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Isolation and characterization of trypsin from pyloric caeca of Monterey sardine *Sardinops sagax caerulea*

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Abstract

Trypsin from pyloric caeca of Monterey sardine was purified by fractionation with ammonium sulfate, gel filtration, affinity and ionic exchange chromatography. Fraction 102, obtained from ionic exchange chromatography, generated one band in sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and isoelectric focusing. The molecular mass of the isolated trypsin was 25 kDa and showed esterase-specific activity on *N*α-*p*-tosyl-L-arginine methyl ester (TAME) that was 4.5 times greater than amidase-specific activity on *N*-benzoyl-L-arginine-*p*-nitroanilide. The purified enzyme was partially inhibited by the serine-protease phenyl-methyl-sulfonyl fluoride (PMSF) inhibitor and fully inhibited by the soybean trypsin inhibitor (SBTI) and benzamidine, but was not inhibited by the metallo-protease inactivator EDTA or the chymotrypsin inhibitor tosyl-L-phenylalanine chloromethyl-ketone. The optimum pH for activity was 8.0 and maximum stability was observed between pH 7 and 8. A marked loss in stability was observed below pH 4 and above pH 11. Activity was optimum at 50 °C and lost activity at higher temperatures. The kinetic trypsin constants K_m and k_{cat} were 0.051 mM and 2.12 s^{-1}, respectively, while the catalytic efficiency (k_{cat}/K_m) was 41 s^{-1} mM^{-1}. General characteristics of the Monterey sardine trypsin resemble those of trypsins from other fish, especially trypsins from the anchovy *Engraulis japonica* and *Engraulis encrasicholus* and the sardine *Sardinops melanostica*.

Keywords: Characterization; Chromatography; Electrophoresis; Monterey sardine; Purification; Pyloric caeca; Trypsin; Viscera

1. Introduction

The main fishing industry in Mexico is the Monterey sardine, yielding about 300,000 metric tons annually (SAGARPA, 2003). It is processed as fish meal (62.5%), canned (30%) and frozen (7.5%) (Ornelas-Días, 2001). This processing generates wastes, mainly stick-water, viscera, heads and processing effluents, which are not treated and have no value. They are discarded directly to the sea, causing pollution (Doode, 1996). This contamination is an acute problem in Mexico, so alternatives, including commercial uses for the by-products and waste are urgently needed. Biotechnology provides a means for transforming such materials into valuable products, such as enzymes.

Fish viscera, accounting for 5% of total mass, have wide biotechnological potential as a source of digestive enzymes, especially digestive proteases that have high activity over a wide temperature range (Gildberg, 1992; Simpson and Haard, 1987; Martinez and Serra, 1989; Cancre et al., 1999). Detailed research on the biochemical characteristics from the main digestive enzymes of viscera of a wide variety of fish is still needed for advancing commercial use.

The Monterey sardine is a small pelagic teleost susceptible to abdominal autolytic degradation after death.

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Deterioration is very rapid and is carried out mainly by proteolytic enzymes in the digestive tract, which suggests that sardine viscera might be a good source of these enzymes (Martínez and Gildberg, 1988). Several studies on digestive enzymes in a variety of fish, like *Engraulis japonica*, *Brevoortia tyranus* and *Katsuwonus vagans*, have revealed that serine proteases, enzymes distributed widely in fish intestines, are responsible for autolytic degradation of abdominal tissue (Heu et al., 1995; Pyeun et al., 1988, 1990). Fish digestive enzymes in viscera might have important industrial applications because they are available in significant quantities and possess high activity, especially in the sardine industry. Recovered enzyme extracts could be used in management of fishery wastes, such as stick-water.

The most important digestive proteases of fish viscera are acid stomach enzymes and alkaline intestine enzymes. The main alkaline enzymes in fish viscera are trypsin, chymotrypsin and elastase, all belonging to the serine-protease family (E.C. 3.1.21.x). They are characterized by serine, histidine and aspartic residues at the active site. As a group, serine proteases are inhibited by di-isopropyl phosphofluoride (DFP), are active at neutral and alkaline pH, and inactive or unstable in acid pH (Simpson, 2000).

