TRYPSIN FROM JUMBO SQUID (Dosidicus gigas) HEPATOPANCREAS: PURIFICATION AND CHARACTERIZATION

Ana Gloria Villalba-Villalba, Enrique Márquez-Ríos, Marina Josafat Esquerra-Brauer and Francisco Javier Castillo-Yáñez

SUMMARY

Trypsin was purified from the hepatopancreas of Dosidicus gigas by fractionation with ammonium sulfate (30-70% saturation), gel filtration, affinity and ion exchange chromatography. The molecular weight of the trypsin obtained was ~23.5kDa according to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and, showed a single band in the zymogram. The enzyme exhibited maximal activity at pH 8.5 and 40°C, using BAPNA as substrate. It was effectively inhibited by phenylmethyl sulfonyl fluoride (PMSF) (98%), and Na-p-Tosyl-L-lysine chloromethyl ketone (TLCK) (99%). Enzyme activity was affected by the following ions in decreasing order: Hg²⁺, Fe³⁺, Cu²⁺, Li⁺, Mg²⁺, K⁺, Mn²⁺, Ca²⁺. Trypsin activity decreased continuously as NaCl concentration increased from 0 to 30%. Km and kcat values were 0.085 ±1.45mM and 1.76 ±0.12s⁻¹, respectively. The results suggest that the purified enzyme a potential agent to be used in biotechnological processes.

Introduction

The marine environment contains the largest pool of diversified genetic material and, hence, represents an enormous potential source of enzymes. Among other enzymes, the possible applications of serine proteases in industry have been studied (Klomklao et al., 2005). The demand for enzymes with specific properties is large, and various enzymes sources are currently being investigated. It has been reported that proteases of marine sources exhibit high catalytic activity (Shahidi et al., 2005). Jumbo squid is an important fishery in Mexico, usually sold as frozen or cooked-frozen gutted mantle (Luna-Raya et al., 2006), after the hepatopancreas is removed, as it represents a source of contamination. This organ could be, however, a good source of enzymes. Jumbo squid hepatopancreas constitutes 2-8% of the body weight and contains 18-20% crude protein (Ezquerra-Brauer et al., 2002). Extracts of hepatopancreas from the Short finned squid, Illex illecebrosus, have been experimentally used in a number of food processing applications, including the preparation of capelin fish sauce; salted, fermented squid mantle; squid tenderization and cheddar cheese ripening (Lee et al., 1982). Fish sauce, with squid hepatopancreas as a fermentation aid, has been commercially produced in Canada for more than 10 years (Raksakulthai et al., 2006). This enzyme cleaves the peptide bond on the carboxyl side of arginine and lysine, has endopeptidase activity and a molecular weight ranging from 22 to 28kDa (Whitaker, 1994). The first studies of proteinases, mainly trypsin and chymotrypsin in vertebrate species, were carried out during the first part of the 20th century, describing kinetic properties, isolation and some molecular characteristics (Muhlía-Almazán et al., 2008). Trypsin has been isolated and characterized thoroughly based on their chemical and enzymatic properties from several aquatic species, such as Siniperca chuatsi (Lu et al., 2008), Balistes capriscus (Jelloul et al., 2009), Sepia officinalis (Balti et al., 2009), Lutjanus vitta (Khantaphant and Benjakul, 2010), Diapterus rhombeus (Silva et al., 2011), Salaria basiliska (Ktari et al., 2012) and Pterygoplichthys disjunctus (Villalba-Villalba et al., 2013). However, most studies have been done in fishes, with very scarce information on trypsin from cephalopods. The aim of this study was to purify and characterize trypsin from the jumbo squid (Dosidicus gigas) hepatopancreas, thus generating basic information about this by-product.

