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# Purification and biochemical characterization of chymotrypsin from the viscera of Monterey sardine (*Sardinops sagax caeruleus*)

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**Abstract**

Chymotrypsin was isolated from the viscera of Monterey sardine by ammonium sulphate fractionation, gel filtration, and ionic exchange chromatography. The approximate molecular weight was 26,000 and its isoelectric point was about 5. Identity as chymotrypsin was established by its catalytic specificity for amide or ester bonds on the synthetic substrates succinyl-L-ala-ala-pro-L-pheil-alanine-*p*-nitroanilide and benzoyl-L-tyrosine-ethyl-ester, showing esterase activity 3.2-fold higher than amidase. It was inhibited by phenylmethylsulfonyl-fluoride and soybean trypsin inhibitor, partly inhibited by the specific chymotrypsin inhibitor *N*-toluenesulfonyl-L-phenylalanine chloromethyl-ketone, but not inhibited by EDTA or Benzamidine. Chymotrypsin showed its maximum activity at pH 8.0 and 50 °C for the hydrolysis of SAAPNA. The Michaelis–Menten constant was 0.074 mM with a catalysis constant of 18.6 $seg^{-1}$, and catalytic efficiency of 252 $seg^{-1}$ $mM^{-1}$. Results indicated that Monterey sardine chymotrypsin is a good catalyst and could be used as a biotechnological tool in food processing and using sardine industry wastes as a material for production of fine reagents.


*Keywords:* Monterey sardine; Chymotrypsin; Biochemical characterization; Viscera; Purification; Chromatography; Enzyme activity

## 1. Introduction

The Gulf of California, Mexico is the habitat of a great variety of marine organisms, including Monterey sardine (*Sardinops sagax caeruleus*), the main commercial fish (SAGARPA, 2003). During processing, large quantities of waste are generated and discarded directly into the sea, causing pollution (Doode, 1996). Viscera are one of the most important by-products of the sardine industry and are recognized as a potential source of digestive enzymes, especially proteases with high activity over a wide range of pH and temperature conditions (Cancre et al., 1999; Gildberg, 1992; Martinez & Serra, 1989; Simpson & Haard, 1987). Proteases from fish viscera could be used in industrial applications, so its recovery from viscera might be a partial solution to the pollution problem generated by the sardine industry.

Monterey sardine, like other small pelagic fish, is very susceptible to fast abdominal deterioration caused mainly by digestive enzymes, suggesting that viscera are a source of digestive enzymes (Martínez & Gildberg, 1988). Fish digestive proteases belong to the aspartic- and serine-proteases families, specifically trypsin and chymotrypsin the main alkaline proteolytic enzymes in fish viscera (Heu, Kim, & Pyeun, 1995; Pyeun, Kim, & Godber, 1990). Most

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studies of fish digestive enzymes have focused on cold-water fish; studies of enzymes of tropical fish are scarce. Among the digestive enzymes, trypsin-like proteases have received more interest while information about chymotrypsins is less available (Kristjansson & Nielsen, 1992). Studies of fish chymotrypsins purified from dogfish (*Squalus acanthias*) (Prahl & Neurath, 1966; Racicot & Hultin, 1987), carp (*Cyprinus carpio*) (Cohen, Gertler, & Birk, 1981a, 1981b), Atlantic cod (*Gadus morhua*) (Asgeirsson & Bjarnason, 1991; Raae & Walther, 1989), rainbow trout (*Oncorhynchus mykiss*) (Kristjansson & Nielsen, 1992) and anchovy (*Engraulis japonica*) (Heu et al., 1995), have revealed that fish chymotrypsins are similar to bovine and porcine chymotrypsins; however, some important differences have been detected in fish chymotrypsin, including higher catalytic activity, lower thermostability, and differences in polypeptide amino acid composition (Cohen et al., 1981a; Racicot & Hultin, 1987; Ramakrishna, Hultin, & Racicot, 1987).

