, in either juice or purified form at pH 4.4, the enzyme was revealed to be very pressure-resistant, up to 700 MPa (*29*). Seyderhelm et al. (*30*) reported the effect of high pressure on PME for commercial orange PME in Tris buffer (pH 7), at 45 °C. The shortest processing time of 2 min was shown to be sufficient to completely inactivate PME at 900 MPa. A degree of inactivation rate of 58% was obtained for purified pepper PME after 15 min of treatment at 800 MPa and 60 °C.

Thermal Inactivation Kinetics of Purified Pepper Pectin Methylesterase. On the basis of results of thermal stability, a detailed kinetic study of thermal inactivation of purified pepper PME dissolved in 20 mM Tris buffer was performed in the range from 55 to 70 °C at atmospheric pressure. **Figure 10A** presents the thermal inactivation curves of pepper PME at pH 7.5, in the temperature range from 55 to 57 °C. A fractional conversion model could accurately describe this inactivation behavior, indicating the presence of a temperature-resistant enzyme fraction that is not affected after a prolonged heating at the preset temperatures. The estimated rate constants, A_∞ values, and activation energy are given in **Table 2**. As expected, the inactivation rate constants increase with increasing temperature.

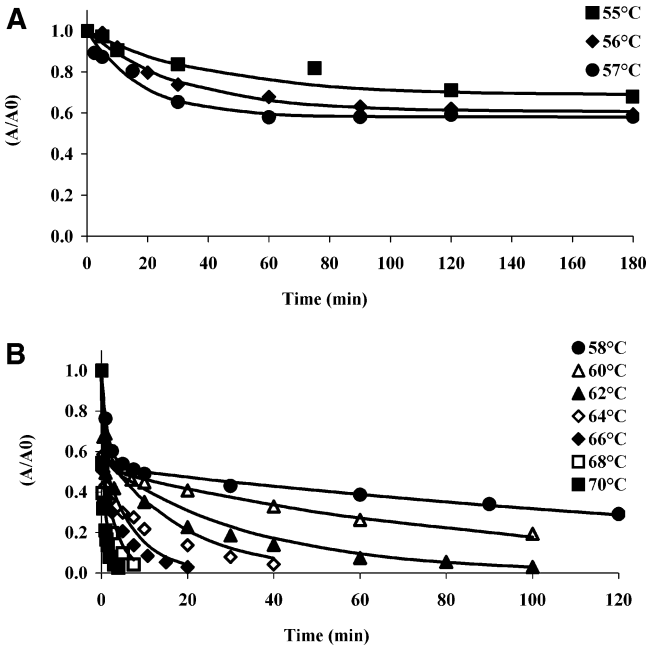

Figure 10. Thermal inactivation of purified green pepper PME dissolved in 20 mM Tris buffer (pH 7.5), for 55–57 °C (**A**) and 58–70 °C (**B**) temperature ranges.

Table 2. Estimated Kinetic Parameters for Thermal Inactivation of Purified Pepper Pectin Methylesterase in 20 mM Tris Buffer, pH 7.5

	A_∞ (%)	k (min^{-1})
55 °C	68.6 ± 1.2[a]	0.0246 ± 0.0029
56 °C	60.7 ± 0.9	0.0330 ± 0.0024
57 °C	58.1 ± 1.5	0.0559 ± 0.0081
E_a (kJ mol^{-1})	369.2 ± 61.2	

	A_L (%)	A_S (%)	k_L (min^{-1})	k_S (min^{-1})
58 °C	47.5 ± 1.2	52.4 ± 0.7	0.6689 ± 0.0413	0.0050 ± 0.0003
60 °C	47.5 ± 3.2	51.6 ± 1.9	0.8084 ± 0.1348	0.0107 ± 0.0013
62 °C	nd[b]	55.6 ± 2.2	nd	0.0293 ± 0.0019
64 °C	nd	61.0 ± 1.1	nd	0.0533 ± 0.0022
66 °C	nd	59.2 ± 2.8	nd	0.1353 ± 0.0117
68 °C	nd	58.3 ± 5.7	nd	0.2913 ± 0.0604
70 °C	nd	61.5 ± 6.9	nd	0.7161 ± 0.1176
E_a (kJ mol^{-1})	388.93 ± 7.47			

[a] Standard error of regression. [b] Not determined.

The temperature dependence of the inactivation rate constants in the temperature range (55−57 °C) was estimated by linear regression analysis (eq 4) as 369.2 kJ mol^{-1}. The residual activity after prolonged heating (A_∞) was ∼62% of the total PME activity. **Figure 10B** illustrates the thermal inactivation curves of pepper PME in a temperature range from 58 to 70 °C. The thermal inactivation of pepper PME at pH 7.5 in this temperature range exhibits a biphasic model, indicating the presence of a heat-labile and a heat-resistant fraction of PME, both showing first-order inactivation mechanisms. Labile and resistant forms of PME have been shown to occur in a number of other fruits and vegetables including oranges (*18*, *31*, *32*), grapefruits (*33*), sweet cherries (*8*), persimmon (*9*), tomatoes (*28*), and green beans (*34*). At the higher temperatures, the inactivation of the heat-labile fraction proceeds very quickly so that the inactivation rate constants cannot be accurately estimated. The activity and the inactivation rate constants of the stable fraction were estimated using nonlinear regression analysis and the activation energy using linear regression analysis (**Table 2**). Purified pepper PME, like carrot PME, is less stable toward thermal treatment when compared to other PMEs from different sources. The inactivation rate constant for the labile and stable PME fraction of purified pepper PME at 60 °C were, respectively, 0.8084 and 0.0107 min^{-1}, and that for the stable PME fraction at 70 °C was 0.7161 min^{-1}. Ly-Nguyen et al. (*12*) reported a $k_{60°C} = 0.6814$ min^{-1} for purified carrot PME, whereas Anthon and Barret (*35*) obtained 0.654 and 0.684 min^{-1} for carrot juice, considering as reference temperatures 65 and 70 °C, respectively. The thermostable pepper PME fraction contributed ∼57% of the total activity. The inactivation rate constants of the thermostable fraction increase with increasing temperature. The Arrhenius plot for the thermal inactivation of the thermoresistant fraction in the temperature range of 58−70 °C (**Figure 11**) showed a linear behavior ($r^2 = 0.998$) and yielded an activation energy of 388.9 kJ mol^{-1}. This value is in the same range found for commercial orange PME, 301.4−350.5 kJ mol^{-1} (*19*), but higher than the one found by Ly-Nguyen et al. (*12*) for purified carrot PME (289.2 kJ mol^{-1}).

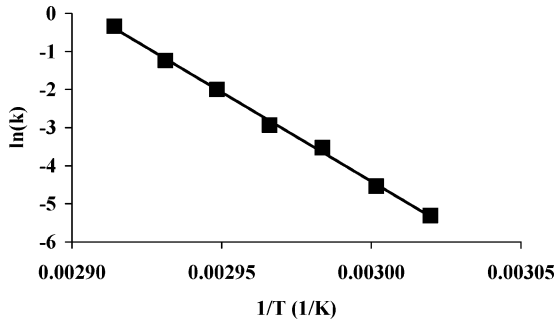

Figure 11. Temperature dependence of inactivation rate constant for thermal inactivation of the thermostable fraction of purified pepper PME.

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ORIGINAL PAPER

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Process stability of *Capsicum annuum* pectin methylesterase in model systems, pepper puree and intact pepper tissue

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Abstract Process stability studies towards temperature and/or pressure on pepper pectin methylesterase (PME) were carried out in different systems (purified form, crude extract, pepper pieces and puree) at pH 5.6. Within the temperature range studied (22–80 °C, 5 min), pepper PME in pure form and crude extract was gradually inactivated showing a biphasic inactivation behaviour, indicating the presence of isoenzymes of different thermostability. Pepper samples heated for 15 min showed a maximum of residual PME activity around 55 °C. Isothermal inactivation of pepper PME in purified form and crude extract at pH 5.6 could be described by a biphasic inactivation model for the temperature range studied (62–76 °C). A stable behaviour towards high-pressure/temperature treatments (400–800 MPa/25–60 °C) was observed for crude extract and purified pepper PME. PME in pepper puree samples revealed to be very pressure stable. Mild temperatures combined with pressure treatments seem to increase the extractability from PME in pepper tissue, probably due to the effect on the cell structure.

Keywords Pectin methylesterase · *Capsicum annuum* · Pepper pieces · Puree · Thermal/high-pressure treatments

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Introduction

Nowadays, there is an increasing demand for ready-to-eat fruits and vegetables, which retain their fresh-like characteristics. However, there is a problem concerning to their short shelf life: they have to reach in time both market distribution and direct consumption by the consumers. Green bell peppers, like any other fruit or vegetable, are perishable and therefore not suitable for long-term storage, even at low temperatures [1, 2]. Blanching of bell pepper fruits, prior to freezing and frozen storage, aims to decrease the contaminating flora to accepted levels to ensure safe raw consumption. Nevertheless, it is well reported in literature that thermal treatments can cause enzymatic, colour, texture and flavour changes and decrease the nutritional value. High-pressure processing can be used as an alternative to conventional thermal processing, such as water blanching, since it offers some advantages particularly in maintaining food quality on the abovementioned quality attributes [3].

Pressure–temperature inactivation of enzymes from different sources has been investigated in different model systems, i.e. buffer system, juices, puree and intact food products—in situ [4–9]. These systems were studied on a kinetic basis under isobaric/isothermal conditions to evaluate the impact of this novel technology on the (in)activation of food quality related enzymes (i.e., pectin methylesterase, polygalacturanase, polyphenoloxygenase, lipoxygenase). Pectin methylesterase (PME, EC 3.1.1.11), which catalyses the de-esterification of pectin, yielding methanol and pectin with a lower degree of esterification, plays a central role in processed food texture by creating the possibility of calcium binding between pectin polymers. The control of PME activity, through knowledge of dependence on parameters such as temperature and/or high-pressure, is of great practical importance in the food industry for protecting and improving the texture and firmness of several processed fruits and vegetables [10]. Stolle-Smitts et al.[11] showed that blanching beans at 70 °C resulted in a greater firmness than blanching at 90 °C, which could be explained by the action of PME on

the pectic substances. Several studies regarding carrot tissue demonstrated that thermal pre-treatments of carrot tissue prior to cooking revealed an enhanced cell–cell adhesion and a reduction of the extended softening, due to an increase in the thermal stability of the calcium cross-linked pectic polysaccharides [12, 13].

The objective of this work was to determine thermal and high-pressure stability of green bell pepper PME in different systems, from buffer solutions to pepper pieces.

Materials and methods

Raw material

Green bell peppers (*Capsicum annuum*) were purchased from a local auction (Mechelen, Belgium). After washing and cleaning the peppers, the stalk and the seeds were removed.

Enzyme preparation

The peppers were cut into small pieces and extracted at 4 °C during 2 h, with 0.2 M Tris(hydroxymethyl)-aminomethan buffer (i.e., Tris buffer) (pH 8.0) containing 1 M NaCl. Afterwards, the extract was subjected to an ammonium sulphate precipitation from 30 to 80% and the resulting precipitate was re-suspended in 20 mM citrate buffer (pH 5.6). In order to obtain purified PME, the crude extract was mixed with CNBr-Sepharose 4B-PME-inhibitor gel and submitted to a purification step by affinity chromatography, using the procedure described by Ly Nguyen et al. [14]. Purified pepper PME fractions were pooled and concentrated using Centricon plus 20 ultrafilters (Millipore). The fractions were dissolved in 20 mM citrate buffer (pH 5.6). The citrate buffer was chosen due to the relatively low negative ionization volume of this buffer [15] and the natural occurrence of citric acid in this vegetable [16, 17]. The pH 5.6 was the pH-value found in pepper homogenate.

Pepper samples preparation

Peppers were cut into small sticks of 1–1.5 cm of length and 6–7 cm width. Each sample contained 7–8 small sticks, placed in a double plastic bag and sealed under vacuum (ca. 55 g). To create a homogenous sample for thermal and high-pressure processing, 6–7 peppers (ca. 200 g each) were used to prepare the individual samples. Puree samples were prepared from small pieces of pepper, followed by homogenisation (Buchi Mixer B-400), during 5 s (×3). Pepper juice was separated from the pulp by centrifugation (10,000×*g*, 30 min) and, if necessary, the pH was adjusted to 5.6 with 0.1 M NaOH. The samples were reconstituted by adding pepper juice. Two puree samples were prepared with different ratio of pulp and juice (w/w). The ratio between the juice and the pulp was 1:2 and 2:1, further designated as puree A and B, respectively.

Moisture content determination

The dry matter content of the samples was determined by drying a known weight of homogenized samples overnight at 70 °C, followed by 3 h at 105 °C. After cooling to room temperature, the samples were re-weighted and the dry matter and moisture content were calculated. The moisture content was 95.9±0.1%, 97.6±0.5% and 87.4±0.6% for pieces, and puree samples (A and B), respectively.

Pectin methylesterase activity assay

The PME activity was measured by automatic titration of the acid production per time unit, using 0.01 N NaOH and an automatic pH-stat (Metrohm, Switzerland). The purified PME samples after the treatments were mixed with 3.5 mg/ml apple pectin solution (DE 75%, 30 mL) containing 0.117 M NaCl (pH 7.0), at 22 °C. The activity unit (U) of PME is defined as the amount of enzyme required to release 1 μmol of carboxyl group per min, under the aforementioned assay conditions.

Thermal treatments

The crude extract and purified enzyme solution (20 mM citrate buffer, pH 5.6) were enclosed in glass capillaries (Hirschmann, Germany), followed by immersion in a temperature-controlled water bath, at constant temperature ranging from 22–80 °C (5 min). The isothermal treatments of purified pepper PME (pH 5.6) were investigated within a temperature range of 62–76 °C. After the treatments, the capillaries were immediately cooled in ice-water bath. Residual activities of PME were measured within 60 min of storage in an ice-water bath, as described in the previous section.

For pepper pieces and puree samples, the temperature range studied was from 25–80 °C, for 15 min. Heat inactivation kinetics was studied in detail at 68 °C by isothermal inactivation experiments. For thermal treatments, the puree samples were evenly distributed within plastic bags and placed in a rack in such a way that they received the same heat treatment. After the treatments, the samples were immediately cooled in a ice-water bath, followed by freezing in liquid nitrogen and storage at −80 °C, for further PME extraction with the extraction buffer (0.2 M Tris buffer, pH 8.0, 1 M NaCl), for 2 h at 4 °C, and enzyme activity determination. Each treatment was performed in duplicate as well as the PME activity measurements.

Combined thermal and high-pressure treatments

Pressure treatments of crude extract and purified pepper PME were conducted in an 8×8 ml multivessel high-pressure apparatus (Resato, Roden, The Netherlands), according to Castro et al. [18]. The pressure medium is a glycol–oil mixture (TR-15, Resato). To enclose the enzyme solution, flexible microtubes of 0.3 ml were used (Elkay, Leuven, Belgium). The microtubes were placed in the pressure vessels, already equilibrated at 25 and 60 °C. Pressure was built up slowly (100 MPa/min) to minimize temperature increases due to adiabatic heating. After 15 min the pressure was released, the samples were immediately cooled in an ice-water bath and the residual activities of PME were measured within 60 min of storage in ice water. For the combined thermal and high-pressure treatments, the puree samples were placed in plastic tubes (50 ml). A single vessel high-pressure equipment (590 ml volume, i.d. 50 mm, height 300 mm), with a maximum operating pressure of 600 MPa and a working temperature ranging from −30;C to 100 °C (SO. 5-7422-0, Engineered Pressure Systems International EPSInt, Belgium) was used. The pressure medium is a mixture of propylene and glycol (60% Dowcal N, Switzerland). After each treatment, the samples were treated as described for thermal treatments.

Data analysis

Enzyme inactivation can often be described by a first-order kinetic model [19]. When there are several isozymes present, which show different behaviour towards temperature and/or pressure, i.e., labile and stable fraction, and both inactivating according to a first-order kinetic model [20–24], a biphasic kinetic model (Eq. 1), or distinct isozyme model can be used [18]. There is a fast inactivation period followed by a decelerated decay.

$$A = A_L \exp(-k_L \cdot t) + A_S \exp(-k_S \cdot t) \quad (1)$$

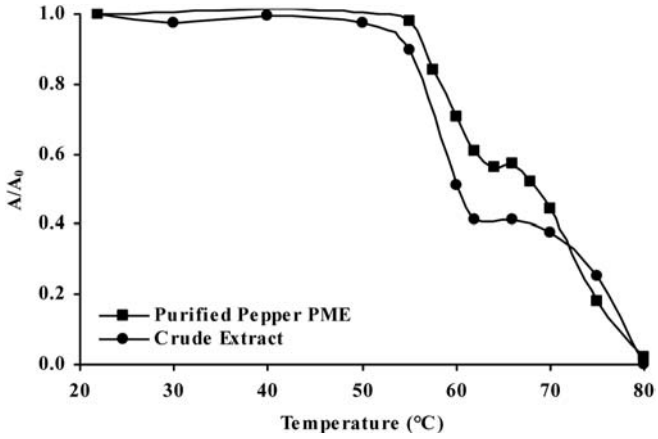

Fig. 1 Thermal stability of PME in pepper crude extract and purified pepper PME, at pH 5.6. Residual activity was measured after 5-min treatment time

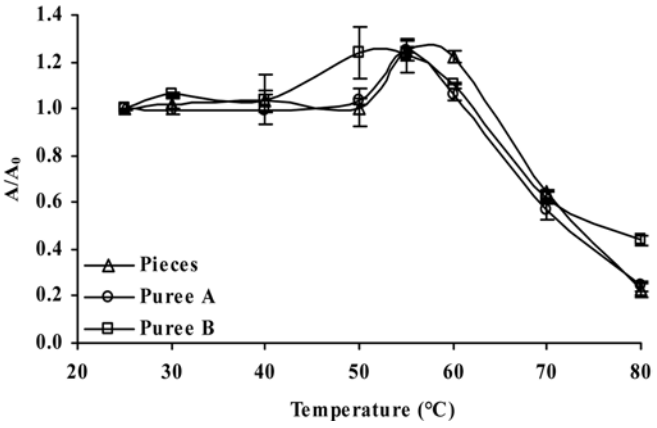

Fig. 2 Thermal stability of pepper PME in pieces and puree samples, after 15 min of heating time. The bars represent the standard deviation (n=2)

Where the subscript L and S means labile and stable enzyme fraction, respectively. The residual activity from the labile and stable fractions (A_L and A_S) as well as the inactivation rate constants of both fractions (k_L- and k_S-value) can be estimated by non-linear regression analysis [25]. Once the k-values are estimated, its temperature dependence can be derived for the labile and the stable fractions. The temperature dependence of k-value is given by the activation energy (E_a) as indicated by the Arrhenius model (Eq. 2):

$$\ln(k) = \ln(k_{ref}) + \left[\frac{E_a}{R}\left(\frac{1}{T_{ref}} - \frac{1}{T}\right)\right] \quad (2)$$

Where T and T_{ref} are the absolute temperature and the reference temperature (K), respectively; k_{ref} (min^{-1}) is the rate constant at T_{ref}; E_a is the activation energy (kJ mol^{-1}), R (8.314 J mol^{-1} K^{-1}) is the universal gas constant. The activation energy can be obtained by linear regression analysis when the natural logarithm of the rate constant is plotted versus the inverse of absolute temperature. This relation is valid at constant pressure.

