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Biochemical characterization of an isoform of chymotrypsin from the viscera of Monterey sardine (*Sardinops sagax caerulea)*, and comparison with bovine chymotrypsin

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ABSTRACT

Chymotrypsin II from the viscera of Monterey sardine was characterized as an isoform of chymotrypsin I previously characterized from the same source and compared with bovine chymotrypsin. Chymotrypsin II had a molecular weight of 25,500 Da, similar to bovine chymotrypsin. The isoform identity as chymotrypsin was established by its catalytic specificity on the specific substrates succinyl-L-ala-ala-pro-L-phenylalanine-*p*-nitroanilide and benzoyl-L-tyrosine ethyl ester, showing higher specific activity than bovine chymotrypsin. Both enzymes showed maximal activity at pH 8.0, chymotrypsin II being stable at alkaline pH while bovine chymotrypsin was stable at acid and alkaline pH. Optimum temperature was 45 °C for chymotrypsin II and 55 °C for bovine chymotrypsin. Both enzymes were inhibited by phenylmethylsulfonyl-fluoride and soybean trypsin inhibitor, and partially by *N*-toluenesulfonyl-L-phenylalanine chloromethyl-ketone. This is valid only in specific conditions of this work. K_m and k_{cat} for chymotrypsin II were 0.048 mM and 4.8 s^{-1}, and 0.09 mM and 1.9 s^{-1} for bovine chymotrypsin. Catalytic efficiency of chymotrypsin II was 4.8-fold higher than bovine chymotrypsin.

1. Introduction

Monterey sardine (*Sardinops sagax caerulea*) is the main commercial fish caught in the Gulf of California, Mexico (SAGARPA, 2003); it is considered a cold adapted organism because its habitat temperature ranges from 7 °C to 22 °C. Cold adapted organisms usually live at low temperatures and have developed a molecular adaptation to cold temperatures, especially structural adapted enzymes (Feller & Gerday, 1997). Adaptation to low temperatures generally involves an increment in the catalytic efficiency of the enzymes, and this is usually associated to a high molecular flexibility and less thermal stability. This allows for conformational changes to occur during catalysis with a smaller activation energy in cold adapted organisms compared to their mammalian enzyme homologues (Hochachka & Somero, 1984; Hultin, 1980; Somero, 1995).

The study of digestive enzymes from marine cold adapted organisms like anchovy (*Engraulis japonica* and *E. encrasicholus*), sardine (*Sardinops melanostica* and *S. sagax caerulea*), Atlantic cod (*Gadus morhua*), carp (*Cyprinus carpio*), rainbow trout (*Oncorhynchus mykiss*) and menhaden (*Brevoortia tyranus*) have showed that trypsin and chymotrypsin have increased catalytic efficiency at low temperatures, cold stability and lower thermostability than their homologous enzymes from terrestrial mammals (Asgeirsson & Bjarnason, 1991; Castillo-Yañez, Pacheco-Aguilar, García-Carreño, & Navarrete-Del Toro, 2005; Cohen, Gertler, & Birk, 1981a, 1981b; Heu, Kim, & Pyeun, 1995; Kristjansson & Nielsen, 1992; Martinez, Olsen, & Serra, 1988; Murakami & Noda, 1981; Pyeun, Kim, & Godber, 1990; Raae & Walther, 1989).

The main digestive enzymes in fish viscera are trypsin, chymotrypsin, and elastase, all belonging to the serine-protease family (E.C. 3.4.21.X). They are characterized by having a catalytic triad comprised of serine, histidine, and aspartic residues at the active site. As a group, serine-proteases are inhibited by di-isopropyl phospho-fluoride (DFP), are active at neutral and alkaline pH, and inactive or unstable in acid pH (Simpson, 2000). Fish chymotrypsins are endopeptidases that cleave the peptide bond of proteins on the carboxyl side of Phe, Tyr, and Trp and also synthetic substrates, such as succinyl-L-Ala-Ala-Pro-L-Phe-*p*-nitroanilide (SAAPNA) and benzoyl-L-Tyr ethyl ester (BTEE). Likewise, they are susceptible to specific inhibitors such as *N*-toluenesulphonyl-L-Phe chloromethyl-ketone (TPCK) and *N*-carbobenzoxi-L-Phe chloromethyl-ketone (ZPCK) (De Vecchi & Coppes, 1996; Simpson, 2000). These chymotrypsins have molecular weights in the range of 22,000–30,000 Da, with an optimum activity range of pH and

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temperature at 7.5–9 °C and 45–55 °C, respectively, they are unstable at temperatures >55 °C and acidic conditions (Cohen et al., 1981a; Heu et al., 1995).