Trypsins are present in fish as isoenzymes and all have essentially the same specificity. They cleave the peptide bond on the carboxyl side of arginine and lysine, and have endopeptidase activity and molecular weight ranging from 22 to 28 kDa. They hydrolyze synthetic substrates like *N*α-benzoyl-L-arginine-*p*-nitroanilide and tosyl-arginine methyl ester and are susceptible to the serine-protease inhibitors phenyl-methyl-sulfonyl fluoride (PMSF), soybean trypsin inhibitor (SBTI) and aprotonin. They are optimally active at pH 7.5–10 and 35–45 °C. They are unstable at lower temperatures and extreme pH (Whitaker, 1994).

The main objectives of the research were to extract and purify trypsin from Monterey sardine viscera and generate basic information about its main biochemical and kinetic characteristics that might contribute to the commercial application of this by-product.

2. Materials and methods

2.1. Reagents

Casein, PMSF, N-tosyl-L-phenylalanine chloromethyl ketone (TPCK), benzamidine, SBTI, ethylenediamine tetraacetic acid (EDTA), *N*α-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA), *N*α-*p*-tosyl-L-arginine methyl ester (TAME), glycin, citric acid, Tris buffer, trichloroacetic acid (TCA), ammonium sulphate and molecular weight markers (14,000–66,000) were purchased from Sigma. Sodium dodecyl sulphate (SDS), acrylamide, ammonium persulphate (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, benzamidine-Sepharose 4 fast flow, Sephadex G-75 and dialysis tubing were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). All reagents were of analytical grade.

2.2. Samples

Monterey sardine *Sardinops sagax caerulea* specimens were provided by Productos Pesqueros de Guaymas (Guaymas, Mexico). Sardines were collected within 6 h of death from a fishing vessel's storage vault, where they were stored at 8 °C. Samples were placed between layers of crushed iced and transported to the CIAD Seafood Products Laboratory in Hermosillo, Mexico. The viscera were extracted at low temperature, placed in sealed polyethylene bags, immediately frozen and kept at −80 °C.

2.3. Preparation of enzyme extract

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).

2.4. Purification procedure

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 and Rydén, 1998) and loaded into a 1×80 cm Sephadex G-75 gel filtration chromatography column (Amersham Pharmacia Biotech). Buffer A was used as a mobile phase at 0.25 ml/min flow rate and 5-ml fractions were collected. Fractions with trypsin and chymotrypsin activity were pooled and loaded into a benzamidine-Sepharose 4B column (1.1×10 cm) equilibrated with buffer A. Retained trypsins were eluted by changing the mobile phase pH from 7.3 to 3.0, using a 50 mM Gly·HCl, pH 3.0 buffer. Fractions with trypsin activity were combined and dialyzed against 6-l 20 mM Tris·HCl, pH 7.5 buffer (García-Carreño and Haard, 1993; Cohen et al., 1981; Simpson and Haard, 1984).

Dialyzed fractions were loaded into a DEAE-Sepharose column (1.6×20 cm) (Amersham Pharmacia Biotech) 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 500-ml linear gradient ranging from 0 to 0.4 M NaCl (García-Carreño and Haard, 1993; Cohen et al., 1981; Amersham Pharmacia Biotech, 1999).

Protein concentration was evaluated using $Abs_{280\ nm}$ and the method of Bradford (1976). Trypsin- and chymotrypsin-

Table 1
Summary of Monterey sardine trypsin purification

Fraction	Total vol. (ml)	Total protein (mg)	Protein (mg/ml)	Total act. (U)[a]	Sp. act. (U/mg)	Yield (%)	Purif. fold
Crude extract	250	217.5	0.87	108.7	0.5	100	1.0
AS fraction[b]	32	121.6	3.8	79.0	0.65	73	1.3
Gel filtration	40	44.8	1.12	76.0	1.7	70	3.4
Affinity	8	8.0	1.0	19.2	2.4	18	4.8
Ionic exchange	5	1.0	0.2	6.5	6.5	6	13.0

[a] U=micromoles of nitroaniline released per minute.
[b] AS=ammonium sulphate.

specific activities in the eluted fractions were also evaluated using specific substrates according to Erlanger et al. (1961) and Tsai et al. (1986). Electrophoretic patterns obtained for fractions were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and proteolytic activity in gel was evaluated by the substrate gel electrophoresis (Laemmli, 1970; García-Carreño et al., 1993).

2.5. Trypsin characterization

2.5.1. Electrophoresis

Gels of 14% polyacrylamide with 0.1% SDS were used to investigate trypsin 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.

