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RESEARCH REPORT

Characterization of protease activity from the digestive tract and tentacles of *Isostichopus fuscus* sea cucumber

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Abstract

Sea cucumbers possess evisceration mechanisms and regeneration capacity. The function of tentacles is to collect food particles. From our results, we suggest these organs could also be part of the digestive system. Therefore proteases in the digestive tract and tentacles of *Isostichopus fuscus* were partially characterized by histological and biochemical methods. Digestive cells and regions, and secretory granules were observed by histological methods in both organs. Proteolytic extracts of the digestive tract and tentacles showed peak activity at pH 6 and 8. The digestive tract extract had peak activity at 40 and 70 °C, whereas the tentacle extract peak activity was at 60 °C. Both extracts showed activity at 0 to 10 °C. The extracts retained 67 to 75 % residual activity when incubated at 60 °C for 1 h. The effect of different ions and specific inhibitors suggested the presence of cysteine- and metallo-proteases in both organs. SDS-PAGE showed 6 proteins of approximately 40, 43, 49, 76, 106, and 147 kDa in the digestive tract extract, and 5 proteins of approximately 44, 60, 81, 108, and 150 kDa in the tentacle extract. Native-PAGE and zymography assays confirmed the presence approximately 100 kDa proteases in both extracts. The tentacle extract had the highest proteolytic activity, suggesting that this organ could contribute to the digestion process of *I. fuscus*.

Key Words: proteolytic enzymes; proteolytic extract; brown sea cucumber; holothurians; digestive system

Introduction

Sea cucumbers are marine invertebrates of phylum Echinodermata, class Holothuroidea. These holothurians play an important ecological role in the structure and functioning of marine benthic communities by consuming organic material (Uthicke, 2001). Sea cucumbers have acquired commercial importance as food items due to its high nutritional value (56 % protein, 2 % fat dry weight). They are specially demanded by consumers in South East Asia (Bordbar *et al.,* 2011; Purcell *et al*., 2012). *Isostichopus fuscus* is the most common sea cucumber commercial species found in the Pacific coast of the Americas, from Baja California, Mexico to Ecuador (Maluf, 1991). Its morphology consists in an elongated body. The mouth, located in the anterior section, is surrounded by 15 to 20 peltated tentacles. The anus is located in the posterior end (Solís-Marín *et al*., 2009) (Fig. 1). The digestive system is formed by a digestive tract (mouth, pharynx, esophagus, stomach, intestine and cloaca) and anus (Yang *et al*., 2015). Tentacles function is related to selection and capture of food particles, the adhesive forces and shape of tentacles select these feeding materials (Jaeckle and Strathmann, 2012; Yang *et al*., 2015). Sea cucumbers are able to eviscerate as a defense mechanism against predators, although evisceration can also be activated by environmental and mechanical factors, such as high temperatures, low oxygen levels, and handling. This process represents economic losses when these animals are produced by aquaculture methods. Even though, several holothurians have the ability of viscera regeneration (García-Arrarás and Greenberg, 2001; Wu *et al*., 2013). Proteases, or proteolytic enzymes, (EC 3.4.) catalyze the hydrolysis of peptide bonds during protein digestion and other biological functions. Over the years, marine animals adapted to different environmental conditions, developing endogenous proteases with

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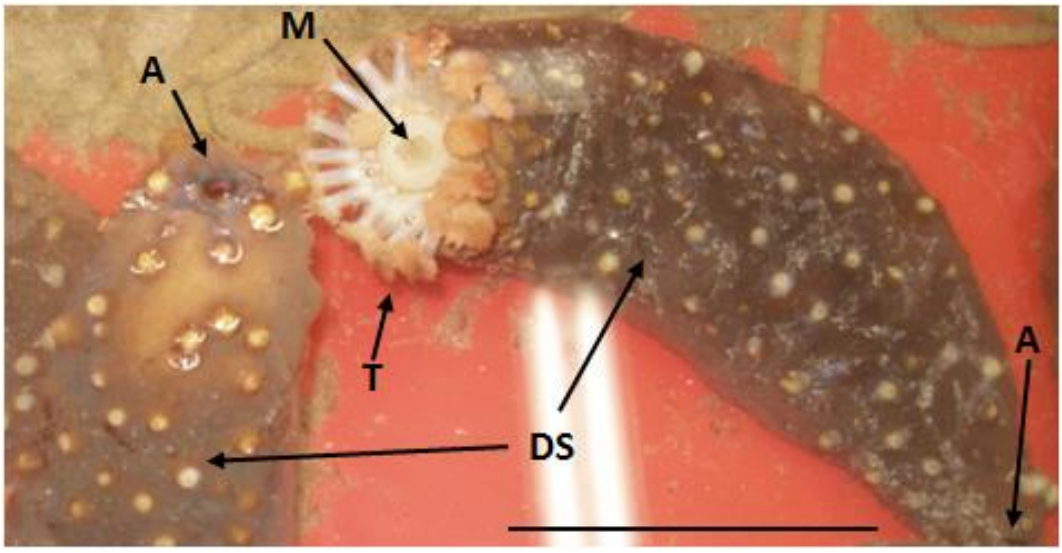

Fig. 1 Morphology of *I. fuscus*. Two sea cucumbers show the dorsal surface (DS); it shows mouth (M) surrounded by 20 peltate tentacles (T) in the anterior section and anus (A) in the posterior section. Scale bar = 10 cm.

unique properties. Some of their distinctive features include high catalytic efficiency at low reaction temperatures, stability to extreme temperatures, and substantial catalytic activity/stability at neutral to alkaline pH (Haard and Simpson, 2000). Marine proteases are classified as serine-, cysteine-, aspartyl-, and metallo-proteases (Whitaker, 1994). In the present work, proteases in the digestive tract and tentacles of *I. fuscus* sea cucumber were identified and partially characterized by histological and biochemical methods to contribute understanding the digestive system of these holothurian.

Materials and Methods

Reagents

The following reagents were purchased from Sigma Chemicals (St. Louis, Missouri, USA): hematoxylin, eosin, casein, hemoglobin, bovine serum albumin (BSA), β-mercaptoethanol, pepstatin A, phenylmethylsulfonyl fluoride (PMSF), N-α-tosyl-L-lysine chloromethyl ketone hydrochloride (TLCK), trypsin inhibitor (TI), ethylenediaminetetraacetic acid (EDTA). Molecular weight markers were purchased from BioRad (Richmond, California, USA). All reagents were analytical grade.

