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Antioxidant Activity of Protein Hydrolysates of Fish and Chicken Bones

G.S. Centenaro, M.S. Mellado and C. Prentice-Hernández
Laboratory of Food Technology, School of Chemical and Food,
Federal University of Rio Grande (FURG), P.O. Box 474, Eng. Alfredo Huch 475,
Postal Code 96201-900, Rio Grande, RS, Brazil

Abstract: Argentine croaker (*Umbrina canosai*) and chicken (*Gallus domesticus*) bones were hydrolyzed with different proteases (Flavourzyme, α-Chymotrypsin and Trypsin) in order to obtain peptides whit antioxidant activity. The hydrolysates showed different degrees of hydrolysis and antioxidant activity. The antioxidant power of the hydrolysates was evaluated through inhibition of the peroxidation of linoleic acid, hydroxyl radical scavenging, DPPH free radical scavenging, ABTS free radical scavenging and reducing power. The hydrolysates of the fish (FF) and chicken (CF) bones produced with Flavourzyme had high activity of lipid peroxidation inhibition (77.3 and 61.6%, respectively) and moderate DPPH free radical scavenging, ABTS scavenging and hydroxyl radical scavenging activity. The fraction <3000 Da was the main constituent of the six hydrolysates followed by the fraction <1000 Da. The results of this study suggest that protein hydrolysates of fish and chicken bones are good sources of natural antioxidants. FF showed better performance e can be used as antioxidant substance.

Key words: Antioxidant activity, argentine croaker, bones, chicken, enzymatic hydrolysis

INTRODUCTION

Each year, a considerable amount of by-products is generated by the fish and chicken processing industries. Annually the offer of by-products of animal origin in Brazil is equal to 8,8 million tons (Sincobesp, 2010). Bones, heads and viscera are commonly used for animal feed production, due to their low commercial value or they are discarded. However, the inadequate treatment of industrial residues can cause environmental problems. Thus, an alternative for utilization of such residues is to develop new products with higher value added. Fish and chicken bones are important sources of protein and minerals and one of the ways to exploit them is by recovering the proteins using enzymatic hydrolysis, which is widely used to improve and enhance nutritional and functional properties.

Lipid oxidation is of great concern to the food industry and consumers because it leads to the development of undesirable odors and flavors and potentially toxic reaction products (Lin and Liang, 2002). Moreover, the production of free radicals from oxidation may be associated with the onset of many diseases such as cancer, neurodegenerative and coronary diseases (Halliwell and Gutteridge, 1984; Diaz *et al*., 1997). Many synthetic antioxidants such as Butylated Hydroxyanisole (BHA), Butylated Hydroxytoluene (BHT), tert-Butylhydroquinone (TBHQ) and Propyl Gallate (PG) are used as food additives to prevent lipid peroxidation in many fields, especially in food (Wanita and Lorenz, 1996). However, the use of these antioxidant chemical compounds is restricted because of potential risks to health (Park *et al*., 2001). Therefore, the search for natural antioxidants as alternatives to synthetic ones is of great interest to researchers.

The antioxidant activity of proteins and peptides may be the result of scavenging of specific radicals formed during peroxidation, scavenging of compounds containing oxygen, or the chelating capacity of metals (Kristinsson and Rasco, 2000). Several studies have described the antioxidant activity of protein hydrolysates from animal sources like, egg yolk (Park *et al*., 2001), yellowfin sole (Jun *et al*., 2004), chicken essence (Wu *et al*., 2005), porcine collagen (Li *et al*., 2007), shrimp processing discards (Guerard *et al*., 2007), chicken crest (Rosa *et al*., 2008), cobia skin (Yang *et al*., 2008), tuna liver (Je *et al*., 2009), casein (Rossini *et al*., 2009), sardinelle by-products (Bougatef *et al*., 2010), backbone of Baltic cod (Zelechowska *et al*., 2010), and marine skin gelatins (Alemán *et al*., 2011). The objective of this study was to investigate the antioxidant activity of protein hydrolysates from fish and chicken bones, which are usually discarded during the processing.