2.5.2. Electrofocusing

Isoelectric point of isolated enzyme was evaluated by analytical electrofocusing in thin-layer polyacrylamide flat gel (LKB ampholyne PAG plate) containing ampholynes in the range pH 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 et al. (1990).

2.5.3. Specific activity

The amidase activity of isolated trypsin was evaluated according to Erlanger et al. (1961), using BAPNA as substrate with slight modifications: 10 µl enzyme solution were mixed with 990 µl 1 mM BAPNA 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. BAPNA hydrolysis units (*U*) were calculated with the following equation: $U=A(410)/\text{min}\times1000\times1/8800\times\text{mg}$ enzyme, where 8800 is the *p*-nitroaniline molar extinction coefficient. Esterase activity was evaluated according to Hummel (1959) using TAME as substrate. Briefly, 20 µl enzyme solution was mixed with 980 µl 1 mM TAME in 50 mM Tris·HCl pH 8.0, 10 mM $CaCl_2$ buffer at 25 °C. Production of *p*-tosyl-arginine was measured by monitoring the increment in $Abs_{247\ nm}$ every 30 s for 10 min. TAME units (*U*) were calculated with the following equation: $U=A(247)/\text{min}\times1000\times1/540\times\text{mg}$ enzyme, where 540 is the *p*-tosyl-arginine molar extinction coefficient.

2.5.4. 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, such as the serine-protease inhibitors PMSF and SBTI, the chymotrypsin-specific inhibitor TPCK, the 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 (1-mM BAPNA 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, considering activity in the absence of inhibitor as 100%.

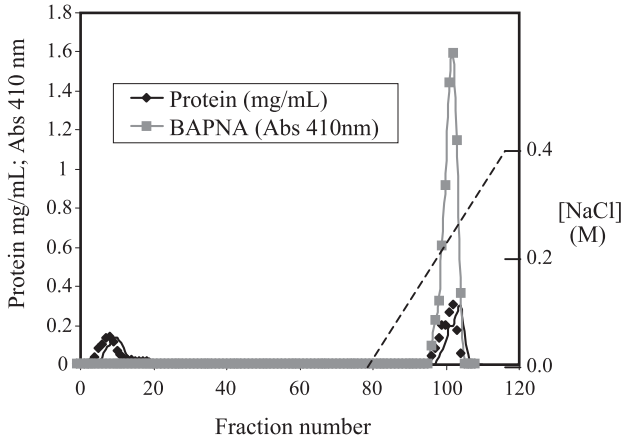

Fig. 1. Ion exchange chromatography. Equilibration buffer 20 mM Tris HCl, pH 7.5. Elution with linear gradient with NaCl concentration increment from 0 to 0.4 M in equilibration buffer (fractions 80–110). Protein content is expressed in Abs 280 nm and activity in terms of ΔAbs 410 nm/10 min.

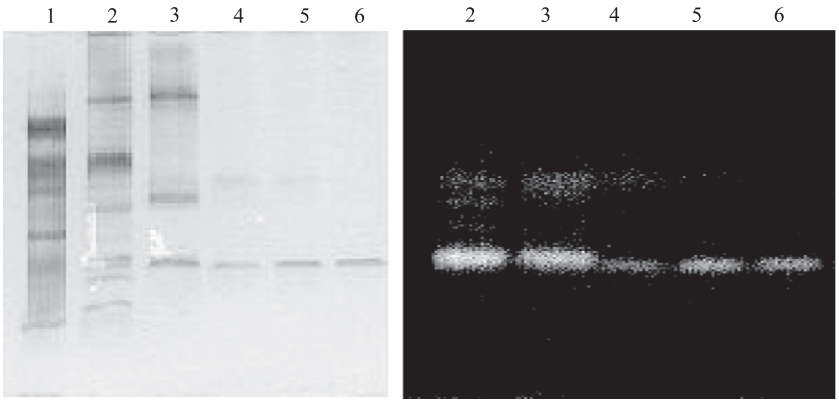

Fig. 2. SDS-PAGE and substrate gel SDS-PAGE. Lane 1 MWM; lane 2 ammonium sulphate fraction; lane 3 affinity chromatography fraction; lanes 4–6: fractions 100, 101 and 102 from ion exchange chromatography.