Most studies of fish chymotrypsins have shown that it is common to find two isoforms with the same specific activity. Like mammalian chymotrypsins, they are endopeptidases that cleavage the peptide bond of proteins on the carboxyl side of phenylalanine, tyrosine, and tryptophane and also synthetic substrates, such as SAAPNA and BTEE. Likewise, they are susceptible to specific inhibitors such as *N*-toluenesulfonyl-L-phenylalanine chloromethyl-ketone (TPCK) and *N*-carbobenzoyi-L-phenylalanine chloromethyl ketone (ZPCK) (De Vecchi & Coppes, 1996; Simpson, 2000). They have molecular weight in the range of 22,000 and 30,000 Da, with an optimum activity range of pH 7.5–9 and 45–55 °C; are unstable at temperatures >55 °C and acidic conditions (Cohen et al., 1981a; Heu et al., 1995). This study generated data on purification and the main biochemical characteristics of chymotrypsin from Monterey sardine viscera to contribute alternatives for the commercial use of this by-product.

## 2. Materials and methods

### 2.1. Reagents

Phenyl methyl sulfonyl fluoride (PMSF), *N*-toluenesulfonyl-L-phenylalanine chloromethyl ketone (TPCK), benzamidine, soybean trypsin inhibitor (SBTI), ethylene-diamine-tetracetic acid (EDTA), succinyl-L-ala-ala-pro-L-phenylalanine-*p*-nitroanilide (SAAPNA), benzoyl-L-tyrosine ethyl-ester (BTEE), *N*-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA), glycine, citric acid, TRIS buffer, trichloroacetic acid (TCA), ammonium sulfate, sodium dodecyl sulphate (SDS)–polyacrylamide gel electrophoresis (PAGE), and molecular weight markers (14,000–66,000) were purchased from Sigma (Mexico). Sodium dodecyl sulphate (SDS), acrylamide, ammonium persulfate (APS), tetramethyl ethylene diamine (TEMED), 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) for 1 min. The homogenate was incubated for 8 h at 25 °C, 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 sulfate 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 dialyzed against 6 l 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 (Amersham Pharmacia Biotech, 1999; Cohen et al., 1981a; García-Carreño & Haard, 1993).

Protein concentration was evaluated using $Abs_{280\,nm}$ and the method of Bradford (1976). Trypsin- and chymotrypsin-specific activities in the eluted fractions were also evaluated, using specific substrates according to

Erlanger, Kokowski, and Cohen (1961) and Tsai, Chuang, and Chuang (1986). Electrophoretic patterns obtained for fractions were analyzed by SDS–PAGE. Proteolytic activity in gel was evaluated by the substrate gel electrophoresis (García-Carreño, Dimes, & Haard, 1993; Laemmli, 1970).

### 2.4. Chymotrypsin characterization

#### 2.4.1. Electrophoresis

Gels of 14% polyacrylamide with 0.1% SDS were used to investigate chymotrypsin purity (Laemmli, 1970). Electrophoresis assays were run at pH 8.3 and 5 °C. Substrate gel electrophoresis was also used to evaluate proteolytic activity (García-Carreño et al., 1993). Bovine serum albumin (66,000 Da); ovoalbumin (45,000 Da); glyceraldehyde 3-phosphate dehydrogenase 36,000 Da; carbonic anhydrase 29,000 Da; trypsinogen 24,000 Da; trypsin inhibitor 20,000 Da, and α-lactoalbumin 14,200 Da were used as molecular weight markers.

#### 2.4.2. Electrofocusing

The isoelectric point of isolated enzyme was evaluated by analytical electrofocusing in thin-layer polyacrylamide flat gel (LKB ampholyne PAG plate) containing ampholynes in the pH 3.5–9.5 range. 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 et al. (1986), using SAAPNA as substrate with slight modifications: 10 μl 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: $U = A_{(410)}/\text{min} \times 1000 \times 1/8800 \times \text{mg enzyme}$ (8800 = *p*-nitroaniline molar extinction coefficient). 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 units (*U*) were calculated with the following equation: $U = A_{(256)}/\text{min} \times 1000 \times 1/964 \times \text{mg enzyme}$ (964 = benzoyl-tyrosine molar extinction coefficient).

