Muscle lysyl oxidase activity and structural/thermal properties of highly cross-linked collagen in jumbo squid (*Dosidicus gigas*) mantle, fins and arms

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Abstract Muscle from mantle, fins and arms of squid (*Dosidicus gigas*) were compared based on lysyl oxidase activity (LOX), chemical/structural and thermodynamic properties of highly cross-linked collagen. The arms collagen presented the highest temperature (Tp) and enthalpy of transition. The arms collagen thermic properties may be explained by the higher imino amino acid content, proline and lysine hydroxylation degrees. Moreover, among the regions, the collagen from the arms had a more intense β band chain, hydroxymerodesmosine peak in the resonance magnetic nuclear spectra and pyridinoline peak in the Raman spectra. Fins showed the highest LOX activity. The LOX activity was associated with the Tp, proline and lysine hydroxylation degrees. These results implied that the collagen in the arms was more intermolecularly ordered than the mantle and fins, and may provide a theoretical basis for a better understanding of the thermal behaviour of squid tissues during management and processing.

Keywords Chemical structure · Hydroxymerodesmosine · Lysyl oxidase · Squid collagen · Thermal denaturation

Introduction

According to the Food and Agricultural Organisation (FAO), jumbo squid (*Dosidicus gigas*) is one of the most important cephalopods captured in many countries' fishing industries [1]. The main globally traded goods are the mantle, fins and arms. Fresh squid muscle has a viscous thickness and is hard to chew. Moreover, the textural behaviour of squid during ice storage and cooking differs from other seafood products [2, 3]. These properties are attributed to the unique thermodynamic characteristics of its connective tissue [2], proteinaceous rich in collagen [4].

Moreover, due to the distinct function of the muscles in the live organism [5], the physicochemical properties of extracted squid connective tissue proteins depend on their anatomical origin [3, 5–7]. Such properties can be attributed to the highly variable crosslinking degree that depends, among other factors, on the tissue evaluated [8].

The principal collagen fibre crosslinking mechanism reportedly occurs through the oxidation of hydroxylysine (Hyl) by lysyl oxidase (LOX), which is formed from pyridinoline (Pyr) [9]. Pyr is an aromatic molecule that can be covalently linked to up to three collagen chains [10] and its concentration is closely associated with squid muscle thermal stability [11]. However, several factors that dictate the collagen thermal stability, such as LOX activity [12], are intertwined in a complex manner [13]. Thus, it is possible that Pyr is not the only molecule that affects the collagen thermal stability, yet there is scarce information available in the literature, to draw a definite conclusion.

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Another molecule that could affect the collagen thermal stability is hydroxymerodesmosine (HMD). HMD is also formed through LOX activity [14, 15] and is linked to the interaction between α -chains, which form dimers or trimers.

Different fractions of collagen obtained from jumbo squid muscle, have been partially characterized, measuring Pyr concentration and LOX activity [7, 16, 17]; however, these studies have primarily focused on soluble collagen fractions, which is not enough to elucidate the different cross-linking mechanisms in collagen fibers. A comparative research study of the collagen thermal denaturation, Pyr presence, and LOX muscle activity, among the highly cross-linked collagen from the edible anatomical regions of squid species, has not yet been performed. Therefore, the main focus of this study was to compare the thermal denaturation and chemical structure of highly cross-linked collagen, extracted from various anatomical regions of the jumbo squid (*D. gigas*). The possible correlation between the thermal properties of collagen, with tissue LOX activity, was discussed. This work provides an in-depth understanding of the interdependence between squid LOX activity, and collagen structure and thermal stability that can be used to direct future research on squid muscle management and processing.

Materials and methods

Samples and sample preparation

Ten jumbo squid (*D. gigas*) (weight 5–6 kg, length 45–50 cm), captured during the fall of 2016 in the Gulf of California (28.75°N, 112.25°W), were purchased from local fisherman. The gutted organisms were transported on ice to the laboratory, within 8 h of capture. The organisms were skinned. Then, the mantles were separated from the fins and arms, and all samples immediately stored frozen (-25°C), for no more than 7 days, until collagen and LOX extraction.

All reagents used in this work, were reagent grade (Sigma Chemical Co., St. Louis, MO, USA). Liquid chromatographic grade solvents were used in the amino acid analysis.

