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Fractionation of Protein Hydrolysates of Fish and Chicken Using Membrane Ultrafiltration: Investigation of Antioxidant Activity

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Abstract In this work, chicken and fish peptides were obtained using the proteolytic enzymes α-Chymotrypsin and Flavourzyme. The muscle was hydrolyzed for 4 h, and the resulting peptides were evaluated. Hydrolysates were produced from Argentine croaker (*Umbrina canosai*) with a degree of hydrolysis (DH) of 25.9 and 27.6 % and from chicken (*Gallus domesticus*) with DH of 17.8 and 20.6 % for Flavourzyme and α-Chymotrypsin, respectively. Membrane ultrafiltration was used to separate fish and chicken hydrolysates from Flavourzyme and α-Chymotrypsin based on molecular weight cutoff of >1,000, <1,000 and >500, and <500 Da, to produce fractions (F1,000, F1,000–500, and F500) with antioxidant activity. Fish hydrolysates produced with Flavourzyme (FHF) and α-Chymotrypsin showed 60.8 and 50.9 % of peptides with a molecular weight of <3 kDa in its composition, respectively. To chicken hydrolysates produced with Flavourzyme and α-Chymotrypsin (CHC) was observed 83 and 92.4 % of peptides with a molecular weight of <3 kDa. The fraction that showed, in general, higher antioxidant potential was F1,000 from FHF. When added 40 mg/mL of FHF and CHC, 93 and 80 % of lipid oxidation in ground beef homogenates was inhibited, respectively. The composition of amino acids indicated higher amino acids hydrophobic content and amino acids containing sulfuric residues for FHF, which showed antioxidant potential.

Keywords Antioxidant activity · Chicken · Fish · Hydrolysate · Peptides · Ultrafiltration membranes

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Introduction

In recent years, there has been an increasing interest in identifying and characterizing bioactive peptides derived from plants and animals. The bioactive peptides are protein fragments that are inactive in the specific molecular structure of proteins. After being released by enzymatic hydrolysis, they may exert different physiological functions [1]. Meat and fish provide valuable sources of protein for many populations around the world; furthermore, meat and fish proteins offer huge potential as novel sources of bioactive peptides [2] displaying antioxidant effects. The chicken meat contained a considerable amount of histidine-containing dipeptides, carnosine (β-alanylhistidine) and anserine (β-alanyl-L-methylhistidine) [3]. Several endogenous antioxidants, e.g., glutathione, uric acid, spermine, carnosine, and anserine, which are characteristic for meat have been studied [4]. Both carnosine (β-alanyl-L-histidine) and anserine (*N*-β-alanyl-1-methyl-L-histidine) are antioxidative histidyl dipeptides and the most abundant antioxidants in meat [5]. The antioxidant activity of these dipeptides may result from their ability to chelate transition metals [6] and form complexes with, e.g., copper, zinc, and cobalt. These antioxidative peptides have also been reported to play a role in wound healing, recovery from fatigue and prevention of diseases related to stress [7]. Different peptides derived from hydrolyzed food proteins have demonstrated bioactive properties. These peptides have a size of 2 to 20 amino acids [8] and a molecular weight below 6,000 Da [9]. Based on its structural properties, its composition, and amino acid sequence, these peptides may play multiple roles, such as immunomodulatory [10], antimicrobial [11], antithrombotic [12], hypocholesterolemic [13] antihypertensive [14], and antioxidant [15–20]. In addition, several peptides were also characterized for having multifunctional properties [7].

Research focused on the peptides contained in protein hydrolysates of animal proteins showed that they contain molecules that can bring health benefits, which are promising for nutritional and pharmaceutical applications [21]. Free radicals are species containing one or more unpaired electrons, are produced in normal or pathological cell metabolism [22], and are involved in many chronic diseases such as diabetes, cardiovascular and neurodegenerative diseases and cancer. The superoxide ($O_2^{\cdot-}$) and hydroxyl radical ($HO^{\cdot}$), known as reactive oxygen species, are some of the free radicals that are formed in vivo [23] and can cause destructive and lethal cellular effects by oxidizing lipids, proteins, DNA, and enzymes.

In foods, lipid peroxidation generated by free radicals, is a major concern during the industrial processing and storage of products, as, despite decreasing the nutritional quality and food safety [24], they also lead to the development of secondary reaction products which are potentially toxic, and of undesirable flavors and odors and also a change in texture [17, 25].

The synthetic antioxidants such as butylated hydroxyanisole, butylated hydroxytoluene (BHT), propyl gallate, and tert-butyl hydroquinone are used to inhibit or retard the formation of free radicals, preventing lipid oxidation and the resulting deterioration of foods. However, the safety and negative perception of synthetic antioxidants by consumers restrict their applications in food products [26]. Although the synthetic compounds are efficient and of relatively low cost, special attention has been given to natural antioxidants because of a worldwide trend to avoid or minimize the use of synthetic additives [24, 27].

The ultrafiltration (UF) membrane technology has been of great importance for the purification, concentration, and fractionation of several products in various areas such as food, pharmaceutical, and biotechnology industries and has been one of the best available techniques for the enrichment of peptides [28]. However, there is little information about the antioxidant activity of muscle protein hydrolysates of chicken and no paper was found on the antioxidant activity of hydrolysates from Argentine croaker (*U. canosai*). In this research, fish and chicken hydrolysates were produced

enzymatically and fractionated by UF technique using membranes with molecular weight cutoff of 1,000 and 500 Da. Were obtained fractions with different molecular weight that were investigated in their antioxidant activity.

The objective of this study was to obtain peptide fractions from the hydrolysates using membranes UF, characterize the hydrolysates and fractions in relation to molecular weight distribution, and evaluate the antioxidant activity of fractions.