#### 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 deactivator 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

The effect of pH on the activity was evaluated by measuring pure enzyme activity at pH 6–10, using 0.1 mM SAAPNA at 25 °C as the substrate, with the following buffers: 100 mM citrate–NaOH pH 6, 10 mM $CaCl_2$; 100 mM Tris–HCl pH 7–9, 10 mM $CaCl_2$; and 100 mM glycine–NaOH pH 10, 10 mM $CaCl_2$. Enzyme activity was measured according to Tsai et al. (1986) and García-Carreño and Haard (1993).

#### 2.4.6. pH stability

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 at 25 °C using 0.1 mM SAAPNA in 100 mM Tris–HCl buffer pH 8.0, 10 mM $CaCl_2$ as a substrate according to Tsai et al. (1986).

#### 2.4.7. Optimum temperature and thermostability

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 60 °C. Pure chymotrypsin temperature stability was evaluated by incubating chymotrypsin at various temperatures from 30 to 55 °C for 60 min and measuring residual activity every 15 min at 25 °C using 0.1 mM SAAPNA as substrate.

#### 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 0.8 and 0.005 mM. $K_m$ and $V_{max}$ were evaluated by nonlinear regression analysis after plotting velocity against substrate concentration, using the Prism 2 computer program (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

Chymotrypsin purification from the Monterey sardine viscera, based on hydrolysis of SAAPNA, is summarized in Table 1 and Fig. 1. Gel filtration chromatography was effective in the separation of high molecular weight proteins. Subsequently, using ionic exchange chromatography in a DEAE-Sepharose column, chymotrypsin was efficiently isolated. The results of ionic exchange chromatography are shown in Fig. 1. From fractions 1–40, corresponding to the "wash" with equilibration buffer, scarce protein was detected in fractions 5–13, with trypsin and chymotrypsin activity detected in fraction 10. A linear gradient of NaCl was applied to fractions 41 and higher, with most proteins eluted from fractions 55–73, which showed trypsin and chymotrypsin activity in fractions 55–65, while fractions 69–73 had low protein but high specific chymotrypsin activity, and no trypsin activity. Electrophoresis showed that fractions 70–72 had a single broad band that seems to be two joint bands. Fractions 70–72 were mixed and the broad band separated by preparative electrophoresis, yielding two fractions, one of them presented two bands on SDS–PAGE and the other one had only one sharp band, characteristic of a single protein that also showed specific SAAPNA activity (Fig. 2). The latter fraction was designated fraction 71 and after that, purity of chymotrypsin was confirmed by isoelectric focusing, where a single band was obtained as evidence for the homogeneity of chymotrypsin (Fig. 3). The pure chymotrypsin fraction was frozen immediately, lyophilized, and stored at −80 °C, until characterization. The other detected band had the same electrophoretic pattern and chymotrypsin specific activity, suggesting that it may be an isoform of the sardine chymotrypsin characterized here. This appears to agreement with data presented in studies on chymotrypsin in rainbow trout and Atlantic cod, which revealed the presence of two isoforms of chymotrypsin (Asgeirsson & Bjarnason, 1991; Kristjansson & Nielsen, 1992; Raae & Walther, 1989).

