**Anti-inflammatory, antioxidant and antimicrobial effects of underutilized fish
protein hydrolysate**

**Meritaine da Rocha^{a,*}, Ailén Alemán^b, Gyselle Chrystina Bacca^c, M. Elvira
López-Caballero^b, M. Carmen Gómez-Guillén^b, Pilar Montero^b, Carlos Prentice^a.**

^aSchool of Chemistry and Food, Federal University of Rio Grande (FURG), Rio
Grande, Brazil.

^bInstitute of Food Science, Technology and Nutrition (ICTAN-CSIC), Madrid, Spain.

^cInstitute of Health Sciences, Federal University of Bahia, Salvador, Brazil,

*Corresponding author. Tel.: +55-53-32336974; Fax: +55-53-3233-6869; *E-mail
address: Meritaine da Rocha (meritaine@gmail.com)

Abstract

Antimicrobial, anti-inflammatory antioxidant activities of protein hydrolysates from Argentine croaker (*Umbrina canosai*) isolated (CPI) or Argentine croaker myofibrillar protein (CMP) with different degrees of hydrolysis (DH: 10–20%) prepared using Alcalase or Protamex were determined. Results showed that an increase in the DH resulted in higher content of hydrophobic and aromatic amino acids and in a decrease in molecular weight (MW) distribution for all hydrolysates obtained. Furthermore, the enzyme and raw material used influenced the amino acid content and MW determined. Hydrolysates from CPI with a 20% DH by Alcalase had higher ABTS radical scavenging activity, metal chelation and ferric reducing antioxidant power-FRAP ($p < 0.05$). All hydrolysates samples decrease the pro-inflammatory capacity. In all the evaluated microorganisms, only 7 were inhibited, most being Gram-positive. Alcalase was found to exert a considerable influence on antibacterial activity. These hydrolysates are an alternative as natural antimicrobials, anti-inflammatory and antioxidants compounds.

Keywords: Fish protein, enzyme, degrees of hydrolysis, RAW 264.7, inhibition zone, health.

Introduction

Lipid oxidation and microbial growth causes of deterioration in food, leading to loss of nutritional value and potentially toxic product formation. Furthermore, these effects are associated with many diseases, causing health problems (Chalamaiah, Dinesh Kumar, Hemalatha, & Jyothirmayi, 2012; Najafian and Babji, 2012). The uncontrolled use of antimicrobials, has led to a rapid increase in resistant bacteria, and the growing demand for innocuous and natural antimicrobials in food preservation has resulted in

research into novel antimicrobial agents (Najafian and Babji, 2012; Robert et al., 2015; Wald, Schwarz, Rehbein, Bußmann, & Beermann, 2016). Reactive oxygen species (ROS) and free radicals causing the oxidation of fats and oils in food products and are regarded as one of factors in many diseases, such as cancer, diabetes, hypertension, neurodegenerative disorders and aging problems. These compounds has unpaired electrons in the valence shell and attract electrons from other substances, causing oxidative stress in the tissues or cells (Chalamaiah et al., 2012; Chi, Hu, Wang, Li, & Ding, 2015; Ngo, Qian, Ryu, Park, & Kim, 2010; Raghavan and Kristinsson, 2009; Sarmadi and Ismail, 2010; Sila et al., 2015). Several commercial antioxidants exhibit strong antioxidant activity, but their use is regulated because the possibility of health problems. In the past few years, there is a growing interest in studies to identify natural antioxidants from different sources (Ktari et al., 2014; Zeng, Zhang, He, & Shi, 2015).

Several researches have reported the use of hydrolysates with antioxidant activities whereas other bioactive properties such as anti-inflammatory are less studied. The inflammation is an important normal immune response during lesions and infections, it is modulated by matrix of inflammatory mediators and cytokines that are released by activated macrophages (Ahn, Cho, & Je, 2015; Zhou et al., 2008). The study of inflammation *in vitro* is the use of macrophages, that can be activated by lipopolysaccharide (LPS), resulting in the production of different pro-inflammatory inducible nitric oxide synthase (NOS) (Ahn et al., 2015; Dia, Bringe, & De Mejia, 2014). Uncontrolled production of pro-inflammatory compounds such as cytokines can contribute to several acute and chronic diseases (Millán-Linares, Bermúdez, Yust, Millán, & Pedroche, 2014). The control of excessive inflammation is important to maintain health and actually many researches with protein hydrolysate (Ahn et al., 2015; Dia et al., 2014; Millán-Linares et al., 2014) has been develop an alternative for this control. Several biotechniques have been developed to recover bioactive

compounds, such as hydrolysate or peptides, that would help improve human health by providing protection from several diseases, and prolonging food shelf-life. (Memarpoor-Yazdi, Asoodeh, & Chamani, 2012; Millán-Linares et al., 2014; Silva, Aline, & Prentice, 2014).

The peptides are inactive within the sequences of the proteins, but might be released by enzymatic hydrolysis and may exert an infinity of bioactivities, such as antimicrobial, antioxidant, antidiabetic, anti-inflammatory and antihypertensive. (Maturana et al., 2017; Millán-Linares et al., 2014; Nongonierma, Mazzocchi, Paoletta, & FitzGerald, 2017; Raghavan and Kristinsson, 2009; Sila et al., 2015). These properties results from amino acid and peptide composition, degree of hydrolysis and molecular weight profile of protein hydrolysates (Chalamaiah et al., 2012; Najafian and Babji, 2012). Presently, hydrolysates or peptides with antioxidant, anti-inflammatory and antimicrobial activities have been isolated and identified from marine sources such as rainbow trout by-products (Wald et al., 2016), salmon pectoral (Ahn et al., 2015), brownstripe red snapper muscle (Khantaphant, Benjakul, & Kishimura, 2011) and others. According to Lempek, Martins and Prentice (2007), the Argentine croaker (*Umbrina canosai*) is a fish species caught in large amounts in the Southern Brazil coast. However, this species is underutilized and has low commercial value, thus attracts the interest to obtain products with high added value such as protein hydrolysates. However, there is slight information about the antioxidant activity of muscle protein hydrolysates of Argentine croaker and no paper was found on the antimicrobial and anti-inflammatory activities of hydrolysates from this species. Furthermore, the antimicrobial and anti-inflammatory activities from fish were rarely reported. According to Raghavan and Kristinsson (2009), the hydrolysates from muscle protein sources can have several pro-oxidants such as heme and unstable oxidised lipid substrates. These compounds may decrease the stability of protein hydrolysates and

affect their bioactivity. An alternative to remove these compounds is isoelectric precipitation method and the washing process for the removal of the unstable lipid substrates (Limpan, Prodpran, Benjakul, & Prasarnpran, 2010; Nolsøe and Undeland, 2009). Thus, the objectives of this study were: (i) to obtain protein hydrolysates recovered from Argentine croaker muscle proteins by alkali-solubilisation method or by successive washings with different degrees of hydrolysis using the commercial proteases Alcalase and Protamex and (ii) to evaluate the *in vitro* antimicrobial, anti-inflammatory and antioxidant activities of the different hydrolysates obtained.

Materials and Methods

Chemicals

Commercial proteases Alcalase[®] (2.4 AU/g) was provided by Novozymes Latin America (Araucária, Paraná, Brazil) and Protamex[®] (1.5 AU/g) was purchased from Sigma–Aldrich. Other chemicals and reagents used were of analytical grade.

Materials

Argentine croaker protein isolate (CPI) was obtained using pH-shifting process, according to Undeland., Kelleher and Hultin (2002) with some modifications. The CPI was obtained from the homogenisation of Argentine croaker muscle –ACM (from a fish processing company from the city of Rio Grande, Southern Brazil) with distilled water (1:9, w/v) adjusting the pH to 11.2 with 1 M NaOH, at 4 °C for 20 min. After solubilisation, the sample was centrifuged at 14.308 $\times$ g (Hanil, Supra 22 K, Korea) for 20 min at 4 °C. The soluble proteins were subjected to isoelectric precipitation carried out at pH 5.0 (1 M HCl) for 20 min at 4 °C and centrifuged at 14.308 $\times$ g at 4 °C for 20 min. The croaker myofibrillar protein (CMP) was obtained by washing process in

cold distilled water and cold NaCl (50 mM) according to Limpan et al. (2010). The CPI and CMP obtained were lyophilized (Liotop, L108, São Carlos, Brazil), ground in a knife-mill (Tecnal, TE-633, Piracicaba, Brazil), sieved through a N° 42 mesh (0.35 mm) and stored at - 18 °C. The CPI and CMP were analyzed in triplicate to determine the protein and lipids content, using classical method by AOAC (2000) and showed a protein content of 92.1% and 88.7% and lipids content of 1.2% and 3.9%, respectively.

