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Potencial bioativo dos hidrolisados de *Mytilus galloprovincialis*: efeito das condições de hidrólise e processamento de amostras

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Microbiologia, realizada sob a orientação científica da Engenheira Inês Alexandra Casaca Lage de Castro, CEO da empresa Castro, Pinto e Costa, Lda. e coorientação da Professora Doutora Maria Adelaide de Pinho Almeida, Professora Catedrática do Departamento de Biologia da Universidade de Aveiro.

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Dedico este trabalho à minha mãe pelo esforço que tem feito sempre por mim. Obrigada.

o júri

presidente	Prof. Doutora Maria Paula Polónia Gonçalves Professora associada do Departamento de Biologia da Universidade de Aveiro
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palavras-chave

Alcalase, Atividade Anti-hipertensiva, Atividade Antimicrobiana, Atividade Antioxidante, Hidrólise Enzimática, *Mytilus galloprovincialis*, Péptidos Bioativos, Protamex

resumo

Este estágio permitiu a integração no projeto ValorMar, que visa melhorar indústrias de aquacultura e ao mesmo tempo fornecer benefícios de saúde ao homem.

A aquacultura gera grandes quantidades de desperdício e subprodutos, o que constitui um problema económico, de saúde e ambiental. Estes subprodutos são conhecidos por possuírem potencial nutritivo e bioativo, merecedores de exploração para o fabrico de novos alimentos funcionais que possam exercer efeitos benéficos específicos na saúde e bem-estar humano.

Deste modo, o objetivo do presente trabalho foi explorar o potencial bioativo do mexilhão Mediterrâneo (*Mytilus galloprovincialis*), um produto comum na aquacultura europeia e que gera altas quantidades de desperdício, através de hidrólise enzimática, para extrair compostos potencialmente bioativos que possam ser posteriormente aplicados na criação de novos alimentos funcionais.

Homogeneizados de carne de mexilhão foram hidrolisados com Alcalase® e Protamex®, a um pH 7.5, temperatura de 60 °C, com diferentes concentrações de enzima (0, 1, 2 e 3%), de quantidade de substrato (25 e 50 g) e de duração (1 e 2 h). Os hidrolisados resultantes foram posteriormente processados através de liofilização ou ultrafiltração por Amicon®. Condições de pré-tratamento termal, hidrólise e processamento de hidrolisados foram avaliadas para compreender quais levariam à produção de péptidos bioativos.
Os hidrolisados foram testados quanto à sua capacidade antioxidante, através do método de eliminação do radical 2,2-diphenyl-1-picrylhydrazyl (DPPH), atividade antimicrobiana, pelas técnicas de difusão em disco e por poço, e atividade anti-hipertensiva pela sua capacidade em inibir a enzima conversora de angiotensina (iACE).

Os hidrolisados produzidos revelaram que hidrólise com múltiplas enzimas produz um maior potencial bioativo. A capacidade de eliminar o radical DPPH aumentou com o substrato e mostrou ser inferior com a fração >3 kDa, ao contrário do potencial iACE, que aumentou com a fração ultrafiltrada. Em geral, hidrolisados de mexilhão fervido mostraram um alto potencial anti-hipertensivo. No entanto, nenhum efeito antimicrobiano foi observado nas amostras obtidas nas condições testadas.

Estudos posteriores deverão ser realizados, ampliando condições de hidrólise e purificação e testando variados potenciais bioativos, de modo a reunir mais informação acerca do potencial desta espécie como fonte de compostos bioativos. Mais ensaios são requeridos para avaliar a estabilidade dos hidrolisados na matriz do alimento e durante digestão, e o seu efeito nos locais alvos.

keywords

Alcalase, Antihypertensive Activity, Antimicrobial Activity, Antioxidant Activity, Bioactive Peptides, Enzymatic Hydrolysis, *Mytilus galloprovincialis*, Protamex

abstract

This internship allowed integration in the ValorMar project, aimed at improving aquaculture industries while also providing health benefits to humans.

Aquaculture generates great amounts of waste and by-products, which constitutes an economic, health and environmental problem. These by-products are known to possess nutritional and bioactive potential, worthy of exploration for the manufacture of novel functional foods, that can exert specific beneficial effects on human health and wellbeing.

Therefore, the goal of the present work was to explore the bioactive potential of the Mediterranean mussel (*Mytilus galloprovincialis*), a common European aquaculture shellfish product, that generates high amounts of waste, through enzymatic hydrolysis, to extract potentially bioactive compounds that can later be applied in the creation of new functional foods.

Mussel meat homogenates were subjected to hydrolysis with Alcalase® and Protamex®, at pH 7.5, temperature of 60 °C, with different enzyme concentrations (0, 1, 2 and 3%), substrate amounts (25 and 50 g) and duration (1 and 2h). Resultant hydrolysates were further processed through lyophilization or Amicon® ultrafiltration. Pre-thermal-treatment, hydrolysis and hydrolysates processing conditions were evaluated to understand which would lead to the production of bioactive peptides.
Hydrolysates were tested for their antioxidant ability, through 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging method, antimicrobial activity, by disk and well diffusion techniques, and for antihypertensive ability by their capacity in inhibiting angiotensin-converting enzyme (iACE).

Produced hydrolysates revealed that multiple enzyme hydrolysis produces higher bioactive potential. DPPH scavenging ability increased with substrate and revealed lower activity with the >3 kDa fraction, unlike iACE potential, which increased with the ultrafiltered fraction. In general, boiled mussel hydrolysates showed high antihypertensive potential. However, no antimicrobial effect was observed from samples obtained in the tested conditions.

Further studies should be done, widening hydrolysis and purification conditions, testing for different bioactive potential, to gather more knowledge on this species potential as a source of bioactive compounds. Further assays are required to assess hydrolysates stability in food matrices and during digestion, and their effect on target sites.

“In nature, nothing is created and nothing is destroyed, everything is transformed”

Antoine Lavoisier

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List of Abbreviations

ACE	Angiotensin converting enzyme
AMPs	Antimicrobial peptides
BHI	Brain Heart Infusion
BMHs	Boiled mussel hydrolysates
BPs	Bioactive peptides
CLSI	Clinical and Laboratory Standards Institute
CMHs	Lyophilised commercial mussel hydrolysates
DH	Degree of hydrolysis
DPPH	2,2-diphenyl-1-picrylhydrazyl
E/S	Enzyme concentration
G10	Gentamicin (10 µg)
LAB	Lactic acid bacteria
LNHs	Lyophilized non-boiled mussel hydrolysates
MWCO	Molecular weight cut-off
NMHs	Non-boiled mussel hydrolysates
SN	Supernatant

CHAPTER 1

1. Work developed at the company

The present dissertation results from the work developed during my internship at Castro, Pinto & Costa, Lda. (CPC), within the scope of the Master's in Microbiology of University of Aveiro.

The company is based in Maia, Porto, since 2000, focusing on the food field, mostly food security and quality control. Activities range from professional training in these areas, audit and consultancy services, implementing quality management systems. The company provides other services, as supplying equipment and accessories for food and medical industries, production of Oleotest®, a rapid test to assess the quality of food frying oils, and food and water microbiological and physicochemical analyses. The company also takes part in R&D, collaborating in food security projects, alongside other companies and public entities.

During this internship (October 2019 - July 2020) I participated in several of these activities, from preparation of stock products and equipment, production of Oleotest® kits, contribution in digital marketing, creating newsletters and content for social media, and mostly in the laboratory, doing routine food and water analyses and in the ValorMar project, the R&D project in which the company is currently collaborating, and that contributed for the development of the present work.

1.1. Routine analysis

LabMaia laboratory, part of the CPC Group, is responsible for conducting physicochemical and microbiological analyses to water, used in food processing, from public water supplies, wells, and pools, and to food, from restaurants, coffee shops, self-manufacturing companies, slaughterhouses, as well as food contact surfaces, handlers, and air microbiological quality. Frequent laboratory quality controls are also executed.

1.1.1. Food physicochemical parameters

Physicochemical analyses are conducted to determine nutritional composition of foods and evaluate their accordance with the stipulated health and safety values. The most common parameters evaluated are lipids (NP 1613:1979), salt (NP 2929:2009), sugars (DL 290/2003), protein (NP 1612:2006), fibre, ash (AOAC 920.153 and AOAC 923.03), and humidity (ISO 1442:1997). Detection of allergens (gluten) and sensorial activity analyses are also performed. Solid samples are previously grinded and homogenised and for each analysis a control assay is performed with a standard food (known nutritional values) with physicochemical resemblance to the sample.

Food lipids are treated with boiling hydrochloric acid, after which they are extracted with petroleum ether in the Soxhlet, followed by rotative evaporation. Salt content is assessed through titration with ammonium thiocyanate, after precipitation with silver nitrate. Total sugars are determined through the Luff-Schoorl method. Protein content measured by the Kjeldahl method. Food fibre is determined using the total dietary fibre assay kit (Megazyme Bray, Ireland). Ash, corresponding to food's total mineral content, and humidity are determined according to a gravimetric method.

1.1.2. Food microbiological parameters

Food microbiological analyses are usually conducted on the day of their reception. Food is weighed onto a sterilized bag and mixed with peptone water using the Stomacher® for mother dilution preparation. Most frequently conducted analyses are total microbial load determination, mould and yeast enumeration, and identification and quantification of lactic acid bacteria (LAB), sulfite-reduction bacteria, *Clostridium perfringens*, *Escherichia coli*, coliforms, Coagulase-Positive Staphylococci (CPS), *Staphylococcus aureus*, *Salmonella* spp. and *Listeria* spp.. Sample categorization and analyses are done according to the National Health Institute Doutor Ricardo Jorge (INSA) – "Valores-guia INSA", which were adapted in September 2019.

Total microbial load assay is conducted according to ISO 4833-1:2013 for colony count at 30 °C by the pour plate technique. It counts aerobic and facultative anaerobic

microorganisms that grow at 30 °C in non-selective nutritive media. Mother culture and respective dilutions are incorporated in Plate Count Agar (PCA), incubated for 72 h ± 3 h.

Moulds and yeasts enumeration done according to ISO 21527-1:2008, Part 1: Colony count technique in products with water activity greater than 0.95 at 25 °C, for mesophilic aerobic moulds and yeasts that grow on the surface of the medium, not intended for spores, flora identification, heat-resistant fungi, or mycotoxins. Sample is spread on the surface of the selective medium Dichloran Rose-Bengal Chloramphenicol (DRBC), that inhibits and reduces spread and growth of moulds and bacteria, incubated at 25 °C ± 1 °C for 5 days.

Quantification of LAB is done according to ISO 15214:1998, colony count technique for mesophilic lactic acid bacteria enumeration at 30 °C in selective medium. Sample is incorporated in the De Man, Rogosa and Sharpe Agar (MRS), selective for Lactobacilli growth, inhibiting competitive bacteria. Plates are incubated at 30 °C ± 1 °C for 72 h ± 3 h.

Sulfite-reducing bacteria are enumerated following ISO 15213:2003, Part 1: Enumeration of sulfite-reducing *Clostridium* spp., by colony count technique, in selective and differential medium, at 37 °C in anaerobiosis. After a heat treatment at 75 °C for 20 min, sample is incorporated on Iron Sulphite Agar (ISA), following addition of a second layer. Plates are incubated at 37 °C ± 1 °C, for 24 h to 48 h, in an anaerobiosis chamber, and resultant positive colonies present black colour and dark halo.

Identification and quantification of *C. perfringens* is done according to ISO 7937:2004, using the colony count technique. Sample is incorporated in the selective medium Tryptose Sulphite Cycloserine (TSC), followed by addition of a second layer, and incubation at 37 °C for 20 h ± 2 h. Typical dark coloured colonies, with possible opaque zones, are quantified, and selected for confirmation tests.

Enumeration of *E. coli* follows ISO 16649-2:2001, Part 2: Colony count technique for enumeration of β-glucuronidase positive *E. coli*, using 5-bromo-4-chloro-3-indolyl β-D-glucuronide, at 44 °C. Sample is incorporated in Tryptone bile-X-glucuronide (TBX), a selective and differential chromogenic medium, distinguishing between *E. coli* blue green β-glucuronidase positive colonies and pink-purple coliforms.

Enumeration of CPS is done according to ISO 6888-1:1999/Amd 1:2003, Part 1: Technique using Baird-Parker selective medium for the enumeration of Coagulase-Positive Staphylococci (*S. aureus* and other species). Plates are incubated at 37 °C ± 1 °C for 24 h to 48 h, after which typical bright, convex, black colonies with opalescent borders and non-typical dark ones are transferred to Brain Heart Infusion (BHI) medium and submitted to conformation tests using rabbit blood.

Detection of *Salmonella* is conducted as mentioned in ISO 6579-1:2017/Amd 1:2020 – Part 1, for detection of *Salmonella* spp., able to detect most *Salmonella* serovars. As these bacteria are fastidious microorganisms with slow growth rate, this method follows two enrichment phases, in non-selective and selective media, after which bacteria are incubated by streak technique in the differential and selective Xylose Lysine Deoxycholate agar (XLD). Resultant presumptive and typical colonies are later confirmed by biochemical assays.

Listeria spp. is detected according to ISO 11290-1:1996/Amd 1:2004 and ISO 11290-1:2017 and quantified as to ISO 11290-2:1998/Amd1:2004 and ISO 11290-2:2017. This method also requires two enrichment phases in selective Half-Fraser broth followed by Fraser broth and subsequential inoculation by spread and streak techniques in the differential and selective Agar Listeria Ottaviani and Agosti (ALOA), for enumeration and detection, respectively. Blue-green coloured colonies, with possible opaque halo (*L. monocytogenes*), are selected for conformation tests.

1.1.3. Water physicochemical parameters

Most frequent physicochemical parameters analysed are smell, chlorine, pH, conductivity, and turbidity, conducted immediately after sample arrival. Others include determination of nitrite, nitrate, iron, oxidability, hardness and suspended solids. Ammonia and aluminium can also be performed, depending on the type of analysis requested.