Results and discussion

Thermal stability

Crude extract and purified pepper PME

Thermostability studies were performed in crude extract and for purified pepper PME (20 mM citrate buffer, pH 5.6), at different temperatures, after 5 min of heating time. The residual PME activity was considered as the ratio between the enzyme activity after a given treatment (A) and the enzyme activity of a non-treated sample (A_0). In Fig. 1, relative residual PME activity is plotted as a function of inactivation temperature. For temperatures higher than 55 °C, pepper PME in both systems was gradually inactivated. Two inactivation phases can be observed, which could indicate the presence of isoenzymes of different thermostabilities, which is in agreement with previous studies related with purified pepper PME at pH 7.5 [18]. The first phase starts to be inactivated at 55 °C, while the inactivation of the second starts at 66 °C. The PME fraction that seems to be more thermostable contributes for 41 and 57% of the total activity in crude extract and purified form, respectively. At 60 °C, around 29 and 49% of the initial PME activity are inactivated in purified pepper PME and crude extract, respectively; while at 80 °C there is complete inactivation. Castro et al. [18] observed a decrease on the initial activity, up to 50%, when purified pepper PME was thermally treated at 60 °C for 5 min, at the optimal pH for purified pepper PME activity (pH 7.5). Purified pepper PME seems to be more thermostable at acidic pH. Although other studies related to tomato, orange and papaya purified PME seem to prove that acidification of the media increases the PME inactivation [24, 26]. Sun and Wicker [27] concluded that pH affected the stability and accessibility of hydrophobic domains to solvent regarding the thermolabile form of grapefruit PME while the thermostable form seemed to be more resistant to conformational changes at low pH. In order to better understand why pH plays a different role in purified pepper PME, further studies on the three-dimensional structure of PME should be considered.

Pepper samples

To investigate PME thermostability *in situ*, pepper pieces and puree samples were heated at different temperatures, for a constant time of 15 min (Fig. 2). After extraction, the relative enzyme activity in these systems was considered as the ratio between the enzyme activity after a given treatment (A, U/100 g pulp×ml) and the enzyme activity of a non-treated sample (A_0, U/100 g pulp×ml). The relative PME activity of the samples after each treatment was characterized by an increase in the residual activity from 50 °C to 55 °C, followed by a fast decay above 60 °C. The increase in residual PME activity around 55 °C may be explained by the thermally induced cell-wall damage and loss of membrane selective permeability in the plant tissue, but insufficient to inactivate the enzyme. Therefore, PME would be more easily ex-

tracted and, at the same time, activated by simultaneous cation diffusion. These studies seemed to be in agreement with other related in situ PME investigations from different sources. Residual PME activity after low-temperature/long-time blanching of potatoes registered a maximum at 60–65 °C, after 45 min of treatment time [28]. More recent studies proved that there is an induced thermal activation of PME between 60 °C and 70 °C when potato tubers undergo blanching treatments (5 min) and that the temperature distribution in the sample affects the degree of PME activity retention during processing [29]. The same authors showed that PME activation takes place as soon as the tissue reaches an average of temperature above 55 °C. Conventional pasteurisation (72 °C, 4 min) of tomato puree induced a two-fold increase of PME activity when compared to untreated samples [30]. Vu et al. [13] also obtained an increase of the activity of extracted PME with the heating time when carrots cylinders were heat treated at 50 °C and 60 °C.

Thermal inactivation kinetics

On the basis of the thermostability results, kinetic studies of thermal inactivation of PME in crude extract and purified pepper PME (citrate buffer, pH 5.6) were performed within the range of 62–76 °C at atmospheric pressure (Figs. 3A, B). In both systems, two different fractions were observed: a heat labile and a heat stable. Labile and resistant forms of PME have been shown to occur in a wide variety of fruits and vegetables including acerola [31], banana [14], grapefruits [27, 32], oranges [7, 24], persimmon [33], carrots [8, 34], cucumbers [35, 36], tomatoes [37], green beans [38] and green bell peppers [18]. The thermostable pepper PME fraction contributed to about 52.0±5.8% of the total activity in purified enzyme solution, which seems to be in agreement with the thermal stability studies performed in green peppers. Castro et al.[18] found around 57–62% the thermostable pepper PME fraction at pH 7.5. It seems that there is a slight increase of the thermostable fraction when crude extract (40.3±3.1%) is submitted to the purification step, which could be due to the interactions of several components present in the media.

Since both fractions showed a first-order inactivation mechanism, a biphasic model (Eq. 1) was used to estimate

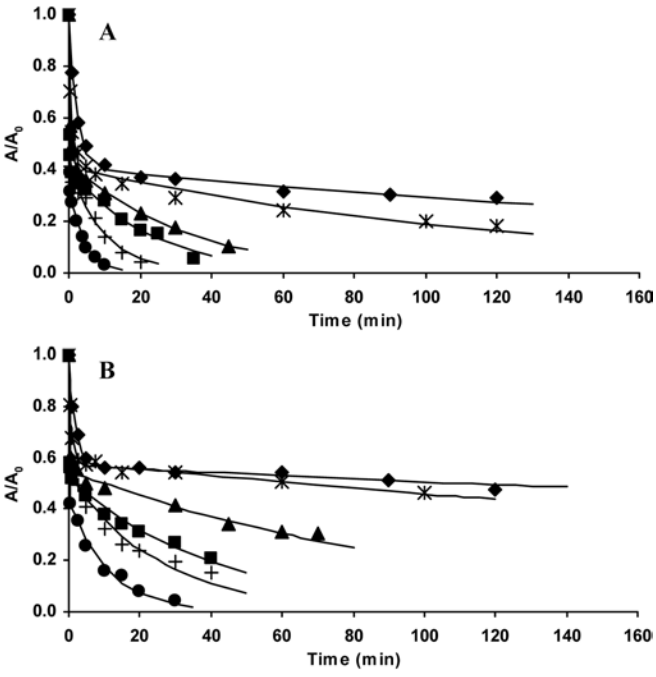

Fig. 3 Thermal inactivation of PME in pepper crude extract (**A**) and purified pepper PME (**B**), at pH 5.6. (◆) 62 °C, (*) 64 °C, (π) 68 °C, (■) 70 °C, (+) 72 °C, and (●) 76 °C

the kinetic inactivation parameters (Table 1). At pH 5.6, the inactivation rate constant for both labile and stable PME fractions of crude extract and purified pepper PME were reported up to 70 °C. At higher temperatures, the inactivation of the heat-labile fraction proceeds very fast and therefore the corresponding *k*-value cannot be estimated. A *k*-value at 62 °C of 0.548±0.048 min^{-1} was obtained for the labile fraction of purified pepper PME at pH 5.6, while Castro et al. [18] stated a similar value (0.669±0.041 min^{-1}) at 58 °C and pH 7.5, indicating a lower thermostability at pH 7.5 compared to pH 5.6. In Table 1, it can also be seen that the resistant fraction was hardly affected at temperatures up to 64 °C. The *k*-values for the thermostable fraction are higher for crude extract while the *k*-values for thermolabile fraction are statistically higher for purified form ($P<0.10$), with the exception of $k_{64\ °C}$. The pepper PME fractions show different thermostabilities in different media. When evaluating the inactivation rate constant of thermostable fraction from

Table 1 Estimated kinetic parameters for thermal inactivation of purified pepper PME and PME in crude extract, pH 5.6

	Purified PME		Crude extract	
	k_L (min^{-1})	k_S (min^{-1})	k_L (min^{-1})	k_S (min^{-1})
62 °C	0.548±0.048[a]	0.0011±0.0002	0.452±0.041	0.0033±0.0006
64 °C	1.337±0.099	0.0023±0.0003	1.377±0.158	0.0078±0.0010
68 °C	3.935±0.686	0.0100±0.0009	2.515±0.247	0.0316±0.0025
70 °C	7.649±1.817	0.0251±0.0025	5.705±0.648	0.0454±0.0037
72 °C	nd	0.0396±0.0036	nd	0.1002±0.0097
76 °C	nd	0.0912±0.0074	nd	0.2403±0.0131
Ea (kJ/mol)	303.5±19.5	318.0±20.8	271.1±47.3	297.4±15.0
R^2	0.992	0.983	0.943	0.990

[a] Standard error of regression; nd: not determined

different sources, i.e. cucumber (pH 6.7, 55–65 °C) and papaya (pH 4.0, 55–70 °C), it seems that the thermostable fraction from green bell pepper is more heat resistant under the studied conditions [26, 35].

The temperature dependence of the *k*-values for thermal inactivation of pepper PME in purified form and crude extract are also shown in Table 1. The E_a-values for thermal inactivation for both fractions in purified form and in crude extract are not statistically different ($P<0.01$). Considering the E_a-value for the thermostable fraction at pH 5.6 (318.0±20.8 kJ/mol), it is similar to orange PME (pH 4.2, 292.6±14.6 kJ/mol) [39], lower than the one found for purified pepper PME (pH 7.5, 388.9±7.5 kJ/mol) [18], but higher than the one reported for cucumber PME (water, 260.2–302.9 kJ/mol) [36] and papaya PME (pH 4.0, 257 kJ/mol) [26]. The activation energy of the thermolabile fraction for crude extract and purified pepper PME are also similar respectively 271.1±47.3 and 303.5±19.5 kJ/mol.

Pepper samples were thermally treated at 68 °C and different times, ranging from 1–40 min, in order to evaluate the inactivation behaviour of PME in the tissue. The residual PME activity was considered as the ratio between the enzyme activity after a given treatment (A) and the enzyme activity of a non-treated sample (A_0). As in purified form and crude extract, PME in pepper pieces and puree samples exhibited a biphasic inactivation behaviour. The estimated kinetic parameters for thermal inactivation at 68 °C of PME in these pepper model systems were also calculated. The *k*-values for the thermostable fraction were 0.029±0.002, 0.063±0.006, and 0.014±0.002 min^{-1} for pieces and puree *A* and *B* samples, respectively. At this temperature, pepper PME is more stable in purified form (citrate buffer, pH 5.6) than in the other systems (crude extract, puree and pieces); whereas thermal inactivation studies related with carrot PME revealed that PME in situ is much more thermostable than PME in carrot juice and purified PME (citrate buffer, pH 6.0) [40]. When thermal treatments are applied, the cell membranes disrupt and a wide diversity of components can interact more easily with the enzyme, influencing the extend of the enzyme inactivation. The thermostable pepper PME fraction contributed to about 49.8±2.0%, 57.1±3.5% and 44.0±2.9% of the total activity in pieces, puree A and B, respectively, which is similar to the amount found for the other model systems. It should be mentioned that the *k*-values of the thermolabile PME fraction in the pepper model systems were not reported since the inactivation of the thermolabile PME faction occurred (partially) during non-isothermal heating conditions.

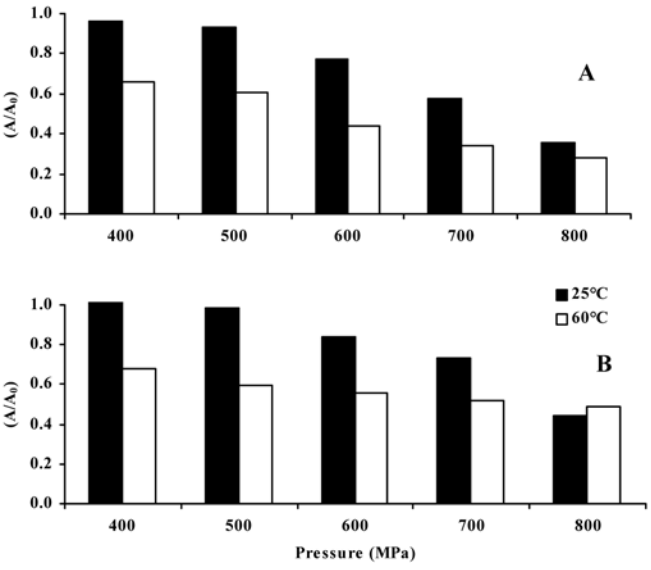

Fig. 4 Pressure stability of PME in pepper crude extract (**A**) and purified pepper PME (**B**), at pH 5.6. Residual activity was measured after 15 min of treatment

High-pressure stability

Crude extract and purified pepper PME

Pressure stability at 25 and 60 °C of PME in pepper crude extract and purified pepper PME was screened by pressurizing samples from 400–800 MPa during 15 min, and plotting residual PME activity as a function of the pressure treatment (Figs. 4A, B). The residual PME activity was considered as the ratio between the pressure/temperature-treated sample and the non-treated sample. As in thermal stability studies, pepper PME seems to be more stable in purified form than in crude extract. There is a decrease up to 56% and 51% of the initial activity of purified PME in the pressure range studied for 25 °C and 60 °C, respectively. This decrease in PME activity could be related not only to the adiabatic effect during the pressure build up for both temperatures, but also to the heat inactivation of the thermolabile fraction at 60 °C. This effect seems to be more notorious in crude extract since the amount of thermolabile fraction is higher than in purified pepper PME. When the 15 min-treated sample was compared to a blank (pressure-released 2 min after the desired pressure), it appears that during the combined pressure/temperature conditions for 15 min (pH 5.6), pepper PME is rather pressure stable (data not shown). In addition, both pressure and thermal stability of purified pepper PME under the studied conditions seem to increase upon media acidification, even though similar studies with orange PME at acidic pH showed that pressure–temperature inactivation was accelerated by lowering the pH [24].

Pepper samples

Figure 5A–C illustrates the effect of combined thermal and high-pressure treatments on PME activity [U/(100 g of pulp weight × ml)] of several pepper samples. At 25 °C, all pepper systems show an increase in the residual PME activity with the pressure level. At 40 °C, the re-

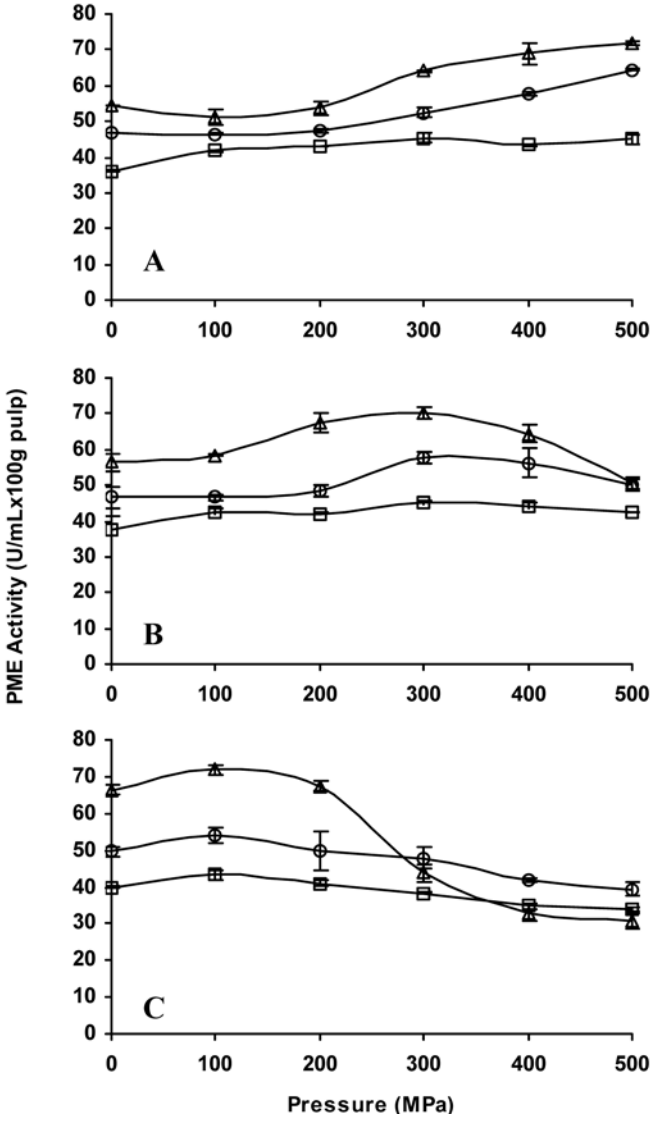

Fig. 5 Pressure stability of pepper PME at 25 °C (**A**), 40 °C (**B**) and 60 °C (**C**), during 15 min treatment. (△) pieces, (◯) puree A, and (❑) puree B. The bars represent the standard deviation of the treatment (n=2)

sidual PME activity of treated pepper pieces increases up to 300 MPa, followed by a decrease probably due to (partial) inactivation of the labile fraction of PME. At 60 °C, the residual PME activity for all pepper samples decays with increased pressure up to around 40% for intact tissue at higher pressures (P>300 MPa), which could be associated with the inactivation of the labile PME fraction. In puree samples, PME seemed to be more stable than in intact pepper tissue under the temperature/pressure conditions studied. In line with these observations, some authors [41] observed an increase in PME activity in tomato (*L. esculentum*) cell cultures with pressure (up to 150 MPa), explained by a more effective extraction of the enzyme due to damage of plant cell membrane/wall and changes in cell wall association of enzyme. The extractability of other components (i.e., carotenoids) in tomato puree (*L. esculentum Mill.*) seems also to be affected by different pressure levels [42]. Further investigations regarding the effect of high-pressure in cherry tomato revealed that a major disruption on the tissue histological structure at 400 MPa (20 °C, 20 min) occurred, whereas at 200 MPa, under the same conditions, the tissue shows minor cell injury, having a similar texture to the non-pressurised sample [43]. Cellular structure of cauliflower when submitted to 400 MPa (5 °C, 30 min) was damaged, as well as loss of turgour and presence of fluid in the intercellular spaces [44].

Conclusions

Pepper PME seemed to be rather thermal and pressure stable under the studied conditions for the different models. It was found that purified PME in citrate buffer is more stable than PME in crude extract and in pepper model systems. This could be possibly attributed to the fact that in situ PME is bound to the cell wall and/or presence of other factors present in peppers that may influence the stability of the enzyme. The last effect could be more important for puree model systems since the cells were previously damaged and the natural components would be in close contact with PME. Combined mild thermal/high-pressure treatments seemed to increase the residual PME activity in the different pepper models, which could be beneficial for structure improvement of processed peppers by enhancing PME activity.

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Inactivation of pepper (*Capsicum annuum*) pectin methylesterase by combined high-pressure and temperature treatments

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Abstract

Pressure and/or temperature inactivation (at mild temperature, 10–64 °C, in combination with high-pressure, 0.1–800 MPa) of the labile fraction of purified pepper pectin methylesterase (PME) was studied in a model system at pH 5.6. Inactivation of the labile fraction under mild-heat and high-pressure conditions could be accurately described by a fractional conversion model, while a biphasic model was used to estimate the inactivation rate constant of both fractions at more drastic conditions of temperature/pressure. At lower pressures ($P \leqslant 300$ MPa) and high temperatures (>54 °C), an antagonistic effect of pressure and temperature was observed. Pressure and temperature dependence of the inactivation rate constants of the labile fraction was quantified using the Eyring and Arrhenius relations, respectively. A third-degree polynomial model (derived from the thermodynamic model) was successfully applied to describe the temperature/pressure dependence of the inactivation rate constants of the labile pepper PME fraction.

