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Sea By-Products as Real Material: New Ways of Application, 2010: 89-115
ISBN: 978-81-7895-485-1 Editor: Estelle Le Bihan

7. Antioxidant and antimicrobial peptide fractions from squid and tuna skin gelatin

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Abstract. Gelatin extracted from tuna skins and giant squid tunics were hydrolysed with Alcalase at 50ºC for 3h. Two peptide fractions (1-10K and ≤1K) were obtained from each gelatin hydrolysate by subjecting them to centrifugal ultrafiltration using successively a 10 kDa and a 1 kDa membrane. The peptide fractions were characterized in terms of amino acid composition and Fourier transform infrared (FTIR) spectroscopy. Antioxidant properties were tested according to the Ferric Reducing Antioxidant Power (FRAP) assay and the radical scavenging capacity (ABTS) assay. A disk diffusion test was performed to test antimicrobial action against a panel of Gram-positive and Gram-negative pathogenic and fish spoilage-associated microorganisms. Although antioxidant and antimicrobial properties could be detected in all tested peptide fractions, the lowermost molecular weight fraction from squid hydrolysate presented the highest reducing and radical scavenging capacities, whereas microbial growth inhibition was found to be specifically related to the type of microorganism.

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1. Introduction

Skins and bones, consisting primarily of collagen, make up about 30 % of waste from fish filleting in the seafood industry. Collagen in most fish species is composed of ~33% glycine and 16-18% amino acids (Pro and Hyp). The collagen molecule, from which gelatin is derived, exists as a triple helix, made up of three discrete α-chains that adopt a three-dimensional structure. This particular structure is due to the almost continuous repetition of the Gly-X-Y- sequence, which is required for close packing of the three chains since Gly residues are buried at the triple helix. The X and Y positions are exposed on the surface and can sterically accommodate any residue, but are frequently occupied by Pro and Hyp, which serve to stabilize the triple helix via hydrogen bonds by promoting the polyproline II conformation (Ledward, 1986). There are also non-helical terminal regions (telopeptide zones) at each end of the molecule, which are involved in covalent cross-linking of the single α-chains, both inter- and intra-molecularly. The collagen rod is extracted in acid and solubilised without altering its original triple-helix configuration. Subsequent thermal treatment cleaves hydrogen and covalent bonds; this destabilizes the triple helix by means of a helix-to-coil transition, leading to conversion into soluble gelatin (Djabourov, Lechaire, & Gaill, 1993). Collagenous material from fish skins is characterized by a low degree of intra and interchain covalent cross-linking, mainly involving lysine and hydroxylysine (Hyl) residues, along with aldehyde derivatives (Montero, Borderías, Turnay, & Leyzarbe, 1990). Squid skin gelatin has been shown to present much higher cross-linking compared to fish gelatins mainly due to the high degree of Lys hydroxylation that participates in different types of cross-links via the Schiff-base formation (Gómez-Guillén, Turnay, Fernández-Díaz, Ulmo, Lizarbe, & Montero, 2002).

In the last decade there has been a growing interest in the recovery of fish waste by enzymatic hydrolysis of protein residues (Jeon, Byun, & Kim, 1999; Guérard, Dufossé, De La Broise, & Binet, 2001; Rajapakse, Mendis, Byun, & Kim, 2005). Dietary proteins are a source of biologically active peptides, which are inactive within the sequence of parent proteins but can be released during the digestion or food processing. Once bioactive peptides are liberated, they may act as regulatory compounds with hormone-like activity (Korhonen, Pihlanto-Leppälä, Rantamäki & Tupasela, 1998). A great deal of attention has been paid to the production of antioxidant hydrolysates and peptides from skin gelatin of different fish species, such as Alaska pollack (Kim, Kim, Byun, Nam, Joo, & Shahidi, 2001), hoki (*Johnius belengerii*)

(Mendis, Rajapakse, & Kim, 2005a), cobia (*Rachycentron canadum*) (Yang, Ho, Chu, & Chow, 2008) or sole (Giménez, Alemán, Montero, & Gómez-Guillén, 2009a), as well as from several squid species, such as Giant squid (*Dosidicus gigas*) (Mendis, Rajapakse, Byun, & Kim, 2005b; Giménez et al., 2009a), Jumbo flying squid (*Dosidicus eschrichitii* Streenstrup) (Lin & Li, 2006) or squid (*Todarodes pacificus*) (Nam, You, & Kim, 2008).

A number of commercial proteases have been used for the production of antioxidant gelatin hydrolysates, including trypsin, chymotrypsin, pepsin, Properase E, Pronase, collagenase, bromelain and papain (Kim et al., 2001; (Mendis et al., 2005a; Lin & Li, 2006; Yang et al., 2008). Alcalase, which is a commercial protease from a microbial source, has been shown to be one of the most efficient in the hydrolysis of fish protein (Guérard, Dufossé, De La Broise, & Binet, 2001). It has been reported that this enzyme also produces high hydrolysis activity in gelatin from the skins of Alaska pollack, squid *Todarodes pacificus* or giant squid (Kim et al., 2001); Nam et al., 2008); Giménez et al., 2009a).

The average molecular weight of protein hydrolysates is one of the most important factors, which determines their functional properties (Jeon, Byun, & Kim, 1999; Park, Jung, Nam, Shahidi, & Kim, 2001). An ultrafiltration membrane system could be a useful method for obtaining peptide fractions with a desired molecular size and enhanced biological activity (Jeon et al., 1999). This system has been successfully applied in the fractionation and functional characterisation of squid skin gelatin hydrolysates (Lin & Li, 2006); and also as a first step in the isolation and further purification of antioxidant peptides from similar sources (Kim et al., 2001; Mendis et al., 2005a).

Generally, the quenching of free radicals by natural antioxidants has been reported as taking place through hydrogen donation. Certain peptides are electron donors and can react with free radicals to terminate the radical chain reaction (Park et al., 2001). Even though the exact mechanism by which peptides act as antioxidants is not clearly known, some aromatic amino acids and His are reported to play a vital role in this activity (Chen, Muramoto, Yamauchi, Fujimoto, & Nokihara, 1998). Mendis et al., (2005a) reported on the substantial presence of hydrophobic amino acids in gelatin peptide sequences for observed antioxidant activities. Besides amino acid composition and specific peptide sequences, functional properties of antioxidant peptides are also highly influenced by molecular structure and mass (Jeon et al., 1999; Suetsuna, Ukeda, & Ochi, 2000). Moreover, peptide conformation can lead to both synergistic and antagonistic effects in comparison with the antioxidant activity of amino acids alone (Hernández-Ledesma, Dávalos, Bartolomé, & Amigo, 2005).

Infrared spectroscopy (FTIR) provides information about the chemical composition and conformational structure of food components (Willard, Merritt, Dean & Settle, 1981). It has also been used to study changes in the secondary structure of fish collagen and gelatin (Muyonga, Cole, & Duodu, 2004). Its application to the characterization of protein hydrolysates is very limited, nevertheless, FTIR spectra of whey and casein hydrolysates have been found to correlate to various functional properties such as emulsion and foam forming capacities (Van der Ven, Muresan, Gruppen, De Bont, Merck, & Voragen, 2002). Although IR spectroscopy is not suitable for identifying specific peptides, according to these authors, some properties like peptide length and influence of amino acid composition could be reflected in FTIR spectra.

