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Antioxidant activity of several marine skin gelatins

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ABSTRACT

Gelatins obtained from the skins of tuna and halibut, and from the tunics of jumbo flying squid were hydrolysed by Alcalase to produce antioxidant peptides. Hydrolysis yielded an increase in the antioxidant capacity of gelatins of around two-fold when measured by the Fe reducing capacity (FRAP) method and even more when measured by the ABTS radical scavenging method. When both squid and tuna gelatins were hydrolysed with different enzymes (collagenase, trypsin, pepsin), Alcalase and pepsin gave the hydrolysates with the highest and lowest ABTS radical scavenging ability, respectively. FRAP assay showed that the squid hydrolysates prepared using Alcalase were the most effective in reducing ferric ions, whereas trypsin gave rise to the tuna hydrolysates with the highest iron reducing ability. When the amino acid composition of the gelatins was related to the antioxidant properties, ABTS radical scavenging was observed to be negatively correlated to the total content of hydrophobic amino acids and imino acids in all the samples, while Fe reducing power (FRAP) was strongly correlated with Hyl and degree of hydroxylation.

1. Introduction

Only about 25–30% of the fish and seafood catch is eaten, the rest is used for fishmeal or discarded as waste, representing high economic and environmental costs. With a view to reducing environmental waste, several strategies over the past 10 years have evolved to develop high-quality functional by-products that can be used as food for human consumption or even be biologically active with nutritional and medical benefits, which could increase their market value. Most of the waste is protein, mainly collagen, the principal component of skin, bones, blood vessels and cartilage. Gelatin has conventionally been extracted from the skin and bone collagens of certain mammalian species, primarily cows and pigs. But due to socio-cultural and safety considerations associated with these sources, other alternative sources like marine species have come into use. For technological purposes gelatin has been extensively employed as an ingredient to improve the elasticity, consistency and stability of foods, but it may also give rise to biologically active peptides by protease hydrolysis, which have shown a potential to act as inhibitors of angiotensin I converting enzyme or as antioxidants (Kim, Byun, Park, & Shahidi, 2001; Lin & Li, 2006).

Lipid oxidation occurring in food products causes the deterioration of food quality and shortening of shelf life. Furthermore, consuming oxidative foods is thought to cause serious diseases (Ames, 1983). Bioactive peptides with antioxidant properties derived from various proteins by enzymatic hydrolysis have become a topic of great interest for pharmaceutical, health, food and processing/preservation industries, since synthetic antioxidants such as BHT, BHA or n-propyl gallate exhibit strong antioxidant activity, but they are under strict regulations because of potential health hazards. Antioxidant activity has been reported for protein hydrolysates prepared from various fish protein sources such as whole capelin, tuna cooking juice, yellowfin sole frame, Alaska pollack frame, round scad muscle or Pacific hake muscle (Amarowicz & Shahidi, 1997; Jao & Ko, 2002; Je, Park, & Kim, 2005; Jun, Park, Jung, & Kim, 2004; Samaranayaka & Li-Chan, 2008; Thiansilakul, Benjakul, & Shahidi, 2007). For fish gelatin-derived peptides, there are also some reports dealing with their antioxidant properties (Giménez, Alemán, Montero, & Gómez-Guillén, 2009; Je, Qian, Byun, & Kim, 2007; Kim, Kim, et al., 2001; Lin & Li, 2006; Mendis, Rajapakse, Byun, & Kim, 2005; Mendis, Rajapakse, & Kim, 2005). The biological properties of peptides are to a large extent influenced by their molecular structure and weight, which are in turn greatly affected by processing conditions. Enzymatic hydrolysis is a useful method to obtain biological active peptides and modify protein functionality. The enzymes that can be selected for use alone or in combination result in a range of possible biological properties for the corresponding hydrolysates.

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The objective of this study was to evaluate the antioxidant ability of gelatin hydrolysates obtained by Alcalase from different marine species (squid, tuna and halibut) by means of different assays (Fe reducing capacity (FRAP) and ABTS radical scavenging ability). Furthermore, squid and tuna gelatins were hydrolysed with different enzymes (collagenase, trypsin, pepsin) and the antioxidant activity of the resulting hydrolysates was evaluated.

