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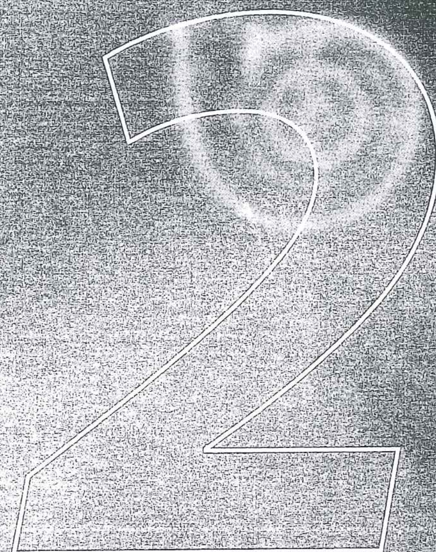


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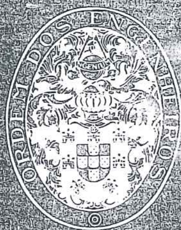

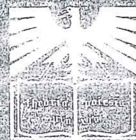

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# Analysis of the inactivation and reactivation kinetics of horseradish peroxidase in mixtures of phosphate buffer/dimethylformamide

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## Abstract

In this work, the kinetics of the thermal inactivation and reactivation of horseradish peroxidase were analysed in buffered solutions of dimethylformamide at several concentrations (up to 40% v/v) and temperatures ranging from 70 to 85 °C. The experimental data of the inactivation and reactivation processes showed a biphasic behaviour, which was well described by a series-type model. The significant activity regain at low temperature of the thermal inactivated enzyme had a strong influence on the estimated value for the $\alpha$ parameter of the model, thus reflecting a clear change of the profile of the experimental curves.

## Introduction

The analysis of the regain of peroxidase activity following limited heat treatment found major advances when food researchers correlated its occurrence to loss of food quality, namely changes in flavour of canned fruits and vegetables after storage (Nebesky *et al.*, 1950; Esselen & Anderson, 1956). More recently, however, it was found that this enzyme was not directly responsible for food deterioration and its adequacy as a quality indicator of the efficiency of blanching of fruit and vegetables was questioned. Although being a very heat-stable enzyme, its inactivation assure that the other enzymes present in the food product would not remain active, but may induce, in most cases, over-heating and thus loss of food quality (Whitaker, 1991). Moreover, the measurement of peroxidase activity should carefully follow standard procedures that would enable to avoid increasing intensity of heating (in terms of both time and temperature) with consequent loss of food quality, whenever instantaneous reactivation might be expected to occur.

Thermal stability of enzymes in organic solvents has been widely reported (Schulze & Klibanov, 1991; Volkin *et al.*, 1991; Blanco *et al.*, 1992; Saraiva *et al.*, 1996a), although one could not found any study correlating the presence of solvents in the reaction media to the kinetics of the spontaneous reactivation process of enzymes. Therefore, the main objectives of this work were (1) to study experimentally the inactivation and reactivation processes of horseradish peroxidase, over a wide range of inactivation temperatures (70-85 °C) and dimethylformamide concentrations (0 to 40% v/v) and (2) to assess the adequacy of a series-type model to describe the reversible inactivation process.

## Materials and methods

*Enzyme solutions*

Aqueous solutions of 0.08 mg $mL^{-1}$ of horseradish peroxidase (EC 1.11.1.7) were prepared in 0.1 mol $L^{-1}$ sodium phosphate buffer, pH 7.0, (Dawson *et al.*, 1986) or in mixtures of this

buffer with N,N-dimethylformamide (DMF) to the final concentrations of 5, 10 and 40% (v/v). The water-miscible solvent was stored over 3Å molecular sieves.

*Thermal treatment*

The enzyme solutions (1.5 mL) were introduced in eppendorf tubes of 3 mL and immersed in a thermostatic water bath, previously equilibrated at the desired temperatures of inactivation: 70, 75, 80 and 85 °C. At pre-determined time intervals, aliquots were removed from the tubes and immediately diluted in cold sodium phosphate buffer 0.1 mol $L^{-1}$, pH 7.0, in eppendorf tubes and immersed in an ice solution. Replicated samples were used.