Materials and Methods

Jumbo squid samples

Jumbo squid specimens were captured in the Gulf of
TRIPSINA DEL HEPATOPÁNCREAS DE CALAMAR GIGANTE (Dosidicus Gigas): PURIFICACIÓN Y CARACTERIZACIÓN
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RESUMEN
Se logró purificar tripsina del hepatopáncreas de Dosidicus gigas por fraccionamiento con sulfato de amonio (30-70% de saturación), cromatografía de filtración en gel, afinidad e intercambio iónico. El peso molecular de la tripsina fue de 23.5 kDa, según la electroforesis SDS-PAGE y mostró una sola banda en el zymograma. La enzima mostró máxima actividad a pH 8.5 y 40°C, usando BAPNA como sustrato. La actividad de la enzima se afectó de manera efectiva por el metil fenil sulfonil fluoro (PMSF) (98%) y por Na-p-tosil-L-lisina clorometil cetona (TLCK) (99%). Además la actividad de la enzima fue afectada por los siguientes iones en orden decreciente: Hg²⁺, Fe²⁺, Cu²⁺, Li⁺, Mg²⁺, K⁺, Mn²⁺, Ca²⁺. Por otro lado la actividad de la enzima disminuyó de manera progresiva a medida que aumentó la concentración de NaCl de 0 a 30%. Los valores de Km y kcat fueron de 0.085 ± 1.45 mM y 1.76 ± 0.12 s⁻¹, respectivamente. Según los resultados obtenidos se sugiere que la enzima presenta potencial de uso biotecnológico.

TRIPSINA DO HEPATOPÂNCREAS DE LULA GIGANTE (Dosidicus gigas): PURIFICACIÓN E CARACTERIZAÇÃO
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RESUMO
Foi possível purificar tripsina do hepatopâncreas de Dosidicus gigas por fracionamento com sulfato de amônia (30-70% de saturação), cromatografia de filtração em gel, afinidade e troca iônica. O peso molecular da tripsina foi de 23.5 kDa, segundo a eletroforese SDS-PAGE e mostrou uma só faixa no zimograma. A enzima mostrou máxima atividade em pH 8.5 e 40°C, usando BAPNA como substrato. A atividade da enzima foi afetada de maneira efetiva por metil-fenil-sulfonil fluoreto (PMSF) (98%) e por Na-p-tosil-L-lisina clorometil cetona (TLCK) (99%). Também a atividade da enzima foi afetada pelos seguintes íons em ordem decrescente: Hg²⁺, Fe²⁺, Cu²⁺, Li⁺, Mg²⁺, K⁺, Mn²⁺, Ca²⁺. Por outro lado, a atividade da enzima diminuiu de maneira progressiva na medida em que aumentou a concentração de NaCl de 0 a 30%. Os valores de Km e kcat foram de 0.085 ± 1.45 mM e 1.76 ± 0.12 s⁻¹, respectivamente. Segundo os resultados obtidos, sugere-se que a enzima apresenta potencial de uso biotecnológico.

California (27°55’S, 110°54‘W). Immediately after capture, specimens were stored in an ice bed system and transported to the seafood laboratory, Universidad de Sonora in Hermosillo, Mexico. The time from capture to the dissection process was under 12h.

Preparation of crude enzyme extract
At the laboratory, thawed jumbo squid specimens were dissected, their hepatopancreas removed, immediately frozen and kept -80°C until further analysis. Hepatopancreas (100g) were homogenized with 200ml of 50mM Tris-HCl buffer, pH 7.5, and 0.5M NaCl at 20,000×g for 2min, and then centrifuged at 18,000×g for 30min at 2–4°C. After centrifugation, most lipids (upper layer) were removed manually and the supernatant was filtered through gauze to further remove them. Finally, the supernatant (enzyme extract, without lipids) was frozen and kept at -80°C until further analysis (Heu et al., 1995).