2. Materials and methods

2.1. Gelatin extraction

Frozen inner and outer tunics of jumbo flying squid (*Dosidicus gigas*) and tuna (*Thunnus* spp.) and halibut (*Hypoglossus* spp.) skins were provided by frozen fish processing companies (squid tunics: PSK Océanos, S.A., Vigo, Spain; tuna and halibut skins: Serpeska, S.A., Madrid, Spain). They were mechanically collected and stored at −20 °C until use (1 month), when they were thawed at 4 °C. The fish gelatins (from tuna and halibut) were extracted according to the method of Gómez-Guillén and Montero (2001). The squid gelatin was extracted according to the method of Giménez, Gómez-Estaca, Alemán, Gómez-Guillén, and Montero (2008). Briefly, the tunics were subjected to enzymatic digestion with pepsin from porcine stomach (EC. 3.4.23.1; 662 units/mg solid; Sigma–Aldrich, Inc., St. Louis, MO, USA) during a swelling step in 0.5 M acetic acid (2 °C/72 h). Subsequent gelatin extraction was done in distilled water at 60 °C/18 h. All the gelatin extracts were dried by heating at 45 °C in a forced-air oven until brittle sheets were formed.

2.2. Chemical composition

Moisture, ash, protein and lipid contents were determined according to the methods 950.46, 900.02A, 992.15 and 960.39, respectively (AOAC, 2005). Protein content was calculated by multiplying the total protein nitrogen by 5.4 (Giménez et al., 2008; Muyonga, Cole, & Duodu, 2004). The carbohydrate content was estimated by difference.

2.2.1. Amino acid composition

An amount of 1 mg/mL of dry gelatin was dissolved in distilled water and 50 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 containing 0.1% phenol with norleucine (Sigma–Aldrich, Inc.) as the internal standard. After hydrolysis, samples were again vacuum-dried, dissolved in application buffer, and injected onto a Biochrom 20 amino acid analyzer (Pharmacia, Barcelona, Spain). A mixture of amino acids was used as the standard (Sigma–Aldrich, Inc.). The percentage of nitrogen recovered was 98.5%. Determinations were performed in triplicate and the data corresponds to mean values. Standard deviations were in all cases lower than 6%.

The total degree of hydroxylation of the Pro and Lys in the gelatins was calculated as $[(\text{Hyp} + \text{Hyl})/(\text{Pro} + \text{Lys} + \text{Hyp} + \text{Hyl})] \times 100$. The degrees of Pro and Lys hydroxylation of the gelatins were calculated as $[\text{Hyp}/(\text{Pro} + \text{Hyp})] \times 100$ and $[\text{Hyl}/(\text{Lys} + \text{Hyl})] \times 100$, respectively.

2.2.2. Molecular weight distribution

An amount of 4 mg/mL of gelatin preparation was dissolved in loading buffer (10 mM Tris–HCl, 10% Glycine, 2.5% SDS, 5% mercaptoethanol, and 0.002% bromophenol blue) at 60 °C. Protein samples were heat-denatured (90 °C for 5 min). Gelatins were analysed by glycine-SDS-PAGE according to Laemmli (1970) using a 4% stacking gel and 6% resolving gel. The samples were run on a Mini Protean II unit (Bio-Rad Laboratories, Hercules, CA, USA) at 25 mA/gel. Loading volume was 15 μL in all lanes. Protein bands were stained with Coomassie Brilliant Blue R250. A high molecular weight-SDS calibration kit (Amersham Pharmacia Biotech, Uppsala, Sweden) comprising myosin (212 kDa), α_2-macroglobulin (170 kDa), β-galactosidase (116 kDa), transferrin (76 kDa), and glutamic dehydrogenase (53 kDa) was used to determine the approximate molecular weights of the gelatin fractions.

2.2.3. Protein hydrolysates

Gelatins (2.5% w/v) were dissolved in sodium phosphate buffer (0.01 M, pH 8) and subjected to enzymatic hydrolysis using Alcalase (EC 3.4.21.14, 2.4 L, 2.64 AU/g, Sigma–Aldrich, Inc., St. Louis, Mo., USA) with an enzyme–substrate ratio of 1:20 (w:w) at the optimal conditions for enzymatic activity (pH 8, 50 °C; Bougatef et al., 2010; Giménez et al., 2009) for 3 h. As squid and tuna Alcalase hydrolysates gave rise to the highest antioxidant activity, enzymatic hydrolysis of gelatins from these species was also carried out with collagenase type IA from *Clostridium histolyticum*, (EC 3.4.24.3, Sigma–Aldrich), trypsin type I from bovine pancreas (EC 3.4.21.4, Sigma–Aldrich) and pepsin (EC. 3.4.23.1, Sigma–Aldrich) with an enzyme–substrate ratio of 1:20 (w:w) for 3 h. The hydrolysis conditions used for these enzymes were as follows: collagenase (37 °C, pH 7.6), trypsin (37 °C, pH 7.5) and pepsin (37 °C, pH 4). The pH of the reaction was kept constant by addition of 1 N NaOH solution to the reaction medium using a pH-stat (TIM 856, Radiometer Analytical, Villeurbanne Cedex, France). For hydrolysis with pepsin, 1 N HCl solution was added to the reaction medium to keep the pH constant. The enzymes were inactivated by heating at 90 °C for 10 min, and the sample was centrifuged at 3000 *g* for 15 min (Heraeus Multifuge 3L, DJB Labcare Ltd., Buckinghamshare, England). The supernatants comprised the hydrolysates and were lyophilized and stored at −80 °C for further assays.