2.5.5. Optimum pH

The effect of pH on the activity was evaluated by measuring pure enzyme activity at various pH's, using 1 mM BAPNA at 25 °C as the substrate. pH was varied from 6 to 10 using 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 Erlanger et al. (1961) and García-Carreño and Haard (1993).

2.5.6. pH stability

The effect of pH on enzyme stability was evaluated by measuring enzyme residual activity after incubation at various pH's for 60 min at 25 °C. The pH of the enzyme was varied in the range 4–11 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 1 mM BAPNA in 100 mM Tris · HCl buffer pH 8.0, 10 mM $CaCl_2$ as a substrate according to Erlanger et al. (1961).

2.5.7. Optimum temperature and thermostability

Optimum temperature of pure trypsin activity was measured at pH 8.0, using 1 mM BAPNA in 100 mM Tris · HCl buffer pH 8.0, 10 mM $CaCl_2$ as substrate and varying temperature in the range 10–60 °C. Pure trypsin temperature stability was evaluated by incubation at various temperatures from 30 to 55 °C for 60 min and measuring residual activity every 15 min at 25 °C using 1 mM BAPNA as substrate.

2.5.8. 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 BAPNA substrate concentration between 1 and 0.01 mM. K_m and V_{max} were evaluated by non-linear regression analysis after plotting velocity against substrate concentration, using the Prism 4 computer program (Graph Pad Software, 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 (Heu et al., 1995; Copeland, 2000).

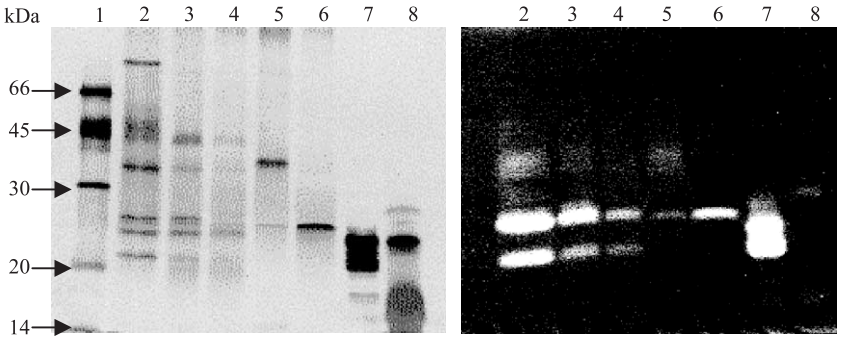

Fig. 3. SDS-PAGE and substrate gel SDS-PAGE. Lane 1 MWM; lane 2 crude extract; lane 3 ammonium sulphate fraction; lane 4 gel filtration fraction; lane 5 affinity chromatography fraction; lane 6 ionic exchange fraction (102); lane 7 porcine trypsin; lane 8 bovine chymotrypsin.

3. Results and discussion

3.1. Purification of trypsin

Results of the purification of trypsin extracted from viscera of the Monterey sardine are summarized in Table 1. Size exclusion chromatography separated the high molecular weight, low trypsin-specific activity (BAPNA) protein group, from low molecular weight, high trypsin-specific activity protein group. Fractions with high activities between 16 and 25 were combined for affinity chromatography.

Affinity chromatography produced 98 fractions; protein was detected in fractions 4–16, but showed no trypsin-specific activity. However, during elution based on pH change, fractions 85 and 86 showed protein concentrations of 3.09 and 0.18 mg/ml, respectively, as well as high trypsin-specific activity. Chymotrypsin activity was also detected in these two fractions (Table 1), which were then selected for further purification.

Results of ionic exchange chromatography are shown in Fig. 1. From fractions 1 to 80, corresponding to the "wash" with equilibration buffer, scarce protein with no enzymatic activity was detected in fractions 7–14. A linear gradient of NaCl was applied to fractions 81 and up, with most protein eluted from fractions 98 to 106, all of them showing BAPNA trypsin activity. Electrophoresis showed that fraction 102 generated only one band, with proteolytic activity revealed by substrate-gel electrophoresis (Fig. 2). Based on its specific activity and on the single band on the protein gel and the zymogram, this fraction contained pure trypsin. All fractions with activity were frozen immediately, lyophilized and stored at −80 °C, then fraction 102 was used for the characterization analysis. A summary of trypsin purification is shown in Fig. 3 and Table 1.