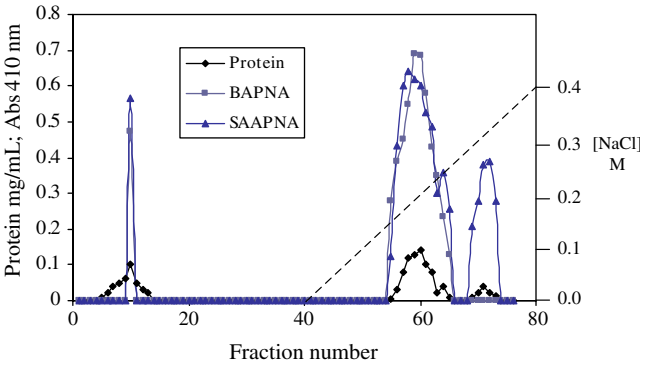


Fig. 1. Ion-exchange chromatography. Equilibration buffer 20 mM Tris–HCl, pH 7.5. Elution with linear gradient with NaCl concentration increment from 0 to 0.4 M in equilibration buffer (fractions 41–80). Protein content is expressed in $Abs_{280\,nm}$ and activity in terms of $\Delta Abs_{410\,nm}$/10 min.

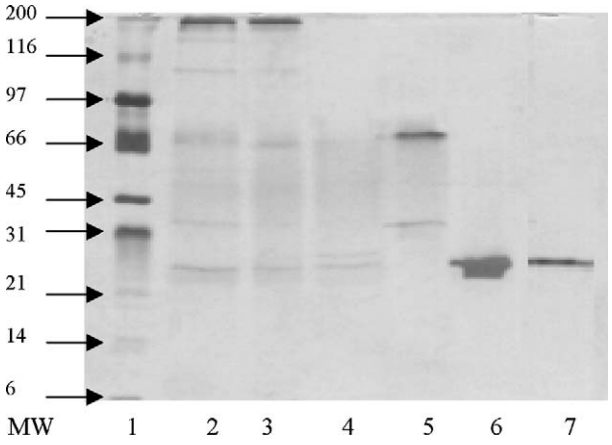


Fig. 2. SDS–polyacrylamide gel electrophoresis (PAGE): (Lane 1) MWM; (Lane 2) crude extract; (Lane 3) ammonium sulfate fraction; (Lane 4) gel filtration chromatography fraction; (Lane 5) fraction 60 from ion exchange chromatography; (Lane 6) fractions 70–72 from ion exchange chromatography; (Lane 7) fraction 71 from preparative electrophoresis.

Table 1
Summary for Monterey sardine chymotrypsin purification

| Fraction | Total volume (ml) | Total protein (mg) | Protein (mg/ml) | Total act. (U)[a] | Sp. act. (U/mg) | Yield (%) | Purif. (fold) |
|---|---|---|---|---|---|---|---|
| Crude extract | 210 | 157.5 | 0.75 | 945 | 6 | 100 | 1.0 |
| AS[b] fraction | 35 | 54.2 | 1.5 | 542 | 10 | 57 | 1.6 |
| Gel filtration (13–19) | 25 | 11.0 | 0.4 | 198 | 18 | 21 | 3.0 |
| Ionic exchange (71) | 5 | 0.24 | 0.08 | 10.3 | 43 | 1.1 | 7.1 |

[a] U, μmol of nitroaniline released per minute.
[b] AS, ammonium sulfate.

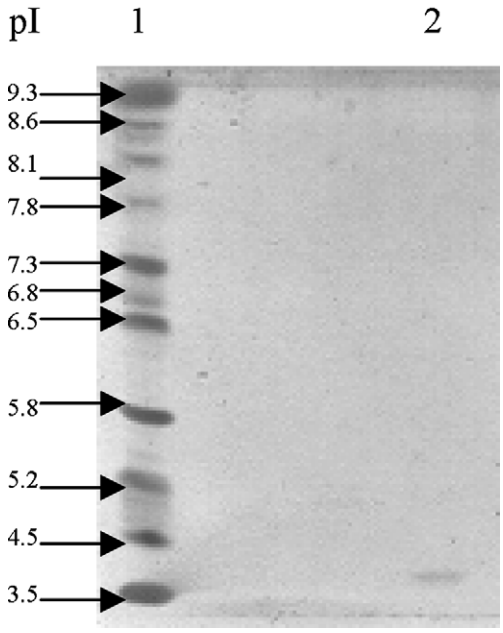


Fig. 3. Isoelectrofocusing of chymotrypsin: (Lane 1) protein markers; (Lane 2) ionic exchange fraction 71.

