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Immunoblotting identification of jumbo squid (*Dosidicus gigas*) LOX isoforms and *in vitro* crosslinking assay over selected collagenous materials

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Abstract

Jumbo squid (*Dosidicus gigas*) muscle hardness has been related to hydroxylysyl-pyridinoline (HP) formation (collagen fibre stabilization) via condensation of oxidized ε-amino groups of lysine and/or hydroxylysine by lysyl oxidase (LOX). Previously published literature has suggested the presence of LOX isoforms in squid muscle. Thus, the objective of the present research was to test the hypothesis that squid LOX isoforms exist in fresh mantle muscle. A semi-pure LOX extract (SPLE) was used to perform an immunoblotting assay using two commercial human-LOX antibodies. Furthermore, SPLE (specific activity of 20 mU/mg of protein) was tested *in vitro* for possible HP formation using several collagenous materials. Immunoblotting assay confirmed the presence of squid LOX at 32 kDa and its isoforms at 34 kDa and 24 kDa. Although LOX oxidized ε-amino groups on collagenous materials, no HP formation was detected. However, the latter could promote interesting collagen functional modifications.

Keywords: jumbo squid; immunoblotting; lysyl oxidase; hydroxylysyl-pyridinoline.

Practical Application: This research contributes to the expansion of existing knowledge about the presence of LOX and their isoforms in jumbo squid muscle. Technological application of the enzyme as a collagen modifier can arise if the optimal *in vitro* kinetic conditions are provided.

1 Introduction

Lysyl oxidase (LOX) has long been studied in human and terrestrial mammals due to its critical role in biogenesis and stabilization of the extracellular matrix (ECM) through a well-documented pyridinoline crosslinking mechanism (Eyre et al., 2008; Kagan & Li, 2003; Kagan & Ryvkin, 2011). The ECM is an interstitial component constituted mainly by collagen and elastin, two connective tissue proteins (Shoulders & Raines, 2009). Its stabilization begins with the initial oxidation of ε-amino groups of lysine and/or hydroxylysine by lysyl oxidase (LOX) following their condensation (crosslinking) within and between individual molecules of collagen and/or elastin (Bailey et al., 1998; Lucero & Kagan, 2006). LOX is known as one member of a family of amino oxidases commonly identified as lysyl oxidase-like enzymes, LOXL-1, 2, 3 and 4 (Vallet & Ricard-Blum, 2019). LOX genetic expression or enzymatic activity in connective tissues differs among species; moreover, it is well known that physiological status (i.e. chronological age, health, sex and size) and environmental factors (i.e. light, temperature and atmosphere) have a strong influence on LOX activity (Consuegra & Johnston, 2006; Geach & Dale, 2005).

Current research in food science has been studying the LOX enzyme, the collagen crosslinking activity of which has been strongly associated with undesirable meat hardness in certain marine species (Tapia-Vasquez et al., 2021). In this sense, pyridinoline crosslinking has been quantified in collagens from red sea bream (*Pagrus major*), yellowtail (*Seriola quinqueradiata*), tiger puffer (*Fugu rubripes*) and squid species (Ando et al., 2001; Ando et al., 2006). Meanwhile, the chemical nature of pyridinoline and its tissue-specific distribution in jumbo squid (*Dosidicus gigas*) connective tissues were documented by Ramirez-Guerra et al. (2015b), suggesting Lys hydroxylation as a critical regulatory step in squid collagen crosslinking. On the other hand, Li et al. (2007) reported a correlation between the fillet firmness of farmed Atlantic salmon (*Salmo salar L.*) and the presence of (hydroxylysyl-pyridinoline, HP) in skeletal insoluble collagen. Similarly, the relationship between muscle texture, LOX activity, and HP concentration in collagen fibres from octopus (*Octopus vulgaris*), guitarfish (*Rhinobatos productus*), and cazon (*Mustelus lunulatus*) was established by Tapia-Vasquez et al. (2019), who found a positive correlation between LOX activity intensity and the total HP quantified, whereas Ramirez-Guerra et al. (2015a) established a relationship between jumbo squid LOX activity, HP content, and the texture of squid muscle stored in ice. In the literature, two findings have been closely linked in reference to squid LOX: first, the use of LOX activity and the collagen crosslinking degree as predictive indicators of changes in the