Determination of pH, conductivity, chlorine, and turbidity require specific equipment that needs frequent calibration. Nitrite (SM 4500-NO_2^-.B) is quantified spectrophotometrically after addition of Griess reagent. Nitrate (SM 4500-NO_3^- B) is measured spectrophotometrically in UV wavelengths. And sulphate (SM 4500-SO_4^{2-}.E) is measured

spectrophotometrically by a turbidimetric method. Iron (SM 3500-Fe. B) is determined by a colorimetric method after phenanthroline addition. Water oxidability (NP 731:1969) evaluates chemical oxygen demand (COD) in waters with low concentration of organic matter, through the consumption of oxygen from potassium permanganate. Total water hardness (SM 2340-C) measures calcium and magnesium ions present in the water using a complexometric method, that involves EDTA titration, in the presence of Eriochrome Black T. And suspended solids (SM 2540-C) correspond to the material left on ceramic crucible after evaporation to dryness on muffle furnace, at 180 °C.

1.1.4. Water microbiological parameters

Water for microbiological assays is collected into sterile recipients, some of which contain sodium thiosulfate for chlorine removal. Analyses are carried out on the day of sampling. Most common parameters evaluated are total microorganisms, enumeration of *C. perfringens*, *Staphylococcus* spp., *Enterococcus* spp., *E. coli* and coliforms. Enumeration of *Pseudomonas aeruginosa* and *Legionella* spp. can also be performed.

Total microorganism count is done according to ISO 6222, for enumeration of cultivable bacteria and molds and yeasts. Sample is incorporated into nutritive PCA medium and incubated at 36 °C ± 2 °C for 44 h ± 4 h and 22 °C ± 2 °C for 68 h ± 4 h, respectively.

Enumeration of *C. perfringens* follows ISO 14189:2013. Water is filtered through a membrane then placed on the differential and selective Tryptose Sulfite Cycloserine agar (TSC) and incubated in anaerobiosis at 44 °C ± 1 °C for 21 h ± 3 h. Colonies with dark black, grey, or brown coloration are selected for incubation in blood agar and the phosphatase test.

Staphylococcus spp. enumeration according to NP 4343, requires sample to be filtered through a membrane then placed in the selective medium Chapman-Mannitol salt agar and incubated at 37 °C ± 1 °C for 48 h ± 4 h. Colonies with yellow coloration and with(out) yellow halo, are selected for confirmation tests.

Enterococcus spp. enumeration is done according to ISO 7899-2-Part 2: Membrane filtration method, for detection and enumeration of intestinal enterococci. Filtration membrane is

placed in differential and selective media Slanetz and Bartley and incubated at 36 °C ± 2 °C for 44 h ± 4 h. Colonies with brown, red or pink coloration are peaked to the Bile Esculin Azide agar (BEA), resulting in positive black coloured colonies.

E. coli detection and total coliform enumeration follows ISO 9038-1:2014/Amd 1:2016 – Part 1: Membrane filtration method for waters with low bacterial background flora. The membrane is put onto the chromogenic coliform agar (CCA), differentiating between presumptive coliform β-D-galactosidase positive red-pink colonies and *E. coli* β-D-galactosidase and β-D-glucuronidase positive violet blue colonies, after incubation at 36 °C ± 2 °C for 21 h to 24 h. Pink colonies are selected for the oxidase test. Total coliforms correspond to the sum of pink and blue oxidase negative colonies.

1.2. ValorMar project

The company is currently working on the research project ValorMar – "Full valorisation of marine resources: potential, technological innovation and new applications", which counts with the participation of 30 more companies and public entities as project co-promoters: Forum Oceano, CIIMAR-UP, ALGAPLUS, Lda., A Poveira S.A., Bivalvia, Lda., CCMAR-UAlg, CVR, CMP, S.A., DocaPesca, S.A., Foodintech, Lda., Fourmag, Lda., Hidromod, Lda., IPLeiria, INEGI, IBET, IPMA, IPVianaCastelo, NAVIA, Necton, S.A., Piscicultura do Vale da Lama, Riasearch, Lda., Sorgal, S.A., Sparos, Lda., SPI S.A., UCP-ESB; UAveiro, UMinho, UPorto (ICBAS, FEUP), and SONAE MC, SGPS, S. A. as project leader and main founder.

This project was designed to respond to the underuse and waste of natural marine resources, as well as to the emergent consumer awareness to food origin, production, and environmental implications and their search for healthier, functional, and sustainable food. Hereby, ValorMar aims to develop innovative technological solutions to enhance marine supplies valorisation and efficient use, in a circular economy logic. Thus, exploring and giving new uses to marine products, creating new functional foods and other applications for biomedical and cosmetic industries.

To achieve this goal, there are minor specific objectives, covered by 5 partial projects from which result distinct products, processes and systems (PPS) (Figure A.I). The development

of new strategies to prolong fish products shelf life, creating innovative snacks and preserves made out of different marine resources (PPS1); development of a new and integrative solution for aquaculture production optimization (PPS2); resort to marine biorefineries, extracting and isolating compounds from marine resources (by-products and biomass), that may present biological activity, for application in food, biomedical and cosmetic products (PPS3); creation of a technological platform to provide information about the entire value chain, to companies and consumers (PPS4); and to divulge the scientific and technological advances obtained during these activities, promoting economic valorisation of the project results (PPS5).

CPC is integrated in PPS3, working on innovative ways to extract, characterize and evaluate potentially bioactive mussel compounds, for later application in smoked fish, to enhance its shelf life and give use to undervalued Portuguese marine species (PPS1), a new aspect of this project.

The project began in 2017, and had an estimated date of completion for September 2020, however, the latter has been postponed due the new coronavirus pandemic.

Project website: https://valormar.pt/en

CHAPTER 2

2. Bibliographic revision

2.1. Food waste in aquaculture

Every year great amounts of food are being wasted, by producers and consumers (Rico *et al.*, 2020), which comprises a rising concern presenting social, economic, health and environmental problems (Silva *et al.*, 2018).

Aquaculture and fish and seafood industries are among those responsible for generating high volumes of waste, discarding more than 50% of the production with the considered non-edible fractions (Ovissipour *et al.*, 2009), as fish heads, skin, viscera, and spines (Sila and Bougatef, 2016). Aquaculture shellfish, especially in mussel production, though being considered very nutritive food sources, present low commercial value, and are responsible for creating great amounts of by-products (Dai *et al.*, 2012), as shells and non-commercialized mussels, as seeds and undersized mussels, or due to damages on the shells (Beaulieu *et al.*, 2013), low meat yields, and presence of barnacles and byssus (Naik and Hayes, 2019).

With increasing world population and higher product demand, so does the amount of waste produced increases, being important to look for alternatives to utilize these natural products instead of disposing them as waste or allocating them into manufacture of low commercial valued products, as feed, fish oil and fertilizer (Ovissipour *et al.*, 2009).

2.1.1. By-products bioactive potential

The hostile marine environment to which marine organisms are constantly being exposed, due to constant temperature changes, presence of predators, pathogens, and toxic chemical compounds, has led them to develop a complex protective and defence mechanism, as the production of bioactive molecules (Beaulieu *et al.*, 2013; Pimentel *et al.*, 2019). Therefore, many of these organisms and aquaculture by-products are known to be important protein and mineral sources (Je *et al.*, 2007), and rich in bioactive molecules (Pimentel *et al.*, 2019). Fish

by-products contain valuable lipids and proteins (Sila and Bougatef, 2016). Macroalgae are important sources of pigments, minerals, fibre, and protein, having already been applied in cosmetics and pharmaceuticals, and consumed directly as food (Pimentel *et al.*, 2019). Seafoods are also rich in many valuable compounds as protein, chitin, collagen, carotenoids, minerals and vitamins, as well as bioactive molecules (Sila and Bougatef, 2016).

Many novel compounds have already been extracted from algae, fish, molluscs, crustaceans, shellfish and their by-products, which have shown to possess bioactive peptides (BPs) (Ngo *et al.*, 2012), molecules displaying various beneficial biological functions, such as antioxidant, antihypertensive, antimicrobial, immunomodulatory, anticancer, antithrombotic, antidiabetic and prebiotic abilities (Aluko, 2017). Thus, the ocean represents a great resource for the discovery of novel molecules that may serve as important sources of nutrients and therapeutic compounds (Ngo *et al.*, 2012).

2.1.2. Mussels bioactive potential

Mussels are the most produced bivalves in European aquaculture systems (Figueras *et al.*, 2019). Species from the Mytilidae family are popular in human diet and provide high levels of protein, omega-3 polyunsaturated fatty acids, iodine, and carbohydrates (Grienke *et al*., 2014).

Their shells are rich in organic minerals, as aragonite and calcite, and proteins. They also possess an important lipid fraction that has been proven to induce bioactive effects (Grienke *et al*., 2014). Their byssal threads, known as mussel's beard, are proteinaceous fibres with strong adhesive capability, widely studied for production of synthetically mimicked products (Grienke *et al*., 2014). They also contain a large portion of muscle tissue, highly rich in protein content, hence widely used for extraction of valuable peptides (Grienke *et al*., 2014).

Most relevant species belong to genera *Mytilus* and *Perna* (Grienke *et al*., 2014). Most produced *Perna* species are the Asian green mussel (*P. viridis*) and the New Zealand green-lipped mussel (*P. canaliculus*), that grow in warmer waters, in Thailand, the Philippines, China and New Zealand (Grienke *et al*., 2014).

On the other hand, *Mytilus* species occur in temperate waters of Europe, Asia, and America, and include the Mediterranean mussel (*M. galloprovincialis*) and the blue mussel (*M. edulis*), both indistinguishable solely on their morphological characteristics, although, Mediterranean mussel specimens are usually larger, flat, with a more concave basal line, and dark coloured shells (FAO, 2019). Its dimensions are greatly influenced by its biotope, when in intertidal zones, shells tend to remain small, while when in deep-water they present larger dimensions (FAO, 2019).

M. galloprovincialis is abundant along extensive areas of the Mediterranean Sea, living on all coasts that possess hard substrates, feeding on phytoplankton and detritus filtered from the surrounding water (FAO, 2019). It is known to present high resistance and tolerance to environmental changes and infectious agents, even more than other bivalve organisms, which is related to its robust immune system, and is also able to bioaccumulate pollutants at higher degrees, thus being extensively applied to assess water quality and in vitro studies of toxic effects (Burgos-Aceves and Faggio, 2017). Studies have also shown this species to naturally possess antimicrobial peptides (AMPs), belonging to various families, such as the defensins, mytilins and myticins, which are present in their haemocytes in the haemolymph (Grienke *et al*., 2014). Furthermore, its protein extracts have revealed to possess additional biological properties (Badiu *et al.*, 2010).

Apart from the latter, other mussel species produce molecules with great biological potential, since they need to adapt to parameters as salinity, wave exposure, substrate, temperature, water quality, and infectious agents or pollutants (Grienke *et al*., 2014). Hence, mussels produce peptides with a plethora of biological functions.

Such as antioxidant and antihypertensive in the green-lipped mussel (Jayaprakash and Perera, 2020). Antihypertensive (Je *et al*., 2005), antiproliferative (Beaulieu *et al.*, 2013), antithrombotic (Qiao *et al.*, 2018), antiadipogenic (Oh *et al*., 2020) and immunomodulatory (Grienke *et al*., 2014) in blue mussel protein extracts, already applied as impotence, menoxenia, and liver and kidney dysfunction treatments (Grienke *et al*., 2014). Or anticancer (Kim *et al.*, 2012) and antioxidant activity (Chen *et al.*, 2019) in the Korean mussel (*M. coruscus*), a species that has been utilized as food and medicine, with great immune system booster capacity and with good ability in regulating liver and kidney functions (Kim *et al.*, 2012).

2.2. Bioactive peptides

As mentioned before, BPs can exert biological effects on the human body. Inactive when present in their parent protein sequence, once released they can induce these bioactivities (Aluko, 2017), such as antihypertensive, antioxidant, antimicrobial, immunomodulatory, antithrombotic, anticancer, antidiabetic, prebiotic and anticholesterolemic (Ryan *et al.*, 2011).

BPs are constituted of 2 to 20 amino acid residues, and the fact that they are low molecular compounds facilitates their absorbance through the intestine into the circulatory system, to exert various functional effects on their target sites, or even produce local effects in the digestive tract (Ryan *et al.*, 2011; Lorenzo *et al.*, 2018).

Their bioactivity depends on their structural properties, as chain length, amino acid composition and physicochemical characteristics (Lorenzo *et al.*, 2018), some have even shown to exhibit multifunctional properties, exerting more than one significant bioactive effect (Di Bernardini *et al.*, 2011; Udenigwe and Aluko, 2012).

Presence of such beneficial compounds in food wastes and by-products and their vast physiological potential, also accompanied by an increase search for healthy and nutritious foods, pushes forward the search to explore such protein-rich produce and their ability to promote and exert beneficial effects on the human body (Galanakis, 2017). This way, increasing research for possible ways to extract and later apply these molecules into the production of functional foods, food that, when consumed regularly, delivers benefits beyond basic nutrition, exerting specific and multifunctional purposes (Gil-Chávez *et al.*, 2013).

2.2.1. Extraction of bioactive peptides

Marine by-products as algae, fish, and shellfish, already mentioned to exert biological effects, can thus be taken advantage and used as protein sources to obtain BPs (Aluko, 2017), and converted into value added innovative products, such as enriched functional foods, assuring complete utilization of the whole food (Silva *et al.*, 2018). This way posing a low-cost prime raw material that can reduce industrial residues and waste associated

environmental problems (Rivero-Pino *et al.*, 2020), while also serving as novel nutrient and therapeutic sources with good benefits to human health, alleviating many existing malnutrition and health issues (Kim and Mendis, 2006).