Keywords: *Capsicum annuum*; Pectin methylesterase; High-pressure; Inactivation; Thermodynamic model

1. Introduction

Preservation processes, which are of major importance to food industry, include procedures to eliminate, inactivate, or reduce microorganisms and enzymes, that would, upon storage, cause deterioration of food or jeopardize the consumer's health. Thermal processing is still one of the most frequently used methods in the food preservation technologies since it allows efficient inactivation of both pathogenic and spoilage microorganisms as well as food quality deteriorating enzymes. For example, in the juice industry thermal treatment (e.g., 90 °C, 1 min) is a conventional method used to inactivate pectin methylesterase (Eagerman & Rouse, 1976) since this enzyme seemed to be more thermoresistant than the relevant spoiling microorganisms (Nath & Ranganna, 1977). On the other hand, these treatments can cause considerable organoleptic and nutritional quality losses. In addition to this factor, the constant consumer demand for high quality, fresh-like, safe food products that are minimally processed continuously stimulates research on novel processing technologies. High-pressure processing is already being applied with success for some food products commercialised in Japan, United States and some European countries (Heremans, 2002), with little detrimental effect on food quality attributes (Cheftel, 1991; Heremans, 1993; Knorr, 1993). However, as some enzymes seem to be very pressure stable (Ly Nguyen et al., 2002; Seyderhelm, Boguslawski, Michaelis, & Knorr, 1996;

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Weemaes, Ludikhuyze, Van den Broeck, & Hendrickx, 1998), high-pressure treatments will most likely be accompanied by other treatments, such as mild heating (Farr, 1990).

Pectin methylesterase (PME, EC 3.1.1.11), which has been found in plants as well as in pathogenic fungi and bacteria, de-esterifies endogenous plant cell-wall pectins, yielding methanol and pectin with a lower degree of esterification. This product, with free carboxylic acid functions, can easily interact with calcium ions, improving the firmness of processed fruits and vegetables (Van Buren, 1979). Consequently, PME can enhance the texture of fruit and vegetable products (Alonso, Canet, & Rodriguez, 1997; Saldaña & Meyer, 1981; Villarreal-Alba, Contreras-Esquivel, Aguilar-Gonzalez, & Reyes-Vega, 2004) increase the extraction yield of juices relatively to conventional methods (Anastasakis, Lindamood, Chism, & Hansen, 1987), and promote the water removal from tissues submitted to drying techniques (Manabe, 1982). Plant PME have been isolated, purified and studied in terms of pressure–thermal processing stability (e.g., Ly Nguyen et al., 2002; Van den Broeck, Ludikhuyze, Van Loey, Weemaes, & Hendrickx, 2000a, Van den Broeck, Ludikhuyze, Weemaes, Van Loey, & Hendrickx, 2000b). High-pressure/thermal treatments can activate or inactivate plant PMEs, depending on the pressure–temperature conditions applied. Both effects can be beneficial in the processing of fruit and vegetable-based products. Pressure–temperature processing stability data for plant PME are of interest in the food industry.

Bell peppers (*Capsicum annuum*) are becoming more popular in recent years due to their chemical composition (e.g., vitamins), their characteristic and exotic flavour, as well as the slightly pungent taste, which makes them an important vegetable crop in the food industry. They can be consumed fresh or processed, as immature (i.e., green) or as mature fruit (i.e., yellow, orange, red). One of the oldest preservation techniques applied to peppers is the thermal blanching. The objective of this work was to gather kinetic information with regard to the inactivation of PME from green pepper at their natural pH (pH 5.6), in order to identify better the potentials of high-pressure compared to thermal processing.

2. Materials and methods

2.1. Materials

Green bell peppers (*C. annuum*) were purchased from a local auction (Mechelen, Belgium). Apple pectin (degree of esterification, DE, 75%) was obtained from Fluka Chemicals Co. (Switzerland). NH-Sepharose 4B resin was purchased from Sigma (USA). All other chemicals were of analytical grade.

2.2. Methods

2.2.1. Pectin methylesterase purification

PME was extracted from peppers using a 0.2 M Tris(hydroxymethyl)–aminomethane buffer (i.e., Tris buffer) (pH 8.0) with 1 M NaCl, followed by purification by affinity chromatography on a NH-Sepharose 4B-PME-inhibitor column, using the procedure as described by Ly Nguyen et al. (2002). The fractions containing PME activity were pooled and desalted.

2.2.2. Pectin methylesterase assay

PME activity was measured by continuous recording of titration of carboxyl groups released from a pectin solution using an automatic pH-stat (Metrohm, Switzerland) and 0.01 N NaOH solution. Routine assays were performed with a 3.5 mg ml^{-1} apple pectin solution (DE 75%, 30 ml) containing 0.117 M NaCl (pH 7.0) at 22 °C. The activity unit (U) of PME is defined as the amount of enzyme required to release 1 μmol of carboxyl groups per minute, under the aforementioned assay conditions.

2.2.3. Thermal Inactivation of Purified Pepper PME

The isothermal treatments were performed by immersing glass capillaries (Hirschmann, Germany) with the enclosed enzyme solution (20 mM citrate buffer, pH 5.6), in a temperature-controlled water bath, during pre-set time intervals. After the treatments, the capillaries were immediately cooled in ice-water bath. Residual activities of PME were measured within 60 min of storage in an ice-water bath. Thermal inactivation of purified pepper PME was investigated between 56 and 64 °C.

2.2.4. High-pressure inactivation of purified pepper PME

Pressure treatments were conducted in a multi-vessel high-pressure apparatus (eight vessels of 8 ml) (Resato, Roden, The Netherlands). The pressure medium is a glycol–oil mixture (TR-15, Resato). To enclose the enzyme solution, flexible microtubes of 0.3 ml were used (Elkay, Leuven, Belgium). The microtubes were placed in the pressure vessels, already equilibrated at the inactivation temperature. Pressure was built up slowly (~100 MPa min^{-1}) to minimize temperature increases due to adiabatic heating (Ludikhuyze, Indrawati, Van den Broeck, Weemaes, & Hendrickx, 1998a). After pressure build-up, an equilibrium period of 2 min was taken into account in order to allow temperature to evolve to its desired value (Van den Broeck et al., 2000b). After the equilibration period, one vessel was decompressed, the sample was immediately cooled in an ice-water bath and the residual activity measured, which corresponds to the blank sample (A_0). The remaining vessels were then decompressed at pre-set time intervals and the total duration of the experiments carried out was chosen

between 40 and 300 min, depending on the conditions of pressure and temperature studied. Combined treatments of pressure and temperature were applied within the range of 100–800 MPa and 10–62 °C.

2.2.5. Kinetic data analysis

Inactivation of enzymes can often be described by a first-order kinetic model (Eagerman & Rouse, 1976). When there are several isozymes present, which show different behaviour towards temperature and/or pressure, i.e., labile and stable fraction, and both inactivating according to a first-order kinetic model (Indrawati, Van Loey, Ludikhuyze, & Hendrickx, 1999, 2000; Ramesh, Sathyanarayana, & Girish, 1998; Van den Broeck et al., 2000a), a biphasic kinetic model or distinct isozyme model can be used (Eq. (1)). There is a fast inactivation period followed by a decelerated decay:

$$A = A_{\mathrm{L}} \exp(-k_{\mathrm{L}} \cdot t) + A_{\mathrm{S}} \exp(-k_{\mathrm{S}} \cdot t), \tag{1}$$

where A_{L} and A_{S} refer, respectively, to activity of labile and stable fractions; and k_{L} and k_{S} are the inactivation rate constants of both labile and stable fraction, respectively. When only the labile fraction inactivates, whereas the activity of the stable fraction does not change, a non-zero residual activity after prolonged thermal and/or pressure treatment is observed and a fractional conversion kinetic model is to be applied (Eq. (2)):

$$A = A_{\infty} + (A_0 - A_{\infty}) \exp(-k \cdot t), \tag{2}$$

where A_0 and A_{∞} refer, respectively, to the initial enzyme activity and to the activity after prolonged treatment time. The inactivation rate constants can be estimated by non-linear regression analysis. It should be stressed that for experiments at constant temperature and/or pressure, the heating and/or pressurizing time should be long enough so that the remaining activity, A_{∞}, is no longer changing with respect to time (Van den Broeck, Ludikhuyze, Van Loey, Weemaes, & Hendrickx, 1999a, 1999b). The temperature dependence of inactivation rate constants can be estimated using the Arrhenius model (Eq. (3)):

$$\ln(k) = \ln(k_0) + \left[\frac{E_{\mathrm{a}}}{R}\left(\frac{1}{T_0} - \frac{1}{T}\right)\right], \tag{3}$$

where T and T_0 are the absolute temperature (K) and the reference temperature (K), respectively; k_0 is the rate constant at T_0; and R (8.314 J mol^{-1} K^{-1}) is the universal gas constant. As a measure for the pressure dependence of the enzyme inactivation, the activation volume can be estimated using the Eyring relationship (Eq. (4)):

$$\ln(k) = \ln(k_0) - \left[\frac{V_{\mathrm{a}}}{RT}(P - P_0)\right], \tag{4}$$

where P and P_0 are the pressure and the reference pressure (MPa), respectively. Both activation energy (E_{a}, kJ mol^{-1}) and activation volume (V_{a}, cm^3 mol^{-1}) can be estimated by linear-regression analysis of the natural logarithm of k-value versus the reciprocal of absolute temperature or versus pressure, respectively.

2.2.6. Mathematical modeling to describe combined pressure temperature dependence of enzyme inactivation

The basic thermodynamic equation governing the behaviour of a system during a pressure and a temperature change has been previously described by Hawley (1971) and later by Morild (1981). It has been often used to understand systems reversible response towards pressure and temperature. This thermodynamic model can be converted into a kinetic model (Eq. (5)) through the transition state theory of Eyring, suggesting that enzyme inactivation is accompanied by formation of a metastable/transition activated state ($\neq$), which exists in equilibrium with the native enzyme (Indrawati, Van Loey, Ludikhuyze, & Hendrickx, 2000; Sonoike, Setoyama, Kuma, & Kobayashi, 1992; Weemaes et al., 1998):

$$\begin{aligned}\ln(k) = \ln(k_0) &- \frac{\Delta V_0^{\neq}}{R_T T}(P - P_0) + \frac{\Delta S_0^{\neq}}{R_T T}(T - T_0) \\ &- \frac{\Delta \kappa^{\neq}}{2R_T T}(P - P_0)^2 + \frac{\Delta C_p^{\neq}}{R_T T}\left\{T\left[\ln\left(\frac{T}{T_0}\right) - 1\right] + T_0\right\} \\ &- \frac{2\Delta \zeta^{\neq}}{R_T T}(P - P_0)(T - T_0),\end{aligned} \tag{5}$$

where P_0 (MPa) and T_0 (K) are reference pressure and temperature, respectively; $k(P,T)$ and k_0 (P_0, T_0) are inactivation rate constants (min^{-1}); $\Delta\kappa^{\neq}$ is the compressibility factor (cm^6 J^{-1} mol^{-1}); $\Delta\zeta^{\neq}$ is thermal expansibility factor (cm^3 mol^{-1} K^{-1}); $\Delta C_p^{\neq}$ is heat capacity (J mol^{-1} K^{-1}); $\Delta V_0^{\neq}$ (cm^3 mol^{-1}) and $\Delta S_0^{\neq}$ (J mol^{-1} K^{-1}) are the volume and entropy change, respectively, between the native and denaturated states at P_0 and T_0; and R_T is the universal gas constant (8.314 J mol^{-1} K^{-1}). Eq. (5) represents the modified thermodynamic model that describes the inactivation rate constant as function of pressure and temperature. Recently, the thermodynamic model has been successfully applied to describe the pressure–temperature inactivation of several enzymes (Indrawati et al., 2000; Ludikhuyze et al., 1997, Ludikhuyze, Indrawati, Van den Broeck, Weemaes, & Hendrickx, 1998b; Ly Nguyen et al., 2003a, 2003b; Van den Broeck et al., 2000a; Weemaes et al., 1998). Smeller (2002) proposed a possible modification in the vicinity of the reference point, resulting in the following second-order approximation (Eq. (6)):

$$T\left(\ln\left(\frac{T}{T_0}\right) - 1\right) + T_0 = \frac{(T - T_0)^2}{2T_0}. \tag{6}$$

Eq. (5) can be written as a second-degree polynomial (elliptic) equation (Eq. (7)):

$$\ln(k) = \ln(k_0) - \frac{\Delta V_0^{\neq}}{R_T T}(P - P_0) + \frac{\Delta S_0^{\neq}}{R_T T}(T - T_0) - \frac{\Delta \kappa^{\neq}}{2R_T T}(P - P_0)^2 + \frac{\Delta C_p^{\neq}}{R_T T}\frac{(T - T_0)^2}{2T_0} - \frac{2\Delta \zeta^{\neq}}{R_T T}(P - P_0)(T - T_0). \quad (7)$$

When $\Delta\kappa$, ΔC_p and $\Delta\zeta$ are temperature and/or pressure dependent (Smeller & Heremans, 1997; Yamaguchi, Yamada, & Akasaka, 1995), an extended analysis of $\ln(k)$ (T, P) was necessary, involving higher-order terms (Eq. (8)). A third-degree polynomial model (derived from the thermodynamic model) was successfully used to describe the heat-pressure inactivation of purified banana and carrot PME (Ly Nguyen et al., 2003a, 2003b). In the present study, we introduced the following high-order terms in Eq. (7):

$$\frac{2\Delta \xi_{2A}^{\neq}}{R_T T}(P - P_0)^2(T - T_0); \quad \frac{2\Delta \xi_{2B}^{\neq}}{R_T T}(P - P_0)(T - T_0)^2; \quad \frac{\Delta \kappa_2^{\neq}}{2R_T T}(P - P_0)^3 \quad \text{and} \quad \frac{\Delta C_p^{\neq}}{2R_T T T_0}(T - T_0)^3.$$

The subscript "2" refers to the coefficients $(\Delta\kappa, \Delta C_p, \Delta\zeta)$ of the higher-order terms. The resulting equation can then be transformed into a linearized equation so that a multi-linear regression analysis can be applied:

$$\ln(k) = a + AX_1 + BX_2 + CX_3 + DX_4 + EX_5 + FX_6 + GX_7 + HX_8 + IX_9, \quad (8)$$

where

$$a = \ln(k_{\text{ref}}),\ X_1 = -\frac{(P - P_0)}{T},\ X_2 = \frac{(T - T_0)}{T},$$

$$X_3 = -\frac{(P - P_0)^2}{T},\ X_4 = \frac{(T - T_0)^2}{T},$$

$$X_5 = -\frac{(P - P_0)(T - T_0)}{T},\ X_6 = \frac{(P - P_0)^2(T - T_0)}{T},$$

$$X_7 = \frac{(P - P_0)(T - T_0)^2}{T},\ X_8 = \frac{(P - P_0)^3}{T},\ X_9 = \frac{(T - T_0)^3}{T}.$$

By using multiple linear regression analysis, multivariate tests across the multiple dependent variables can be performed and the number of significant variables in the model selected. The "forward" selection procedure in the multiple linear regression analysis allows to start with no variables in the model and to calculate the F^* statistic for each independent variable, which will reveal if the contribution of the several variables is significant to the model.

As a measure for the quality of the model fitting to the experimental data, the corrected r^2 and the model standard deviation (SD) were also calculated using Eqs. (9) and (10), respectively:

$$\text{Corrected } r^2 = \left[1 - \frac{(m-1)\left(1 - \frac{\text{SSQ}_{\text{regression}}}{\text{SSQ}_{\text{total}}}\right)}{(m-j)}\right], \quad (9)$$

$$\text{SD} = \sqrt{\frac{\text{SSQ}_{\text{residual}}}{(m-j)}}, \quad (10)$$

where m is the number of observations, j is the number of model parameters, SSQ is the sum of squares, and SD is standard deviation. Besides the corrected r^2 and SD, the Mallow's C_p statistic was also computed; C_p is a measure for the total sum of squared errors and defined as (Eq. (11)):

$$C_p = \left(\frac{\text{SSE}_p}{s^2}\right) - (N - 2p), \quad (11)$$

where s^2 is the mean error sum of squares for the full model; SSE_p is the error sum of squares for a model with p parameters including the intercept; N is the number of observations. The best situation occurs when C_p is close to p.

3. Results and discussion

3.1. Kinetics of pepper PME inactivation due to combined pressure and temperature treatments

Combined treatments of pressure and temperature for inactivation of purified pepper PME at pH 5.6 were investigated within the range of 10–64 °C and 0.1–800 MPa. For the experiments at elevated pressure, purified pepper PME was partially inactivated during pressure build-up due to the increasing temperature as a consequence of adiabatic heating. Depending on the pressure/temperature conditions applied, the activity loss of purified pepper PME could increase up to 64% after the pressure build-up phase. In the present study, 20% of the initial activity of purified pepper PME was found to be thermostable (data not shown), which is less than the amount (57–62%) found during the inactivation study of purified pepper PME at pH 7.5 (Castro, Van Loey, Saraiva, Smout, & Hendrickx, 2004). It is well known that the amount of thermostable PME fraction can vary, among other factors, with cultivars degree of maturity and experimental differences in the procedure (Snir, Koehler, Sims, & Wicker, 1996; Wicker, 1992).

No complete inactivation of purified pepper PME was attained when the enzyme solution was submitted to different combinations of pressure/temperature.

Under mild-heat and high-pressure conditions, only the labile fraction was inactivated, whereas the activity of the stable fraction remained unchanged with respect to time. Therefore, the fractional conversion model was applied to fit the isothermal–isobaric inactivation data (Eq. (2)). More drastic conditions of temperature/pressure led to a fast inactivation period followed by a slight decay. A biphasic model was applied to estimate the inactivation rate constant of the labile fraction (Eq. (1)). Previous kinetic studies on purified pepper PME (pH 7.5) concluded that a fractional conversion model could characterize the isothermal inactivation at mild temperatures (55–57 °C) while for higher temperatures (58–70 °C) a biphasic model proved to be more adequate to describe the experimental data (Castro et al., 2004). The inactivation rate constants for the labile fraction of purified pepper PME (pH 5.6) were estimated by non-linear regression analysis (Proc NLIN, SAS), according to the most adequate inactivation model (Table 1). At room temperature, pressures higher than 600 MPa are required to inactivate the labile pepper PME fraction. The stable fraction of purified pepper PME was also possible to inactivate at 800 MPa. At higher temperatures the inactivation rate accelerated, indicating the synergistic effect of pressure and temperature. But in the high-temperature (>54 °C) and "low"-pressure ($P \leqslant 300$ MPa) region, on the contrary, an antagonistic effect of pressure and temperature was observed. In this range, a pressure increase resulted in a decrease of the inactivation rate constant. For enzyme inactivation/protein denaturation, an antagonistic effect of pressure and temperature has frequently been encountered, although mostly limited to pressures below 300 MPa (Balny & Mason, 1993; Heremans, 1993; Indrawati, Van Loey, Ludikhuyze, & Hendrickx, 2001; Ly Nguyen et al., 2003a, 2003b; Van den Broeck et al., 2000a). Research on α-chymotrypsin and pressure, led Mozhaev, Lange, Kudryashova, and Balny (1996) to the explanation that pressure stabilization against thermal inactivation/denaturation might be due to counteracting effects of pressure and temperature on the formation or disruption of intramolecular interactions and/or their opposing effects on interactions between enzyme and solvent (water). During the initial step of thermal inactivation, proteins can lose a number of essential water molecules, which might lead to structural rearrangements. High-pressure may hinder this process owing to its favourable effect on hydration of both charged and non-polar groups.

