Chymotrypsin isolation from jumbo squid (*Dosidicus gigas*) hepatopancreas: Partial characterization and effect on muscle collagen

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Abstract Chymotrypsin was purified from jumbo squid hepatopancreas (HP) with 2.4-fold and yield 1.9%, and characterized with a molecular weight of 31 kDa, as estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Chymotrypsin effect over collagen extracted from the mantle, fins and arms of the jumbo squid was evaluated. The enzyme exhibited the maximum activity at pH 7 and 65°C using Suc-Ala-Ala-Pro-Phe-p-nitroanilide (SAAPNA) as a substrate and it was identified using the specific inhibitors N-tosyl-L-phenylalaninechloromethyl ketone (TPCK) and phenyl methyl sulfonyl fluoride (PMSF), showing residual activities of 6% and 0%, respectively. Furthermore, high activity was observed in the pH range of 4.0 to 8.0. Purified enzyme showed a moderate *in vitro* activity using muscle collagen as a substrate. Although further research is needed, the results suggest that the enzyme has a potential application where acidic or slightly alkaline conditions are needed.

Keywords: chymotrypsin, *Dosidicus gigas*, hepatopancreas, characterization, activity

Introduction

Approximately 40–50% of the total marine animals caught annually are used for direct human consumption; based on this, one million metric tons of the fish and shellfish captured annually, which are potential sources for singular and useful compounds including marine enzymes, are discarded (1). In recent years it has been studied the possible applications of serine proteases in industry, either as detergents, as well as in the food, pharmaceutical, leather and silk industries (2). It has been reported that there are structural differences and in the functional properties among marine and mammalian proteases (3), where proteases of marine sources have exhibited higher catalytic activity throughout a broad pH and temperature range at relatively low concentrations (4). Chymotrypsin (EC 3.4.21.1), an important digestive enzyme from fish and marine invertebrates, has a range of substrate specificity higher than trypsin, allowing the cleavage of peptide bonds involving non-polar amino acids and amino acids containing bulky side chains such as leucine, phenylalanine, tyrosine, and tryptophan (5). This protease has been

isolated and also characterized from several fish species, such as freshwater and marine fish like crucian carp (*Carassius auratus*) (6), Monterey sardine (*Sardinops sagax caeruleus*) (7), Japanese sea bass (*Lateolabrax japonicus*) (8), and striped seabream (*Lithognathus mormyrus*) (9); however, there is still limited knowledge on the behaviour of this enzyme in cephalopods such as jumbo squid.

Jumbo squid is an important fishery in Mexico and it is normally sold as frozen or cooked gutted mantle (10) from which the hepatopancreas (HP) is discarded. The HP is a potential source of digestive enzymes, such as chymotrypsin (11). It is important to understand the activity and kinetic parameters of jumbo squid chymotrypsin in order to use it during protein hydrolysis in fish industry waste, such as jumbo squid muscle, of which more than 30% is not used as food (12). Enzymatically modified by-products could be used during the manufacture of protein hydrolysates, aquaculture foods, cosmetics or biodiesel (13). One of the principal constituents of squid industry by-products is connective tissue, specifically collagen (14), which has been reported as a substrate for chymotrypsin purified from various species of crustaceans (15,16).

Different types of chymotrypsins from freshwater and marine fish have been identified; however, knowledge regarding this enzyme of jumbo squid is scarce. In addition to contributing to diminishing environmental pollution and providing added value to this fishery, the aim of the present study is to understand the characteristics of chymotrypsin from jumbo squid HP in order to sustain approaches for the potential application of this enzyme at the industrial level.

Materials and Methods

Samples Nine jumbo squid specimens were purchased from local fishermen (Guaymas, Sonora, Mexico). The average weight of each squid ranged from 1.4 to 3.5 kg, and they were approximately 80 cm long. The temperature was maintained at 0°C from capture until the HP was removed (<6 h). The HP was manually removed and separated in three batches ($n=3$). The removed HP was freeze-dried, packed in sealed polyethylene bags, and maintained at 20°C until used.

Crude enzyme extract preparation HP was mixed and homogenized for 2 min with 0.05 M Tris-HCl buffer (pH 8.0) and 0.5 M NaCl (1:2, v/v) and centrifuged at refrigeration temperature for 30 min at 18,000xg; then, the lipids layer was removed manually, and the precipitate was discarded. Finally, the enzyme extract without lipids (supernatant) was stored at -80°C until further analysis (17).