As referred previously, BPs are encrypted in their parent protein, exerting their biological function when released during digestion after their consumption, or through food/protein processing, as microbial fermentation, enzymatic hydrolysis (Udenigwe and Aluko, 2012), ultrasonic-assisted enzymatic extraction, pressurized liquid extraction, supercritical fluid extraction, organic solvent extraction or microwave-assisted extraction (Gil-Chávez *et al.*, 2013).

According to the techniques used to extract these BPs, various molecular weights and amino acid sequences can result, thus producing peptides with different physicochemical properties and biological activities (Kim and Mendis, 2006; Galanakis, 2017). Several of these methods have been used to obtain various protein extracts from mussel meat, which have shown to present numerous bioactive properties (Table 1).

Relatively to pressurized liquid extraction, organic solvents are used at high pressure (1450 to 2175 psi) and temperature (50 to 200 °C), which, despite allowing for a rapid extraction, can provoke deleterious effects on some compounds. In solvent extraction, the use of organic solvents can induce toxic effects (Gil-Chávez *et al.*, 2013), in addition it also presents low selectivity and low extraction efficiency (Najafian and Babji, 2012). Microbial fermentation uses proteolytic systems of bacteria, usually LAB that secrete microbial proteases (Sila and Bougatef, 2016), however, there is seemed to be no reports on BPs production from muscle proteins through this method (Ryan *et al.*, 2011).

Enzymatic hydrolysis is the most common method applied to extract peptides from food wastes (Kim *et al.*, 2012), especially for application in food and pharmaceutical industries, as it avoids the use of chemicals and physical treatments, thus reducing the risk of destroying valuable molecules or the presence of toxic chemicals in the final product (Ryan *et al.*, 2011; Beaulieu *et al.*, 2013; Sila and Bougatef, 2016). This process is easily controlled, produces higher efficiency (Ryan *et al.*, 2011), and can be used to extract BPs from different food matrices, as plants, macroalgae and animal sources, being widely applied in fish and shellfish wastes (Udenigwe and Aluko, 2012).

Table 1. Bioactive potential of different mussel species by-products. Distinct extraction processes that can be applied to obtain several peptides with various biological functions.

By-product (species)	Extraction process	Bioactivity	Reference
Korean thick-shell mussel (*Mytilus coruscus*)	Enzymatic hydrolysis	Anticancer	(Kim *et al.*, 2012)
	Ultrasonic-assisted enzymatic extraction	Antioxidant	(Chen *et al.*, 2019)
New Zealand green-lipped mussel (*Perna canaliculus*)	Enzymatic hydrolysis	Antioxidant Antihypertensive	(Jayaprakash and Perera, 2020)
Blue mussel (*Mytilus edulis*)	Microbial fermentation	Antihypertensive	(Je *et al.*, 2005)
	Enzymatic hydrolysis	Anti-proliferative	(Beaulieu *et al.*, 2013)
		Antioxidant	(Wang *et al.*, 2013)
		Antithrombotic	(Qiao *et al.*, 2018)
		Antiadipogenic	(Oh *et al*., 2020)
Mediterranean mussel (*Mytilus galloprovincialis*)	Isolation of AMPs from haemocytes	Antimicrobial, antifungal, antiprotozoal, antiviral	(Harnedy and FitzGerald, 2012)
	Solvent extraction	Anti-inflammatory	(Badiu *et al.*, 2010)

2.3. Hydrolysis

Enzymatic hydrolysis uses proteolytic enzymes that cleave specific peptide bonds, breaking proteins into small peptides and amino acids. It occurs in two steps, firstly the enzyme associates with and bonds to the substrate and subsequently initiates the catalytic action, cleaving a large number of peptide bonds, resulting in the release of soluble peptides and amino acids, the hydrolysates (Guérard *et al.*, 2001). This process influences the resultant

hydrolysates molecular size and hydrophobicity, which directly affects their functional properties (Kim *et al.*, 2012). These properties can then be enhanced through specific and controlled reaction conditions, as any pre-treatment the protein source has undergone, amount of substrate, enzyme type and concentration, pH, temperature, and reaction time (Kim *et al.*, 2012; Udenigwe and Aluko, 2012).

2.3.1. Reaction conditions

Although longer reaction times allow for enzymes proteolytic activity to take place, an extensive enzymatic hydrolysis may result in decreased activity, due to a high liberation of amino acids, since are peptides that present bioactivity, for their chemical composition and physical properties, or to products recombination (Sila and Bougatef, 2016). Thus, the most important factor is not an extended process, but a protein degradation sufficient to produce BPs of various sizes and bioactive properties (Guerard *et al*., 2002). In addition, reactions that endure for too long may be subjected to microbial contamination, which could potentially lead to hydrolysates deterioration (Xiong *et al.*, 2012).

Substrate should be reduced to small particles and homogenized, this way facilitating interactions with enzyme (Hammed *et al.*, 2013). Agitation is an important factor too, since it increases the contact between enzyme and substrate, which can reduce the need for higher reaction times (Hammed *et al.*, 2013).

For enzymes to maintain their catalytic activity it is crucial that reaction conditions stay adjusted to its optimum values. The solution pH is related to the temperature, hence the optimal temperature must be adjusted prior to pH (Aluko, 2017). For this, it is important to continuously monitor these parameters, mainly pH and especially in the first minutes of reaction (15 to 30 min) (Wang *et al.*, 2018; Tkaczewska *et al.*, 2020), since protein hydrolysis is accompanied by the release of H^+, that accumulate and lead to decreases in pH. Thus, to maintain values at enzyme optimum is necessary to add a base to the reaction, usually NaOH (Aluko, 2017).

2.3.2. Proteolytic enzymes

The specificity of the enzyme is one of the most important factors, since they are the ones to dictate cleavage patterns, thus, different proteases produce several types of hydrolysates with different amino acid sequences and chain lengths (Najafian and Babji, 2012; Sila and Bougatef, 2016).

Conducting hydrolysis relying solely on the action of the substrate endogenous enzymes has proven to be useful in the production of BPs from marine by-products, since they possess high enzymatic content (Marciniak *et al.*, 2018). However, such autolytic reactions are difficult to control, due to the existence of many factors leading to differences among organisms, even within the same species, as seasonality, environmental conditions and stress, inducing in the sample different chemical compositions, altering protein content and changes in enzyme content, concentration and activity (Bhaskar *et al.*, 2008). All these factors pose an obstacle, producing different reaction effects that are unable to control and reproduce (Bhaskar *et al.*, 2008; Sila and Bougatef, 2016; Marciniak *et al.*, 2018). On the other hand, hydrolysis conducted with exogenous commercial enzymes allows for better reaction control and hydrolysates production in less time (Bhaskar *et al.*, 2008; Sila and Bougatef, 2016).

For this reason, it is usual to apply a pre-treatment to the substrate prior hydrolysis, generally heating samples at 85 °C for 20 min, to inactivate endogenous enzymes (Guérard *et al.*, 2001; Ovissipour *et al.*, 2009). In addition, this step promotes fat removal and protein recovery (Bhaskar and Mahendrakar, 2008), and enhances hydrolysis by inducing molecular and conformational rearrangements in the original protein, which allows enzymes to access to new cleavage sites, increasing enzyme-substrate interactions (Udenigwe and Aluko, 2012; Pimentel *et al.*, 2019).

When using exogenous enzymes, especially to produce hydrolysates with the aim for application as food additives, enzyme selection must consider they need to be food-grade, commercially available and with an acceptable price (Tkaczewska *et al.*, 2020).

Though the most common approach is for single enzyme hydrolysis, they can also be applied in a multiple enzyme way, where enzymes can be added simultaneously or sequentially (Aluko, 2017).

Enzymes can be classified into two categories according to their cleavage mode of action, endopeptidases and exopeptidases. Endopeptidases break peptide bonds on non-terminal amino acids, cleaving bonds within the molecules (Tan *et al*., 2019). Whereas, exopeptidases break the outer bonds, hydrolysing proteins at the terminal bonds (Fonseca *et al.*, 2016).

Several enzymes can be applied for this hydrolysis process, existing a vast diversity, some from animal or plant origin (Kim and Mendis, 2006), others from microbial organisms, which present several advantages relatively to the previous ones, since they possess a wide variety of available catalytic activities and greater pH and temperature stability (Ovissipour *et al.*, 2009). Many enzymes have been applied for hydrolysis of foods, the most applied in these extraction processes are Alcalase®, Protamex®, Neutrase®, Flavourzyme®, papain, trypsin and pepsin (Tan *et al*., 2019). In Table 2 are presented the origin, type of cleavage and range of optimal conditions for these enzymes.

Table 2. Proteolytic enzymes. Origin, mode of action and reported optimal range of pH and temperature conditions (Samarakoon and Jeon, 2012; Aluko, 2017).

Enzyme	Origin	Mode of action	pH	Temperature (°C)
Alcalase	Microbial	Endopeptidase	7 – 9.5	50 – 60
Neutrase	Microbial	Endopeptidase	6.5 – 8	50 – 60
Protamex	Microbial	Endo and exopeptidase	6 – 7	40 – 50
Flavourzyme	Microbial	Endo and exopeptidase	6 – 7	50 – 60
Papain	Plant	Endo and exopeptidase	6 – 7	37 – 70
Trypsin	Animal	Endopeptidase	5 – 8	37 – 50
Pepsin	Animal	Endopeptidase	2	37 – 50

Most applied enzymes for hydrolysis of marine protein are Alcalase and Protamex (Sila and Bougatef, 2016). Alcalase is an endopeptidase from *Bacillus licheniformis*, mainly constituted with subtilisin A (Ovissipour *et al.*, 2009), is an alkaline, food-grade, commercially available enzyme that has been widely studied, namely for preparation of fish and seafood hydrolysates with low bitterness (Bhaskar and Mahendrakar, 2008; Salwanee *et al.*, 2013), also presenting benefits in terms of enzyme cost per activity (Bhaskar and Mahendrakar, 2008). Protamex is another microbial protease, with endo and exopeptidase activity, from *Bacillus* spp. (Silva *et al*., 2010), commercially available with food-grade characteristics, also producing non-bitter hydrolysates (Beaulieu *et al.*, 2013).

It is also important to introduce control reactions, where no enzyme is added to the solution, especially when not having performed a pre-treatment for endogenous enzymes inactivation, this way assessing their potential contribution to the hydrolysis (Aspevik *et al*., 2016) and sample behaviour under selected reaction conditions.

2.3.3. Degree of hydrolysis

Hydrolysis of protein is usually measured in terms of degree of hydrolysis (DH), an estimate of the total number of broken peptide bonds (Aluko, 2017), and is considered an important parameter to determine functional and biological properties of the protein hydrolysates preparations, since reaction conditions exert an influence on the DH and the produced hydrolysates (Sila and Bougatef, 2016).

The DH can be calculated through several methods, based on peptides solubility in trichloroacetic acid (TCA), through colorimetric methods based on binding of certain compounds, such as trinitrobenzenesulfonic acid (TNBS) and orthophtaldialdehyde (OPA), to the free amino acids, producing changes in reaction colour, which is directly proportional to the number of amino acids released, and the pH-stat method, one of the most applied to evaluate hydrolysis reactions (Aluko, 2017).

The pH-stat method is based on the amount of alkaline solution consumed to maintain a constant pH throughout the entire reaction, which allows for a continuous measurement of the hydrolytic process (Aluko, 2017). However, due to constant addition of base to the solution, the final product ends up having high levels of cations (Aspevik *et al*., 2016).

2.3.4. Hydrolysates purification

Reactions can be terminated by several methods, as altering the solution pH to levels that inactivate the enzyme (4 to 5 pH), through increases in temperature at levels that denature the enzyme, normally above 80 °C, for 10 to 15 min, after which the resultant solution is cooled at room temperature or with the aid of an ice bath (Wang *et al.*, 2018), enzymes can also be inactivated by storing the solution in cold temperatures, usually <-20 °C (Aluko, 2017).

After this step, resultant hydrolysates go through processing to isolate BPs from the complex mixture of molecules (Udenigwe and Aluko, 2012), containing a wide range of peptides, with different chain lengths, hydrophobicity, charge, and activity, for this reason, several separation methods can be applied to isolate the desired portions (Aluko, 2017).

Once the reaction is terminated, the mixture is usually centrifuged to separate the remaining meat tissue and debris in the sludge bottom, from the supernatant (SN), the phase containing the hydrolysates, thus, the SN is selected for further processing, and the *pellet* is normally discarded (Aluko, 2017).

To obtain high-yield hydrolysates product with potent bioactivity, the SN needs to carry additional purification steps that can concentrate peptides of defined weight ranges, being important to obtain low molecular weight peptides, typically <10 kDa, as this is related not only to an ability to withstand digestion and absorption, but reports have also associated smaller molecular weights to their bioactive potential (Udenigwe and Aluko, 2012).

This additional purification can be done through several approaches, such as high-pressure liquid chromatography (HPLC), fast protein liquid chromatography (FPLC) and membrane filtration, that separates peptides according to their peptide chain length (Aluko, 2017).

Membrane filtration techniques include microfiltration, nanofiltration, reverse osmosis and ultrafiltration, being the latter the most applied to separate protein hydrolysates. This technique uses specific membranes with certain molecular weight cut-offs (MWCO), usually of 3, 5 and 10 kDa, that can separate hydrolysates into more tighter ranges of molecular weights that may express different activities (Safari and Yaghoubzadeh, 2020). The most common equipment used for the effect are Amicon® separation membranes, resulting in two

fractions, the permeate, containing the lower molecular weight peptides, and the retentate with the higher molecular weight peptides (Aluko, 2017).

After this process, hydrolysates showing the highest bioactive potential can be submitted to further purification, through reverse phase high performance liquid chromatography (RP-HPLC), gel permeation chromatography (Ryan *et al.*, 2011) or liquid chromatography/tandem mass spectrometry (LC/MS/MS), to identify individual peptides (Aluko, 2017).