Among fish, trypsin-like proteases have been the most studied and information about chymotrypsin and elastase is less available. Most of the research on fish chymotrypsins has shown that it is common to find two isoforms with the same specific activity (Asgeirsson & Bjarnason, 1991; De Vecchi & Coppes, 1996; Kristjansson & Nielsen, 1992). Several studies on these chymotrypsins have found that biochemical characteristics of these enzymes are similar to bovine and porcine chymotrypsins; however, some important differences have been observed. Specifically, the fish enzymes showed higher catalytic activities and lower thermostability than bovine and porcine chymotrypsins (Cohen et al., 1981a; Racicot & Hultin, 1987; Ramakrishna, Hultin, & Racicot, 1987).

In this study, biochemical characteristics of an isoform of chymotrypsin from Monterey sardine are presented and compared with bovine chymotrypsin. The aim of this work is to contribute to a better understanding of this kind of enzymes and to generate basic information about its main biochemical and kinetic characteristics which might contribute to the commercial application of the viscera of Monterey sardine.

2. Materials and methods

2.1. Reagents

PMSF, TPCK, benzamidine, soybean trypsin inhibitor (SBTI), ethylene diamine tetraacetic acid (EDTA), SAAPNA, BTEE, BAPNA, glycine, citric acid, Tris buffer, trichloroacetic acid (TCA) and ammonium sulphate were purchased from Sigma (Mexico). Sodium dodecyl sulphate (SDS), acrylamide, ammonium persulfate (APS), tetramethyl ethylene diamine (TEMED), molecular weight markers (6000–200,000 Da) and Coomassie Blue G and R were obtained from Bio-Rad Laboratories (Mexico). Diethylene amino ethyl sepharose (DEAE-Sepharose) fast flow, Sephadex G-75, and dialysis tubing were purchased from Amersham Pharmacia Biotech (Sweden). All reagents were of analytical grade.

2.2. Samples

Monterey sardine specimens were obtained from Productos Pesqueros de Guaymas, S.A. (Guaymas, Mexico). Sardines were collected within 6 h of death from the fishing vessel's storage vault, where they were stored at 8 °C. Samples were placed in a portable cooler between layers of crushed ice and transported to the CIAD Seafood Products Laboratory in Hermosillo, Mexico. The viscera were extracted at low temperature, placed in hermetically sealed polyethylene bags, immediately frozen, and kept at −80 °C until analysis.

2.3. Purification procedure

Portions of pyloric caeca (50 g) were separated from the viscera and homogenized with 250 mL extraction buffer (50 mM Tris–HCl pH 7.5, 10 mM $CaCl_2$, 0.5 M NaCl), using a tissue homogenizer Tizzumizer Tekman model SDT 1810 (Tecman Co., West Germany) for 1 min. The homogenate was incubated for 8 h at 25 °C for activation of chymotrypsinogen, defatted with 50 mL of CCl_4, and centrifuged at 26,000*g* for 30 min at 2–4 °C. The supernatant was considered the crude enzyme extract (Heu et al., 1995; Whitaker, 1994).

The crude enzyme extract was mixed with ammonium sulphate and the fraction between 30% and 70% saturation was collected. After 2 h in an ice-bath, this fraction was centrifuged at 20,000*g* for 20 min. The pellet was dissolved in 30 mL buffer A (50 mM Tris·HCl pH 7.5, NaCl 0.5 M) (Janson & Rydén, 1998), and loaded into a 1 × 80 cm Sephadex G-75 gel filtration chromatography column (Amersham Pharmacia Biotech, Uppsala, Sweden). Buffer A was used as a mobile phase at 0.5 mL/min flow rate and 5 mL fractions were collected. Fractions with chymotrypsin activity were combined and dialysed overnight in Sigma dialysis tubing 25 mm width and 5000 Da molecular weight cut-off, against two changes of 6 L of 20 mM Tris·HCl, pH 7.5 buffer (Cohen et al., 1981a; García-Carreño & Haard, 1993; Simpson & Haard, 1984).

Dialyzed fractions were loaded into a DEAE-Sepharose column (1.6 × 20 cm) and equilibrated with 20 mM Tris–HCl pH 7.5 buffer. Unabsorbed protein was washed with equilibration buffer, and the column was eluted with a 400 mL linear gradient ranging from 0.0 to 0.4 M NaCl in equilibration buffer (Cohen et al., 1981a; García-Carreño & Haard, 1993).