Purification procedure The crude enzyme extract was fractionated with 30-70% ammonium sulfate, centrifuged at 20,000xg for 20 min at 4°C and the precipitate was collected and dissolved in 20 mL of 50 mM Tris-HCl buffer (pH 8.0, buffer A). This solution was loaded onto an anion-exchange column, using DEAE-Sepharose matrix (Amersham Pharmacia Biotech, Little Chalfont, UK) pre-equilibrated with buffer A, and eluted at flow rate of 0.3 mL/min, with a linear gradient of NaCl from 0 to 0.5 M. Fractions with chymotrypsin activity were collected for further analysis as described below.

Quantitative assessment of proteins The protein concentrations in the crude extracts and at a measured absorbance of 280 nm during anion-exchange chromatography were determined using the Bradford method (18) with bovine serum albumin as a standard (1 mg/mL).

Electrophoresis analysis To determine the presence of chymotrypsin, the active fractions were analyzed by sodium dodecyl sulfate-12% polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions (19). The gels were stained depending on the protein concentration, with Coomassie Brilliant Blue R-250 (crude extract) or silver nitrate (purified protein). Finally, images of protein bands on electrophoresis gels were captured by using image densitometer (Model GS-700; Bio-Rad Laboratories, Hercules, CA, USA).

Assay for amidase activity The activity was measured by colorimetric

detection at 410 nm (20), using 0.1 mM Suc-Ala-Ala-Pro-Phe-p-nitroanilide (SAAPNA) as the specific substrate, as follows: 990 μ L of solution were prepared containing 0.1 mM SAAPNA and mixed with 10 μ L of enzyme extract. The reaction mixture was incubated for 10 min at 25°C and the production of *p*-nitroaniline was measured by monitoring the increase in absorbance every 30 s. The enzymatic unit activity was expressed as the amount of enzyme needed to release 1 μ M *p*-nitroaniline/min using 8800 M⁻¹ cm⁻¹ as the extinction coefficient of *p*-nitroaniline at 410 nm.

Effect of pH The optimal pH of chymotrypsin activity was evaluated at 25°C for 15 min using a universal buffer (pH 4-10), and pH stability was studied by incubation of the enzyme for 60 min at the same pH and temperature values mentioned above (21). Percent of enzyme activity was estimated considering 100% the highest activity detected.

Effect of temperature The optimal temperature of chymotrypsin activity was measured from 20 to 70°C for 15 min in Tris-HCl buffer under optimal pH. Thermal stability was studied by incubation of the enzyme at 20-70°C for 60 min. Percent of enzyme activity was estimated considering 100% the highest activity detected.

Inhibition assays A series of inhibitors were evaluated to explain their effects on purified chymotrypsin activity. PMSF (phenyl methyl sulfonyl fluoride; 100 mM in 2-propanol), TPCK (N-tosyl-L-phenylalanine chloromethyl ketone; 5 mM in methanol) and pepstatin A (1 mM in dimethyl sulfoxide) were prepared and used according to the method of Villalba-Villalba *et al.* (21). Enzyme was incubated with an equal volume of inhibitors (50 μ L) at 25°C for 60 min; then, amidase activity was measured under standard assay conditions. Residual enzyme activity was expressed as a percentage of the activity without inhibitors.

Hydrolysis of muscle collagen The ability of jumbo squid chymotrypsin to hydrolyze muscle collagen was analyzed by extracting pepsin-solubilized collagen from the jumbo squid muscle (mantle, fins and arms) according to Torres-Arreola *et al.* (12), with slight modifications. The muscle was subjected to a preliminary extraction, homogenized for 2 min with five volumes (v/w) of 0.1 M NaOH, stirred overnight and centrifuged at 10,000xg to obtain the connective tissue (precipitate). The collagen was extracted with pepsin (10 mg/g tissue in acetic acid) from the connective tissue and lyophilized for analysis. The amount of collagen was estimated by determining the hydroxyproline (Hyp) content (22).