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texture profile of squid meat during its post-catch handling and second, the explanation of the high thermal stability and poorly solubility of the isolated squid crosslinked collagen that have been previously reported by other researches (Ezquerra-Brauer et al., 2018; Sarabia-Sainz et al., 2017; Tapia-Vasquez et al., 2021). Thus, it seems important to inquire about the existence of jumbo squid LOX variants for future research involving LOX enzyme activity and cross-linkages produced in jumbo squid muscle. LOX variants have been confirmed for terrestrial animals and humans (Kagan & Ryvkin, 2011; Vallet & Ricard-Blum, 2019). As for Jumbo squid muscle, a slight molecular weight variation (from 29 to 34 kDa) in jumbo squid LOX was estimated by SDS-PAGE densitometric analysis (Ramirez-Guerra et al., 2015a; Torres-Arreola et al., 2011, 2012), supporting the hypothesis that enzyme variants exist in jumbo squid muscle. Since the enzymatic method developed by Palamakumbura & Trackman (2002) only shows the presence or absence of LOX enzymes in a sample but not of its isoforms, the objective of the present research was to test this hypothesis in a semi-pure LOX extract (SPLE) using immunoblotting assays against two commercial human-LOX antibodies. Moreover, a potential technological application of jumbo squid LOX through HP synthesis *in vitro* using SPLE and several collagenous materials was explored.

2 Materials and methods

2.1 Materials

1,5-Diaminopentane dihydrochloride, horseradish peroxidase type II (5000 units), β-aminopropionitrile fumarate salt (BAPN), urea, sodium tetraborate decahydrate, hydrogen peroxide 30%, trizma hydrochloride, potassium phosphate monobasic, potassium phosphate dibasic, acid acetic, 2-mercaptoethanol, heptafluorobutyric acid (HBFA), type I and V collagens from bovine Achilles tendon, bovine gelatin, fish gelatin, monoclonal Anti-LOX (Cat. SAB4100014) antibody produced in mouse, polyclonal anti-lysyl oxidase (Cat. L4794) antibody produced in rabbit, anti-mouse IgG (Cat. A5362) and anti-rabbit IgG (Cat. A3687) alkaline phosphatase coupled antibodies were purchased from Sigma Aldrich (St. Louis, MO, USA). Acrylamide, N, N, N′, N′-tetramethylethylenediamine (TEMED), sodium dodecyl sulphate (SDS), ammonium persulphate, the alkaline phosphatase conjugate substrate kit, Quick Start Bradford 1x Dye Reagent, broad range protein standard and the bovine gamma globulin (BGG) standard set were purchased from Bio-Rad (Bio-Rad Laboratories, Hercules, CA, USA). Polyvinylidene fluoride (PVDF) microporous membranes and Ultra-0.5 mL centrifugal filters were purchased from Millipore (Merc KGaA, Darmstad, GER). Amplex red was purchased from Invitrogen™ (Thermo Fisher Scientific, Inc., USA). Standard hydroxylysyl-pyridinoline (HP) was purchased from Wako Pure Chemicals Co. (Richmond, VA, USA) and CHROMABOND® Crosslinks polypropylene columns were purchased from Macherey-Nagel (Bethlehem, PA, USA).

2.2 Preparation of semi pure LOX extract (SPLE)

Fresh jumbo squid (*Dosidicus gigas*) specimens 20-40 cm in length were obtained from a local fish market and stored at −80 °C. All extractions and enzyme purification steps were carried out between 0 and 4 °C. The partially thawed mantle (350 g) was skinned, chopped into small pieces and then mixed with 200 mL of buffer A (13 mM K_2HPO_4/3 mM KH_2PO_4, pH 7.8). Then, the homogenate was centrifuged at 34,000 × g for 15 min and the supernatant discarded. This procedure was repeated once more. Then, the pellet was washed twice with 200 mL of buffer B (13 mM K_2HPO_4/3 mM KH_2PO_4/0.4 M NaCl, pH 7.8) and centrifuged as before. Finally, the pellet was homogenized with buffer C (13 mM K_2HPO_4/3 mM KH_2PO_4/6 mM Urea, pH 7.8) at a 1:1 (p/v) ratio, stirred for 12 h and centrifuged as before. The obtained supernatant, called crude extract (CE), was collected and loaded onto a pre-packed DEAE Fast Flow anion exchange column, previously equilibrated with buffer C. After the column was washed with five volumes of buffer C, bound proteins were eluted using a gradient of salt concentration (from 0 to 0.25 M NaCl, in buffer C), at a flow rate of 1 mL/min. The amino oxidase activity of all collected fractions were monitored by a fluorescence-based assay using Amplex™ Red fluorophore (*see enzyme activity section*) (Palamakumbura & Trackman, 2002). Active fractions were pooled and concentrated with 10-kDa Amicon® Ultra-0.5 mL centrifugal filters (Millipore) and then loaded onto a HiPrep 16/60 Sephacryl S-200 HR exclusion column, previously equilibrated with buffer C. Proteins were eluted at 0.5 mL/min with buffer C. Active fractions were pooled (SPLE), concentrated as indicated above and then subjected to electrophoresis and western blot analysis.