Based on the estimated k-values from the isothermal–isobaric experiments, a pressure–temperature kinetic diagram, an isorate contour (SAS, 1994), for purified pepper PME (pH 5.6) was made (Fig. 1a). The lines

Table 1
k-Values (10^2 min^{-1}) for combined pressure–temperature inactivation of labile fraction of purified pepper PME in citrate buffer; pH 5.6 (pressure, MPa; temperature, °C)

P/T	10	25	30	40	50	54	56	60	62	64
0.1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	3.22 ± 0.26[a]	15.35 ± 0.71[a]	37.41 ± 1.12[a]	79.22 ± 3.27[a]
100	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	2.95 ± 0.16[a]	8.28 ± 1.19[a]	11.38 ± 0.74[b]	15.63 ± 5.48[b]
200	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	2.04 ± 0.14[a]	4.17 ± 1.05[a]	n.d.	8.33 ± 2.64[b]
300	n.d.	n.d.	n.d.	n.d.	n.d.	1.05 ± 0.38[a]	1.82 ± 0.77[a]	2.83 ± 1.88[b]	2.94 ± 0.62[b]	6.63 ± 1.71[b]
400	n.d.	n.d.	n.d.	n.d.	1.01 ± 0.51[a]	2.04 ± 0.53[a]	2.86 ± 0.48[a]	7.71 ± 2.02[b]	7.95 ± 3.87[b]	10.79 ± 2.70[b]
500	n.d.	n.d.	n.d.	0.78 ± 0.20[a]	2.05 ± 0.29[a]	3.52 ± 0.80[b]	3.73 ± 0.70[b]	11.61 ± 1.62[b]	15.38 ± 8.22[b]	n.d.
600	0.56 ± 0.07[a,c]	2.22 ± 0.36[a]	2.81 ± 0.31[a]	3.85 ± 1.07[b]	6.38 ± 1.63[b]	8.84 ± 0.80[b]	13.49 ± 4.69[b]	n.d.	n.d.	n.d.
700	2.19 ± 0.72[a]	7.14 ± 1.94[a]	7.661.95[a]	17.66 ± 1.15[b]	20.72 ± 1.84[b]	n.d.	30.69 ± 4.97[b]	n.d.	n.d.	n.d.
800	7.42 ± 1.07[b]	17.16 ± 3.71[b]	21.42 ± 2.69[b]	39.40 ± 2.81[b]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

n.d.: Not determined.
[a] Fractional conversion model.
[b] Biphasic model.
[c] Asymptotic standard error.

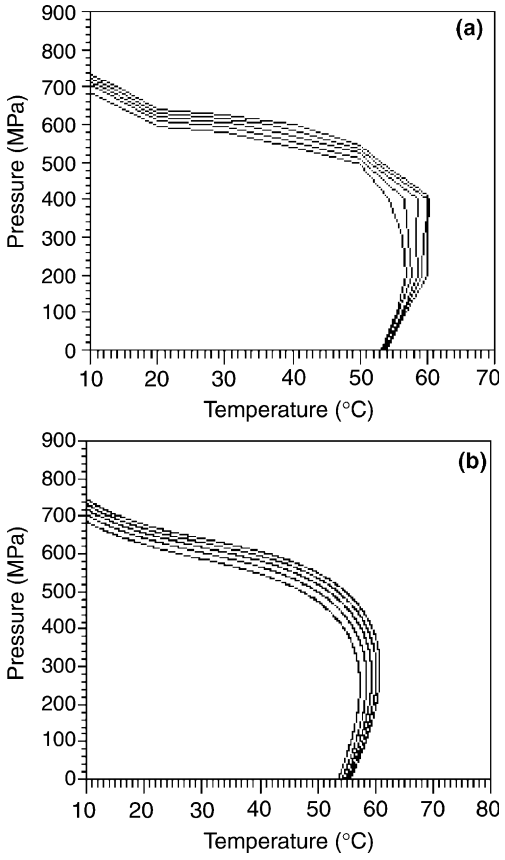

Fig. 1. Pressure–temperature kinetic diagram for the P/T inactivation of labile fraction of purified pepper PME in citrate buffer (pH 5.6): (a) raw data; (b) Eq. (12). The inner and the outer lines represent P/T combinations for which $k = 0.02$ and $0.04\ \text{min}^{-1}$ (by 0.005), respectively.

represent several combinations of pressure–temperature, resulting in the same inactivation rate constant of the labile fraction. The synergistic effect of pressure and temperature in the high-pressure region ($P \geqslant 300$ MPa) and the antagonistic effect of pressure and temperature in the "low"-pressure–high-temperature domain can be deduced from the shape of this kinetic diagram.

Temperature and pressure coefficient models include the Arrhenius and Eyring equations, which respectively describe the temperature and pressure dependence of the experimental k-values. The Arrhenius relation was valid in the whole pressure domain studied; the inactivation rate constant could be enhanced with increasing temperature, at a constant pressure. By plotting the natural logarithm of the inactivation rate constant as a function of reciprocal absolute temperature for the different pressure levels, the activation energy can be determined by linear regression analysis (Eq. (3)). The estimated activation energies are presented in Table 2. At atmospheric pressure, the activation energy of purified pepper PME was $371.6 \pm 7.4\ \text{kJ mol}^{-1}$, similar to the one found for the thermostable fraction of purified pepper PME at pH 7.5 ($388.93 \pm 7.47\ \text{kJ mol}^{-1}$) (Castro et al., 2004).

Table 2
E_a-values for thermal inactivation of labile fraction of purified pepper PME (20 mM citrate buffer, pH 5.6), at atmospheric and elevated pressure

P (MPa)	E_a (kJ/mol)	R^2
0.1	371.6 ± 7.4[a]	0.999
100	193.6 ± 17.2	0.985
200	162.3 ± 0.4	1.000
300	143.3 ± 25.8	0.911
400	158.7 ± 10.7	0.980
500	111.6 ± 13.3	0.946
600	47.3 ± 3.8	0.968
700	42.4 ± 3.8	0.968
800	40.5 ± 1.7	0.996

[a] Standard error of regression.

An increase of pressure resulted in a decrease of the activation energy. A linear relation between the natural logarithm of activation energy (E_a) and pressure (P) could be could be established ($R^2 = 0.91$). In the high-pressure region ($\geqslant 600$ MPa), a value of $43\ \text{kJ mol}^{-1}$ (average) was found, which is similar to commercial orange PME in distilled water ($\sim$60–70 kJ mol^{-1}) and purified orange at pH 3.7 ($\sim$25 to 38 kJ mol^{-1}) (Van den Broeck et al., 2000a).

Due to the observed antagonistic effect of pressure and temperature, the Eyring relation was not valid for the entire pressure domain. By evaluating the pressure dependence, two regions were observed: for pressures exceeding 300 MPa, the inactivation rate constant increased with pressure increase; while below 300 MPa, the inactivation rate constant decreased with pressure increase (ascribed to the antagonistic effect of pressure and temperature). As a result, the estimation of the activation volume values (V_a) was restricted to the higher pressure region ($\geqslant 300$ MPa). By plotting the natural logarithm of the inactivation rate constant as a function of pressure for the different temperature levels, the activation volume can be determined by linear regression analysis (Eq. (4)). At temperatures below 54 °C, V_a-values were situated between -35 and $-25\ \text{cm}^3\ \text{mol}^{-1}$, and no real trend in the variation with temperature was observed (Table 3). At temperatures where at atmospheric

Table 3
V_a-values for pressure inactivation of labile fraction of purified pepper PME (20 mM citrate buffer, pH 5.6), at different temperatures

T (°C)	V_a ($\text{cm}^3\ \text{mol}^{-1}$)	R^2
10	−30.33 ± 0.93[a]	0.999
25	−25.35 ± 2.08	0.993
30	−25.60 ± 0.19	1.000
40	−34.60 ± 3.48	0.980
50	−27.40 ± 2.13	0.988
54	−19.01 ± 1.09	0.993
56	−19.71 ± 2.88	0.940
60	−19.55 ± 4.74	0.944
62	−19.60 ± 4.69	0.946

[a] Standard error of regression.

pressure inactivation occurs (>54 °C), the pressure sensitivity of the inactivation rate constants was significantly reduced ($\sim -19\ cm^3\ mol^{-1}$). When compared to other PME sources, purified pepper PME seemed to be less pressure sensitive than carrot PME (Tris–HCl buffer, pH 7.0; $-32.1 \pm 3.7\ cm^3\ mol^{-1}$) but more than orange PME (citrate buffer, 3.7; $-13.77 \pm 2.92\ cm^3\ mol^{-1}$) at temperatures at which at atmospheric pressure inactivation occurs (Ly Nguyen et al., 2003a; Van den Broeck et al., 2000a).

3.1.1. Mathematical modelling to describe combined pressure temperature dependence of k-values

The relevance to include third degree terms in the polynomial equation (Eq. (8)) to describe the k-value as a function of pressure and temperature, was statistically evaluated. All the variables significantly contribute to the model, with the exception of variable X_7 ($Pr > F^*$ is 0.4881), which was removed from the model, yielding Eq. (12):

$$\ln(k) = a + AX_1 + BX_2 + CX_3 + DX_4 + EX_5 + FX_6 + HX_8 + IX_9. \quad (12)$$

The isocontour plots based on the raw data and simulated by the third-degree model are visualized in Fig. 1. The simulated isorate plot based on Eq. (12) (Fig. 1b) has a similar shape than the experimental one (Fig. 1a). Smeller and Heremans (1997) discussed that when one applies the thermodynamic model with first- or second-order terms, the shape of the contour plot will be elliptic or hyperbolic. But when one includes higher-order terms, which are temperature and/or pressure dependent, the contour plot will be a distorted ellipse. In Fig. 1, the antagonistic effect of pressure and temperature, as previously discussed, can be observed at lower pressure values ($P \leqslant 300$ MPa) and in the high temperature domain (>54 °C). The estimated model parameters of Eq. (12) are presented in Table 4. The model under study resulted in a good accuracy of the estimated model parameters for the inactivation of purified pepper PME. The relatively low accuracy of some parameters could be partially explained by the need of more data within the range of higher pressure and temperature to be applied in the computerized curve-fitting. This requirement is from experimental point of view not always possible to accomplish. The Mallow's C_p statistic was also calculated and as previously mentioned, the best situation occurs when C_p is close to p. For the considered equation (Eq. (12)), C_p was found to be equal to the number of variables, including the intercept ($p = 9$).

For the model studied, no trend in residuals (differences between experimental- and predicted k-values) was noticed as a function of temperature, pressure, experimental k-value, and predicted k-value (data not shown). A parity plot of the natural logarithm of the predicted k-values based on Eq. (12) compared with the natural logarithm of the experimental k-values was established (Fig. 2). The divergence from the bisector can be considered as an indicator for the inaccuracy of the considered model and its parameters; the more the experimental and the estimated values differ, the less successful the model is. A good correlation between the natural logarithm of the predicted k-values and that of the experimental k-values was found ($R^2 = 0.960$). Graphically, the third degree model shows good fitting within the temperature–pressure range studied (Fig. 3).

Table 4
Estimated model parameters for purified pepper PME inactivation based on Eq. (12) at reference pressure of 600 MPa and reference temperature of 329.15 K (56 °C)

Parameter	Estimated value	RSE (%)
a	0.1100 ± 0.0085[a]	7.73
A	-223.5 ± 16.3	7.29
B	$(2.197 \pm 0.312) \times 10^3$	14.20
C	$-(85.80 \pm 31.59) \times 10^{-2}$	36.82
D	$(2.517 \pm 0.862) \times 10^7$	34.25
E	0.455 ± 0.254	55.82
F	$(2.885 \pm 0.561) \times 10^{-3}$	19.45
H	$-(6.651 \pm 4.157) \times 10^{-4}$	62.50
I	$(3.5281.295) \times 10^5$	36.71
	Corrected r^2	0.950
	SD	0.245
	C_p	9

[a] Standard error of regression.

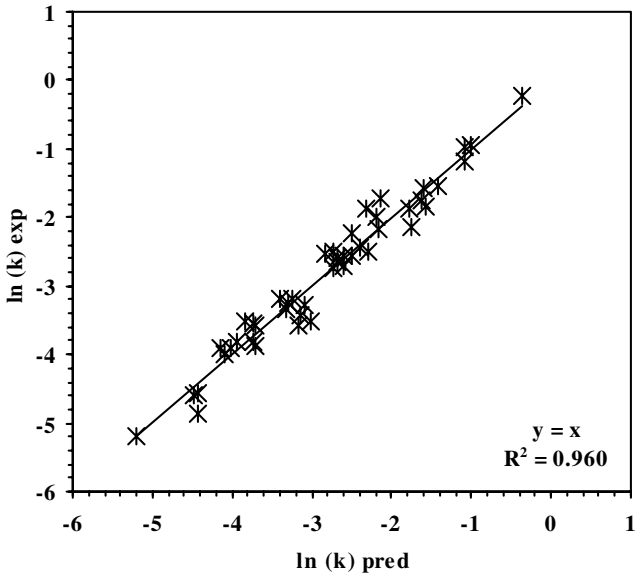

Fig. 2. Correlation between the predicted k-values of labile fraction of purified pepper PME (citrate buffer, pH 5.6) determined from experimental isothermal–isobaric inactivation work and the k-values estimated using Eq. (12).

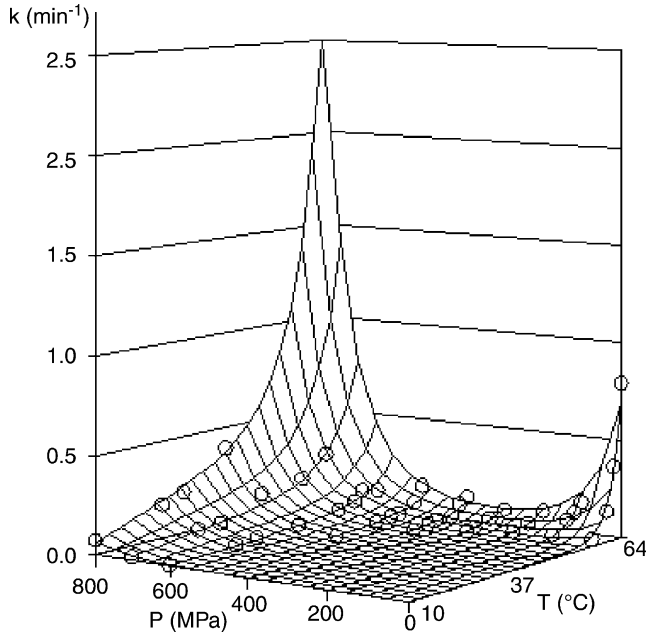

Fig. 3. 3D plot for heat-pressure inactivation of labile fraction of purified pepper PME (citrate buffer, pH 5.6), based on the third-degree polynomial model (Eq. (12)); (○) raw data points.

4. Conclusions

Pressure and/or temperature inactivation of the labile fraction of purified pepper PME was studied in a model system at pH 5.6. Knowing the benefits of high pressure processing compared to thermal processing (i.e., better food quality retention), this work provides valuable insight to implement high-pressure technology for pepper-based products. Since an antagonistic effect of pressure and temperature was observed at lower pressures ($P \leqslant 300$ MPa) and high temperatures (>54 °C), high pressure processing for pepper PME inactivation is only beneficial above 300 MPa. Considering the estimated k-values within the temperature–pressure range studied, a mathematical model describing the impact of combined temperature–pressure process on the inactivation of the labile fraction of pepper PME was devised. Such mathematical models for pressure–temperature inactivation of several food characteristics (e.g., microorganisms, enzymes) form the food engineering basis for design and optimisation of high pressure–thermal processes. Further research on the stable pepper PME fraction as well as on PME inactivation in real pepper-based products would be interesting for food processors in order to fully evaluate the potential of high-pressure processing of these products.

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Identification of pressure/temperature combinations for optimal pepper (*Capsicum annuum*) pectin methylesterase activity

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Abstract

Pectin methylesterase (PME) was extracted from green bell peppers and purified by affinity chromatography. The optimal pectin and salt concentrations for the PME catalysed reaction were investigated. Purified pepper PME activity was studied during combined high-pressure/temperature treatments (18–65 °C, 0.1–600 MPa) in a model system of pectin at pH 5.6. The activity of purified pepper PME showed a maximum at 200 MPa and 55 °C. A third-degree polynomial model (derived from a thermodynamic model) was successfully used to describe the heat–pressure dependence of the initial rates of purified pepper PME-catalyzed methanol formation.

Keywords: Green bell pepper; Pectin methylesterase activity; Thermal and high-pressure treatments; Thermodynamic model

1. Introduction

With their characteristic and exotic flavour, as well as the slightly pungent taste, C*apsicum annuum* has become a commercially important vegetable crop. Although commonly thought as vegetables, capsicums are botanical fruits and undergo a process of ripening similar to those of fruits [1]. The major post-harvest problem of this crop is the excessive softening that may cause shrinkage, drying and pathological disorders leading to a reduction in quality and consumers acceptability. The consumption of bell pepper is mainly fresh, although their commercialisation as frozen, dried or canned is also an aspect to take into consideration regarding their quality attributes. Therefore, additional to post-harvest softening, the effect of preservation technologies on texture loss deserves proper attention. Blanching, as one of the oldest technologies of pre-processing of fruits and vegetables during preservation/processing, known to induce undesirable quality changes in the products, has been thoroughly studied. In regular blanching (high-temperature/short-time, i.e., 90 °C, 1–5 min) vegetable tissue softens, largely as a result of turgor loss and occluded air, thermal degradation of pectins and other cell wall polysaccharides [2–4]. Structurally, the cell separation is the most obvious result of thermal destabilization. This is accompanied by an increase in the solubility of pectic polysaccharides, probably as a result of β-eliminative degradation [5]. However, low-temperature/long-time blanching treatments have been reported to result in a positive effect on the textural quality of several fruits and vegetables [6–10]. For example, after 40 min at 60 °C, jalapeño peppers seemed to increase their firmness [11]. This firming effect around the optimal temperature for pectin methylesterase (PME) activity, has been frequently associated to the action of this enzyme on the pectic substances. According to Bartolome and Hoff [7], such thermal treatments also cause loss of membrane selective permeability, giving rise to diffusion of ions to the cell wall. The increased presence of these ions in the cell wall, especially divalent cations (calcium and magnesium), would activate the PME enzyme, producing

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free carboxyl groups on the pectin chain. The formation of bridges between galacturonic residues of adjacent pectin molecules can act as cementing material and, therefore, creating more rigid structures and increasing the firmness of plant tissue. Other modifications within the pectin structure can often occur due to the action of another endogenous pectin degrading enzyme, i.e., polygalacturonase (PG). The action of PME makes the pectin more susceptible for further degradation by PG since this enzyme acts on segments of the pectin chain that have been demethylated by PME. The combined action of PME and PG can lead to drastic textural changes of fruits and vegetables (i.e., [12]). Enzyme inactivation studies have shown that, under suitable high-pressure/temperature conditions, PG can be inactivated, whereas PME can remain active [13–15]. At the same time, commercial tomato PME was found to be activated under lower pressure treatment at mild temperature (∼300 MPa, 60–65 °C) [16].