Enzyme purification
Crude extract was fractionated with ammonium sulfate at 30 and 70% saturation, according to Whitaker (1994). Each time sample was centrifuged at 20,000×g for 20min at 4°C. The precipitate was dissolved in 20ml of 50mM Tris-HCl buffer, pH 7.5 (buffer A), loaded into a 1.6 x 120cm Sephadex G-75 gel filtration chromatography column (Amersham Pharmacia Biotech), eluted at 0.3ml/min and equilibrated with buffer A, collecting 3ml fractions. Then, fractions with trypsin-like activity were pooled and loaded into a 1.1 x 15cm Benzamidine-Sepharose 4 Fast Flow column (Amersham Pharmacia Biotech) equilibrated with the same buffer. The retained fractions were eluted by changing the mobile phase pH from 7.5 to 3.0, using a 50mM Gly-HCl, pH 3.0, buffer (after elution, 200µl Tris-HCl 1M, pH 9.0, were added to each tube). Fractions eluted with trypsin-like activity were combined, dialyzed against 6l of 20mM Tris-HCl, pH 7.5 (buffer B), and pooled for further analysis. For analyses such as optimal pH and temperature (and their respective stabilities), inhibitors and NaCl effect, purified trypsin was used.

Protein concentration determination
Protein concentration in peaks that showed absorbance at 280nm during protein purification was determined according to Bradford (1976) using bovine serum albumin as standard.

SDS-PAGE and zymography
Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried according to the method of Laemmli (1970) using 4% stacking gel and 14% separating gel. For protein band analysis, a volume of enzyme extract was mixed with two volumes of sample buffer (125mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 10% β-mercaptoethanol, 0.004% bromophenol blue), heated at 95°C for 4min, cooled immediately and loaded into the gel. After electrophoresis, gels were stained with 0.25% Coomassie brilliant blue R-250 (CBB) in 40% methanol, 10% acetic acid or silver staining, depending on the purification process (protein concentration). Bovine serum albumin (66kDa), ovalbumin (45kDa), glyceraldehyde 3-phosphate dehydrogenase (36kDa), carbonic anhydrase (29kDa), trypsinogen (24kDa), trypsin inhibitor (20kDa) and lactoalbumin (14.2kDa) were used as molecular weight markers.

Zymograms were performed according to the procedure of Laemmli (1970) except that samples were not heated and
no reducing agents were added. After electrophoresis, the gel was soaked for 30 min in 1.25% casein in 50 mM Tris-HCl, pH 8.0, at 4°C. Then, the gel was immersed in the same solution at 37°C for 60 min, soaked in trichloroacetic acid for 30 min to stop the reaction, washed in distilled water, fixed and stained with 0.05% Coomassie Blue solution, and finally destained with 40% methanol and 10% acetic acid. Native-PAGE was performed according to the procedure of Laemmli (1970) except that samples were not heated and no SDS and reducing agents were added.

**Trypsin amidase activity**

Trypsin amidase activity was evaluated according to Erlanger et al. (1961) using 10 µl of enzyme extract combined with 990 µl N-benzoyl-DL-arginine p-nitroanilide (BAPNA) solution (1 mM BAPNA dissolved in 50 mM Tris-HCl, pH 7.5 buffer) at 25°C. Production of p-nitroaniline was measured by monitoring the increment in absorbance at 410 nm (ε = 8,800 M^-1 cm^-1) from Lineweaver-Burk plots (Stauffer, 1989). Enzyme solution temperature stability was evaluated by incubation at various temperatures (25, 35, 45, 55, and 65°C) for 60 min.

**Effect of inhibitors**

In order to elucidate the effect of inhibitors on trypsin activity, a series of inhibitors were evaluated at different concentrations. Phenylmethylsulfonyl fluoride (PMSF; 100 mM in 2-propanol), Nα-p-tosyl-L-lysine chloromethyl ketone (TLCK; 10 mM in dimethyl sulfoxide, DMSO), benzamidine (10 mM in DMSO), and N-tosyl-phenylalanine chloromethyl ketone (TPCK; 5 mM in methanol), ethylenediamine tetra-ace- tic acid (EDTA; 10 mM in distilled water) and pepstatin A (1 mM in DMSO) were used for the analysis. A mixture of 50 µl inhibitor solution and 50 µl enzyme extract was incubated for 60 min at 25°C; then 950 µl of substrate solution (1 mM BAPNA in 50 mM Tris-HCl buffer, pH 7.5) was added and residual activity was measured. Control tests were performed in absence of inhibitors.