Corresponding Author: G.S. Centenaro, Laboratory of Food Technology, School of Chemical and Food, Federal University of Rio Grande (FURG), P.O. Box 474, Eng. Alfredo Huch 475, Postal Code 96201-900, Rio Grande, RS, Brazil

MATERIALS AND METHODS

Materials and chemicals: Bones from the Argentine croaker, produced after the filleting process (containing 15.1% protein) were donated by Pescal S/A Co. (Rio Grande, RS, Brazil). Chicken bones (containing 18.1% protein) mechanically separated from the muscle were donated by Minuano Food Co. (Lajeado, RS, Brazil). Both of the raw materials were ground in a knife mill (Thomas - Wiley Mill) and stored at -20ºC in plastic bags until ready to use. The study was carried out between 2009 and 2010 in the Food Technology Laboratory of School of Chemical and Food, Federal University of Rio Grande, Brazil. Flavourzyme 1000L® (EC 3.4.11.1, mixture of endoproteinase and exopeptidase of *Aspergillus oryzae*) was donated by Novozymes Latin America (Araucária, PR, Brazil). Trypsin (EC 3.4.21.4) and α-Chymotrypsin (EC 3.4.21.1), endopeptidases obtained from bovine pancreas, were supplied by Sigma-Aldrich Co. (St. Louis, MO, USA). All other reagents and solvents were analytical grade.

Removal of non-collagenous protein and demineralization process: The non-collagenous proteins were removed from the bones according to the procedure described by Skierka *et al*. (2006), with some modifications. The milled samples were mixed with 0.1 M NaOH (1:2, w/v) solution, homogenized at 600 rpm in mechanical stirrer (IKA®, RW 28, Germany) and kept at 4ºC for 24 h. The mixture was centrifuged for 20 min at 9,000 × g using a centrifuge (Biosystems, MPW-350/350R, Brazil) and the supernatant was discarded. This procedure was repeated twice. The material, after alkaline extraction, was washed thoroughly with cold tap water to remove remaining muscle proteins and NaOH until the wash water reached neutral pH. The deproteinised samples were demineralized with 1.0 M HCl solution (1:4, w/v). Firstly the mixture of bones and hydrochloric acid was homogenized at 600 rpm in a mechanical stirrer (IKA®, RW28, Germany) for 2 min and kept for 48 h at 4ºC. The solution was changed every 24 h and then filtered in cotton-cloth. The demineralized bones were stored at -20ºC in plastic bags until use.

Preparations of protein hydrolysates: The demineralized chicken and fish bones were homogenized in mechanical stirrer (IKA®, RW 28, Germany) with 0.2 M phosphate buffer solution at a ratio of 1:3 (w/v) considering the protein content. Before the beginning of the reaction, the mixtures were pre-incubated for 20 min at the optimum conditions for each enzyme (50°C and pH 7.0 for Flavourzyme and 37°C and pH 8.0 for Trypsin and α-Chymotrypsin). The hydrolysis reaction began by adding 1% (w/w) enzyme under agitation at 600 rpm for 8 h. Samples were taken at pre-established time intervals (0, 15, 30, 60, 120, 240, 360 and 480 min) to measure the degree of hydrolysis (DH) according to the trinitrobenzenesulfonic acid (TNBS) method described by Adler-Nissen (1979). After the reaction, the enzyme was thermally inactivated at 85ºC for 15 min with occasional shaking. The hydrolysates were centrifuged for 20 min at 3,500×*g* using a centrifuge (Biosystems, MPW-350/350R, Brazil), to remove non-hydrolyzed residues. The supernatant was lyophilized (lyophilizer Edwards, Micro Modulyo, UK). A leucine calibration curve was prepared (0-1.6 mM). Six hydrolysates were produced and called: fish bone hydrolyzed with Flavourzyme (FF), fish bone hydrolyzed with α-Chymotrypsin (FC), fish bone hydrolyzed with Trypsin (FT), chicken bone hydrolyzed with Flavourzyme (CF), chicken bone hydrolyzed with α-Chymotrypsin (CC), chicken bone hydrolyzed with Trypsin (CT).

Determination of molecular weight distribution of the hydrolysates: The hydrolysates were characterized by gel filtration using an FPLC AKTA system (Amersham Biosciences, Sweden). The molecular weight distribution of the protein hydrolysates was determined on a 10/300 GL Superdex peptide (Amersham Biosciences, Sweden) column with 0.1% TFA in 30% acetonitrile as eluent. The flow rate was 0.5 mL/min and readings were carried out at 280 nm. Void volume was estimated with blue dextran 2000. A calibration curve with ribonuclease A (13700 Da), aprotinin (6500 Da), angiotensin I (1296 Da) and tryptophan (204 Da) was prepared.