Protein concentration was evaluated using $Abs_{280\ nm}$ and the method of Bradford (1976) using bovine serum albumin as a standard. All assays were done by triplicate. Trypsin- and chymotrypsin-specific activities in the eluted fractions were also evaluated, using specific substrates. Electrophoretic patterns obtained for fractions were analysed by SDS–PAGE.

2.4. Chymotrypsin characterization

2.4.1. Electrophoresis

Gels of 14% polyacrylamide with 0.1% SDS were used to analyse chymotrypsin purity (Laemmli, 1970). Electrophoresis assays were run at pH 8.3 and 5 °C. Myosin (200,000 Da); β-galactosidase (116,250 Da); phosphorylase b (97,400 Da); bovine serum albumin (66,200 Da); ovalbumin (45,000 Da); carbonic anhydrase (31,000 Da); trypsin inhibitor (21,500 Da); lysozyme (14,400 Da) and aprotinin (6500 Da) were used as molecular weight markers.

2.4.2. Isoelectric point

The isoelectric point of the isolated enzymes was evaluated by analytical electrofocusing in thin layer polyacrylamide flat gel (LKB ampholyne PAG plate) containing ampholynes in the pH range 3.5–9.5. An isoelectric focusing calibration kit (Amersham Pharmacia Biotech), containing 11 proteins with known isoelectric points, was used as a reference. Proteins were stained with Coomassie Brilliant Blue as described by Gildberg, Olsen, and Bjarnasson (1990).

2.4.3. Specific activity

Amidase activity of isolated chymotrypsin was evaluated according to Tsai, Chuang, and Chuang (1986), using SAAPNA as substrate with slight modifications: 10 μL of enzyme solution was mixed with 990 μL 0.1 mM SAAPNA in 50 mM Tris·HCl pH 8.0, 10 mM $CaCl_2$ buffer at 25 °C. Production of *p*-nitroaniline was measured by monitoring the increment in $Abs_{410\ nm}$ every 30 s for 10 min. SAAPNA hydrolysis units (*U*) were calculated with the following equation: $U = [A_{(410)}/\text{min} \times 1000 \times 1]$ divided by (8800), where 8800 = *p*-nitroaniline molar extinction coefficient in $M^{-1}\,cm^{-1}$, 1000 is the factor to convert M to μmoles in 1 mL which is the volume of the reaction mixture (Erlanger, Kokowski, & Cohen 1961). Esterase activity was evaluated according to Hummel (1959), using BTEE as substrate, where 20 μL enzyme solution was mixed with 980 μL 1 mM BTEE in 50 mM Tris·HCl pH 8.0, 10 mM $CaCl_2$ buffer at 25 °C. Production of benzoyl-tyrosine was measured by monitoring the increment in $Abs_{256\ nm}$ every 30 s for 10 min. BTEE hydrolysis units (*U*) were calculated with the following equation: $U = [A_{(256)}/\text{min} \times 1000 \times 1]$ divided by (964), where 964 = benzoyl-tyrosine molar extinction coefficient in $M^{-1}\,cm^{-1}$, and 1 is the volume of the reaction mixture. All assays were done by triplicate.

2.4.4. Effect of inhibitors

Inhibition was measured according to García-Carreño and Haard (1993) and García-Carreño (1996). Enzyme extracts were incubated with different specific protease inhibitors: serine-protease inhibitors PMSF and SBTI, chymotrypsin-specific inhibitor TPCK, trypsin specific inhibitor benzamidine, and the metallo protease inhibitor EDTA. A mixture of 10 μL inhibitor solution and 10 μL enzyme extract was incubated for 60 min at 25 °C, and then 980 μL substrate solution (0.1 mM SAAPNA in 50 mM Tris–HCl pH 8.0, 10 mM $CaCl_2$ buffer) was added and residual activity was measured. Appropriate blanks and inhibitor solvents were used as controls. Percentage activity in inhibition assays was reported, using activity in the absence of an inhibitor as 100%.

2.4.5. Optimum pH at 25 °C

The effect of pH on the activity was evaluated by measuring pure enzyme activity at pH 4–11, using 0.1 mM SAAPNA at 25 °C as the substrate, with the following buffers: 100 mM citrate·NaOH pH 4–6, 10 mM $CaCl_2$, 100 mM Tris–HCl pH 7–9, 10 mM $CaCl_2$; and 100 mM glycine·NaOH pH 10–11, 10 mM $CaCl_2$. Enzyme activity was measured according to Tsai et al. (1986).