Materials and Methods

Raw Materials

The fish species used was croaker (*U. canosai*), donated by Pescal S/A industry, located in Rio Grande, RS, Brazil. After capture, the fish was fillet and the muscle (containing 18.2 % protein) was minced uniformly and stored frozen at −20 °C in polyethylene bags until use. The frozen chicken muscle (containing 21.4 % protein) was donated by Minuano Food Co. located in Lajeado, RS, Brazil, and stored frozen at −20 °C in polyethylene bags until use.

Chemicals and Enzymes

The enzymes used were Flavourzyme 1000 L® (mixture of endoprotease and exopeptidase from *Aspergillus oryzae*), donated by Novozymes Latin America (Araucaria, Brazil) and α-Chymotrypsin (endopeptidase obtained from bovine pancreas) provided by Sigma-Aldrich Co. (St. Louis, MO, USA). The other reagents used in this study were of analytical grade.

Preparations of Protein Hydrolysates

The muscle was homogenized (IKA®, RW28, Staufen, Germany) with phosphate buffer 0.2 mol/L at a ratio of 1:3 (*w*/*v*), considering the protein content. Before the reaction, suspensions were pre-incubated in jacketed glass reactor at optimum conditions for each enzyme (50 °C and pH 7.0 for Flavourzyme and 37 °C and pH 8.0 for α-Chymotrypsin) for 20 min. The hydrolysis reaction initiated by adding enzyme in the amount of 1 % (*w*/*w*) with stirring at 600 rpm (IKA®, RW28, Staufen, Germany) for 4 h.

Samples were taken at preset times (zero, 15, 30, 60, 120, and 240 min) to measure the DH according to the method trinitrobenzenesulfonic acid (TNBS) described earlier [29] for determining percentage of hydrolysed peptide bonds by measuring the number of free amino groups in the supernatant, by the reaction with TNBS. A standard leucine curve was prepared from a concentration of 0 to 1.6 mmol/L.

At the end of reaction time, the enzyme was thermally inactivated at 85 °C for 15 min with occasional shaking. The hydrolysates were centrifuged at 3,500×*g* (centrifugal Biosystems, MPW-350/350R, PR, Brazil) for 20 min to remove unhydrolysed material and the obtained supernatant was lyophilized (freeze dryer MicroModulyo, Edwards, Sussex, UK). Fish and chicken hydrolysates were produced with Flavourzyme (FHF; CHF) and α-Chymotrypsin (FHC; CHC) enzymes.

Electrophoresis

For the separation of peptides by molecular weights the technique of electrophoresis on polyacrylamide gel containing sodium dodecyl sulfate (SDS-PAGE) in a concentration of

12 % [30] was used. All samples were treated with β-mercaptoethanol (5 %). Gels were run at a constant voltage of 0.02 A for about 1 h and later it was increased to 0.04 A for more 1 h. The staining of the gels occurred in a solution of methanol (50 %, *v*/*v*), acetic acid glacial (6.8 %, *v*/*v*), and Coomassie Brilliant Blue-R (1 mg/mL) for 3 h. The gels were distaining in a solution of acetic acid (7.5 %, *v*/*v*) and methanol (5 %, *v*/*v*), renovating the solution until a clear gel was obtained. The molecular weight of peptides was determined by comparison with standards of molecular weight of 10–220 kDa (Bench MarkTM Protein Ladder, Invitrogen, SP, Brazil).

Fractionation by UF

The hydrolysates were fractionated using an UF cell (Advantec, UHP-76, Japan) under magnetic stirring with a capacity of 450 mL. UF membranes regenerated cellulose, 76 mm diameter (Amicon Inc., Beverly, MA, USA) with molecular weight cutoff of 500 and 1,000 Da were used. During filtration process pressure was applied with nitrogen, as indicated by the manufacturer of the membranes. A sample volume (10 mg/mL) was passed through each membrane where three fractions were collected: F1,000 retentate (fraction, >1,000 Da), permeate and retentate F1,000–500 (fraction, <1,000 and >500 Da), and F500 permeate (fraction, <500 Da). All fractions were then lyophilized and analyzed.

Molecular Weight Distribution by Gel Filtration

The molecular weight distribution of the protein hydrolysate was estimated by gel filtration chromatography on a fast performance liquid chromatography (FPLC AKTA-Amersham Biosciences, Sweden) equipped with a Superdex peptide 10/300 GL, containing 30 % acetonitrile and trifluoroacetic acid 0.1 % as eluent. The void volume of the column was determined with blue dextran 2000. The flow rate was 0.5 mL/minute and readings were made at 280 nm. For quantitative determination of the molecular weights, areas of peaks of the chromatograms were integrated. A calibration curve with ribonuclease A (13,700 Da), aprotinin (6,500 Da), angiotensin I (1,296 Da), and tryptophan (204 Da) was prepared as described earlier [31].

Antioxidant Activity

The antioxidant activity of the fractions of the hydrolysates of fish and chicken were evaluated using different tests that included the sequestration ability of the hydroxyl radical ($OH^{\cdot}$), the ability to sequester free radical 2,2-diphenyl-1-picryl-hydrazyl (DPPH), the sequestration ability of the ABTS free radical and reducing power.