**Effect of metal ions**

Samples (30 µl) of the purified enzyme were pre-incubated with 70 µl of various metal ion solutions (KCl, LiCl, CaCl₂, MnCl₂, MgCl₂, CuSO₄, FeSO₄ and HgSO₄ at 5 mM) for 60 min at 25°C. Thereafter, 900 µl of substrate solution (1 mM BAPNA in 50 mM Tris-HCl buffer, pH 8.0) was added and the residual activity measured (Souza et al., 2007; Ktari et al., 2012). Control tests were performed in absence of metal ions.

**Effect of NaCl on enzyme activity**

Trypsin activity was assayed in the presence of NaCl at varying final concentrations (0, 5, 10, 15, 20, 25, and 30% w/v). Residual activity was determined at 25°C at pH 7.5 using 1 mM TAME as a substrate. The activity of the enzyme in the absence of NaCl was taken as a control.

**Kinetic parameters**

The Michaelis-Menten constant (Kₘ) and catalysis constant (kcat) were evaluated. The initial velocity of the enzymatic reaction was evaluated at 25°C by varying BAPNA substrate concentration (0.01, 0.05, 0.1, 0.25, 0.5, 0.75 and 1.0 mM). Kₘ and Vₘₐₓ were calculated from Lineweaver-Burk plots (Lineweaver and Burk, 1934). The value of the turnover number or kcat was calculated from kcatVₘₐₓ/[E], where [E] is the active enzyme concentration (Copeland, 2000).

**SDS-PAGE and zymography**

Purified trypsin showed a single band on both SDS and zymography (Figure 2a, b) suggesting their high purity and showing only one dominant isofrom of trypsin in Dosidicus gigas hepatopancreas. Trypsins have been reported to have molecular masses between 20 and 30 kDa in mammals and aquatic species. The purified trypsin enzyme from Dosidicus gigas

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**TABLE I**

**A SUMMARY OF PURIFICATION OF TRYPsin FROM THE HEPATOPANCREAS OF Dosidicus gigas**

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total protein (mg)</th>
<th>Total activity (U)</th>
<th>Specific activity (U/mg)</th>
<th>Purification fold</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>7265.35</td>
<td>6102.89</td>
<td>8.48</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Ammonium sulfate</td>
<td>1268.22</td>
<td>5871.86</td>
<td>4.63</td>
<td>5.51</td>
<td>96.21</td>
</tr>
<tr>
<td>Gel Filtration</td>
<td>563.14</td>
<td>4775.43</td>
<td>8.48</td>
<td>10.10</td>
<td>81.33</td>
</tr>
<tr>
<td>Affinity</td>
<td>145.21</td>
<td>2629.75</td>
<td>18.11</td>
<td>21.56</td>
<td>55.07</td>
</tr>
<tr>
<td>Ionic exchange</td>
<td>33.34</td>
<td>1674.00</td>
<td>50.21</td>
<td>59.77</td>
<td>63.66</td>
</tr>
</tbody>
</table>

**Results**

Results on purification steps of trypsin from jumbo squid (*Dosidicus gigas*) hepatopancreas are summarized in Table I. As observed, trypsin was purified 59.77 fold, achieving a 63.66% yield. Gel filtration chromatography (G-75 Sephadex) separated the high molecular weight protein group, with low trypsin specific activity, from the low molecular weight protein group showing higher trypsin specific activity. Fractions with high trypsin activity were combined for affinity chromatography (Benzamidine-Sepharose). This last chromatography detected protein in fractions 4 to 10, but with no trypsin specific activity (Figure 1a); however, fractions 40 to 48 showed high trypsin specific activity and were combined for ionic exchange chromatography (DEAE-Sepharose) (Figure 1b). Washing (equilibration) buffer conditions (fractions 1 to 77) showed scarce protein (fractions 4 to 11) with no enzymatic activity detected. The NaCl linear gradient, applied after fraction 73, eluted most of the protein with trypsin activity (fractions 78 to 81).