2.4.6. pH stability at 25 °C

The effect of pH on enzyme stability was evaluated by measuring enzyme residual activity after incubation at a range of pH 4–11 for 60 min at 25 °C, using the following buffers: 100 mM citrate·NaOH pH 4–6, 10 mM $CaCl_2$; 100 mM Tris–HCl pH 7–9, 10 mM $CaCl_2$, and 100 mM glycine·NaOH pH 10–11, 10 mM $CaCl_2$. Enzyme residual activity was measured according to Tsai et al. (1986).

2.4.7. Optimum temperature and thermostability at pH 8

Optimum temperature of pure chymotrypsin activity was measured at pH 8.0, using 0.1 mM SAAPNA in 100 mM Tris·HCl buffer pH 8.0, 10 mM $CaCl_2$ as substrate and varying temperature ranging from 10 to 80 °C. Chymotrypsin temperature stability was evaluated by incubating chymotrypsin at various temperatures from 20 to 90 °C for 60 min and measuring residual activity according to Tsai et al. (1986).

2.4.8. Kinetic parameters

The Michaelis–Menten constant (K_m), maximum velocity (V_{max}), and catalysis constant (k_{cat}) were evaluated. The initial velocity of the enzymatic reaction was evaluated at 30 °C by varying SAAPNA substrate concentration between 1.0 and 0.005 mM. K_m and V_{max} were evaluated by non-linear regression analysis after plotting velocity against substrate concentration, using the Prism 2 computer programme (Graph Pad Software Inc., San Diego, CA). Turnover number or k_{cat} was obtained by dividing V_{max} by enzyme molar concentration, which was estimated, using its molecular weight as determined by SDS–PAGE (Copeland, 2000; Heu et al., 1995).

3. Results and discussion

3.1. Purification of chymotrypsin isoform

Chymotrypsin I and the newly described isoform chymotrypsin II were isolated from Monterey sardine viscera and the purification procedure of the former was described in a previous paper (Castillo-Yañez, Pacheco-Aguilar, García-Carreño, Navarrete-Del Toro, & Félix-López, 2006). Both chymotrypsins showed the same electrophoretic behaviour and specific chymotrypsin activity, and the broad band obtained from ionic exchange chromatography was separated by preparative electrophoresis in two single bands being named as chymotrypsin I and chymotrypsin II, respectively (Fig. 1). The characterization of the chymotrypsin isoform (chymotrypsin II) is presented here and its catalytic characteristics are compared with those of chymotrypsin I and bovine chymotrypsin.

3.2. Biochemical characterization

3.2.1. Molecular mass

The molecular mass of chymotrypsin II was 25.5 kDa (Fig. 1), inside the range of 22–30 kDa determined for chymotrypsins purified from other fish species, and very similar to chymotrypsin I (26 kDa) and bovine chymotrypsin which has a molecular mass of 25 kDa (Castillo-Yañez, Pacheco-Aguilar, García-Carreño, Navarrete-Del Toro, & Félix-López, 2006; Simpson, 2000; SIGMA chemical, Co., Los Angeles, CA). Very little differences were found in the molecular masses in sardine chymotrypsins which are in agreement with results showed by Asgeirsson and Bjarnason (1991) and Kristjansson and Nielsen (1992) for two chymotrypsin isoforms characterized from rainbow trout and Atlantic cod, respectively. The presence of two isoenzymes is common in mammalian organisms (Bender & Killheffer, 1973) and two or three in fish species like those mentioned above and carp (*C. carpio*), all of them with slight differences in molecular mass (Cohen et al., 1981a).

3.2.2. Isoelectric point

Monterey sardine chymotrypsin II migrated as a single band during isoelectric point analysis confirming its purity (Fig. 2). The isoelectric point (pI) of chymotrypsin II was approximately 5.8 and 4.3 for chymotrypsin I (Fig. 2), which indicated that these enzymes are anionic proteins at neutral pH. These data support the assumption that Monterey sardine has two chymotrypsin isoenzymes in agreement with results presented by Asgeirsson and Bjarnason (1991); Kristjansson and Nielsen (1992) and Cohen et al. (1981a).

3.2.3. Specific activity

Amidase (SAAPNA) and esterase (BTEE) specific activities in chymotrypsin I, chymotrypsin II and bovine chymotrypsin were evaluated. Making a comparison of the sardine and bovine chymotrypsins, the three enzymes have a faster hydrolysis for the esterase substrate (BTEE) than the amidase substrate (SAAPNA). Monterey sardine chymotrypsin I and II showed specific activities of 168 U/mg of enzyme and 109 U/mg of enzyme on the ester