2.2.4. Degree of hydrolysis (DH)

When gelatin hydrolysis was performed by Alcalase, collagenase and trypsin, the DH, defined as the percentage of peptide bonds cleaved with respect to the total number of peptide bonds, was calculated by the method described by Adler-Nissen (1977) as follows:

$$\text{DH} = \left[(B \cdot N_b)/(\alpha \cdot M_p \cdot h_{tot})\right] \cdot 100$$

where B was the amount of alkali consumed to keep the pH constant during the reaction, N_b was the normality of the alkali, M_p was the mass of the protein substrate in the reaction (determined as N × 5.4), α was the average degree of dissociation of the α-NH_2 groups released during hydrolysis ($\alpha = 10^{(pH-pka)}/1 + 10^{(pH-pka)}$), and h_{tot} was the total number of peptide bonds per unit weight of gelatin, calculated from the amino acid composition.

In the case of the hydrolysis with pepsin, the DH was determined by the ratio of the percentage of 10% trichloroacetic acid (TCA)-soluble nitrogen to total nitrogen in the sample (Hoyle & Merritt, 1994). Aliquots were removed after hydrolysis and mixed with TCA to the final concentration of 10%. After 30 min at 4 °C, the mixture was centrifuged at 3000 *g* for 20 min and the supernatants were analysed for nitrogen using a LECO FP-2000 nitrogen/protein analyzer (LECO Corp., St. Joseph, MI, USA).

2.2.5. Antioxidant activities of the gelatins and their hydrolysates

The FRAP and ABTS assays were used to measure the antioxidant activity of the samples. FRAP is a measure of the reducing ability of samples and was performed according to the method described by Pulido, Bravo, and Saura-Calixto (2000). Both gelatins and hydrolysates were dissolved in distilled water. A 30 μL dissolved sample was incubated (37 °C) with 90 μL of distilled water and 900 μL of FRAP reagent (containing tripiridiltriazine (TPTZ, Sigma–Aldrich) and $FeCl_3$). Absorbance values were read at 595 nm after 30 min.

Results were expressed as μmol $FeSO_4 \cdot 7H_2O$ equivalents/g of sample (gelatin or hydrolysate) based on a standard curve of $FeSO_4 \cdot 7H_2O$, which relates the concentration of $FeSO_4 \cdot 7H_2O$ (μM) to the absorbance at 595 nm.

The ABTS radical (2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)) scavenging capacity of the gelatins and hydrolysates was evaluated according to the method of Re et al. (1999). The stock solution of ABTS radical (7 mM ABTS in potassium persulfate 2.45 mM) was kept in the dark at room temperature for 12–16 h. An aliquot of stock solution was diluted with distilled water to prepare the working solution of ABTS radical with absorbance of 0.70 ± 0.02 at 734 nm. A 20 μL aliquot of sample was mixed with 980 μL of ABTS reagent. The mixture was then left to stand at 30 °C for 10 min and absorbance values were read at 734 nm. Results were expressed as mg Vitamin C Equivalent Antioxidant Capacity (VCEAC) per g of sample (gelatin or hydrolysate) based on a standard curve of vitamin C (Sigma–Aldrich), which relates the concentration of vitamin C to the amount of absorbance reduction caused by vitamin C. All determinations were performed at least in triplicate.

2.2.6. Statistical analysis

Statistical tests were performed using the SPSS® computer program (SPSS Statistical Software, Inc., Chicago, IL, USA). Data were expressed as mean ± standard error of the mean ($n = 3$). Tukey's test was used at the level of significance at $p \leq 0.05$.

Multivariate analysis was run to assess the influence of the different amino acids on the antioxidant properties, independently of gelatin origin. The data from the amino acid analysis of the different species and those from ABTS and FRAP were used as the data matrix in the principal component analysis. Varimax rotation of principal components was used.

3. Results and discussion

3.1. Gelatins

The proximate composition of the gelatins is shown in Table 1. All the extracted dried gelatins had similar water content, lower than 10%. The fat content in the gelatins was not detectable. This is attributed to the extraction process, in which the skin/tunics were stirred three times in a salt solution to remove protein and fat residues before extracting the gelatin (Sarabia, Gómez-Guillén, & Montero, 2000). In addition, the fat content has been reported to be very low in the skins of other species, such as *Sebastes mentella* (Wang et al., 2008) and *Gadus morhua*, *Salmon salar*, and *Clupea harengus* (Kolodziejska, Skierka, Sadowska, Kolodziejski, & Niecikowska, 2008). The ash content varied considerably with gelatin origin despite the fact that the extraction process was similar for the three fish gelatins, and ash may partially consist of the sodium chloride used to remove the non-collagenous material during extraction. The estimated carbohydrate content was higher in the squid gelatin, suggesting that squid collagen is more glycosylated, although carbohydrates were also present in the other gelatins (Table 1). Nam, You, and Kim (2008) also observed a noticeable carbohydrate content in the skin of the squid (*Todarodes pacificus*), especially in the inner skin (~3.9%).