Preparation of protein hydrolysate

Hydrolysates were obtained according to Raghavan and Kristinsson (2009) by enzymatic hydrolysis using two different enzymes, Alcalase and Protamex. The optimum hydrolysis conditions used for these enzymes were as follows: Alcalase (pH 8 and 50°C) and Protamex (pH 7.0 and 50 °C). CPI or CMP (2.0%, w/v) were dissolved in distilled water and homogenised at a speed of 300 rpm for 1 min. The dispersion was equilibrated to the optimum pH and temperature conditions corresponding to the enzyme used for hydrolysis. The hydrolysis reaction was started by the addition of the enzyme at 30 U/g (enzyme/protein ratio) according to protease activity of Alcalase (13.3 U/mg protein) and Protamex (14 U/mg protein). The DH of proteins was determined by assay according to the pH-stat method (Adler-Nissen, 1986) at achieve a 10 and 20% DH. After the desired DH was reached, the enzymes were rendered inactive by heating at 90 °C for 10 min. The samples were then centrifuged at 9000 rpm (Hanil, Supra 22K, Korea) for 20 min at 4 °C. The supernatants were lyophilized and stored at -18 °C for further analysis. Eight hydrolysates were obtained 1: CPI hydrolysate sample by Alcalase with a 10% DH (CPHA1); 2: CPI hydrolysate sample by Alcalase with a 20% DH (CPHA2); 3: CPI hydrolysate sample by Alcalase with a 20% DH (CMHA1); 4: CMP hydrolysate sample by Alcalase with a 20% DH using (CMHA2); 5: CPI hydrolysate sample by Protamex with a 10% DH (CPHP1); 6: CPI hydrolysate sample

by Protamex with a 20% DH (CPHP2); 7: CMP hydrolysate sample by Protamex with a 10% DH (CMHP1); 8: CMP hydrolysate sample by Protamex with a 20% DH (CMHP2).

Amino acid composition

The hydrolysate (5 mg/mL) was homogenised in distilled water and 20 µL of sample was dried and hydrolysed in vacuum-sealed glass tubes at 110 °C for 24 h in the presence of 6 N HCl with 0.1% phenol using norleucine as internal standard. The samples were again vacuum-dried, dissolved in application buffer, and injected in an amino acid analyzer (Biochrom 20, Pharmacia, Barcelona, Spain) as standard a mixture of amino acids was used. The determinations were performed in triplicate.

Molecular weight distribution

Molecular weight (MW) distribution of different hydrolysates was determined according to Sila et al. (2015) by size exclusion - HPLC (SPE-MA10AVP, Shimadzu, Kyoto, Japan). The injection volume was 10 µL of hydrolysate was loaded into a Superdex Peptide PC 3.2/30 column (GE Health-care Bio-Sciences, Barcelona, Spain), with a 100 - 7000 Da separation range, at a flow rate of 2.5 µL/min using acetonitrile (30%; v/v) with trifluoroacetic acid - TFA (0.01%; v/v) as mobile phase. The molecular weight standards were glycine (75 Da), hippuryl-L -histidyl-L-leucine (429 Da), vitamin B₁₂ (1340 Da), aprotinin (6512 Da) and bovine serum albumin (BSA, 6700 Da). Optical density was monitored at 214 nm.

Antioxidant properties of hydrolysates

ABTS radical scavenging activity

ABTS radical was generated by incubation of 7mM ABTS with 2.45 mM of potassium persulfate at a ratio of 1:1 (v/v) (Re et al., 1999). The working solution was prepared by dilution with distilled water to absorbance of 0.70 ± 0.02 at 734 nm. The aliquot of 20 μ L of the hydrolysate (2 mg/mL) was mixed in 980 μ L of ABTS solution. The mixture was incubated at 30 °C for 10 min in the dark and absorbance values were read at 734 nm. ABTS radical scavenging activity was expressed as mg Vitamin C Equivalent Antioxidant Capacity (VCEAC)/g of sample based on L -ascorbic acid standard curve (0.02 – 0.2 mg/mL). All determinations were performed at least in triplicate.

Ferric reducing antioxidant power (FRAP assay)

The ability of the samples to reduce ferric ion (Fe^{3+}) was evaluated by the method of Pulido et al. (2000) with some modifications. The FRAP reagent consisted of 25 ml of acetate buffer (pH 3.6), 2.5 ml of TPTZ (2,4,6-tripyridyl-s-triazine) (10 mM) in HCl (40 mM) and 2.5 ml of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (20 mM) (St. Louis MO, USA). An aliquot of 30 μ L of hydrolysate dissolved in distilled water (20 mg/mL) was incubated with 90 μ L of distilled water and 900 μ L of FRAP reagent at 37 °C for 30 min. Absorbance was read at 595 nm and FRAP was calculated from the $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ standard curve (0.1 – 0.8 μ M). The results were expressed as μ mol $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ equivalents/g sample. All determinations were performed at least in triplicate.

Metal chelating activity

The Fe^{2+} chelating activity of hydrolysates at 5, 25 and 50 mg/mL assay concentration was determined by method of Chung et al. (2002) and Giménez, Alemán, Montero, and Gómez-Guillén (2009) with some modifications. The sample (800 μ L) was mixed with 10 μ L of FeCl_2 (2mM) and 20 μ L of ferrozine (5mM). The mixture was

vortexed and incubated at room temperature for 10 min prior to evaluation of the absorbance at 562 nm. As control, a mixture composed by 800 μ L of water, 10 μ L of FeCl_2 (2 mM) and 20 μ L of ferrozine (5 mM) was used. All determinations were performed at least in triplicate.

DPPH radical scavenging assay

The DPPH scavenging activity at 2.5, 5.0 and 10 mg/mL assay concentration was measured according to the method described by Ye and Hsieh (1995) and Memarpour-Yazdi et al. (2012). Briefly, 200 μ L of hydrolysate was homogenised with 600 μ L of methanol and 200 μ L of DPPH (0.15 mM) for 2 min. This was left in the dark at room temperature for 30 min. The absorbance of the dispersion was determined at 517nm. The control sample was composed of 200 μ L of distilled water, 600 μ L of methanol, 200 μ L of DPPH (0.15 mM). All determinations were performed at least in triplicate.

Anti-inflammatory properties of hydrolysates

The anti-inflammatory activity of the hydrolysates was evaluated according to Greiss Reagent System by nitrite (NO_2^-) determination using Griess's reagent (1% sulfanilamide and 0.1% N-1-(naphthyl) ethylenediamine-diHCl in 2.5% H_3PO_4 - Promega, G2030, Madison, USA). The RAW 264.7 macrophages cell lines obtained from the American Type Culture Collection (ATCC TIB-71) were cultured in plastic culture flask in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin under 5% CO_2 at 37 °C. Thus, was added 15 mL of DEMEN in red phenol into plastic culture flask. An aliquots of 200 μ L of them and 50 μ L of each samples (at concentrations in each well were 5.00 and 1.25 mg/mL) in phosphate saline buffer (0.1 M; pH 7) were added to each well followed by

cell activation with lipopolysaccharide (LPS, 10 µg/mL) incorporation. The measure of NO_2^- was evaluated after 24 h of incubation. Approximately 50 µl of the samples supernatant were added in 50 µL of sulfonamide solution (1% of sulfanilamide in 5% phosphoric acid) and incubated in the dark for 10 min. Then, an aliquot of 50 µL of NED Solution (0.1%, N-1-naphthylethylenediamine dihydrochloride) was added to all wells and incubated at room temperature for 10 min, protected from light. A negative control with ethanol and a positive control were used. The absorbance was measured within 30 minutes in a plate reader with a filter between 520 and 550 nm and the results were expressed as NO_2^- (µM).

Antimicrobial properties of hydrolysate

The antimicrobial activity of hydrolysate samples was determined by the inhibition zone test method as described by Gómez-Guillén et al. (2010) with some modifications. The bacterial strains, culture media and incubation conditions used for determination of antibacterial activity are showed in Table 1. The antimicrobial activity measurement spread plates, were inoculated with 100 µL of these microorganisms grown overnight ($\sim 10^6$ CFU/mL). Sterile filter paper disks (0.5 cm diameter) soaked with 40 µl of hydrolysate in different concentrations (1.25; 2.50; 5.00 and 7.50 mg/mL) were put onto the plate surface and incubated in the conditions described in Table 1 for 24 h. As control, Polylysine and Nisin (Nisaplin[®], Danisco, Denmark) were used. The antibacterial activity was measured as the diameter of the clear zone around the disc in CorelDRAW Graphics Suite X6 software. All determinations were performed at least in triplicate and the results were expressed in cm.

Statistical analysis

Except for the molecular weight distribution, all determinations were performed in triplicate. Means were compared by Tukey's test at 5% level of significance by analysis of variance (ANOVA) using the software Statistica (Version 5.0, by StatSoft, Inc., Tulsa, USA).