Antimicrobial peptides are found throughout nature; they are important components of innate defences, because in addition to killing microoganisms, they are able to modulate inflammatory responses (Devin and Hancock, 2002). In this respect, Gobbetti, Minervini & Rizzello (2004) described the total antibacterial effect of milk as higher than the sum of individual contributions made by protein defences, which could be attributed to the synergistic activity between natural proteins and peptides and peptides from precursors (Clare and Swaisgood 2000). In the search for new preservation methods, antimicrobial peptides can be used in natural biopreservation to control spoilage and pathogenic microorganisms (Sirtori, Motta & Brandelli, 2008).

In previous work, tuna and giant squid skin gelatins have been shown to have very different physicochemical properties, mainly based on differences in amino acid composition (higher Hyp and Hyl content in squid) and molecular weight distribution (absence of cross-linked α-chains in squid) (Gómez-Estaca, Montero, Fernández-Martín, & Gómez-Guillén, 2009; Giménez, Gómez-Estaca, Alemán, Gómez-Guillén, & Montero, 2009b). Working with these two gelatins, so different one from the other, (taken from both tuna and giant squid skins), the aim of this work has been to hydrolyse the said gelatins under identical conditions and then obtain from each one, using an ultrafiltration membrane system, two peptide fractions with antioxidant and antimicrobial properties. Subsequently a multivariate analysis was carried out to show correlations between amino acid composition, main FTIR events and antioxidant properties.

2. Materials and methods

2.1. Gelatin extraction

The tuna-skin gelatin was prepared in our laboratory according to the method described by Gómez-Guillén and Montero (2001). Giant squid

(*Dosidicus gigas*) inner and outer tunics were obtained after mechanical removal from mantles. Squid gelatin extraction was carried out according to the method described by Giménez et al., (2009b), submitting the skins to an enzymatic digestion with pepsin from porcine stomach mucosa (EC 3.4.23.1; 662 units/mg solid; Sigma-Aldrich, Inc., St. Louis, Mo., USA) at the rate of 1/8000 (w/w) during the swelling step in 0.5 M acetic acid. Subsequent gelatin extraction was done in distilled water at 60 ºC. Once a first-step-gelatin was extracted, the collagenous residues were swollen again in 0.5 M acetic acid for 24 h and a second-step-gelatin extraction was carried out at 60 ºC. Both, the tuna-skin gelatin and the second-step-gelatin from squid tunics were dried by heating at 45 ºC in a forced-air oven until brittle sheets were formed.

2.2. Gelatin hydrolysis

Tuna and squid gelatins were dissolved (2.5% w/w) in 10mM phosphate buffer (pH 8) and submitted to enzymatic hydrolysis in a pH-stat (TIM 856, Radiometer analytical, Villeurbanne, France) using Alcalase (2.4 L, 2.64 AU/g, Sigma-Aldrich, USA) at 50 ºC for 3 h with an enzyme-substrate ratio of 1:20 (w:w). The pH of the reaction was kept constant by continuous adding a 1N NaOH solution to the reaction medium. Afterwards, the enzyme was inactivated by heating the mixture at 90 ºC for 10 min and then centrifuged at 3800 rpm for 15 min at 4 ºC (Heaerus Labofuge 400, DJB Labcare, Bucks, UK). The degree of hydrolysis (DH) was calculated according to Adler-Nissen (1986), as described in a previous work (Giménez et al., 2009a).

2.3. Fractionation of gelatin hydrolysates

The supernatants of gelatin hydrolysates were collected. Part of them was studied as whole hydrolysates, and the rest was fractionated through a series of centrifugal ultrafiltration filters with molecular weight cut-off (MWCO) membranes of 10 and 1 kDa (Macrosep® Centrifugal Devices 10 K and 1K, Pall Corporation, NY, USA). Each hydrolysate solution was first passed through a filter with 10 kDa MWCO. The resulting ultrafiltrate was subsequently passed through a 1 kDa MWCO membrane, obtaining then two peptide fractions: (i) the resulting ultraconcentrate with theoretically main components ranging between 10 and 1 kDa MW was considered as the PF10 fraction, and (ii) the ultrafiltrate with theoretically main components below 1 kDa MW was considered as the PF1 fraction. Whole hydrolysates and peptide fractions were freeze-dried and stored at -80 ºC.

2.4. Amino acid composition

Dry tuna and squid gelatin hydrolysates, and their corresponding peptide fractions (PF10 and PF1), were dissolved in distilled water at 1 mg/ml and 50 µl of each sample were dried and hydrolysed in vacuum-sealed glass tubes at 110 ºC for 24 h in the presence of constant boiling 6N HCl containing 0.1% phenol and using norleucine (Sigma-Aldrich, Inc., St. Louis, Mo., USA) as the internal standard. After hydrolysis, samples were again vacuum-dried, dissolved in application buffer and injected into a Biochrom 20 amino acid analyser (Pharmacia, Barcelona, Spain). Determinations were performed in triplicate and data correspond to mean values. Standard deviations were in all cases lower than 6%.

2.5. FTIR-ATR spectroscopy

Infrared spectra between 4000 and 650 cm^{-1} were recorded using a Perkin Elmer Spectrum 400 Infrared Spectrometer (Perkin Elmer Inc, Waltham, MA, USA) equipped with an ATR prism crystal accessory. The spectral resolution was 4 cm^{-1}. Measurements were performed at room temperature using approximately 25 mg of the freeze-dried hydrolysates and peptide fractions, which were placed on the surface of the ATR crystal, and pressed with a flat-tip plunger under spectra with suitable peaks were obtained. All experiments were performed at least in duplicate. Background was subtracted using the Spectrum software version 6.3.2 (Perkin Elmer Inc.). The spectra were baseline corrected at 1812 cm^{-1} and normalized (mean normalisation option) for comparison purposes. The amide I band was Fourier self-deconvoluted using a resolution enhancement factor of 1.4. The area of the main FTIR bands was calculated and the values were used for the multivariate analysis.

2.6. FRAP assay

The FRAP method (Ferric Reducing Ability of Plasma) described by Benzie & Strain (1996), was used to measure the ferric ion reducing capacity of the tuna and squid hydrolysates and peptide fractions. It is based on the increase in absorbance at 595 nm due to the formation of the complex tripiridiltriazine (TPTZ)-Fe(II) in the presence of reducing agents at 37 ºC. Both hydrolysates and peptide fractions were dissolved in distilled water. Absorbance was read at 30 min using an UV-1601 spectrophotometer (Model CPS-240, Shimadzu, Kyoto, Japan). 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, was performed. Results were expressed as EC1: concentration having the

same antioxidative effect as 1mM de Fe^{2+} per gram of protein. At least three replicates were carried out for each sample.

2.7. ABTS assay

The ABTS radical (2, 2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)) scavenging capacity of the tuna and squid hydrolysates, and their peptide fractions, was determined according to a modified version of the method of Re, Pellegrini, Proteggente, Pannala, Yang, & Rice-Evans (1999). The stock solution of ABTS radical consisted of 7mM ABTS in potassium persulphate 2.45mM, kept in the dark at room temperature for 16 h. An aliquot of stock solution was diluted with distilled water in order to prepare the working solution of ABTS radical with absorbance at 734 nm of 0.70 ± 0.02. Both hydrolysates and peptide fractions were dissolved in distilled water. A 20 µl aliquot of sample (dissolved hydrolysate) or distilled water (in case of the control) was mixed with 980 µl of ABTS radical working solution, and the reduction of absorbance at 734 nm was measured after incubation at 37 ºC for 10 min in the dark. A standard curve of vitamin C, which relates the concentration of vitamin C to the amount of absorbance reduction caused by vitamin C, was performed. Antioxidant capacity was expressed as mg Vitamin C Equivalent Antioxidant Capacity (VCEAC)/g protein. All determinations were performed at least in triplicate.