Hydroxyl Radical Scavenging Activity

The ability of hydrolysates fractions to inhibit the hydroxyl radicals, formed by Fenton reaction by degradation of 2-deoxy-D-ribose, was determined [32]. A mixture containing 0.2 mL of 10 mmol/L $FeSO_4 \cdot 7H_2O$, 0.2 ml of 10 mmol/L EDTA, 0.2 mL of 10 mmol/L 2-deoxy-D-ribose, 0.2 mL of sample (1 mg/mL), and 1 mL of phosphate buffer solution (0.2 mol/L, pH 7.4) was homogenized with 0.2 mL of 10 mmol/L H_2O_2 and incubated at 37 °C for 4 h. Then 1 mL of 2.8 % TCA and 1 mL of 1 % TBA was added, and the samples were heated in a water bath at 100 °C for 10 min. Then cooled by immersion in a water bath and ice for 5 min and the absorbance (Abs) was read at 532 nm on a

spectrophotometer UV/VIS (ATI UNICAM Helios, Alpha, UK). The percentage inhibition was calculated as follow:

$$\text{Inhibition}(\%) = \left[\left(\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}\right)/\text{Abs}_{\text{control}}\right] \times 100$$

DPPH Free Radical Scavenging Activity

The free radical scavenging effect of DPPH was measured as described earlier [33] with some modifications. Samples of 1.0 mL with different hydrolysed fractions (zero, 0.5, 1.0, 2.5, and 5.0 mg/mL) were added in 1.0 mL of DPPH (0.1 mmol/L) in 95 % ethanol. The mixture was homogenized with a vortex (Phoenix, AP-56, Brazil) and kept 30 min at room temperature. The Abs of the resulting solution was measured at 517 nm in a spectrophotometer UV/VIS (ATI UNICAM Helios, Alpha, UK). Lower Abs represents a higher sequestering activity of DPPH, which was calculated according to equation 1. The values of the effective concentration (concentration able to inhibit 50 % of the oxidation (EC_{50})) were calculated from the inhibition percentage.

ABTS Radical Antioxidant Activity

The antioxidant activity was measured according to Re et al. [34] with some modifications. 2,2-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) was dissolved in water at a concentration of 7 mmol/L. The radical cation ($ABTS^{\cdot+}$) was produced by reaction of ABTS stock solution with potassium persulphate (2.45 mmol/L, final concentration) and keeping the mixture in the dark at room temperature for 16 h before use. The $ABTS^{+}$ solution was diluted in sodium phosphate buffer pH 7.4 (5 mmol/L) to an Abs of 0.7±0.02 to 734 nm. After addition of 20 mL of sample (5 mg/mL) to 2 mL of diluted $ABTS^{+}$, the mixture was homogenized with a vortex (Phoenix, AP-56, Brazil) and incubated in a water bath at 30 °C for 6 min. The Abs reading was carried out at 734 nm in a spectrophotometer UV/VIS (Hitachi U-2001, Tokyo, Japan). The synthetic antioxidant Trolox (soluble derivative of α-tocopherol) in concentrations 0–1,500 μM (in ethanol) was used as standard and the results were expressed as Trolox equivalent antioxidant capacity in mmol/g sample.

Reducing Power

The ability of hydrolysed fractions to reduce Fe^{3+} to Fe^{2+} was measured spectrophotometrically [35]. A volume of 2 mL of sample (5 mg/mL) was added in 2 mL phosphate buffer 0.2 moL/L (pH 6.6) and 2 mL of 1 % potassium ferricyanide. The mixture was incubated at 50 °C for 20 min, and thereafter was added 2 mL of 10 % TCA. A 2-mL aliquot was mixed with 2 mL of distilled water and 0.4 mL of 0.1 % ferric chloride in test tubes. After 10 min of reaction, the Abs of the resulting solution was read at 700 nm on a spectrophotometer UV/VIS (ATI UNICAM Helios, Alpha, UK). Ascorbic acid (AA) was used as standard for the construction of a calibration curve and reducing power is expressed as mol AA/g of sample.

Antioxidant Activity in Ground Beef

The antioxidant activity in ground beef was determined as described by Sakanaka [36]. Minced meat (5 g) was homogenized with 25 mL of 50 mmol/L HEPES buffer (pH 7.0). The mixture containing 0.8 mL of suspension of minced meat and 0.2 mL HEPES buffer or a

sample of a solution of a hydrolysate (hydrolysate in HEPES buffer) at final concentrations of 2.5, 5, 10, 20, and 40 mg/mL were incubated at 37 °C for 60 min. After incubation, the mixture was evaluated for the formation of substances that react with thiobarbituric acid reactive substances (TBARS). A stock solution of TBA in TCA was prepared containing 0.9 mol/L TCA and 0.03 mol/L TBA in 0.25 mol/L HCl. After slight warming and stirring to dissolve the components, 3 mL of 20 g/L BHT in absolute ethanol were added to 100 mL TCA/TBA stock solution. At appropriate intervals, aliquots of 1.0 mL of the mixture were added to tubes containing 2 mL TCA/TBA stock solution and immediately homogenized with a vortex (Phoenix, AP-56, Brazil). The samples were then heated to boiling for 10 min in a water bath, cooled to room temperature and centrifuged at 1,710×*g* (Biosystems, MPW-350/350R, PR, Brazil) for 10 min. The Abs of the supernatant was measured at 532 nm. TBARS were quantified from a standard curve of malondialdehyde using tetramethoxypropane.

Amino Acid Composition of Hydrolysate

The determination of the amino acid composition of samples of croaker muscle hydrolysate with Flavourzyme and chicken with α-Chymotrypsin were conducted by the Faculty of Medicine of Ribeirão Preto (UNESP, Ribeirão Preto, Brazil). The amino acids were obtained by hydrolysis of the peptides with HCl 6 mol/L for 22 h at 110 °C±1 °C [37].

Statistical Analysis

All determinations were performed in triplicate. The data for the different parameters were compared using analysis of variance using the program STATISTICA© version 7.0 (Statsoft Inc., Tulsa, OK, USA). Significant differences ($p<0.05$) between the results were identified using the Tukey test.