Capture of specimens

Twelve *Isostichopus fuscus* adult male specimens (385 g average weight, 23.25 cm average length), apparently healthy, were captured at Espíritu Santo Island shore (24° 24´ N, 110° 18´ W and 24° 36´ N, 110° 27´ W), Gulf of California, Mexico, at 3 to 15 m depth. Organism collection was carried out according to official regulations (Mexican Official Regulation, NOM-059-SEMARNAT-2010). The corresponding authorities issued the respective collection license (license number SGPA/DGVS/00616/11). Living sea cucumber specimens were placed in seawater at 10 to 15 °C for 24 h. After killing the animals by anoxia, the digestive tract and tentacles were dissected and washed with distilled water to remove organic residues and extra tissue, and immediately processed for histological characterization and preparation of protease extracts.

Histological characterization

Samples of the studied organs were fixed in 70 % ethanol for 24 h, and imbibed in paraffin using an automatic tissue processor (Leica TP1020, Leica Biosystems, Nussloch, Germany). The method consisted in sample dehydration for 6 h with increasing ethanol concentrations (50 to 100 %), subsequent clearing for 70 min with ethanol-xylene and xylene, and infiltration for 2 h 15 min with paraffin-xylene and paraffin (Leica EG1140 modular tissue embedding). 7 to 12 µm-thick sections were obtained using a Microm HM315 rotary microtome (Thermo Fisher Scientific, Walldorf, Germany). Paraffin was removed from tissue sections by 25 min sequence with 100 % xylene, 100 to 70 % ethanol and distilled water. Samples were then stained with hematoxylin-eosin (HE), hematoxylin for 10 min, 15 min sequence with acid ethanol solution, water, ammonium water, distilled water and 80 % ethanol, and eosin for 5 min, followed by a sequence of 96 to 100 % ethanol, ethanol-xylene and xylene for 17.5 min total time (Grant and Tyler, 1983). Preparations were mounted in a synthetic resin, and observed with a light microscope. Multiple slides and observations were registered but only the representative ones were reported. Brown staining was considered as an indicator of the digestive enzyme activity, including proteases, due that waste accumulation is stained in brown (Byrne, 2001).

Preparation of proteolytic extracts

Digestive tract and tentacle proteolytic extracts were obtained by homogenizing 30 g with 60 mL-20 mM phosphate buffer at 4 °C, pH 7 (anti-proteases were not used during tissue homogenization), and centrifuged at 10,000*g* for 30 min, 4 °C. The supernatant was separated and stored at -20 °C until used. Protein content was analyzed by the method reported by Lowry *et al.* (1951), using bovine serum albumin as standard.

Determination of proteolytic activity

It was analyzed according to a modification of the method reported by Anson (1938) for acid proteases, and by Kunitz (1946) for alkaline proteases. Acid protease analysis was based on the hydrolysis of 1 % (w/v) hemoglobin; the hemoglobin solution contained 0.025 M boric acid, 0.025 M phosphoric acid, and 0.25 M acetic acid. Alkaline proteases were analyzed by casein hydrolysis (1 %, w/v casein in 20 mM phosphate buffer, pH 7). 80 µL proteolytic extract and 250 µL substrate (casein or hemoglobin at given pH) was incubated for 15 min at a given temperature. The reaction was stopped by adding 175 µL 25 % (w/v) trichloroacetic acid. Samples were then centrifuged at 10,000*g* for 10 min, 4 °C. The supernatant containing peptides resulting from substrate hydrolysis was read at 280 nm. Results were reported as specific activity, expressed in U/mg protein, where one proteolytic activity unit (U) was defined as the amount of active enzyme necessary to change the absorbance 0.001 unit/min at 280 nm, at the given experimental conditions (Yamaguchi *et al*., 1983). Therefore: U = ($Abs_{280\ nm}$) (dilution factor)/ (0.001) (reaction time, min).

Effect of pH

The extract proteolytic activity was measured on hemoglobin or casein substrate at a pH range from 2 to 10, 37 °C. At pH 2 to 5, the activity was analyzed using 1 % (w/v) hemoglobin; at pH 6 to 10 the substrate was 1 % (w/v) casein. Enzymatic stability at a given pH was assayed by incubating the extracts at 25 °C for 1 h in the following buffers: 0.1 M KCl-HCl (pH 2); 0.2 M Gly-HCl (pH 3, 4); 0.1 M phosphate buffer (pH 5 - 8); and 0.1 M Tris-HCl (pH 9 - 10) (Fu *et al*., 2005). Specific activity was analyzed after incubating the samples at the optimal pH and temperature, by the method described before and reported as residual activity (RA, %). Therefore: RA = (final specific activity) (100)/(initial specific activity), where the initial specific activity was obtained at optimal pH and temperature before incubation.

Effect of temperature

Proteolytic activity was also analyzed in extracts incubated from 0 °C to 80 °C, at pH of peak activity. Activity was measured by the method described above. Once the temperature of peak activity was obtained, the extract activity of tentacles and digestive tracts were assayed at optimal pH and temperature conditions. Results were reported as residual activity (RA).

Effect of protease inhibitors

The following protease inhibitors were studied: 5 mM PMSF and 10 mM TLCK for serine-proteases; 10 mM EDTA for metallo-proteases; 0.6 g/L TI for trypsin; 1 µM pepstatin A for aspartyl-proteases; and 1 mM β-mercaptoethanol (reducing agent). The inhibitory effect was analyzed by incubating the proteolytic extracts and a given inhibitor (1:1, v/v) for 1 h at 25 °C (García-Carreño, 1992; Fu *et al.*, 2005). Proteolytic activity was also analyzed at the optimal pH and temperature conditions by the method described above. Results were reported as residual activity (RA).

Effect of ions

The following ions were studied (10 mM): Mn^{+2} and Cu^{+2} (sulfates), and K^{+}, Ca^{+2}, Na^{+2}, Zn^{+2}, Mg^{+2}, Ba^{+2}, Hg^{+2} (chlorine salts). Proteolytic extracts were incubated together with a given ion (1:1, v/v) for 1 h at 25 °C (Qi *et al*., 2007; Zhu *et al*., 2008; Sun *et al*., 2011; Wu HL *et al*., 2013). The activity was analyzed at optimal pH and temperature by the method described above. Results were reported as residual activity (RA).