Results and discussion

Amino acid composition

The amino acid compositions of the hydrolysates obtained are shown in Table 2. All hydrolysates contained glutamic acid, aspartic acid, lysine, leucine, arginine and alanine as the major amino acids present. Similar to this study, the major amino acids present in brownstripe red snapper muscle (*Lutjanus vitta*) hydrolysates (Khantaphant et al., 2011) were glutamic acid, aspartic acid, alanine, lysine and leucine.

According to Nasri et al. (2013), fish protein hydrolysates present variation in their amino acids composition that depends on several factors such as enzyme source, fish species and hydrolysis conditions. In this study, an increase from 10 to 20% in DH results in an improve in the content of hydrophobic amino acid - HAA ($p < 0.05$). According to Panyam and Kilara (1996), this occurs because the hydrolysis reaction results in exposure of hydrophobic groups which were protected in the original structure of the protein. The hydrolyzed samples 1, 2, 3 and 4 of CPI or CMP by Alcalase with 10 or 20% of DH showed a higher HAA content of 363.91; 365.44; 361.87 and 364.24 mg/g protein than those prepared from Protamex, respectively. Alcalase has been reported to preferably hydrolyse hydrophobic amino acid residues (Intarasirisawat, Benjakul, Wu, & Visessanguan, 2013; Wiriyaphan, Chitsomboon, & Yongsawadigul, 2012). The HAA of hydrolysate can improve their interaction with lipid targets or entry into target organs through hydrophobic associations, which is favorable for antioxidant

and antimicrobial properties (He, Girgih, Malomo, Ju, & Aluko, 2013; Sarmadi and Ismail, 2010).

According to Najafian and Babji (2012), the protein hydrolysates with antimicrobial activity generally have a cationic amino acids in its composition. The positively charged amino acid (PCAA) content has not been influenced by DH. However, a tendency to increase the PCAA content was verified, with the increase from 10 to 20% in the DH for the CPI hydrolysed by Alcalase. In contrast, a decrease in negatively charged amino acid (NCAA) content with an increase in DH from 10 to 20% was observed. With the same raw material and DH utilized, however, Protamex showed a significant increase ($p < 0.05$) in NCAA content than that obtained by Alcalase. The NCAA (aspartic and glutamic acid) have strong antioxidant effects due to the presence of excess electrons, which can easily be donated to quench free radicals (He et al., 2013). The significant increase ($p < 0.05$) in levels of the aromatic amino acids (AAA) were observed when the DH increased for hydrolysate with same raw material and enzyme. However, this increase was pronounced in the hydrolysed by Protamex, where samples 7 and 8 showed an increase of 67.67 to 81.81 mg/g protein. The presence of AAA is important for antioxidant activity, because it can stabilize the ROS through direct electron transfer, maintaining its stability through resonance structures. In addition, some AAA contain phenolic and indolic groups which can act as hydrogen donors (Wiriyaphan et al., 2012).

Molecular weight distribution

The molecular weight (MW) distribution of the different hydrolysates is showed in Figure 1. The MW of the hydrolysates with the same DH and raw material produced by Alcalase, Fig, 1a, 1b, 1c and 1d, were generally lower than those by Protamex. Protamex is a mixture of exo- and endo-peptidases characterized as a mixture of

different catalytic types such as metallo and aspartic proteases. In these catalytic types, the nucleophile attack is mediated by a water molecule activated by two aspartic acid residues, one glutamic acid residue and a metal ion, respectively (Benítez, Ibarz, & Pagan, 2008). On the other hand, Alcalase is a serine protease and does not require a metal ion for the activation of the nucleophilic attack as Protamex, which could be associated with the higher activities of the former (Damodaran, Parkin, & Fennema, 2010; Klompong, Benjakul, Kantachote, & Shahidi, 2007).

The results show that Alcalase and Protamex exhibited higher affinity to hydrolyse the CMP as substrate at the same DH, resulting in peptides with lower MW compared to those obtained by CPI. According to Silva et al. (2014), the protein isolation process may damage the protein structure making it difficult for the enzyme action because the structure may be affected by any agents capable of causing conformational changes in the structure such as pH. The lower MW distribution was revealed at DH 20% for all samples. Raghavan and Kristinsson (2009) reported that increasing hydrolysis from 7.5 to 25% increased the amount of low molecular weight peptides of tilapia (*O. niloticus*) protein hydrolysates. According to Panyam and Kilara (1996), the extensively hydrolysed proteins result in a decreased MW of the peptides obtained. The hydrolysates with the highest DH are preferred in order to ensure the presence of lower peptides that have been reported as potent antioxidant and antimicrobial agents (Chalamaiah et al., 2012; Najafian and Babji, 2012).

Antioxidant properties of hydrolysates

ABTS radical scavenging activity

ABTS radical scavenging activities of CPI and CMP hydrolysates are shown in Fig. 2(a). The increase in the DH for all hydrolysates results in a highest ABTS radical

scavenging activity ($p < 0.05$). Khantaphant et al. (2011) verified that brownstripe red snapper (*L. vitta*) muscle hydrolysates by Alcalase had increased ABTS scavenging activity with increasing DH. According to Raghavan, Kristinsson and Leeuwenburgh, (2008), peptides with lower MW or higher DH, have a greater ability to scavenge ROS generated by mononuclear cells. The hydrolysate 2, obtained from CPI with DH 20% by Alcalase, exhibited the highest ABTS scavenging activity (62.79 mg VCEAC/g of sample) ($p < 0.05$). This sample showed a lower MW distribution (1083 Da, Fig. 1b) and a higher content of AAH (365.44 mg/g protein, Table 3). Giménez et al. (2009) reported that skin gelatin hydrolysates by Alcalase with a DH of 50% showed an ABTS radical scavenging activity of 34.50 mg de VCEAC/g of sample, lower than that found in this study. ABTS radical scavenging activity is based on the ability of antioxidants to donate a hydrogen atom (Re et al., 1999). Samples rich in HAA, such as hydrolysate 2, are expected to inhibit lipid peroxidation as proton donors to hydrophobic peroxy radicals (Chalamaiah et al., 2012; Giménez et al., 2009; Saiga, Tanabe, & Nishimura, 2003). The hydrolysate 2 showed a high content of Tyr and according to Wiriyaphan et al. (2012), the Trp, Tyr and His residues contain the indolic, phenolic, and imidazole groups, respectively, which serve as hydrogen donors. The protein hydrolysates by Alcalase showed higher ABTS radical scavenging activity, Fig. 2a, in comparison with samples hydrolysed by Protamex, with the same DH and raw material. These results can be associated with higher HAA content that were significantly highest ($p < 0.05$) in hydrolysed by Alcalase. Furthermore, the Alcalase showed hydrolysates with a lower MW distribution than that presented by Protamex, with the same raw material and DH, enhancing the antioxidant activity of these samples.

Ferric reducing antioxidant power (FRAP assay)

All hydrolysates obtained prepared using Alcalase and Protamex had increases in FRAP when the DH increased ($p < 0.05$) (Fig.2b). An increase in the reducing power with increase in the DH has been reported in hydrolysed brownstripe red snapper muscle obtained by different proteases (Khantaphant et al., 2011). Hydrolysates with lower MW are expected to expose more side chains which can donate electrons and become more accessible by the Fe^{3+} -TPTZ complex and could contribute to high FRAP value (Wiriyaphan, Xiao, Decker, & Yongsawatdigul, 2015).

In general, at the same DH and substrate, hydrolysates prepared using Alcalase showed a higher activity than those produced using Protamex, except at 20% DH utilising the CMP as raw material (hydrolysate 8). Senphan and Benjakul (2014) observed that hydrolysates from seabass skin prepared using Alcalase presented a higher FRAP. Hydrolysate 8 was more effective as FRAP ($20.75 \mu\text{mol FeSO}_4 \cdot 7\text{H}_2\text{O/g}$ sample). This sample has a higher AAA content ($81.81 \text{ mg/g protein}$) ($p < 0.05$). Moreover, it had a Phe level significantly higher ($p < 0.05$) than the other hydrolysates. According to Wiriyaphan et al. (2012), AAAs such as Phe and Tyr can stabilize the ROS, by transferring electrons, maintaining their stability through resonance structures. The FRAP results obtained in this study were higher than those obtained for squid skin gelatin hydrolysates by Alcalase with a DH of 50% that presented $16.50 \mu\text{mol FeSO}_4 \cdot 7\text{H}_2\text{O/g}$ sample.

Metal chelating activity

The MCA of different hydrolysates is shown in Fig. 2c. All hydrolysates showed dose-dependent MCA as it increased from 5 to 50 mg/mL resulting in higher metal chelation ($p < 0.05$). The MCA results suggest that different hydrolysates may act as chelators of metal ions, which ultimately reduce the susceptibility of lipids to oxidative peroxidation and associated negative health effects (Giménez et al., 2009).

The MCA of peptides in hydrolysates had different metal ion chelating capacity that depend on the amino acid sequences and chain length of peptide fragments (Senphan and Benjakul, 2014).