Table 1
Proximate composition (g/100 g) of the gelatins.

	Crude Protein	Crude fat	Moisture	Ashes	Carbohydrates
Squid	84.4 ± 0.6	–	9.65 ± 0.47	1.54 ± 0.18	4.44 ± 0.10
Tuna	89.6 ± 1.0	–	8.72 ± 0.98	1.45 ± 0.07	0.27 ± 0.03
Halibut	86.6 ± 1.8	–	9.12 ± 1.05	3.13 ± 0.10	1.20 ± 0.05

The molecular weight profiles of the gelatins were analysed by SDS-PAGE (Fig. 1). There were noticeable differences in the electrophoretic profiles between the gelatins. The native α chain ratio α1:α2 was accessed visually as approximately 2 in the tuna gelatin, but there was a predominance of α2 in the squid gelatin, and only one kind of α chain was predominant in the halibut skin gelatin, although it was difficult to determine whether it was the α1 or α2 chain. There were other essential differences among the gelatins. The tuna gelatin displayed a prevalence of high molecular weight bands (abundant β components with γ components and highly cross-linked aggregates). In contrast, the squid gelatin contained low molecular weight components (≤α chain up to low oligopeptides), attributed to the action of the pepsin during swelling prior to gelatin extraction at 60 °C. A similar electrophoretic profile was reported for squid tunic-derived gelatin obtained by heating at 80 °C without prior enzymatic digestion with proteases (Gómez-Guillén et al., 2002), although lesser amounts of α-chains were extracted and, as a consequence, the extraction yield was lower. The molecular weight distribution of halibut skin gelatin was intermediate, showing a high dispersion of molecular weights between γ and α components, as well as a significant content of low oligopeptides. The electrophoretic profiles described for gelatins from the skins of tuna, cod, hake or flatfish are generally characterized by the presence of β components and higher molecular weight components, as well as α1 and α2 chains (Carvalho et al., 2008; Gómez-Guillén, Ihl, Bifani, Silva, & Montero, 2007; Gómez-Guillén et al., 2002), and in the case of flatfish by an α1:α2 chain ratio of around two (Carvalho et al., 2008). However, according to the current results, gelatin from halibut (which is a flatfish species) did not exhibit this ratio. Very likely, the processing conditions before gelatin extraction altered or partially degraded the collagen in the halibut skin. Collagen has been reported to undergo proteolysis during chilled storage (Montero & Mackie, 1992). Kolodziejska et al. (2008) also observed partial degradation of skin gelatins from processed (salted and marinated) fish.

The amino acid composition of all the gelatins was typical of the collagen molecule (Table 2), with 33–34% Gly. The tuna gelatin had

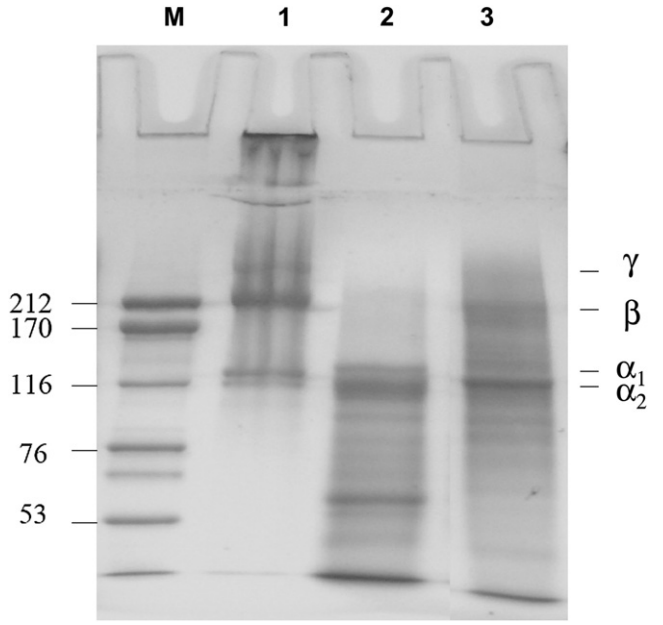

Fig. 1. Electrophoretic profiles of the gelatin preparations (SDS-PAGE) in the presence of 2-β-mercaptoethanol. M, molecular weight markers; 1, tuna gelatin; 2, squid gelatin; 3, halibut gelatin.