*Enzyme reactivation*

Peroxidase was found to regain activity after removal from heat, within the range of temperatures tested, and storage at 4 °C. Therefore, the activity of all the samples was always monitored after 5 min from heat treatment and daily until no further significant difference was found between consecutive readings of activity, which was achieved after 24 hours at 4 °C.

*Analysis of the enzyme activity*

The peroxidase activity was measured according to the Worthington's procedure (Worthington, 1978). The increase in optical density upon addition of an aliquot of the enzyme solution to the substrate solution was measured at 510 nm and 25 °C using a UV/VIS kinetics spectrophotometer, every second over 1 minute. The slope of the straight line obtained was used as a measure of the activity.

*Data analysis*

Biphasic and multiphasic patterns of the inactivation experimental data of different enzymes have been reported and adequately described by a series-type model (Henley & Sadana, 1985; Saraiva *et al.*, 1996b). The series-type model considers a homogeneous native enzyme population ($E_N$), which inactivates by a mechanism that includes the formation of an intermediate form, partially inactivated ($E_I$), that can have different thermal resistance and specific activity compared to the native form, to yield an irreversibly inactivated form ($E_D$):

$$E_N \xrightarrow{k_1} E_I \xrightarrow{k_2} E_D \quad (1)$$

In this model, the conversions of $E_N$ to $E_I$ and $E_I$ to $E_D$ are considered to be first-order. The activity decay is therefore described by (Henley & Sadana, 1985):

$$\frac{A}{A_0} = \left(1 - \frac{\alpha * k_1}{k_1 - k_2}\right) * \exp(-k_1 * t) + \frac{\alpha * k_1}{k_1 - k_2} * \exp(-k_2 * t) \quad (2)$$

where $\alpha$ is the ratio between the specific activities of the intermediate and native forms (respectively, $\xi I$ and $\xi N$) and $k_1$ and $k_2$ are, respectively, the reaction rate constants of the native and intermediate forms, as expressed in equation (1).

Parameter estimation and model building, to study the effects of temperature and solvent concentration on the inactivation and reactivation of the enzyme, were performed by non-linear regression using Stata 5.0 (Computing Research Centre, Santa Monica, USA).

Temperature dependence of the rate parameters followed an Arrhenius-type relationship according to:

$$k = k_{ref} * \exp\left[-\frac{Ea}{R} * \left(\frac{1}{T} - \frac{1}{T_{ref}}\right)\right] \quad (3)$$

where k is the rate parameter at temperature T, $k_{ref}$ is the rate parameter at a reference temperature $T_{ref}$ (K), Ea is the activation energy (kJ $mol^{-1}$) and R is the universal gas constant (8.314 $Jmol^{-1}K^{-1}$). The use of a finite reference temperature is most important, as it decreases the correlation between the pre-exponential factor, $k_{ref}$, and the activation energy, Ea, thus improving parameter estimation (Haralampu *et al.*, 1985). In this work, the reference temperature chosen was the mean value of the temperatures studied, 77.5 °C.

## Results and discussion

Figures 1 and 2 show the inactivation profiles of the horseradish peroxidase in phosphate buffer and 40% (v/v) buffer-dimethylformamide and corresponding fitting of the series-type