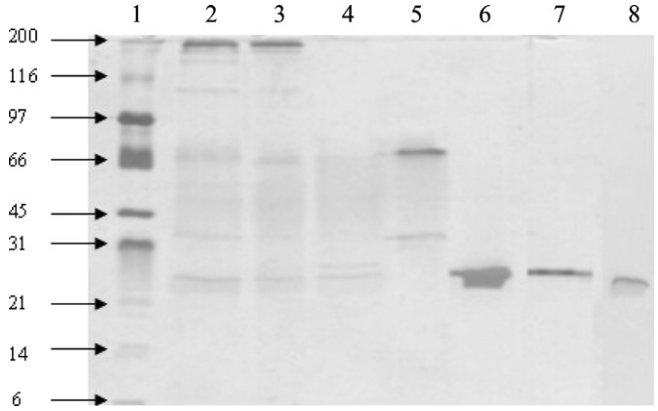

Fig. 1. SDS–polyacrylamide gel electrophoresis (PAGE). Lane 1, MWM; Lane 2, crude extract; Lane 3, ammonium sulphate fraction; Lane 4, gel filtration chromatography fraction; Lane 5, fraction 60 from ion exchange chromatography; Lane 6, fractions 70, 71, and 72 from ion exchange chromatography; Lane 7, chymotrypsin I from preparative electrophoresis; Lane 8, chymotrypsin II from preparative electrophoresis.

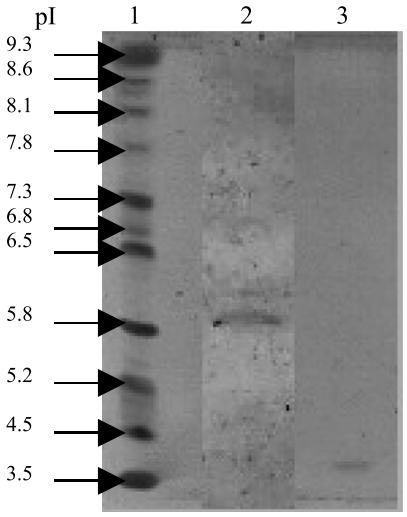

Fig. 2. Isoelectrofocusing of Monterey sardine chymotrypsins. Lane 1, protein markers; Lane 2, chymotrypsin II; Lane 3, chymotrypsin I.

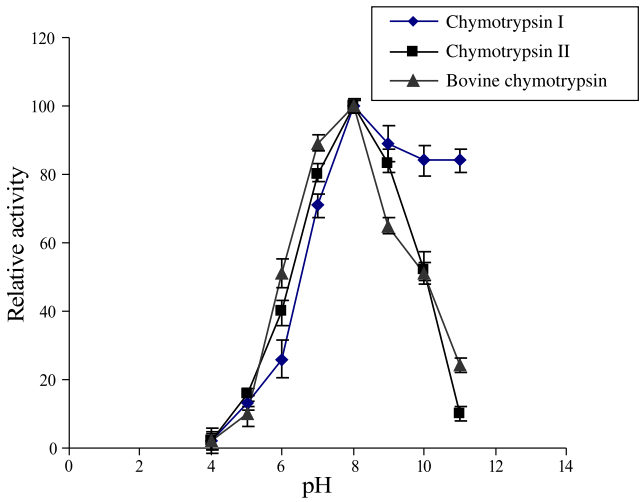

Fig. 3. Effect of pH on activity. Activity was measured in a series of buffers using SAAPNA 0.1 mM as substrate at 25 °C and varying pH from 4 to 11. Percentage of enzyme activity was estimated considering 100% of the highest activity detected in this assay. Error bars are SD from the mean of three determinations.

substrate, and of 58 U/mg of enzyme and 39 U/mg of enzyme on the amide substrate, respectively, whereas bovine chymotrypsin showed specific activity values of 84 U/mg and 36 U/mg of enzyme, respectively. Similar behaviour has been reported for many serine-proteases studied with both esterase and amidase substrates (Mihalyi, 1978).

3.2.4. Effect of inhibitors

The effect of different inhibitors on chymotrypsin I, chymotrypsin II and bovine chymotrypsin is shown in Table 1. The three enzymes were fully inhibited by the serine-protease inhibitors PMSF and SBTI; however, they were partially inhibited by the specific inhibitor of chymotrypsin, TPCK. On the other hand, chymotrypsin I and bovine chymotrypsin were not inhibited by the specific inhibitor of trypsin, benzamidine and the metallo protease inhibitor EDTA. A noticeable difference was showed by chymotrypsin II which was more susceptible to EDTA and benzamidine. However, the inhibition profile amongest three chymotrypsins was similar to chymotrypsins from other fish species such as anchovy (*E. japonica*) and rainbow trout (*O. mykiss*) (Heu et al., 1995; Kristjansson & Nielsen, 1992).