**Optimum pH and temperature**

The effect of pH on trypsin activity was evaluated using a universal buffer from pH 4 to 11 at 25°C for 15 min (Stauffer, 1989). In order to study the effect of temperature on enzyme activity, the extract was incubated at 20, 25, 30, 35, 40, 50, 55, 60, and 70°C for 15 min in 50 mM Tris-HCl buffer under optimal pH.

**pH and thermal stability**

The effect of pH and temperature on enzyme stability was evaluated by measuring the residual activity after incubation at various pH (from 4 to 11) for 60 min at 25°C using universal buffers (Stauffer, 1989). Enzyme solution temperature stability was evaluated by incubation at various temperatures (25, 35, 45, 55, and 65°C) for 60 min.
Optimum pH and pH stability

Optimum pH for *Dosidicus gigas* trypsin activity was 8.5 (Figure 3), although high activity was observed throughout the pH range of 6.5 to 11. The optimum pH falls in the range for alkaline aquatic species digestive proteases such as Monterey sardine (*Sardinops sagax caerulea*; pH 8.0; Castillo-Yañez et al., 2005), mandarin fish (*Siniperca chuatsi*; pH 8.5; Lu et al., 2008), silver mojarra (*Diapterus rhombeus*; pH 8.5; Silva et al., 2011), hybrid catfish (*Clarias macrocephalus × Clarias gariepinus*; pH 8.0; Klomklao et al., 2011). A considerable loss of activity, although still some of it remained, was observed from pH from 5.5 to 4.0, with 36 to 13% of activity. Trypsin stability at different pH remained remarkable high over a broad pH range, from 7.0 to 11.0, maintaining more than 80% of activity; however, the enzyme was unstable at lower pH (4.5 and 4.0). Similar results were found by (Klomklao et al., 2006; Khishimura et al., 2008; Lu et al., 2008; Jellouli et al., 2009) for tongol tuna (*Thunnus tonggol*), walleye pollock (*Theragra chalcogramma*), mandarin fish (*Siniperca chuatsi*), and grey triggerfish (*Balistes capriscus*), respectively.

Optimum temperature and thermal stability

The optimum temperature for *Dosidicus gigas* trypsin activity under experimental conditions was 40ºC (Figure 4). The same result was obtained for anionic trypsin from grey triggerfish (*Balistes capriscus*; Jellouli et al., 2009) and mandarin fish (*Siniperca chuatsi*; Lu et al., 2008) while higher results were found for cod (*Gadus ogac*; 55ºC; Simpson and Haard, 1987), anchovy (*Engraulis encrasicholus*; Martínez et al., 1988), silver mojarra (*Diapterus rhombeus*; 55ºC; Silva et al., 2011), hybrid catfish (*Clarias macrocephalus × Clarias gariepinus*; 60ºC; Klomklao et al., 2011) and zebra blenny (*Salaria basilisca*; 70ºC; Ktari et al., 2012). The difference in optimal temperatures might be related to the aquatic species inhabiting environment.

The thermal stability profile (Figure 5) showed that *Dosidicus gigas* trypsin is highly stable at 25 and 35ºC, maintaining more than 80% of its activity; however, its activity decreases sharply at temperatures above 55ºC. The enzyme was inactivated after 15min of incubation at 65ºC. The thermostability profile shown by this enzyme is similar to that of other fish trypsins, like sardine (*Sardinops sagax* 2007), walleye pollock (24kDa; Kishimura et al., 2008), sardine (*Sardinella aurita*; 28.8kDa; Khaled et al., 2011) and zebra blenny (*Salaria basilisca*; 27kDa; Ktari et al., 2012).

showed a molecular weight of 23.5kDa, similar to several fish trypsin such as rainbow trout (25.7kDa; Kristjasson, 1991), common carp (28kDa; Cao et al., 2000), Monterey sardine (*Sardinops sagax caerulea*; 25kDa; Castillo-Yañez et al., 2005), Atlantic bonito (*Sarda sarda*; 29kDa; Klomklao et al., 2007), and grey triggerfish (*Balistes capriscus*), respectively.