Electrophoretic analysis

Extracts were analyzed by SDS-PAGE, according to the method reported by Laemmli (1970), using a Mini-Protean II electrophoresis cell (BioRad, Richmond, California, USA), with 8 to 10 % stacking gels and 4 to 5 % concentration gels. Previous to analysis, extracts were boiled for 1 min in order to denature proteins. 5 mg/mL sample and 200 to 45 kDa MW markers (myosin: 200 kDa, β-galactosidase: 116.3 kDa, phosphorylase b: 97.4 kDa, BSA: 66.2 kDa, and ovalbumin: 45 kDa) were applied to the gels. Analysis were carried out at 150 V constant voltage, 30 mA, and 4 °C. The gels were stained with 0.1 % (w/v) Coomassie blue R-250 solution (40 % methanol and 10 % acetic acid). Image analysis were performed with a Gel-Doc 2000 image analyzer (BioRad), fitted with a Quantity One software (version 4.0, BioRad). Native-PAGE was also performed at 15 mA/gel constant amperage, 150 V, 4 °C. 66 to 14 kDa molecular weight markers (BSA: 66.2 kDa, ovalbumin: 45 kDa, glyceraldehyde-3-phosphate dehydrogenase: 36 kDa, carbonic anhydrase: 29 kDa, trypsinogen: 24 kDa, trypsin inhibitor: 20 kDa, and α-lactalbumin: 14.2 kDa) were used. Zymography assays were performed on native-PAGE by a modification of the method reported by García-Carreño (1993). The gels were immersed in 2 % casein solution (pH 6, 4 °C) for 1 h, incubated for 2 h at 60 °C, and washed with distilled water. Finally, the gels were stained with Coomassie blue R-250 and analyzed using a Gel-Doc analyzer, as described above.

Statistical analysis

The results were subjected to Analysis of Variance (ANOVA), complying with ANOVA assumptions (homogeneity of variances and normal distribution), and Tukey's multiple comparison tests using SPSS statistical package for Windows (version 15.0). All analyses were carried out in

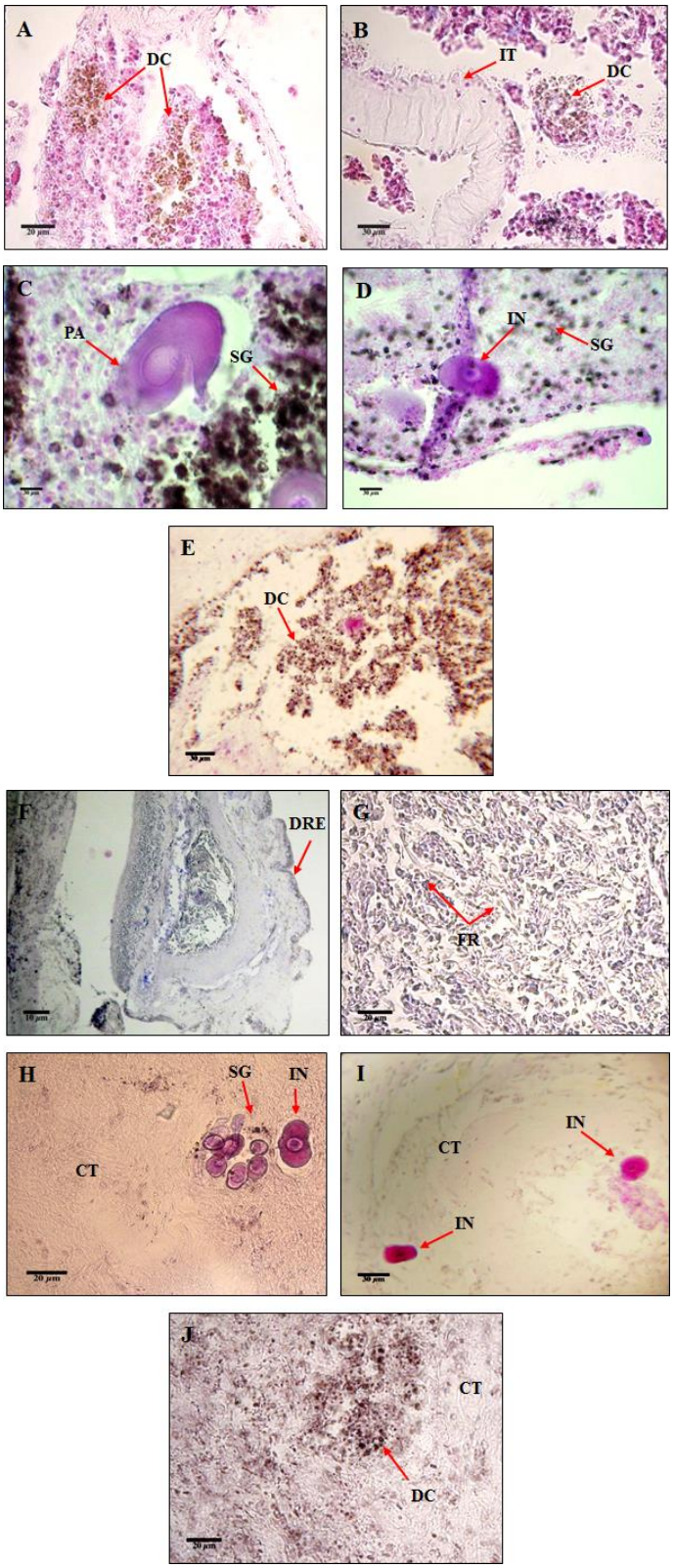

Fig. 2 Histological sections of digestive tract and tentacles of *I. fuscus*. Hematoxylin-eosin staining. Digestive tract: (A) Intestine section with digestive cells (DC). (B) Intestinal tube (IT) and digestive cells (DC). (C) Possible parasite (PA) in basophile staining and secretory granules (SG). (D) Secretory granules (SG) close to an inclusion (IN). (E) Digestive cell (DC) region. Tentacles: (F) Digestive region epithelium (DRE). (G) Possible food remains (FR). (H) Inclusion (IN) aggregates in connective tissue (CT) in basophile staining and secretory granules (SG). (I) Inclusions (IN) in connective tissue (CT) in basophile staining. (J) Digestive cell (DC) region in connective tissue (CT). Scale bars: A, G, H, J, 20 µm; B, C, D, E, I, 30 µm; F, 10 µm.