Results and Discussion

Enzymatic Hydrolysis

In this study, croaker muscle proteins and chicken were separately hydrolyzed with α-Chymotrypsin and Flavourzyme for the production of protein hydrolysates. The hydrolysates were fractionated by UF membranes. The three fractions obtained for each hydrolysate produced had their antioxidant activity evaluated. The extent of hydrolysis of proteins by proteolytic enzymes was estimated by the DH yielding in the hydrolysate fish values of 25.9 and 27.6 % and the chicken hydrolysate, 17.8 and 20.6 %, with Flavourzyme and α-Chymotrypsin, respectively. There was a rapid initial increase in DH, as the time increased, indicating a very high protein cleavage in the early hours of reaction. Then there was a stabilization of the hydrolysis reactions. The stabilization or reduction in the rate of reaction can be explained by inhibition of the enzyme by products that are formed in high levels of DH [38]. These products compete with the substrate causing none digestion or partial digestion of proteins. The shape of the curves of hydrolysis was similar to that reported in previous studies with salmon protein [39], tuna [40], chicken [41] and tilapia [28]. With the same substrate and the same amount of enzyme, α-Chymotrypsin showed higher DH values for hydrolysates of fish and chicken than Flavourzyme. The highest levels of DH obtained with α-Chymotrypsin suggest that this enzyme has more affinity for the substrate and thus more efficient than Flavourzyme for the production of protein hydrolysates of fish muscle and poultry. Generally,

the alkaline proteases such as α-Chymotrypsin exhibit higher activity than acid or neutral proteases [42, 43].

Electrophoresis

The hydrolysates were analyzed through electrophoresis (SDS-PAGE) in order to determine the molecular weight profile and also to confirm that hydrolysis had been reached. The electrophoretic profiles showed that the enzymatic treatments were effective in the degradation of both fish and chicken muscle, resulting in small peptides as shown in Fig. 1.

After hydrolysis, the presence of two peptide bands was observed, with an estimated molecular weight of 10 kDa and one below 10 kDa, for the fish muscle hydrolysate with the enzyme Flavourzyme. Rossini et al. [44] also observed the presence of two peptide bands with molecular weights estimated at 9 and 14 kDa for peptides with antioxidant activity of casein produced with Flavourzyme. Five peptide bands were observed for the chicken muscle hydrolysate with the enzyme α-Chymotrypsin. Four of these of higher molecular weight, being estimated in the range of 35, 27.5, and 20 kDa and the remaining bands with a molecular weight estimated at 15 kDa and less than 10 kDa [44].

In the study of Souissi et al. [38], the electrophoretic profiles of hydrolysates of *Sardinella aurita* showed a molecular weight range less than 14.2 kDa and two bands with molecular weights in the range of 55 and 30 kDa. According to these authors, this may be due to larger proteins present in the raw material or proteins that were not totally hydrolyzed by the enzyme.

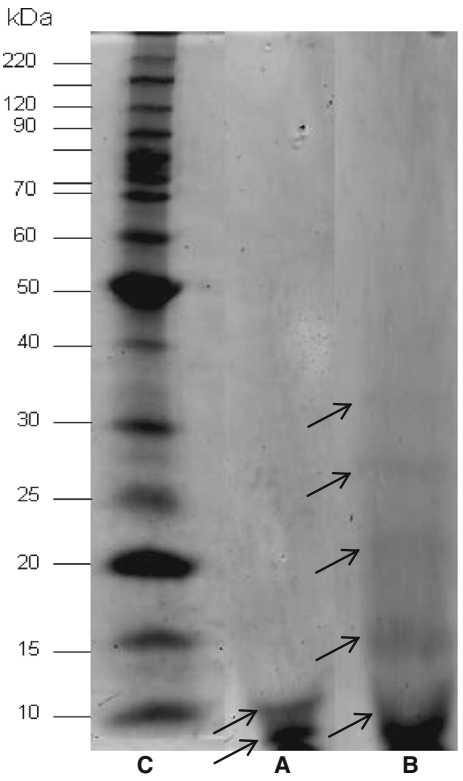

Fig. 1 Electrophoretic profile of fish hydrolysates with Flavourzyme (**a**), chicken hydrolysates with α-Chymotrypsin (**b**), and molecular weight standards (**c**)

During the hydrolysis, cleavage of the protein involves greater structural change, in which the protein is hydrolyzed into smaller peptide units [45]. Comparing the electrophoretic profiles, it was found that FHF (DH=25.9 %) showed bands with molecular weights lower than the CHC (DH=20.6 %), indicating that the enzyme Flavourzyme favored the hydrolysis of the muscle proteins.

Fractionation by UF

The use of the UF membrane is suitable to obtain protein hydrolysates with ranges of desired molecular weight [46]. During the UF process in two stages, three different fractions (from F1,000, F1,000–500, and F500 Da) were obtained using membranes with a cutoff of 1,000 and 500 Da. The ultrafiltrated (F1,000–500 and F500 Da) and retentate (F1,000 Da) were rich in peptides with molecular weights in accordance with the cutoff of its respective UF membrane used. However, a small percentage of peptides, above or below the cutoff of each membrane were found in the analyzed fractions.

According to some authors, the membranes don't perform separations with total efficiency, and some classes of molecular weights can be found in several fractions. A complicated phenomenon because of the physical and chemical factors occurs during the filtration of solutions containing proteins and proteinaceous solids, soluble and insoluble, which causes a concentration polarization and fouling/clogging of the membrane, and decrease in flow [47]. Given their hydrophobicity, interactions between proteins and membranes may occur and are inherent in the process. Depending on particle size, the proteins can enter the pores of the membrane or remain as a layer in gel form forming a deposit on the surface, significantly increasing the resistance to filtration [28].