### 3.2. Biochemical characterization

#### 3.2.1. Molecular weight

The molecular weight of chymotrypsin was estimated by SDS–PAGE as 26,000 Da (Fig. 2), which is similar to those of mammalian and fish chymotrypsin reported in the literature (22,000–30,000 Da), and very close to those reported for chymotrypsin from anchovy (*Engraulis japonica*) and Atlantic cod (*Gadus morhua*) (Asgeirsson & Bjarnason, 1991; Heu et al., 1995; Simpson, 2000).

#### 3.2.2. Isoelectric point

The results suggest that Monterey sardine chymotrypsin had a pI about 4.3 (Fig. 3), supporting the assumption that this enzyme is anionic in nature, like other fish chymotrypsins (Asgeirsson & Bjarnason, 1991; Kristjansson & Nielsen, 1992).

#### 3.2.3. Specific activity

Monterey sardine chymotrypsin showed activity on amide (SAAPNA) and ester (BTEE) specific substrates containing phenylalanine and tyrosine, respectively, but did not display any activity against trypsin substrates. This indicated that chymotrypsin was not contaminated with trypsin-like enzymes. Monterey sardine chymotrypsin showed a specific activity of 168 U/mg enzyme on the ester substrate and 52 U/mg enzyme on the amide substrate, indicating a 3.2-fold faster hydrolysis of BTEE than SAAPNA. These results are in agreement with those of chymotrypsin from anchovy, which showed an esterase specific activity 3.4-fold higher than amidase specific activity (Heu et al., 1995).

#### 3.2.4. Effect of inhibitors

The effect of several inhibitors on Monterey sardine chymotrypsin activity was determined and the results summarized in Table 2. The pattern of inhibition of these inhibitors is characteristic of the inhibition effect of other chymotrypsins, supporting the identity of this enzyme as chymotrypsin. Monterey sardine chymotrypsin was almost completely inhibited by the serine-protease inhibitors PMSF and SBTI, inhibited more than 50% by chymotrypsin specific inhibitor TPCK, and not affected by the metallo-protease inactivator EDTA or the trypsin specific inhibitor benzamidine. Similar inhibition pattern was observed in Atlantic cod, anchovy, and rainbow trout (Asgeirsson & Bjarnason, 1991; Heu et al., 1995; Kristjansson & Nielsen, 1992).

#### 3.2.5. Effects of pH on activity and stability

The optimum pH for hydrolysis of SAAPNA at 25 °C by Monterey sardine chymotrypsin activity was 8.0, showing high relative activity in the pH range from 8 to 10 (Fig. 4). Optimum pH for other fish chymotrypsins range between pH 7.5 and 9. Anchovy chymotrypsin showed an optimum pH of 8.0; chymotrypsins from less related fish were around 9.0 (Asgeirsson & Bjarnason, 1991; Heu et al., 1995; Kristjansson & Nielsen, 1992).

Table 2
Effect of inhibitors on Monterey sardine chymotrypsin activity

| Inhibitor | (mg/ml) | Residual enzyme activity (%) |
|---|---|---|
| PMSF[a] | 1.4 | 1 |
| SBTI[b] | 0.5 | 6 |
| TPCK[c] | 1.0 | 56 |
| Benzamidine | 2.0 | 103 |
| EDTA[d] | 0.25 | 98 |

[a] PMSF, phenyl-methyl-sulfonyl-fluoride.
[b] SBTI, soybean trypsin inhibitor.
[c] TPCK, toluenesulfonyl-phenylalanine chloromethyl ketone.
[d] EDTA, etilenediamine tetracetic acid.