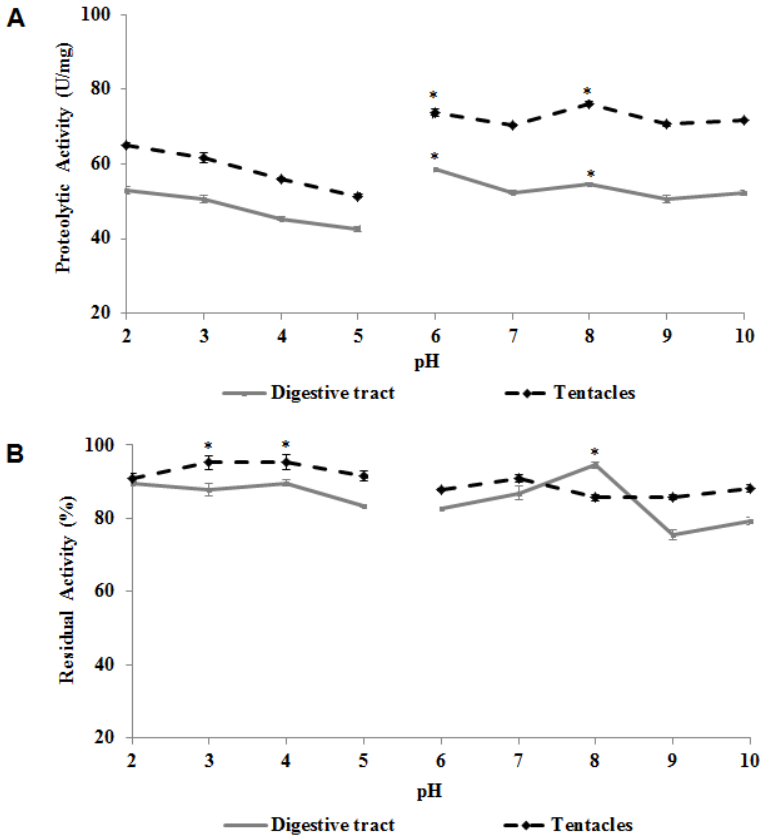

Fig. 3 Effect of pH on proteolytic extracts obtained from the digestive tract and tentacles of *I. fuscus*. (A) pH effect at 37 °C. (B) pH stability (optimal activity conditions). Results reported the mean of three replicates ± SD. Statistically significant at *$p < 0.05$ level.

triplicate, in three independent experiments; mean values ± standard deviations were reported. Differences were statistically significant at $p < 0.05$ level.

Results

Histological characterization

Several structures were observed in the digestive tract and tentacles of *I. fuscus*, such as a dense connective tissue layer, dermal epithelium, cell aggregates, digestive regions, and secretory granules. The digestive regions stained in brown with HE were present around damaged cells, observed as deformed cells, foreign material, and possible pathogenic agents.

The digestive tract showed the presence of cells with digestive activity, observed in brown staining (Fig. 2A). Intestinal tubules were observed (Fig. 2B); a possible platyhelminth-type parasite showing digestive activity due to secretory granules close to it, observed in brown staining (Fig. 2C); secretory granules close to a cell type in basophilic staining (Fig. 2D) named “inclusion” in the present paper; and a digestive cell region was observed in brown (Fig. 2E).

Tentacles showed digestive regions in the epithelium (Fig. 2F), included cells that stained brown, similar to the secretory cells in the digestive epithelium; food residues, possibly algae (Fig. 2G); secretory granules observed in brown staining close to different cell types in basophilic staining (“inclusions”) of ovoid or spherical shape forming aggregates, immersed in connective tissue (Fig. 2H); “inclusions” isolated in connective tissue (Fig. 2I); and cells with digestive activity observed in brown (Fig. 2J). The brown staining can be interpreted as digestive activity in both tissues.

Effect of pH

Proteolytic activity showed a peak ($p < 0.001$) at pH 6 and 8, 37 °C, with respect to other studied pH in proteolytic extracts obtained from the digestive tract and tentacles of *I. fuscus*. Comparing both organs, tentacle extracts had a higher proteolytic activity ($p < 0.001$) than digestive tract extracts at pH 2 to 10 (Fig. 3A). The digestive tract extracts were highly stable ($p < 0.001$) at pH 8, 94.5 ± 1.0 % residual activity (RA) whereas tentacles extract showed higher stability at pH 3 to 4, 95.3 ± 1.9 % RA (Fig. 3B).

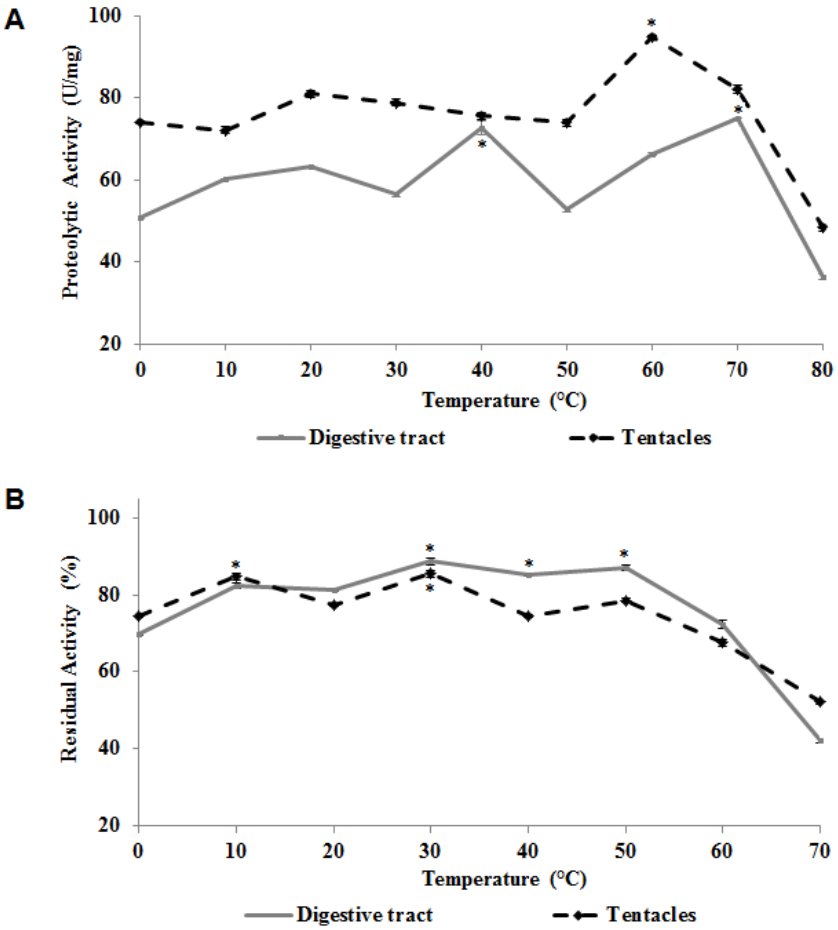

Fig. 4 Effect of temperature on proteolytic extract activity obtained from the digestive tract and tentacles of *I. fuscus*. (A) Temperature effect (pH 6). (B) Thermal stability (optimal activity conditions). Results are reported as the mean of three replicates ± SD. Statistically significant at *$p < 0.05$ level.

Effect of temperature

Peak proteolytic activity ($p < 0.001$) of digestive tract extracts was observed at 40 and 70 °C (assayed from 0 to 80 °C), whereas peak activity of tentacle extracts was at 60 °C. However, extracts of both organs showed proteolytic activity even at temperatures as low as 0 to 10 °C (Fig. 4A). Digestive tract and tentacles extracts were partially stable when incubated at 60 °C for 1 h, retaining 72.3 ± 1.0 % and 67.5 ± 0.1 % initial proteolytic activity, respectively. As compared to other studied temperatures, significantly higher stability ($p < 0.001$) was observed at 30 to 50 °C (86.9 ± 0.6 to 88.6 ± 0.9 % RA) in the digestive tract extract. Significantly higher stability of the tentacle extract ($p < 0.001$) was at 10 °C (84.7 ± 0.9 % RA) and 30 °C (85.5 ± 0.6 % RA) (Fig. 4B).