Molecular Weight Distribution

The gel filtration chromatography, using FPLC system was used to study the distribution profiles of the molecular weight of fractions of fish (FHF and FHC) and chicken (CHF and CHC) protein hydrolysates obtained through UF membranes. From the chromatographic data (Figs. 2 and 3), there was a reduction of high molecular weight peptides.

Considering the whole hydrolysates before the UF process (data not shown), it can be seen that 60.8 % of the FHF and 50.9 % of the FHC consisted of small peptides, smaller than 3 kDa. Similar values were reported in hydrolysates obtained from silver carp with pancreatin and Neutrase, showing that 69.3 and 64.7 % of the peptides had molecular weight less than 3 kDa, respectively [48]. However, for CHF and CHC, 83 and 92.4 % of all peptides had molecular weight less than 3 kDa, respectively.

These results also agree with data published earlier [49], who report that hydrolysates of *Pollachius virens*, a species of the cod family produced with the enzyme Alcalase, consisted of 90 % of peptides less than 3 kDa. In general, the peptide fractions obtained from chicken muscle (Fig. 3) showed a higher percentage of low molecular weight peptides in relation to peptide fractions of the hydrolysate obtained from fish muscle (Fig. 2). Although the relative proportions of the peaks varied according to the porosity of the membrane used, the molecular weight distributions were similar when comparing the fractions with the same enzymes, both as fish and chicken peptides. From these results, it is verified that the muscle fish and chicken hydrolysates were probably formed from a mixture of peptides of small size.

All fractions, both fish and chicken hydrolysates showed high concentrations of peptides (above 80 %) according to the cutoff of the membrane used, except for F1,000, which retained

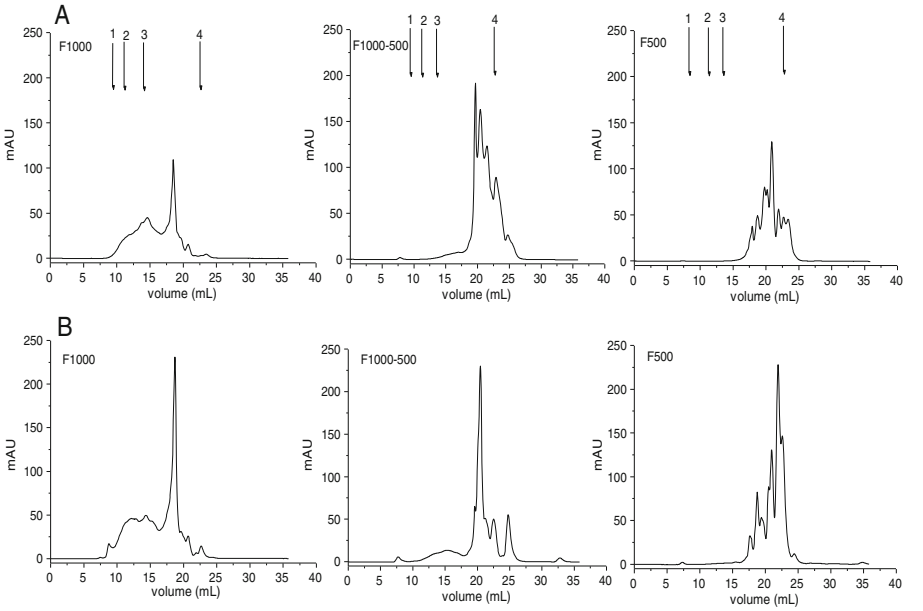

Fig. 2 Molecular weigth distribution of fraction of fish hydrolysates (F1,000, F1,000–500, and F500) with Flavourzyme (**a**) and α-Chymotrypsin (**b**). *1*, Ribonuclease A (13,700 Da); *2*, aprotinin (6,500 Da); *3*, angiotensin I (1,296 Da); and *4*, tryptophan (204 Da)

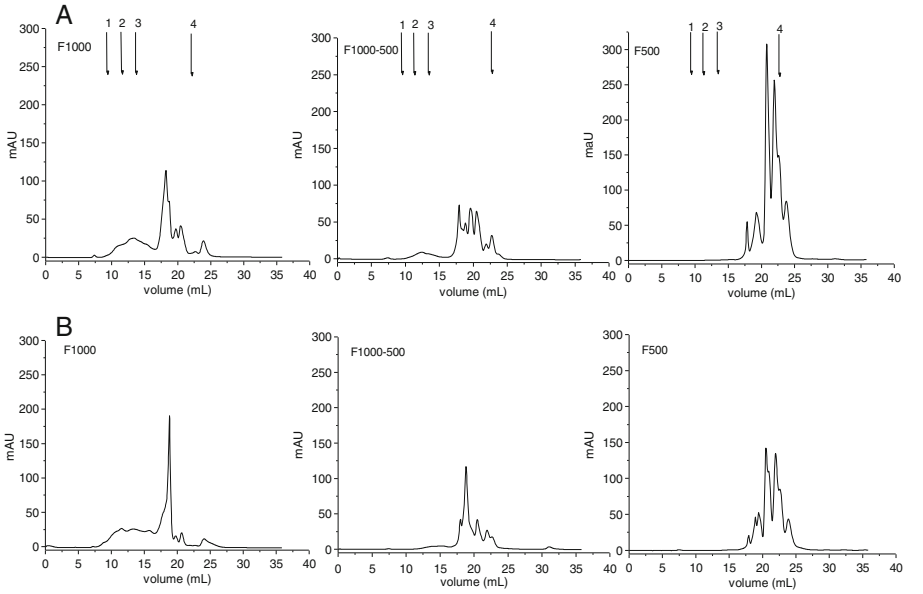

Fig. 3 Molecular weigth distribution of fraction of chicken hydrolysates (F1,000, F1000–500, and F500) with Flavourzyme (**a**) and α-Chymotrypsin (**b**). *1*, Ribonuclease A (13,700 Da); *2*, aprotinin (6,500 Da); *3*, angiotensin I (1,296 Da); and *4*, tryptophan (204 Da)

a high concentration of peptides with smaller molecular weight than the porosity of the membrane. This is probably due to the membrane fouling.