Drying processes, as freeze-drying/lyophilization or spray-drying, are often applied to extend products shelf-life through reduction in water activity (Rivero-Pino *et al*., 2020), thus, the obtained hydrolysates are frequently subjected to dehydration processes to obtain powdered forms with high protein content and stability (Bhaskar and Mahendrakar, 2008).

2.4. Bioactivity

2.4.1. Antioxidant activity

Free radicals and reactive oxygen species (ROS) attack membrane lipids, proteins, and DNA, being responsible for aging and many disorders such as cardiovascular disease, diabetes, cancer, Alzheimer's disease, and other neurodegenerative and inflammatory illnesses (Kedare and Singh, 2011). These molecules are also involved in food deterioration (Ryan *et al.*, 2011), being oxidation of foods a great concern for food industries and consumers, since it leads to development of off-flavours, odours, and even potentially toxic reaction products, generating great amounts of waste and possibly side effects in consumers (Bougatef *et al.*, 2010).

Antioxidants protect against these molecules, they can retard or prevent oxidation by chelating metals, scavenging free radicals, and substituting electron doners (Lorenzo *et al.*, 2018). The currently available synthetic antioxidants present some potential health risks, thus being important to search for new and safer substitutes (Ryan *et al.*, 2011). Natural compounds derived from food waste sources are good alternatives and do not seem to present any side effects (Bougatef *et al.*, 2010; Rivero-Pino *et al*., 2020). Some reports have inclusively shown seafood extracted BPs to exhibit antioxidant activity with great potential

to be applied as alternatives to the currently available compounds (Bougatef *et al.*, 2010; Kim *et al.*, 2012).

Antioxidant peptides are amongst the most studied, yet no specific assays for their bioactive potential have been developed or standardized, because of the complexity of oxidative processes occurring in food and biological systems and the different antioxidant mechanisms by which various compounds act, hence, many assays can be applied to measure antioxidant potential from food protein hydrolysates (Sila and Bougatef, 2016). Usually more than one method is applied, since when performed in isolation they fail to reflect sample's exact antioxidant ability (Lorenzo *et al.*, 2018), as an antioxidant compound can produce different results depending on the assay performed, because of differences in the mechanism of antioxidative action being measured or even reaction conditions, thus is common and important to apply more than one assay (Najafian and Babji, 2012).

Antioxidant activity assays can be divided into two major categories (Di Bernardini *et al.*, 2011), methods that involve hydrogen atom transfers, as total radical-trapping antioxidant parameter (TRAP) and oxygen radical absorption capacity (ORAC), that measures the decrease in fluorescence, being useful when analysing coloured foods (Lorenzo *et al.*, 2018). The other category involves electron transfer, measuring radical scavenging activity of the antioxidant compound through colour changes (Di Bernardini *et al.*, 2011), as trolox equivalent antioxidant capacity (TEAC), ferric reducing antioxidant power (FRAP), that measures the ability to reduce ferric salt to a blue ferrous complex in acidic conditions (Lorenzo *et al.*, 2018), and ABTS and DPPH radical scavenging, possibly the ones most commonly applied and that also combine hydrogen atom transfers (Lorenzo *et al.*, 2018).

The ABTS method uses the free radical chromophore 2,2-azino-bis(ethylbenzo-thiazoline-6-sulfonic acid) diammonium salt (ABTS), being one of the most rapid and reproducible assays (Lorenzo *et al.*, 2018).

The DPPH method uses the substrate 2,2-diphenyl-1-picrylhydrazyl (DPPH), a stable nitrogen purple coloured radical, with an absorbance wavelength between 515 to 520 nm. Reduction of this substrate is monitored through decrease in absorbance when in the presence of a hydrogen atom from the antioxidant, in ethanol solution, hence, colour change to yellow and decrease in absorbance are directly proportional to the antioxidant effect. It is

a rapid, practical, stable, and inexpensive method, that requires simple equipment and can measure multiple samples, usually able to react to the whole sample and provides enough time for the DPPH to react slowly, even with the weak antioxidant samples (Kedare and Singh, 2011; Lorenzo *et al.*, 2018). Generally, with incubations of 30 min in the dark, but that depends on the antioxidant compound, as reaction rates vary. In addition, samples may sometimes interfere with the absorbance measurement (Kedare and Singh, 2011).

2.4.2. Antimicrobial activity

Food spoilage and food borne diseases are generally associated with bacterial contamination, especially Gram-negative bacteria as *Salmonella typhi*, *P. aeruginosa* and *E. coli*, and Gram-positive as *S. aureus* and *B. cereus*. Preventive methods are usually achieved through chemical preservatives, however, their constant application results in accumulation across the food chain, possible side effects on human health, and acquisition of microbial resistance (Mostafa *et al.*, 2018). An emergent grave health issue, therefore, the urgent need to search for new antimicrobial agents from natural sources such as bacteria, plants, and animals, as well as food wastes (Balouiri *et al*., 2016). This search for novel antimicrobial compounds is further emphasised by the critical clinical health issues arriving from microbial infections, increasingly difficult to handle (Balouiri *et al*., 2016).

AMPs are natural non-enzymatically hydrolysed peptides that have been reported to be extracted from fish and marine animals, being involved in their defence mechanism, thus many of these organisms produce such molecules as a primary innate immune strategy (Najafian and Babji, 2012). As mentioned before, mussels have shown to possess many families of AMPs, that are extracted from their haemolymph, some studies have reported their presence in the Mediterranean mussel (Grienke *et al*., 2014).

Methods for in vitro testing of antimicrobial activity in samples and extracts include the Kirby-Bauer agar disk diffusion, the official method used in clinical microbiology for routine antimicrobial susceptibility testing, where the approved standards are published by the Clinical and Laboratory Standards Institute (CLSI).
Disk diffusion method is based on the inoculation of agar plates with a standardise inoculum, compared to the 0.5 McFarland standard solution, a suspension of barium sulfate. Followed

by disposal of filter paper discs, usually of 6 mm in diameter, containing the testing agent, which generally diffuses through the agar and inhibits growth of a specific microbial organism, creating inhibition zones around the discs. A clear halo with no observed growth, corresponding to an indirect measure of the compound ability to inhibit growth of a given microorganism, and allows to categorize bacteria as susceptible, intermediate, or resistant to the given substance.
This is a simple method that presents further advantages, as it is low cost and capable of being used for a variety of microorganisms and antimicrobial compounds, being also easy to interpret (Hudzicki, 2009; Balouiri *et al*., 2016).

Other commonly used method is agar well diffusion, with a similar principle to the previous one, based on diffusion of the antimicrobial agent from the wells, created on the surface of the agar, with 6 to 8 mm in diameter, into the medium, inhibiting growth of the tested microorganism, forming a halo around the well (Balouiri *et al*., 2016).

Another widely applied method is the dilution technique for determination of the minimal inhibitory concentration (MIC), that allows to estimate the lowest concentration of the tested compound that can inhibit growth of a given organism (Balouiri *et al*., 2016).

2.4.3. Antihypertensive activity

Cardiovascular disease is an important cause of mortality in industrialized societies (Guerrero *et al.*, 2012), hypertension is one of the most relevant factors for the development of this disease, and is influenced by genetics, aging, overweight, nutrition and lifestyle (Pihlanto *et al*., 2010; Ngo *et al.*, 2012).

Hypertension, and other conditions as sarcoidosis, infertility, anaemia, and migraine have been reported to be related to the renin-angiotensin system (Sentandreu and Toldrá, 2006), a key factor in the maintenance of blood pressure (Guerrero *et al.*, 2012). One of the most important components of this system is the angiotensin converting enzyme (ACE), that catalyses the conversion of the inactive form of angiotensin I into angiotensin II, a potent vasoconstrictor, that is also involved in the deactivation of bradykinin, a vasodilator, therefore, this enzyme promotes vasoconstriction and prevents vasodilation, thus increasing blood pressure (Ryan *et al.*, 2011).

The existing synthetic ACE inhibitors are widely used for cardiovascular and renal disease treatment, however, they can induce some side effects, being important to investigate new and natural compounds that could be used to inhibit ACE with potential antihypertensive effect (Sentandreu and Toldrá, 2006; Guerrero *et al.*, 2012).

The current antihypertensive drugs act by directly blocking the action of the enzyme, whereas these novel natural compounds inhibit ACE by interacting with it, through competition methods, binding to its active site and preventing it to exert any effect on angiotensin/substrate, or by binding to an inhibitor site located at the enzyme, altering its conformation and inducing formation of an inactive complex (Ryan *et al.*, 2011; Ngo *et al.*, 2012; Udenigwe and Aluko, 2012).

Determination of ACE inhibition ability is important in the discovery of novel ACE inhibitor peptides with antihypertensive potential. Among the most applied methods to assess this is the one based on hydrolysis of the synthetic peptide hippuryl-L-histidyl-L-leucine (HHL) by ACE and spectrophotometric determination of hippuric acid, one of the resultant products (Jayaprakash and Perera, 2020).

Other common method uses the subtract o-aminobenzoylglycyl-p-nitro-L-phenylalanyl-L-proline (Abz-Gly-p-nitro-Phe-Pro-OH), an internally quenched fluorescent compound that when cleaved releases fluorescence (Sentandreu and Toldrá, 2006). This method presents some benefits comparing to the previous one, it is a more rapid, accurate and sensitive assay, that relies in the enzyme ability to cleave the substrate and produce fluorescence (Sentandreu and Toldrá, 2006). This fluorescence is then easily quantified using appropriate equipment and wavelengths of excitation (355 to 375 nm) and emission (400 to 430 nm) (Sentandreu and Toldrá, 2006).

2.5. Objective

With this information in mind, the goal of the project is to give value to marine resources, otherwise discarded as waste, in the manufacture of enriched products, cosmetics, pharmaceuticals, and food with nutritional and biological functions, offering benefits to the human body. The present work aims to determine the optimal hydrolysis and sample/hydrolysates processing conditions, that can result in peptides with high bioactivity

(antioxidant, antimicrobial and antihypertensive) and thus, confirm that mussel by-products, especially Mediterranean mussel, can be used as a valuable resource of bioactive compounds able to be applied in functional foods production.

CHAPTER 3

3. Material and methods

To obtain potentially bioactive hydrolysates from mussel meat, different variables were studied, starting from type of mussel, to reaction conditions and hydrolysates purification processes, all with the aim to determine the best combination of conditions to produce compounds with biological activity.

Reagents used to conduct these assays were the proteolytic enzymes Alcalase® (≥0.75 AU mL^{-1}, density of 1.17 g mL^{-1}) and Protamex® (>1.5 AU g^{-1}), purchased from Merck (Darmstadt, Germany). The free radical DPPH and the rabbit lung ACE (1 UN) from Sigma-Aldrich (St. Lois, MO, USA), and the ACE substrate Abz-Gly-p-nitro-Phe-Pro-OH from Bachem (Bubendorf, Switzerland). Bacterial species *E. coli* NCTC 9001, *B. cereus* NCTC 7464 and *S. aureus* NCTC 6571 were acquired from Frilabo (Maia, Portugal).

3.1. Mussels

Mussel samples belong to the species *M. galloprovincialis* and were provided by Testa & Cunhas – Fishing and Aquaculture. Aquaculture by-products with different size arrays, meat content and shell integrity, thus not used for commercial purposes.

Samples were washed with distilled water to remove any dirt or unwanted objects, some were also submitted to a boiling bath, at 85 °C for 20 min. Shells and byssus were manually removed, has they have been reported to alter enzymatic hydrolysis (Beaulieu *et al.*, 2013). The obtained meat was minced into a homogenized paste, divided onto different plastic bags (≅100 g), and stored at -18 °C until use.

Also, to note that to test the hydrolysis protocol, and due to delays in mussel delivery, first trials were done with commercial mussels (*Mytilus* spp.), already without shells and possibly having undergone a previous treatment.

Meat chemical composition (proteins, lipids, ash, humidity, carbohydrates) was also determined for the different homogenates, using the methods implemented at the company.

This is an important step, as it not only allows to evaluate mussels' characteristics and heat treatment effect, but the protein content is required for later determination of the DH.

3.2. Hydrolysis

For the enzymatic reaction it was assembled a hydrolysis reactor (Figure 1) containing a heat plate for temperature and agitation control, a 250 mL beaker with the homogenised sample, previously defrosted at 4 °C and weighed to the desired mass, distilled water for a solution total of 150 g mussel/water (1:3 and 1:6 ratio), and a magnet for constant agitation, two electrodes to monitor temperature and pH, and a titration burette containing the base (NaOH 1 mol L^{-1}).

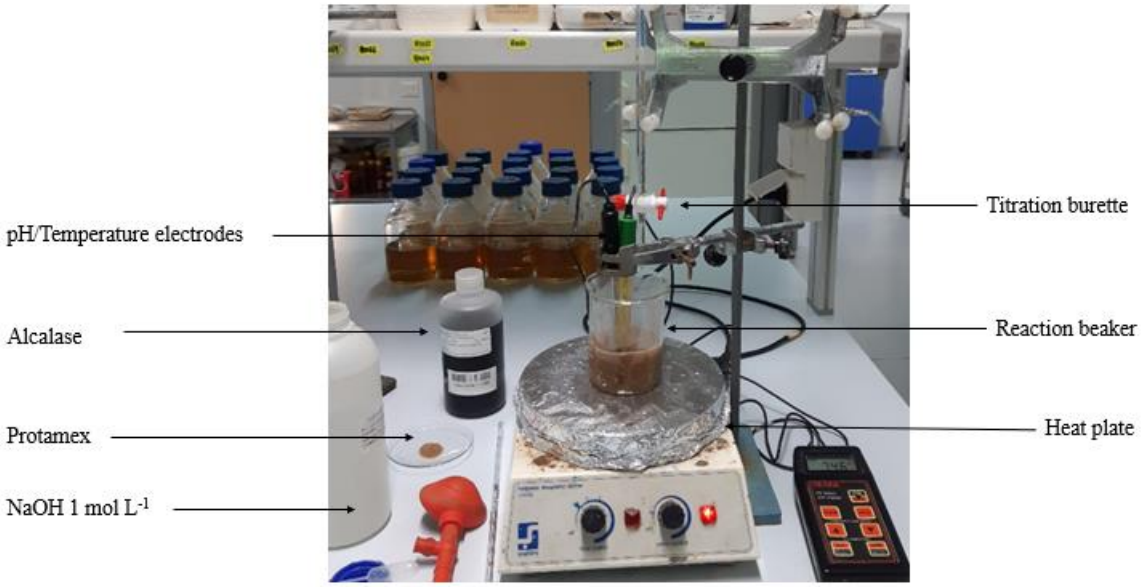

Figure 1. Experimental montage of the enzymatic hydrolysis reaction with *M. galloprovincialis*.