Collagenolytic and electrophoretic detection: The collagenolytic activity of jumbo squid chymotrypsin was assessed according to Burgos-Hernandez *et al.* (23). Briefly, 100 μ L of purified chymotrypsin was incubated for five hour with 6 mg of lyophilized collagen (extracted from the muscle) at 25°C in 1.25 mL of 0.1 M TES buffer (pH 7.5) containing 0.35 mM CaCl₂. After incubation, the reaction mixture was centrifuged at 4,000xg for 5 min. Then, 0.2 mL of the

supernatant was mixed with 0.5 mL of 1.5% ninhydrin and incubated at 100°C for 15 min, followed by the addition of 2.5 mL of 50% ethanol. The absorbance was recorded at 600 nm, and collagenolytic activity was expressed in micromoles of leucine released, using a standard L-leucine curve where one Unit (U)= μmol of L-leucine equivalent liberated from the substrate over 5 h. For electro-phoretic detection, appropriate aliquots were collected before and after collagen/enzyme incubation and compared using SDS-PAGE, as described above, using a 10% separating gel.

Statistical analysis The statistical design of the present study was planned to reduce variation by replication. The reported data of chymotrypsin activity during purification and characterization were based on the average of three determinations. The data were assessed using the analysis of variance (ANOVA). The mean differences were established using Tukey's test with a variation by replication of <5%. The data were analyzed using the JMP program 5.0 (StatSoft Inc., Tulsa, OK, USA).

Results and Discussion

Purification of chymotrypsin The results of the purification steps of chymotrypsin from jumbo squid HP are summarized in Table 1.

Chymotrypsin was purified 2.4-fold, achieving a 62.81% yield. While the unabsorbed fractions from DEAE-Sepharose showed chymotrypsin activity, the absorbed fractions (eluted with NaCl linear gradient) did not show this enzyme activity (fraction 26 to 34) (Fig. 1). This is one of the first reports regarding chymotrypsin purification in jumbo squid HP. Ezquerro-Brauer *et al.* (11) reported high chymotrypsin activity in the hepatopancreas from jumbo squid; however, the enzyme was not purified. Moreover, cysteine proteinase identified from this by-product (HP) was confirmed to be cathepsin L (24). Chymotrypsin is the main protease in aquatic animals and has been studied in a wide variety of species, such as Monterey sardine (*Sardinops sagax caerulea*), carp (*Cyprinus carpio*), sailfin catfish (*Pterygoplichthys disjunctivus*), and rainbow trout (*Oncorhynchus mykiss*) (6,21,25,26). Its concentration and activity vary according to the evaluated species and the environmental conditions (fat content, feeding habits, age, etc.) (25,27,28).

Estimation of molecular mass SDS-PAGE showed that after final purification procedure chymotrypsin appeared as one single band with a molecular weight of 31 kDa (Fig. 2C), suggesting its high purity. The results show that there is one dominant isoform of chymotrypsin in jumbo squid HP, although three isoforms have been reported (A, B, and C) in terrestrial animals (29). However, only chymotrypsin A (anionic) and B (cationic) have been found in aquatic organisms (25).

Table 1. Summary of the purification procedure to obtain the chymotrypsin from jumbo squid (*Dosidicus gigas*) hepatopancreas. Activity was evaluated using 0.1 mM SAAPNA as substrate

Purification steps	Total protein (mg)	Total activity (U) ¹⁾	Specific activity (U/mg protein)	Purification (fold)	Yield (%)
Crude extract	253.42	126.21	0.50	1	100
Ammonium sulfate	123.90	82.37	0.66	1.32	65.26
DEAE-Sepharose	49.84	79.28	1.59	2.4	62.81

¹⁾Units (U)=Activity of releasing 1 μM *p*-nitroaniline/min using 8800 $\text{M}^{-1} \text{cm}^{-1}$ as the extinction coefficient of *p*-nitroaniline at 410 nm. Protein content was calculated by Bradford method.

Results represent an average of 3 determinations. Standard deviations were in all cases lower than 5%.

Fig. 1. Chromatographic (DEAE-Sepharose) purification of chymotrypsin from jumbo squid (*Dosidicus gigas*) hepatopancreas.

Fig. 2. SDS-PAGE of chymotrypsin activity containing fractions, extracted from jumbo squid (*Dosidicus gigas*) hepatopancreas. (A) Molecular weights markers, kDa; (B) Crude extract; (C) DEAE-SEPCHAROSE (fractions 6-14).