the highest Hyp contents, while the halibut gelatin had the lowest. Cysteine was only detected in the squid gelatin. The presence of cysteine is considered to reflect the presence of certain stroma proteins such as elastin (Morales, Montero, & Moral, 2000). The total number of imino acid (Pro + Hyp) residues was between 160 and 184 residues per 1000 residues, more similar to those values published for collagen from cold-water fish species (16–18%) (Wang et al., 2008). Hyp plays a key role in stabilizing the triple-stranded collagen helix through the hydrogen bonding ability of its hydroxyl group. The degree of Pro hydroxylation was similar in all the gelatins, independently of the source species. In contrast, the Hyl content and degree of Lys hydroxylation were sharply higher in the gelatin from the squid tunics. The values for the total degree of hydroxylation of the Pro and Lys in the gelatins were 47.4% for the squid, 38.8% for the tuna and 38.0% for the halibut. All these values were higher than those for other skin collagens reported for *S. mentella* (Wang et al., 2008), brownstripe red snapper (*Lutjanus vitta*) (Jongjareonrak, Benjakul, Visessanguan, Nagai, & Tanaka, 2005), bigeye snapper (*Pricanthus tayenus*) (Kittiphattanabawon, Benjakul, Visessanguan, Nagai, & Tanaka, 2005); and similar to those reported for Nile perch (*Lates niloticus*) (Muyonga et al., 2004). The degree of hydroxylation of Pro and Lys influences the thermal stability of collagen (Kimura, Zhu, Matsui, Shijoh, & Takamizawa, 1988), although Wang et al. (2008) attributed the lower denaturation temperature for deep-sea redfish collagens more to the lower imino acid content than to the degree of hydroxylation.

Pro and Ala were the most abundant hydrophobic amino acids in all the species, although there were clear differences. The tuna gelatin had the highest values of Pro, though the ratio with respect to the total hydrophobic amino acid content was steady. Hydrophobic amino acids have been observed in several antioxidant peptide sequences, and, in addition, certain researchers have suggested that the presence of hydrophobic amino acids in the peptide sequences in jumbo squid skin gelatin contributed greatly to its antioxidant properties (Mendis, Rajapakse, Byun, et al., 2005; Rajapakse, Mendis, Byum, & Kim, 2005).

Table 2
Amino acid composition of the gelatins.

Amino acid	Number of residues/1000 residues		
	Squid	Tuna	Halibut
Hyp	74	78	67
Asp	61	44	49
Thr	26	21	22
Ser	43	48	65
Glu	83	71	71
Pro	89	107	94
Gly	332	336	365
Ala	82	119	108
Cys	10	–	–
Val	37	28	15
Met	9	16	12
Ile	11	7	8
Leu	26	21	22
Tyr	8	3	3
Phe	10	13	13
His	7	7	5
Lys	12	25	25
Arg	61	52	50
Hyl	17	6	6
Imino acids (Pro + Hyp)	163	184	160
Pro hydroxylation (%)	45	42	42
Lys hydroxylation (%)	58	19	18
Pro + Lys hydroxylation (%)	47.4	38.8	38.0
Σ Hydrophobic aa[a]	596	647	637

[a] Σ Hydrophobic aa = Gly + Pro + Ala + Val + Met + Ile + Leu + Phe.

Table 3
Degree of hydrolysis (%) of squid, tuna and halibut gelatins after 3 h of enzymatic digestion.

	Alcalase	Collagenase	Trypsin	Pepsin
Squid	30.9 ± 0.6	12.1 ± 0.9	11.7 ± 0.5	29.0 ± 0.4
Tuna	25.6 ± 2.0	9.82 ± 0.43	8.55 ± 1.04	28.2 ± 1.2
Halibut	18.8 ± 1.5	–	–	

3.2. Protein hydrolysates

The hydrolysis of the squid, tuna and halibut gelatins with Alcalase resulted in about 31, 26 and 19 DH respectively, after 3 h at 50 °C (Table 3). In all cases, the hydrolysis rates were fast in the initial stage (15–20 min), and then gradually decreased until reaching a stationary phase where no apparent hydrolysis took place (data not shown). This kinetic profile is similar to those of classic protease-induced hydrolysis of protein (Gbogouri, Linder, Fanni, & Parmentier, 2004) and has been previously reported for different protein substrates such as fish proteins (Benjakul & Morrisey, 1997; Bougatef et al., 2009; Guérard, Guimas, & Binet, 2002; Klompong, Benjakul, Kantachote, & Shahidi, 2007), whey proteins (Mutilangi, Panyam, & Kilara, 1995) or wheat gluten (Kong, Zhou, & Qian, 2007). The molecular weight distribution of the starting gelatins does not seem to influence the accessibility of the gelatin chains to the enzyme attack. Thus, tuna gelatin, with high molecular weight components (abundant β components with γ components and highly cross-linked aggregates) gave rise to a DH higher than halibut gelatin, with a significantly lower amount of high molecular weight bands.