2.8. Bacterial strains and culture media

The antimicrobial activity of the peptide fractions was tested over the following bacteria strains: *Aeromonas hydrophila* CECT 839T, *Bacillus cereus,* CECT148, *Bifidobacterium animalis subsp. lactis* DSMZ 10140, *Brochothrix thermosphacta* CECT 847*, Citrobacter frenduii* CECT 401, *Clostridium perfringens* CECT 486, *Escherichia coli* CECT 515*, Lactobacillus acidophilus* CECT 903*, Listeria innocua* CECT 910*, Photobacterium phosphoreum* CECT 4192, *Pseudomonas aeruginosa* CECT 110*, Pseudomonas fluorescens* CECT 4898, *Salmonella cholerasuis* CECT 4300*, Shewanella putrefaciens* CECT 5346T, *Shigella sonnei* CECT 4887*, Staphylococcus aureus* CECT 240*, Vibrio parahaemolyticus* CECT 511T and *Yersinia enterocolitica* CECT 4315. The strains were stored at -80 ºC in Brain Heart Infusion Broth (Oxoid, Basingstoke, UK) with 25 % glycerol (Panreac, Moncada i Reixac, Barcelona, Spain) until use. Then the strains were grown in BHI broth (Oxoid), (supplemented with 3% NaCl for *V. parahaemolyticus* and 1% NaCl for *P. phosphoreum*). Organisms were incubated at 37 ºC excepting *A. hydrophila*, *P. fluorecens*, *S. putrefaciens*, incubated at 30 ºC; *B. thermosphacta* at 25 ºC

and *P. phosphoreum* at 15 ºC. In addition, *L. acidophilus* was incubated under CO_2 flow and *C. perfringens* and *B. animalis subsp. lactis* under anaerobic conditions (Gas-Pack, Anaerogen, Oxoid).

For antimicrobial activity measurement, spread plates of BHI agar (Oxoid) were inoculated with 100 µL of overnight bacterial cultures grown (~10^8 CFU/mL). BHI agar containing 1% and 3% NaCl was used for *P. phosphoreum and V. parahaemolyticus,* respectively. Filter paper disks (0.5 cm diameter), soaked with 40 µl of each peptide fraction (2 mg/mL w:v) were put onto the plate surface and after incubation (the temperature of incubation was as described before) the clear zone surrounding the circles –inhibition zones- was considered as a measurement of the antimicrobial activity. The measurement of the inhibition zones was performed with specific software for digital image analysis (MIP 4 ADV, Consulting de Imagen Digital, S.L. & Microm, España). The measurement of the inhibition was considered as the difference between the diameter of the inhibition zone and the diameter of the disk, expressed according to the following classification: +++: > 1 cm; ++ ≥ 0.5 cm, +: 0.25-0.5 cm, ±: <0.25 cm and -: 0.1 cm. Lectures were done at 24 and 48 h. A PAL standard 50 Hz monochrome video system was used for image digitations. Analyses were performed in triplicate. In addition, another concentration of each peptide fraction (0.2 mg/ml w:v) was also tested for *B. animalis subsp. lactis* DSMZ 10140, *L. acidophilus* CECT 903, *P. phosphoreum* CECT 4192 and *S. putrefaciens* CECT 5346T.

2.9. Statistical analysis

Statistical tests were performed using the SPSS® computer program (SPSS Statistical Software, Inc., Chicago, Ill.) One-way analysis of variance was carried out. The difference of means between pairs was resolved by means of confidence intervals using a Tukey-b test at a level of significance of p≤0.05. A multivariate analysis on aminoacid compositions, main FTIR bands and antioxidant properties (FRAP and ABTS values) was performed by Principal Component Analysis, and after Varimax rotation, the two main factors were represented.

3. Results and discussion

3.1. Degree of hydrolysis

The dissolved gelatins were hydrolysed with Alcalase at 50ºC for 3h, the pH being controlled by means of a pH-stat, as reported previously (Giménez, et al., 2009a). The changes produced in the degree of hydrolysis with respect to the incubation period of the gelatin with the enzyme are shown in Fig. 1.

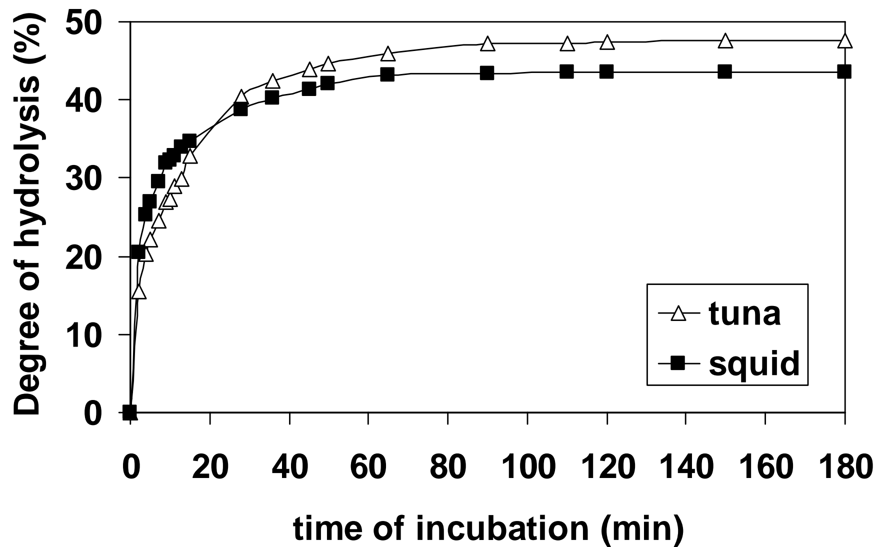

Figure 1. Degree of hydrolysis (DH) of gelatin hydrolysates from tuna skins and squid tunics.

In spite of their different physicochemical properties, both gelatins offered very similar hydrolysis profiles with Alcalase, registering a maximum value for DH of 47.52% after 150 min of incubation, and 43.46% after 110 min, for tuna and squid gelatins respectively. The kinetics of gelatin hydrolysis is very rapid, with a DH of about 25-30% in both species in the first 5 minutes. After 30 minutes of incubation the tuna gelatin had reached a value of DH which was ~78% of the maximum value, whilst the squid gelatin reached ~90% in the same period. The faster hydrolysis of the squid gelatin is mainly due to the greater protein degradation of the original squid gelatin, with a clear predominance of components with a molecular weight of less than 100 KDa (Giménez et al., 2009b), that facilitated enzyme access to the substrate. However, the tuna skin gelatin hydrolysate reached a slightly higher DH than in squid, which is attributed to a higher availability of hydrolysable peptide bonds (Guérard et al., 2001), in accordance with the high amount of cross-linked alpha-chains (γ- and β-components) in its molecular weight distribution (Gómez-Estaca et al., 2009).