Yang *et al.* (6) reported the presence of these two chymotrypsins from crucian carp (*Carassius carassius*), whereas in this work, adsorbed fractions with chymotrypsin activity in DEAE-Sepharose suggest its anionic nature (greater proportion of acidic than basic amino acids). In another study, Díaz-Tenorio *et al.* (30) showed that isochymotrypsins were 42.6, 34.9, and 33.2 kDa in *C. bellicosus*, whereas two isochymotrypsins at 30.8 and 28.4 kDa were found in *C. arcuatus*. It has been reported that bovine chymotrypsin splits into various polypeptide chains (A, B, and C) under reduced conditions (31), suggesting a different evolution of these enzymes. Moreover, Martínez *et al.* (32) reported chymotrypsin activity in crude extract from wild octopuses using SAAPNA as a specific substrate. However, it was not possible to determine the weight of the enzyme.

Optimum pH and pH stability Jumbo squid chymotrypsin showed optimum activity at pH 7.0 with SAAPNA as a substrate at 25°C (Fig. 3). However, at a more alkaline pH (7.5-8.5), the activity was still approximately 84-88%. The optimum pH for chymotrypsin extracted from marine animals is within the alkaline range (15). In Monterey sardine, two types of chymotrypsin have been purified at an optimum pH of 8.0 (26), which is similar to that from terrestrial sources (25). Moreover, Bibó-Verdugo *et al.* (15) reported an optimum pH for chymotrypsin from the gastric juice of California spiny lobster (*Panulirus interruptus*) between 7 and 8. Chymotrypsin readily hydrolyzes a number of basic proteins with larger nonpolar and aromatic groups (25,33). This information is consistent with the high relative activity observed at pH values ranging from 7.5 to 8.5. Based on the above discussion, chymotrypsin from jumbo squid HP could be used principally at an alkaline pH. However, from pH 5.5 to 7, the remaining activity is between 50 and 60%, which allows the enzyme to be used in a wide range of applications in food processing where protein hydrolysis is needed.

Fig. 3. Optimum pH and pH stability of purified chymotrypsin from jumbo squid (*Dosidicus gigas*) hepatopancreas.

Regarding stability, chymotrypsin showed remarkable stability over a broad pH range (from 4.0 to 8.0), with remaining activities of 84 (pH 4.0), 88 (pH 5.0), 90 (pH 6.0), 98 (pH 7.5) and 87% (pH 8.0). It was observed a considerable decrease of activity at pH 9.0-10.0 (32-24% residual activity). Fish chymotrypsin has been reported to be stable only under alkaline pH conditions (26). However, in jumbo squid HP, the stability range includes acidic pH conditions, which is consistent with that reported for shrimp chymotrypsin where the enzyme retained 75% activity for 120 min at pH 4 (16). The stability of an enzyme is usually higher in a pH range near the optimal pH; a reduced stability corresponds to the protein unfolding that occurs at extreme pH conditions, thus affecting its tertiary structure (7).

Because jumbo squid chymotrypsin is stable and shows high activity at pH range from 4 to 8, the protease could be used during those conditions in food industry, such as fermentation or protein hydrolysis.

Optimum temperature and thermal stability The optimum temperature for chymotrypsin activity under experimental conditions was 65°C (Fig. 4), which is higher than that reported for vermiculated sailfin catfish, crucian carp, and Monterey sardine (50°C) (6-7,21). This difference could be related to adaptation of the enzyme systems to the warmer temperature of the jumbo squid.

Figure 4 shows that chymotrypsin extracted from jumbo squid HP is highly stable at temperatures above 55°C, and strongly inactivated at temperatures below 45°C. This thermal stability profile differs from that of fish chymotrypsins, which show stability at approximately 30°C and lability at 55°C (6-7,17). Moreover, chymotrypsin extracted from lobster is rapidly inactivated when the temperature is above 50°C (15). This suggests that both fish chymotrypsin and crustacean chymotrypsin are thermolabile. However, Navarrete-Del-Toro *et al.* (16) reported high stability of shrimp chymotrypsin incubated for 120 min at 50°C (the enzyme retained 90% of its activity), which is in agreement with the results presented in this work. This thermal

Fig. 4. Optimum temperature and thermal stability of purified chymotrypsin from jumbo squid (*Dosidicus gigas*) hepatopancreas.

stability could be attributed to a fewer polar hydrogen bonds and higher proportion of charged residues (29).