2.3 Protein quantification

Protein content was quantified according to Bradford (1976) using the Quick Start™ Bradford Protein Assay (Bio-Rad Laboratories), following a microplate assay protocol. The total protein concentration was calculated using two standard curves that were constructed using bovine α-globulin as a standard (1.25-25 μg/mL and 125-1,500 μg/mL).

2.4 Amino oxidase activity

A fluorescence-based assay protocol was carried out to measure amino oxidase activity using the Amplex™ Red assay kit according to Palamakumbura & Trackman (2002), which uses 1,5-diaminopentane as the substrate. The reaction was performed as follows: 150 μL of a solution containing 1.2 M Urea, 50 mM sodium borate, pH 8.2, 1 U/mL horseradish peroxidase, 20 μM Amplex™ Red and 10 mM 1,5-diaminopentane were loaded onto a 96-well plate (Corning® Life Sciences, NY, USA). The reaction was initiated by adding 50 μL of SPLE and incubated for 30 min at 37 °C. The amount of hydrogen peroxide produced by the action of amino oxidase enzymes present in the SPLE was determined by comparing fluorescence changes to a standard plot, relating the change in fluorescence to nmol of hydrogen peroxide added to the control (no sample). One unit was expressed as the amount of hydrogen peroxide released *per* mg protein.

2.5 SDS-PAGE analysis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (1970) using

a 4% stacking gel and 12% resolving gel. After electrophoresis, gels were stained with Coomassie Blue (Bio-Rad Laboratories). For the analysis, 30 μg of protein were loaded into each well of polyacrylamide gels. A broad-range molecular weight protein standard (Bio-Rad Laboratories, Hercules, CA, USA) was used containing the following proteins: myosin (200 kDa), β-galactosidase (116.3 kDa), phosporylase b (97.4 kDa), serum albumin (66.2), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21.5 kDa), lysozyme (14.4 kDa) and aprotinin (6.5 kDa). Images of electrophoresis gels were captured by using an image densitometer (Bio-Rad Model GS-700).

2.6 Western blot analysis

SDS-PAGE resolved proteins were electrophoretically transferred to PVDF membranes in transfer buffer (25 mM Tris, 192 mM Glycine, 20% methanol, pH 8.3) using a mini-Trans-Blot apparatus run at 30V and 4 °C overnight. Membranes were blocked overnight with 1% albumin from bovine serum at 4 °C. Monoclonal anti-LOX antibody and anti-lysyl oxidase polyclonal antibody were individually tested overnight at a 1:200 dilution and room temperature. Each membrane was treated overnight with its respective alkaline phosphatase-conjugated secondary antibody (anti-mouse IgG and anti-rabbit IgG, respectively) at a 1:150,000 dilution at room temperature and developed using an Alkaline Phosphatase Conjugate Substrate Kit (Bio-Rad Laboratories, Inc., CA, USA). Images were captured using an image densitometer (Bio-Rad Model GS-700).