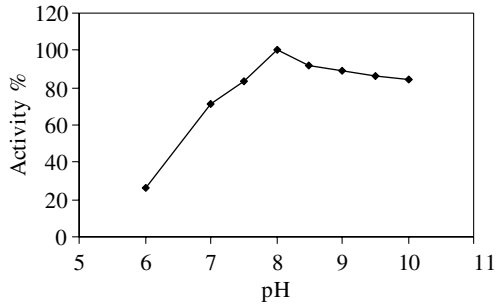


Fig. 4. Effect of pH on activity. Activity was measured in a series of buffers using 0.1 mM SAAPNA as substrate at 25 °C and varying pH from 6 to 10. Percentage of enzyme activity was estimated using the highest activity detected in this assay as 100%.

Monterey sardine chymotrypsin showed high pH stability in the range 7–8 and was unstable below pH 5 (Fig. 5). Likewise, a loss in stability below pH 5 had been observed in chymotrypsins from carp, rainbow trout and Atlantic cod (Asgeirsson & Bjarnason, 1991; Cohen et al., 1981a; Kristjansson & Nielsen, 1992). This reduced stability corresponds with a change of their net charge that occurs when the enzymes are at pH below their pI. This affects their tertiary structure. Monterey sardine chymotrypsin retained more than 80% activity over a pH range 5–10, which agrees with data reported for chymotrypsins from Atlantic cod and rainbow trout (Asgeirsson & Bjarnason, 1991; Kristjansson & Nielsen, 1992).

### 3.2.6. Effect of temperature on activity and stability

The optimum temperature for activity against SAAPNA detected in Monterey sardine chymotrypsin was about 50 °C. At temperatures above the optimum, activity quickly decreases (Table 3). The relative activity of the enzyme at 25 °C was 60% of maximum activity, indicating considerable activity is maintained even at ambient temperatures. This behavior is similar to anchovy chymotrypsin, which maintained about 50% of its maximum activity at ambient temperature but different from that of rainbow trout with maintained only 20% at the same temperature (Heu et al., 1995; Kristjansson & Nielsen, 1992).

Thermostability profile of the isolated chymotrypsin was similar to other fish chymotrypsins. The enzyme is labile at relatively high temperatures ($\geqslant 55$ °C) and stables at $\leqslant 30$ °C (Fig. 6). Chymotrypsins from anchovy, rainbow trout, carp, Atlantic cod, and dogfish are reported to be stable at $\leqslant 40$ °C and rapidly inactivated at $\geqslant 50$ °C (Asgeirsson & Bjarnason, 1991; Cohen et al., 1981a; Heu et al., 1995; Kristjansson & Nielsen, 1992; Ramakrishna et al., 1987).

### 3.2.7. Kinetic characteristics

The kinetic constants $K_m$ and $k_{cat}$ for Monterey sardine chymotrypsin, calculated from a Michaelis–Menten plot (Fig. 7), are listed in Table 4, along with the corresponding values for other fish chymotrypsins obtained from the literature. $K_m$ and $k_{cat}$ for the sardine chymotrypsin were 0.074 mM and 18.6 $s^{-1}$, respectively, and similar to those reported for anchovy chymotrypsin. In contrast, these values were far apart from those reported for carp and rainbow trout (Cohen, Gertler, & Birk,

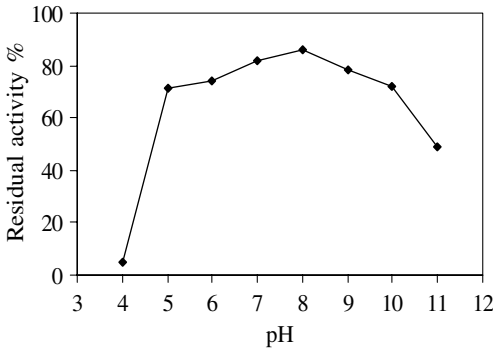


Fig. 5. pH stability. Residual activity was measured after incubation of enzyme extracts with substrate solution pH varying from 4 to 11 for 60 min at 25 °C; 100% of enzyme activity is the activity of enzyme without incubation.