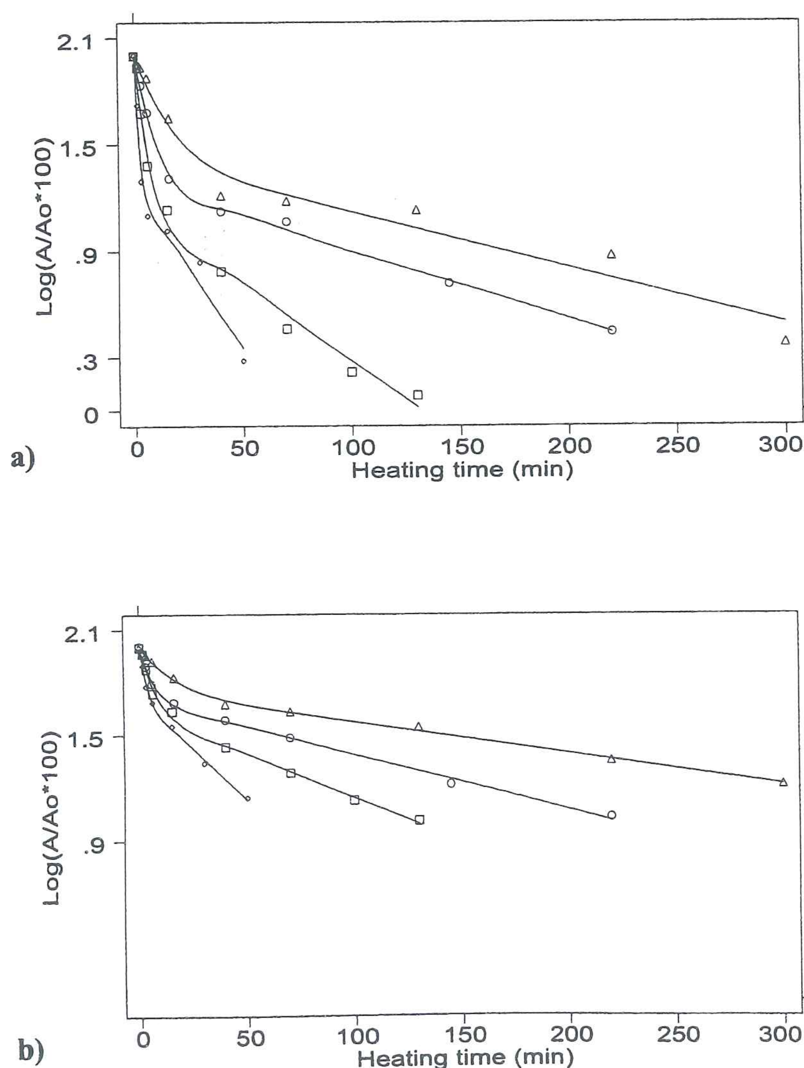


**Figure 1.** Kinetics of both the (a) inactivation and (b) reactivation processes of peroxidase in sodium phosphate buffer at 70 °C (ρ), 75 °C (O), 80 °C (⊠) and 85 °C (♬) and fitted curves of the series-type model.