Color determination: The color of the hydrolysates was measured with a colorimeter (Macbeth, Color-Eye® 3000, USA). Prior to measurements the equipment was standardized to a specific color blank. The samples were evaluated through the parameters L* (lightness) and chroma a* (green, - or red, +) and b* (blue, - or yellow, +). Whiteness (W) was calculated by the equation 1, such as described by Fujii *et al*. (1973):

$$W = 100 - [(100 - L^*)^2 + a^{*2} + b^{*2}]^{1/2} \quad (1)$$

Inhibition of the peroxidation of linoleic acid: The lipid peroxidation inhibition activity of hydrolysates was measured in a linoleic acid emulsion system according to the method of Osawa and Namiki (1985) with some modifications. Briefly, the hydrolyzed sample (5.0 mg) or standard α-tocopherol was dissolved in 10 mL of 50 mM phosphate buffer (pH 7.0), and added to a solution of 0.13 mL linoleic acid and 10 mL of 99.5% ethanol. The mixture was homogenized and the final volume was adjusted to 25 mL with deionized water. A control reaction was prepared using 50 mM phosphate buffer (pH 7.0). The mixture was incubated in screw-cap tubes at 40±1ºC in the dark. The degree of oxidation of linoleic

acid was measured according to Mitsuda *et al*. (1966), at intervals of 24 h for seven days. Aliquot (0.1 mL) of the incubated solution was mixed with 4.7 mL of 75% ethanol, 0.1 mL of 30% ammonium thiocyanate and 0.1 mL of 0.02 M ferrous chloride solution in 3.5% HCl. After 3 min, the degree of color development, which represents the oxidation of linoleic acid was measured by reading the absorbance at 500 nm on a spectrophotometer (Biospectro UV, SP-22, Brazil) and calculated according to Eq. (2).

$$\text{Inhibition (\%)} = [1 - (\text{Absorbance}_{\text{sample}}/\text{Absorbance}_{\text{control}})] \times 100 \quad (2)$$

Hydroxyl radical scavenging activity: The ability of the hydrolysates to inhibit hydroxyl radical-mediated 2-deoxy-D-ribose degradation was determined spectrophotometrically by the method of Chung *et al.* (1997). The reaction mixture containing 0.2 mL of $FeSO_4.7H_2O$ 10 mM, 0.2 mL of EDTA 10 mM, 0.2 mL of 2-deoxy-D-ribose 10 mM, 0.2 ml of sample (1 mg/mL) and 1 mL of phosphate buffer solution (0.2 M, pH 7.4) was mixed. Then 0.2 mL of H_2O_2 10 mM were added to the reaction mixture and incubated at 37ºC for 4 h. Thereafter 1 mL of TCA 2.8% and 1 mL of TBA 1% were added to the tubes. The samples were mixed and heated in a water-bath at 100ºC for 15 min. The mixture was cooled by immersion for 5 min in an ice/water bath. The absorbance was read at 532 nm in a UV/VIS spectrophotometer (ATI UNICAM Helios, Alpha, UK). The percentage of inhibition was calculated using Eq. (3).

$$\text{Inhibition (\%)} = [(\text{Absorbance}_{\text{control}} - \text{Absorbance}_{\text{sample}}) / \text{Absorbance}_{\text{control}}] \times 100 \quad (3)$$

DPPH free radical scavenging activity: The scavenging effect on the 2,2-diphenyl-1-picryl hydrazyl (DPPH) free radical was measured as described by Shimada *et al.* (1992), with modifications. A volume of 1.0 mL of sample (hydrolysate) in different concentrations (0.5, 1.0, 2.5 and 5.0 mg/mL) was added to 1.0 mL of 0.1 mM DPPH in 95% ethanol. The mixture was homogenized in a vortex (Phoenix, AP-56, Brazil) and kept for 30 min at room temperature in the dark. The absorbance of the solution was measured at 517 nm in a UV/VIS spectrophotometer (ATI UNICAM Helios Alpha, UK). The percentage inhibition was calculated using Eq. (4).

$$\text{DPPH radical scavenging activity (\%)} = \frac{(\text{Absorbance}_{\text{control}} - \text{Absorbance}_{\text{sample}})}{\text{Absorbance}_{\text{control}}} \times 100 \quad (4)$$