The DH may significantly influence the antioxidative activity of the resulting hydrolysates, since DH greatly influences the peptide chain length as well as the exposure of the terminal amino groups of products obtained (Thiansilakul et al., 2007). Fig. 2 depicts the antioxidant activity of squid tunic gelatin Alcalase hydrolysate with different DH obtained during the course of the hydrolysis. ABTS radical scavenging ability was sharply increased by increasing the DH, with the highest activity at 31% DH, whereas the degree of increase of Fe reducing power was not noticeable. Therefore, the hydrolysis most likely increased the antioxidative activity of the resulting hydrolysate via the enhancement of radical scavenging activity. Other studies (Raghavan, Kristinsson, & Leeuwenburgh, 2008; Thiansilakul et al., 2007) have also reported that fish protein hydrolysates with a higher degree of hydrolysis showed greater antioxidant activity, likely due to the presence of high amounts of low molecular weight peptides.

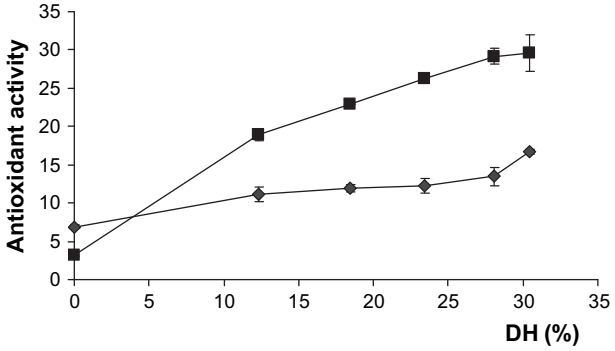

Fig. 2. Fe reducing ability (FRAP) (◆), expressed as μmol $FeSO_4 \cdot 7H_2O$ equivalents/g gelatin, and ABTS radical scavenging capacity (■), expressed as mg of ascorbic acid equivalents/g gelatin, with respect to the percentage DH of the squid gelatin hydrolysate.

Regarding the hydrolysis of squid and tuna gelatins with various enzymes, Alcalase was more efficient than collagenase and trypsin. After 3 h of hydrolysis, the DH assessed for squid gelatin by the pH-stat method reached about 12% for collagenase and trypsin, and 31% for Alcalase, whereas the DH for tuna gelatin attained about 10%, 9% and 26% for collagenase, trypsin and Alcalase, respectively (Table 3). Alcalase is a bacterial protease with low specificity and is a relatively effective enzyme preparation for hydrolysis of fish protein (Benjakul & Morrisey, 1997). This enzyme preparation's broad specificity and efficiency, under these assay conditions, are the reasons for the high degree of hydrolysis usually obtained (Ravallec-Plé, Gilmartin, van Wormhoudt, & Le Gal, 2000). Thus, hydrolysis of yellowfin tuna stomach protein (*Thunnus albacores*) with 45.3 AU/kg Alcalase during 6 h gave rise to DH about 23% (Guérard, Dufossé, De La Broise, & Binet, 2001). Ravallec-Plé et al. (2000) also observed extensive hydrolysis of cod muscle (about 31%) with 1% Alcalase. In the case of pepsin, a similar DH was reached in both squid and tuna gelatins after 3 h of hydrolysis (about 29% and 28% for squid and tuna gelatins, respectively).

3.3. Antioxidant properties of the gelatins and their hydrolysates

The antioxidant activity of the gelatins and the hydrolysates obtained after digestion with Alcalase for 3 h was evaluated on the basis of Fe reducing capacity (FRAP) (Fig. 3a) and ABTS radical scavenging ability (Fig. 3b). All the gelatins, but especially the squid gelatin, showed certain antioxidant properties as measured by radical scavenging ability and Fe reducing capacity. Gelatin has been observed to possess certain antioxidant properties in previous work, but the mechanism is uncertain (Giménez et al., 2009). The hydrolysis of gelatin chains gave rise to a noticeable increase in the antioxidant capacity for both measurement methods, FRAP and especially ABTS. Squid, halibut and tuna hydrolysates showed approximately a 2-fold higher Fe reducing ability than the corresponding gelatins ($p \leq 0.05$). For ABTS, hydrolysates also showed a significantly higher Vitamin C Equivalent Antioxidant Capacity (VCEAC) than the starting substrates, especially the squid hydrolysate (10-fold increase), followed by tuna (7.5-fold increase) and halibut hydrolysates (3-fold increase). For the hydrolysis of squid and tuna gelatins with various enzymes (Fig. 4), Alcalase and pepsin gave hydrolysates with the highest and lowest ABTS radical scavenging ability, respectively (Fig. 4b). In the case of squid, the ability of the hydrolysate to scavenge ABTS radicals significantly decreased in the order Alcalase > collagenase > trypsin > pepsin ($p \leq 0.05$). The hydrolysates obtained from tuna gelatin showed lower radical scavenging capacity than squid hydrolysate in most cases, and this capacity decreased in the order Alcalase > trypsin > collagenase > pepsin ($p \leq 0.05$). On the other hand, all the squid hydrolysates showed a significantly higher Fe reducing power (FRAP) than the corresponding tuna hydrolysates (Fig. 4a). FRAP values decreased in the order Alcalase > trypsin > collagenase ~ pepsin in the case of squid hydrolysate and trypsin > pepsin > Alcalase ~ collagenase in the case of tuna hydrolysate.