3.2.5. Optimum pH at 25 °C

The effect of pH on the rate of SAAPNA hydrolysis was examined and the results are presented in Fig. 3. Chymotrypsin I, chymotrypsin II and bovine chymotrypsin showed the same behaviour between pH 4 and 8 and from pH 8 to 11 chymotrypsin I showed more activity than chymotrypsin I and bovine chymotrypsin (Fig. 3). The amidase activity was increased at higher pH, a very common characteristic of alkaline proteases. Optimum pH for hydrolysis of SAAPNA at 25 °C by Monterey sardine chymotrypsin isoforms and bovine chymotrypsin was 8.0. These enzymes showed low activity at acidic pH (4–5), considerably activity at pH from 6 to 9 whilst chymotrypsin I showed the highest activity toward the alkaline pH (7–11). pH dependence of the three enzymes is the expected for serine-proteases which share the same hydrolysis mechanism. Apparent pH activity profiles of sardine chymotrypsins for amidase substrate are similar to those reported for pH profiles of chymotrypsin from anchovy (Heu et al., 1995), rainbow trout (Kristjansson & Nielsen, 1992), Atlantic salmon (Asgeirsson & Bjarnason, 1991) and carp (Cohen et al., 1981a).

3.2.6. pH stability at 25 °C

Monterey sardine chymotrypsin I and II and bovine chymotrypsin exhibited different pH stability. Sardine chymotrypsins I and II showed optimum stability over a pH range of 6.0–11.0 (Fig. 4), whilst bovine chymotrypsin has stability at acidic and alkaline

Table 1
Effect of inhibitors on chymotrypsin I, chymotrypsin II and bovine chymotrypsin

Inhibitor	Conc. (mg/mL)	Residual enzyme activity (%)		
		Chymotrypsin I	Chymotrypsin II	Bovine chymotrypsin
PMSF	1.4	1	2	4
TPCK	1.0	56	60	53
SBTI	0.5	0.5	0	17
Benzamide	2.0	103	71	117
EDTA	0.25	98	65	96

PMSF, phenyl-methyl-sulphonyl-fluoride; TPCK, tosyl-phenylalanine chloromethyl-ketone; SBTI, soybean trypsin inhibitor; EDTA, ethylenediamine tetraacetic acid.

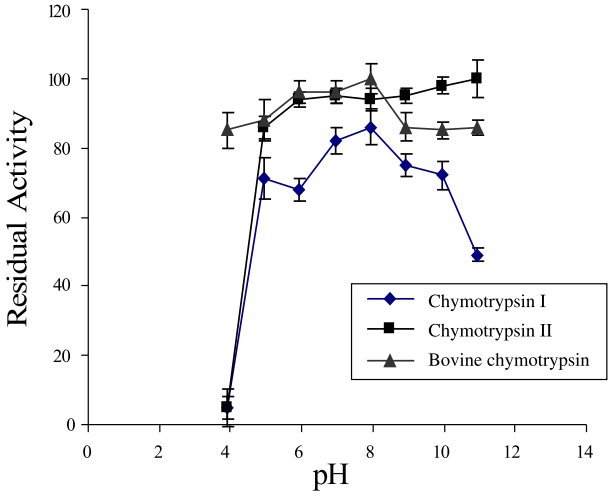

Fig. 4. pH stability. Residual activity was measured after 60 min incubation of enzyme extracts with substrate solution pH varying from 4 to 11 at 25 °C; 100% of enzyme activity is the activity of enzyme without incubation. Error bars are SD from the mean of three determinations.

pHs. Accordingly, chymotrypsins from warm blooded organism are extremely stable at acidic and alkaline pHs whilst fish chymotrypsins are stable only to alkaline pH (Simpson, 2000). pH stability of sardine chymotrypsins I and II is similar to that reported for other marine organisms chymotrypsins, where is commonly found great stability at alkaline pH and an irreversibly inactivation at pH values below 5.0 (Kimoto, Yokoi, & Murakami, 1985; Raae & Walther, 1989; Tsai et al., 1986).