ABTS radical antioxidant activity: The antioxidant activity was determined according to Re *et al.* (1999), with some modifications. ABTS, 2,2-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) was dissolved in water at a concentration of 7 mM. ABTS cation radical ($ABTS^{\bullet+}$) was produced by reacting ABTS stock solution with 2.45 mM sodium persulfate (final concentration), and allowing the mixture to stand in the dark at room temperature for 16 h before using. The stock solution is used for a maximum of 3 days. In the moment of use, the solution of $ABTS^{\bullet+}$ was diluted with 5 mM sodium phosphate buffer (pH 7.4) until absorbance of 0.7 ± 0.02 at 734 nm. Samples were diluted (5 mg/mL) and 20 μL mixed with 2 mL of solution diluted $ABTS^{\bullet+}$, and then shaken in a vortex (Phoenix, AP-56, Brazil) and incubated in a water bath at 30ºC for 6 min. The absorbance was read at 734 nm in a UV/VIS spectrophotometer (Hitachi, U-2001, Japan). The standard curve of Trolox (0 a 1.5 mmol/L) was prepared and the activity was expressed as mmol Trolox equivalent (TE)/g protein.

Reducing power: The ability of the hydrolysates to reduce $Ferro^{3+}$ to $Ferro^{2+}$ was measured spectrophotometrically by the method of Oyaizu (1988) with some modifications. A volume of 2 mL of sample (5 mg/mL) was mixed with 2 mL of 0.2 M phosphate buffer (pH 6.6) and 2 mL of 1% potassium ferricyanide. The mixture was incubated at 50ºC for 20 min, and 2 mL 10% TCA was added to the reaction. Then 2 mL from each incubated mixture was mixed with 2 mL of distilled water and 0.4 mL of 0.1% ferric chloride in test tubes. After a 10 min reaction, the absorbance of the resulting solution was measured at 700 nm in a UV/VIS spectrophotometer (ATI UNICAM Helios, Alpha, UK). The increase in absorbance of the reaction indicates an increased reducing power.

Statistical analysis: The results are expressed as means ± standard deviation (SD), of three repetitions. All data were subjected to Analysis of Variance (ANOVA) and significant differences ($p<0.05$) between the results were identified using Tukey's test. The STATISTICA© program version 6.0 (Statsoft Inc, Tulsa, OK, USA) was used for data analysis.

RESULTS AND DISCUSSION

Enzymatic Hydrolysis: In this study, fish and chicken bones were separately hydrolyzed by Flavourzyme, α-Chymotrypsin and Trypsin to produce antioxidant peptides. The extent of protein degradation by proteolytic enzymes was measured by assessing the degree of hydrolysis. The degree of hydrolysis (DH) is the most widely used indicator for comparing different protein hydrolysates (Bougatef *et al*., 2010). DH values of 9.7, 8.5 and 6.5% were found for the fish bone hydrolysates with Flavourzyme, α-Chymotrypsin and Trypsin and 13.1,13.0 and 5.6% for the chicken bone hydrolysates

Table 1: Color parameters of protein hydrolysates of fish and chicken bones

Amostras	*L**	*a**	*b**	(W)
FF	90.6±0.14^{a,b}	-0.0445±0.02^{a}	6.8±0.05^{a}	88.4±0.08^{a}
FC	92.4±0.49^{c}	-0.3705±0.08^{b,d}	4.9±0.003^{b}	90.9±0.41^{b}
FT	90.4±0.28^{a}	-0.2135±0.08^{a,d}	7.4±0.04^{c}	87.9±0.20^{a}
CF	91.1±0.01^{b}	-0.5030±0.02^{c,d}	4.5±0.001^{d}	90.0±0.01^{c}
CC	91.2±0.15^{b}	-0.5275±0.01^{c,d}	5.9±0.10^{e}	89.3±0.07^{d}
CT	90.4±0.11^{a}	-0.3695±0.11^{d}	5.8±0.21^{e}	88.8±0.20^{a,d}

Values represent the mean±SD of three determinations. Different letters indicate significant differences ($p<0.05$)
FF = fish bone hydrolyzed with Flavourzyme; FC = fish bone hydrolyzed with α-Chymotrypsin; FT = fish bone hydrolyzed with Trypsin; CF = chicken bone hydrolyzed with Flavourzyme; CC = chicken bone hydrolyzed with α-Chymotrypsin; CT = chicken bone hydrolyzed with Trypsin

with Flavourzyme, α-Chymotrypsin and Trypsin, respectively (Fig. 1). The DH values for FF and FC were significantly equal ($p < 0.05$), as were CF and CC, which indicates that the enzymatic hydrolysis with Flavourzyme and α-Chymotrypsin resulted in increased soluble protein after 8 h, with possible release of free amino acids. The hydrolysates produced with Trypsin had lower DH values. Hinsberger and Sandhu (2004) reported that may be due to the enzyme selectivity, which catalyzes only the hydrolysis of the peptide bonds of the carbonyl group in the basic amino acids arginine or lysine.