The hydrolysate 2 (CPHA2), obtained from CPI with a 20% DH by Alcalase, exhibited MCA of 90.7, 81.0 and 64.1% at concentrations of 50, 25 and 5 mg/mL respectively, which was significantly ($p < 0.05$) higher than those of the other hydrolysates. However, these results were lower than those by Giménez et al. (2009) who reported an MCA of 7.2, 26.9, 80.9, 99.5 and 99.2% at concentrations of 0.02, 0.1, 0.2, 2.5 and 5.0 mg/mL from skin gelatin hydrolysates obtained from squid (*D. gigas*) by Alcalase with a DH of 50%. According to Klompong et al. (2007), the MCA of hydrolysate from yellow stripe trevally (*Selaroides leptolepis*) muscle prepared by both Alcalase and Flavourzyme increased with increasing DH. However, in this study it was verified that with the increase in DH from 10 to 20%, there was a significant decrease ($p < 0.05$) in the MCA for the samples hydrolysed by Protamex at the same hydrolysate concentration. Khantaphant et al. (2011) reported that MCA decreased with an increase in DH in hydrolysed brownstripe red snapper (*L. vitta*) muscle using Alcalase and Flavourzyme as enzymes.

According to Intarasirisawat et al. (2012), a marked decrease in the metal chelating activity was found when DH was 50% in defatted skipjack tuna (*Katsuwonus pelamis*) roe using Alcalase. Nevertheless, they found that the MCA increased with increasing DH up to 40% ($p < 0.05$), when a concentration of 20 mg/ml was used. According to Khantaphant et al. (2011), short chain peptides may result in loss of ability to form a complex with metals. In this study, it was found that the increase in DH, showed the opposite behaviour to the sample CPI hydrolyzed by Alcalase. These results indicate that partial hydrolysis of proteins causes the MCA compared to extensive hydrolysis. The strong metal chelating properties of long-chain peptides may be due to

synergistic effects of higher number of amino acid residues when compared to the shorter peptides (He et al., 2013). The amino acid Asp is present in higher content ($p < 0.05$) in protein hydrolysates obtained from CPI for both enzymes. According to Saiga et al. (2003), the carboxyl and amino groups of the amino acid side chains, such as Glu, Asp, His, Lys and Arg, can interact with metal ions and inactivate their pro-oxidant activity. Evaluating the Asp content in the hydrolysates with potential MCA, it might be suggested that the antioxidant activity is strongly linked to it.

DPPH radical scavenging assay

DPPH radical scavenging activities of CPI and CMP hydrolysates prepared using Alcalase and Protamex with different DH are showed in Fig. 2d. The results clearly indicate that all hydrolysates showed a dose-dependent relation since an increase from 2.50 to 10 mg/mL resulted in higher DPPH radical scavenging activities ($p < 0.05$). Hydrolysates from CPI or CMP produced by Protamex exhibited decreased DPPH radical scavenging activity as the DH increased at different concentrations evaluated. However, this DPPH radical scavenging activity is not verified in the hydrolysate prepared using Alcalase ($p < 0.05$). These results indicate that antioxidative peptides were more produced by Alcalase especially with the increasing cleavage of peptide bonds from 10 to 20%. Senphan and Benjakul (2014) observed that hydrolysates from seabass skin prepared using Alcalase did not present any difference in DPPH radical scavenging activity between hydrolysates prepared using Alcalase with a DH of 10%, 20% and 30%.

Furthermore, the results suggest that the efficiency in hydrogen donation of the peptides produced was influenced by enzymes used for hydrolysis. Samples hydrolyzed by Protamex, had higher DPPH radical scavenging activity ($p < 0.05$) than those prepared by Alcalase. It has been known that many antioxidative peptides include

hydrophobic amino (He et al., 2013; Najafian and Babji, 2012; Sarmadi and Ismail, 2010). The hydrolysates obtained by Alcalase, with the same raw material and DH, presented a higher HAA content as shown in Table 2. Furthermore, the lower MW distribution of Alcalase hydrolysate suggested the formation of shorter chain peptides than that of hydrolysates by Protamex as shown in Fig 1. Nevertheless, those peptides had less efficacy in DPPH radical scavenging activity. Furthermore, the hydrolysates prepared by Protamex were poor in hydrophobic amino acids when compared to Alcalase hydrolysates. According to Ngo et al. (2010), a peptide obtained from Nile tilapia (*O. niloticus*) scale gelatin showed a higher DPPH radical scavenging activity and was poor in aromatic aminoacids and rich in glycine and proline. These results suggested that the observed potent DPPH radical scavenging activity could be due to non-aromatic amino acids such as Pro and others. In this study, all hydrolysates by Protamex showed the highest Pro content ($p < 0.05$) in general. Hydrolysates 5 and 7, obtained from CPI and CMP with 10% DH using Protamex showed the highest DPPH radical scavenging activity ($p < 0.05$) at the different concentrations utilized. These samples showed an MW distribution of 1921 Da and 1845 Da, Fig. 1e and 1g, higher than the other samples. In this study, the protein hydrolysates were homogenised in methanol for DPPH radical scavenging activity evaluation. According to Karadag, and Ozcelik (2009), when proteins are homogeneised in an alcoholic solution, they can precipitate or present the solubility difference. In this study, the hydrolysates were homogeneised in distilled water, and the DPPH in methanol. This may have influenced the different results obtained in comparison with ABTS radical scavenging activity, ferric reducing antioxidant power (FRAP) and metal chelating activity.

Anti-inflammatory properties of hydrolysate

The cells were treated with different concentrations of CPI and CMP hydrolysates samples followed by LPS stimulation. After 24 h incubation, NO production in the culture medium was quantified using Griess's reagent as shown in Fig. 3. These results indicate that the most samples tested showed a decrease in NO production in a dose-dependent manner. The highest concentration (5.00 mg/mL) are able to inhibit the inflammatory response in macrophage cells compared to control among of sample tested ($p < 0.05$). However, as depicted in Fig. 3c, the suppression of NO varied among hydrolysates samples and notably, the sample 3 (from CMP produced by Protamex with 10% DH) possessed the strongest inhibition at 5.00 mg/mL of NO release from RAW 264.7 macrophages ($p < 0.05$). This hydrolysate sample showed a MW of 1245 Da as shown in Fig. 1. According to Ahn et al. (2015) low molecular weight anti-inflammatory peptide from salmon by-product protein hydrolysate exhibited strong NO inhibition activity in LPS-stimulated RAW 264.7 macrophages. However, this study showed other hydrolysates samples with lower MW, as shown in Fig. 1, than that hydrolysate sample 3, but with less NO inhibition. This result is in agreement with Ahn et al. (2015) which verified the NO inhibitory activity of the peptide fraction with higher MW is higher compared to the effect of salmon by-product protein hydrolysate fraction with lower molecular weight. These results indicate that among of hydrolysates samples of this study tend to decrease the pro-inflammatory capacity of activated macrophages by decreased NO production, establishing a trend of regulation of the inflammatory process.

Antimicrobial properties of hydrolysate

The antimicrobial activity was verified on 7 microorganisms tested as showed in Table 3. Most of the microorganisms inhibited by different hydrolysates were Gram-positive bacteria (*B. thermosphacta*, *L. innocua*, *L. monocytogenes* and *S. aureus*)

followed by Gram-negative bacteria (*A. hydrophila* and *Y. enterocolitica*) and yeast (*D. hanseii*). Moreover, the hydrolysates tested did not exhibit an inhibitory effect against some microorganisms such as probiotics: *B. bifidum*, *L. acidophilus* and *L. helveticus*. These results indicate that hydrolysates can be used in food formulations that contain these probiotic microorganisms. Jemil et al. (2014) elaborated fermented fish meat protein hydrolysates from sardinelle (*Sardinella aurita*), zebra blenny (*Salaria basilisca*), goby (*Zosterisessor ophiocephalus*) and ray (*Dasyatis pastinaca*) and evaluated the antimicrobial activity at 200 mg/mL. These hydrolysates inhibited most Gram-positive microorganisms (*S. aureus*, *Micrococcus luteus*, *B. cereus* and *Enterococcus faecalis*) and only one Gram-negative microorganism (*E. coli*). The Gram-negative bacteria are generally more resistant with respect to Gram-positive bacteria because they have an additional structure on the cell wall that acts as a barrier to limit the diffusion of harmful compounds to the interior cell, such as lipopolysaccharide (LPS) (Chalamaiah et al., 2012; Najafian and Babji, 2012).