3.2. Amino acid composition

The amino acid compositions of the hydrolysates and PF10 and PF1 fractions are shown in Table 1. Just as described by Kim et al. (2001), the amino acid composition in both hydrolysates is very similar to that of the parent gelatins, both being rich in residues of Gly, Ala, Pro, Hyp, Glx

(glutamic acid + glutamine), Arg and Asx (aspartic acid + asparagine). For the most part, the data were comparable with that for other amino acid compositions reported for other fish gelatin hydrolysates (Mendis et al., 2005a). Our results showed that Hyp and Hyl residues were noticeably higher in the squid gelatin hydrolysate as well as in their corresponding peptide fractions, in comparison with the tuna samples. In contrast, Pro and Lys residues were noticeably higher in all tuna samples. This reveals a much higher degree of Pro and Lys hydroxylation in squid hydrolysate, as a result of a higher degree of collagen cross-linking in the squid gelatin, which was in fact, the product of a second-step gelatin extraction. Hyp has been shown to play a singular role in the stabilization of the triple-stranded collagen helix due to its hydrogen bonding ability (Burjandze, 1979). Hyl residues, and their aldehyde derivatives, are involved in both intra- and inter-molecular covalent cross-links, mainly located in the telopeptide regions which do not form triple helical structures (Bateman, Lamandé & Ramshaw, 1996). Moreover, Hyl is normally glycosylated in invertebrate collagens, sugar moieties contributing to the establishment of additional covalent cross-links, which make glycoproteins and glycopeptides more stable when faced with heat denaturation, freezing and protease digestion (Dwek, 1996). A noticeably higher degree of Pro and Lys hydroxylation has been previously reported for Giant squid (*Dosidicus gigas*) skin gelatin hydrolysates when compared to hydrolysates obtained under identical conditions from sole skin gelatin (Giménez et al., 2009a). As mentioned above, the squid gelatin used in the present work comes from a second-step extraction, where slightly higher cross-linked gelatin was obtained. In this regard, in a previous work the amino acid composition of both types of squid gelatin hydrolysates was found to be very similar showing that hydrolysate from gelatin obtained from second-step extraction has a slightly higher degree of Pro hydroxylation (Giménez et al., 2009a,c)

Both in squid and tuna gelatin the Hyp content was relatively lower in their corresponding hydrolysates, as well as in the successive fractions of less molecular weight, being especially evident in the PF1 from both species. This suggests a tendency on the part of Hyp to remain in the molecularly larger peptides, probably because its presence is associated with small residual fragments in triple helix which hinder Alcalase activity. In contrast, Hyl residues in squid samples is highly concentrated in PF10 and even more in PF1, while in the case of tuna, it is only concentrated in PF1. In the same way, Arg residues which appear in similar quantities in both species, showed a clear tendency to concentrate in PF1.

The squid hydrolysate was also characterised by a significantly lower Ala content, the most abundant hydrophobic residue, which hardly varied in the

Table 1. Aminoacid composition of gelatin, whole hydrolysate and peptide fractions (PF10 and PF1) from tuna skins and squid tunics.

	TUNA n° residues/1000				SQUID n° residues /1000			
	Gelatin	Hydrolysate	PF10	PF1	Gelatin	Hydrolysate	PF10	PF1
Hyp	78	64	64	56	94	90	89	72
Asx	44	45	48	26	64	64	54	43
Thr	21	21	22	24	28	28	32	33
Ser	48	49	50	50	45	48	46	52
Glx	71	70	74	40	85	85	69	54
Pro	107	115	111	94	76	82	83	76
Gly	336	340	317	296	326	321	308	299
Ala	119	119	110	119	87	86	80	88
Val	28	24	25	21	23	22	29	26
Met	16	15	16	19	16	15	13	14
Ile	7	7	11	12	13	14	13	13
Leu	21	21	23	27	30	32	30	34
Tyr	3	4	10	10	8	7	13	10
Phe	13	13	16	20	12	12	11	12
His	7	7	13	14	7	7	6	7
Lys	25	25	29	31	14	13	12	16
Arg	52	52	53	75	58	57	56	74
Hyl	6	11	8	66	14	16	54	75
% Pro hydroxylation	42.13	36.00	36.59	37.08	55.42	52.33	51.60	48.65
% Lys hydroxylation	18.64	30.86	20.60	67.83	50.00	54.90	81.37	82.39

Determinations were performed in triplicate and data correspond to mean values. Standard deviations were in all cases lower than 6%.
Asx = Asp + Asn; Glx = Glu + Gln.

peptide fractions. This fact, together with the lower Pro content, makes squid hydrolysate slightly less hydrophobic than its tuna counterpart. Gómez-Guillén, Turnay, Fernández-Díaz, Ulmo, Lizarbe, & Montero, (2002) also found squid gelatin to be less hydrophobic than gelatins extracted from other fish species such as sole, megrim, cod or hake skins. Nevertheless, it should be noted that the higher amounts of other minor but highly hydrophobic residues (Ile, Leu and Tyr) in the squid hydrolysate, remained practically the same in the corresponding peptide fractions whereas these residues tended to increase in the tuna PF10 and PF1 fractions.

The squid hydrolysate as well as its peptide fractions was significantly richer in Asx and Glx residues, which confirms the more hydrophilic nature of squid samples, when compared with their tuna counterparts. In both species there is a sharp decline in the quantity of these residues in the lowermost molecular weight peptide fraction (PF1). These results indicate that independently of the initial amino acid composition of the gelatin, the ratio of hydrophobic to hydrophilic amino acids in the peptide fractions can be modified depending on the molecular size of the predominant peptides.

3.3. FTIR spectroscopy

IR spectroscopy has been used to provide further information about chemical composition and conformational structure of the lyophilised gelatin hydrolysates and their corresponding peptide fractions. FTIR spectra ranging from 4000 to 650 cm^{-1} are presented in Figure 2.

3.4. Changes in the amide A band

The amide A band, in both squid and tuna gelatins and hydrolysates, exhibited a broadened absorbance with a maximum value ranging between 3283-3275 cm^{-1} and a minor peak at ~3072 cm^{-1}. These bands were attributed fundamentally to OH and NH stretching vibrations and, as reported by Yakimets, Wellner, Smith, Wilson, Farhat, & Mitchell, (2005) in hydrated gelatin films, could be in part attributed to adsorbed water molecules. The amide A band appears with more intensity in squid hydrolysate which is attributed to a greater hygroscopicity in this hydrolysate in accordance with

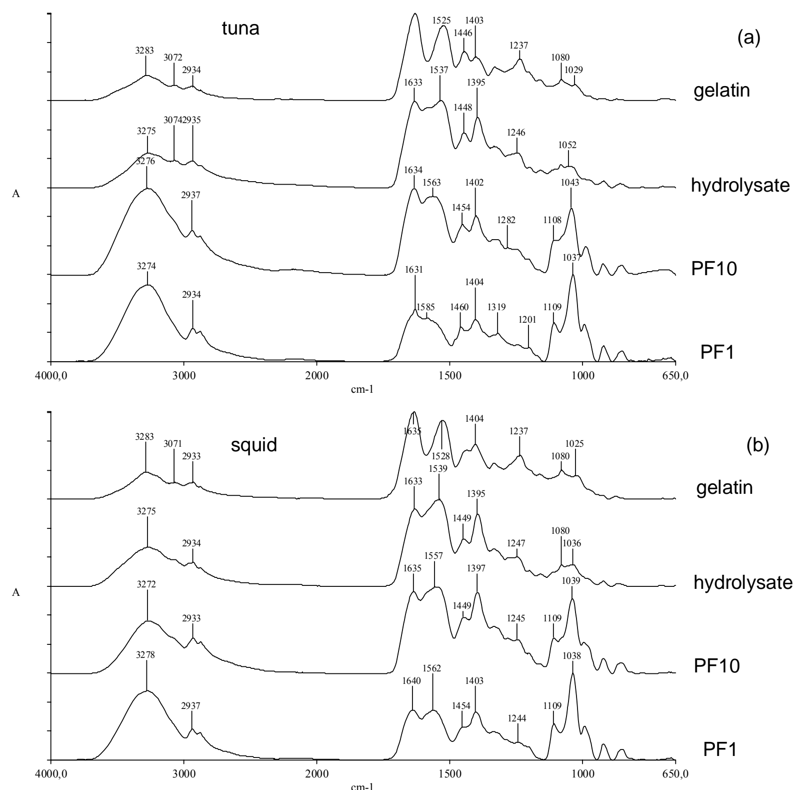

Figure 2. FTIR spectra of gelatin, whole hydrolysate and peptide fractions (PF10 and PF1) from a) tuna skins and b) squid tunics.