Both effects, activation and inactivation of pectinases, can be beneficial in processing fruit and vegetable based products. Since it has been reported for early stages of ripening of *Capsicum* fruits higher activity of PME and lower [17] or even no PG activity [1] has been reported for early ripening stages of *Capsicum* fruits, one might be interested in promoting the activity of PME in these fruits in their early ripening stage in order to improve its texture.

Pressure–temperature processing stability and activity data for PME from different sources are of interest to the food industry. The aim of this work was to study the effect of combined temperature/high-pressure treatments on the activity of purified pepper PME at acidic pH (pH 5.6). The importance of this work is related to the possibility to replace traditional thermal treatments by combined temperature/pressure treatments.

2. Materials and methods

2.1. Materials

Green bell peppers (*Capsicum annuum*) were purchased from a local auction (Mechelen, Belgium). Apple pectin (degree of esterification [DE], 75%) was obtained from Fluka Chemicals Co. (Switzerland). NHS-Sepharose 4B resin was purchased from Sigma (USA). All other chemicals were of analytical grade.

2.2. Methods

2.2.1. Pectin methylesterase purification

PME was extracted from peppers using a 0.2 M Tris(hydroxymethyl)-aminomethane buffer (i.e., Tris buffer) (pH 8.0) with 1 M NaCl, followed by purification using affinity chromatography on a NHS-Sepharose-PME inhibitor column, according to the procedure previously described by Ly-Nguyen et al. [18]. The PME fractions (2 mL) were pooled together and concentrated with Centricon Plus-20 (PL-10, Millipore).

2.2.2. Pectin methylesterase activity measurement

2.2.2.1. Titration method. A standard assay for PME activity is based on the titration of carboxyl groups released from a pectin solution using an automatic pH-stat (Metrohm, Switzerland) and 0.01 N NaOH solution. Routine assays were performed with a 3.5 g L^{-1} apple pectin solution (DE 75%, 30 mL) containing 0.117 M NaCl (pH 7.0) at 22 °C. The activity unit of PME is defined as the amount of enzyme required to release 1 μmol of carboxyl groups per minute, under the aforementioned assay conditions. The activity of PME is proportional to the rate of NaOH consumption ($\Delta V_{NaOH}/\Delta t$).

2.2.2.2. Colorimetrical method. The PME activity during thermal and combined pressure/temperature treatments was determined by measuring the release of methanol, produced during the action of the enzyme on pectin solution, as a function of time. The amount of methanol formed was determined colorimetrically according to Klavons and Bennett [19]. In this method, the methanol formed is oxidized to formaldehyde by alcoholoxidase (E.C. 1.1.3.13, Sigma, product no. A-2404), followed by condensation with 2,4-pentanedione in ammonium acetate and acetic acid to obtain 3,5-diacetyl-1,4-dihydro-2,6-dimethylpyridine [20].

2.2.3. Effect of pectin and NaCl concentration on PME activity

The effect of substrate concentration (apple pectin, DE 75%, 30 mL) was determined by measuring the activity of purified pepper PME in the presence of various pectin concentrations (0.1–15.0 g L^{-1}), with 0.117 M NaCl (pH 5.6). The K_m and maximum rate (V_{max}) were determined by non-linear regression analysis using the Michaelis–Menten equation. The effect of NaCl concentration on purified pepper PME activity was also studied, within the range of 0–1.0 M.

2.2.4. Thermal and combined high-pressure/temperature treatments

The temperature range applied for determination of purified pepper PME activity was from 18 to 65 °C. The isothermal experiments were performed in a water bath with temperature control. The enzymatic reaction started by adding 250 μL of purified pepper PME to 30 mL of pectin solution (4.0 g L^{-1}). During the enzymatic reaction, pH was controlled by dissolving the pectin in 0.2 M citrate buffer (pH 5.6), containing 0.4 M NaCl. Individual pyrex glass tubes were filled with 1.5 mL of this pectin-PME solution within 3 min and incubated in a thermostatic water bath, for pre-set time intervals. The first sample was withdrawn after 3 min. For isothermal/isobaric experiments, the enzyme-substrate solution was enclosed in 0.4 mL flexible microtubes (Biozym, Netherlands) within 4 min and enclosed in the pressure vessels (4 min), already equilibrated

at a pre-set temperature. Pressure was built up slowly (100 MPa min^{-1}) to minimize temperature increase due to adiabatic heating. After pressure build-up, an equilibrium period of 3 min to allow temperature to evolve to its desired value was taken into account, and one vessel was decompressed and the product formation of this sample was considered as a time zero sample. Afterwards, the vessels were decompressed after preset time intervals. The studied conditions during combined thermal and high-pressure treatments were from 200 to 600 MPa, and from 30 to 60 °C. After each thermal and thermal/high-pressure treatment, the enzymatic reaction was quenched by a heat shock (85 °C, 2 min), followed by cooling in an ice-water bath. The amount of methanol produced during the treatments was quantified in duplicate by the alcoholoxidase test [19]. The amount of methanol formed in a pectin solution, without PME addition, was also evaluated due to the possibility of chemical hydrolysis.

2.2.5. Data analysis

The PME activity (μg MeOH/mL pectin solution, min) was estimated from the initial linear part of the curve obtained by plotting the amount of methanol formed as a function of time and denoted as V_0. Due to small variations of the diluted solution of purified pepper PME daily prepared, it was necessary to divide V_0 by the amount of PME (U) determined under standard assay conditions (measured titrimetrically at pH 7.0, 22 °C). The normalized PME activity is denoted as V_0' ($V_0' = V_0$/PME activity (U)). Once the initial rate of purified pepper PME-catalyzed methanol formation (V_0') is known at different temperatures, the temperature dependence of V_0' at given pressure, expressed by the activation energy (E_a, kJ mol^{-1}), can be estimated in the temperature area in which the reaction accelerates, using the Arrhenius equation (Eq. (1)):

$$V_0' = V_{0\,\mathrm{ref}}' \exp\left[\frac{E_a}{R}\left(\frac{1}{T_{\mathrm{ref}}} - \frac{1}{T}\right)\right] \tag{1}$$

where T_{ref} and T are the reference and the absolute temperature (K), respectively; R (8.314 J mol^{-1} K^{-1}) is the universal gas constant. As a measure of pressure dependence of V_0' at a given temperature, the activation volume (V_a, cm^3 mol^{-1}) can be estimated using the Eyring equation (Eq. (2)):

$$V_0' = V_{0\,\mathrm{ref}}' \exp\left[\frac{-V_a}{RT}(P - P_{\mathrm{ref}})\right] \tag{2}$$

P and P_{ref} are the pressure and the reference pressure (MPa), respectively. Both activation energy and activation volume can be estimated by linear regression analysis of the natural logarithm of V_0' versus the reciprocal of absolute temperature or versus pressure, respectively.

Different approaches have been applied in order to describe the elliptical contour diagram depicting combined pressure temperature dependence of protein denaturation/enzyme inactivation. The elliptical pressure temperature kinetic diagram for enzyme inactivation can be approached on a thermodynamic basis [21] or on an empirical mathematical basis using a modified Arrhenius relation [22,23], a modified Eyring equation [24] or an elliptical equation [25,26]. The most useful thermodynamic-based kinetic model governing the behaviour of a system during pressure–temperature changes, and successfully applied as a generic model for a number of enzyme inactivation data, is described in Eq. (3) [27].

$$\ln(k) = \ln(k_{\mathrm{ref}}) - \frac{\Delta V^{\neq}}{R_T T}(P - P_0) + \frac{\Delta S_0^{\neq}}{R_T T}(T - T_0) - \frac{\Delta \kappa^{\neq}}{2R_T T} \times (P - P_0)^2 + \frac{\Delta C_p^{\neq}}{R_T T}\left\{T\left[\ln\left(\frac{T}{T_0}\right) - 1\right] + T_0\right\} - \frac{2\Delta \xi^{\neq}}{R_T T}(P - P_0)(T - T_0) \tag{3}$$

where P denotes pressure (MPa); T the absolute temperature (K); P_0 and T_0 the reference pressure (MPa) and absolute temperature (K), respectively; V_0 and S_0 the volume change (cm^3 mol^{-1}) and entropy change (J mol^{-1} K^{-1}) between native and denaturated states, respectively; $\Delta\kappa$ the compressibility factor (cm^6 J^{-1} mol^{-1}); ΔC_p the heat capacity (J mol^{-1} K^{-1}); $\Delta\zeta$ the thermal expansibility factor (cm^3 mol^{-1} K^{-1}); k the inactivation rate constant (min^{-1}); k_0 the inactivation rate constant at P_0 and T_0 (min^{-1}); R_T the universal gas constant (8.314 J mol^{-1} K^{-1}). A modification in the vicinity of the reference point has been recently proposed by Smeller [28], which results in the following second-order approximation (Eq. (4)):

$$T\left(\ln\left(\frac{T}{T_0}\right) - 1\right) + T_0 = \frac{(T - T_0)^2}{2T_0} \tag{4}$$

Hence, Eq. (3), a second-degree polynomial (elliptic) equation, can be rearranged into Eq. (5):

$$\ln(k) = \ln(k_{\mathrm{ref}}) - \frac{\Delta V^{\neq}}{R_T T}(P - P_0) + \frac{\Delta S_0^{\neq}}{R_T T}(T - T_0) - \frac{\Delta \kappa^{\neq}}{2R_T T}(P - P_0)^2 + \frac{\Delta C_p^{\neq}}{R_T T}\frac{(T - T_0)^2}{2T_0} - \frac{2\Delta \xi^{\neq}}{R_T T}(P - P_0)(T - T_0) \tag{5}$$

High-order terms can also be considered if $\Delta\kappa$, ΔC_p and $\Delta\zeta$ are temperature and/or pressure dependent [29,30]. Eq. (6) is the result of the introduction of the above-mentioned terms and as a consequence the ellipse can be distorted.

$$\ln(k) = \ln(k_{\mathrm{ref}}) - \frac{\Delta V^{\neq}}{R_T T}(P - P_0) + \frac{\Delta S_0^{\neq}}{R_T T}(T - T_0) - \frac{\Delta \kappa^{\neq}}{2R_T T} \times (P - P_0)^2 + \frac{\Delta C_p^{\neq}}{R_T T}\frac{(T - T_0)^2}{2T_0} - \frac{2\Delta \xi^{\neq}}{R_T T}(P - P_0) \times (T - T_0) + \text{higher-order terms} \tag{6}$$

So far, the initial rate of purified pepper PME-catalyzed methanol formation (V'_0) has not been analysed in terms of pressure–temperature dependency. Therefore, Eqs. (5) and (6) were used as a basis to model the combined effect of pressure and temperature on the initial rate of purified pepper PME-catalyzed methanol formation [$\ln(V'_0)(P, T)$], in the same way than temperature/pressure inactivation rate constants [$\ln(k)(P, T)$].

3. Results and discussion

Different concentrations of enzyme and substrate were assayed so that it was possible to identify an appropriate experimental set-up. The enzyme concentration was adjusted so that the amount of methanol formed by PME increased linearly with time, during at least 7 min, excluding the dynamic phase. Therefore, purified pepper PME activity could be accurately estimated from the slope of methanol formation versus treatment time.

3.1. Effect of pectin and NaCl concentration on PME activity

Purified pepper PME activity was measured as a function of pectin concentration, at acidic pH (Fig. 1). The pectin concentration was gradually increased until substrate saturation was attained. The kinetic parameters K_m and the maximum rate (V_{max}) were determined by non-linear regression analysis as 1.614 ± 0.065 mg mL^{-1} and 0.417 ± 0.005 mL min^{-1}, respectively. For neutral pH, lower values for K_m and V_{max} were found, 0.329 mg mL^{-1} and 0.272 mL min^{-1}, respectively [31]. Purified pepper PME has higher substrate affinity at pHs closer to the optimum pH, around 7.5, than at acidic pH. Purified pepper PME activity was also studied as a function of NaCl concentration, since it is well known that addition of salts can, in general, improve the PMEs activity. At pH 5.6, purified pepper PME activity increased up to a maximum of 0.4 M of NaCl concentration, followed by a decay in activity (Fig. 2). For a 3.4-fold increase in salt concentration up to 0.4 M, there was a 4.3-fold increase in enzyme activity. According to Castro et al. [31], maximal activity

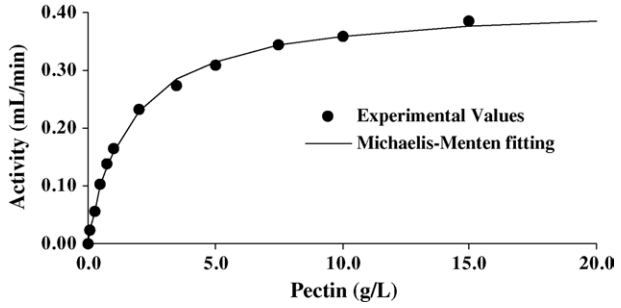

Fig. 1. Activity of green pepper PME as a function of substrate concentration. Assay conditions: 0.117 M NaCl, pH 5.6, at 22 °C.

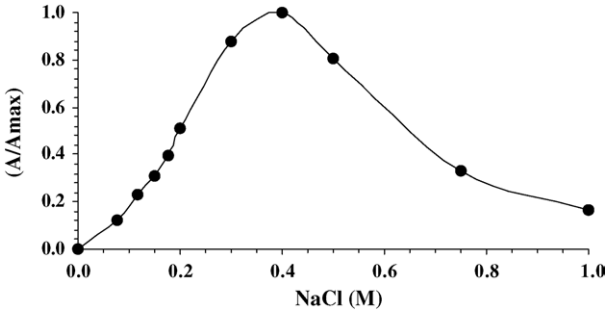

Fig. 2. Activity of green pepper PME as a function of salt concentration. Assay conditions: 4.0 g/L apple pectin (DE 75%), pH 5.6, at 22 °C.

for purified pepper PME was reached at 0.13 M of NaCl, at neutral pH. Also, Denés et al. [32] concluded that the concentration of salt needed for maximal activity of purified apple PME increased when pH decreased. The effect of combined temperature/high-pressure treatments on the activity of purified pepper PME was studied in the presence of 4.0 g L^{-1} apple pectin substrate, with 0.4 M NaCl, at acidic pH (pH 5.6).

3.2. Activity of purified pepper PME during thermal and combined high-pressure/temperature treatments

Under all temperatures and high-pressure conditions tested (18–65 °C, atmospheric pressure to 600 MPa), no chemical hydrolysis of pectin at pH 5.6 was observed. Renard and Thibault [33] have already concluded that at room temperature spontaneous demethoxylation of pectin can occur but at mild alkaline conditions. At pH 5.6 and atmospheric pressure, the optimal temperature for activity of purified pepper PME was found within the range of 50–55 °C (Fig. 3). The temperature optimum for commercial and purified tomato PME was 55 °C at neutral pH, and 45 and 35 °C at pH 8.0 and 4.4, respectively [15,16]. The optimal temperature for methanol formation catalysed by purified pepper PME at elevated pressure was in a broader range (50–60 °C) as

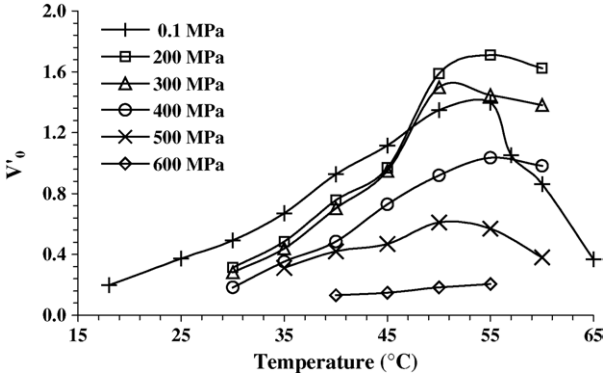

Fig. 3. Purified pepper PME activity (V'_0) as a function of temperature at pH 5.6.

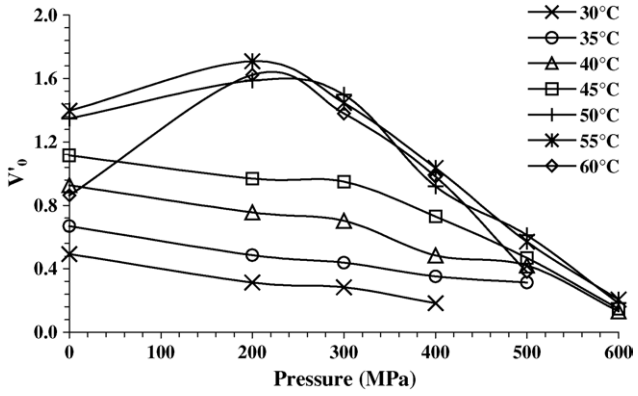

Fig. 4. Purified pepper PME activity (V'_0) as a function of pressure at pH 5.6.

compared to atmospheric pressure. A shift in the optimal temperature at elevated pressures has been observed for commercial and purified tomato PME [15,16]. In Fig. 4, it can be noticed that for temperatures lower than the optimal temperature (i.e., $T < 50\,^{\circ}C$), PME activity decreased with increasing pressure. But for temperatures higher than 50 °C, there was an enhancement of PME activity up to 200–300 MPa, followed by a decrease in activity for higher pressures. The temperature dependence of V'_0 at a given pressure in the temperature range in which the reaction is accelerated, and the pressure dependence of V'_0 at the different temperatures (V_a), could be described by Arrhenius and Eyring equations, respectively. At atmospheric pressure, an activation energy for purified pepper PME activity between 18 and 50 °C of 46.45 kJ mol^{-1} was estimated (Table 1). Thus, within this temperature range, the rate of methanol formation accelerated with increasing temperature. The activation energy increased slightly until 300–400 MPa, followed by a decrease. By evaluating the pressure dependence, two different regions were observed as previously mentioned. Therefore, the V_a-values were estimated in the pressure–temperature domain where V'_0 decreases (Table 1). From the V_a-values, it can be noted that the initial rate of purified pepper PME-catalyzed reaction is less pressure sensitive at mild temperatures (up to 50 °C).