Color: The color parameters of the hydrolysates are shown in Table 1. The color is an extremely important attribute for selling food, and is considered to be the primary criterion for acceptance or rejection of a product. In general, the fish bone hydrolysates were cream in color, slightly darker than the hydrolysates of chicken bones, which were white. FF and CF were equal ($p>0.05$) in *L** and FC, FT, CF and CC were similar in *a** parameter. CF had the lowest value of *b** (4.5) while FT was the most yellow with *b** value of 7.4. According to Zelechowska et al. (2010) the more pronounced color in the fish hydrolysates may be due to the presence of residual muscle protein that has not been completely removed, even after deproteinization and demineralization treatments. Furthermore, the characteristics of each raw material may also contribute to this difference, since they are two different animal species. The hydrolysates had significant differences ($p<0.05$) in W values, but there was no clear relationship between the raw materials and the enzymes used.

Antioxidant activity of protein hydrolysates: Protein hydrolysates that are produced using different enzymes have differently sized peptides and amino acid sequences that can determine their antioxidant capacity. It is well known that the radical system used for antioxidant evaluation may influence the experimental results, and two or more radical systems are required to investigate the radical scavenging capacities of a selected antioxidant (Yu *et al*., 2002). The fish and chicken bone hydrolysates were lyophilized and their antioxidant activities were evaluated using different assays including inhibition of

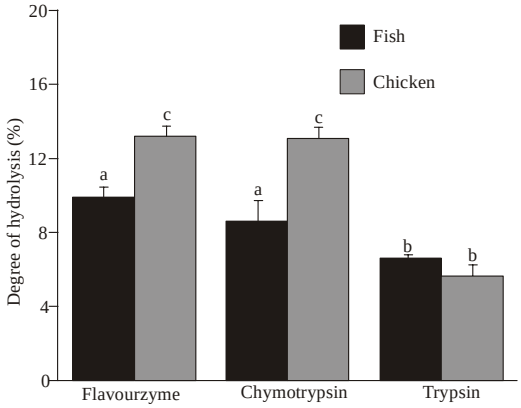

Fig. 1: Degree of hydrolysis (DH) of protein hydrolysates of fish and chicken bones produced with different enzymes, after 8 h of reaction. Values represent the mean±SD of three determinations. Different letters indicate significant differences ($p < 0.05$).

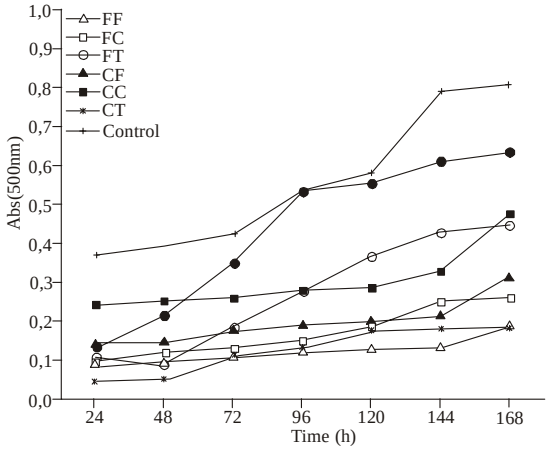

Fig. 2: Lipid peroxidation inhibition activity of hydrolysates and α-tocopherol. Values represent the mean±SD of three determinations.
Toco = α-Tocopherol,
FF = fish bone hydrolyzed with Flavourzyme
FC = fish bone hydrolyzed with α-Chymotrypsin
FT = fish bone hydrolyzed with Trypsin
CF = chicken bone hydrolyzed with Flavourzyme
CC = chicken bone hydrolyzed with α-Chymotrypsin
CT = chicken bone hydrolyzed with Trypsin

linoleic acid peroxidation, hydroxyl radical scavenging activity, DPPH free radical scavenging capacity, ABTS free radical scavenging capacity and reducing power.