the more hydrophilic nature of its amino acid composition, when compared with the tuna hydrolysate. The PF10 and PF1 fractions showed an increase in the absorbance of the main component at ~3275 cm^{-1}, whereas the shoulder at 3072 cm^{-1} tended to disappear, in both squid and tuna samples.

The IR spectra also showed a peak at 2934 cm^{-1}, which as reported by Muyonga, Cole, & Duodu, (2004) in Nile perch skin gelatins, could be related to CH_2 stretching vibrations. This peak is more pronounced in the peptide fractions, than in the gelatins and hydrolysates. According to Muyonga et al., (2004) and Kemp (1987), the larger number of intermolecular associations between carboxylic acid groups in the gelatin and also in the whole hydrolysates would cause amide A bands to merge with the CH_2 stretch peak in those samples.

3.5. Changes in the amide I band

The amide I band presents maximum IR absorbance at ~1633 cm^{-1}, arising predominantly from protein amide C=O stretching vibrations. A similar band has been previously reported for pure calf-skin collagen at 1658 cm^{-1} (Mohd Nasir, Raha, Kadri, Sahidan, Rampado, & Azlan, 2006), Nile perch gelatin at ~ 1650 cm^{-1} (Muyonga et al., 2004) and bovine skin gelatin films at 1660 cm^{-1} (Yakimets et al., 2005). Figure 3 shows in more detail the deconvolution spectra of the amide I band. All curves showed several components between 1634 and 1645 cm^{-1}, as well as a remarkable one at 1652 cm^{-1}. Payne & Veis, (1988) resolved three main peaks near 1633 cm^{-1}, 1643 cm^{-1} and 1660 cm^{-1} after deconvolution of their collagen and gelatin ATR-FTIR spectra, which they assigned, respectively, to hydrogen bonding between water and glycine residues, hydrogen bonding between water and imide residues and hydrogen bonding between polypeptide chains. According to Prystupa & Donald, (1996), an absorption band at ~ 1630 cm^{-1} may correspond mainly to imide residues hydrogen bonded to water molecules, whereas the 1645 cm^{-1} band would reflect the typical random coil frequency corresponding mainly to amine residues. A band around 1630 cm^{-1} has also been reported in both collagen and gelatin from calf skin (Tsunoda et al., 2001) and Nile perch skin (Muyonga et al., 2004), indicating a disordered structure. The band around 1650 cm^{-1} has been assigned to gelatin random coils, while the hydrogen bonded triple helix structure is reportedly shown at ~1660 cm^{-1}. It seems, therefore, that both squid and tuna gelatins have few triple helix structures, with random coils predominating, as a result of a high degree of collagen denaturation. Collagen to gelatin denaturation has been reported as producing an increase in the intensity of the C=O group at 1633

cm^{-1} (disordered structure) to the detriment of the C=O group at 1660 cm^{-1} (triple helix) (Tsunoda et al., 2001).

With regards to the hydrolysates and peptide fractions, the amide I band shows a notable reduction in IR absorbance and a loss in definition of the peaks in the lowermost molecular weight peptide fractions (PF1), denoting an evident secondary structure loss. The peaks at 1634 and 1651 cm^{-1} clearly persist in the hydrolysates and peptide fractions, however the peak at 1645 cm^{-1} related to amine residues involved in the random coils practically disappears. In a study using a synthetic peptide of 30 amino acids made up exclusively of Pro and Gly [(Pro-Pro-Gly)$_{10}$] residues, the peak at 1645 cm^{-1}, reflecting mainly the contribution of the Gly-Pro peptide bond, has been proposed as a possible marker because it is highly sensitive to secondary structure changes produced by thermal transition (Bryan, Brauner, Anderle, Flach, Brodsky, & Mendelsohn, 2007). The study shows the Pro-Pro link of the said peptide, registered at 1629 cm^{-1}, as the most stable in the face of conformational changes.

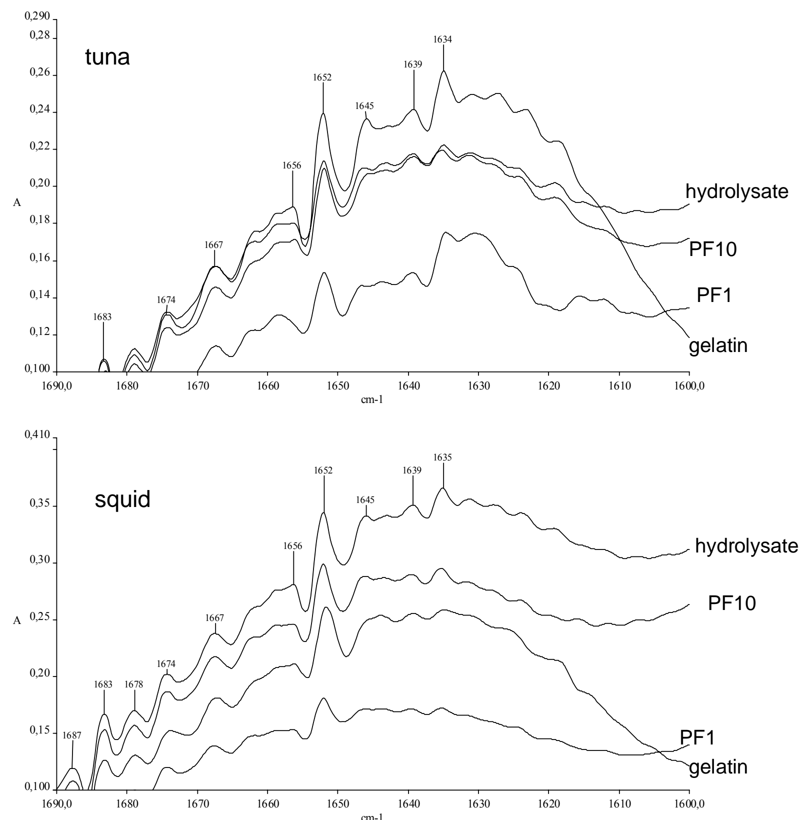

Figure 3. Deconvoluted FTIR spectra of amide I band of gelatin, whole hydrolysate and peptide fractions (PF10 and PF1) from a) tuna skins and b) squid tunics.