Table 3
Optimum temperature for activity of Monterey sardine chymotrypsin

| Temperature (°C) | Enzyme activity (%)[a] |
|---|---|
| 10 | 20 |
| 20 | 43 |
| 30 | 74 |
| 35 | 80 |
| 40 | 94 |
| 45 | 98 |
| 50 | 100 |
| 55 | 65 |
| 60 | 4 |

[a] Percentage of enzyme activity was estimated, based on the highest activity detected in this assay as 100%.

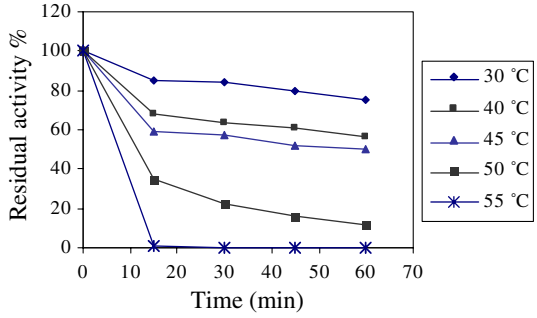


Fig. 6. Temperature stability. Residual activity at pH 8 after incubation of enzyme extract with 0.1 mM SAAPNA for 60 min at temperatures from 30 to 55 °C. Percentage of enzyme activity is based on the activity of enzyme without incubation as 100%.

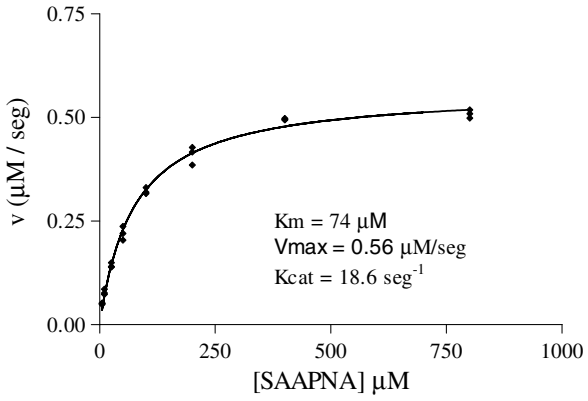


Fig. 7. Michaelis–Menten plot for chymotrypsin kinetics. SAAPNA concentrations (0.8–0.005 mM); enzyme concentration: 0.03 µM; buffer: 50 mM Tris–HCl pH 8.0 + $CaCl_2$ 10 mM; 30 °C; ($y = V_{max}[x]/K_m + [x]$; $R^2 = 0.99$).

Table 4
Kinetic constants from Monterey sardine chymotrypsin and other fish chymotrypsins[A]

| Chymotrypsin | $K_m$ (mM) | $k_{cat}$ ($seg^{-1}$) | $k_{cat}/K_m$ ($seg^{-1}$ $mM^{-1}$) |
|---|---|---|---|
| Monterey sardine (*S. sagax c.*) | 0.074 | 18.6 | 251 |
| Anchovy (*E. japonica*)[a] | 0.089 | 14.7 | 165 |
| Rainbow trout (*O. mykiss*)[b] | 0.035 | 2.2 | 62.8 |
| Carp (*C. carpio*)[c] | 0.300 | 4.4 | 15 |

[A] *Substrate*. Succinyl-L-ala-ala-pro-L-phenilalanine-*p*-nitroanilide (SAAPNA).
[a] Heu et al. (1995).
[b] Kristjansson and Nielsen (1992).
[c] Cohen et al., 1981a, 1981b.

1981b; Heu et al., 1995; Kristjansson & Nielsen, 1992). The catalytic efficiency ($k_{cat}/K_m$) of Monterey sardine chymotrypsin (251 $s^{-1}$ $mM^{-1}$) was higher than other previously cited fish chymotrypsins (Table 4) (Cohen et al., 1981b; Heu et al., 1995; Kristjansson & Nielsen, 1992).

In summary, this study showed that Monterey sardine chymotrypsin is an efficient catalyst. Further research is needed to determine its properties as a possible biotechnological tool in food processing.

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