Collagen extraction

The connective tissue from the main anatomical regions of jumbo squid muscle (mantle, fins and arms) was extracted with 0.1 M NaOH (1:3 w/v, 4°C , 24 h), followed by washing with Milli-Q water (to remove excess NaOH). Once centrifuged (32,200g), the connective tissue was frozen at -40°C , lyophilised and, then, fractionated with

pepsin solution (10 mg g^{-1} tissue in 0.5 M acetic acid, 1:5 w/v, 4°C , 24 h). After centrifugation (32,200g, 40 min), the precipitate (50 mg) was homogenised (4°C , 24 h) in 15 mL of 50 mM sodium acetate, pH 4.8, containing 6 M urea. The sample was passed through a cation exchange [18] HiTrap CM Sepharose FF column (GE Healthcare, Uppsala, Sweden), coupled to a ÄKTA purifier system (GE Healthcare). Highly cross-linked collagen was eluted with a linear gradient from 0 to 0.5 M NaCl, in a total volume of 20 mL (flow rate 1.0 mL min^{-1}). The effluent was monitored at 280 nm. The fractions were pooled, dialysed against 0.05 M acetic acid, lyophilised and stored at -40°C , until further analysis.

Gel electrophoresis

The purity of collagen was analysed by sodium dodecyl sulphate 7.5% polyacrylamide gel electrophoresis (SDS-PAGE) [19]. The highly cross-linked collagen (12 μg) was dissolved in 0.05 M Tris, pH 7.4, containing 1% SDS. The separation gel used, contained 5.5% acrylamide. The electrophoresed gels were stained with Coomassie Blue R-250 and imaged, using a densitometer (Bio-Rad GS-700, Hercules, CA).

Amino acid analysis

The amino acid composition of collagen, was determined by reverse-phase high-performance liquid chromatography (Agilent Technologies Inc., Palo Alto, CA) [20]. The samples were hydrolysed in 6 M HCl containing sodium thioglycolate (1:1, v/v), at 150°C for 60 min. After hydrolysis, the samples were derivatised at 60°C for 5 min, in the presence of 0.5 M citrate containing 2 mg mL^{-1} 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole in methanol. The analysis was performed in triplicate and the results were expressed in % (w/w).

Raman spectroscopy

A Raman spectrometer (Perkin-Elmer GX), equipped with a Nd:YAG laser (excitation wavelength 1064 nm), was used to analyse the collagen [21] and to detect the presence of Pyr in the samples [22]. The backscattered light was collected at 180°C . The equipment was adjusted to 100 mW and 128 scans were collected at 4 cm^{-1} . The spectra were analysed using the program equipment's analysis.

¹H nuclear magnetic resonance (¹H NMR)

¹H NMR analyses of the collagen were measured at 25 °C on a Bruker Avance 400 spectrometer (Billerica, MA, USA), operating at 400 MHz [23]. Lyophilised samples (1 mg) were dissolved in 0.5 mL of deuterated water (D₂O at 99.6% atom 2H, Sigma-Aldrich, St Louis, MO, USA) and 0.5 mL of deuterated potassium hydroxide solution (KOD 40% in D₂O). Dimethylsilapentane-5-sulphonic acid (DSS) was used as the reference, using a 20-ppm spectral window, 90° pulse and an acquisition time of 3.98 s for 128 scans.

Differential scanning calorimetry (DSC)

The thermal profile of the collagen was analysed, using DSC, from 25 to 180 °C [7]. The transition temperature, onset temperature (T_o) and peak temperature (T_p), and the transition enthalpy (ΔH) of the collagen fractions, were measured using a 1020 Series DSC thermal analysis system (PerkinElmer, Norwalk, CN). Data acquisition and evaluation were done with a computer, using a Perkin Elmer PE Nelson 1022 integrator.

LOX extraction

LOX was sequentially extracted from the fresh muscle of the mantle, fins and arms, with phosphate buffer (pH 7.8) containing 16 mM KH₂PO₄, 16 mM K₂HPO₄, 0.4 M NaCl, and phosphate-buffered saline (1:2 w/v, 4 °C), followed by 4 M urea (4 °C, 3 h) [24]. The homogenate was centrifuged (39,000g, 40 min) and the supernatant was precipitated with 4 M urea. The urea fraction was resuspended in phosphate-buffered saline and frozen at − 80 °C.

LOX activity

A Cary Eclipse G9800A fluorescence spectrophotometer (Agilent Technologies, USA) was used, to determine the fluorescent Amplex red (*N*-acetyl-3,7 dihydroxyphenoxazine) compound, generated from the LOX activity in the samples [16]. The reaction mixture (1000 μL) consisted of 10 mM 1,5-diaminopentane (synthetic substrate), 50 mM sodium borate (pH 8.2), 1.2 M urea, Amplex red and 1 U mL^{−1} horseradish peroxidase. For the reaction, 50 μL of the enzymatic extract was added to the mixture, followed by incubation at 37 °C for 30 min. The reaction was stopped by immersion in an ice bath for 5 min. The excitation wavelength was 560 nm and the emission wavelength was 590 nm. The amount of hydrogen peroxide

(H₂O₂) produced by the action of LOX, was determined by comparing the fluorescence intensity with an H₂O₂ standard curve. The specific activity was expressed as enzyme units per mg of protein (U mg^{−1}). Each trial was performed in triplicate.

