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Squid By-product Gelatines: Effect on Oxidative Stress Biomarkers in Healthy Rats

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Abstract

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Gelatines from three different jumbo squid (*Dosidicus gigas*) by-products (fins, arms, and skin) were compared based on their chemical and biochemical properties (amino acid composition, molecular weight distribution, and *in vitro* digestibility), antioxidant capacity ($ABTS^+$ and ORAC assays) and effect on oxidative stress biomarkers (serum antioxidant capacity (TAC) and serum malondialdehyde levels (MDA)) in healthy rats. Gelatine from the skin showed the highest polar and imino amino acid contents and a higher proline hydroxylation degree. Gelatine β-component was not detected in either fins or arms. These differences may explain the higher *in vitro* digestibility and higher antioxidant capacity (before and after digestibility) of the skin gelatine. Fin gelatines decreased TAC-ORAC values. All obtained gelatines decreased the MDA levels. Jumbo squid gelatine, administered during feeding, may help decrease a breakdown product of spontaneous lipid peroxidation in serum.

Keywords: colagen derivates; *Dosidicus gigas*; antioxidant capacity; *in vitro* assays

In recent years, the search for naturally occurring molecules that can act as antioxidants has become one of the fastest growing fields of study in food chemistry worldwide (Gil-Chavez *et al.* 2013) because of the increase in the number of oxidative stress-related diseases (Joudalová & Réblová 2012). The unbalance between free radicals and antioxidants is one of the primary causative agents, and improving the antioxidant status will decrease the risks of developing several types of illnesses (Joudalová & Réblová 2012). Analyses such as the serum total antioxidant capacity (TAC) indicate the cumulative effect of all antioxidants in blood (Prior & Cao 1999). High TAC values may suggest greater protection against oxidative stress-related diseases; however, it may also be part of an adaptation process to an increase in oxidative stress at early stages (Prior & Cao 1999) of DNA and protein damage due to the production of radicals; by-products in lipid peroxidation, mainly malondialdehyde (MDA) is an example. Low levels of serum MDA are frequently linked to low probabilities of developing diseases that are associated with oxidative stress (Niedernhofer *et al.* 2003).

Meanwhile, one of the most frequently studied components of marine products is collagen, which is a stromal protein. Since collagen shows resistance to hydrolysis, working with a digestible protein such as gelatine is preferable. Gelatine is a soluble protein whose functionality is observed in both the native form of the protein and its hydrolysates. They have been proved to have antioxidant, antihypertensive, and anticancer activities, among many other characteristics (Alemán *et al.* 2011). The main by-product from jumbo squid that has been studied is the skin; however, fins and arms, which are also considered discards, are rich in collagen and other proteins (Torres-Arreola *et al.* 2008). In the literature, there is scarce information about the functional properties of

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collagen derivatives from jumbo squid fins and arms, and about the effect of this protein in *in vivo* assays. Production of gelatines may represent an added-value approach for jumbo squid by-products that are currently considered wastes.

This study aims to determine and compare the chemical and biochemical properties of jumbo squid gelatine from three anatomical regions and their effects on two oxidative stress-related biomarkers (TAC and MDA) in healthy rats.

MATERIAL AND METHODS

Gelatine extraction. Gelatine from the skin, fins, and arms of the jumbo squid was separately obtained using the method of Jongjareonrak *et al.* (2010) with some modifications. Briefly, 100 g of each washed squid by-product was soaked with 0.8% NaCl, 15 min, 25°C, 1 : 6 ratio (w/v) and centrifuged at 7000 *g* for 30 min, 4°C. The precipitate (P1) was retained and soaked in 0.2 M NaOH for 45 minutes. Then, the precipitate (P2) was separated after centrifugation and soaked with 0.05 M acetic acid, 90 min, 25°C, to obtain the collagen (P3). P3 was soaked in distilled water, 65°C, 12 h, 1 : 4 ratio (w/v) to obtain the gelatine (P4). P4 was lyophilised and stored at –80°C until use. Crude protein (% N × 5.4) was measured using the Dumas method.