Antioxidant Activity of Fractions

Although there are many methods to evaluate the antioxidant capacity in food systems, none of these can be used as an official standard method. Therefore, it is suggested that each evaluation be done using various measurement techniques, in different media and oxidation conditions [50]. In general, considering the evaluation methods employed, it was observed that all fractions exhibited antioxidant activity.

Hydroxyl Radical Scavenging Activity

The sequestration ability of the hydroxyl radical was investigated by the method of oxidation of 2-deoxyribose. Figure 4 shows the effect of sequestration of the hydroxyl radical presented by the fractions of the hydrolysates of fish and chicken muscle. The fractions obtained from the hydrolysates of fish were more effective than the fractions obtained from chicken hyrdrolysates in the sequestration ability of the hydroxyl radical. F1,000–500, obtained from the FHF has reached 55.3 % inhibition with 1 mg/mL (Fig. 4a). Of the three fractions obtained for each hydrolysate using UF membranes, F1,000–500 showed the highest inhibition for both and fish chicken hydrolysates, except for the F1,000–500 of CHF (Fig. 4b). Furthermore, fish hydrolysates obtained with Flavourzyme were more efficient in sequestering ability of the radical $OH^{\cdot}$ than those obtained with α-Chymotrypsin (Fig. 4a). As for the chicken hydrolysates, the behavior was different and opposite where the α-Chymotrypsin produced fractions with more efficient antioxidant power.

DPPH Free Radical Scavenging Activity

Figure 5 shows the sequestration ability of DPPH of fractionated hydrolysates. The fraction F1000 for both fish and chicken hydrolysates produced with Flavourzyme and α-Chymotrypsin showed greater ability to sequester most of the DPPH radical concentrations. The EC_{50} values capable of inhibiting 50 % of the oxidation were 2.1 and 1.4 mg/mL for the

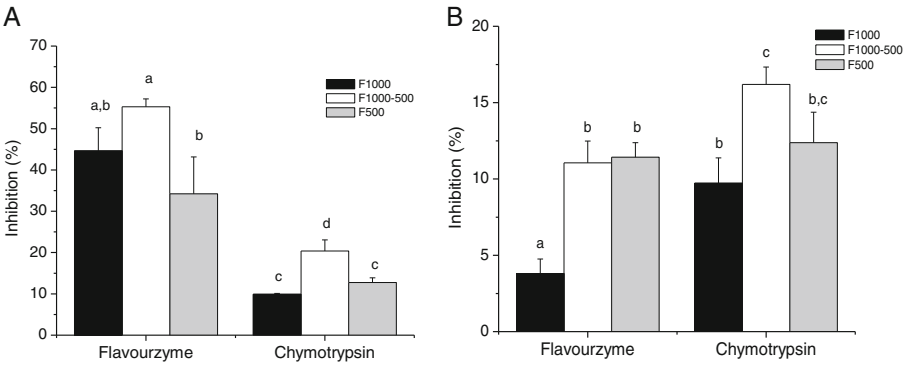

Fig. 4 Hydroxyl radical scavenging activity of fractions of fish (**a**) and chicken (**b**) hydrolysates. Values represent the mean±SD of three determinations. *Different letters* indicate significant differences ($p<0.05$)

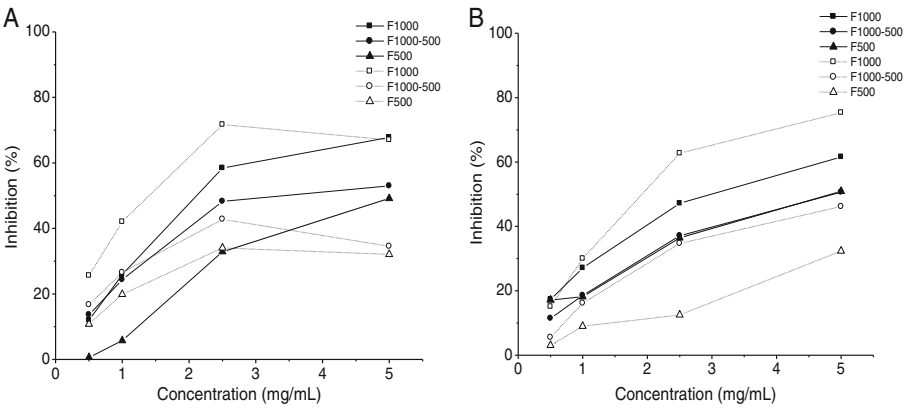

Fig. 5 DPPH radical scavenging activity of fractions of fish (**a**) and chicken (**b**) hydrolysates. *Solid line*, Flavourzyme; *dotted line*, α-Chymotrypsin. Values represent the mean of three determinations

F1,000 of FHF and FHC, respectively (Fig. 5a). For F1,000, of the CHF and CHC, the EC_{50} values were 2.9 and 1.9 mg/mL, respectively (Fig. 5b).

It was also observed that the ability to sequester the DPPH radicals presented by fractions of CHF, CHC, and FHF was dose dependent, in agreement with earlier results [51]. This tendency was not observed in the fractions obtained from the FHC, i.e., when the sample concentrations were increased above 2.5 mg/mL, the power of inhibition was not increased because at higher concentrations hydrolysates may have caused a pro-oxidant effect.