Statistical analysis

Data were analysed by one-way analysis of variance (ANOVA), to establish differences among the samples of the mantle, fins and arms. The normality of the data was assessed by the Kolmogorov–Smirnov test. Differences between least-squares means were examined by Tukey–Kramer's honestly significant difference (HSD) test ($p < 0.05$, in all cases). JMP version 4.04 (Statsoft, Tulsa, OK) was used for all statistics. Correlation values for LOX activity with T_p, and proline (Pro) and lysine (Lys) hydroxylation degrees, were calculated. All assays were performed in triplicate and the results were presented as mean ± standard deviation.

Results and discussion

Highly cross-linked collagen concentration

The yields of connective tissue in mantle, fins and arms were 0.8, 1.3 and 2.0 g/100 g muscle, respectively. In jumbo squid, connective tissue concentrations usually vary between 1 and 18 g depending on the anatomical region and the age of the organism [4]. In these extracts, the highly cross-linked collagen concentration varied significantly ($p < 0.05$). The greatest yield was obtained from the arms (51%), followed by the mantle (28.7%) and fins (22.3%). This behaviour is similar to that observed in previous works, which found that, depending on the anatomical region, there is variability in muscle collagen content [4, 5, 7].

SDS-PAGE electrophoresis

The electrophoretic patterns of the purified collagen samples obtained from each anatomical region (Fig. 1), demonstrated that the collagen comprised α- and β-chains as the main constituents, with a low content of γ-chains, indicating that Lys-derived crosslinks were present [26]. The similarity in the electrophoretic migration and amino acid composition (Table 1) for collagen type I of *D. gigas* with *Illex coindetii*, *Todaropsis eblanae* and *Todarodes pacificus* [5, 27], confirmed the presence of type I collagen in the analysed tissues. The band intensity (optical density)

Fig. 1 SDS-PAGE patterns of collagen obtained from the mantle (A), fins (B) and arms (C) of giant squid

of the crosslinked chain (β dimer) was higher ($p < 0.05$) in collagen from the arms (3040 ± 211) than fins (2280 ± 210) and mantle (1520 ± 214). This trend was confirmed by the Pyr Raman analysis.

Amino acid analysis

The amino acid compositions of purified collagen from the mantle, fins and arms, are shown in Table 1. The glycine (Gly) content represented 29–30% of the total amino acids residues in all the collagen samples. These Gly contents are typical for collagen [25]. In addition, low proportions of leucine (Leu), Lys and serine (Ser) and high proportions of alanine (Ala) and hydroxyproline (Hyp) were detected. Based on the study by Morales et al. [5], these results implied that the three anatomical regions contain type I collagen.

The analysis of the total imino acid (Pro + Hyp) content revealed that, collagen from the arms had a higher ($p < 0.05$) Pro + Hyp content than that obtained either from mantle or fins. Furthermore, the Pro and Lys hydroxylation extents (%), tended to be higher ($p < 0.05$) in the arms collagen. The differences in the percentage of hydroxylation of these amino acids, suggest that each anatomical region possesses different covalent crosslinks. Therefore, under the conditions of this study, this result demonstrated that the tissue collagen derived from the arms, possessed a higher degree of crosslinks than the

Table 1 Amino acid composition (%) of highly cross-linked collagen purified from the mantle, fins and arms of jumbo squid