The microorganisms *B. thermosphacta*, *L. innocua* and *S. aureus* were inhibited by all hydrolysates tested, as shown in Table 3. However, *L. monocytogenes*, *A. hydrophila*, *Y. enterocolitica* and *D. hanseii* were inhibited by hydrolysates from CPI or CMP in different DH (10 - 20%) using Alcalase as enzyme. These samples had lower MW distribution, 1285 Da, 1083 Da, 1245 Da and 1066 Da, than those obtained by Protamex, as shown in Fig. 1a, 1b, 1c and 1d. Moreover, the hydrolysates prepared from CPI or CMP by Alcalase had higher content of HAA ($p < 0.05$), as shown in Table 2. According to Gómez-Guillén et al. (2010), the molecular weight could be related to the elimination of aggregates and better exposure of the amino acids and their charges as well as structure acquisition, thus facilitating the interaction with bacterial membranes. According to Najafian and Babji (2012), the antimicrobial peptides usually have less than 50 amino acids, of which nearly 50% are hydrophobic and have a molecular weight

below 10 kDa. The HAA peptides can enter the membrane, as the positive charge, would initiate the peptide interaction with the surface of the negatively charged bacteria.

The DH is imperative to control and optimize the hydrolysis of proteins to obtain reproducible and highly biologically active hydrolysates. However, in this study the DH did not show a clear influence on microorganism inhibition, as shown in Table 4. Wald et al. (2016) reported that the antibacterial effect of rainbow trout by-products hydrolysates increased significantly with an increasing DH. This suggests that the biological antimicrobial activity of the hydrolysates obtained in this study can be associated with amino acid composition and/or sequence (Memarpour-Yazdi et al., 2012; Najafian and Babji, 2012). The inhibition zones were more pronounced in the hydrolysates elaborated by Alcalase compared to Protamex. As previously mentioned, the raw material hydrolysed by Alcalase presents a higher HAA, reportedly responsible for acting on the microorganism wall.

In this study, a tendency of increase in inhibition zones with increase in the concentration from 1.25 to 7.50 mg/mL was verified for some of microorganisms tested. This was verified for *A. hydrophila*, *B. thermospacta*, *D. hanseii* and *L. innocua* when the hydrolysates 1, 2, 3 and 4 were used in some of them. The hydrolysate 1, from CPI at DH 10% using Alcalase, showed the greatest inhibition of *D. hanseii* and *L. innocua* at 7.50 mg/mL. However, at the same concentration, Nisin showed the highest inhibition against *D. hanseii*. Gómez-Guillén et al. (2010) obtained hydrolysates from tuna skin gelatin in fractions with MW of 10 kDa and 1 kDa at 2 mg/mL that showed an inhibition zone of 0.25 cm of *L. innocua*. At a concentration of 2.50 mg/mL, hydrolysate 1 showed an inhibition zone higher than that found by these authors. Hydrolysate 2, showed a higher HAA content (365.44 mg/g protein) (Table 3) and a MW distribution of 1083 Da (Fig. 1b) that could improve the antimicrobial activity in accordance with some authors (Memarpour-Yazdi et al., 2012; Najafian and Babji,

2012). This hydrolysate inhibited some microorganisms that are important food pathogens such as *A. hydrophila*, *Y. enterocolitica* and *S. aureus* and because of this, the application of the same as an antimicrobial agent is interesting. Furthermore, from these results these hydrolysates may be considered to be a natural preservative against food-borne pathogens for the food industry or an alternative as natural antimicrobial.

CONCLUSION

The protein hydrolysates recovered from Argentine croaker muscle proteins, by alkali-solubilisation method and by successive washings with different degrees of hydrolysis using enzymatic hydrolysis by Alcalase and Protamex, were used to generate antioxidant, anti-inflammatory and antibacterial properties. The raw material, enzyme and degree of hydrolysis presented an influence in antioxidant activity. The hydrolysate from Argentine croaker protein isolate with a 20% DH by Alcalase showed the highest antioxidant activity evaluated in three of the four methods. The antimicrobial activity was verified on seven microorganisms tested. The protease used had great influence on the antimicrobial activity, as it was observed that the samples hydrolyzed by Alcalase exhibited greater inhibition zones than those by hydrolyzed by Protamex. The results of anti-inflammatory indicate that the most samples tested showed a decrease in NO production in a dose-dependent manner. Furthermore, these hydrolysates can serve as food preservatives or an alternative of antimicrobial and natural antioxidants and anti-inflammatory compounds. Nevertheless, more detailed research is required, such as on the indication of amino acid sequence as well *in vivo* antioxidant, antimicrobial and anti-inflammatory activities.

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REFERENCES

- Adler-Nissen, J. 1986. *Enzymatic hydrolysis of food proteins*. ElsevierApplied Science Publishing, London.
- Ahn, C. B., Cho, Y. S., and Je, J. Y. 2015. Purification and anti-inflammatory action of tripeptide from salmon pectoral fin byproduct protein hydrolysate. *Food Chem.* 168: 151–156.
- AOAC. 2000. *Official methods of analysis*.
- Karadag, A. and Ozcelik, S. S. 2009. Review of Methods to Determine Antioxidant Capacities. *Food Analy. Meth.* 2: 41–60.
- Benítez, R., Ibarz, A., and Pagan, J. 2008. Protein hydrolysates: Processes and applications. *Acta Bioq. Clín. Latinoam.* 42: 227–236.
- Chalamaiah, M., Dinesh Kumar, B., Hemalatha, R., and Jyothirmayi, T. 2012. Fish protein hydrolysates: Proximate composition, amino acid composition, antioxidant activities and applications: A review. *Food Chem.* 135: 3020–3038.
- Chi, C. F., Hu, F. Y., Wang, B., Li, T., and Ding, G. F. 2015. Antioxidant and anticancer peptides from the protein hydrolysate of blood clam (*Tegillarca granosa*) muscle. *J. Funct. Foods.* 15: 301–313.
- Chung, Y. C., Chang, C. T., Chao, W. W., Lin, C. F., and Chou, S. T. 2002. Antioxidative activity and safety of the 50% ethanolic extract from red bean fermented by *Bacillus subtilis* IMR-NK1. *J. Agricult. Food Chem.* 50: 2454–2458.
- Damodaran, S., Parkin, K.L., and Fennema, O. R. 2010. *Química de Alimentos de*

- 590 Fennema (4th ed.). Porto Alegre.
- 591 Dia, V. P., Bringe, N. A., and De Mejia, E. G. 2014. Peptides in pepsin-pancreatin
592 hydrolysates from commercially available soy products that inhibit
593 lipopolysaccharide-induced inflammation in macrophages. Food Chem. 152: 423–
594 431.
- 595 Giménez, B., Alemán, A., Montero, P., and Gómez-Guillén, M. C. 2009. Antioxidant
596 and functional properties of gelatin hydrolysates obtained from skin of sole and
597 squid. Food Chem. 114: 976–983.
- 598 Gómez-Guillén, M.C, López-Caballero. M.E., Alemán, A., Giménez, B., and Montero,
599 P. 2010. Antioxidant and antimicrobial peptide fractions from squid and tuna skin
600 gelatin. Sea by-Products as Real Material, 661(2), 89–115.
- 601 He, R., Girgih, A. T., Malomo, S.A., Ju, X., and Aluko, R. E. 2013. Antioxidant
602 activities of enzymatic rapeseed protein hydrolysates and the membrane
603 ultrafiltration fractions. J. Funct. Foods. 5: 219–227.
- 604 Intarasirisawat, R., Benjakul, S., Visessanguan, W., and Wu, J. 2012. Antioxidative and
605 functional properties of protein hydrolysate from defatted skipjack (*Katsuwonus*
606 *pelamis*) roe. Food Chem. 135: 3039–3048.
- 607 Intarasirisawat, R., Benjakul, S., Wu, J., and Visessanguan, W. 2013. Isolation of
608 antioxidative and ACE inhibitory peptides from protein hydrolysate of skipjack
609 (*Katsuwana pelamis*) roe. J. Funct. Foods., 5: 1854–1862.
- 610 Jemil, I., Jridi, M., Nasri, R., Ktari, N., Ben Slama-Ben Salem, R., Mehiri, M., and
611 Nasri, M. 2014. Functional, antioxidant and antibacterial properties of protein
612 hydrolysates prepared from fish meat fermented by *Bacillus subtilis* A26. Process
613 Biochem. 49: 963–972.
- 614 Khantaphant, S., Benjakul, S., and Kishimura, H. 2011. Antioxidative and ACE
615 inhibitory activities of protein hydrolysates from the muscle of brownstripe red