3.3. Modeling of combined temperature–pressure dependence of initial rates of purified pepper PME-catalyzed reaction

In a first attempt, Eq. (5) was used to describe the pressure–temperature dependence of initial rate of purified pepper PME-catalyzed methanol formation (V'_0). To estimate the model parameters, a non-linear regression analysis (Proc NLIN, SAS) was used involving an iterative numerical procedure based on the minimal sum of squares. According to the model parameters estimated (data not shown), the second-degree polynomial equation was not suitable to describe V'_0 as a function of pressure and temperature. Recently, Ly-Nguyen et al. [34,35] successfully suggested the introduction in Eq. (6) of third-degree terms for pressure–temperature inactivation studies of carrot and banana PME, respectively. In the present work, the relevance of third-degree terms in the thermodynamic model to describe V'_0 as a function of pressure and temperature was also considered (Eq. (7)):

$$\ln(V'_0) = \ln(V'_{0\,\mathrm{ref}}) - \frac{\Delta V_0^{\neq}}{R_T T}(P - P_0) + \frac{\Delta S_0^{\neq}}{R_T T}(T - T_0) - \frac{\Delta \kappa^{\neq}}{2R_T T}(P - P_0)^2 + \frac{\Delta C_p^{\neq}}{R_T T}\frac{(T - T_0)^2}{2T_0} - \frac{2\Delta \xi^{\neq}}{R_T T} \times (P - P_0)(T - T_0) + \frac{2\Delta \xi_{2A}^{\neq}}{R_T T}(P - P_0)^2(T - T_0) + \frac{2\Delta \xi_{2B}^{\neq}}{R_T T}(P - P_0)(T - T_0)^2 + \frac{\Delta \kappa_2^{\neq}}{2R_T T}(P - P_0)^3 + \frac{\Delta C_p^{\neq}}{2R_T T T_0}(T - T_0)^3 \quad (7)$$

Table 1
Initial rate of purified pepper PME-catalysed pectin demethylation at different thermal and high-pressure conditions (pH 5.6)

T (°C)	V'_0							
	0.1 MPa	200 MPa	300 MPa	400 MPa	500 MPa	600 MPa	V_a (cm^3/mol)	R^2
18	0.199 ± 0.007[a]	n.d.[b]	n.d.	n.d.	n.d.	n.d.		
25	0.373 ± 0.009	n.d.	n.d.	n.d.	n.d.	n.d.		
30	0.493 ± 0.025	0.314 ± 0.077	0.283 ± 0.044	0.183 ± 0.037	n.d.	n.d.	5.87 ± 0.90	0.96
35	0.670 ± 0.025	0.486 ± 0.007	0.439 ± 0.013	0.353 ± 0.015	0.313 ± 0.070	n.d.	3.93 ± 0.19	0.99
40	0.927 ± 0.035	0.757 ± 0.009	0.703 ± 0.019	0.485 ± 0.019	0.422 ± 0.030	0.132 ± 0.024	5.54 ± 1.01	0.94
45	1.116 ± 0.022	0.969 ± 0.020	0.949 ± 0.053	0.730 ± 0.029	0.469 ± 0.028	0.170 ± 0.021	6.45 ± 1.76	0.87
50	1.393 ± 0.028	1.588 ± 0.051	1.371 ± 0.077	0.919 ± 0.044	0.611 ± 0.048	0.184 ± 0.013	9.01 ± 1.77	0.93
55	1.398 ± 0.030	1.710 ± 0.075	1.447 ± 0.051	1.035 ± 0.031	0.570 ± 0.042	0.206 ± 0.016	17.59 ± 2.97	0.95
57	1.052 ± 0.072	n.d.	n.d.	n.d.	n.d.	n.d.		
60	0.863 ± 0.041	1.623 ± 0.039	1.381 ± 0.024	0.982 ± 0.079	0.380 ± 0.016	0.190 ± 0.020	19.11 ± 2.29	0.97
65	0.397 ± 0.111	n.d.	n.d.	n.d.	n.d.	n.d.		
E_a (kJ/mol)	46.45 ± 2.42	58.20 ± 4.14	66.86 ± 2.24	64.61 ± 6.09	34.95 ± 4.08	26.30 ± 2.41		
R^2	0.99	0.98	1.00	0.97	0.97	0.98		

[a] Standard error regression.
[b] Not determined.

The subscript "2" refers to the coefficients of the higher-order terms. With the following substitutions,

$$a = \ln(V'_{0\,\mathrm{ref}}), \qquad X_1 = -\frac{(P-P_0)}{T}, \qquad X_2 = \frac{(T-T_0)}{T},$$

$$X_3 = -\frac{(P-P_0)^2}{T}, \qquad X_4 = \frac{(T-T_0)^2}{T},$$

$$X_5 = -\frac{(P-P_0)(T-T_0)}{T}, \qquad X_6 = \frac{(P-P_0)^2(T-T_0)}{T},$$

$$X_7 = \frac{(P-P_0)(T-T_0)^2}{T},$$

$$X_8 = \frac{(P-P_0)^3}{T}, \qquad X_9 = \frac{(T-T_0)^3}{T},$$

Eq. (7) can be rewritten into Eq. (8):

$$\ln(V'_0) = a + AX_1 + BX_2 + CX_3 + DX_4 + EX_5 + FX_6 + GX_7 + HX_8 + IX_9 \quad (8)$$

To select the number of significant variables in the model, we have performed multivariate tests across the multiple dependent variables. The "forward" selection procedure in the multiple linear regression analysis allows to start with no variables in the model and for each of the independent variable, F^* statistic is calculated to evaluate whether the contribution of the several variables are significant to the model. Based on this selection procedure, all the variables contribute significantly to the model. Graphically, the third-degree model adequately predicts the raw data (Fig. 5). The model parameter estimates of Eq. (8) are presented in Table 2. All model parameters could be adequately estimated. In literature, no mathematical model describing the combined pressure–temperature dependence of the catalytic activity of an enzyme has been reported yet.

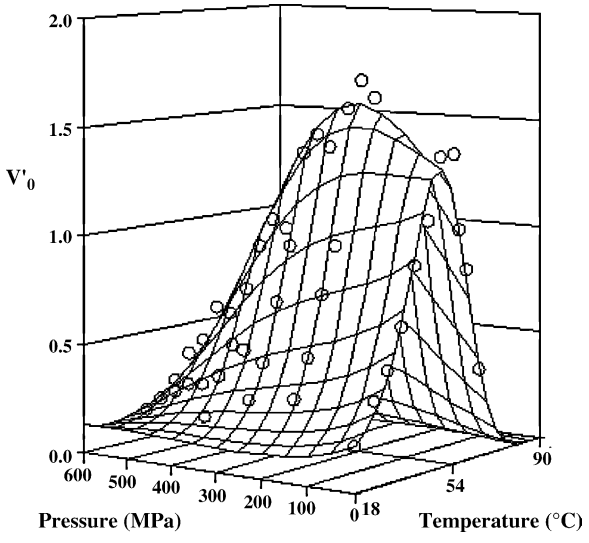

Fig. 5. 3D plot for the V'_0-values of the isobaric-isothermal of purified pepper PME-catalyzed methanol formation (pH 5.6) based on equation (8); (◯) raw data.

Table 2
Predicted model parameters for purified pepper PME-catalysed pectin demethylation at pH 5.6 based on Eq. (8) at reference pressure of 300 MPa and reference temperature of 318.15 K (45 °C)

Model parameters	Estimated value	R.S.E. (%)
a	1.012 ± 0.037	3.65
A	43.357 ± 7.156	16.50
B	$(1.513 \pm 0.082) \times 10^3$	5.42
C	0.732 ± 0.053	7.24
D	$-(1.901 \pm 0.214) \times 10^7$	11.26
E	0.115 ± 0.076	66.09
F	$-(0.250 \pm 0.030) \times 10^{-2}$	12.00
G	$(2.930 \pm 1.160) \times 10^{--2}$	39.59
H	$-(0.120 \pm 0.030) \times 10^{-2}$	25.00
I	$-(6.406 \pm 1.202) \times 10^5$	18.76
Quality of fitting		
Corrected r^2	0.970	
S.D.	0.126	
C_p	10	

The pressure–temperature range was from 0.1 to 600 MPa and from 18 to 65 °C.

As a measure for the quality of the model fitting to the experimental data, the corrected r^2 and the model standard deviation (S.D.) were also calculated using Eqs. (9) and (10), respectively.

$$\text{Corrected } r^2 = \left[1 - \frac{(m-1)(1-\mathrm{SSQ}_{\mathrm{regression}}/\mathrm{SSQ}_{\mathrm{total}})}{(m-j)}\right] \quad (9)$$

$$\text{S.D.} = \sqrt{\frac{\mathrm{SSQ}_{\mathrm{residual}}}{(m-j)}} \quad (10)$$

where m is the number of observations, j the number of model parameters, SSQ the sum of squares, and S.D. the deviation. A corrected r^2 of 0.970 and a model standard deviation of 0.126 were calculated, indicating a good model fitting. In addition to the corrected r^2 and S.D., the Mallow's C_p statistic was computed for the 10-terms model; C_p is a measure for the total sum of squared errors and defined as (Eq. (11)):

$$C_p = \left(\frac{\mathrm{SSE}_p}{s^2}\right) - (N - 2p) \quad (11)$$

where s^2 is the mean error sum of squares for the full model; SSE_p the error sum of squares for a model with p parameters including the intercept; N is the number of observations. The best situation occurs when C_p is close to p. For Eq. (8), C_p was equal to p, namely 10.

In addition, no tendency was found by plotting the residuals (differences between experimental and predicted V'_0-values, respectively) as a function of temperature, pressure, experimental V'_0-value, and predicted V'_0-value (data not shown). A parity plot of the natural logarithm of predicted V'_0 based on Eq. (8) versus the natural logarithm of the experimental V'_0 values, respectively, was established (Fig. 6). The deviation from the bisector can be considered as an indi-

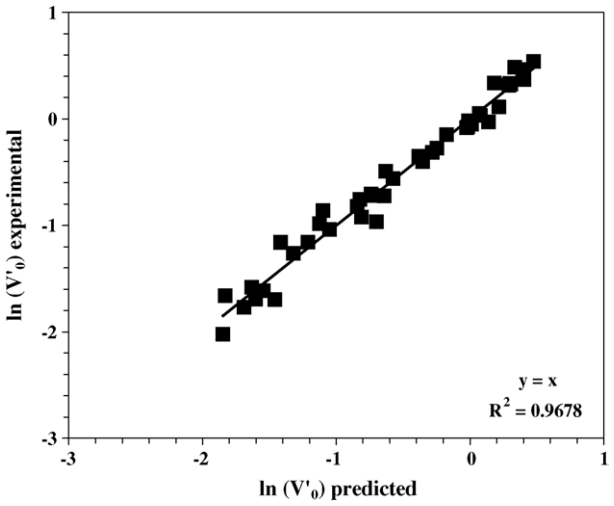

Fig. 6. Correlation between the natural logarithm of the experimental V_0' values of the isobaric-isothermal of purified pepper PME-catalyzed methanol formation (pH 5.6) and the natural logarithm of the predicted V_0' values according to Eq. (8).

cator for the inaccuracy of the models. Good correlation between the natural logarithm of predicted V_0' and that of the experimental V_0' values was observed [$R^2 = 0.9678$]. The isocontour plot (Fig. 7) results from the insertion of the model parameters of Table 2 into Eq. (8), and allows identification of pressure–temperature combinations resulting in a preset initial rate of purified pepper PME-catalyzed methanol formation. The initial rate of purified pepper PME increased to an optimum at 200 MPa and 55 °C. At pressure levels above 200 MPa combined with 55 °C, PME catalytic activity decreased again. At atmospheric pressure, the initial rate of purified pepper PME-catalyzed methanol formation is similar at 30 °C and at around 64 °C.

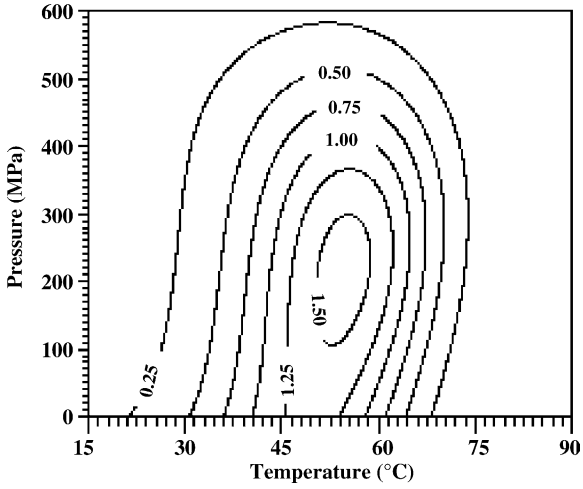

Fig. 7. Heat–pressure isocontour plot based on the third-degree polynomial model (Eq. (8)).

4. Conclusions

At atmospheric pressure, the highest enzymatic activity is noted at 50–55 °C. The increase of pressure in this temperature domain, up to 200 MPa, proves to be very efficient in stimulating catalytic activity. At elevated pressure, a broader optimal temperature range (50–60 °C) was observed. A combination of pressure of 200 MPa and mild-heat treatment (∼55 °C) seems to be optimal. The heat–pressure behaviour of purified pepper PME in presence of pectin substrate (pH 5.6) was successfully described by a third-degree polynomial model (derived from a thermodynamic model).

The obtained results clearly illustrate the potential of combined high pressure–temperature treatments in increasing the activity and stability of enzymes, which could be an add-value to high-pressure processing of fruits and vegetables regarding textural properties.

Acknowledgements

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ORIGINAL PAPER

Effect of temperature, pressure and calcium soaking pre-treatments and pressure shift freezing on the texture and texture evolution of frozen green bell peppers (*Capsicum annuum*)

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Abstract The firmness of green bell pepper (*Capsicum annuum*) was studied under different processing conditions. Thermal texture degradation kinetics of pepper tissue between 75 and 95 °C could be accurately described by a fractional conversion model. The firmness of pre-processed pepper increased when the samples were submitted to several heat, pressure, and combinations of heat/pressure and calcium soaking pre-treatments. Pre-heating at 55 °C during 60 min and mild heat/high-pressure treatments (200 MPa at 25 °C, 15 min) yielded the best results, which were further improved when combined with calcium soaking. These pre-treatments significantly slowed down thermal texture degradation of pepper at 90 °C, a typical temperature used for pepper blanching prior to freezing. The above-mentioned pre-treated samples showed a significant reduction in firmness when frozen by regular freezing at 0.1 MPa. The same samples showed no changes in firmness when frozen by high-pressure shift freezing at 200 MPa. When freezing was carried out by high-pressure shift and after frozen storage (− 18 °C) for 2.5 months, pressure pre-treated pepper showed a better retention of texture than thermal pre-treated pepper.

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Introduction

Plant cell walls constitute key structural components of plants and many plant-based foods. They are highly complex structures performing a diversity of functions during the life of the plant. Texture is one of the most prominent quality attributes of vegetable food products to which cell walls significantly contribute [1]. Research has been carried out on plant cell walls in relation to texture (e.g. [1–6]), in particular on the mechanical properties of tissue systems.

Thermal processing and freezing, even though considered to be efficient and widespread methods of preserving food products, including fruits and vegetables, affect firmness of plant-based products. Nowadays, the consumer demand for natural, fresh-like, more convenient and, at the same time, safe food products has driven the food industry to apply minimal (mild) efficient techniques in processing plant products. Consequently, there is a need for optimization of conventional technologies as well as their combination with new emerging technologies, like high hydrostatic pressure (e.g., combined thermal/high-pressure treatments and high-pressure assisted/shifted freezing/thawing), in order to produce high-quality (including firmness) processed fruit and vegetable products.

The possibility of using milder pre-processes for preservation of tissue integrity and the improvement of quality has been explored in the last years. Pre-heating of some vegetable tissues at temperatures between 50 and 60 °C before canning can reduce canning-induced softening in several plant tissues [4, 7–10]. It has been suggested that the firming effect ob-

tained from mild heat treatments alone or even combined with calcium chloride treatments [11–13] may be attributed to the action of heat-activated pectin methylesterase (PME, EC 3.1.1.11), a well-known cell-wall bound enzyme, and/or to increased Ca^{2+} diffusion into the tissue. Treatments that increase PME activity would in particular have an effect on improving the texture of fruits and vegetables [14, 15]. High-pressure pre-treatments alone or even combined with temperature seem to have a similar positive effect when compared to thermal treatments [16].

The treatments applied to preserve fresh peppers are brining and pasteurization or freezing, with prior blanching. During pepper brining, calcium addition has been shown to reduce the softening process and the leaching out of phytochemicals from pepper fruit during pasteurization and storage [17, 18]. Calcium seems to firm plant tissues by forming ionic cross-linkages with polysaccharide polyelectrolytes, especially galacturonans [19], resulting in a structure that retards both enzymatic [20] and non-enzymatic softening [21]. Interestingly, the ability of Ca^{2+} to cross-link pectic polysaccharides and thereby reducing their solubilization provides a dual role for this ion as a pectin degradation promoter through β-elimination and at the same time as a texture-enhancing agent through cross-linking [22].

Fresh fruits and vegetables, when properly frozen, maintain their organoleptic characteristics like flavour, colour and their nutritional value. But unfortunately, formation of ice crystals, which is an integral part of the freezing process, can damage the fragile membranes causing release of enclosed contents and changes in the microenvironment of food tissues and, as a consequence, affecting the food product texture. Softening caused by freezing/thawing can sometimes be minimized by pre-treatments of the tissue with $CaCl_2$ [23] in combination with mild heat treatments [24, 25]. For example, a stepwise blanching before freezing seems to preserve texture of frozen potatoes [26].

In addition, a conventional freezing process can be combined with other processing techniques in order to improve the texture quality of plant-based food products. It is well known that “slow freezing” induces the formation of large ice crystals that may cause mechanical damage, while “rapid freezing” enhances nucleation and the formation of smaller crystals [27]. However, very rapid freezing (e.g., liquid nitrogen) may also cause macroscopic cracks due to non-homogeneous volume expansion. Slow freezing of fruits and vegetables leads to large extracellular ice crystals, an increase in extracellular concentration of solutes and, therefore, to cellular dehydration. After thawing, excessive drip loss and texture softening is observed. Detrimental reactions are also enhanced by solute concentration effects and enhanced enzyme–substrate interactions. More recently, high-pressure freezing has been applied with success to several food products. High-pressure freezing promotes uniform and rapid ice nucleation and growth through the whole sample due to the high and uniform degree of supercooling [28–30]. Fuchigami et al. [31, 32] reported an improvement in both textural and histological structure of frozen carrots when pressures of 200, 340 and 400 MPa where applied during the high-pressure freezing.

The aim of this work was (1) to study the thermal texture degradation kinetics of green bell pepper; (2) to evaluate the effect of several pre-treatment conditions regarding temperature, high-pressure and calcium soaking on pepper texture; and (3) to determine the effect of thermal and pressure pre-processing and subsequent freezing (regular and high-pressure shift freezing) and frozen storage on pepper texture. The results obtained are applicable to the texture improvement of frozen bell peppers.

Materials and methods

Pepper tissue preparation

Green bell peppers (*Capsicum annuum*) obtained from a local auction (Mechelen, Belgium), were washed, the stems were cut off, and the core removed by hand. To create a homogeneous sample for thermal and high-pressure processing, six to seven peppers (ca. 200 g each) were used. Individual samples were prepared by cutting small pepper tissue circles of 20 mm diameter. Each sample containing six small circles was placed in a double plastic bag and vacuum sealed.