Amino acid profile. The amino acid content was determined according to Vázquez-Ortíz *et al.* (1997) by RP-HPLC. Dry squid gelatines were hydrolysed (6 M HCl, 150°C, 6 h). Gelatine was diluted (0.4 M sodium borate buffer), derivatised (4-chloro-7-nitro-2,1,1-benzoxadiazole), and heated (60°C, 5 min). Fluorescence was monitored (330 nm (excitation) and 418 nm (emission) wavelengths).

Polyacrylamide gel electrophoresis. Molecular masses of gelatine extracts were analysed using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE: 4% stacking gel and 10% separating gel) (Laemmli 1970) under non-reducing conditions. Gelatine extracts (25 ml) were diluted (1 : 1) in sample buffer, and 15 ml containing approximately 12 mg of protein was loaded onto a vertical electrophoresis device (Bio-Rad Mini Protean III).

In vitro digestibility analysis. In order to test the susceptibility of gelatines to be broken down by the digestive system of a living organism a prediction assay was carried out. Four enzymes were used to digest *in vitro* the gelatines from squid skin, fins, and arms: trypsin, chymotrypsin, carboxypeptidase A, and subtilisin (Satterlee *et al.* 1982). Gelatines were conditioned (pH 8.0, 37°C) and to determine digestibility (%), the final pH was measured after 20 min of adding the enzyme. The enzymes were inactivated by heating (90°C, 15 min). In order to corroborate the enzymatic hydrolysis, the electrophoretic profile of the digestion products was evaluated.

Gelatine intervention assay. Collagen shows resistance to hydrolysis, therefore it is preferable to convert it into a form suitable for its application, gelatine being one of them. The effect of jumbo squid by-product gelatine on serum total antioxidant capacity was studied in rats that were cared and managed according to the guidelines for the housing of rats in scientific institutions (Animal Ethics Infolink: http://www.animalethics.org.au). A group of 10 healthy female 100-g Sprague-Dawley rats from the animal house of the University of Sonora were distributed in individual cages, and were administered a 0.5% water-gelatine solution (jumbo squid fins-, arms-, or skin-derived according to the group to which they belonged) instead of distilled water (control group). The volume of gelatine solution was registered daily during a 30-day period. Chow pellets were equally given to rats, regardless of the treatment group. After the period of gelatine intervention, a blood sample was taken via cardiac puncture, and the animal was humanly euthanised. The blood was centrifuged and the serum was used to determine the total antioxidant activity and malondialdehyde levels.

Antioxidant analyses of collagen, gelatine, and healthy-rat serum. The $ABTS^{+}$ radical scavenging assay (collagen, gelatine, and serum) was performed according to a method described by Re *et al.* (1999), and the oxygen radical absorbance capacity (ORAC) assay (gelatine and serum) was performed using 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) as the precursor of the reactive oxygen species (Garret *et al.* 2010). The results were expressed as mmol Trolox equivalent (TE)/mg protein (collagen and gelatine) and mmol TE/ml (serum).

MDA levels. The MDA levels were determined using an MDA commercial kit. The serum was mixed with 42 mmol sulphuric acid and 10% phosphotungstic acid to promote the exposure of MDA molecules. Thiobarbituric acid (TBA) was added to form an MDA-TBA adduct after incubation at 96°C (1 h), and the concentration of MDA in the samples was obtained by comparing the absorbance readings with an MDA standard curve.

doi: 10.17221/320/2015-CJFS

Statistical analysis. A total random design was used in the *in vivo* assay. All data were subjected to the one-way ANOVA method ($P < 0.05$). Statistical tests were performed using the JMP software. Tukey's test was used to determine the level of significance ($P < 0.05$). All measurements were performed in triplicate and results are expressed as mean ± standard deviation.