Several reactions were performed to evaluate the effect different values of the chosen variables would have on the DH, hydrolysates production and resultant peptide bioactivity. A total amount of 20 reactions were conducted to test all the variables: type of mussel [*Sample* – commercial (J), non-boiled, boiled (F)]; type of enzyme [*Enzyme* – Alcalase (A), Protamex (P) and mixture, added sequentially (1→1), or simultaneously (1+1)]; enzyme concentration [*E/S* - 1, 2, 3% and no added enzyme for control]; substrate mass [*Substrate* - 25 and 50 g of mussel paste]; and time of reaction (*time* – 1 and 2 h). Reaction temperature

and pH were maintained constant throughout hydrolysis and among all the assays performed, at 60 °C ± 1.0 °C and 7.5 ± 0.02, respectively. Conditions tested in each reaction are discriminated in Table 3.

Table 3. Hydrolysis conditions for each reaction performed (type of mussel sample, enzyme type, enzyme concentration (E/S), mass of substrate, and time of reaction).

Assay code	Sample	Enzyme	E/S (%)	Substrate (g)	time (h)
JC	Commercial	-	0	25	1
J1		Alcalase	3	25	1
J2			2	50	2
J3			3	25	2
C25	Non-boiled	-	0	25	1
C50		-	0	50	1
A1		Alcalase	1	25	1
A2			2	25	1
A3			3	25	1
A4			2	50	1
P1		Protamex	1	25	1
P2			2	25	1
P3			3	25	1
P4			2	50	1
A2P1		Combination	2 (1→1)	25	1
AP			2 (1+1)	25	1
FC	Boiled	-	0	25	1
FA		Alcalase	2	25	1
FP		Protamex	2	25	1
FAP		Combination	2 (1+1)	25	1

Once the desired temperature and pH were reached, the enzyme was introduced and time count started, during which base was added to maintain the pH. In assay A2P1 Alcalase was added at 30 min of reaction. The base pipetted volume was recorded at 5, 10, 15, 30, 45 and 60 min of reaction (90 and 120 min for some commercial mussel reactions), to determine

the DH through the pH-stat method according to Adler-Nissen *et al*. (1983), using the following equation:

$$DH\ (\%) = B\ \times\ N_b\ \times \frac{1}{\alpha} \times \frac{1}{MP} \times \frac{1}{h_{tot}} \times 100$$

Where:

B = base consumption

N_b = the base normality (1 mol L^{-1})

α = average degree of dissociation of the α-NH_2 groups

MP = mass of protein (N × 6.25)

h_{tot} = total number of peptide bonds in the protein substrate (meqv g^{-1})

After the stipulated time, reactions were terminated through thermal enzyme inactivation, by increasing the temperature to 85 °C ± 0.5 °C for 10 min, followed by cooling at room temperature for 5 min.

As for the control reactions (JC, C25, C50 and FC), same conditions were maintained, apart from enzyme addiction, any decrease in pH was due to endogenous activity, and it was adjusted with NaOH. They also suffered an inactivation process.

3.3. Hydrolysates

The resultant solution was then submitted to a series of processing to obtain the desired purified hydrolysates. Firstly, it was centrifuged with 8 000 G, at 25 °C for 30 min, after which different phases could be observed, a dark *pellet* at the bottom (Figure 2a), containing the remaining mussel tissue, rests of enzyme (Protamex) and other debris, and the SN, the liquid fraction with the hydrolysates and a thin upper oil layer. The *pellet* was discarded, and the SN selected for additional purification.

To further concentrate the hydrolysates, the recovered SN (Figure 2b) was subjected to a filtration process, the majority of samples were only filtered through filter paper with 1.2 µm pore, however, samples from boiled mussel were also passed through a glass membrane filter with 0.45 µm pore and then through an ultrafiltration membrane (3 kDa MWCO

Amicon® Ultra-15 centrifugal filter, Millipore®). The retentate containing hydrolysates with molecular weight >3 kDa was selected for the bioactivity assays.

In addition, part of the commercial (50 mL) and non-boiled mussel (25 mL) hydrolysates were lyophilised, at Minho University. Unlike non-boiled mussel samples, commercial ones were transformed into a viscous paste, rather than a clear white-yellow powder (Figure 2c).

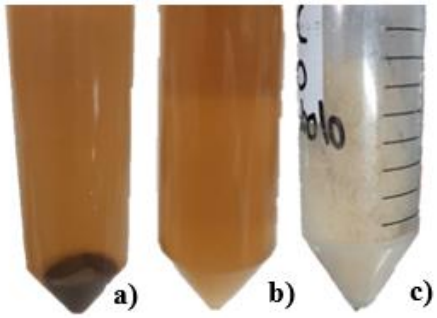

Figure 2. Mussel hydrolysates. Solution after centrifugation with apparent *pellet* formation (a); SN containing hydrolysates (b); lyophilized hydrolysate powder (c).

Samples were distributed into several 1 mL aliquots and stored at -18 °C until use. Lyophilized samples were kept inside 50 mL Falcon® tubes and stored in the desiccator at room temperature. Right before the bioactivity assays, to each tube containing lyophilized commercial mussel samples, were added 5 mL of sterile distilled water, vortexed and divided into 1 mL aliquots. Non-boiled mussel ones were distributed into 200 µL aliquots in a concentration of 0.025 mg μL^{-1}.

To add that, to dissolve lyophilized samples, especially those from commercial mussel, the hydrolysate concentration process was probably reverted, due to the amount of water required, as samples were quite viscous.

3.4. Bioactivity

The various hydrolysate samples obtained were subsequently subjected to a series of tests to determine their bioactive potential, in terms of antioxidant, antimicrobial and antihypertensive activity. These preliminary assays serve to evaluate sample behaviour in the executed protocols and to understand what the best conditions are to produce compounds with bioactive ability.

3.4.1. Antioxidant activity

To determine hydrolysates antioxidative potential, it was used the DPPH radical scavenging method. A transparent 96 well flat bottom microtiter was filled with 10 µl of sample and 200 µl of DPPH reagent for the sample wells, 10 µl of sample and 200 µl of ethanol 50% in distilled water, for colour control wells, and 10 µl of ethanol 50% and 200 µl of DPPH for the blank wells. After adding the free radical agent, with the aid of a multichannel pipette, plates were incubated in the dark for 20, 30, 40, 60 or 120 min, depending on the samples, since different hydrolysates reacted at different rates with the reagent. After the incubation period, microtiters were placed inside the microplate reader (EZ Read 2000, Biochrom®) and absorbance was measured at a wavelength of 515 nm, with initial 20 sec of automatic agitation, since samples were dense and precipitated at the bottom of the well.

Even with the agitation, commercial mussel hydrolysates precipitated to the bottom of the well, not properly mixing with the reagent. After being diluted in a 1:10 ratio with sterile distilled water, or even with 10 µL of tween 80, samples continued to present a slight agglomeration, which may have affected the results.

Antioxidant activity of the hydrolysates was then calculated according to the following equation (Jayaprakash and Perera, 2020):

$$DPPH\ radical\ scavenging\ ability\ (\%) = \left[1 - \frac{Abs_{sample} - Abs_{colour\ control}}{Abs_{blank}}\right] \times 100$$

Where:

Abs_{sample} = sample absorbance

$Abs_{colour\ control}$ = absorbance of sample in ethanol (50%)

Abs_{blank} = absorbance on blank wells

3.4.2. Antimicrobial activity

For the antimicrobial assays, three species of food pathogenic bacteria were selected, the Gram-negative *E. coli*, and the Gram-positive *B. cereus* and *S. aureus*, to test the antimicrobial ability of the obtained hydrolysates, according to the disk and well diffusion methods. As previously mentioned, both methods have the same principle, the diffusion of the antimicrobial compound from the dampened disk or well through the agar.

For this assay, the protocol was adapted from Hudzicki (2009) and Nalawade *et al*. (2016). For the inoculum preparation, a pure culture was prepared in BHI broth and incubated at 35 °C ± 2 °C for 24 h, after which the culture was transferred, by the streak method, to Trypticase soy agar, for 24 h at 35 °C ± 2 °C, for growth of isolated colonies. With the help of an inoculating loop, 4 to 5 colonies were transferred to a sterile saline diluent and then vortexed to create a homogenised suspension, its turbidity was then adjusted to the absorbance at 600 nm of a 0.5 McFarland standard solution, to obtain an optical density between 0.8 to 0.11 OD.

Once the inoculum was prepared, 100 µL were pipetted onto the surface of the agar and spread with a sterile swab, streaking in three different directions and across the plate borders to remove any excess. Sterile discs were placed on top of the media with the aid of a sterile tweezers, and wells created by perforating the agar, samples were then pipetted onto the discs and wells (20, 40 or 60 µL).

For negative control, discs and wells were left empty or with the same amount of sterile distilled water, for positive control were used discs with 10 µg of the antibiotic Gentamicin (G10). Assays were performed in duplicate for each method.

Plates were left to incubate at 35 °C ± 2 °C, for 18 h ± 4 h, after which any zone of inhibition formed around the discs or wells, with no observed bacterial growth to the unaided eye, was measured.

3.4.3. Antihypertensive activity

To determine the antihypertensive potential of the hydrolysates, samples were tested at School of Biotechnology of the Catholic University of Porto (ESB-UCP), following the protocol there implemented, adapted from Sentandreu and Toldrá (2006).

For the assay, was used Abz-Gly-p-nitro-Phe-Pro-OH as the ACE substrate. Due to the UV interference in this assay, a black flat-bottom microtiter is required to inhibit the background fluorescence. Wells were filled with 40 µL of ACE and 40 µL of sample (using dilutions from a 1:4 to 1:128 ratio, determined after trial assays), for the sample wells, 40 µL of sample and 40 µL of distilled water for the colour control, 40 µL of water and 40 µL of ACE for the control wells, 80 µL of water for the blank wells, and finally, 160 µL of substrate on each well with the multichannel pipette.

The microplate was placed in the reader (Synergy H1 Hybrid reader, BioTek ™) using 350 nm and 420 nm, as excitation and emission wavelengths, respectively. Absorbance was recorded for 30 min to produce the kinetics of the ACE inhibition reaction through the emitted fluorescence. Results, expressed as percentage of ACE inhibition were given automatically in iACE%, as well as the kinetic curves.

Hydrolysates samples used for this assay were only the ones produced from the boiled mussel hydrolysis, using both ultrafiltered and non-filtered fractions.

CHAPTER 4

4. Results and discussion

The mussel homogenate, from different origins and pre-treatments, was firstly subjected to its chemical composition analysis, and then used to extract possible BPs through hydrolysis reaction, for later evaluation of their bioactive potential.

4.1. Nutritional composition

Protein content from mussel homogenates is expressed in Table 4. Sample from boiled mussel showed the highest protein content (13.64%), this boiling process prior to hydrolysis, besides inactivating endogenous enzymes, also induces alterations in protein content, as well as reduction in humidity and fat content (Bhaskar *et al.*, 2008). As seen in previous assay, with mussel boiled without shells, the initial protein content of 12.6% increased to 18.9% after boiling, humidity went from 81.4% to 74.8% and lipids from 1.7% to 1.3% (Ramos, 2019). Lipid content in commercial and non-boiled samples was also inferior to 2% (Table A.I). Other studies obtained protein content of approximately 10% and 1% lipids in blue (Beaulieu *et al.*, 2013) and Asian green mussel (Normah *et al.*, 2013). Decrease in lipid content may pose as an advantage, as it promotes sample stability, since they become less susceptible to lipid oxidation (Ovissipour *et al.*, 2009).

Differences observed among samples, in protein content and the other chemical characteristics (Table A.I), may be due to their distinct origins, thus possessing an inherent variability due to factors such as life cycle, seasonality, and environmental conditions (Guerard *et al.*, 2007).

Table 4. Protein content present in each mussel sample.

Mussel sample	Protein (%)
Commercial	13.04 ± 0.59
Non-boiled	12.24 ± 0.15
Boiled	13.64 ± 0.09

4.2. Hydrolysis

4.2.1. Hydrolysis degree

The DH obtained for each hydrolysis are presented in Table 5, and range from 0.47%, in boiled mussel control reaction (FC), to 41.13% in the FAP assay, with boiled mussel and enzyme mixture.

The highest values were generated in reactions conducted with boiled mussel, which can be explained by the fact that the heat treatment alters protein conformation and may facilitate the binding of the enzyme to its cleavage site in the substrate, increasing enzyme-protein interactions, thus enhancing hydrolysis (Udenigwe and Aluko, 2012; Pimentel *et al.*, 2019).

On the other hand, control essays were the ones that presented the lowest DH, therefore showing that the action of the endogenous enzymes is negligible when compared with the exogenous commercial ones, as they need more time of reaction to be able to acquire similar values (Bhaskar *et al.*, 2008; Sila and Bougatef, 2016). Additionally, as mentioned before, the FC essay had the lowest DH, indicating that thermal treatment prior hydrolysis affects endogenous enzymes, inactivating or decreasing its activity (Guérard *et al.*, 2001; Ovissipour *et al.*, 2009).