ABTS Free Radical Scavenging Activity

The method of capture of the ABTS radical is widely used to determine antioxidant activity and is reported as an assay applicable to discoloration of hydrophilic compounds and lipophilic compounds, indicating a reduction of color and reduction of the ABTS radical [52]. Figure 6 shows the ability and sequestration of ABTS radical of fractionated hydrolysates. The fraction F1,000, both for FHF and CHF showed greater ability to sequester ABTS radical, while for CHC and FHC the higher antioxidant potential is displayed by F1,000–500.

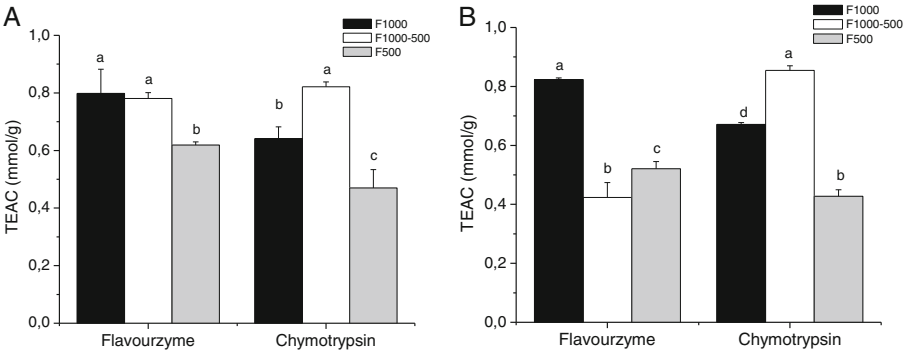

Fig. 6 ABTS radical scavenging activity of fractions of fish (**a**) and chicken (**b**) hydrolysates. Values represent the mean±SD of three determinations. Different *letters* indicate significant differences ($p<0.05$)

Peptides produced from the hydrolysis with different enzymes may vary in their ability to capture ABTS free radical. These authors also found that the ability to sequester free radical ABTS of *Priacanthus macracanthus* hydrolysates was higher in the fraction that contained peptides with molecular weight of 1,700 Da [53].

Reducing Power

The reducing power is the most effective method to assess the ability of an antioxidant in donating electrons [54]. Figure 7 shows the reducing power of the fractions of the fish and chicken hydrolysates. All fractions of the hydrolysates showed some antioxidant activity. The fractions of the hydrolysates prepared with fish muscle showed the greatest reducer potential than the fractions of the hydrolysates prepared with chicken muscle. This is probably due to higher DH values displayed by the fish hydrolysates (25.9 and 27.6 % for FHC and FHF respectively) or due to increased availability of hydrogen ions produced during the hydrolysis of protein [54].

According to these results, we can say that F1,000 obtained from FHF and CHF was the part that most contributed to the activity of these hydrolysates. Moreover, F1,000–500 was the fraction that showed the greatest contribution to the antioxidant activity of hydrolysates produced with α-Chymotrypsin, following the same trend as the antioxidant activity measured with ABTS radical assay. In this study, strong antioxidant activity in F1,000 and F1,000–500 Da was observed. In general, for the fractions obtained from the hydrolysates of fish higher values of antioxidant activity of the enzyme Flavourzyme were observed. However, for the fractions obtained from chicken hydrolysates, better results were presented by the enzyme α-Chymotrypsin. These observations seem to agree with the findings for the whole hydrolysates. Small peptides are often related to higher antioxidant activity, however this was not observed in this study [55]. However, hydrolysates of mackerel (*Scomber austriasicus*) with molecular weight of approximately 1,400 Da, showed greater in vitro antioxidant activity against lipid peroxidation, sequestration ability of radical DPPH and reducing power, than peptides with molecular weights in the range of 900–200 Da [56].

Hake bones hydrolysates was produced and was observed that the fraction with molecular weight in the range of 1,000–3,000 Da, obtained after UF, showed the highest antioxidant potential [57]. These results suggest that the antioxidant activity of the protein or peptide depends, not only on its molecular weight, but also on nature and the composition of the

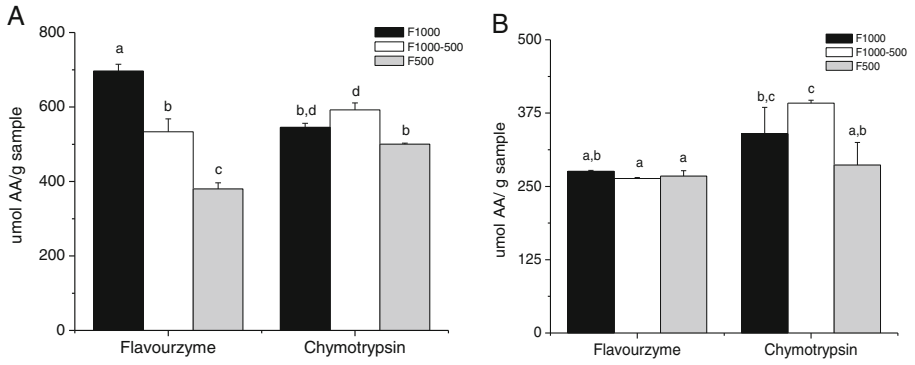

Fig. 7 Reducing power of fractions of fish (**a**) and chicken (**b**) hydrolysates. Values represent the mean±SD of three determinations. *Different letters* indicate significant differences ($p<0.05$)

different peptide fractions produced, properties such as hydrophobicity, the ability to transfer electrons from the amino acid residues in a sequence and hydrolysis conditions [27, 49]. The presence of the antioxidant activity using in vitro assays is based on different mechanisms and reflects the antioxidant properties of the multifunctional peptide. However, neither the structure activity nor antioxidant mechanism are yet fully understood [58].