RESULTS AND DISCUSSION

Obtained gelatines. The gelatine yield after extraction, which is expressed as grams of dry gelatine per 100 g of by-product, slightly varied between the fins (12%) and arms (11.8%). Both yields were lower than that obtained from the skin (14.5%). This result was higher than those previously obtained from the same squid species (Gómez-Guillén *et al.* 2002; Uriarte-Montoya *et al.* 2011). The difference in gelatine recovery can be attributed to the increase in the time of the NaOH soaking period, which can improve the removal of contractile proteins mainly in the muscle-rich regions (fins and arms). The same concentration of true collagen was detected between fins (65.4%) and arms (65.4%). Those values were higher than that of the skin (62.1%). These differences suggest that collagen from the skin differs in the cross-linking degree from that obtained from fins and arms, which is determined by the number of bonds susceptible to hydroxylamine as well as by their solubility in salt solutions and buffers (Sadowska & Sikorski 1987).

Amino acid profile. Gelatines from the 3 different sources had different amino acid profiles (Table 1). Skin gelatine contained 29% Gly and 16% imino amino acids (Pro + Hyp). These values were higher than those of fin and arm gelatines (both of which had 25% Gly and 13% imino amino acids). Moreover, skin gelatine had higher content of polar amino acids and lower contents of charged and aromatic amino acids than those from the fins and arms. All obtained gelatines had a lower concentration of Gly and Hyp than those reported for the jumbo squid mantle and skin (Rajapakse *et al.* 2005; Giménez *et al.* 2009; Alemán *et al.* 2011). Histidine residues are not common in collagen, and they might suggest the presence of elastin or actin in the extracted collagen. Nevertheless, the presence of this amino acid may have improved the antioxidant activity of the gelatines (Chen *et al.* 1998).

SDS-PAGE. The SDS-PAGE profile of gelatine from the skin was different from that of gelatine from fins and arms (Figure 1). All three gelatines showed a band at approximately 116 kDa, which was associated with collagen's α-chain, and two other bands in the continuous position with molecular weights of approximately 70 and 65 kDa, which may be related to a process of collagen degradation. Skin gelatine (Figure 1, Line D) also exhibited a thin band at 190 kDa, which is associated with a β-chain and another band at 40 kDa, which may be because of the presence of elastin (Gómez-Guillén *et al.* 2002). The electrophoretic pattern was similar to those observed for other squid species (Torres-Arreola *et al.* 2008; Giménez *et al.* 2009).

Table 1. Amino acid composition of gelatine from jumbo squid by-products (number of residues/1000 residues)

Amino acid	Fins	Arms	Skin
Hyp	55	55	67
Gly	254	256	285
Ala	62	58	58
Val	52	45	34
Leu	44	48	29
Ile	18	18	15
Pro	88	84	87
Asp	65	65	58
Glu	98	107	82
Ser	27	24	33
Thr	30	37	106
Met	37	39	58
Lys	77	79	21
Arg	38	27	43
His	14	13	11
Phe	21	19	15
Tyr	19	22	18
Total	1000	1000	1000
Imino amino acids (Pro + Hyp)	143	139	154

Determinations were performed in triplicate and data corresponded to mean values

In vitro digestibility. The highest degree of digestibility was observed for the skin gelatine ($P < 0.05$) (Table 2). Consistently with the results discussed above, the skin gelatine showed a lower true-collagen content than that present in either fins or arms, which led to an increase in its digestibility. The lower digestibility results observed in fin and arm gelatines, compared to skin gelatine, might be mainly associated with the presence of lysinoalanine, among others, a known product of the protein that has undergone a heat treatment (Gilani & Sepehr

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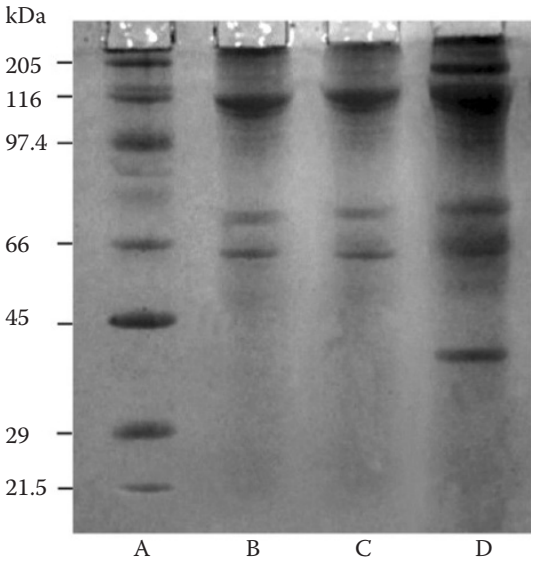