Effect of protease inhibitors

In order to preliminary characterize the protease type, several protease inhibitors were assayed (Fig. 5). The digestive tract extract was significantly inhibited ($p < 0.001$) at pH 6 by PMSF (79.4 ± 1.1 % RA) and pepstatin A (80.5 ± 1.9 % RA), followed by TLCK (86.2 ± 1.9 % RA). TI, EDTA, and β-mercaptoethanol (a reducing agent) did not inhibited proteases ($p > 0.05$) at pH 6 (92.0± 1.9 to 98.8 ± 2.3 % RA). A significant inhibition effect ($p < 0.001$) was observed at pH 8 with TI and EDTA (71.2 ± 1.9 and 74.9 ± 1.8 % RA, respectively). Tentacle proteolytic enzymes were inhibited ($p < 0.001$) at pH 6 by TLCK (79.4 ± 0.9 % RA) and pepstatin A (83.3 ± 1.2 % RA); TI, PMSF, EDTA and β-mercaptoethanol had no effect at pH 6 ($p > 0.05$) (93.1 ± 1.2 to 99.7 ± 0.12 % RA). TI and EDTA inhibited ($p < 0.001$) proteolytic enzymes (81.9 ± 1.6 and 81.1 ± 2.3 % RA, respectively) at pH 8.

Effect of ions

Various ions were also assayed to preliminary characterize the proteases (Fig. 6). The digestive tract extract proteolytic effect was significantly ($p < 0.001$) inhibited by Ba^{+2} (74.8 ± 0.4 % RA), whereas Ca^{+2} increased the proteolytic activity by 1 %. Other studied ions (Cl^{-}, Na^{+}, Zn^{+}, Mn^{+2}, Cu^{+2}, Mg^{+2}, and Hg^{+2}) had no effect ($p > 0.05$) (90.8 ± 0.4 to 95.3 ± 1.2 % RA). Tentacle proteolytic extracts retained 70.8 ± 0.1 % RA ($p < 0.001$) initial proteolytic activity

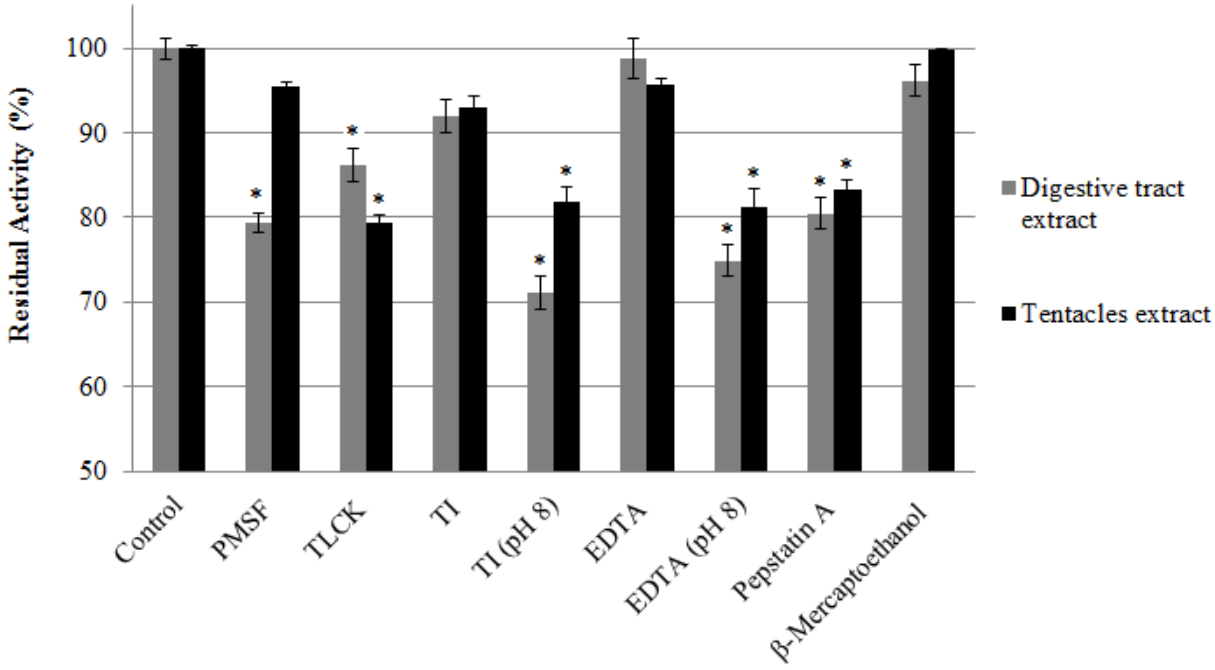

Fig. 5 Effect of protease inhibitors (pH 6) on proteolytic extracts obtained from the digestive tract and tentacles of *I. fuscus*. Results are reported as mean of three replicates ± SD. Statistically significant at $*p < 0.05$ level.

when was incubated with Ba^{+2}. Conversely, these extracts were partially inhibited by Mg^{+2}, Cu^{+2}, Mn^{+2}, Zn^{+2}, Hg^{+2}, and Ca^{+2} (81.3 ± 0.3 to 88.5 ± 0.2 % RA). Cl^- and Na^+ showed no significant effect ($p > 0.05$) (90.5 ± 0.2 % RA).

Electrophoretic analysis

Six proteins of 40, 43, 49, 76, 106, and 147 kDa apparent molecular weight were observed in SDS-PAGE of the digestive tract extract. Similarly, tentacle extract SDS-PAGE showed five proteins (44, 60, 81, 108, and 150 kDa) (Fig. 7). Native-PAGE of the digestive extract showed three proteins of 39, 42, and 109 kDa apparent molecular weight; whereas the tentacle extract showed three proteins of 39, 68, and 106 kDa (Fig. 8A). 2 % casein-zymograms (pH 6, 60 °C) confirmed the presence in the digestive tract and tentacle extracts of 100 kDa-proteolytic enzymes (approximately 109 and 106 kDa, respectively) (Fig. 8B).

Discussion

Some species of sea cucumber, mainly *Stichopus japonicas,* had been studied by histological and biochemical methods (Yang *et al*., 2015). However, there are scatter reports regarding *I. fuscus* proteolytic enzymes (Hernández-Sámano *et al*., 2015).