Thermal texture degradation kinetics

For thermal texture degradation kinetic studies, the samples were heated in a thermostated water bath at temperatures from 75 to 90 °C, and in a thermostated oil bath at 95 °C, in both cases using metallic grids to immerse the samples. After thermal treatments, the samples were cooled immediately in an ice-water bath for 5 min and equilibrated at room temperature before texture measurements. To account for the come-up time, the first sample (time-zero sample) was taken after 5 min of heating. This heating lag time was experimentally determined by measuring the temperature profile at the centre of five pepper samples with calibrated thermocouples connected to a data acquisition system (data not shown).

Thermal pre-treatments combined with/without calcium soaking

The samples were kept for 20, 40, 60 and 90 min in a thermostated water bath at 55 °C. After the pre-set time interval, the samples were immediately cooled in an ice-water bath

for 5 min and equilibrated at room temperature before texture measurements. The different pre-treatments conditions investigated in this study were:

- no pre-treatment (control);
- pre-heating at 55 °C (60 min);
- pre-heating at 55 °C (60 min), followed by soaking in $CaCl_2$-solution (0.25 and 0.50%, w/v) for 30 and 60 min (room temperature);
- pre-heating at 55 °C (60 min), followed by soaking in 0.25% (w/v) $CaCl_2$-solution for 60 min (room temperature) and a further 60 min holding period (t_H) at room temperature;
- soaking in 0.50% (w/v) $CaCl_2$ for 60 min at 55 °C;
- soaking in $CaCl_2$ (0.25 and 0.50%, w/v) for 30 and 60 min, at room temperature;
- soaking in 0.50% (w/v) $CaCl_2$ (60 min), followed by pre-heating at 55 °C (60 min).

For calcium soaking, pepper circles were removed from the sealed plastic bags and immersed in a beaker with $CaCl_2$-solution (0.25 and 0.50%, w/v) during different periods. The samples, treated simultaneously with temperature and calcium, were soaked in a $CaCl_2$-solution, previously equilibrated at the required temperature.

Combined thermal/high-pressure pre-treatments with/without calcium soaking

The pepper samples were submitted to different pressures, ranging from atmospheric pressure to 500 MPa, at 25 and 55 °C, for 15 min. After each treatment, the samples were left at room temperature for 45 min, to allow PME catalysed de-esterification of the methylated pectin-substrate, not only during the treatments [33], but also after the release of the pressure [15, 34]. The combined thermal/high-pressure treatments were performed in a single vessel high-pressure equipment (590 mL volume, i.d. 50 mm, height 300 mm), with a maximum operating pressure of 600 MPa and a working temperature ranging from − 30 to 100 °C (SO. 5-7422-0, Engineered Pressure Systems International EPSInt, Belgium). The pressure medium used was a glycol water mixture (60% Dowcal N, Switzerland). The combination of calcium soaking (0.50%, w/v) with thermal/high-pressure pre-treatments was also evaluated.

Influence of pre-treatments on texture degradation kinetics at 90 °C

The effect of the following pre-treatments on pepper texture degradation kinetics at 90 °C was also studied:

- no pre-treatment (control);
- pre-heating at 55 °C (60 min);
- pre-heating at 55 °C (60 min) followed by soaking in 0.50% (w/v) $CaCl_2$ (60 min);
- soaking in 0.50% (w/v) $CaCl_2$ (60 min) at room temperature;
- soaking in 0.50% (w/v) $CaCl_2$ (60 min) followed by pre-heating at 55 °C (60 min);
- combined thermal/high-pressure pre-treatment (200 MPa, 25 and 55 °C) for 15 min, followed by a 45 min of standing time at room temperature;
- combined thermal/high-pressure pre-treatment (200 MPa, 25 and 55 °C) for 15 min, followed by soaking in 0.50% (w/v) $CaCl_2$ (45 min).

Freezing treatments

The effect of the freezing process on texture of peppers was evaluated for non- and pre-treated samples. The pre-treatment conditions chosen were:

- no pre-treatment (control);
- pre-heating at 55 °C (60 min) followed by soaking in 0.50% (w/v) $CaCl_2$ (60 min);
- combined thermal/high-pressure pre-treatment (200 MPa, 25 °C) for 15 min, followed by a 45 min of standing time at room temperature;
- combined thermal/high-pressure pre-treatment (200 MPa, 25 °C) for 15 min, followed by soaking in 0.50% (w/v) $CaCl_2$ for 45 min.

Pepper samples were frozen by cryogenic freezing (CF) and high-pressure shift freezing (HPSF). A programmable cryogenic freezer (Nicool PC Plus, Air. Liquide, Paris, France) was used to freeze the pepper samples. Liquid nitrogen was supplied by a self-pressurized (0.04 MPa) vessel (TP 60, Air Liquide, Paris, France). The CF conditions were established at pre-set temperatures of − 18 and − 40 °C. The temperature profile at the core of pepper samples was registered in triplicate (average values are reported). The frozen samples were kept overnight in a conventional freezer at the respective temperature and thawed at room temperature before texture measurements.

The HPSF experiments were performed in the same high-pressure equipment used for combined temperature/high-pressure treatments. The pepper samples at room temperature were placed inside the vessel, with the pressure medium already at − 25 °C (by external cooling) and the pressure was built up to 200 MPa. When the temperature at the centre of the pepper circles reached − 18 °C, pressure was released to atmospheric pressure to freeze the pepper samples. Due to the pressure release, the sample temperature increases until the initial freezing point at atmospheric pressure. The samples were kept in the system until they reached − 18 °C. The frozen samples were transferred to a conventional freezer at − 18 °C, were stored for the appropriate time (overnight or

2.5 months at − 18 °C), thawed at room temperature and used for texture measurements.

Texture measurements

The texture of the pepper samples was measured by a texture analyser (TA-XT2i, Stable Micro System), using a plate with a 7 mm diameter hole, with the following parameters: 5 kg force load cell, 2 mm diameter aluminium cylinder probe, and 2.0 mm s^{-1} test speed. The property "firmness" (hardness), the maximum force applied to puncture the pepper tissue (*TP*), was measured as an indicator of texture. From the force–time curves, hardness is determined as the maximal force produced upon compression of the sample, which is very similar to the one performed by mastication, which takes part during eating. For this reason, firmness has been the textural characteristic most used to quantify texture degradation of vegetables [35]. The measurements were done on both sides of the pepper tissue, that is, from the skin and the flesh sides. In Fig. 1, a typical curve of force (kg) versus time (s) of pepper tissue firmness measurement is shown. When the measurement was done from the skin side, there was a steady increase up to a maximum force as the probe was driven into the flesh, until the skin was disrupted (full arrow, Fig. 1), followed by a reduction required to drive the probe further into the fruit flesh and, finally, the tissue failure occurred. From the flesh side, there was an initial maximum force, prolonged in time, corresponding to the rupture of the first internal layers of tissue (dashed arrow, Fig. 1), followed by another maximum force, due to rupture of the skin from the flesh side. Rupture of the skin from the flesh side required a lower force when compared with the same action from the skin side. An average value of firmness from nine puncture measurements (skin and flesh sides) was calculated for each experimental condition. Texture analysis of pepper pre-treated samples was carried within 1 h after the pre-treatments have been applied, and the samples were kept at 4 °C during this period. All the measurements were conducted at room temperature. It should be mentioned that, even though, the texture measurement performed from the skin side were easier to carry out than those from the flesh side, the latter gave more information (the maximum force registered varied more with the treatments applied) associated to the different conditions under study, probably due to a higher cell heterogeneity and heat sensibility [36]. Therefore, in some cases, the data obtained for the skin-side measurements are not shown for all the considered treatments.

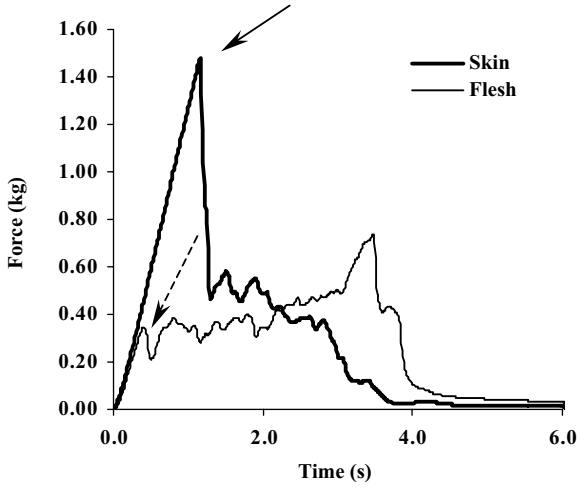

Fig. 1 Typical force (kg) curve versus time (s) for skin and flesh sides of pepper tissue using a puncture test. *Arrows* indicate the maximum force applied to puncture the pepper tissue measured from the skin (⟶) and flesh side (⇢)

Data analysis

Several mechanisms have been used to describe thermal texture degradation. Most of the earlier published studies have indicated that texture degradation of vegetables followed a first-order kinetic model. It should be mentioned that the heating time selected in most of these studies was relatively short since some products became unacceptably soft after prolonged heating. When testing longer heating times, Huang and Bourne [37] proposed two simultaneous first-order reactions, at two different rates to describe texture degradation kinetics. Other studies reported a similar trend in softening when different vegetables were heated at long processing times [38–40]. Rizvi and Tong [35] proposed a fractional conversion model to better explain vegetable tissue texture degradation kinetic data, where the second mechanism takes in consideration that the textural property (e.g., firmness) being measured no longer changes with respect to time, for prolonged heating periods. The fractional conversion factor, *f*, can be defined as the following equation:

$$f = \frac{TP_0 - TP_t}{TP_0 - TP_\infty} \tag{1}$$

where TP_0 is the initial firmness, TP_t is the firmness at time *t*, and TP_∞ is the non-zero equilibrium firmness after prolonged treatment time. For most irreversible first-order reactions TP_∞ approaches to zero. But even when the vegetable and fruit texture becomes very soft after a long heating-period, a measurable degree of firmness can be kept [35, 41, 42]. For a first-order reaction [43], a plot of the logarithm of $(1 - f)$ against time is linear, and the rate constant (k) is the negative slope value (Eq. (2)):

$$\ln(1 - f) = \ln\left(\frac{TP_t - TP_\infty}{TP_0 - TP_\infty}\right) = -kt \tag{2}$$

The firmness as a function of heating time at a constant temperature can be expressed by rearranging Eq. (2):

$$TP_t = TP_\infty + (TP_0 - TP_\infty)\exp(-kt) \quad (3)$$

The texture degradation rate constant (k) and the non-zero equilibrium firmness after prolonged treatment time (TP_∞) were estimated using non-linear regression analysis on Eq. (3) [44]. The temperature dependence of k-value is given by the activation energy (E_a) as indicated by the Arrhenius model:

$$k = k_{ref}\exp\left[-\frac{E_a}{R}\left(\frac{1}{T} - \frac{1}{T_{ref}}\right)\right] \quad (4)$$

where E_a is the activation energy (kJ mol^{-1}); R (8.314 J mol^{-1} K^{-1}) is the universal gas constant; T and T_{ref} are the absolute temperature (K) and the absolute reference temperature (K), respectively; k and k_{ref} are the texture degradation rate constants (min^{-1}) at T and T_{ref}, respectively. The E_a was estimated by linear regression analysis of the natural logarithm of the rate constant versus the inverse of the absolute temperature.

Statistical analysis

In order to statistically compare the effect of the different treatments on firmness, ANOVA and bilateral Tukey's test were carried out.

Results and discussion

Thermal texture degradation kinetics

Thermal texture degradation of peppers was investigated up to 210 min for temperatures ranging from 75 to 95 °C. Even though long heating times were included in this study in order to estimate TP_∞ correctly, at temperatures higher than 80 °C, shorter periods ought to be considered due to excessive softening of the pepper tissue, which made it difficult to measure the texture of the samples. The relative firmness, that is the ratio between the firmness of the sample at a certain time (TP_t) and the firmness of the sample at time-zero (TP_0), is plotted as a function of the heating time (Figs. 2A and B). As previously mentioned, the time-zero sample was considered to be 5 min, since lack of such a heating lag-time correction has been pointed out by Rao and Lund [45] as the main constraint in many vegetables tissues softening studies. It should also be mentioned that during this period, the firmness of the pepper samples measured from the flesh side, when heated at 75 and 80 °C, increased, respectively, 12 and 6.5% when compared to fresh sample (data not shown),

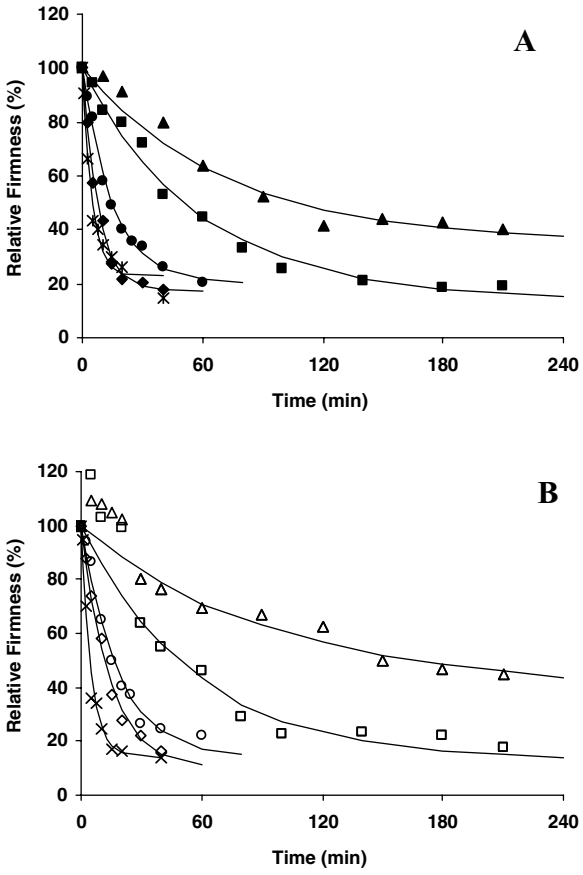

Fig. 2 Thermal texture degradation of pepper modeled using a fractional conversion model of (**A**) skin and (**B**) flesh. (▲, △) 75 °C; (■, □) 80 °C; (●, ○) 85 °C; (◆, ◇) 90 °C; (∗, ×) 95 °C

probably due to an increase of PME activity during the non-isothermal conditions, which is consistent with findings of other researchers for other vegetables treated under mild conditions [7, 11–13, 25, 46]. For 75 and 80 °C, the flesh-side firmness values were similar to the ones obtained for untreated pepper samples only after 20 min of heating, after which the softening process is monitored (Fig. 2B). The first experimental data points obtained for 75 and 80 °C were not considered to estimate the respective kinetic parameters due to the above-mentioned effect.

The kinetic parameters of thermal texture degradation of pepper at different heating temperatures are presented in Table 1. As can be observed, an increase in temperature from 75 to 95 °C corresponds to a 15–20-fold increase in the thermal texture degradation rate constant (k), for skin and flesh, respectively. With the exception of 90 °C, the k-values are very similar for skin and flesh, indicating an identical rate of texture degradation. The relative final value of the texture parameter (TP_∞/TP_0) decreases about one third for both skin and flesh, when the temperature increases from 75 to 80 °C. This sudden decrease in TP_∞/TP_0 value can be explained by the fact that from 75 to 80 °C the TP_∞ value

Table 1 Kinetic parameters (± standard error of regression) for thermal loss of firmness of pepper tissue at different heating temperatures

	$k \times 10^2$ (min^{-1})		Relative firmness at t_∞ (TP_∞/TP_0, %)	
	Skin	Flesh	Skin	Flesh
Temperature (°C)				
75	1.42 ± 0.29	1.00 ± 0.17	35.7 ± 4.6	38.2 ± 5.1
80	1.70 ± 0.11	1.79 ± 0.21	12.3 ± 1.9	12.9 ± 3.6
85	6.57 ± 0.44	5.38 ± 0.68	19.9 ± 2.1	13.8 ± 4.8
90	13.0 ± 0.93	7.16 ± 0.72	17.4 ± 1.8	10.1 ± 3.9
95	20.8 ± 2.72	20.0 ± 3.35	22.0 ± 3.1	14.0 ± 4.7
E_a (kJ mol^{-1})	157.7 ± 20.1	157.5 ± 13.9		

decreases to a much larger extent than the TP_0 value leading to a decrease in the TP_∞/TP_0 value. For 85–95 °C, TP_∞/TP_0 value increased for the skin side, with an average value of about half of that at 75 °C, while for the flesh side kept constant in relation to 80 °C. While for 75 and 80 °C, the relative firmness parameter (TP_∞/TP_0) was similar for skin and flesh, for 85–95 °C higher values were found for skin.

Activation energy for thermal texture degradation of pepper

The temperature dependence of the texture degradation rate constant is illustrated in Fig. 3. Both regression analysis for skin and flesh showed good correlations (skin: $R^2 = 0.974$; flesh: $R^2 = 0.979$). The activation energy (E_a) estimated from the Arrhenius equation (Eq. (4)) was 158 kJ mol^{-1} for both skin and flesh, indicating a similar sensitivity of reaction rates towards temperature. Paulus and Saguy [47] reported E_a-values of 92–117 kJ mol^{-1} for thermal texture degradation (90–120 °C) for different carrot varieties, and more recently, Vu et al. [42] reported an activation energy of 118 kJ mol^{-1} for thermal softening of carrots between 80 and 110 °C. A higher E_a-value implies that the k-value is more sensitive to temperature increase, therefore reactions that might be involved in the thermal texture degradation of green bell pepper seem to be more sensitive to temperature than the ones related to carrot softening. According to Lund [48], the E_a-values for plant tissues are in the range associated with chemical reactions such as hydrolysis of cell-wall constituents, swelling due to gas expansion, and heat-induced changes in water holding capacity that can affect the plant tissue.

Effect of pre-treatments on pepper texture

Thermal pre-treatments

PME activity for different plant sources shows a maximum around 50–60 °C [49–51], which could be explained by the thermally induced cell-wall damage and loss of membrane selective permeability in the plant tissue while the enzyme under these conditions is not inactivated. Therefore, PME would be more easily extracted and, at the same time activated by simultaneous cation diffusion. Previous process stability studies related to pepper PME indicated that after heat treatments at 50–55 °C relative PME activity of the pepper samples (intact tissue and puree) increased, followed by a fast decay above 60 °C [52]. Therefore, 55 °C was chosen as a thermal pre-treatment in order to study the effect of time (20, 40, 60 and 90 min) on pepper tissue texture (Fig. 4). Treatments of 40, 60 and 90 min caused an increase in firmness compared to not pre-treated peppers ($P < 0.05$), for both skin and flesh, up to a maximum of 40%. For each treatment no differences were found ($P > 0.05$) when firmness was measured from the skin or flesh side. These results are in agreement with those obtained in other low temperature pre-treatment studies for *C. annuum* fruits [8–10]. For example, Domínguez et al. [9] found that the highest resistance force to puncture pepper tissue was observed when diced green bell peppers were heated at 69 °C for 49 min. Other vegetables (sprouting broccoli, asparagus, lettuce and mustard) also showed relative firmness increments up to a maximum value, when heated at 50–55 °C for 30 min [7].