Most researchers agree that the antioxidant activity of peptides may not be attributable to a single antioxidant mechanism, probably because the properties derive from the presence of different amino acids favouring one mechanism over others. For instance, generally speaking, peptides rich in hydrophobic amino acids are expected to inhibit lipid peroxidation both as proton donors to hydrophobic peroxyl radicals and as chelators of metal ions. In this sense, peptides containing His in their sequences have been reported to act as metal ion chelators (Chen, Muramoto, Yamaguchi, Fujimoto, & Nokihara, 1998; Megías et al., 2007), perhaps because of their characteristic ring structure. Saiga, Tanabe, and Nishimura (2003) observed that the carboxyl and amino groups in the side chains of acidic and basic amino acids are thought to play an important role in chelating metal ions. Due to their abundance in skin gelatin hydrolysates, Gly, Pro and Hyp merit special consideration. Mendis, Rajapakse, Byun, et al. (2005) postulated that the antioxidant reactivity of squid skin gelatin was

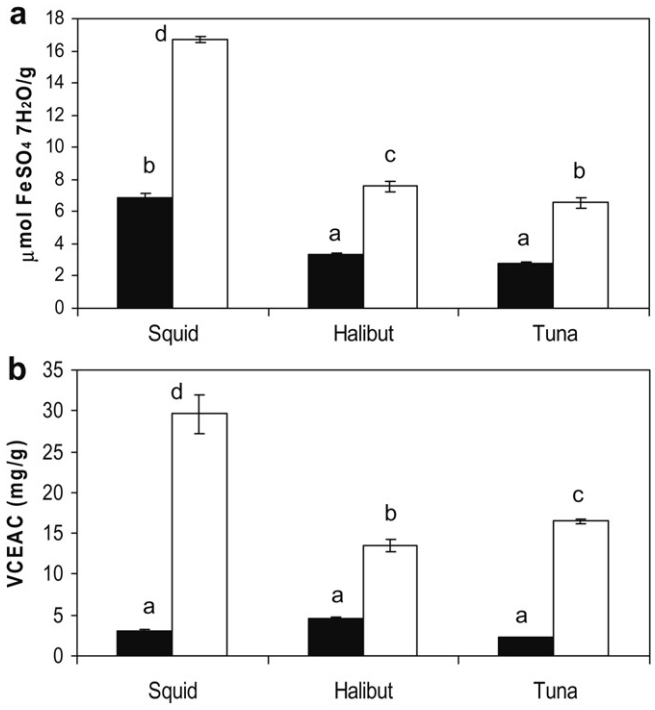

Fig. 3. Fe reducing ability (FRAP) (3a) and ABTS radical scavenging capacity (3b) of the gelatins (■) and the corresponding hydrolysates (□). Different letters (a, b, c, d) indicate significant differences ($p \leq 0.05$).

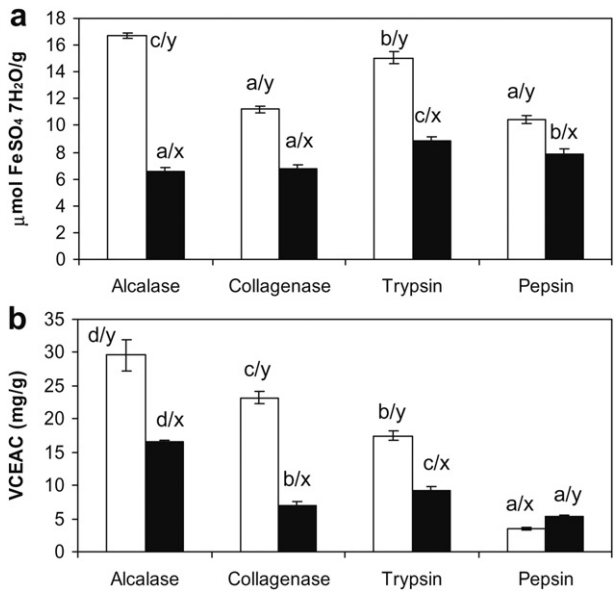

Fig. 4. Fe reducing ability (FRAP) (4a) and ABTS radical scavenging capacity (4b) of the squid (□) and tuna (■) hydrolysates obtained using different enzymes. Different letters from the beginning of the alphabet within the same species indicate significant differences ($p \leq 0.05$). Different letters from the end of the alphabet within the same enzyme indicate significant differences ($p \leq 0.05$).