3.6. Changes in the amide II band

The amide II band is a main component at 1528 cm^{-1} in squid gelatin and at 1525 cm^{-1} in tuna gelatin, but tended to shift to higher wavenumbers as the molecular weight of the peptide fraction decreased (Fig. 2). This band has been associated with amide N-H bending and C-N stretching vibrations, and has been reported to appear at 1554 cm^{-1} in calf skin collagen (Mohd Nasir et al., 2006), and at 1542 cm^{-1} in acid soluble collagen and gelatin from Nile perch skins (Muyonga et al., 2004). The amide II band is less sensitive to secondary structure than the amide I, but it is very much influenced by hydration. Yakimets et al., (2005) reported an up-shift from 1534 to 1565 cm^{-1} in gelatin films as a result of increasing levels of protein backbone hydration. A similar shift was observed in the present work, which could be indicative of an increased hygroscopicity in the peptide fractions, in agreement with the increased absorbance in the amide A range. On the other hand, a shift of the amide II band to lower wavenumbers has also been reported to be indicative of collagen self-assembly (Jakobsen, Brown, Hutson, Fink, & Veis, 1983), which would presumably be higher in gelatin samples, and would be reduced as the molecular weight of the peptide fractions decreased.

In both gelatin spectra (squid and tuna), two bands at ~1448 and ~1400 cm^{-1} appear with less intensity. The peak at 1400 cm^{-1} showed an increased intensity of absorbance in the hydrolysates and peptide fractions. This band which is associated with C-N stretching vibrations of primary amides is more intense in the squid samples.

3.7. Changes in the amide III band

The amide III band showed a main component at 1237 cm^{-1} in both squid and tuna gelatins spectra, which lost intensity in the hydrolysate whilst peptide fractions appeared slightly displaced to higher frequencies (Fig. 2). A similar component has also been reported by Muyonga et al. (2004) in gelatin and collagen samples from Nile perch skins. Mohd Nasir et al (2006) reported a main amide III peak at 1240 cm^{-1} in calf skin collagen, together with two smaller peaks at 1204 and 1283 cm^{-1}, which also appeared in the squid and tuna gelatin spectra. The amide III could be described as a complex peak associated with N-H in plane bending and C-N stretching from amide linkages, as well as CH_2 wagging vibrations from the glycin backbone and proline side-chains (Mohd Nasir et al., 2006; Jackson, Choo, Watson, Halliday, & Mantsch, 1995). Therefore, the decrease in intensity and the slight up-shift of the main amide III peak displayed by the hydrolysates and peptide fractions could be largely attributed to a reduced degree of

intermolecular interactions between collagen-like peptides. Yakimets et al., (2005) reported an intensity loss at 1210 cm^{-1} combined with a frequency shift towards 1240 cm^{-1} as a result of increasing the hydration level in gelatin films. In this sense, the peak at ~1208 cm^{-1} was found to disappear in the lowermost molecular weight peptide fractions from both species, confirming the higher hydration level of this fraction.

A special feature of IR spectra from squid and tuna PF10 and, especially, PF1 fractions is the sharp increase in the intensity of the peak at ~1040 cm^{-1}. An appreciable absorption in this region was also observed by Muyonga et al. (2004) in Nile perch bone gelatins, and according to Jackson et al. (1995), this could be attributed to C-O vibration due to carbohydrates. It is well known that the presence of carbohydrates in collagen is associated with protein glycation, preferentially at lysine and hydroxylysine residue levels. In this regard, the calculated area of the ~1040 cm^{-1} peak in the peptide fractions showed a strong correlation with the relative number of Hyl (r^2=0.85) and Arg (r^2=0.99) residues, which were considerably increased in the squid and tuna peptide fractions. The accumulation of reducing sugars in bone tissue has been associated with the formation of stable cross-links derived from non-enzymatic glycation of collagen. Pentosidine, which is a pentose derived crosslink formed between lysine and arginine residues is the most widely advanced glycation end (AGE) product described in type I collagen. It has also been found in bovine hide collagen and in Nile perch bone gelatins, although not in any appreciable amount in fish skin gelatin (Muyonga et al., 2004). Pentosidine has been reported in human collagen hydrolysates (Iijima, Murata, Takahara, Irie, & Fujimoto, 2000). These authors also identified N(ω)-carboxymethylarginine (CMA) to be a major AGE product in hydrolysates from glycated collagen. They found that CMA gradually increases during incubation with glucose, yielding a concentration even higher than that of pentosidine. According to that study, both the unexpected increase in the number of Arg residues in the peptide fractions, and such a high correlation with the presumptive carbohydrate FTIR event, might be due to the formation of some kind of AGE product involving Arg residues, produced mainly during the fractionation process, in the presence of sugars that are also supposedly concentrated in the these fractions.

3.8. Antioxidant activity

Results of anti-oxidative activity, in terms of overall reducing (FRAP) and radical scavenging (ABTS) capacities of whole and fractionated hydrolysates are shown in Table 2. Both FRAP and ABTS values were substantially increased after gelatin hydrolysis, and were more than 2-fold

higher in the squid hydrolysate and its corresponding peptide fractions, as compared to tuna samples. In all cases, the lowermost molecular weight peptide fraction (PF1) registered the highest anti-oxidative activity, except in tuna samples where no significant differences ($p<0.05$) in the ABTS values were observed. Obviously, the results reveal the presence of antioxidant peptides in all the fractions, and a clear tendency to concentrate in the fractions with a lower molecular weight, especially in those from squid gelatin hydrolysate. Giant squid (*Dosidicus gigas*) skin gelatin has already been shown to be an excellent source of antioxidant peptides, after hydrolysis with trypsin, α-chymotrypsin or pepsin, with tryptic hydrolysate exhibiting the highest lipid peroxidation inhibition (Mendis et al., 2005b). These authors attributed these potent scavenging activities to the relative abundance of non-aromatic amino acids such as Pro, Ala, Val and Leu, as well as to their positioning in the peptide sequence. The high content of hydrophobic amino acids in squid gelatin hydrolysate, reported by those authors, was put forward as the main cause for its high lipid peroxidation inhibition, due to their high affinity with linoleic acid. A similar argument was proposed by Mendis et al. (2005a) and Rajapakse et al. (2005b) for hoki skin gelatin and giant squid muscle peptide fractions, respectively. Peptide fractions isolated from hydrolysed gelatin from Jumbo flying squid (*Dosidicus eschrichitii* Streenstrup) skins using Properase E and pepsin also showed a considerably high radical scavenging capacity (Lin & Li, 2006). Our work confirmed the high antioxidative capacity of whole and fractionated alcalase hydrolysate from second-step extraction gelatin from giant squid skin that was, noticeably higher than that obtained from tuna skins, under the same hydrolysis conditions. However, as the total number of hydrophobic residues was noticeably higher in the tuna hydrolysate, that line of reasoning does not explain the mechanism responsible for the observed higher reducing and scavenging capacities in the squid hydrolysate. It is possible that in the said hydrolysate certain more active specific sequences predominate than in the tuna hydrolysate, probably involving Leu and Ile residues at the C- or N-terminus (Mendis et al. 2005b; Suetsuna, et al., 2000; Park et al., 2001; Chen, et al., 1995), which were slightly more abundant in squid samples (Table 1). Alcalase has also been shown to induce the production of antioxidant peptide fractions from squid (*Todarodes pacificus)* collagen (Nam et al., 2008). Kim et al., 2001) proposed that the principal sequence responsible for their high antioxidative capacity was the repeating motif Gly-Pro-Hyp of peptides isolated from Alaska pollack skin hydrolysates and treated sequentially with alcalase, pronase E and collagenase, This result was to be expected, given the unique high amino acid content of these skin gelatin hydrolysates. In this connection, Lin & Li (2006) also attributed, to a large extent, the high