2.7 In vitro crosslinking assay

The ability of jumbo squid LOX to oxidize Lys and Hyl, producing HP crosslinks on squid mantle collagens, commercial Type I and V bovine collagens, and bovine and fish gelatin was tested by using two experimental procedures. First, crude collagen from squid muscle (CC) was obtained according to Ramirez-Guerra et al. (2015b) and subsequently fractionated to obtain both the 0.5 M acetic acid-soluble collagen (ASC) and insoluble collagen (IC) by centrifugation. Then, in order to confirm the squid LOX oxidative function over the above collagenous materials, each of these were used as substrate (by substitution of 1,5-diaminopentane) in the *in vitro* amino oxidase activity assay. The positive reaction was confirmed by visual examination of the red colour developing (Amplex™ Red oxidation). For the crosslinking assays, all substrates were suspended in 50 mM borate buffer, pH 8.2 at a 5:1 (p/v) ratio and incubated with 100 μL of squid SPLE at 37 °C for 30 min. The reaction was stopped by cooling the solution at 0 °C. Finally, the incubated collagens were lyophilized for HP analysis.

2.8 Hydroxylysyl-pyridinoline (HP) analysis

Collagens and gelatines that had previously been incubated with squid SPLE were later subjected to HP analysis according to Ramirez-Guerra et al. (2015b). One hundred milligrams of lyophilized sample was hydrolysed with 3 mL 6 M HCl at 150 °C for 6 h. Then, the hydrolysed sample was pre-fractionated by solid-phase extraction (SPE) using a Chromabond® Crosslinks column to remove interfering molecules. Briefly, 1.6 mL of hydrolysed samples were diluted with an equal volume of 90% acetic acid and mixed with 2.5 mL of acetonitrile in a 5-mL glass tube. Then, the solution was transferred to a SPE column previously equilibrated with 2.5 mL of washing buffer prepared with acetonitrile, acetic acid and DI water (8:1:1, v/v/v). The column was washed extensively 4 times with 2.5 mL of washing buffer, and a final wash was performed with 400 μL of deionized (DI) water. The column was drained, and HP crosslinks were eluted with 200 μL of 1% heptafluorobutyric acid (HBFA) directly on a HPLC micro-insert vial. HP crosslinks were separated using a Series 1100 HPLC system (Hewlett Packard Co. Waldbrom, Germany) coupled to a fluorescence detector. An automatic injection system equipped with a 100-μL syringe was used to load 20 μL of each sample to an ODS C18 Microsorb-MV column (100 C_{18}, 4.6 mm ID × 250 mm, Microsorb, Rainin, CA, USA), and separation was performed at a flow rate of 1 mL/min at 40 °C. The mobile phase consisted of 0.12% HBFA in DI water (solution A) and 50% acetonitrile (solution B). The column was equilibrated with 20% of solvent B prior to use, achieving chromatographic separation with a gradient elution from 20% to 30% solvent B in 20 min. The HP crosslinks were monitored for fluorescence at λ_{ex} = 297 nm and λ_{em} = 395 nm. The HP content was estimated as moles *per* mole of collagen. A factor of 7.14 was used to convert the mass of hydroxyproline to the mass of collagen, and the HP concentration was calculated assuming a molecular mass of 3.38×10^5 g/mol for collagen.

2.9 Statistical analysis

All data generated in crosslinking assays were analysed using one-way ANOVA within the statistical software package NCSS 2007 (Kaysville, UT). A Tukey test was used when significant differences were detected ($P < 0.05$) between the means. At least three replicates (n = 3) were carried out for each analysis. The experiment was repeated three times.

3 Results and discussion

3.1 Specific activity of semi-pure squid LOX extract (SPLE)

The sequential anion exchange and gel filtration chromatographies used to purify LOX from jumbo squid mantle muscle are shown in Figure 1 (A and B, respectively). Most DEAE-bounded proteins, in which amino oxidase enzymatic activity was detected (fractions 7-9), were separated during the first half of the salt gradient (0-0.1 M NaCl) (Figure 1A). After DEAE chromatography, the squid LOX purification yield was 2.45%, with an increment of 41 times the enzyme specific activity with respect to the CE (Table 1). Subsequently, major protein contaminants present in the DEAE pooled fraction were eliminated by molecular size exclusion chromatography as shown in Figure 1B. Here, amino oxidase activity was registered in fractions 29-31, and after they were pooled, its specific activity was enhanced 115.7 times relative to the first chromatographic step. The results of the final purification step of squid LOX from mantle muscle are summarized in Table 1. As the results showed, it was possible to recover a semi-pure LOX extract (SPLE) with specific activity of 20 mU *per* mg protein at the end of the purification procedure.