Figure 1. SDS-PAGE of non-hydrolysed gelatine from jumbo squid by-products: (A) standard molecular weight (SMW); (B) fin gelatine; (C) arm gelatine; (D) skin gelatine

SMW: myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase B (97.4 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), soybean trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa)

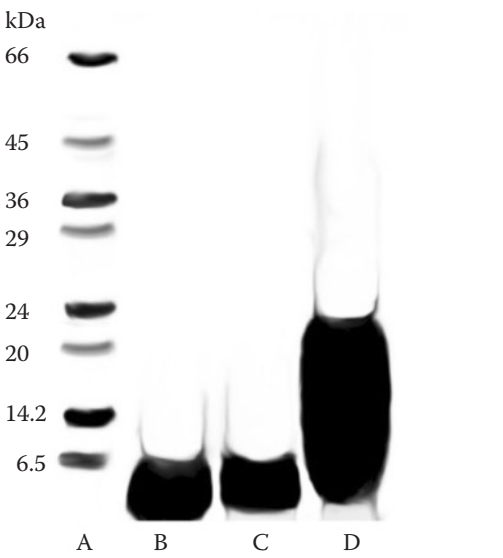

Figure 2. SDS-PAGE of hydrolysed gelatine from jumbo squid by-products: (A) standard molecular weight (SMW); (B) fin hydrolysed gelatine; (C) arm hydrolysed gelatine; (D) skin hydrolysed gelatine

SMW: albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), soybean trypsin inhibitor (21.5 kDa), α-lactalabumin (14.4 kDa), and aprotinin (6.5 kDa)

2003). Molecular weights of digested gelatine products were lower than 6.5 kDa for all gelatines from jumbo squid (Figure 2). These results could be associated with an increase of the antioxidant capacity shown by digested products in comparison with that determined in undigested gelatines.

Antioxidant capacity of non-hydrolysed gelatine and hydrolysed gelatine. All collagen showed antioxidant properties ($ABTS^+$ assay) (Table 2). The transformation of collagen into gelatine gave rise to a noticeable increase in the antioxidant capacity, due to better gelatine solubility. During the heat treatment, the triple helix molecular structure of collagen unfolds, and collagen is dissolved into random peptide chains which improve its biological properties. The order of antioxidant capacity of the obtained gelatines ranked as follows: skin > arms > fins (ORAC assay) and skin > fins = arms ($ABTS^+$ assay) (Table 2). The higher antioxidant capacity of skin gelatine can be attributed to the higher total content of imino acids, mainly Hyp, which is associated with the biological capacity of the squid collagen product (Alemán *et al*. 2011).

Based on previous reports (Giménez *et al*. 2009; Alemán *et al*. 2011), digested products had higher

Table 2. Antioxidant capacity and *in vitro* digestibility percentage of native gelatines and antioxidant capacity of hydrolysed gelatine

Product	Assay	Fins	Arms	Skin
	digestibility (%)	78.5 ± 0.7^b	77.4 ± 0.1^b	81.1 ± 0.5^a
Collagen	TEAC (mmol ET/mg)	0.7 ± 0.05^a	0.8 ± 0.05^a	0.8 ± 0.05^a
Gelatins	TEAC (mmol ET/mg)	2.8 ± 0.1^b	3.1 ± 0.1^b	3.3 ± 0.1^a
	ORAC (mmol ET/mg)	1.6 ± 0.1^c	2.0 ± 0.2^b	2.6 ± 0.1^a
Hydrolyesed gelatine	TEAC (mmol ET/mg)	6.8 ± 0.1^a	5.7 ± 0.1^b	6.6 ± 0.1^a
	ORAC (mmol ET/mg)	6.3 ± 0.5^b	4.6 ± 0.3^c	7.2 ± 0.3^a