scavenging effects on radicals of jumbo flying squid skin gelatin hydrolysates to the existence of Pro residues in the peptide sequence. When comparing the amino acid composition of our squid and tuna hydrolysates, the amount of Pro was noticeably lower in squid samples, whereas the quantity of Hyp was higher. Even so, the number of Hyp residues cannot be proposed either as the main factor for higher anti-oxidative activity, since their relative quantities showed a clear tendency to diminish in the most anti-oxidative peptide fractions (PF1) from both species. Significantly higher ferric iron reducing and radical scavenging capacities were previously found in giant squid skin gelatin hydrolysates in comparison with sole, suggesting that the higher degree of Pro and Lys hydroxylation together with the higher content of glycopeptides play an important role (Giménez, et al., 2009a). The anti-oxidative properties of whole hydrolysates from giant squid gelatins from both a first-extraction and a second- extraction step were found to be similar, thus in agreement with their similitude in amino acid composition (Giménez, Gómez-Estaca, Alemán, Gómez-Guillén, & Montero, 2009c).

It is well documented that, although antioxidant activity is normally widely observed in every different whole and fractionated hydrolysate, the lowermost molecular weight peptide fractions always exhibit the strongest

Table 2. Ferric ion reducing capacity (FRAP assay) and radical scavenging capacity (ABTS assay) of gelatin, whole hydrolysate and peptide fractions (PF10 and PF1) from tuna skins and squid tunics.

	FRAP (EC1)	ABTS (VCEAC/g protein)
TUNA		
Gelatin	5.14 ± 0.54 a	3.57 ± 0.12 a
Hydrolysate	7.88 ± 0.27 b	16.32 ± 0.06 b
PF10	9.12 ± 0.38 b	17.07 ±0.21 b
PF1	11.55 ± 0.68 c	17.57 ±0.05 b
SQUID		
Gelatin	12.03 ± 0.06 c	2.65 ± 0.18 a
Hydrolysate	17.01 ± 0.75 d	37.81 ± 0.06 c
PF10	16.67 ± 0.26 d	41.96 ± 0.84 d
PF1	27.57 ± 1.84 e	48.24 ± 4.33 e

Different letters (a,b,c..) in the same column indicate significant differences ($p<0.05$)
VCEAC: mg Vitamin C Equivalent Antioxidant Capacity
EC1: concentration having the same antioxidative effect as 1mM de Fe^{2+} per gram of protein

activity (Kim et al., 2001; Rajapakse, et al, 2005); Yang et al., 2008). It therefore seems clear that, besides specific amino acid sequences, peptide antioxidative activity is strongly related to their molecular weight (Jeon et al., 1999; Park et al., 2001).

3.9. Antimicrobial properties

The antimicrobial activity of the peptide fractions with different molecular weights for several bacteria is shown in Table 3. For the 18 strains, the bacteria manifesting the most sensitivity to the fractions were *L. acidophilus*, *B. lactis S. putrafaciens and P. phosphoreum*. It is generally agreed that peptides need to interact with cell membranes as part of their action against microbes (Hancock and Patrzykat, 2002). Some of these interacting antimicrobial peptide mechanisms have already been reviewed (Gobetti et al. 2004; Hancock and Patrzykat, 2002; Patrzykat and Douglas 2005), and describe the tendency to form discrete channels in lipid bilayers, disturbance of the lipid bilayer because of carpet-like peptide binding, specific peptide-lipid interaction that results in phase separation, detergent-like solubilization of the membrane and even peptides that enter the cell leaving the membrane intact.

Despite the majority of the α-helical peptides being cationic or amphiphatic, the hydrophobic α-helical peptides were also reported to have antimicrobial activity (Eppand & Voguel, 1999). Peptides from fish gelatin have repeated unique Gly-Pro-Ala sequences in their structure (Kim & Mendis, 2006) and therefore their properties could be related to their particular amino acid composition. Although the structure of the fractions obtained in our study has not been established yet, the fractions contained hydrophobic amino acids (Table 1). Wieprecht et al. (1997) reported that this hydrophobic character would let peptides enter the membrane, as the positive charge would initiate the peptide interaction with the surface of the bacteria (negatively charged). On the other hand, Floris, Recio, Berkhout & Visser (2003) found that the differences existing in membrane composition have implications for the mode of action and the specificity of the antibacterial compounds. For example, the first bacterial membrane encountered by peptides acting on Gram-negative bacteria is the lipopolysaccharide (LPS)-containing outer membrane. Patrzykat and Douglas (2005) reported that the extent of LPS binding ability is neither directly nor inversely proportional to peptide activity; that once the outer membrane has been broken, peptide activity would depend on its ability to interact with bacterial cytoplasmic membranes, in which the sequence and concentration of the peptide and the composition of the bacteria membrane would influence the mode of

interaction. However, our results showed that the antimicrobial activity of the fractions was found both on Gram-positive and Gram-negative bacteria (Table 3).

In some cases, irrespective of the fish species (tuna or squid), PF1 fractions seemed to be more active than PF10 ones. This fact can be seen for example in *B. cereus*, *E. coli* and *S. putrefaciens* (Table 3). The molecular weight could be related to the elimination of aggregates and better exposure of the amino acids and their charges as well as structure acquisition, thus facilitating the interaction with bacterial membranes. As described before, squid and tuna have a different amino acidic composition, since the degree of Pro and Lys hydroxylation was higher in squid than in tuna. These characteristics give squid fractions a greater antioxidant potential. Even so, with respect to antimicrobial activity, there was no clear evidence that fractions from one species were more active than those from the other

Table 3. Antimicrobial activity of peptide fractions (2 mg/ml) from tuna skins and squid tunics.

Strains	Fractions			
	Tuna		Squid	
	PF10	PF1	PF10	PF1
L. acidophilus	++	++	+/-	++
A. hydrophila	-	-	-	+
P. fluorescens	-	-	-	+/-
S. putrefaciens	+	+	+	++
B. thermosphacta	-	+/-	+/-	-
P. phosphoreum	++	++	++	++
B. lactis	+++	+++	+++	+++
B. cereus	-	+/-	-	+/-
C. freundii	-	-	-	-
E. coli	-	+/-	-	+/-
L. innocua	+/-	-	-	+/-
P. aeruginosa	+/-	+/-	-	-
S. cholerasuis	+/-	+/-	-	-
S. sonnai	-	-	-	+/-
S. aureus	-	+/-	-	+/-
Y. enterocolitica	+	-	-	+
V. parahaemolyticus	+/-	-	+/-	+
C. perfringens	+	-	-	+

Inhibition zones: +++: > 1 cm; ++ ≥ 0.5 cm, +: 0.25-0.5 cm, ±: <0.25 cm and -: 0.1 cm.

Table 4. Antimicrobial activity of peptide fractions (0.2 mg/ml) from tuna skins and squid tunics.

Strains	Fractions			
	Tuna		Squid	
	PF10	PF1	PF10	PF1
L. acidophilus	++	+	+	++
S. putrefaciens	++	++	+	++
P. phosphoreum	++	+	++	+++
B. lactis	+++	+++	+++	+++

Inhibition zones: +++: > 1 cm; ++ ≥ 0.5 cm, +: 0.25-0.5 cm, ±: <0.25 cm and -: 0.1 cm.

species. Thus, fractions from tuna seemed to be more active than squid ones for *L. acidophilus*, *P. aeruginosa and S. cholerasuis*; whereas squid fractions were more effective at inhibiting, for example the growth of *A. hydrophila* (Table 3).