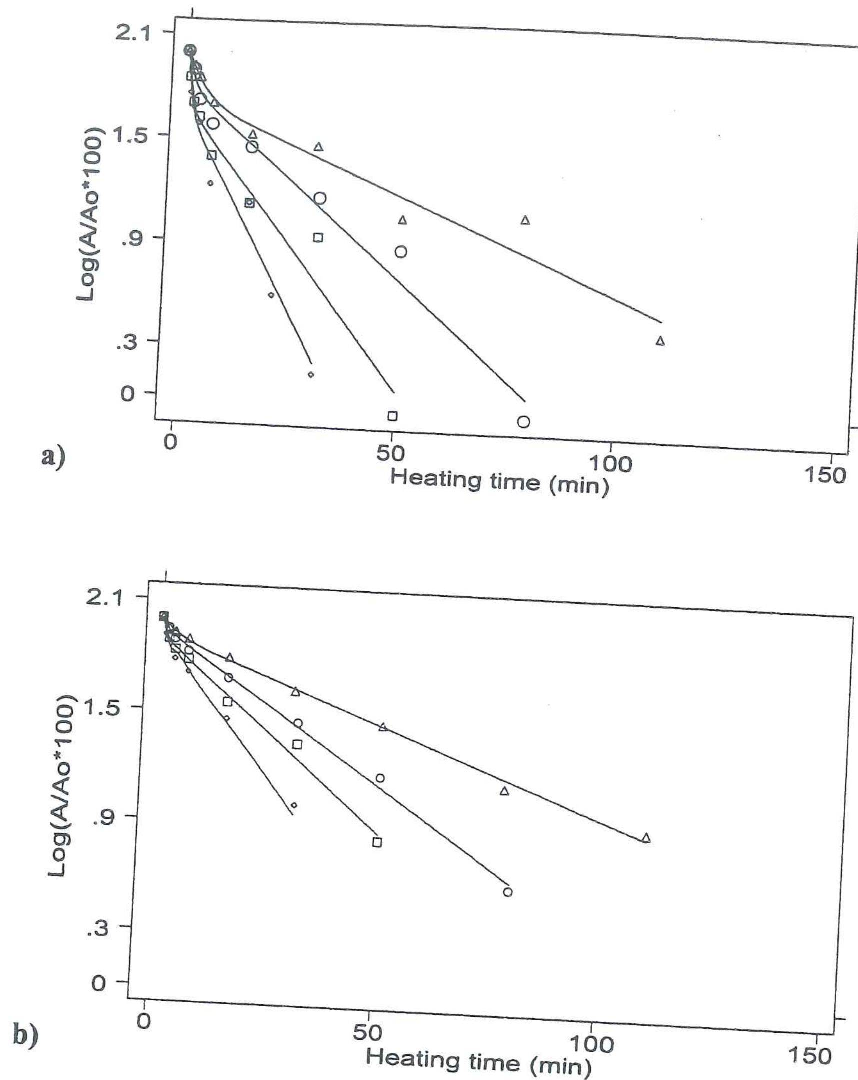


**Figure 2.** Kinetics of both the (a) inactivation and (b) reactivation processes of peroxidase in a mixture of phosphate buffer-DMF (40% v/v) at 70 °C (ρ), 75 °C (O), 80 °C (⊠) and 85 °C (♬) and fitted curves of the series-type model.

model to the experimental data. It can be seen that the inactivation features were found to be biphasic in all the cases studied, although reactivation of the enzyme yielded less pronounced biphasic experimental curves, particularly in the presence of dimethylformamide.

The estimated kinetic parameters for the series-type model of both the inactivation and reactivation of peroxidase in buffer and mixtures of buffer-dimethylformamide are described in Tables 1 and 2 and the coefficients of determination ($R^2$) were always above 0.99. The residuals proved to yield constancy of variance and tended to follow a normal distribution, thus showing the adequacy of the model. The rate parameters of the model, $k_1$ and $k_2$, showed

**Table 1.** Kinetic parameters (and their 95% confidence interval) for inactivation of horseradish peroxidase in mixtures of buffer-DMF.

| DMF (% v/v) | T (°C) | α | $k_{1ref}$*10 ($min^{-1}$) | $Ea_1$ ($kJ\ mol^{-1}$) | $k_{2ref}$*100 ($min^{-1}$) | $Ea_2$ ($kJ\ mol^{-1}$) |
|---|---|---|---|---|---|---|
| 0 | 70 | 0.259±0.11 | 2.20±1.36 | 177.4±94.0 | 1.44±0.53 | 146.1±57.5 |
| | 75 | 0.185±0.02 | | | | |
| | 80 | 0.130±0.03 | | | | |
| | 85 | 0.170±0.06 | | | | |
| 5 | 70 | 0.263±0.05 | 3.89±1.87 | 134.3±76.0 | 2.19±0.95 | 139.0±68.5 |
| | 75 | 0.197±0.03 | | | | |
| | 80 | 0.211±0.06 | | | | |
| | 85 | 0.319±0.04 | | | | |
| 10 | 70 | 0.305±0.07 | 7.59±3.54 | 145.0±73.0 | 2.91±1.12 | 98.0±64.0 |
| | 75 | 0.284±0.04 | | | | |
| | 80 | 0.257±0.03 | | | | |
| | 85 | 0.345±0.05 | | | | |
| 40 | 70 | 0.500±0.19 | 8.63±4.76 | 74.4±95.0 | 5.79±1.16 | 87.3±34.1 |
| | 75 | 0.582±0.25 | | | | |
| | 80 | 0.444±0.19 | | | | |
| | 85 | 0.420±0.17 | | | | |

**Table 2.** Kinetic parameters (and their 95% confidence interval) for reactivation of horseradish peroxidase in mixtures of buffer-DMF.