Inhibition of the peroxidation of linoleic acid: The hydrolysates were evaluated using the model of lipid peroxidation inhibition in linoleic acid in emulsion as described above. As shown in Fig. 2, all hydrolysates can act as retarders of lipid peroxidation since they are capable of inhibiting oxidation. However, the antioxidant activity of these products was lower than that of α-tocopherol. Lower absorbance values at 500 nm represent a greater inhibition of lipid peroxidation. The control (no antioxidant) showed the highest absorbance values which indicate the higher degree of oxidation among samples. Among the fish bones hydrolysates, FF had the highest inhibition (77.3%) after 7 days of evaluation, similar to the natural antioxidant α-tocopherol (77.6%). Among the chicken bones hydrolysates, FF had the highest inhibition power (61.6%), which indicates that this hydrolysate might contain antioxidant peptides (data not shown). Moreover, it was found that the ability of the hydrolysates to inhibit lipid oxidation was influenced by DH. The protein hydrolysates produced with Flavourzyme presented better acting in the inhibition of the lipid peroxidation that specific enzymes α-Chymotrypsin and Trypsin. Hydrolysates with the highest DH values (FF, FC and CF) had a higher antioxidant activity, possibly due to factors such as the size and composition of peptides, which play an important role in the ability to delay or inhibit oxidation. CT stood out as having the lowest antioxidant power. Pihlanto (2006) reported that this might be due to the possibility that hydrolysates may contain both antioxidative and pro-oxidative components making them a less efficient antioxidant system against lipid oxidation.

Hydroxyl radical scavenging activity: Hydroxyl radical is a powerful oxidant particularly of lipids. The ability of quenching hydroxyl radicals by an antioxidant appears to be directly related to the prevention of propagation of lipid peroxidation process (Batista *et al*., 2010). The results in Fig. 3 show that the hydrolysates with higher DH, i.e., those prepared with Flavourzyme, had a significantly higher hydroxyl radical scavenging activity ($p<0.05$) than those obtained with α-Chymotrypsin and Trypsin, but CF did not significantly differ from CC. The percentage inhibition was 28.1 and 12.8%, respectively for FF and CF. The FF hydrolysates stood out, because of their higher scavenging capacity. This difference can be attributed to the presence of specific amino acids and peptides and their composition (Wu *et al*., 2003). The hydrolysates produced whit Flavourzyme from fish bones showed larger power scavenging when compared with chicken bones hydrolysates. Among the hydrolysates

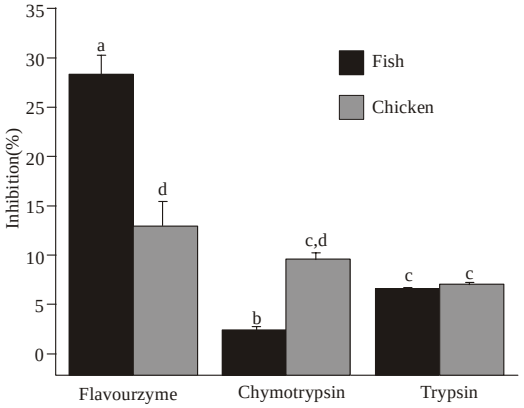

Fig. 3. Hydroxyl radical scavenging activity of fish and chicken bone hydrolysates. Values represent the mean ± SD of three determinations. Different letters indicate significant differences ($p<0.05$).

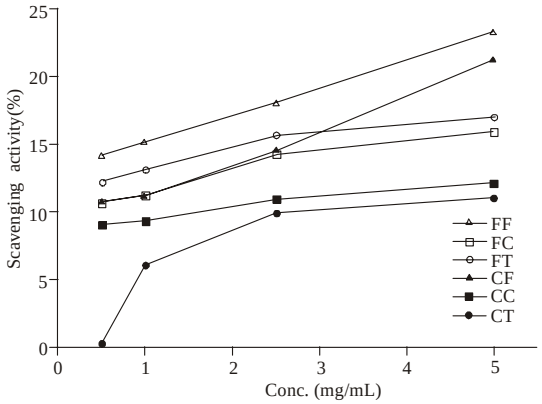

Fig. 4: DPPH radical scavenging activity of protein hydrolysates from fish and chicken bones. Values represent the mean ± SD of three determinations (SD ≤ 1%)

FF = fish bone hydrolyzed with Flavourzyme
FC = fish bone hydrolyzed with α-Chymotrypsin
FT = fish bone hydrolyzed with Trypsin
CF = chicken bone hydrolyzed with Flavourzyme
CC = chicken bone hydrolyzed with α-Chymotrypsin
CT = chicken bone hydrolyzed with Trypsin

produce with α-Chymotrypsin, larger scavenging effect it was reached with the of chicken bones hydrolysates. Trypsin showed the same effect on the two studied species.