The lower concentration of the peptide fraction (0.2 mg/ml *versus* 2 mg/ml) maintained the antibacterial properties, since the profile of the two concentrations was very similar for the two species (Tables 3 and 4). In this connection, Isracidin, an antibacterial peptide derived from α_{sl}-CN-treated with chymosin (Hill et al. 1974) was found to inhibit in vitro the growth of lactobacilli and other Gram-positive bacteria at 0.1-1 g/ml; however, this peptide was strongly protective *in vivo* against some pathogenic bacteria when administrated at very low doses (10µg).

3.10. Multivariate analysis

A multivariate analysis of the principal components has been carried out with the aim of establishing possible correlations between amino acid composition, the main FTIR events and antioxidant properties, by considering the gelatins, the hydrolysates and the peptide fractions of both squid and tuna together. As a result of our analysis we were able to pinpoint two principal components that together explain 77% of all the variance; the first of which represents 40.6% (PC1) and the second 36.4% (PC2) of all the explainable cases. The rates of antioxidant activities studied, the reducing capacity (FRAP) and radical scavenging (ABTS), presented a high positive correlation between

them only through PC1 (Fig. 4). In fact, PC2 does not indicate any effect related to antioxidant activity, but rather reveals relationships of a structural nature. The amino acids that show a greater positive correlation with antioxidant activity were Thr>Leu>Ile>Hyl>Tyr>Arg. In contrast, a larger content of Pro, Gly and Ala, in that order, worked in detriment to antioxidant activity, as shown by the high negative correlation that they manifested with respect to PC1. The composition of the remaining amino acids had little to offer of any relevance in connection with the parameters of the antioxidant activity studied. Even though Lys and Hyp residues were not closely correlated with FRAP and ABTS values, the degree of Lys hydroxylation to a large extent, and also to a lesser extent that of Pro, reveals a high positive correlation with both activities, which can be attributed in part to the high reactivity of the additional OH group.

The most significant positive contribution of the main bands of FTIR spectra to anti-oxidative properties through PC1 is that the components are registered at 2934 cm^{-1}, followed by bands at 1040 cm^{-1}, around 1400 cm^{-1} and 3275 cm^{-1}. A decrease in the CH_2 stretching vibrations (2934 cm^{-1}), largely related to a reduced number of intermolecular associations between the carboxylic acid groups in the peptide fractions, would favour anti-oxidative capacity as a result of the increased availability of free functional groups for radical scavenging. On the other hand, the multivariate analysis shows a clear correlation between anti-oxidative capacities and presumptive carbohydrates (1040 cm^{-1}), most likely in the form of glycopeptides or glycosylated amino acids. Moreover, figure 4 shows a close correlation between this FTIR event and the number of Arg and Hyl residues, which, as discussed above may be covalently linked to sugars. Glycosylated peptides have been shown to present a high bioactive potential. As reported by Oh & Lim, (2008), the high polarity in the carbohydrate part of the glycoprotein molecule could be a principal factor in making glycoproteins capable of scavenging free radicals. Regarding the band around 1400 cm^{-1}, its close correlation with PC1 could reveal a significant contribution of primary amides to the anti-oxidative activity. The squid peptide fractions were characterized by a higher absorbance at the 1400 cm^{-1} peak when compared with tuna samples, in agreement with their higher antioxidant properties. Squid samples showed a greater Asx and Glx residue content in their amino acid composition, however, the multivariate analysis did not show any significant contribution of these residues to the anti-oxidative activity, as their relative presence decreases significantly in peptide fractions with the lowermost molecular weight in both, squid and tuna samples. One might think that it is the amino acids which are more closely correlated with antioxidant activities that in turn are involved in the formation of the said amides.

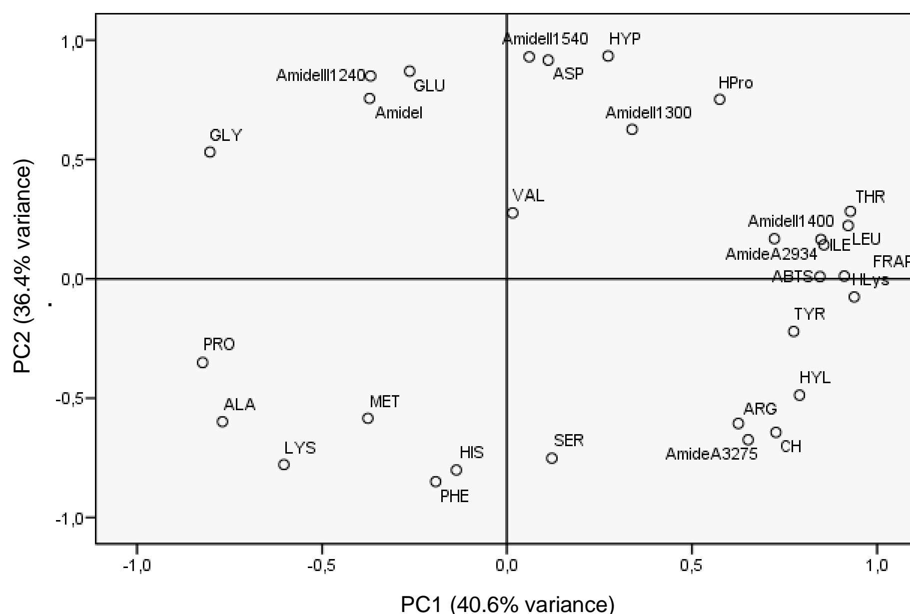

Figure 4. Principal Component Analysis of aminoacid composition, FRAP values, ABTS values and absorbance of main FTIR bands, of all studied samples from tuna skins and squid tunics. CH = presumptive carbohydrates.

On the contrary, the amide I band and the amide III component at around 1240 cm^{-1} were highly correlated the one with the other, showing a clear tendency towards inverse correlation to both FRAP and ABTS values. Both changes in the amide I and amide III components revealed molecular interactions between collagen-like peptides, consequently, as suggested above, reduced inter- and intra-molecular associations in lower molecular weight peptide fractions would favour especially both reducing and radical scavenging capacities. The amide II component at ~1525 cm^{-1} showed the highest correlation with PC2, and so it is the FTIR event that seems to have the least involvement with the antioxidant capacity. Even so, it is worth noting that for this analysis the peak areas have been considered, but not possible changes in the wavenumber.

In conclusion, peptide fractions from hydrolysates of tuna and squid gelatins presented both antioxidant and antimicrobial properties. The production of enzymatic hydrolysates with biological activities is an interesting alternative to the use of waste from the fishing industry. Separated peptide fractions from hydrolysates of tuna and squid gelatins presented both antioxidant and antimicrobial properties. These capacities were found to be higher mainly in the lowermost molecular weight fractions, especially in squid samples. Further work is going to be performed to isolate the bioactive peptides from the hydrolysates, eliminating the non-active residues. Studies

in depth are needed in order to elucidate the nature and structure of the fractions and thus discern the mechanisms of action.

Acknowledgements

This study was supported by the Spanish “Ministerio de Educación y Ciencia” (project AGL2005-02380/ALI and AGL2008-02135/ALI).

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