- 616 snapper prepared using pyloric caeca and commercial proteases. *Process Biochem.*
617 46: 318–327.
- 618 Klompong, V., Benjakul, S., Kantachote, D., and Shahidi, F. 2007. Antioxidative
619 activity and functional properties of protein hydrolysate of yellow stripe trevally
620 (*Selaroides leptolepis*) as influenced by the degree of hydrolysis and enzyme type.
621 *Food Chem.* 102: 1317–1327.
- 622 Ktari, N., Nasri, R., Mnafigui, K., Hamden, K., Belguith, O., Boudaouara, T., and Nasri,
623 M. 2014. Antioxidative and ACE inhibitory activities of protein hydrolysates from
624 zebra blenny (*Salaria basilisca*) in alloxan-induced diabetic rats. *Process Biochem.*
625 49: 890–897.
- 626 Lempek, T.S., Martins, V.G., Prentice, C. 2007. Rheology of Surimi-Based Products
627 from Fatty Fish Underutilized by the Industry. *J. Aquatic Food Prod. Technol.* 16:
628 27–44.
- 629 Limpan, N., Prodpran, T., Benjakul, S., and Prasarnpran, S. 2010. Properties of
630 biodegradable blend films based on fish myofibrillar protein and polyvinyl alcohol
631 as influenced by blend composition and pH level. *J. Food Engin.* 100: 85–92.
- 632 Maturana, P., Martinez, M., Noguera, M., Santos, N. C., Disalvo, E. A., Semorile, L.,
633 and Hollmann, A. 2017. Lipid selectivity in novel antimicrobial peptides:
634 implication on antimicrobial and hemolytic activity. *Coll. Surf. Biointerf.* 153,
635 152–159.
- 636 Memarpour-Yazdi, M., Asoodeh, A., and Chamani, J. 2012. A novel antioxidant and
637 antimicrobial peptide from hen egg white lysozyme hydrolysates. *J. Funct. Foods.*
638 4: 278–286.
- 639 Millán-Linares, M. D. C., Bermúdez, B., Yust, M. D. M., Millán, F., and Pedroche, J.
640 2014. Anti-inflammatory activity of lupine (*Lupinus angustifolius* L.) protein
641 hydrolysates in THP-1-derived macrophages. *J. Funct. Foods.* 8:, 224–233.

- 642 Najafian, L., and Babji, A. S. 2012. A review of fish-derived antioxidant and
643 antimicrobial peptides: Their production, assessment, and applications. *Pept.* 33:
644 178–185.
- 645 Nasri, R., Younes, I., Jridi, M., Trigui, M., Bougatef, A., Nedjar-Arroume, N., and
646 Karra-Châabouni, M. 2013. ACE inhibitory and antioxidative activities of Goby
647 (*Zosterisessor ophiocephalus*) fish protein hydrolysates: Effect on meat lipid
648 oxidation. *Food Res. Internat.* 54: 552–561.
- 649 Ngo, D. H., Qian, Z. J., Ryu, B., Park, J. W., and Kim, S. K. 2010. In vitro antioxidant
650 activity of a peptide isolated from Nile tilapia (*Oreochromis niloticus*) scale gelatin
651 in free radical-mediated oxidative systems. *J. Funct. Foods.* 2: 107–117.
- 652 Nolsøe, H., and Undeland, I. 2009. The acid and alkaline solubilization process for the
653 isolation of muscle proteins: State of the art. *Food Bioproc. Technol.* 2: 1–27.
- 654 Nongonierma, A. B., Mazzocchi, C., Paoletta, S., and FitzGerald, R. J. 2017. Release of
655 dipeptidyl peptidase IV (DPP-IV) inhibitory peptides from milk protein isolate
656 (MPI) during enzymatic hydrolysis. *Food Res. Internat.* 94: 79–89.
- 657 Panyam, D., and Kilara, A. 1996. Enhancing the functionality of food proteins by
658 enzymatic modification. *Trends Food Sci. Technol.* 7:120–125.
- 659 Pulido, R., Bravo, L., and Saura-Calixto, F. 2000. Antioxidant activity of dietary
660 polyphenols as determined by a modified ferric reducing/antioxidant power assay.
661 *J. Agricult. Food Chem.* 48: 3396–3402.
- 662 Raghavan, S., and Kristinsson, H. G. 2009. ACE-inhibitory activity of tilapia protein
663 hydrolysates. *Food Chem.* 117: 582–588.
- 664 Raghavan, S., Kristinsson, H.G., Leeuwenburgh, C. 2008. Radical scavenging and
665 reducing ability of tilapia (*Oreochromis niloticus*) protein hydrolysates. *J. Agricult.*
666 *Food Chem.* 56: 10359–10369.
- 667 Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., and Rice-Evans, C. 1999.

- 668 Antioxidant Activity Applying an Improved Abts Radical Cation Decolorization
669 Assay. *Free Rad. Biol. Medic.* 26: 1231–1237.
- 670 Robert, M., Zatylny-Gaudin, C., Fournier, V., Corre, E., Le Corguillé, G., Bernay, B.,
671 and Henry, J. 2015. Molecular characterization of peptide fractions of a Tilapia
672 (*Oreochromis niloticus*) by-product hydrolysate and in vitro evaluation of
673 antibacterial activity. *Process Biochem.* 50: 487–492.
- 674 Saiga, A., Tanabe, S., and Nishimura, T. 2003. Antioxidant activity of peptides obtained
675 from porcine myofibrillar proteins by protease treatment. *J. Agricult. Food Chem.*
676 51: 3661–3667.
- 677 Sarmadi, B. H., and Ismail, A. 2010. Antioxidative peptides from food proteins: A
678 review. *Pept.* 31: 1949–1956.
- 679 Senphan, T., and Benjakul, S. 2014. Antioxidative activities of hydrolysates from
680 seabass skin prepared using protease from hepatopancreas of Pacific white shrimp.
681 *J. Funct. Foods.* 6: 147–156.
- 682 Sila, A., Martinez-Alvarez, O., Haddar, A., Gómez-Guillén, M. C., Nasri, M., Montero,
683 M. P., and Bougatef, A. 2015. Recovery, viscoelastic and functional properties of
684 Barbel skin gelatine: Investigation of anti-DPP-IV and anti-prolyl endopeptidase
685 activities of generated gelatine polypeptides. *Food Chem.*, 168: 478–486.
- 686 Silva, C. M., Aline, R., and Prentice, C. 2014. Comparing the hydrolysis degree of
687 industrialization byproducts of Withemouth croaker (*Micropogonias furnieri*)
688 using microbial enzymes. *Food Researc. Int.* 21: 1757–1761.
- 689 Undeland I., Kelleher S.D, and Hultin. H. 2002. Recovery of functional proteins from
690 herring (*Clupea harengus*) light muscle by an acid or alkaline solubilization
691 process. *J. Agricult. Food Chem.* 50: 7371–7371.
- 692 Wald, M., Schwarz, K., Rehbein, H., Bußmann, B., and Beermann, C. 2016. Detection
693 of antibacterial activity of an enzymatic hydrolysate generated by processing

- 694 rainbow trout by-products with trout pepsin. Food Chem. 205: 221–228.
- 695 Wiriyaphan, C., Chitsomboon, B., and Yongsawadigul, J. 2012. Antioxidant activity of
696 protein hydrolysates derived from threadfin bream surimi byproducts. Food Chem.
697 132: 104–111.
- 698 Wiriyaphan, C., Xiao, H., Decker, E. A., and Yongsawatdigul, J. 2015. Chemical and
699 cellular antioxidative properties of threadfin bream (*Nemipterus* spp.) surimi
700 byproduct hydrolysates fractionated by ultrafiltration. Food Chem. 167: 7–15.
- 701 Yen, G. C., and Hsieh, P. P. 1995. Antioxidative activity and scavenging effects on
702 active oxygen of xylose–lysine Maillard reaction-products. J. Sci. Food Agricult.
703 67: 415–420.
- 704 Zeng, W.-C., Zhang, W.-H., He, Q., and Shi, B. 2015. Purification and characterization
705 of a novel antioxidant peptide from bovine hair hydrolysates. Process Biochem. 50:
706 948-954.
- 707 Zhou, H. Y., Shin, E. M., Guo, L. Y., Youn, U. J., Bae, K., Kang, S. S., and Kim, Y. S.
708 2008. Anti-inflammatory activity of 4-methoxyhonokiol is a function of the
709 inhibition of iNOS and COX-2 expression in RAW 264.7 macrophages via NF- κ B,
710 JNK and p38 MAPK inactivation. Europ. J. Pharmacol. 586: 340–349.
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- 712

Table 1.- Bacterial strains, culture media and incubation conditions used for determination of antibacterial activity of hydrolysates obtained from Argentine croaker.