Histological characterization

Sea cucumber insoluble collagen content is around 70 % total protein (Saito *et al*., 2002). Therefore, the animal's connective tissue contains a considerable amount of collagen, responsible of cellular binding and support (San Miguel-Ruíz and García-Arrarás, 2007). Our histological observations were similar to those reported by Mashanov *et al*. (2004) in *Eupentacta fraudatrix*. Brown staining of aggregates in tentacles and digestive tract indicated that these cells main function is secreting proteases as digestive enzymes in both tissues. Byrne (2001) reported waste deposits in the connective tissue, also stained in brown. Cell aggregates with digestive activity have been mainly described in the intestine of other holothurians (Estrada-Flores *et al*., 1982; Smiley, 1994; Vergara and Rodríguez, 2015). This is a defense mechanism around damaged cells or foreign particles, and protection against microbial invasion (Vergara and Rodríguez, 2015). For instance, *Cladolabes schmeltzii* sea cucumber showed abundant brown-stained cells in the luminal epithelium and conjunctive tissue of three regions of the digestive tract (Vergara and Rodríguez, 2015). These authors reported that it is likely that amebocytes are involved in the formation of brown cells. Amebocytes are cells acting as a defense against external agents (Rodriguez *et al*., 2000). Clumps of material stained in brown are probably a result of defense functions by these amebocytes.

Secretory granules, also stained in brown, function as storage compartments for secreted products. These are the main organelles involved in regulated secretion. Transport from the formation to export sites is a multiple step process (Pavelka and Roth, 2010). The observed secretory granules are sphere-shaped structures of heterogeneous content, as reported by Mashanov *et al*. (2004). These granules stain positive for neutral mucopolysaccharides and proteins in paraffin preparations.

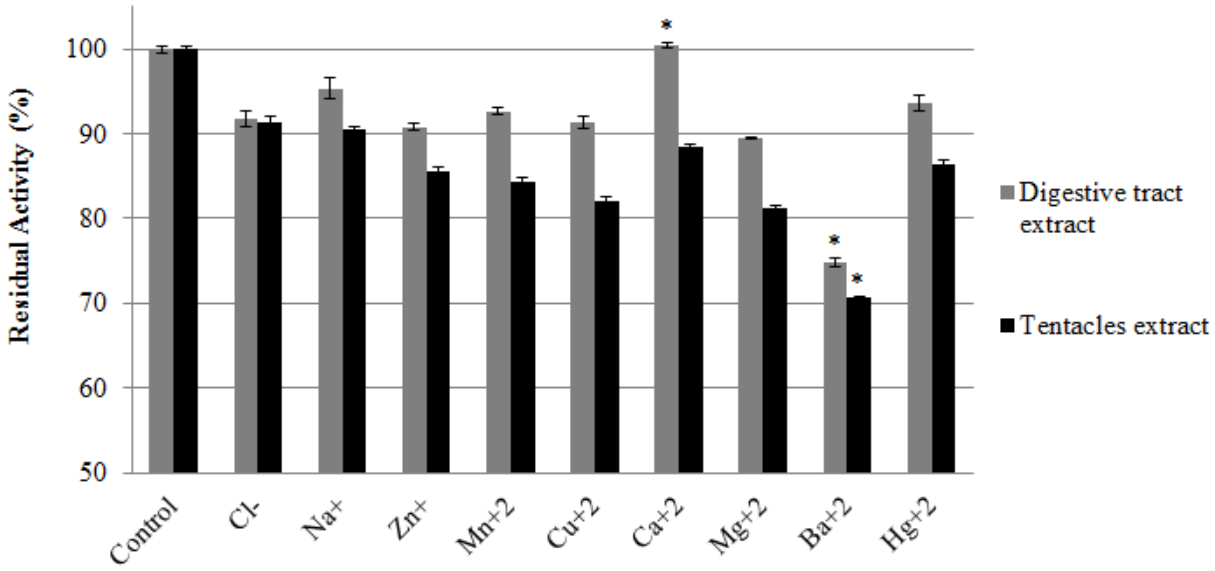

Fig. 6 Ion effect (10 mM) on proteolytic extracts obtained from the digestive tract and tentacles of *I. fuscus* (optimal activity conditions). Results are reported as the mean of three replicates ± SD. Statistically significant at *$p < 0.05$ level.

Figure 2 showed the possible presence of a parasite. These are frequent in sea cucumbers as the habitat preference of holothurians is rocky, sandy or muddy environments, also a suitable substrate for parasites. It was reported that one third of parasites found in echinoderms lives on, or inside sea cucumbers, mainly in the digestive system and celom (Jangoux, 1990). Therefore, the observed structure is a possible parasite in the digestive tract of *I. fuscus*. Moreover, "inclusions" may be proteolytic cystic parasites. The cells are presumably amebocytes.

Sea cucumber feed mainly of algae or microalgae such as *Rhodomonas* and *Dunaliella*. Histological sections also showed particles of these feeding material, in agreement to the reports on sea cucumber's diet (Lovatelli *et al*., 2004).

The function of tentacles is the selective uptake of particles (Schmidt-Rhaesa, 2007). However, proteolytic activity in *I. fuscus* tentacles can also be related to the autolytic character and regeneration mechanisms of sea cucumbers. The autolytic character is favored by the presence of highly active endogenous proteases (Sun *et al*., 2011). Likewise, it has been reported that the regeneration process in holothurians involves mechanisms including serine-, cysteine-, and metallo-proteases (Quiñones *et al*., 2002; Lamash and Dolmatov, 2013). Based on our reported results reported in the present work, we suggest that the proteolytic activity of *I. fuscus* tentacles can be an additional digestive mechanism when evisceration takes place as a response to stress or defense against predators (García-Arrarás and Greenberg, 2001). That is, during the time the internal organs takes to regenerate (3 to 4 weeks) (San Miguel-Ruíz and García-Arrarás, 2007), sea cucumbers could utilize the digestive activity located in the tentacles as part of the digestion system. This conclusion is based in the fact that only the middle part of the digestive tube is ejected through the anal opening, characteristic of order Aspidochirotida. Nevertheless, the anterior (pharynx and esophagus) and posterior (cloaca) regions are retained. Regeneration after evisceration includes only the transformation of intestinal mesentery and the retained broken ends of esophagus and cloaca (Vergara and Rodríguez, 2015).

The information obtained by means of histological techniques was mainly qualitative, indirectly showing the presence of proteolytic activity. Proteolytic characterization complemented this findings.