DPPH free radical scavenging activity: DPPH is a stable free radical that shows maximal absorbance at 517 nm in ethanol. When DPPH encounters a proton-donating substance (H^+), the radical is scavenged by changing color from purple to yellow and the absorbance is reduced. In our DPPH test, the protein hydrolysates reduced the DPPH radical to a yellow-colored compound due to the

DPPH radical accepting an electron or hydrogen to become a stable diamagnetic molecule (Liu *et al*., 2010). Based on this principle, the antioxidant activity of a substance can be expressed as its ability to scavenge the DPPH radical. Figure 4 shows the results of the DPPH radical scavenging capacity of the fish and chicken bone hydrolysates, using different concentrations. It was observed that the scavenging effect increased with the concentration of hydrolysate, i.e., the results show a dose-dependent activity. The hydrolysates with the highest DH values produced with Flavourzyme had higher DPPH radical scavenging activity (23.3 and 21.3% for FF and CF, respectively), at 5 mg/mL. This indicates that the DH can strongly influence the antioxidant properties of the peptides. Similar results were reported for tuna bone protein (Je *et al.*, 2007) and cobia (*Rachycentron canadum*) skin (Yang *et al*., 2008). At concentrations below 2.5 mg/mL, no hydrolysate had DPPH radical scavenging activity above 20%, which is a relatively low value when compared with tuna liver hydrolysates produced by Je *et al*. (2009). The Flavourzyme again presented an effective effect in the hydrolysates production with DPPH radical scavenging activity, larger than the enzymes α-Chymotrypsin and Trypsin.

ABTS free radical scavenging activity: ABTS radical assay is a widely used method to measure antioxidant activity in which the radical is quenched to form ABTS radical complex (Binsan *et al*., 2008). The $ABTS^{\bullet+}$ is a relatively stable radical that is easily reduced by an antioxidant (Miller *et al*., 1993). By reducing the color of the ABTS radical, protein hydrolysates from various sources have been identified as potential antioxidants (Miliauskas *et al*., 2004; Rossini *et al*., 2009). In our study, the peptides from fish and chicken bones presented free radical scavenging activity and the results are shown in Fig. 5. This indicates that the peptides or free amino acids in the hydrolysates have the ability to donate hydrogen atoms to the free radicals, slowing the propagation of lipid peroxidation process (Faithong *et al.*, 2010). The TE values ranged from 0.109 to 0.248 mmol/g and the highest potentials were found in the hydrolysates obtained with the enzyme Flavourzyme (0.248 and 0.225 mmol/g for CF and FF, respectively), although FF did not significantly differ ($p>0.05$) from FC and CT. Similar results were reported by Faithong *et al.* (2010) using fermented shrimp products. This result indicates that the peptides produced may have a different amino acid compositions, sequences and chain lengths (Khantaphant Benjakul, 2008). Comparing the results of hydrolysates produced with the enzyme Flavourzyme (FF and CF) at concentrations of 1 mg/mL, it is evident that the hydroxyl radical scavenging activity was more effective than that of the DPPH radical. However, when comparing the antiradical activity of DPPH with ABTS, FF and CF

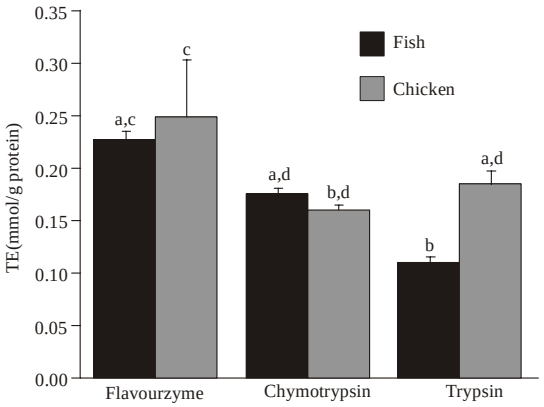

Fig. 5: ABTS radical scavenging activity of protein hydrolysates from fish and chicken bones. Values represent the mean ± SD of three determinations. Different letters indicate significant differences ($p<0.05$).