Respectively to the commercial mussel, the higher DH was obtained for J1 (E/S= 3%; S= 25 g; t= 1 h; DH= 30.56%), superior to J3 (E/S= 3%; S= 25 g; t= 2 h; DH= 27.97%), which only differed in hydrolysis duration, seeming that the extended reaction time led to a decrease in the DH. The same was observed by Liu *et al.* (2010), explaining that it could be due to recombination of degraded peptides.

As to the non-boiled mussel, the highest DH were obtained in assays P3>A2P1>A3>AP, showing that multiple enzyme hydrolysis, especially when added sequentially, and higher enzyme concentrations, contribute to higher DH. The same was observed in Xu *et al.* (2019) that obtained higher DH for reactions conducted with a combination of Alcalase and Flavourzyme, Nchienzia *et al*. (2010) and Justus *et al.* (2019) also obtained higher DH for multiple enzyme reactions with Alcalase and Flavourzyme, being higher for the sequential addition over simultaneous one. Guérard *et al.* (2001), Klompong *et al.* (2007) and Liu *et al.* (2010) also obtained higher DH with increasing enzyme concentrations.

Table 5. Degree of hydrolysis (DH) obtained for each reaction, performed under specific conditions of enzyme type, enzyme concentration (E/S), substrate quantity and time of reaction.

Assay code	Sample	Enzyme	E/S (%)	Substrate (g)	Time (h)	DH (%)
JC	Commercial	-	0	25	1	0,50
J1		Alcalase	3	25	1	30,56
J2			2	50	2	26,58
J3			3	25	2	27,97
C25	Non-boiled	-	0	25	1	0,52
C50		-	0	50	1	0,63
A1		Alcalase	1	25	1	16,71
A2			2	25	1	17,02
A3			3	25	1	18,80
A4			2	50	1	15,77
P1		Protamex	1	25	1	13,16
P2			2	25	1	16,92
P3			3	25	1	19,74
P4			2	50	1	16,76
A2P1		Combination	2 (1→1)	25	1	19,63
AP			2 (1+1)	25	1	18,69
FC	Boiled	-	0	25	1	0,47
FA		Alcalase	2	25	1	31,01
FP		Protamex	2	25	1	28,57
FAP		Combination	2 (1+1)	25	1	41,13

When analysing results obtained for non-boiled mussel and comparing the effect type of enzyme and concentration and amount of substrate had in the resultant DH (Figure 3, left), it is visible that an increase in enzyme concentration increases the DH, as was mentioned before. We can also observe that for the same concentration (2%) the use of less substrate (25 g) produces higher DH. The same was observed by Liu *et al.* (2010) that obtained higher DH for inferior ratios of matter/water, describing as a possible reason the decrease in fluidity, in which higher amounts of substrate decreases fluidity, affecting binding between enzyme and substrate, hence decreasing hydrolysis. In results obtained from previous study, with

mussel boiled without shell, the DH would increase with increases in enzyme concentration and with inferior amounts of substrate (Ramos, 2019).

In addition, for lower enzyme concentrations, hydrolysis conducted with Alcalase produced higher DH, but for 3% of enzyme and 50 g of substrate was Protamex that contributed for higher DH, being this discrepancy more explicit for 1% of enzyme. Shen *et al.* (2012), Piotrowicz and Mellado (2015), Tan *et al*. (2019) and Tkaczewska *et al.* (2020) also obtained DH in Alcalase reactions superior to those conducted with Protamex, the same was observed by Dai *et al.* (2012) in blue mussel hydrolysis. This demonstrates that variations in substrate specificity in proteolytic enzymes leads to different cleavage patterns, hence different DH (Tan *et al*., 2019).

Comparing results obtained for boiled and non-boiled mussel (Figure 3, right), as mentioned previously, DH increases with the heat treatment. In terms of enzyme, for 2% concentration, both types of mussel reveal the same pattern, i.e., that the combination of enzymes results in a higher DH, followed by Alcalase, that contributes to a higher DH than Protamex, as it was stated before.

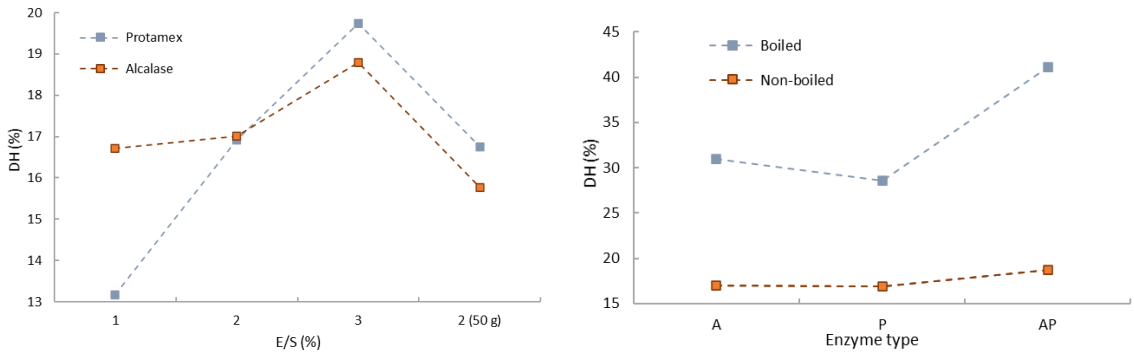

Figure 3. Degree of hydrolysis of boiled and non-boiled mussel. Effect of heat treatment (boiled, non-boiled mussel), type of enzyme (Alcalase, Protamex, combination), enzyme concentration (1, 2 and 3%) and mass of substrate (25 and 50 g). DH depending on enzyme concentration for non-boiled mussel (left) and type of enzyme for boiled and non-boiled mussel with 2% concentration (right).

Recording NaOH titrated volume, in specific reaction times, allowed to evaluate the DH evolution over the course of reaction, and draw hydrolysis kinetic curves. As shown in Figure 4, we can see a rapid initial reaction progression, due to greater enzymatic activity

leading to higher amounts of amino acids and peptides being released, after this exponential phase, there is a decrease in reaction rate, enzymatic activity slows down and base consumption stabilizes, leading to a *plateau* stage. The same has been observed in several hydrolysis studies for any type of substrate (Guérard *et al.*, 2001; Bhaskar *et al.*, 2008; Ovissipour *et al.*, 2009; Tan *et al.*, 2019). Similar curve patterns were seen in brown mussel (*Perna perna*) hydrolysis with Protamex (Silva *et al.*, 2010) and with green-lipped mussel (Jayaprakash and Perera, 2020). Dai *et al.* (2012) attained a steady state after 30 min of blue mussel hydrolysis, and previous study with mussel boiled without shell, where hydrolysis endure for 1, 2 and 3 h, confirmed little to no evolution after 1 h of reaction (Ramos, 2019).

This reduction in hydrolysis rate has been associated to competition that may occur between unhydrolyzed protein and peptides that are being released, by the decrease in substrate/peptide bonds available for enzymes to cleave (Tkaczewska *et al.*, 2020), enzyme inhibition by inhibitor peptides that are continuously being produced, and enzyme deactivation due to low stability at high temperatures for a certain amount of time (Guerard *et al.*, 2002; Ovissipour *et al.*, 2009).

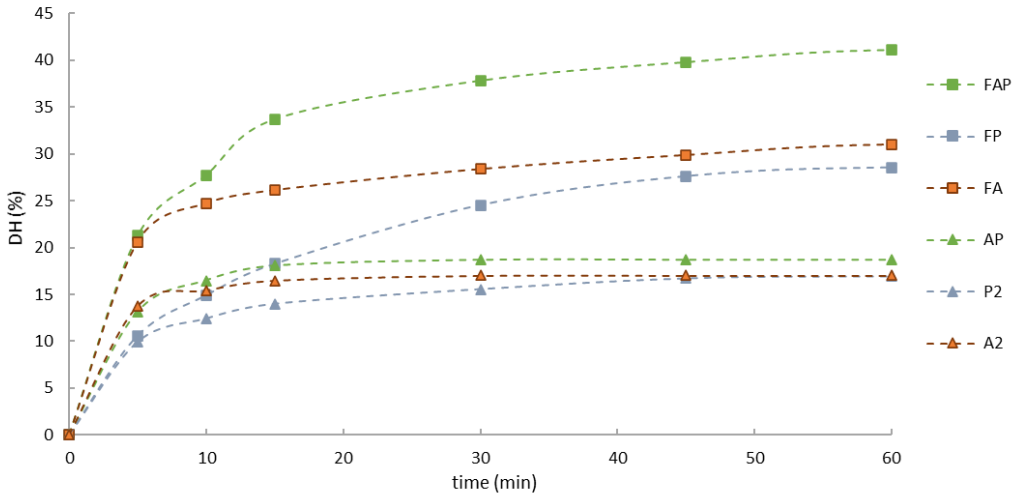

Figure 4. Hydrolysis kinetics. Evolution of the hydrolysis degree during reactions with boiled (FAP, FP, FA) and non-boiled (AP, P2, A2) mussel, with combination of enzymes, Protamex and Alcalase, respectively, 2% enzyme concentration and 25 g of mussel paste.

Other aspect worth mentioning is the continuous evolution in reactions FAP, FP and P2, unlike those carried out only with Alcalase, which stabilized earlier, around 15 to 20 min of hydrolysis, these continued to increase, showing that Protamex, though producing lower DH,

has a steady activity, whereas Alcalase seems to have high cleavage action in the beginning of the reaction which soon stabilizes. This is an important fact for reaction A2P1, where Alcalase is added at 30 min of hydrolysis, after which there is a considerable increase in NaOH consumption, the combination of a relatively steady action of Protamex through great part of the reaction and an initial high cleavage rate of Alcalase allowed it to have a continuous DH increase throughout the entire hydrolysis (Table A.II).

4.3. Bioactivity

Hydrolysates produced belonged to five different categories: lyophilized commercial mussel hydrolysates (CMHs); non-boiled mussel hydrolysates (NMHs); lyophilized non-boiled mussel hydrolysates (LNHs); boiled mussel hydrolysates (BMHs); and ultrafiltered boiled mussel hydrolysates (>3kDa). This allowed to evaluate not only hydrolysis conditions (type of enzyme, enzyme concentration, mass of substrate), but also to compare effects of the heat treatment, lyophilization process, and membrane ultrafiltration on hydrolysates bioactivity.

4.3.1. Antioxidant activity

Hydrolysates tested by DPPH method to determine their antioxidant ability (Table A.III), showed a radical scavenging activity ranging from 5.82% in C50 with non-boiled mussel, to 46.29% in J2 with commercial mussel.

Relatively to CMHs, the ones produced in reaction J2 (E/S= 2%; S= 50 g; t= 2 h), which had the lower DH (26.58%), apart from control, revealed the highest antioxidant activity (46.29%), on the other hand, reaction J1(E/S= 3; S= 25 g; t= 1 h), which had the highest DH (30.56%), produced hydrolysates with the lowest antioxidant activity (24.48%), even inferior to the hydrolysates produced in the control reaction (30.05%). In fact, Guerard *et al.*, 2007 obtained no significant differences in antioxidant activity between hydrolysates produced from control reaction and with Alcalase. There also seems to be a lack of correlation between the DH and the bioactivity expressed by respective hydrolysates.

When analysing hydrolysates produced from non-boiled mussel (Figure 5) and comparing the effect enzyme type and concentration and lyophilization had on resultant bioactivity, it

is possible to observe that little differences were obtained between hydrolysates from control reactions and those of other reactions, as commented before for CMHs.

We can also see that lyophilization decreased hydrolysates activity, instead of what was being expected. A decrease in hydrolysates bioactivity was also observed by Abdul-Hamid *et al*. (2002) and Bhaskar and Mahendrakar (2008), after dehydrating fish waste hydrolysates through spray drying, possibly due to losses during this processing, that led to a decrease in protein content. Though being a different processing treatment than lyophilization, the fact that samples were submitted to thermal treatments and dehydration may induce changes in peptides and their bioactivity, since this processing though inducing extended shelf-life and stability, as they reduce water activity, might also modify peptides, hence, altering their bioactive properties (Rivero-Pino *et al*., 2020).

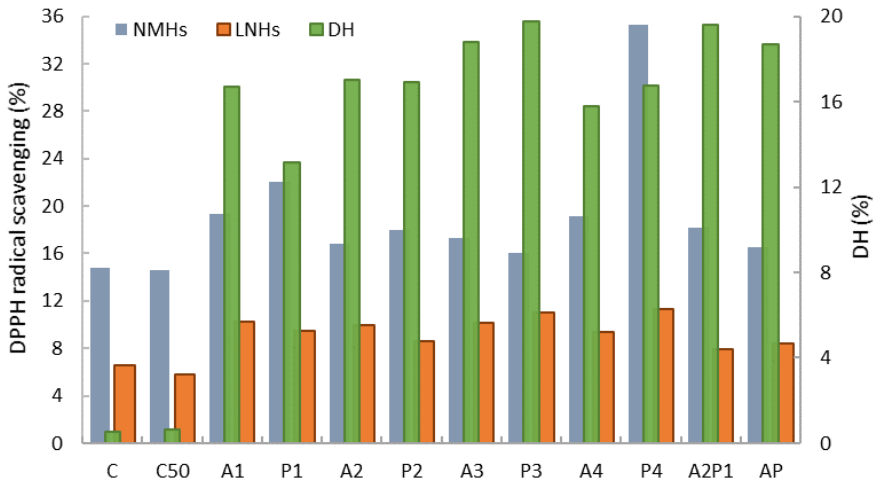

Figure 5. DPPH radical scavenging activity of non-boiled mussel hydrolysates. Comparing activity of lyophilized hydrolysates (LNHs) with liquid samples (NMHs), as well as reaction conditions (enzyme type, enzyme concentration and substrate), and the corresponding degree of hydrolysis.