In general, the fractionation improved the antioxidant properties of hydrolysates. Although antioxidant properties were detected in all tested peptide fractions, the highest molecular weight fractions from hydrolysates produced with Flavourzyme and α-Chymotrypsin presented the highest DPPH scavenging activity.

Antioxidant Activity in Ground Beef

The FHF and CHC hydrolysates, which showed good response to the antioxidant activity in vitro were tested in a model system of minced beef. The hydrolysates were added to the homogenized minced beef in different concentrations, and lipid oxidation was measured. The reaction was measured by monitoring the TBARS, and the results are shown in Fig. 8. According to some authors, the homogenized minced beef can be an effective means to investigate the protective effects of water-soluble antioxidants against lipid peroxidation [59]. The addition of minced beef, FHF and CHC inhibited respectively, 72 and 73.8 % of lipid oxidation added at a concentration of 20 mg/mL. When 40 mg/mL of CHC and FHF was added, 93 and 80 % of lipid oxidation was inhibited, respectively. It was observed that the hydrolysate produced with Flavourzyme was more efficient at inhibiting the oxidation and thus can be used as a good source natural antioxidant in meat systems. The study of Sakanaka et al. [35] showed 69.7 % inhibition of the oxidation as minced beef with peptides obtained from calcium caseinate using the same concentration. Whereas Rossini et al. [44] showed 100 % inhibition of oxidation using 20 mg/mL casein peptides obtained with Flavourzyme. Lee and Hendricks [58] reached 76.2 % inhibition with 20 mmol/L of carnosine (a dipeptide endogenous found in skeletal muscle of most vertebrates).

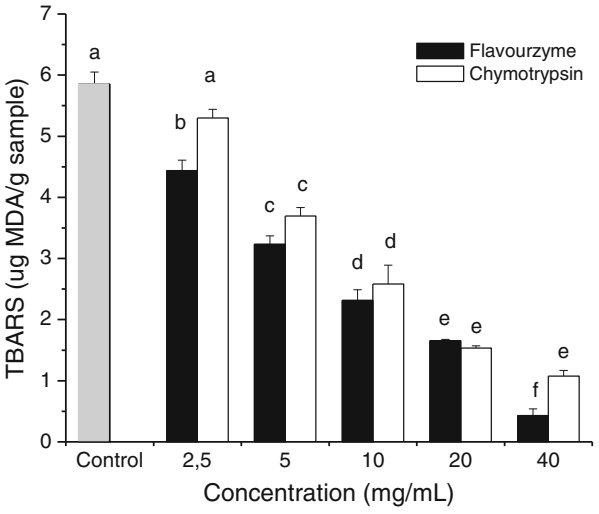

Fig. 8 Effect of fish (FHF) and chicken (CHC) hydrolysates on the formation of TBARS in ground beef homogenates. Values represent the mean±SD of three determinations. Different *letters* indicate significant differences ($p<0.05$)

Amino Acid Composition of Hydrolysate

The amino acid composition of the fish muscle hydrolysate obtained from Flavourzyme (FHF) and chicken muscle prepared with α-Chymotrypsin (CHC) is shown in Table 1. It was found that 34.0 and 32.9 % of the amino acids in the FHF and CHC, respectively, were comprised of hydrophobic amino acids. For hydrolysates of proteins and peptides, the greater the hydrophobicity, the higher the lipid solubility, and therefore the antioxidant activity increases [60]. The highest amount of histidine was observed in CHC. Histidine has the ability to sequester radical lipid by the imidazole ring [61]. However, the amount of amino acid containing sulfur residues (cysteine and methionine) was higher for FHF than CHC, which may have contributed more intensely to the higher antioxidant activity of this hydrolysate along with the higher content of hydrophobic amino acids, especially leucine, alanine, valine, and isoleucine. Similar results were observed for casein hydrolysates with Flavourzyme, which the amount of hydrophobic amino acids was 35.6 % (excluding tryptophan) [44]. It was found that 39.1 and 40.8 % of hydrophobic amino acids in protein hydrolysates of silver carp muscle (*Hypophthalmichthys molitrix*) were prepared with Flavourzyme for 1.5 and 4 h of hydrolysis, respectively [62].

Conclusions

The different fractions obtained showed appreciable levels of antioxidant activity and radical sequestering ability. The results showed that the fraction of fish hydrolysate obtained with the enzyme Flavourzyme with a molecular weight greater than 1,000 Da, presented the highest antioxidant activity. The hydrolysate obtained from fish muscle with Flavourzyme had a

Table 1 Amino acid composition of the fish (FHF) and chicken (CHC) hydrolysates (milligrams per gram protein)

Amino acids (%)	FHF[a]	CHC[a]
Aspartic acid	91.9±2.6	82.8±0.6
Threonine	43.5±2.6	40.6±0.2
Serine	45.3±1.2	41.7±3.6
Glutamic acid	167.8±0.8	162.3±2.5
Glycine	48.8±0.5	33.9±0.1
Alanine	63.3±0.1	52.3±0.3
Valine	50.6±1.1	47.0±3.0
1/2 cystine	4.6±0.1	3.3±0.3
Methionine	35.7±1.7	31.1±3.3
Isoleucine	45.6±1.5	45.2±0.7
Leucine	85.1±3.2	83.1±1.2
Tyrosine	19.8±1.4	29.5±3.7
Phenylalanine	40.4±0.3	40.9±0.7
Lysine	100.2±1.4	105.9±3.5
Histidine	24.5±0.6	61.7±0.7
Tryptophan	_[b]	_[b]
Arginine	47.3±0.7	71.48±1.0
Proline	85.7±1.4	64.27±0.6

[a]Amino acid composition of whole hydrolysates
[b]Not determined

higher content of hydrophobic amino acids and sulfur amino acids, leading to greater inhibition of lipid oxidation when used in a meat system.

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