Microorganismos	Type Culture Collection	ID	Cultive medium	Incubation temperature (°C)
<i>Aeromonas hydrophila</i>	CECT 839T	AH	BHI	30
<i>Aspergillus niger</i>	CECT 2088	AN	PDA	30
<i>Bacillus cereus</i>	CECT 148	BC	PDA	37
<i>Bifidobacterium bifidum</i>	DSMZ 20215	BB	BHI	*AN 37
<i>Brochothrix thermosphacta</i>	CECT 847	BT	BHI	20 - 25
<i>Citrobacter freundii</i>	CECT 401	CF	BHI	37
<i>Clostridium perfringens</i>	CECT 486	CP	BHI	*AN 37
<i>Debaryomyces hanseii</i>	CECT 11364	GH	BHI (3% NaCl)	20
<i>Enterococcus faecium</i>	DSM 20477	EF	BHI	37
<i>Escherichia coli</i>	CECT 515	EC	BHI	37
<i>Lactobacillus acidophilus</i>	CECT 903	LA	MRS	** CO ₂
<i>Lactobacillus helveticus</i>	DSM 20075	LH	BHI	37
<i>Listeria innocua</i>	CECT 910	LI	BHI	37
<i>Listeria monocytogenes</i>	CECT 4032	LM	BHI	37
<i>Photobacterium phosphoreum</i>	CECT 4191	PP	BHI (1% NaCl)	15
<i>Pseudomonas fluorescens</i>	CECT 4898	PF	BHI	30
<i>Salmonella choleraesuis</i>	CECT 4300	SC	BHI	37
<i>Shewanella putrefaciens</i>	CECT 5346	SP	BHI	30
<i>Shigella sonnei</i>	CECT 4887	SS	BHI	37
<i>Staphylococcus aureus</i>	CECT 240	SA	BHI	37
<i>Vibrio parahaemolyticus</i>	CECT 511 T	VP	BHI (3% NaCl)	37
<i>Yersinia enterocolitica</i>	CECT 4315	YE	BHI	37
<i>Penicillium expansum</i>	DSMZ 62841	PE	PDA	30
<i>Colletotrichum lindemuthianum</i>	CECT 2119	CL	PDA	25
<i>Mucor rouxii</i>	CECT 2655	MR	PDA	25
<i>Fusarium oxysporum</i>	CECT 2869	FOX	PDA	25

The bacterial strains were conserved at -80 °C in Brain Heart Infusion (BHI) Broth (Oxoid, Basingstoke, UK) containing 25% glycerol (Panreac, Barcelona, Spain). BHI: Brain Heart Infusion (BHI) Agar; BHI (3% NaCl): BHI supplemented with 3% NaCl; BHI (1% NaCl): BHI supplemented with 1% NaCl; PDA: Potato dextrose agar; MRS: MRS agar (DE MAN, ROGOSA, SHARPE); *under anaerobic conditions (Gas-Pack, Anaerogen, Oxoid); **incubated under CO₂ flow.

Table 2 - Amino acid composition of hydrolysates obtained from Argentine croaker.

Amino acids	Composition (mg/gprotein)							
	1	2	3	4	5	6	7	8
Alanine (Ala)	59.40 ± 0.67 ^{bcd}	58.40 ± 0.04 ^{cde}	58.04 ± 1.14 ^{de}	57.18 ± 1.06 ^e	60.06 ± 0.36 ^{bc}	62.31 ± 0.35 ^a	60.93 ± 0.27 ^{ab}	61.18 ± 0.06 ^{ab}
Arginine (Arg)	60.85 ± 0.08 ^{bc}	61.01 ± 0.46 ^{bc}	62.06 ± 1.25 ^{ab}	63.28 ± 0.78 ^a	63.03 ± 0.11 ^a	59.62 ± 0.04 ^c	62.09 ± 0.08 ^{ab}	57.83 ± 0.13 ^d
AsparticAcid (Asp)	137.02 ± 1.10 ^a	136.93 ± 0.38 ^a	131.28 ± 1.11 ^d	132.61 ± 0.31 ^{cd}	135.27 ± 0.70 ^{ab}	136.73 ± 0.60 ^a	133.70 ± 0.24 ^{bc}	131.42 ± 0.27 ^d
Cysteine (Cys)	4.40 ± 0.08 ^b	4.16 ± 0.01 ^b	4.11 ± 0.01 ^b	4.11 ± 0.11 ^b	4.05 ± 0.05 ^b	4.96 ± 0.04 ^a	4.03 ± 0.05 ^b	4.00 ± 0.43 ^b
GlutamicAcid (Glu)	195.50 ± 1.91 ^b	188.60 ± 1.37 ^c	196.16 ± 2.37 ^b	190.66 ± 1.48 ^c	200.50 ± 0.22 ^a	196.19 ± 0.33 ^b	201.20 ± 0.43 ^a	198.25 ± 0.18 ^{ab}
Glycine (Gly)	33.78 ± 0.61 ^b	35.87 ± 0.28 ^a	32.62 ± 0.64 ^c	33.40 ± 0.44 ^{bc}	32.50 ± 0.26 ^c	36.25 ± 0.12 ^a	34.23 ± 0.16 ^b	35.56 ± 0.15 ^a
Histidine (His)	24.81 ± 1.38 ^{ab}	24.66 ± 0.14 ^{ab}	24.69 ± 0.47 ^{ab}	24.91 ± 0.53 ^{ab}	23.28 ± 0.22 ^c	24.69 ± 0.10 ^{ab}	23.00 ± 0.03 ^b	26.39 ± 1.32 ^a
Isoleucine* (Ile)	31.26 ± 0.33 ^a	31.00 ± 0.09 ^a	31.46 ± 1.24 ^a	31.14 ± 0.57 ^a	29.21 ± 0.32 ^b	26.24 ± 0.09 ^c	28.76 ± 0.10 ^b	25.42 ± 0.11 ^c
Leucine* (Leu)	84.96 ± 0.13 ^a	83.96 ± 0.19 ^{ab}	83.09 ± 1.03 ^{bc}	82.35 ± 0.55 ^{cd}	84.45 ± 0.34 ^a	81.28 ± 0.30 ^c	82.36 ± 0.13 ^{cd}	78.84 ± 0.13 ^e
Lysine* (Lys)	92.30 ± 7.23 ^c	96.07 ± 0.36 ^{bc}	100.21 ± 1.35 ^{ab}	98.58 ± 0.87 ^{abc}	103.87 ± 0.18 ^a	97.98 ± 0.05 ^{abc}	101.41 ± 0.32 ^{ab}	99.16 ± 0.40 ^{abc}
Methionine* (Met)	39.78 ± 0.31 ^{bc}	41.68 ± 0.48 ^a	39.55 ± 0.57 ^{bc}	40.34 ± 0.28 ^b	37.80 ± 0.31 ^d	39.33 ± 0.21 ^c	39.14 ± 0.03 ^c	39.49 ± 0.11 ^{bc}
Phenylalanine* (Phe)	38.43 ± 1.27 ^c	38.67 ± 0.01 ^c	38.42 ± 0.70 ^c	39.71 ± 0.48 ^{bc}	35.32 ± 0.25 ^d	40.92 ± 0.22 ^b	33.82 ± 0.05 ^d	43.88 ± 0.13 ^a
Proline (Pro)	32.42 ± 0.13 ^{cd}	33.37 ± 0.46 ^{bc}	33.05 ± 0.06 ^{bc}	33.57 ± 0.58 ^b	31.11 ± 0.06 ^e	31.71 ± 0.45 ^{de}	34.88 ± 0.46 ^a	32.95 ± 0.28 ^{bc}
Serine (Ser)	46.00 ± 0.26 ^c	46.04 ± 0.40 ^c	45.09 ± 0.01 ^d	45.83 ± 0.08 ^c	46.84 ± 0.10 ^b	49.08 ± 0.41 ^a	46.22 ± 0.30 ^{bc}	49.72 ± 0.02 ^a
Threonine (Thr)	45.85 ± 0.08 ^{ab}	45.38 ± 0.62 ^{ab}	46.01 ± 1.31 ^{ab}	46.50 ± 0.99 ^a	45.86 ± 0.40 ^{ab}	44.54 ± 0.15 ^b	45.86 ± 0.08 ^{ab}	45.40 ± 0.01 ^{ab}
Tyrosine* (Tyr)	36.30 ± 0.71 ^c	38.32 ± 0.11 ^b	37.68 ± 0.20 ^b	39.88 ± 0.09 ^a	31.48 ± 0.01 ^e	34.15 ± 0.09 ^d	33.84 ± 0.06 ^d	37.93 ± 0.02 ^b
Valine* (Val)	37.00 ± 0.42 ^a	35.87 ± 0.02 ^{abc}	36.47 ± 1.08 ^{ab}	35.94 ± 0.32 ^{ab}	35.39 ± 0.41 ^{bc}	34.02 ± 0.21 ^d	34.52 ± 0.01 ^{cd}	32.58 ± 0.46 ^e
HAA	363.91 ± 1.50 ^{ab}	365.44 ± 0.34 ^a	361.87 ± 0.70 ^b	364.24 ± 0.20 ^a	348.85 ± 0.16 ^e	354.92 ± 0.15 ^c	352.29 ± 0.13 ^d	356.28 ± 0.13 ^c
AAA	74.71 ± 2.00 ^c	77.00 ± 0.11 ^c	76.10 ± 0.90 ^{cd}	79.60 ± 0.39 ^b	66.80 ± 0.25 ^e	75.07 ± 0.13 ^d	67.67 ± 0.10 ^e	81.81 ± 0.15 ^a
NCAA	332.51 ± 3.01 ^{abc}	325.53 ± 1.75 ^{de}	327.44 ± 3.47 ^{cde}	323.27 ± 1.79 ^e	335.77 ± 0.47 ^a	332.92 ± 0.92 ^{ab}	334.90 ± 0.19 ^{ab}	329.67 ± 0.45 ^{bcd}
PCAA	177.95 ± 5.93 ^c	181.74 ± 0.68 ^{bc}	186.97 ± 2.13 ^{ab}	186.76 ± 1.12 ^{ab}	190.18 ± 0.07 ^a	182.29 ± 0.09 ^{bc}	186.50 ± 0.43 ^{ab}	183.37 ± 0.80 ^{bc}
*EAA	394.36 ± 4.98 ^{ab}	390.23 ± 0.28 ^{ab}	391.57 ± 2.78 ^{ab}	392.86 ± 1.11 ^{ab}	395.17 ± 0.85 ^a	389.00 ± 0.40 ^b	388.88 ± 0.75 ^b	391.16 ± 0.92 ^{ab}