In addition, highest DPPH radical scavenging activity was obtained in NMHs from reaction P4 (E/S= 2%; S= 50 g) with 35.25% activity, followed by reactions P1 (E/S= 1%; S= 25 g; DPPH= 22.09%)>A1 (E/S= 1%; S= 25 g; DPPH= 19.31%)>A4 (E/S= 2%; S=50 g; DPPH= 19.12%). Similarly, in case of lyophilised samples, LNHs from reaction P4 revealed the highest scavenging activity (11.32%), followed by P3 (E/S= 3%; S= 25 g; DPPH= 11.01%)>A1 (E/S= 1%; S= 25 g; DPPH= 10.25%)>A3 (E/S= 3%; S= 25 g; DPPH= 10.12%).

These results seem to indicate that higher substrate amounts led to higher scavenging activity, however, they appear to show an absence of correlation between enzyme type and concentration and resultant bioactivity. In previous study with mussel boiled without shells, were attained higher antioxidant activities with higher amounts of substrate (75 g and 50 g), however, no association between enzyme concentration and resultant activity could also be drawn (Ramos, 2019).

We can also observe from Figure 5 that, as seen for commercial samples (CMHs), these also lack correlation between their antioxidant activity and respective degree of hydrolysis. Which was also obtained in other studies, as from Wang *et al*. (2013) with mackerel hydrolysates with Alcalase and Protamex, or Tonon *et al.* (2016), that observed DH and antioxidant activity had opposite behaviours, higher the degree the lower would be the resultant bioactivity. And Klompong *et al.* (2007) that obtained no changes in DPPH scavenging with different DH with Flavourzyme and increase in activity with decreases in DH with Alcalase. The same absence of correlation was obtained in previous study (Ramos, 2019).

When analysing hydrolysates from boiled mussel (Figure 6), both whole hydrolysates solution (BMHs) and ultrafiltered ones (>3 kDa) reveal the same pattern, i.e., FC<FP<FA<FAP, where control reaction hydrolysates had lower activity, whereas, hydrolysates from reaction conducted with both enzymes had the highest antioxidant activity, with 30.53% and 27.14%, for BMHs and >3 kDa fraction, respectively, followed by Alcalase hydrolysates, with higher scavenging activity than Protamex ones. Justus *et al.* (2019) also obtained higher DPPH scavenging activity with multiple enzyme hydrolysis.

In addition, BMHs seem to possess higher levels of activity when compared to filtered ones. This purification step does not always guarantee increase in bioactivity (Aluko, 2017), some hydrolysates mixtures have even shown to possess higher bioactivity than purified peptides (Grienke *et al*., 2014). In this case, where hydrolysates fraction used presents a molecular weight >3 kDa, in terms of antioxidant activity, low molecular weight fractions, usually <3 kDa (Jun *et al.*, 2004; Mendis *et al*., 2005; Rajapakse *et al.*, 2005) and even <1 kDa (Dong *et al.*, 2008; Ryan *et al.*, 2011), have shown to express higher activity. Wang *et al.* (2013) showed blue mussel hydrolysates from the whole mixture to possess higher DPPH radical scavenging activity than >3 kDa hydrolysates, being the ones with the highest activity from

the <3 kDa fraction. Therefore, this ultrafiltration process may have led to the loss of potentially antioxidant peptides.

When comparing bioactivity results to the respective DH, unlike previous samples, these seem to express a correlation between antioxidant activity and the respective DH.

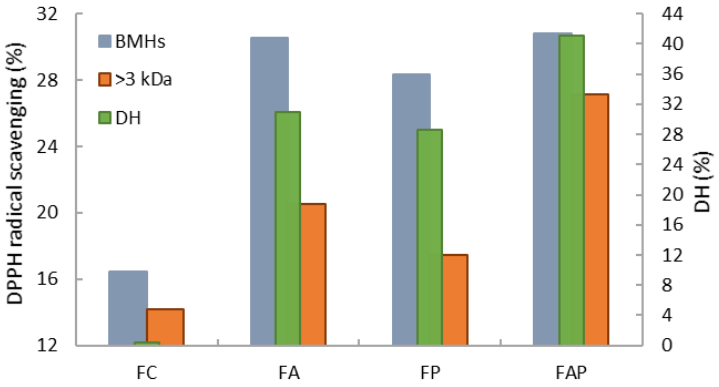

Figure 6. DPPH radical scavenging ability of boiled mussel samples, both whole (BMHs) and ultrafiltered (>3 kDa) fractions. Analysing the effect membrane ultrafiltration, catalytic enzymes and the DH exert on the expressed activity.

Unlike with commercial samples (CMHs), the remaining hydrolysates revealed the lowest activity to belong to control reactions. Boiled mussel hydrolysates revealed a correlation between their antioxidant activity and respective DH, however, the same failed to occur for commercial and non-boiled mussel hydrolysates.

With the exposed, it is possible to say that hydrolysates produced in tested conditions revealed low antioxidant potential. Which can also be confirmed by Figure 7, a model of how microplates looked after 20 to 120 min of incubation, in this case with >3 kDa hydrolysates, showing a very subtle difference in colour change, corresponding to low DPPH reduction. Other observed aspect is the close resemblance of control reaction (FC) wells to the blank (B) ones, since these hydrolysates were the ones with inferior antioxidant activity.

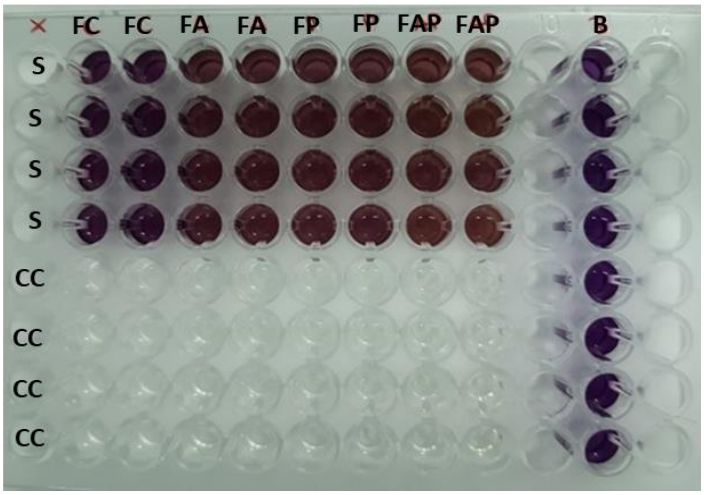

Figure 7. DPPH radical scavenging reaction in microplate. Antioxidant activity of >3 kDa hydrolysates, after 120 min contact with DPPH. Microplate containing wells with blank (B), colour controls (CC) and samples (S).

4.3.2. Antimicrobial activity

As for the antimicrobial activity of the resultant hydrolysates, there were no positive results for the tested conditions in both disk and well diffusion methods. Hydrolysates showed no capacity to inhibit growth of the three tested bacterial species, no inhibition zone was observed, apart from the halo resultant from the positive controls with G10 paper discs as it is shown in Figure 8, in this case with >3 kDa hydrolysates fraction, against *B. cereus*.

According to CLSI guidelines, a bacterium can be classified as resistant, intermediate, or susceptible to an antimicrobial substance according to the formed halo size. In this present work, inhibition zones formed for G10 positive controls were compared to the CLSI standards (Hudzicki, 2009) (Table A.IV). For *E. coli* was obtained a 14 mm diameter halo, corresponding to an intermediate resistance, as for *S. aureus* it was formed a 12 mm halo, corresponding to a resistant strain.

Hydrolysates obtained in previous study, with mussel boiled without shell, generated inhibition zones against *B. cereus* and *S. aureus*, with halos of 17 mm and 20 mm diameter, respectively. As for antibiotic controls, halo dimensions against same bacterial species differed, *E. coli* was found to have intermediate resistance as well, but *S. aureus* on the other hand, was considered susceptible. Comparing this antimicrobial activity with the respective DH, no correlation was observed (Ramos, 2019).

Though same species have been used in both studies, discrepancies in results obtained may be a result of different mussel sampling, as they were harvested with almost a year apart, thus collected samples contained a different mixture of mussels at various life cycles and chemical compositions, thus altering resultant hydrolysates bioactive potential, further affected by pre-thermal-treatment, hydrolysis conditions and hydrolysates processing.

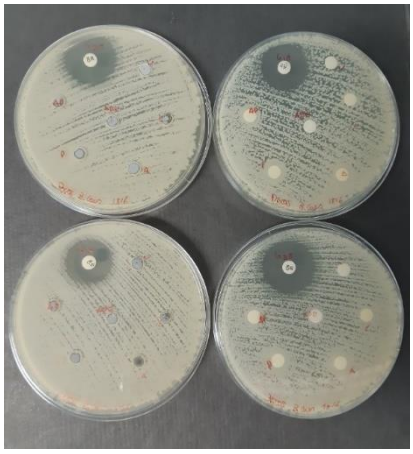

Figure 8. Antimicrobial activity test by well (left) and disk (right) diffusion methods, using >3 kDa hydrolysates fraction against *B. cereus*. Observed halos correspond to the positive control discs with gentamicin (G10).

These results seem to indicate that tested hydrolysis conditions and any hydrolysates processing performed, generated hydrolysates with no antimicrobial activity against these bacterial species. Several studies have also reported the occurrence of higher antimicrobial activity in hydrolysates with molecular weight <1 kDa (Fernandes *et al.*, 2015; Wang *et al.*, 2018).

In addition, mussels have been reported to be rich in AMPs, non-hydrolysed peptides naturally present in these organisms, thus, some believe there is no need for all this processing to break down larger proteins to obtain peptides with antimicrobial capacity (Grienke *et al*., 2014), hence, majority of papers reporting the existence of antimicrobial activity in mussels were carried out using solvent extraction to obtain these AMPs from mussel's haemolymph (Jayaprakash and Perera, 2020).

4.3.3. Antihypertensive activity

To test for antihypertensive activity, only boiled mussel hydrolysates were used. This assay was carried out with both BMHs and >3 kDa serial diluted (1:4 to 1:128) fractions.

Results obtained seem to indicate that the produced hydrolysates possess high ability to inactivate ACE (Table A.V), with values rounding 100% of inhibition for dilutions 1:4 and 1:8. High iACE% was also observed in green-lipped mussel pepsin hydrolysis extracts, that revealed an ACE inhibitory activity of approximately 90% (Jayaprakash and Perera, 2020) and in blue mussel Alcalase hydrolysates, with iACE% of approximately 80% (Dai *et al.*, 2012).

Hence hydrolysates from 1:128 diluted fraction were selected to analyse enzyme and ultrafiltration effect on resultant activity and are expressed in Figure 9.

As it is visible, >3 kDa hydrolysates showed higher levels of ACE inactivation. Though this purified fraction, containing the highest molecular weight hydrolysates, had showed to possess a superior iACE% than BMHs, most papers have reported an association of higher antihypertensive activity to low molecular weight hydrolysates fractions (Ryan *et al.*, 2011).

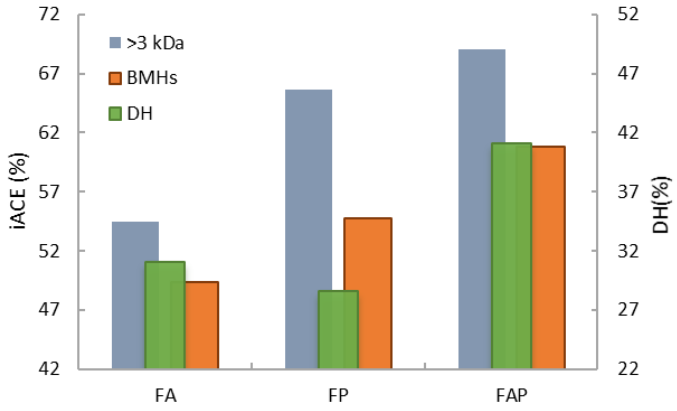

Figure 9. Antihypertensive activity of boiled mussel hydrolysates. Whole (BMHs) and ultrafiltered (>3 kDa) fractions ability to inactivate ACE. Comparing the effect of membrane ultrafiltration, enzymes (Alcalase, Protamex and combination) on resultant hydrolysates bioactivity, and corresponding hydrolysis degree (DH).

We can also observe that hydrolysates from the enzymatic combined reaction (FAP) revealed higher iACE%, and the ones produced simply with Alcalase (FA) had the lowest levels of activity, being this observed for both the filtered (>3 kDa) and non-filtered (BMHs)

samples. Dai *et al.* (2012), on the other hand, obtained a higher antihypertensive activity for Alcalase hydrolysates rather than for Protamex.

As for control reaction (FC) hydrolysates, ACE inhibition activity was very low, and not detected for the highest dilutions (Table A.V).

When comparing these results to the corresponding degree of hydrolysis we can observe, once again, the lack of correlation between both parameters. Dai *et al.* (2012) observed the same with his blue mussel hydrolysates, where their antihypertensive potential would decrease with increases in DH.

CHAPTER 5

5. Conclusions

Projects like ValorMar and related studies are gaining notoriety, not just in academia, but in many food and fish-seafood industries, due to the amounts of waste and by-products generated, that, not only represent economic lost, as they also induce great environmental problems. Thus, recognition of their nutritious and bioactive potential, and as valuable sources of potent compounds, with ability to be introduced in the manufacture of functional foods, that can exert specific beneficial effects on human body and prevent their waste, is of outmost importance.

From this work was possible to redraw some conclusions as to the seemingly favourable hydrolysis conditions for the attainment of a higher DH, even though this parameter does not seem to have a correlation with the bioactive ability of the resultant hydrolysates. The same way, it was possible to assume which are the better conditions, among the tested, that produce hydrolysates with greater biological activity.

The pre-thermal-treatment enhances protein content and enzyme/substrate interactions, thus producing higher DH. As for hydrolysis conditions, an exogenous enzyme activity is a more profitable approach to autolytic hydrolysis, in terms of reaction control and resultant bioactivity. Combination of relatively smaller quantities of substrate and higher enzyme concentrations produce higher DH. An extended reaction time is not productive, as it may negatively affect peptides, in addition enzymatic activity starts to stabilize, so, more time-consuming reactions with possible deleterious effects on bioactive potential for a slightly higher DH is not favourable.