Effect of pH

Proteolytic activity has been demonstrated in a variety of echinoderms (Jangoux and Lawrence, 1982). Extracts obtained from the digestive tract and tentacles of *I. fuscus* also showed proteolytic activity, in agreement to other authors (Fu *et al*., 2006) who previously demonstrated that protease activity of *S. japonicus* varies according to the anatomical region. Peak activity at the studied pH range was similar to that reported for digestive tract crude extracts of *S. japonicus* (pH 5 and 8, at 37 °C). Proteolytic enzymes where reported as metallo- and serine- proteases (Fu *et al*., 2005). The presence of serine proteases, as trypsin and chymotrypsin (alkaline proteases) in holothurians, was fully demonstrated (Fish, 1967). It has also been reported that collagenases (metallo-proteases) in the digestive tract show both trypsin- and chymotrypsin-like activities with peak activity at pH 6.5 to 8.0 (Yoshinaka *et al*., 1987; Zefirova *et al*., 1996; Fu *et al*., 2005).

Our results were in agreement to data reported by Wu *et al.* (2013) and Wu *et al.* (2013). These

authors found peak activities of proteases of *S. japonicus* at pH 6, 46 °C for cysteine-proteases at pH 8 to 9, 37 °C for metallo-proteases. To the best of our knowledge, there are no reports on protease activity of sea cucumber tentacles. It is suggested that proteolytic enzymes in this organ are of the same type as in the digestive tract, probably in order to start food digestion from the oral cavity, or as part of an alternative digestive pathway to the digestive tract. Previous studies reported digestive enzyme activity in various fish species, depending on food habits (Hidalgo *et al*., 1999). Carnivore species have higher concentrations of acid proteases as compared to omnivorous or herbivorous species (Jonas, 1983). Sea cucumber is a detritivorous organism, *i.e*. obtains nutrients by consuming detritus (organic material, it typically including fragments of plants and animals as well as feces). Therefore, alkaline and acid proteases are very active in this species.

In our study, proteolytic stability was similar to that reported for *S. japonicus* cysteine-proteases (Qi *et al.,* 2007). However, our results in the alkaline region differ from these authors. Protease activity in *S. japonicus* notably decreased at high pH, whereas it remains stable for *I. fuscus*. Conversely, *I. fuscus* protease stability at basic pH was similar for *S. japonicus* metallo-proteases (Wu *et al*., 2013). *I. fuscus* proteases were stable at a wide pH range. The behavior of these digestive enzymes could be also affected by pH due to sea cucumber's habitat (Fu *et al*., 2006). *I. fuscus* protease stability at alkaline pH was related to a basic environment where this holothurian develops (pH 8.4 to 8.5) (Mercier, 2004). This pH is higher than the optimal for *S. japonicus* cultivation (pH 7.8) (Wang and Cheng, 2004; Liu *et al*., 2004) and to species adaptation to a continuous feeding system involving ingestion of large amounts of seawater at neutral pH (Guillaume and Choubert, 1999).

Consequently, *I. fuscus* proteases showing peak activity at pH 6 and 8 can be possibly cysteine- and metallo-proteases, respectively, as those reported for *S. japonicas*. These results were confirmed by studies with protease inhibitors and ions, as well as electrophoretic analysis, discussed below.

Effect of temperature

The optimal proteolytic temperature range was similar to the data reported by Qi *et al*. (2007), Zhu *et al*. (2008), and Wu *et al*. (2013) who observed that peak activity for *S. japonicus* cysteine-proteases was at 46 to 50 °C. Fu *et al*. (2005) also reported that metallo-proteases peak activity occurred at 37 °C. The optimal temperature range depends on the habitat's physicochemical conditions. The environmental temperature of *S. japonicus* is as low as 3 to 4 °C, up to 24 to 26 °C, whereas *I. fuscus* lives at 8 to 12 °C, up to 30 °C and ever higher (Lovatelli *et al*., 2004; Lluch-Cota *et al*., 2007). The higher water temperature causes *I. fuscus* proteases having optimal temperatures higher than for *S. japonicus* proteases.

I. fuscus proteases also showed proteolytic activity at 0 to 10 °C. To the best of our knowledge, other studies were carried out from 20 °C

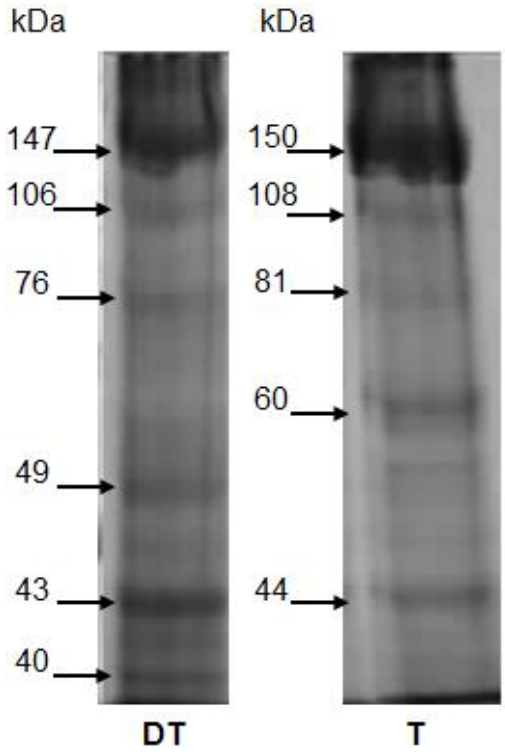

Fig. 7 SDS-PAGE of proteolytic extracts from *I. fuscus*. Digestive tract extract (DT), tentacles extract (T).

upwards (Fu *et al*., 2005; Qi *et al*., 2007; Zhu *et al*., 2008; Wu *et al*., 2013), but not as low as < 10 °C as in the present work, although (Haard and Simpson, 2000) reported that marine proteases are highly active at low reaction temperature.

The effect of water temperature on enzyme activity was also reported in *S. japonicus* by Gao *et al*. (2009). Our research team reported that purified enzymes of aquatic animals have considerable higher activity than similar enzymes in land animals (García Barrientos *et al.,* 2006). From this information, it was concluded that, among a number of factors making aquatic enzymes more active than their counterparts in land animals, is the fact that aquatic enzymes are active *in vivo* at lower temperatures than enzymes from warm-blooded land animals (Guerrero-Legarreta *et al*., 2009).

Our results on proteolytic stability agreed to those reported in previous studies for acid and alkaline proteases of *S. japonicus* digestive tract extract (Fu *et al*., 2005) and for cysteine-proteases, stable at 20 to 50 °C of the same species (Qi *et al*., 2007; Zhu *et al*., 2008; Sun *et al*., 2011). Even though, these authors found a significant reduction in proteolytic activity at temperatures above 50 °C (Fu *et al*., 2005; Qi *et al*., 2007; Zhu *et al*., 2008; Sun *et al*., 2011), as opposite to our results where residual activity was as high 86 to 88 % RA at 50 °C. From our results, it was concluded that proteolytic enzymes resistant to heat denaturation were present in the extracts obtained from *I. fuscus*.