![Figure 1. Chromatographic purification of trypsin from hepatopancreas of *Dosidicus gigas*. a: Affinity chromatography. b: DEAE-Sepharose chromatography. Trypsin activity in BAPNA.](image)

![Figure 2. SDS-PAGE (a) and Native-PAGE (b) zymography of purified trypsin from hepatopancreas of *Dosidicus gigas*. Lane 1: molecular weighs markers, lane 2: crude extract, lane 3: DEAE-Sepharose fraction, lane 4: bovine trypsin, lane 5: crude extract, lane 6: DEAE-Sepharose fraction.](image)

![Figure 3. Optimum pH and pH stability of purified trypsin from viscera of *Dosidicus gigas*.](image)
Figure 4. Optimum temperature of purified trypsin from hepatopancreas of Dosidicus gigas.

Figure 5. Thermal stability of purified trypsin from hepatopancreas of Dosidicus gigas.

carula; Castillo-Yañez et al., 2055), bogue (Boops boops; Barkia et al., 2010) and zebra blenny (Salarias basiliscia; Ktari et al., 2012).

Effect of inhibitors and metal ions

Proteases can be classified by their sensitivity to various inhibitors (North, 1982). The effect of several proteinase inhibitors on Dosidicus gigas trypsin activity is summarized in Table II. The enzyme was 98% inhibited by PMSF, a serine-protease inhibitor. Trypsin activity was strongly inhibited by TLCK (83%) and benzamidine (85%), both specific inhibitors of trypsin. EDTA (a metallo protease inactivator), TPCK (a chymotrypsin inactivator), and pepstatin A (an aspartic proteases inhibitor) did not affect trypsin activity (<10% inhibition). These results confirm that the single band detected by SDS and zymogram corresponds to trypsin. Similar results were observed for trypsins from Monterrey sardine (Castillo-Yañez et al., 2005), Pacific cod (Gadus macrocephalus; Fuchise et al., 2009) and brownstripe red snapper (Lutjanus vitta; Khantaphant and Benjakul, 2010).

The effect of metal ions over enzyme activity is also shown in Table II. It can be observed that Ca2+ ion did not have an effect over proteolytic activity; however, the enzyme was slightly inactivated by Mn2+, K2+, Mg2+, Li+ and Cu2+ ions, down to 94, 91, 82, 78 and 68% residual activity, respectively. On the other hand, Fe2+ and Hg2+ ions greatly affected trypsin activity, showing only 29 and 23% residual activity, respectively. Similar results were observed in a trypsin from Sardinella aurita (Khaled et al., 2011) and bogue (Boops boops; Barkia et al., 2010). It has been reported that Hg2+ ions bind to -SH groups on the enzymes, inhibiting their action (Klee, 1988).

Effect of NaCl on enzyme activity

The effect of NaCl on the activity of Dosidicus gigas trypsin showed a continuous decreased in activity with increasing NaCl concentration. However, it remained very active even at high salt concentrations, showing 87, 72, 61, 53, 44 and 32% residual activity at 5, 10, 15, 20, 25 and 30% of NaCl, respectively.

Kinetic studies

The kinetic constants, Km and kmw of purified Dosidicus gigas trypsin, hydrolyzing BAPNA at 25°C, were determined using a Lineaweaver-Burk plot. The values of Km and kmw presented by the purified trypsin were 0.085mM and 1.76s⁻¹, respectively. Km was similar to that reported for B. capriscus (0.07mM; Jellouli et al., 2009), sardinelle (Sardinella aurita; 0.125mM; Khaled et al., 2009), but lower than those from snapper (P. macracanthus; Hau and Benjakul, 2006) and brownstripe red snapper (Lutjanus vitta; Khantaphant and Benjakul, 2010). The kmw value was similar to those found in trypsins from anchoveta, of 1.55s⁻¹ and sniper, of 1.06s⁻¹ (E. japonica; Heu et al., 1995 and P. macracanthus; Hau and Benjakul, 2006).