1: CPI hydrolysate sample by Alcalase with 10% DH (CPHA1); 2: CPI hydrolysate sample by Alcalase with 20% DH (CPHA2); 3: CPI hydrolysate sample by Alcalase with 20% DH (CMHA1); 4: CMP hydrolysate sample by Alcalase with 20% DH using (CMHA2); 5: CPI hydrolysate sample by Protamex with 10% DH (CPHP1); 6: CPI hydrolysate sample by Protamex with 20% DH (CPHP2); 7: CMP hydrolysate sample by Protamex with 10% DH (CMHP1); 8: CMP hydrolysate sample by Protamex with 20% DH (CMHP2); AAH: hydrophobic amino acids (alanine, valine, isoleucine, leucine, tyrosine, phenylalanine, proline, methionine, and cysteine); AAA: aromatic amino acids (phenylalanine, tryptophan and tyrosine); AACP: positively charged amino acids (arginine, histidine, lysine); AACN: negatively charged amino acids (aspartic acid, glutamic acid); * EEA: essential amino acids (phenylalanine, valine, threonine, isoleucine, methionine, histidine, leucine and lysine). Different letters (a, b, c) in the same row indicate significant differences (p < 0.05). Data reported are mean values ± SD.

Table 3- Antimicrobial activity of different hydrolysates obtained from Argentine croaker.

Hydrolysates	Concentrations (mg/mL)	Microorganisms						
		AH	BT	DH	LI	LM	SA	YE
1 CPHA1	7.50	+/-	+/-	++++	++-	NI	+/-	NI
	5.00	--	+/-	+++-	++	NI	+/-	NI
	2.50	-	+	+++-	+-	+/-	++	NI
	1.25	NI	--	+++-	+-	NI	++	NI
2 CPHA2	7.50	++++	+	+++	+/-	NI	NI	+/-
	5.00	+/-	+/-	++	+/-	NI	++-	+/-
	2.50	NI	+/-	++	--	NI	+	NI
	1.25	NI	--	++-	--	NI	++-	NI
3 CMHA1	7.50	--	+/-	+++	--	+/-	++	NI
	5.00	--	+/-	++	+/-	+/-	+	NI
	2.50	NI	+/-	++	+/-	+/-	++	NI
	1.25	NI	+/-	++	--	--	+/-	NI
4 CMHA2	7.50	NI	+/-	+++	NI	NI	+/-	NI
	5.00	NI	+/-	++-	+/-	--	+	NI
	2.50	NI	--	++	+/-	--	++	NI
	1.25	NI	+/-	+++	++	--	+/-	NI
5 CPHP1	7.50	NI	NI	NI	NI	NI	+/-	NI
	5.00	NI	+/-	NI	NI	NI	++	NI
	2.50	NI	+	NI	+/-	NI	NI	NI
	1.25	NI	--	NI	+/-	NI	NI	NI
6 CPHP2	7.50	NI	+/-	+	+	NI	+	NI
	5.00	NI	+	+	NI	NI	+	NI
	2.50	NI	+/-	++-	NI	NI	++	NI
	1.25	NI	--	++-	NI	NI	++	NI
7 CMHP1	7.50	NI	+/-	NI	+/-	NI	+/-	NI
	5.00	+/-	+/-	NI	+	NI	+	NI
	2.50	--	++	NI	++	NI	+	NI
	1.25	--	+/-	NI	+	NI	++	NI
8 CMHP2	7.50	NI	+/-	++	++	NI	NI	NI
	5.00	NI	+/-	++-	+	NI	NI	NI
	2.50	NI	+/-	++-	+/-	NI	++	NI
	1.25	NI	+	++-	++-	NI	+	NI
Polylysine	7.50	+/-	+/-	NI	+	+	+/-	+
	5.00	--	+	NI	+/-	+/-	+/-	+/-
	2.50	--	--	NI	+/-	--	++	--
	1.25	--	NI	NI	+/-	--	++	--
Nisin	7.50	NI	+++++	+++++	+/-	+/-	+/-	NI
	5.00	NI	+++++	+++-	+/-	--	+	NI
	2.50	NI	++++	+++	+/-	+++++	+	NI
	1.25	NI	++++	++-	--	-	+	NI

++++: 2.75 – 3.00 cm; +++: 2.00 – 2.75 cm; ++: 1.75 – 2.00 cm; +: 1.50 – 1.75 cm; +/-: 1.25 – 1.50 cm; -: 1.00 – 1.25 cm; -: 0.75 – 1.00 cm; -: 0.50 – 0.75 cm; -: 0.25 – 0.50 cm; -: < 0.25 cm; NI: no inhibition; 1: CPI hydrolysate sample by Alcalase with 10% DH (CPHA1); 2: CPI hydrolysate sample by Alcalase with 20% DH (CPHA2); 3: CPI hydrolysate sample by Alcalase with 20% DH (CMHA1); 4: CMP hydrolysate sample by Alcalase with 20% DH using (CMHA2); 5: CPI hydrolysate sample by Protamex with 10% DH (CPHP1); 6: CPI hydrolysate sample by Protamex with 20% DH (CPHP2); 7: CMP hydrolysate sample by Protamex with 10% DH (CMHP1); 8: CMP hydrolysate sample by Protamex with 20% DH (CMHP2).

Figure 1 - Molecular weight distribution of different hydrolysates. The optical density was measured at 214 nm.

1: CPI hydrolysate sample by Alcalase with 10% DH (CPHA1); 2: CPI hydrolysate sample by Alcalase with 20% DH (CPHA2); 3: CPI hydrolysate sample by Alcalase with 20% DH (CMHA1); 4: CMP hydrolysate sample by Alcalase with 20% DH using (CMHA2); 5: CPI hydrolysate sample by Protamex with 10% DH (CPHP1); 6: CPI hydrolysate sample by Protamex with 20% DH (CPHP2); 7: CMP hydrolysate sample by Protamex with 10% DH (CMHP1); 8: CMP hydrolysate sample by Protamex with 20% DH (CMHP2).

Figure 2 - Antioxidative activities of protein hydrolysate obtained from Argentine croaker. Bars represent the standard deviation (n=3).

Different lowercase letters within the different hydrolysates at same concentration indicate significant differences ($p < 0.05$). Different capital letters within the same hydrolysates indicate significant differences ($p < 0.05$) between the concentrations (c-d). 1: CPI hydrolysate sample by Alcalase with 10% DH (CPHA1); 2: CPI hydrolysate sample by Alcalase with 20% DH (CPHA2); 3: CPI hydrolysate sample by Alcalase with 20% DH (CMHA1); 4: CMP hydrolysate sample by Alcalase with 20% DH using (CMHA2); 5: CPI hydrolysate sample by Protamex with 10% DH (CPHP1); 6: CPI hydrolysate sample by Protamex with 20% DH (CPHP2); 7: CMP hydrolysate sample by Protamex with 10% DH (CMHP1); 8: CMP hydrolysate sample by Protamex with 20% DH (CMHP2).

Figure 3 - Anti-inflammatory activities of protein hydrolysate obtained from Argentine croaker. Bars represent the standard deviation (n=3).

Different letters indicate significant differences ($p < 0.05$) between the different concentrations tested. 1: CPI hydrolysate sample by Alcalase with 10% DH (CPHA1); 2: CPI hydrolysate sample by Alcalase with 20% DH (CPHA2); 3: CPI hydrolysate sample by Alcalase with 20% DH (CMHA1); 4: CMP hydrolysate sample by Alcalase with 20% DH using (CMHA2); 5: CPI hydrolysate sample by Protamex with 10% DH (CPHP1); 6: CPI hydrolysate sample by Protamex with 20% DH (CPHP2); 7: CMP hydrolysate sample by Protamex with 10% DH (CMHP1); 8: CMP hydrolysate sample by Protamex with 20% DH (CMHP2).