TEAC – Trolox equivalent antioxidant capacity; ORAC – oxygen radical absorbance capacity; data expressed as the mean ± SD for three determinations; the same letters in the same row are not significantly different ($P < 0.05$)

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antioxidant capacity than native gelatines (Table 2). Furthermore, the hydrolysis process modified the order of antioxidant capacity of the gelatines: skin > fins > arms (ORAC assay), skin = fins > arms ($ABTS^+$ assay). The differences in the antioxidant capacity behaviour which were mainly observed between the fin and arm gelatines might have been due to the amino acids in the peptides which were produced after hydrolysis (Kim & Mendis 2006).

Gelatine intervention bioassays. The analysis of growth results showed a low degree of variation between replicates. No differences in weight gains between rats fed a non-hydrolysed gelatine-supplemented diet and control group were detected (data not shown). This result suggests that the feeding of jumbo squid gelatine did not affect the normal growth of healthy rats.

TAC and MDA levels. The TAC-ABTS assay was expected to increase because of the inclusion of antioxidant compounds in the diets (Kambayshi *et al.* 2009). However, the TAC-ABTS remained identical among all groups ($P > 0.05$) after gelatine administration; moreover, the TAC-ORAC values significantly decreased ($P < 0.05$) by approximately 14% in the rats that were fed fin gelatine compared to the control group (Table 3). The observed decrease in TAC-ORAC values in rats that were fed fin gelatine may be attributed to the compositional complexity of the serum, containing compounds that might behave as antioxidants (uric acid, bilirubin, etc.) and may mask the effect of the inclusion of gelatine in the rats' diet (Prior & Cao 1999).

Regarding the serum MDA levels, the rats to which any of the obtained gelatines was administrated had lower MDA (by approximately 13%) than those in the control group (Table 3). Liang *et al.* (2010) found that feeding to Sprague-Dawley rats a diet supplemented with marine collagen peptides that were derived from the chum salmon skin reduced the age-related lipid peroxidation. The presence of Gly, Pro, and Hyp in the gelatines may explain the shielding action for some cell components such as lipid membranes (Udenigwe & Aluko 2011). These results concur with the improvement of the oxidative state of healthy rats. Even though the differences between gelatine groups were not statistically significant, the fact that MDA levels were lower compared to the control diet indicates the protective effect of squid gelatines against lipid peroxidation *in vivo*.

Table 3. Effects of squid by-product gelatines on total antioxidant capacity (TAC) and malondialdehyde (MDA) levels in healthy rats

Treatment	TAC (mmol/ml)		MDA (μmol/μl)
	TEAC	ORAC	
Control	2.8 ± 0.1[a]	13.1 ± 0.9[a]	0.53 ± 0.01[a]
Fins	2.8 ± 0.2[a]	11.3 ± 0.7[b]	0.46 ± 0.01[b]
Arms	2.8 ± 0.2[a]	13.3 ± 0.8[a]	0.47 ± 0.01[b]
Skin	3.0 ± 0.2[a]	12.1 ± 0.9[a]	0.48 ± 0.01[b]

TEAC – Trolox equivalent antioxidant capacity; ORAC – oxygen radical absorbance capacity; data expressed as the mean ± SD for ten determinations; the same letters in the same column are not significantly different ($P < 0.05$)

CONCLUSION

Under the conditions of this study, the obtained gelatine from each studied by-product of jumbo squid (fins, arms, and skin) showed different chemical properties and *in vitro* antioxidant capacity. No positive effect of the administration of any gelatine on the antioxidant status *in vivo* determined by TAC assay was observed, but all of them decreased the malondialdehyde serum levels in healthy rats. The *in vitro* antioxidant capacity of the three extracts appears to be associated with positive effects on the oxidative stress of an organism, which is mainly evidenced by the MDA values. Further studies must be focused on the establishment of the mechanism for antioxidant peptide capacity in living organisms.

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