| DMF (% v/v) | T (°C) | α | $k_{1ref}$*10 ($min^{-1}$) | $Ea_1$ ($kJ\ mol^{-1}$) | $k_{2ref}$*100 ($min^{-1}$) | $Ea_2$ ($kJ\ mol^{-1}$) |
|---|---|---|---|---|---|---|
| 0 | 70 | 0.533±0.13 | 1.48±0.59 | 132.5±63.0 | 0.91±0.43 | 146.1±74.2 |
| | 75 | 0.484±0.06 | | | | |
| | 80 | 0.404±0.06 | | | | |
| | 85 | 0.473±0.06 | | | | |
| 5 | 70 | 0.636±0.07 | 3.73±3.32 | 142.6±138.0 | 1.94±0.76 | 94.0±66.0 |
| | 75 | 0.620±0.10 | | | | |
| | 80 | 0.611±0.07 | | | | |
| | 85 | 0.578±0.05 | | | | |
| 10 | 70 | 0.624±0.17 | 9.64±3.04 | 217.2±46.3 | 2.59±0.95 | 110.4±60.1 |
| | 75 | 0.719±0.07 | | | | |
| | 80 | 0.770±0.05 | | | | |
| | 85 | 0.787±0.07 | | | | |
| 40 | 70 | 0.770±0.13 | 10.03±3.85 | 94.3±64.2 | 4.12±0.74 | 75.3±31.0 |
| | 75 | 0.830±0.26 | | | | |
| | 80 | 0.764±0.17 | | | | |
| | 85 | 0.772±0.17 | | | | |

an Arrhenius-type dependence on temperature. The rate parameter of the reaction for the formation of the intermediate ($k_{1ref}$) for both the inactivation and reactivation processes appears to increase when the solvent concentration is increased from 0 to 10% (v/v), although its value remains constant when the concentration reaches 40% (v/v). Note, however, that greater errors are associated to the parameters of the first reaction, $k_{1ref}$ and $Ea_1$. Because the intermediate forms fast, particularly at higher temperatures, therefore the number of experimental points relevant to the description of the first reaction is comparatively small, the results have poor statistical significance and cannot be analysed in detail. On the other hand, the reaction for the inactivation of the intermediate was found to be slower than that of its formation ($k_{2ref}$ values were one order of magnitude smaller than $k_{1ref}$ values) and it can also be seen that significantly greater values for the $k_{2ref}$ parameter were obtained when comparing the results in buffer alone and 40% (v/v) dimethylformamide. In general, after reactivation, there is indication that the $k_{2ref}$ parameter might yield slightly smaller values.

The $\alpha$ parameter, which defines the ratio between the specific activities of the intermediate and native forms of the enzyme, had in most cases significantly higher values when reactivation of the enzyme occurred, which thus reflect the change of the experimental curves profile into a less pronounced biphasic pattern, as earlier mentioned. This parameter was also affected by the concentration of dimethylformamide in the aqueous systems analysed, especially when comparing the results in buffer alone to 40% (v/v) dimethylformamide. It is interesting to note, however, that temperature might not influence the values of the parameter.

In spite of the statistical uncertainty associated to the estimated values of the activation energy for the reaction of formation of the intermediate ($Ea_1$), above discussed, it appears that sensitivity to temperature after thermal inactivation decreased from pure buffer to the solution with 40% (v/v) buffer-dimethylformamide ($Ea_1$ decreased from 177 kJ $mol^{-1}$ to 74 kJ $mol^{-1}$). This same feature may be found after reactivation, although with a maximum value at 10% (v/v) buffer-dimethylformamide. In the case of inactivation of the intermediate, the values of the activation energy ($Ea_2$) also may decrease from pure buffer to 10% (v/v) buffer-dimethylformamide after inactivation, though no further influence of higher concentration of the solvent was apparently found on the estimated values of this parameter.

## Conclusions

The series-type inactivation model adequately described the inactivation and reactivation processes of peroxidase in buffer and buffer-dimethylformamide. Reactivation mainly affected the values of the $\alpha$ parameter, thus showing that recovery of the enzyme activity was particularly relevant in the region of the experimental curves in which it is expected to find significant amounts of the intermediate form of the enzyme.

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