3.2.7. Optimum temperature and thermostability at pH 8

The activity of sardine chymotrypsin I, chymotrypsin II and bovine chymotrypsin increased with temperature up to an optimum of 50, 45 and 55 °C, respectively, when assayed against SAAPNA at pH 8.0 (Fig. 5). Sardine chymotrypsin I showed high activity in the range from 20 °C to 50 °C and chymotrypsin II from 10 °C to 45 °C while bovine chymotrypsin was more active in the range from 55 °C to 70 °C. It is remarkable that chymotrypsin II showed higher activity at low temperatures (10–45 °C) which could be in response to the change in the temperature habitat of Monterey sardine. It is well known that some species undergoing migrations along a sharp thermal gradient, or occurring over widely different thermal habitats, can express specific isozymes kinetically adapted to a given environment (Feller & Gerday, 1997). This behaviour is in accordance to the fact that mammalian chymotrypsins are more active at higher temperatures than the fish chymotrypsins (Simpson, 2000). Chymotrypsin I and II lose their activity at a faster rate than bovine chymotrypsin at temperatures above their optimum, which is evidence of the high thermostability of the bovine enzyme. Studies on chymotrypsins from other fish species like rainbow trout (*O. mykiss*), Atlantic cod (*G. morhua*), anchovy (*E. japonica*) and carp (*C. carpio*) showed similar profiles on activity toward temperature with slight differences on the optimum temperature (Asgeirsson & Bjarnason, 1991; Cohen et al., 1981a; Heu et al., 1995; Kristjansson & Nielsen, 1992).

Residual activity of chymotrypsins was evaluated after heat treatment for 60 min at various temperatures and results are shown in Fig. 6. Bovine chymotrypsin was significantly more stable than sardine chymotrypsins I and II when temperatures were above 40 °C which confirms the data observed on the optimum temperature for activity assay. Chymotrypsin I was completely inactivated at 55 °C, chymotrypsin II at 50 °C whilst bovine chymotrypsin was inactivated at 60 °C, this data is in accordance to the fact that the mammal chymotrypsins are more stable to temperature than fish chymotrypsins (Asgeirsson & Bjarnason, 1991; Cohen et al., 1981a; Heu et al., 1995; Kristjansson & Nielsen, 1992; Simon, Lászlo, Kotorman, & Szaláni, 2001). Chymotrypsin II was the less stable to high temperatures of the three enzymes which is in accordance with the assumption that cold adapted enzymes improve their catalytic efficiencies in base to a highly flexible structure that provides enhanced abilities to undergo conformational changes during catalysis. Therefore, the thermal instability of cold- adapted enzymes could be regarded as a consequence of their conformational flexibility (Feller & Gerday, 1997).

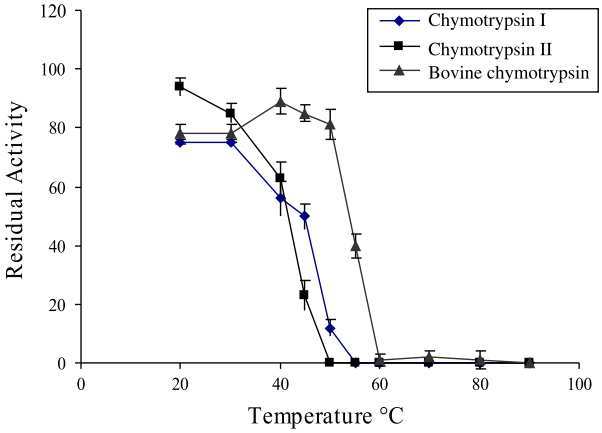

Fig. 6. Temperature stability. Residual activity at pH 8 after incubation of enzymes extracts with 0.1 mM SAAPNA for 60 min at temperatures from 20 to 90 °C. Percentage of enzyme activity is based on the activity of enzyme without incubation as 100%. Error bars are SD from the mean of three determinations.

3.2.8. Kinetic characteristics

Kinetic constants K_m and k_{cat} for Monterey sardine viscera chymotrypsins and bovine chymotrypsin were calculated from a Michaelis–Menten plot (Fig. 7 and Table 2). K_m and k_{cat} were 0.074 mM and 18.6 s^{-1} for chymotrypsin I; 0.048 mM and 4.8 s^{-1} for chymptrypsin II and 0.09 mM and 1.9 s^{-1} for bovine chymotrypsin, respectively. Catalytic efficiencies (k_{cat}/K_m) of Monterey sardine chymotrypsins I and II were significantly higher than that of bovine chymotrypsin, being 251, 100 and 21 s^{-1} mM^{-1}, respectively (Table 2). The higher catalytic efficiencies of the sardine