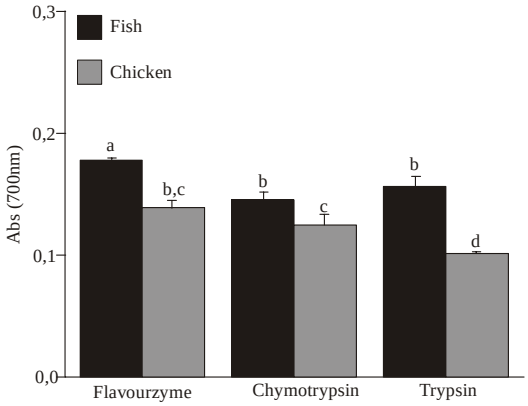

Fig. 6: Reducing power of fish and chicken bones hydrolysates produced with different enzymes. Values represent the mean±SD of three determinations. Different letters indicate significant differences ($p<0.05$).

presented higher ability to inhibit the ABTS radical at concentrations of 5 mg/mL, which suggests that the hydrolysates have different abilities to scavenge the radicals and hydroxyl, DPPH and ABTS.

Reducing power: The reducing capacity of a given compound may serve as a significant indicator of its potential antioxidant activity. In this assay, the presence of antioxidants caused the reduction of the Fe^{3+}/ ferricyanide complex to the ferrous form, and the yellow color of the test solution changed to various shades of green and blue depending on the reducing power of each compound. The Fe^{2+} was then monitored by measuring the formation of Perl Prussian blue at 700 nm (Ferreira *et al*., 2007). In general, the fish bone hydrolysates showed higher reducing power when compared with the chicken bone hydrolysates (Fig. 6). The highest reducing

Table 2: Molecular weight distribution of fish and chicken bones hydrolysates

	Peak area (%)*					
Molecular weight range (kDa)	FF	FC	FT	CF	CC	CT
>6	0.49±0.04	3.9±0.2	2.2±0.37	10.5±0.9	1.1±0.1	3.8±0.1
3-6	50.8±0.4	38.4±1.9	49.9±0.9	17.4±2.6	24.4±1.0	30.6±4.3
<3	48.7±1.3	57.7±6.6	47.8±2.3	72.0±4.0	74.5±2.4	65.6±4.4
<1	48.7±1.3	53.5±6.5	47.6±2.7	72.0±4.0	74.5±2.4	65.6±4.4

*Values represent the mean ± SD of three determinations (SD ≤ 1%).
FF = fish bone hydrolyzed with Flavourzyme; FC = fish bone hydrolyzed with α-Chymotrypsin; FT = fish bone hydrolyzed with Trypsin; CF = chicken bone hydrolyzed with Flavourzyme; CC = chicken bone hydrolyzed with α-Chymotrypsin; CT = chicken bone hydrolyzed with Trypsin

power was observed for FF, which had the highest DH among the fish hydrolysates, followed by FT and FC. Among the chicken hydrolysates, the highest reducing power was observed for CF, which also had the highest DH. However, the results of CF, FC and FT did not differ significantly ($p>0.05$). According to Je *et al.* (2009), higher reducing power can be attributed to the high content of peptides that are electron or hydrogen donors. The absorbance values were not higher than 0.2 using 5 mg/mL. Similar results were reported by Wu *et al.* (2005) with chicken essence extracts.

Molecular weight distribution: The molecular weight distribution of protein hydrolysates was determined using gel filtration chromatography and the results are shown in Table 2. CC had the highest percentage (74.5±2.4%) of peptides <3 and <1 kDa. The percentages of fractions 3-6 and >6 kDa were lower. In this study, all the hydrolysates had the peptide fractions <3 kDa as the main component. It was also found that the most effective protease for the hydrolysis of bone protein from fish and chicken were α-Chymotrypsin and Flavourzyme, followed by Trypsin.

CONCLUSION

This study allow to concluded that the antioxidant activity of the peptides differs according to the type of enzyme used, the degree of hydrolysis and the type of system in which antioxidants are tested. The fish and chicken bone hydrolysates had different molecular weight distributions resulting in different antioxidant behaviors. FF and CF presented high activity of lipid peroxidation inhibition (77.3 and 61.6%, respectively) and can effectively act as antioxidants. The Flavourzyme enzyme was more effective at producing antioxidant hydrolysates than α-Chymotrypsin and Trypsin, however, the highest proportion of low molecular weight peptides, for fish and chicken, was observed in hydrolysates with α-Chymotrypsin. The DH was not entirely related to the molecular weight distribution of hydrolysates, and, therefore, some other factors seem to be involved. Further studies will be carried out in order to fractionate the hydrolysates for future analyses.

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