Amino acid	Mantle	Fins	Arms
Hyp	9.1 ± 0.1	7.9 ± 0.3	11.2 ± 0.4
Asp	5.7 ± 0.1	5.3 ± 0.3	5.4 ± 0.2
Thr	2.9 ± 0.4	2.5 ± 0.1	2.0 ± 0.4
Ser	2.9 ± 0.3	3.9 ± 0.4	2.0 ± 0.4
Glu	8.0 ± 0.5	9.2 ± 0.7	6.8 ± 0.3
Pro	11.0 ± 0.6	10.6 ± 0.6	13.1 ± 0.7
Gly	29.5 ± 0.6	28.9 ± 0.7	30.0 ± 0.6
Ala	12.2 ± 0.7	10.4 ± 1.2	12.4 ± 0.7
Val	2.9 ± 0.4	2.9 ± 0.2	3.0 ± 0.2
Met	1.0 ± 0.2	1.0 ± 0.1	1.0 ± 0.4
Ile	1.1 ± 0.2	1.2 ± 0.3	1.0 ± 0.4
Leu	1.2 ± 0.1	1.9 ± 0.1	1.0 ± 0.2
Tyr	0.4 ± 0.02	0.4 ± 0.04	0.2 ± 0.03
Phe	1.2 ± 0.4	1.2 ± 0.2	1.0 ± 0.2
His	1.0 ± 0.1	1.9 ± 0.1	1.0 ± 0.2
Hyl	1.8 ± 0.5	1.7 ± 0.6	2.0 ± 0.8
Lys	1.9 ± 0.8	2.3 ± 0.3	1.9 ± 0.6
Arg	6.2 ± 0.7	6.1 ± 0.5	5.0 ± 0.9
Imino acid (Pro + Hyp)	20.1 ± 0.4^b	18.5 ± 0.3^c	24.3 ± 0.6^a
Pro hydroxylation (%)	45.3 ± 0.6^c	42.7 ± 0.3^c	46.1 ± 0.3^a
Lys hydroxylation (%)	49.3 ± 0.2^b	43 ± 0.3^c	51 ± 0.3^a
True collagen content/crude protein* (%)	84 ± 0.6^c	75 ± 1.3^b	93 ± 2.4^a

Values are the mean of triplicates $\pm$ standard deviation ($n = 3$)

* True collagen content/crude protein = $[\text{hydroxyproline} \times 8 / \text{crude protein}] \times 100$ (AOAC, Method 990.26, 2000). Different letters in imino acid (Pro + Hyp), Pro hydroxylation, Lys hydroxylation and true collagen denote significant difference ($p < 0.05$)

mantle and fins, as was confirmed by SDS-PAGE and the intensity of the Pyr band, by Raman spectroscopy.

Raman spectroscopy analysis

Raman spectroscopy was applied to analyse the structure of the purified collagens (Fig. 2). In general, relatively neutral bonds (CC, CH, C=C) are strong Raman scatterers. The bands observed at about 1663 and 830 cm^{-1} , are associated with Pyr. The differences in the shape and intensity of these bands, suggest structural differences among the samples [22]. The higher intensity of these bands in collagen from the arms compared to mantle and fins implies a higher proportion of highly cross-linked amino acids in this collagen.

Bands observed in the high-frequency region ($\sim 1246 \text{ cm}^{-1}$) are assigned to amide III. Fewer intense bands in this region were observed in the collagen from the mantle and fins than from the arms, suggesting fewer chain interactions through the hydrogen bonds of the collagen triple helix [21, 28], as well as a lower thermal stability.

The peak at 1326 cm^{-1} [22] is assigned to Pro and 845 cm^{-1} to Hyp [29]. These bands were more prominent in the collagen from the arms than mantle and fins, in accordance with the amino acid profiles. The highest Hyp activity, which was associated with the arms collagen, confirmed that the collagen in this anatomical region possesses a more stable triple helix, and therefore, would be expected to display the highest thermal stability, among the samples.

The 815–935 cm^{-1} region is associated with vibrations of C–C and C–O–C bonds present in covalent crosslinks of collagen molecules including, besides Pyr, HMD,

5-hydroxylysinoxidized, and glucosyl-galactosylhydroxylysinoxidized [14, 30, 31]. These types of bonds derive from the condensation between Lys and histidine (His) residues of the protein side chains. The oxidation of the adjacent Lys residues by LOX activity, results in the condensation of both residues that subsequently react with His residues, to form histidino-dihydroxymerodesmosine [15].

^1H NMR

The chemical structure of collagen from the squid mantle, fins and arms, were also analysed by ^1H NMR (Fig. 3), allowing the assignment of amino acid side chain protons. All extracts were prepared under identical conditions to avoid variability in the NMR spectra. Consequently, any differences in spectra can be attributed to the anatomical region evaluated.

The ^1H NMR analysis of all samples studied, confirmed the presence of water molecules (4.8 at 4.6 ppm) [32]. Proton alpha-Pro (4.5 ppm) chemical displacements were similar in all samples analysed. However, the intensity of this peak varied. Although the method cannot be used for amino acid quantification, a relative estimation can be made. In general, a smaller Pro peak was observed in collagen from the fins than mantle and arms, which is consistent with the above-discussed amino acid compositions. Moreover, the peak at 3.8 ppm, attributed to protons present in hydroxylated carbon and allyl methylene adjacent to the imino group of HMD [14], was more intense in the arms than the other anatomical regions evaluated, suggesting that relatively more Lys residues had been oxidised by LOX, in the collagen from the arms, to form this molecule.