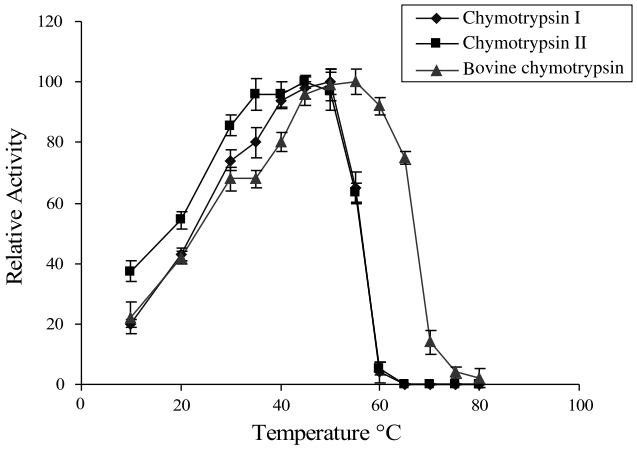

Fig. 5. Optimum temperature. Activity at pH 8 (50 mM Tris·HCl pH 8.0 + $CaCl_2$ 10 mM) was evaluated using 0.1 mM SAAPNA as substrate and changing temperature from 10 to 80 °C. Percentage of enzyme activity was estimated based on the highest activity detected in this assay as 100%. Error bars are SD from the mean of three determinations.

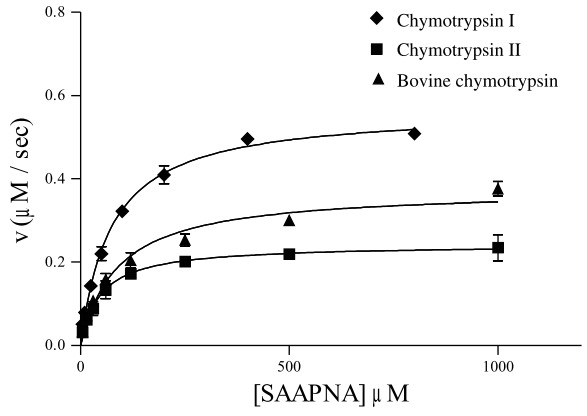

Fig. 7. Michaelis–Menton plot for chymotrypsin I, chymotrypsin II and bovine chymotrypsin kinetics. SAAPNA concentrations (0.8–0.005 mM for chymotrypsin I and 1–0.01 mM for chymotrypsin II and bovine chymotrypsin); enzyme concentration: 0.03 μM for chymotrypsin I and 0.08 μM for chymotrypsin II and bovine chymotrypsin; buffer: 50 mM Tris·HCl pH 8.0 + $CaCl_2$ 10 mM; 30 °C; $y = (V_{max}x)/(K_m + x)$; $R^2 = 0.99$ for chymotrypsin I, 0.99 for chymotrypsin II and 0.97 for bovine chymotrypsin. Error bars are SD from the mean of three determinations.

Table 2
Kinetic constants from Monterey sardine chymotrypsin II, bovine chymotrypsin and other fish chymotrypsins

Chymotrypsin	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})
Monterey sardine chymotrypsin I[a]	0.074	18.6	251
Monterey sardine chymotrypsin II	0.048	4.8	100
Bovine chymotrypsin	0.09	1.9	21
Anchovy (*E. japonica*)[b]	0.089	14.7	165
Rainbow trout (*O. mykiss*)[c]	0.035	2.2	62.8
Carp (*C. carpio*)[d]	0.300	4.4	15

Source: [a]Castillo-Yañez et al. (2006); [b]Heu et al. (1995); [c]Kristjansson and Nielsen (1992); [d]Cohen, Gertler, & Birk (1981a, 1981b).
Substrate: succinyl-L-ala-ala-pro-L-phenylalanine-*p*-nitroanilide (SAAPNA).

chymotrypsins with respect to the bovine chymotrypsin reflect the fact that fish digestive proteases have an enhanced efficiency in response to their low temperature habitats. The higher activity of chymotrypsin II from sardine may be achieved by lowering K_m value and/or by raising the turnover number (k_{cat}), as it has been seen in chymotrypsins from other fish species like rainbow trout (*O. mykiss*), Atlantic cod (*G. morhua*) and anchovy (*E. japonica*) (Asgeirsson & Bjarnason, 1991; Heu et al., 1995; Kristjansson & Nielsen, 1992).

4. Conclusion

Based on SDS–PAGE and isoelectric focusing analyses as well as in its susceptibility to inhibitors, chymotrypsin II is considered to be an isoenzyme of chymotrypsin I. Moreover, chymotrypsin II could be a cold adapted enzyme based on its activity at low temperatures and heat unstability. The dependence of activity on pH and temperature could make chymotrypsin II a biotechnological alternative for food processing when low temperatures are needed like in fish ripening, fish sauce production, fish protein hydrolysate production and other emerging processes. Also, this enzyme together with other proteases from sardine viscera might aid in the enzymatic treatment of stick-water, in which a reduction in viscosity is required for further processing of effluent.

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