The relative firmness increase during pre-cooking conditions can be due to the action of PME on the methoxyl groups of pectin molecules present in the middle lamella between

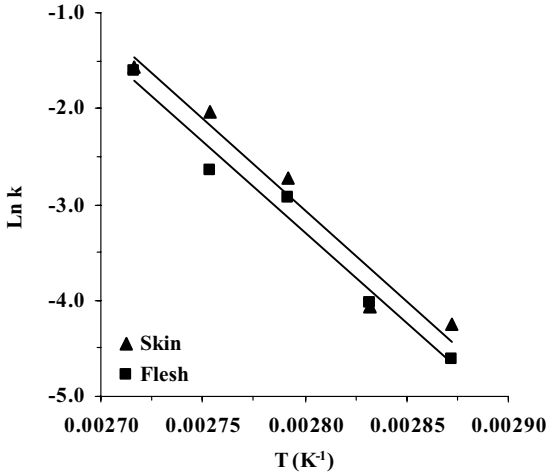

Fig. 3 Plot of the natural logarithm of texture degradation rate constant of green bell pepper (k-value, min^{-1}) as a function of the reciprocal of the absolute temperature (K^{-1})

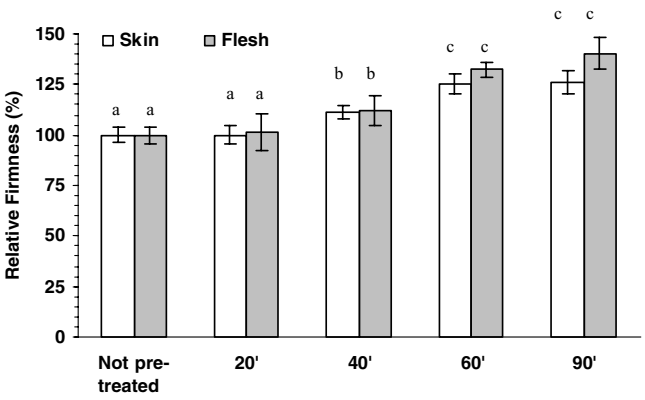

Fig. 4 Relative firmness of not pre-treated and thermally treated pepper samples at 55 °C for different time intervals. The *bars* represent the standard deviation of each sample ($n = 9$). *Bars* marked with different letters are significantly different ($P < 0.05$, Tukey's test)

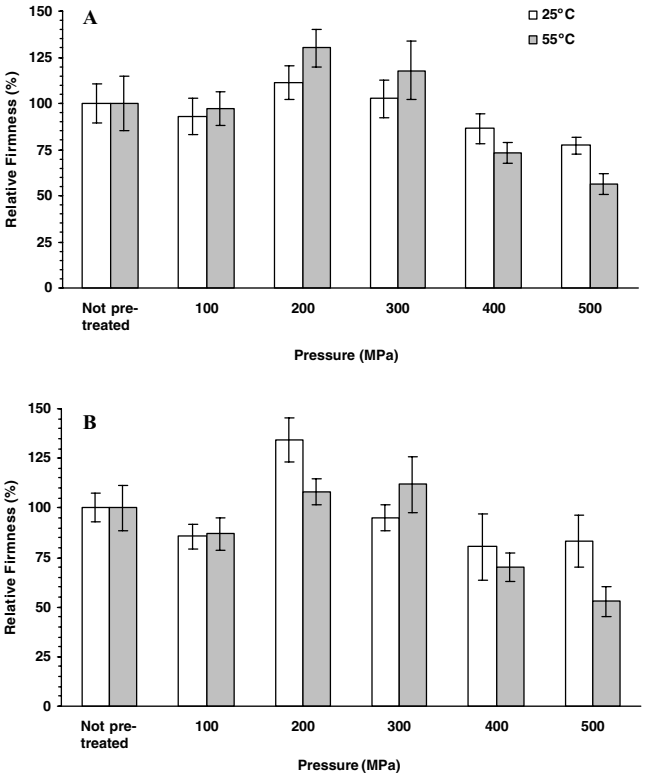

Fig. 5 Relative firmness of not pre-treated and high-pressure treated samples between 100 and 500 MPa, for 15 min for skin (**A**) and for flesh (**B**). The *bars* represent the standard deviation of each sample ($n = 9$)

the cell walls [7, 10]. The free carboxyl groups formed could then interact with Ca^{2+} ions to form bridges between the pectin chains, resulting in higher firmness and resistance of the tissue to further cooking. According to Chang et al. [53], the major forces for maintaining the texture of sweet pepper come from pectin molecules linked by heat labile bonds and covalent bonds, but interactions between pectin molecules and other cell-wall constituents are also reasonable and applicable. Given that the firmness of the pepper tissue was not significantly ($P > 0.05$) affected by an increase of the heating period from 60 to 90 min (Fig. 4) and since an extended heating time can also lead to nutritional losses by leaching [8], the thermal pre-treatment chosen for further investigation, in combination with pressure and calcium soaking, was 55 °C and 60 min.

Combined thermal/high-pressure pre-treatments

The effect of combined thermal/high-pressure treatments was studied between 100 and 500 MPa during 15 min, at 25 and 55 °C (Figs. 5A and B). Statistical analysis revealed a general increase in relative firmness ($P < 0.05$), for the skin side, from 500 MPa < 400 MPa < not pre-treated = 100 MPa < 200 MPa = 300 MPa, and from 500 MPa = 400 MPa < 100 MPa < not pre-treated = 300 MPa < 200 MPa for the flesh side, for both 25 and 55 °C.

The firmness increment caused by pressure might be related to the effect of high-pressure on the cell structure, which leads to an increase in the extractability of PME from the pepper tissue [52], and further contact of the enzyme with the pectin-substrate. At this pressure level, pepper PME seems to be protected from thermal inactivation [54] and when in presence of pectin-substrate, the PME catalyzed reaction exhibited an optimum for enzyme activity [33], leading to an improvement of the relative firmness observed at 200–300 MPa. For 400 and 500 MPa, relative firmness of pepper tissue decreases, especially when combined with 55 °C. At these pressure levels, a severe disruption of the tissue might occur and lead to permanent damages [34] as well as partial inactivation of the PME enzyme [52, 54].

Comparing the firming effect of thermal (Fig. 4) and combined heat-pressure treatments (Fig. 5), it can be concluded that the heat-pressure treatment at 200 MPa, 25 °C for 15 min for the flesh (134 ± 8%) side is similar ($P > 0.05$) to thermal treatment alone at 55 °C for 60 min (132 ± 4%), although it should be noted that high-pressure pre-treatments seemed to cause higher standard deviations than thermal pre-treatments. For flesh, for example, the standard deviation for the thermal pre-treatments at 55 °C at different time intervals was 3–6% (Fig. 4), while for pressure pre-treatments (100–500 MPa) at 25 °C, the standard deviation was between 6 and 17% (Fig. 5A). Since the best results on texture improvement for both skin and flesh were obtained within the range of 200–300 MPa, these pressure levels were further used to study the effect of calcium soaking.

Effect of calcium soaking

Soaking of pepper samples in calcium chloride solutions (0.25 and 0.50%, w/v) was done in combination with ther-

Table 2 Relative firmness (± standard deviation) of pepper samples with different temperature-time-calcium soaking pre-treatment combinations

Pre-treatment	Relative firmness (%)	
	Skin	Flesh
Not pre-treated	100.0 ± 4.5 ad	100.0 ± 15.3
0.25% Ca^{2+} (60′)	99.2 ± 6.1 a	99.8 ± 8.1 a
0.50% Ca^{2+} (60′)	109.1 ± 9.1 aef	111.4 ± 14.2 a
55 °C (60′)	125.0 ± 5.3 bg	132.0 ± 4.2 be
0.50% Ca^{2+} (60′) → 55 °C (60′)	125.0 ± 8.3 bh	136.7 ± 20.6 bf
55 °C + 0.50% Ca^{2+} (60′)	113.6 ± 6.8 ce	118.2 ± 15.5 bd
55 °C (60′) → 0.50% Ca^{2+} (30′)	112.9 ± 9.8 ce	111.4 ± 10.4 ad
55 °C (60′) → 0.50% Ca^{2+} (60′)	120.5 ± 9.1 cgh	150.4 ± 13.1 cef
55 °C (60′) → 0.25% Ca^{2+} (30′)	109.8 ± 5.3 cdf	95.6 ± 8.7 a
55 °C (60′) → 0.25% Ca^{2+} (60′)	119.7 ± 3.8 cgh	122.7 ± 19.9 c
55 °C (60′) → 0.25% Ca^{2+} (60′) → t_H (60′)	112.9 ± 9.8 c	146.0 ± 16.5 cef

t_H means holding time. Lines, within each column, marked with different letters are significantly different ($P < 0.05$, Tukey's test).

mal and thermal/high-pressure pre-treatments. The effect of different combinations of soaking in $CaCl_2$-solution with a thermal pre-treatment at 55 °C on pepper tissue firmness can be seen in Table 2. Immersion time on $CaCl_2$-solution concentration for the flesh side and thermal treatment at (55 °C) are important factors regarding the pepper tissue improvement. While immersion on $CaCl_2$-solution alone did not improve firmness ($P > 0.05$), with the exception of two treatments for the flesh side, all other treatments involving the thermal treatment at 55 °C, caused an increase ($P < 0.05$) in firmness, compared to samples not pre-treated or immersed in $CaCl_2$-solution only. Domínguez et al. [9] also observed that calcium addition to an initial blanching treatment (65 °C, 3 min) and 10–20 min holding without cooling, or even to the second, more severe, blanching step, caused a significant firming effect on the texture improvement of rehydrated dried bell peppers.

The effect of different combinations of pressure–temperature, with/without calcium soaking (0.50%, w/v) is presented in Table 3. Without calcium, with the exception of the pre-treatment at 300 MPa at 25 °C for the flesh side, the other three pressure pre-treatments studied increased pepper firmness for skin and flesh ($P < 0.05$).

The effect of pre-treatments on texture degradation at 90 °C

Industrially, bell pepper is usually frozen, after being thermally blanched, to be consumed in salads after thawing. Therefore, the effect of the pre-treatments yielding the best results in terms of firmness was evaluated for their effect on pepper thermal texture degradation kinetics at 90 °C, a temperature typically used to blanch peppers prior to freezing. The kinetic parameter estimates for thermal texture degradation at 90 °C for pre-treated pepper tissue are presented in Table 4. Texture degradation at 90 °C of pre-treated pepper is significantly slowed down by all the pre-treatment conditions investigated: the k-value decreases by 1.3–6.6-fold. Softness induced by processing at 90 °C was clearly slowed down by mild thermal and mild thermal/high-pressure pre-treatments, as already observed for other vegetables (e.g. [16, 55–57]). As it can be seen in Table 4 from the different selected conditions, pepper samples pre-treated at 200 MPa (15 min), followed by calcium soaking (45 min) yield the lower k-values at 90 °C. From several pre-treatment conditions, Sila and co-workers [16] concluded that pre-pressurizing carrots at 400 MPa and 60 °C was the best combination to reduce thermal texture degradation between 90 and 110 °C.

Since the final value of texture (TP_∞) only becomes important after a very long heating time, unrealistic for industrial applications, the optimisation of texture degradation should be in terms of texture degradation rate constants. Nevertheless, it should be emphasized that, globally, the relative firmness (TP_∞/TP_0) for the skin was not significantly affected, while for flesh, improvements of 1.5–4.1-fold were obtained for all pre-treatments studied. This evidence, together with the observed decrease in the k-value, clearly illustrates that texture of pre-treated pepper, under the studied conditions applied, followed by blanching (heating at 90 °C) was improved.

Influence of freezing and frozen storage on pepper tissue

Three of the pre-treatments studied, showing the most significant beneficial effect on pepper firmness, were further evaluated for the effect of freezing on pepper texture. The results are presented in Fig. 6 (given the similar impact of the three pre-treatments on both skin and flesh measurements, even though more pronounced for the flesh side, the results are only presented for the latter measurements). Statistical analysis revealed that only the pre-treated samples that were frozen by HPSF (200 MPa, −18 °C), showed no detrimental effect on firmness ($P > 0.05$), while those frozen by cryogenic freezing (−18 and −40 °C), presented a significant decrease on firmness ($P < 0.05$), of about 40–60%,

Table 3 Relative firmness (± standard deviation) of pepper samples with different pre-treatment combinations of temperature/high-pressure and calcium soaking

Pre-treatment	Relative firmness (%)	
	Skin	Flesh
Not pre-treated	100.0 ± 4.5[a] a	100.0 ± 15.3 a
200 MPa, 25 °C (15′)	111.4 ± 9.2 b	134.2 ± 11.0 bd
300 MPa, 25 °C (15′)	102.3 ± 10.4 b	95.1 ± 6.5 a
200 MPa, 55 °C (15′)	129.9 ± 14.5 bd	107.9 ± 6.6 c
300 MPa, 55 °C (15′)	117.9 ± 16.1 cd	111.9 ± 14.4 c
200 MPa, 25 °C (15′) → 0.50% Ca^{2+} (45′)	119.7 ± 9.4 bd	158.7 ± 11.5 a
300 MPa, 25 °C (15′) → 0.50% Ca^{2+} (45′)	88.6 ± 17.4 a	153.2 ± 16.7 a
200 MPa, 55 °C (15′) → 0.50% Ca^{2+} (45′)	122.0 ± 11.4 bd	149.4 ± 15.5 cd
300 MPa, 55 °C (15′) → 0.50% Ca^{2+} (45′)	115.2 ± 16.4 b	129.4 ± 13.3 b

Lines, within each column, marked with different letters are significantly different ($P < 0.05$, Tukey's test).

Table 4 Kinetic parameter estimates, texture degradation rate constants, k, and relative final texture value (TP_∞/TP_0 (± standard error of regression)), for thermal (at 90 °C) texture degradation of pepper, submitted to different pre-treatment conditions

Pre-treatment	$k \times 10^2$ (min^{-1})		Relative firmness at t_∞ (TP_∞/TP_0, %)	
	Skin	Flesh	Skin	Flesh
Not pre-treated	13.0 ± 0.93	7.16 ± 0.72	17.4 ± 1.8	10.1 ± 3.9
0.50% Ca^{2+} (60′)	7.17 ± 0.89	4.71 ± 1.27	17.1 ± 3.8	23.5 ± 8.9
55 °C (60′)	8.03 ± 0.84	5.37 ± 0.88	17.3 ± 2.9	14.9 ± 6.0
55 °C (60′) → 0.50% Ca^{2+} (60′)	4.50 ± 0.29	3.04 ± 0.41	20.7 ± 2.3	17.0 ± 4.6
200 MPa, 25 °C (15′)	5.80 ± 0.39	3.90 ± 0.14	21.1 ± 1.6	16.0 ± 1.1
200 MPa, 55 °C (15′)	3.18 ± 0.18	2.92 ± 0.18	18.2 ± 1.8	34.3 ± 1.6
200 MPa, 25 °C (15′) → 0.50% Ca^{2+} (45′)	2.17 ± 0.13	1.60 ± 0.27	20.2 ± 2.2	17.4 ± 6.9
200 MPa, 55 °C (15′) → 0.50% Ca^{2+} (45′)	1.97 ± 0.12	1.95 ± 0.16	12.4 ± 2.5	41.5 ± 2.2

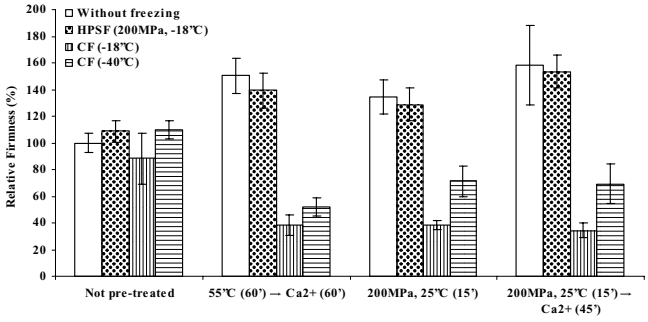

Fig. 6 Effect of freezing on relative firmness of not pre-treated and pre-treated pepper, measured from the flesh side. The *bars* represent the standard deviation of each sample ($n = 9$)

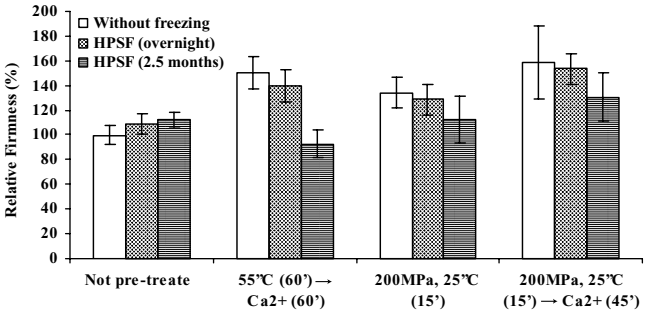

Fig. 7 Effect of frozen storage (overnight and 2.5 months storage) on relative firmness, measured from the flesh side of not pre-treated and pre-treated pepper, frozen by HPSF. The *bars* represent the standard deviation of each sample ($n = 9$)

an effect that was more pronounced for freezing at − 18 °C. This may be due to a lower freezing rate at − 18 °C and as a consequence, the ice crystals become larger, causing more detrimental effects on pepper tissue. For the not pre-treated pepper samples, no significant differences between the three freezing processes were observed, although these samples showed a lower firmness before the freezing process. Our results seem to be in agreement with other studies, where quick freezing of carrots improved the texture of frozen carrots [25, 32].

The effect of frozen storage (overnight and 2.5 months) at − 18 °C (a usual commercial and domestic storage temperature) on fresh pepper tissue, for samples submitted to HPSF (those showing the best texture after freezing), is plotted in Fig. 7. While for overnight storage samples, the three pre-treated samples showed no changes of firmness ($P > 0.05$), after 2.5 months storage, only the pressure pre-treated pepper samples showed no decrease on firmness ($P > 0.05$), clearly indicating the beneficial effect of the pressure pre-treatments to retain texture. The decrease in firmness observed for the thermal pre-treated samples after 2.5 months storage, may be caused by the action of an enzyme. The action of such an enzyme can be due to tissue disruption caused by the pre-treatment, allowing the contact between the enzyme and the substrate.

Conclusions

Texture degradation kinetics of pepper between 75 and 95 °C was adequately described by a fractional conversion model and characterized by an activation energy of 158 kJ mol^{-1}. Texture of pepper was improved by thermal, pressure and combined thermal/pressure pre-treatments, with the best results obtained for 55 °C (60 min) and 200 MPa at 25 °C (15 min). All the above cited pre-treatments reduced significantly the reaction rate for pepper texture degradation at 90 °C (1.3–6.6-fold) and substantially improved (1.5–4.1-fold) firmness. Pepper samples frozen by high-pressure shift freezing at 200 MPa (− 18 °C) showed no changes in texture, while cryogenic freezing at 0.1 MPa (− 18 and − 40 °C) caused pronounced firmness reduction. Pressure pre-treated pepper showed a better retention of firmness upon frozen storage (− 18 °C) for 2.5 months, than thermal pre-treated pepper. These results illustrate the potential of thermal, pressure, and combined thermal/pressure pre-treatments to improve texture of pepper and of high-pressure shift freezing to retain pepper firmness during freezing and frozen storage, compared to cryogenic freezing.

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