In general, combination of enzymes generated high bioactivity, especially ACE inhibition ability and the use of higher amounts of substrate seemed to induce higher antioxidant activity. Sample purification through membrane ultrafiltration produced hydrolysates with molecular weight >3 kDa with high antihypertensive activity, comparatively to the whole hydrolysate's mixture, however, the same was not verified for the DPPH radical scavenging activity, as the latter has been associated to peptides with molecular weight <1 kDa. Thus, it would have been beneficial to study the bioactive behaviour of the <3 kDa permeate and

even fractions from smaller MWCO. It would also be important to test antioxidant activity using additional methods to DPPH radical scavenging.

Having said this, it is important to continue this work, looking to improve the required techniques, as well as to establish which hydrolysis conditions contribute to produce hydrolysates with good bioactive potential to be applied in food, cosmetic and pharmaceutical industries. This way providing a solution to the aquaculture generated wastes and creating novel and enriched products with great benefits for human health and wellbeing.

It would be interesting to widen hydrolysis conditions to be tested, as well as other potential bioactivities, to gather more intel on this species bioactive capacity and potentiality as a valuable source of compounds to be introduced in the making of new functional foods.

It is also important to note that, to exert their activity, these peptides need to remain active after food processing, storage, and digestion, thus, further studies concerning processing effect of different foods containing bioactive peptides is important, as well as peptides ability to exert their function on the target sites.

It was observed among all the studies developed, that there is a great diversity of protocols to obtain and purify bioactive peptides, hampering interpretation and comparison of results. Even with the same starting material/by-product, there is variability in terms of sampling sites and growth environmental conditions that introduces variables prior to any pre-treatment, extraction and purification method applied. However, is this factor that generates many possibilities and widens research perspectives and allows this field of research to be so rich and interesting.

CHAPTER 6

6. References

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ANNEX

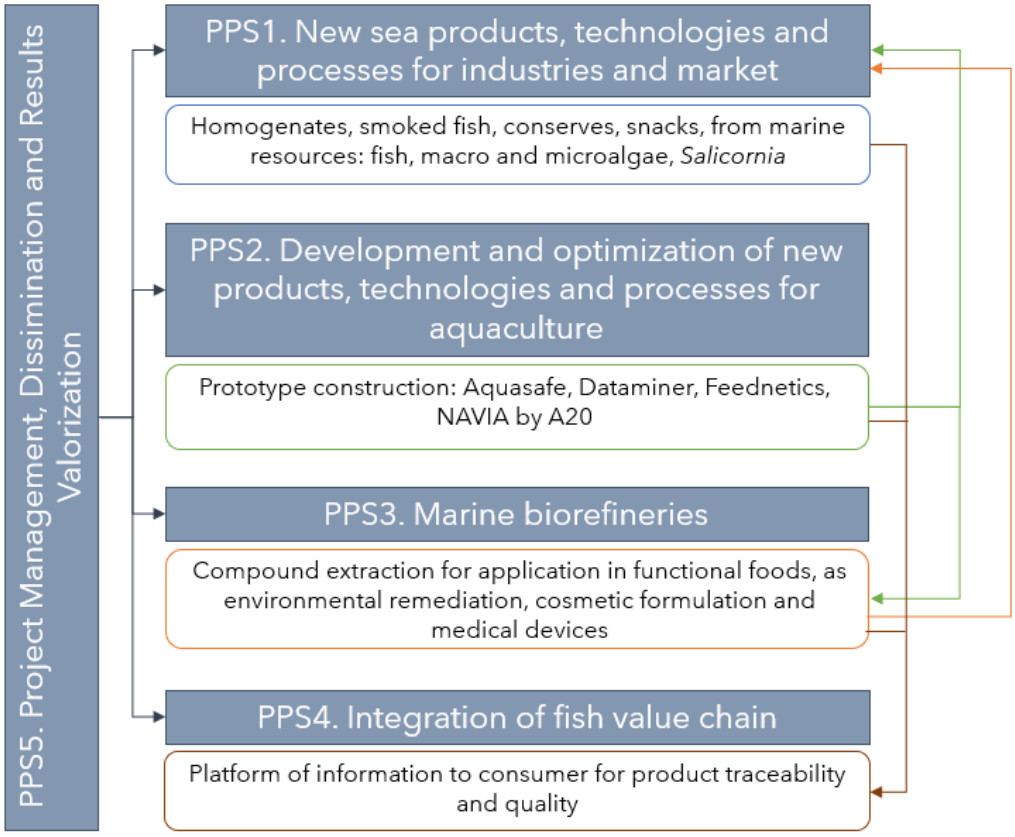

Figure A.I. Articulation of the five PPS of the ValorMar project. Adapted from the technical annex of the project application.

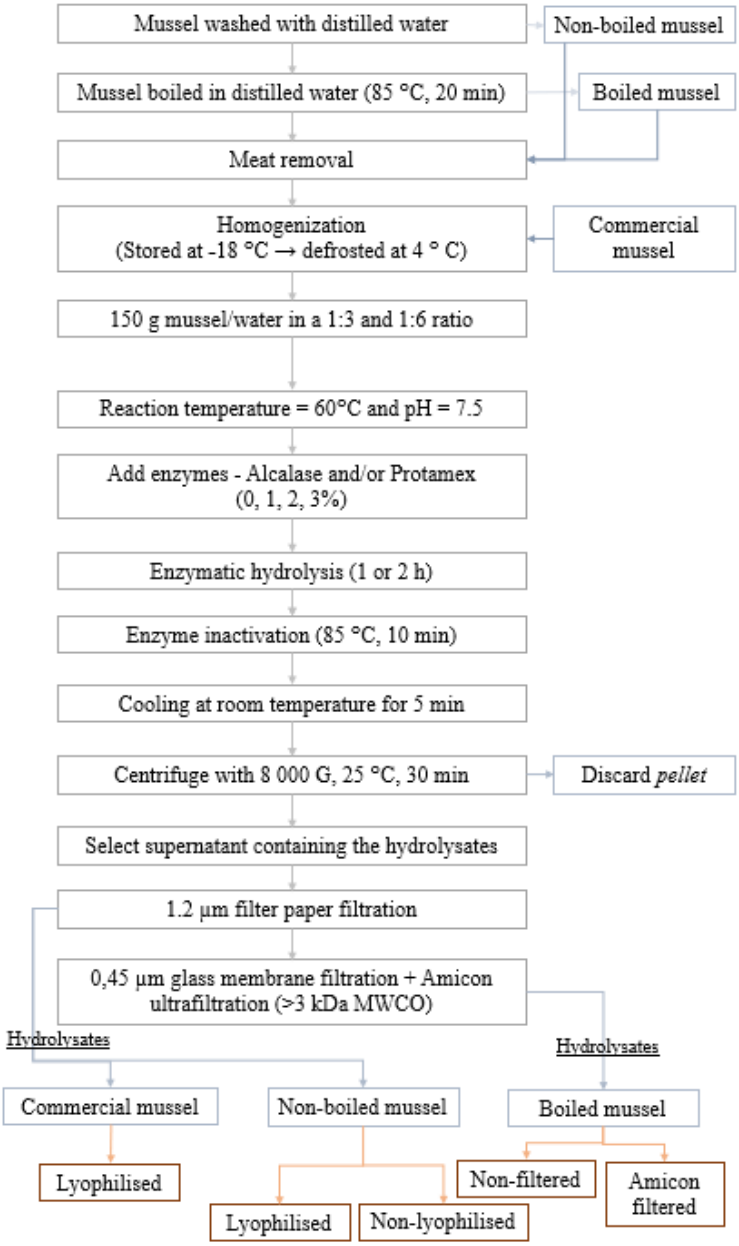

Figure A.II. Flowchart for obtaining hydrolysates. Sample preparation, hydrolysis, and hydrolysates extraction and processing.

Table A.I. Chemical composition of commercial and non-boiled mussel.

Mussel	Nutritional component (%)				
	Lipids	**Carbohydrates**	**Ash**	**Humidity**	**Protein**
Commercial	1.68 ± 0.02	3.42 ± 0.67	3.35 ± 0.01	78.51 ± 0.12	13.04 ± 0.59
Non-boiled	1.58 ± 0.05	2.48 ± 0.24	1.09 ± 0.10	82.62 ± 0.23	12.24 ± 0.15

* Boiled mussel samples were only submitted to protein content determination.

Table A.II. Evolution of hydrolysis degree. Sum of DH values acquired throughout reaction, for each hydrolysis performed.

Assay Code	Sample	Enzyme	E/S (%)	Substrate (g)	time (h)	DH (%)								Total DH (%)
						5	10	15	30	45	60	90	120	
JC	Commercial	-	0	25	1	0,50	0,50	0,50	0,50	0,50	0,50	-	-	0,50
J1		Alcalase	3	25	1	19,31	25,18	27,17	29,07	30,06	30,56	-	-	30,56
J2			2	50	2	14,83	18,56	20,56	23,34	24,59	25,48	26,58	26,58	26,58
J3			3	25	2	17,42	21,40	25,68	26,68	26,88	27,67	27,97	27,97	27,97
C25	Non-boiled	-	0	25	1	0,00	0,00	0,00	0,52	0,52	0,52	-	-	0,52
C50		-	0	50	1	0,00	0,00	0,00	0,37	0,63	0,63	-	-	0,63
A1		Alcalase	1	25	1	11,70	14,10	15,87	16,71	16,71	16,71	-	-	16,71
A2			2	25	1	13,79	15,46	16,50	17,02	17,02	17,02	-	-	17,02
A3			3	25	1	12,85	16,81	17,75	18,80	18,80	18,80	-	-	18,80
A4			2	50	1	11,49	13,89	14,93	15,77	15,77	15,77	-	-	15,77
P1		Protamex	1	25	1	6,16	7,94	9,29	12,01	12,64	13,16	-	-	13,16
P2			2	25	1	9,92	12,43	13,99	15,56	16,71	16,92	-	-	16,92
P3			3	25	1	11,70	15,67	18,49	18,90	19,74	19,74	-	-	19,74
P4			2	50	1	7,15	10,34	12,85	15,72	16,24	16,76	-	-	16,76
A2P1		Combination	2 (1->1)	25	1	8,88	11,38	12,32	14,41	18,49	19,63	-	-	19,63
AP			2 (1+1)	25	1	13,16	16,50	18,07	18,69	18,69	18,69	-	-	18,69
FC	Boiled	-	0	25	1	0,00	0,00	0,09	0,37	0,47	0,47	-	-	0,47
FA		Alcalase	2	25	1	20,61	24,73	26,14	28,39	29,89	31,01	-	-	31,01
FP		Protamex	2	25	1	10,59	14,90	18,27	24,55	27,64	28,57	-	-	28,57
FAP		Combination	2 (1+1)	25	1	21,27	27,73	33,73	37,85	39,82	41,13	-	-	41,13

Table A.III. Antioxidant activity expressed as DPPH radical scavenging ability of each hydrolysate, after 20 min of exposure to DPPH (values presented for the boiled mussel hydrolysates corresponded to readings measured after 60 min of incubation with DPPH).

Assay Code	Sample	Enzyme	E/S (%)	Substrate (g)	time (h)	DH (%)	DPPH (%)	
JC	Commercial	-	0	25	1	0,50	30,05	
J1		Alcalase	3	25	1	30,56	24,48	
J2			2	50	2	26,58	46,29	
J3			3	25	2	27,97	27,93	
C25	Non-boiled	-	0	25	1	0,52	14,79	6,53*
C50		-	0	50	1	0,63	14,60	5,82*
A1		Alcalase	1	25	1	16,71	19,31	10,25*
A2			2	25	1	17,02	16,86	9,95*
A3			3	25	1	18,80	17,35	10,12*
A4			2	50	1	15,77	19,12	9,38*
P1		Protamex	1	25	1	13,16	22,09	9,45*
P2			2	25	1	16,92	17,98	8,61*
P3			3	25	1	19,74	16,03	11,01*
P4			2	50	1	16,76	35,25	11,32*
A2P1		Combination	2 (->1)	25	1	19,63	18,18	7,92*
AP			2 (1+1)	25	1	18,69	16,53	8,45*
FC	Boiled	-	0	25	1	0,47	16,43	14,16**
FA		Alcalase	2	25	1	31,01	30,53	20,50**
FP		Protamex	2	25	1	28,57	28,32	17,43**
FAP		Combination	2 (1+1)	25	1	41,13	30,79	27,14**

* lyophilised samples (LNHs)

** ultrafiltered samples (>3 kDa)

Table A.IV. Inhibition zone diameters (mm) formed by Gentamicin (G10) impregnated discs against the three tested bacterial species, as control positive.

Antibiotic, µg	Bacterial species		
	E. coli	***S. aureus***	***B. cereus***
	diameter (mm)		
Gentamicin, 10	14	12	14

Table A.V. Antihypertensive activity of boiled mussel hydrolysates from whole non-filtered (-) and ultrafiltered (+) hydrolysates. Results are expressed as hydrolysates ability to inhibit ACE enzyme (iACE%).

Assay Code	Membrane Ultrafiltration	Dilutions					
		4	**8**	**16**	**32**	**64**	**128**
FC	+	22,11	4,73	N/D	N/D	N/D	N/D
	-	5,80	N/D	N/D	N/D	N/D	N/D
FA	+	106,32	99,06	87,23	79,69	73,91	54,47
	-	103,76	95,52	80,95	75,92	66,25	49,37
FP	+	109,17	97,34	88,71	80,72	77,63	65,59
	-	109,55	105,07	82,72	79,03	70,64	54,78
FAP	+	109,30	98,88	86,06	85,44	78,40	69,08
	-	105,30	96,72	81,09	78,14	71,65	60,85

* N/D (not detected)