3.2 SDS-PAGE analysis of SPLE

The electrophoretic profile of the SPLE showed the presence of four protein bands with molecular weights of 50 kDa, 34 kDa, 32 kDa and 24 kDa (Figure 2). The electrophoretic analysis indicated the presence of the LOX enzyme, since a protein band at 32 kDa, stained in the gel, was associated with the LOX previously identified in jumbo squid (*Dosidicus gigas*) tentacles by Torres-Arreola et al. (2011, 2012). Similarly, this LOX band was isolated from other species, such as in bovine aorta, rat skin and uteri, piglet skin and human skin and placenta (Kagan & Cai, 1995; Kuivaniemi, 1985; Palamakumbura & Trackman, 2002; Romero-Chapman et al., 1991; Shackleton & Hulmes, 1990).

3.3 Western blot analysis of SPLE

The results of immunoblotting assays of SPLE in PVDF membranes treated with monoclonal Anti-LOX and polyclonal Anti-Lysyl oxidase antibodies are shown in Figure 3. A positive antibody reaction suggests structural homology between squid LOX and human LOX. Both tested antibodies recognized two squid LOX variants with weights of 32 kDa and 34 kDa. Moreover, one LOX fragment of 24 kDa was recognized when semi-pure extract was incubated with the polyclonal antibody. The antigenic determinants recognized by the antibodies (mono- and polyclonal) used in our immunoblotting assays indicate a high degree of identity between human LOX and squid LOX, as well as between squid LOX and its variants. The polyclonal antibody response to squid LOX in its mature form (32 kDa) and the 24 kDa LOX fragment was similar to the one shown by Burbelo et al. (1986) in immunoassays of human LOX purified

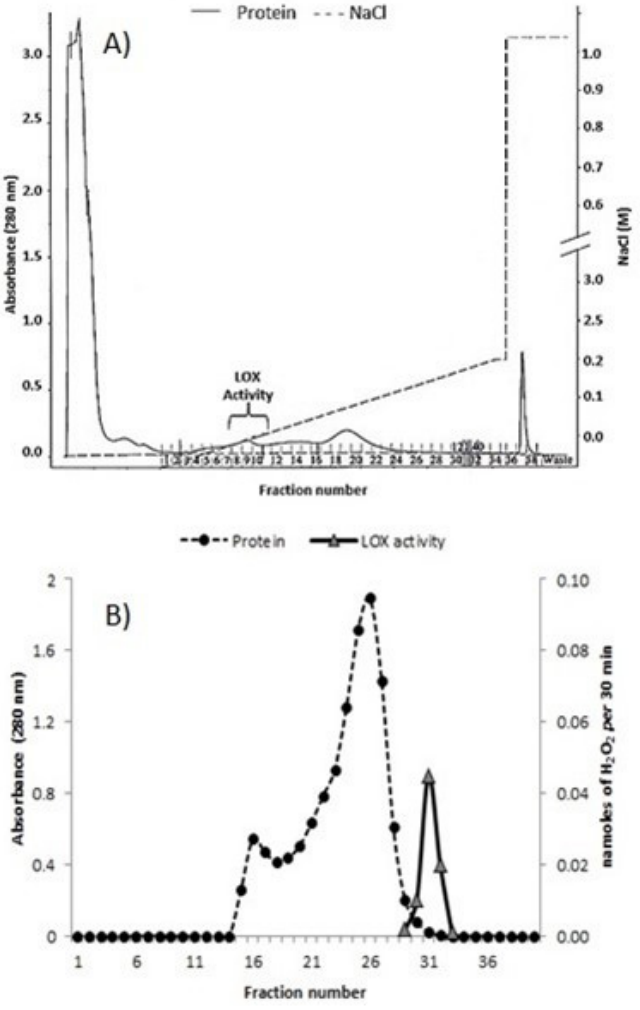

Figure 1. Purification of lysyl oxidase (LOX) from jumbo squid (*Dosidicus gigas*) mantle muscle. (A) Anion exchange chromatography; and (B) Gel filtration chromatography.