Discussion

Based on the behavior observed during DEAE-Sepharose separation, it is concluded that the retained protein, with trypsin activity, is of anionic nature. It has been observed that trypsins from fish viscera have high ratios of acidic to basic amino acids. This characteristic is quite different from mammalian trypsins, which are of basic nature (Heu et al., 1995). Anionic trypsins are common in fish such as salmon (Oncorhynchus keta), sardine (Sardinops melasticta), anchovy (Engralius encrasicholus), sardine (Sardinsops sagax comula) and pez diablo (Pterygoplicthys disjunctivus) (Martinez et al., 1988; Sekisaki et al., 2000; Castillo-Yañez et al., 2005; Villalba-Villalba et al., 2013). The differences in trypsin molecular mass may be due to genetic variation among species, but the possibility that these differences are caused by auto-lytic degradation should not be excluded (Lu et al., 2008).

Trypsins generally belong to the alkaline proteinase group (Simpson and Haard, 1987); thus, under acid conditions the charge distribution and conformation were changed and the enzyme could not bind to substrate properly (Benjakul and Morrissey, 1997). The stability
of enzymes at a particular pH may be related to its net charge at that pH; at low pH, the increment of positive charges on the enzyme destabilizes them, affecting its tertiary structure (Castillo-Yañez et al., 2006). *Dosidicus gigas* trypsin showed a high activity and stability at high alkaline pH conditions, as well as in slightly acid ones, which makes it a potential candidate for application in food processing operations or for its applications in detergents and food industry. That the trypsin showed low thermostability could be related to a high proportion of charged residues and fewer polar hydrogen-bond forming residues as suggested by Benjakul and Morrissey, 1997. High activity of trypsin from *Dosidicus gigas* at low temperatures may be interesting for many biotechnological and food protein processing applications. Besides, this low thermostability can be advantageous, as these enzymes can be easy inactivated by using less heat treatment (Jiang et al., 2010).

The inhibitors employed are highly specific, possessing at their surface one or more peptide bonds made with the α-carboxylic side of arginine known as reactive sites. The reactive site specifically interacts with the active site of the cognate enzyme. Both natural and synthetic inhibitors inhibit trypsin. The extent of the inhibition (K<sub>i</sub> molar) for trypsin goes from millimolar to femtomicromolar, indicating that quite different affinities between them are possible (Zollner, 1993). In the present study, the pattern of inhibition observed inhibitors is characteristic of other trypsins, supporting the finding that he isolated enzymes is, indeed, a trypsin.

Loss of enzyme activity due to the denaturation of trypsin caused by the ‘salting out’ effect (Khaled et al., 2011) could be observed. Similar results were found in trypsin from bogue (*Boops boops*; Klee, 1998). Results show that trypsin from *Dosidicus gigas* may have potential to be used to accelerate hydrolysis of proteins under hypersaline conditions, such as in fermented products like fish sauce.

The catalytic efficiency (k<sub>c</sub>/K<sub>c</sub>) value of *Dosidicus gigas* trypsin (20.71s M<sup>-1</sup>) compared with that of bovine trypsin (3.1s M<sup>-1</sup>; Asgeirsson et al., 1989) revealed a higher catalytic efficiency (6.68 folds at 25°C), which means that *Dosidicus gigas* trypsin is more prompt to attach to and hydrolyze the substrate than the bovine type.

Trypsin shows species-specific characteristics, since there are significant differences in feeding habits, ingested food composition and differences in the protein digestion process, according to the requirements of the species. These differences are adaptive responses to different life styles, environments and mostly, different survival abilities among species. However, trypsins share some general characteristics, mainly related to their catalytic features (Muhlía-Almazán et al., 2008).

**Conclusions**

This paper describes the characteristics of a serine protease obtained from *Dosidicus gigas* hepatopancreas. Based on the results, it can be concluded that the isolated and purified enzyme was trypsin, showing high activity at pH 6.5-11.0, 25-40°C and 30% salt concentration. Therefore, the hepatopancreas of *Dosidicus gigas* can be an important source of trypsin, whose pH stability, activity under high salt concentration and thermal behaviour make it a potential agent to be used in biotechnological processes.

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