Assays of chymotrypsin inhibition The effects of several proteinase inhibitors on chymotrypsin activity from jumbo squid were evaluated. The enzyme was strongly inhibited by a specific chymotrypsin inhibitor (TPCK, 6% residual activity) and by a serine-protease inhibitor (PMSF, 0% residual activity), but was barely inhibited by an aspartic protease inhibitor (pepstatin A, 94.6% residual activity). These results confirm the presence of chymotrypsin. The inhibition pattern presented by these inhibitors was characteristic of other fish chymotrypsins. It has been reported that chymotrypsin isolated from Monterey sardine is strongly inhibited by 0.05 mM TPCK (17.7-35.6% residual enzyme activity) (26), whereas pepstatin had an inhibition effect of less than 10%. In crustaceans, TPCK is not as effective as in fish; in *Penaeus californiensis* and *Panulirus interruptus*, chymotrypsin is slightly inhibited by this synthetic inhibitor (TPCK) (1-25%) (16-17).

Hydrolysis of muscle collagen The collagenolytic activity of chymotrypsin purified from jumbo squid hepatopancreas was evaluated using collagen extracted from the mantle, fins, and arms of the same organism (21.03, 23.21, and 26.59 U/mg protein, respectively; $p \geq 0.05$). Because the enzyme is a serine protease, it showed less activity on the substrate (collagen) compared to that reported by others (150-250 U/mg protein) (23,34). However, those studies were developed with metalloproteases extracted from different fish and shrimp species, which are specific for collagen. Chymotrypsin extracted from pancreatic tissue is an endopeptidase that primarily hydrolyzes proteins with aromatic and non-polar amino acids (5). Therefore, it is capable of acting on a variety of proteins, including collagen, which is a major structural protein in jumbo squid by-products, although its aromatic amino acids content is low (14).

The hydrolysis of muscle collagen was also monitored by SDS-PAGE (Fig. 5), which showed that jumbo squid chymotrypsin cleaves collagen extracted from the muscle. The α chain of collagen was

Fig. 5. SDS-PAGE of muscle collagen hydrolyzed by purified chymotrypsin from jumbo squid (*Dosidicus gigas*) hepatopancreas. (A) Molecular weights markers, kDa; (B) Collagen from mantle; (C) Collagen from mantle incubated with chymotrypsin; (D) Collagen from fins; (E) Collagen from fins incubated with chymotrypsin; (F) Collagen from arms; (G) Collagen from arms incubated with chymotrypsin

identified in the mantle, fins, and arms by determining the hydroxyproline content (data not shown), which was almost completely hydrolyzed after five hour of incubation at 37°C with the purified enzyme. This result correlates well with the measured collagenolytic activity. Bibó-Verdugo *et al.* (15) reported that lobster chymotrypsin showed collagenolytic activity using bovine-soluble collagen as a substrate. On the other hand, serine collagenase-like enzymes extracted from fresh water prawn hepatopancreas were able to hydrolyze soluble collagen extracted from the muscle of the same organism (35). Because of its ability to hydrolyze collagen from marine organisms, the jumbo squid chymotrypsin could be used in the fish industry, specifically the treatment of by-products, which have a large amount of collagen (12).

In conclusion, the results of this study clearly demonstrate that the enzyme purified and characterized from the HP of jumbo squid was chymotrypsin, which shows high activity in the pH range of 4.0-8.0 and the temperature range of 50-65°C, with the ability to hydrolyze collagen extracted from the muscle. These results suggest that the enzyme possesses potential applications in the food, leather and silk industries. The authors found significant differences between the major biochemical properties of chymotrypsin from jumbo squid and chymotrypsins from different fish species. Chymotrypsin from vertebrate fish is more stable at alkaline pH conditions, whereas jumbo squid chymotrypsin shows better stability at neutral or slightly acidic pH conditions. With this information, structural studies of jumbo squid chymotrypsin are needed in order to establish a better understanding of those differences.

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