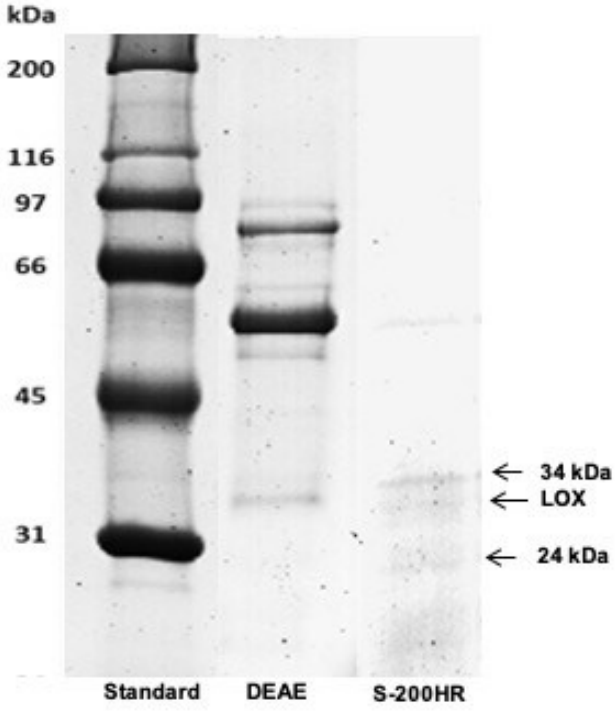

Figure 2. SDS-PAGE analysis of recovered fractions from anion exchange (DEAE) and gel filtration (S-200HR) chromatography.

Table 1. Purification parameters of lysyl oxidase (LOX) from jumbo squid (*Dosidicus gigas*) mantle muscle.

Purification step	Total protein (mg)	Total activity (Units)	Specific activity U (nmol/min)*	Yield (%)	Folding
Crude extract (CE)	2373	1.50	0.000004	100	1
DEAE anion exchange	20	0.07	0.000172	2.45	41
Sephacryl S-200HR	0.075	0.01	0.02	0.02	4746

Units = Activity was expressed as nmol of hydrogen peroxide produced *per* min. *U = Units *per* mg of protein in the assay at 37 °C. Data are average of five determinations with a coefficient of variation <5% among replicates.

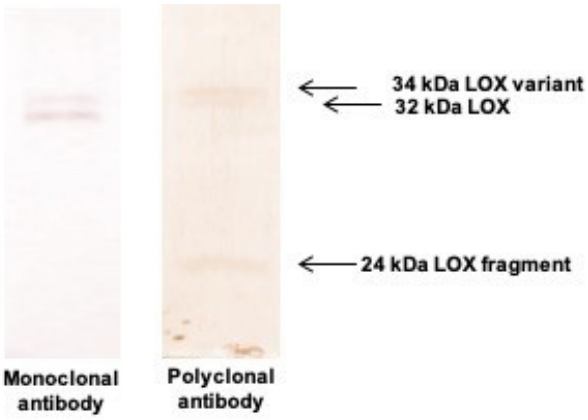

Figure 3. Immunoblotting assay of semi-pure LOX extract (SPLE) with monoclonal and polyclonal human antibodies.

Table 2. Hydroxylysyl-pyridinoline (HP) concentration in collagens and gelatines treated with semi-pure LOX extract (SPLE) from jumbo squid (*Dosidicus gigas*) mantle muscle.

Material	Control	LOX treatment
	HP (mmol/mol collagen)	HP (mmol/mol collagen)
†CC	$1.78 \pm 0.12^{a,w}$	$1.88 \pm 0.02^{a,w}$
†ASC	N.D.	N.D.
†IC	$1.98 \pm 0.09^{a,w}$	$1.89 \pm 0.03^{a,w}$
*Type I collagen	$4.19 \pm 0.27^{a,y}$	$4.29 \pm 0.04^{a,y}$
*Type V collagen	$14.73 \pm 0.29^{a,z}$	$14.61 \pm 0.05^{a,z}$
Bovine gelatin	$1.8 \pm 0.26^{a,x}$	$2.1 \pm 0.15^{a,x}$
Fish gelatin	N.D.	N.D.

Mean ± E.D. (n = 3). Different superscripts between columns and rows indicate significant differences (P < 0.05). †Squid mantle collagens. CC: crude collagen from squid mantle; ASC: acid soluble squid collagen; IC: insoluble squid collagen; *Collagen From bovine Achilles tendon. N.D.: not detected.

from umbilical cords, using monoclonal antibodies produced by mouse hybridoma cells.