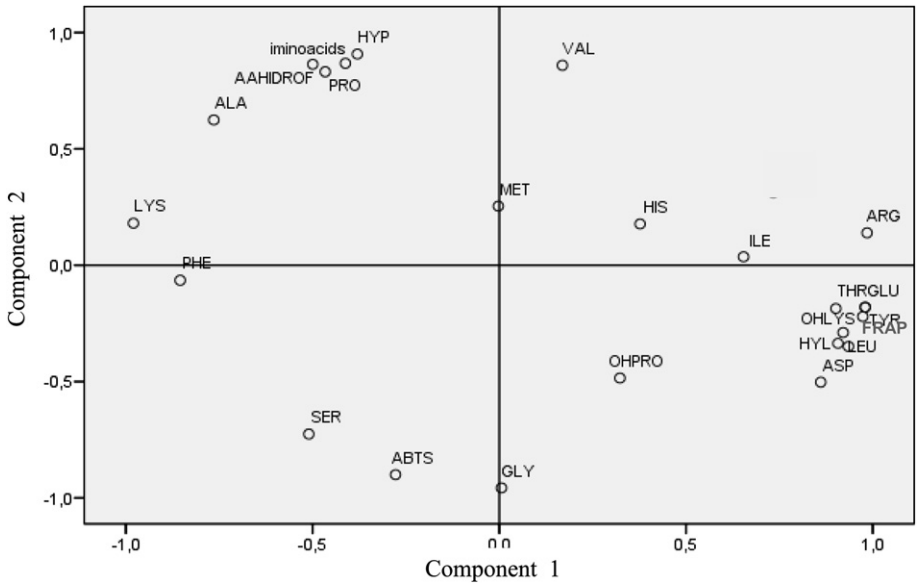

Fig. 5. Principal components from the multivariate analysis of the antioxidant capacities of the hydrolysates and the amino acid composition of the corresponding gelatins.

due to hydrophilic–hydrophobic partitioning in the peptide sequence.

Multivariate analysis was used to evaluate the influence of certain amino acids on antioxidant properties. Two principal components explained 80.6% of the total variance (Fig. 5). Principal component 1 (PC1) explained 56.3% and PC2 explained 24.3%. The rotation matrix showed a high positive correlation of antioxidant properties as measured by FRAP with PC1 (0.935) and with Hyl, the degree of hydroxylation of Lys, Arg, Glu, Tyr, Asp, Thr, Leu, and Ile, and a negative correlation with Lys, Phe, and Ala. Additionally, the ABTS measurement results were indicative of a high negative correlation through PC2 with Hyp, Pro, Σ imino acids, Σ hydrophobic amino acids and Val, and a positive correlation with Gly and Ser. As mentioned above, certain researchers have suggested that the hydrophobic amino acids present in the peptide sequences in jumbo squid skin gelatin contributed significantly to its antioxidant properties, mainly inhibition of lipid peroxidation (Mendis, Rajapakse, Byun, et al., 2005; Rajapakse et al., 2005). In this study, however, no relationship could be established between the total hydrophobic amino acids and the antioxidant properties, although a relationship was found with Leu and Ile, because other factors are likely involved such as molecular weight and peptide sequence. According to Lin and Li (2006), hydrolysates of jumbo flying squid skin gelatin had a scavenging effect on radicals, probably because of the presence of Pro residues in the peptide sequence. On the other hand, Hyl and the percentage hydroxylation, and therefore low Lys content and certain ionic amino acids would seem to be related to the antioxidant properties as measured by FRAP. However, hydrophobic amino acids do not appear to play any special role, since some, like Leu and Ile, contribute positively, while others, like Phe and Ala, contribute negatively, and Ala is one of the most abundant amino acids in skin gelatins.

4. Conclusions

The enzyme Alcalase is effective at producing antioxidant hydrolysates from gelatins from different marine sources. The DH was not wholly related to the molecular weight distribution of the starting gelatins, and hence some other unexplained factors appear to be involved. The hydrolysis increased the antioxidative activity of the resulting hydrolysates presumably mainly via the enhancement of radical scavenging activity. The type of enzyme used, the amino acid composition and likely the degree of glycosylation play a role in the antioxidant activity of the resulting hydrolysates as measured by the ABTS and FRAP methods.

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