Thermal analysis

The thermal stability of collagen, purified from the mantle, fins and arms, was determined using DSC, by measuring the beginning (T_o) and the peak temperatures (T_p) during the protein transition (Table 2). The denaturation temperature was above 100 $^{\circ}\text{C}$ in all cases, in agreement with previous reports [2, 33]; T_o and T_p of collagen extracted from arms were higher ($p < 0.05$) than the transition temperatures of the mantle and fin collagens, indicating that the collagen extracted from the arms was more thermally stable than the other collagens. However, the necessary energy (ΔH) to disrupt the structure of the collagen extracted from the mantle was greater ($p < 0.05$) than in the collagen of fin and arms. In DSC, a single peak may consist of several endothermic and exothermic interactions. Thermally induced protein denaturation results from a

Fig. 2 Raman spectra of collagen obtained from the mantle (A), fins (B) and arms (C) of giant squid, showing the high-frequency fingerprint regions (500 and 3000 cm^{-1})

Fig. 3 ^1H NMR spectra of collagen obtained from the mantle (A), fins (B) and arms (C) of giant squid. Amino acids indicated by their corresponding peaks; Lysine (Lys), threonine (Thr), isoleucine (Ile), glutamate (Glu), proline (Pro), histidine (His) and hydroxymerodesmosine (HMD)

Table 2 Transition temperatures and enthalpies of collagen obtained from mantle, fins and arms of giant squid

Anatomical region	To (°C)	Tp (°C)	ΔH (J g ⁻¹)
Mantle	137 ± 0.6 ^b	153.2 ± 0.6 ^b	33.8 ± 1.4 ^a
Fins	135 ± 0.4 ^c	145.9 ± 0.6 ^c	23.3 ± 1.5 ^b
Arms	138 ± 0.7 ^a	176.1 ± 0.4 ^a	25.8 ± 1.5 ^b

Values are mean of three replicates. Different letter for each column are statistically different ($p < 0.05$)

mixture of reactions such as the endothermic disruption of hydrogen bonds, the exothermic breaking of hydrophobic interactions, and others events occurring during heating [34]. These results showed that the helices of the mantle collagen had a greater number of conformational changes than those of the other collagens.

Among the samples, the lowest Tp and ΔH values were observed in the collagen from fins, consistent with the lower Pro and degree of hydroxylation of Pro and Leu, detected in this anatomical region. Likewise, Torres-Arreola et al. [7] reported that among the anatomical regions, collagen from the fins showed both the lowest Tp and ΔH

and can be attributed to a less stable triple helix structure [12, 35].

LOX activity

The specific LOX activity determined in each anatomical region was significantly different ($p < 0.05$). The highest LOX activity was measured in the fins (6.8×10^{-3} U mg⁻¹), followed by mantle (3.5×10^{-3} U mg⁻¹) and arms (2.3×10^{-3} U mg⁻¹). Among the anatomical regions, the highest LOX activity detected in fins muscle, might suggest that less Lys residues have been oxidised by this enzyme, as confirmed by the relatively lower Hyl content and the lower band intensity of Pry and HMD, by Raman and ^1H NMR, respectively, in this sample.

Finally, significant correlations between Hyl content ($R^2=$), Hyp content ($R^2=$) and Tp ($R^2=$) of collagen, with LOX activity, were observed. Thus, the differences in hydroxylation (%) of Lys and Pro (Table 1), and HMD band intensity (^1H NMR) (Fig. 3), might suggest that Pry itself, partially contributed to the collagen thermal stability. Several factors that dictate the collagen thermal stability, such as LOX activity [12], are interconnected in a complex

manner [13], requiring more studies to decipher the exact intercorrelations.

Under the conditions of this work, it was proven that the collagen from each jumbo squid anatomical region has a different chemical structure. The Tp and ΔH indicated that the fibres of arms collagen are stronger than that of the mantle and fins. The differences detected are due to variations in the crosslinked helix structure, among the collagens derived from the mantle, fins and arms. The detection of the HMD band by Raman indicated that the Pyr is not the only molecule involved in the crosslinking of collagen, which was consistent with the results of the collagen thermodynamic values. Despite the lower LOX activity detected in arms muscle, its collagen seems to have a higher degree of crosslinking than the mantle and fins. However, analysis using additional techniques is important to corroborate this finding. These results may serve as a fundamental basis, for further investigation into the management and processing of jumbo squid tissues.

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