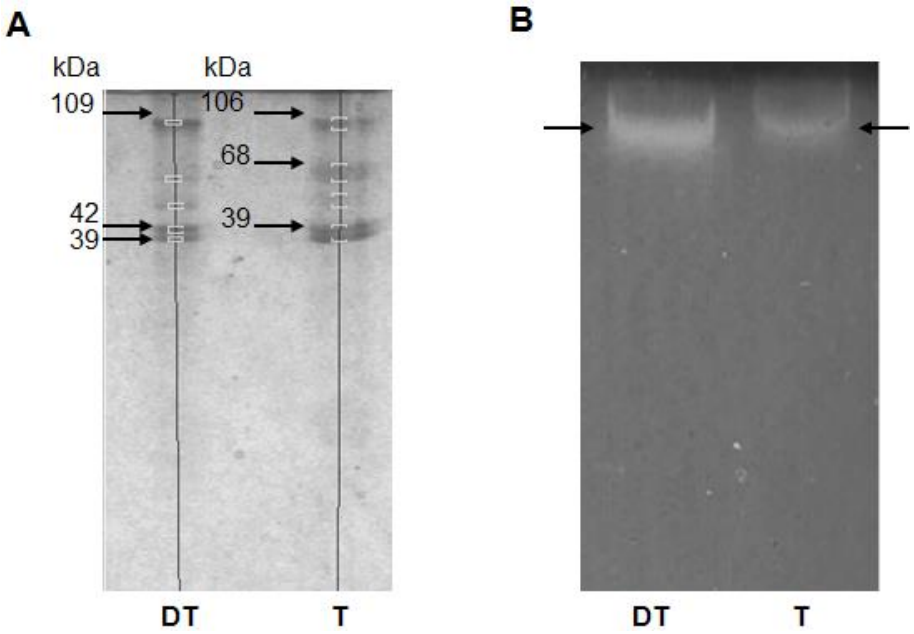

Fig. 8 Native-PAGE and zymography of proteolytic extracts from *I. fuscus*. (A) Native-PAGE. Digestive tract extract (DT), tentacles extract (T). (B) Zymography. Digestive tract extract (DT), tentacles extract (T).

Effect of protease inhibitors

The effect of several inhibitors on digestive tract and tentacle protease extracts, obtained from *I. fuscus,* agreed to other reports (Fu *et al.,* 2005). These authors described Cu^{+2}-dependent serine- and metallo-proteases in *S. japonicus* digestive tract. They also suggested the presence of collagenases (metallo-proteases). Wu *et al.* (2013) also reported that *S. japonicus* metallo-proteases are strongly inhibited by EDTA. Therefore, it was conclude that metallo-proteases are predominant in digestive tract and tentacle extracts of *I. fuscus*. Aspartyl-proteases can also be present due to inhibition by pepstatin A. However, studies with purified proteases are necessary to confirm these results.

Effect of ions

Inhibition of *I. fuscus* proteolytic activity by Ba^{+2} was a possible result of the presence of a metalloprotease matrix (MMP). This is a family of peptidases that regulate cellular behavior by depleting components in the extracellular matrix (collagen, gelatin) (Nagase and Woessner, 1999). Wu *et al*. (2013) reported gelatinolytic metalloproteases, also part of MMP, in *S. japonicus* body wall. This fact suggested its involvement in sea cucumber autolysis. Ziouti *et al.* (2006) reported selective inhibition by barium chloride, removing Ca^{+2} from metalloprotease-2 matrix (MMP-2) catalytic center.

Ca^{+2} activation effect was reported for metallo-proteases in *S. japonicus* (Fu *et al.,* 2005) and for cysteine-proteases in the same species (Sun *et al.,* 2011). The high concentrations of Ca^{+2} and Mg^{+2} in seawater (10 and 50 mM, respectively) is responsible for cell metallo-proteases activation (Mayne and Robinson, 1996).

The presence of endogenous ions in the studied specimens encouraged the proteolytic effect. Therefore, Zn^{+2} net effect was possibly masked. In addition, presence of Zn^{+2}-dependent metallo-proteases in MMP-2 has been demonstrated (Ziouti *et al.,* 2006). The high proteolytic activity observed with Hg^{+2} can also be related to MMP activation mechanism, as MMP can be activated by proteases or *in vitro* by chemical agents, such as $HgCl_2$ (Visse and Nagase, 2003). A further study, previously inhibiting proteases with 5mM EDTA, is recommended.

Currently, there are no reports in the literature related to ion effects on sea cucumber tentacle extract, although our results were similar to those obtained for the digestive tract extracts of the same samples.

Electrophoretic analysis

Electrophoretic profiles of *I. fuscus* digestive tract and tentacle extracts showed protein bands of molecular weight that coincide to previous reports (Fu *et al.,* 2005; Qi *et al.,* 2007; Zhu *et al.,* 2008; Wu *et al.,* 2013; Lamash and Dolmatov, 2013). These authors identified proteins of 35.5, 39.1, 45, 47, 53, 58, 63, 114 and 132 kDa in *S. japonicus* and *E. fraudatrix*. In our study, the highest apparent molecular weights (147 and 150 kDa) were similar for proteins in *S. japonicus*, reported by Saito *et al*. (2002), Cui *et al*. (2007), and Park *et al*. (2012). According to these authors, these molecular weights correspond to collagen chains of 100, 135, and 130 kDa, forming a 1α trimer. Siddiqui *et al*. (2013) also found in *Bohadshia spp.* (Jaeger, 1833) a type I

collagen, with α1 as major component of about 138 kDa.

Protease molecular weights found by zymography (109 and 106 kDa) were similar to those reported by Fu *et al*. (2005) in the digestive tract of *S. japonicus*, a 114 kDa metallo-proteases. Lamash and Dolmatov (2013) also found a 132 kDa metallo-proteases in *E. fraudatrix* gut, concluding that high molecular weight proteases in the digestive tube are not typical of extracellular proteases, but probably indicate the presence of a protein complex. Likewise, proteases above 100 kDa in digestive tract and tentacle extracts of *I. fuscus* were possibly protein complexes, suggesting that their similarity is related to the role of the studied organs digestive processes.

In summary, proteolytic extracts obtained from the digestive tract and tentacles of *I. fuscus* showed presence of thermo-stable, active at low temperature acid and alkaline proteases. The studied proteases showed higher activity at alkaline pH, similarly to *S. japonicas*, the most studied species. It was also concluded that cysteine- and metallo-proteases are possibly in the digestive tract and tentacles of *I. fuscus*. However, studies on purified protease are necessary to confirm the presence of these enzymes. Characterization of tentacle proteases showed that these organs act as tool to start food degradation when the digestive tract has been eviscerated. To the best of our knowledge, there are no reported studies on proteases from tentacles of any sea cucumber.

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