As shown in the present research, immunoblotting assays indicate a significant consensus between the squid LOX and human LOX sequences. Regardless of the nature of the antibody used or its specificity, the results suggest similarities between the posttranslational processing pathways of squid LOX and human LOX. It is important to remark that the tested polyclonal antibody was designed to recognize the N-terminal sequence (YDTYERPRPGGRYRPG, locus 187-202) of the active site of human LOX. Therefore, the findings of the present research will help to support the previous characterization studies of squid LOX developed by Torres-Arreola et al. (2012). LOX is known as a member of the amino oxidase family, which shares a conservative C-catalytic domain with LOX-like enzymes, and their presence in biological samples is commonly monitored by the same amino oxidase activity assay, using 1,5-diaminopentane as a substrate (Barker et al., 2012). However, this last test only tells us about the presence or absence of LOX enzyme and not about possible isoforms. Thus, the implementation of the immunoblotting assays to confirm the presence of LOX and their isoforms is desirable in either squid or other marine species. Although squid LOX variants were revealed in the immunoblotting assays of SPLE, it cannot be guaranteed that these variants are active forms of the enzyme, therefore the authors do not discard the possibility that future research can be directed at their isolation, in order to clarify this issue.

3.4 In vitro crosslinking assays using SPLE

The results obtained in the crosslinking assays using SPLE and collagenous materials are shown in Table 2. SPLE was capable of triggering the initial oxidation of the ε-amino group of Hyl and Lys residues present in all the collagens and gelatines analysed. This was confirmed as the microplate wells containing the amino oxidase assay reaction mixtures turned red (*data not shown*) after incubation time. However, no differences ($P \geq 0.05$) in HP content for all substrates after incubation with SPLE were registered (Table 2). These last results indicated the formation of aldehydes from the ε-amino groups of Hyl and Lys residues by LOX activity, with the concomitant formation of hydrogen peroxide but not their condensation into HP formation. In contrast, the *in situ* formation of HP by squid LOX in collagen from mantle muscle stored on ice for 20 days was confirmed previously by Ramirez-Guerra et al. (2015a). However, in the best approach, these primary modifications could offer an opportunity to modify the functionality (i.e. solubility and gelling properties) of incubated collagenous materials. Nevertheless, more research is needed to improve the *in vitro* optimal kinetic conditions to promote the condensation reactions of these modified materials and thus affect their functionality. Nonetheless, it was interesting to note differences between the HP content in the substrates (Control) previously to crosslinking assays. As expected, after fractionation of crude collagen (CC) from squid mantle, the HP content was mainly distributed in its insoluble collagen (IC). HP crosslinks were not detected in both ASC and commercial fish gelatin, but bovine gelatin registered an HP content of 1.8 ± 0.26 mmol/mol collagen, a similar value ($P \geq 0.05$) to the one registered for CC. On the other hand, the HP content in Type I and V collagens from bovine Achilles tendon was 2.3 and 8.3 times higher than that registered for CC, respectively.

It is well documented that the intramuscular collagen of cephalopod species (*T. pacificus*, *I. coindetii, T. ablanae,* and *E. cirrhosa*) is mainly composed of Type I and V collagens (Morales et al., 2000). Although the isolation and identification of squid collagen types was not considered in the present research, it is very probable that Type I and V are the most representative collagen species in squid muscle. In addition, a greater difference ($P < 0.05$) in HP content between the two commercial collagens from bovine Achilles tendon was found. Type V collagen appears to be insoluble and higher crosslinked material than type I (Table 2).

Finally, intriguingly, the commercial bovine gelatin analysed in this research contained the same amount of HP as the insoluble squid muscle collagen fraction, so, the question remains, how much does the solubility and gelling properties really depend on the degree of crosslinking in collagenous materials?

4 Conclusion

The positive reactions of the two commercial antibodies used in western blot analysis of squid LOX suggest their substantial homology with the human enzyme equivalent. Moreover, the presence of squid LOX variants lends relevance to the above results, although their occurrence and enzymatic activity remain unclear. Under the tested experimental conditions, squid LOX was capable of triggering oxidation of Lys or Hyl residues in collagenous materials but did not promote HP condensation. On the other hand, the crosslinking assay results suggest a possible technological application of squid LOX in the future, provided the optimal *in vitro* kinetic conditions to promote the condensation reactions in the oxidized collagenous materials could be achieved.

Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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