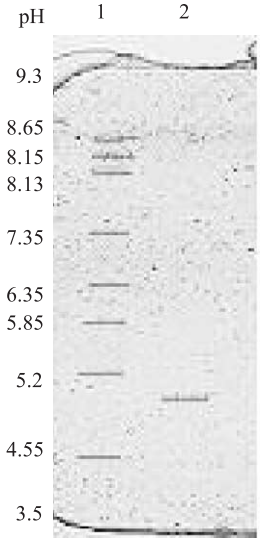

Fig. 4. Isoelectrofocusing of trypsin. Lane 1 protein markers; lane 2 ionic exchange fraction 102.

Table 2
Effect of inhibitors on trypsin activity

Inhibitor	Conc. (mg/ml)	Residual enzyme activity (%)
PMSF	1.4	57
TPCK	1.0	105
SBTI	0.5	0
Benzamidine	2.0	28
EDTA	0.25	86

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

3.2. Biochemical characterization

3.2.1. Molecular mass

The molecular mass of isolated trypsin was 25,000 Da, which is in the range of 22,000–28,000 Da for trypsins purified from other fish species (Fig. 3) (Simpson, 2000).

3.2.2. Isoelectric point

A single band observed during isoelectric point analysis confirmed fraction 102 trypsin purity (Fig. 4). The isoelectric point of isolated trypsin was approximately 5, which indicated that this enzyme is an anionic protein at neutral pH. Anionic trypsins are common in marine organisms such as capelin *Mallotus villosus*, salmon *Oncorhynchus keta*, sardine *Sardinops melanostica* and anchovy *Engraulis encrasicholus*, while mammal trypsins are generally cationic (Heu et al., 1995; Martinez et al., 1988; Murakami and Noda, 1981; Sekizaki et al., 2000).

3.2.3. Specific activity

Amidase (BAPNA)- and esterase (TAME)-specific activities in the pure trypsin were evaluated. Monterey sardine trypsin showed specific activity 29 U/mg enzyme on the

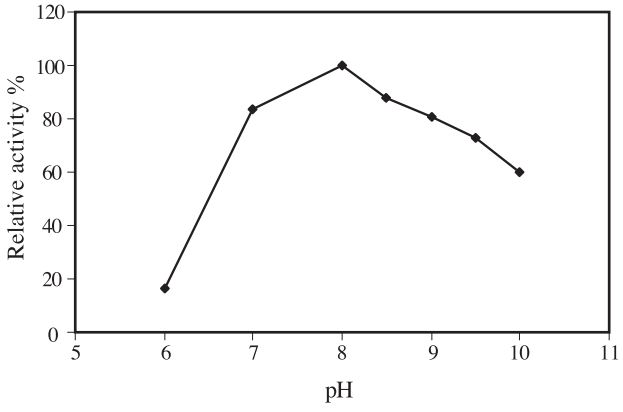

Fig. 5. Effect of pH on activity. Activity was measured in a series of buffers using BAPNA 1 mM as substrate at 25 °C and varying pH from 6 to 10. Percentage of enzyme activity was estimated considering 100% the highest activity detected in this assay.

ester substrate, and 6.5 U/mg enzyme on the amide substrate, indicating 4.5 times faster hydrolysis of TAME than BAPNA. These results are in agreement with trypsin from anchovy *E. encrasicholus* and *E. japonica*, which showed esterase-specific activity 27 and 7 times higher than amidase-specific activity, respectively (Heu et al., 1995; Martinez et al., 1988).

3.2.4. Effect of inhibitors

Inhibition of pure trypsin by specific inhibitors for trypsin SBTI and benzamidine was complete and almost complete, respectively (Table 2). Activity was inhibited almost 50% by the serine-protease inhibitor PMSF, but not affected by the metallo-protease inactivator EDTA or the chymotrypsin-specific inhibitor TPCK. These results confirm that the single band detected by SDS-PAGE and isoelectric focusing corresponds to a serine-protease, specifically trypsin. The inhibition pattern of trypsin from Monterey sardine was similar to trypsins from other fish species, such as anchovy *E. encrasicholus* and menhaden *B. tyranus* (Martinez et al., 1988; Pyeun et al., 1990).

3.2.5. Optimum pH for activity

The optimum pH for sardine trypsin activity was 8.0, but high relative activity was observed at alkaline pH for the hydrolysis of BAPNA at 25 °C (Fig. 5). The optimum pH for this enzyme falls in the range from 7.5 to 10, which is generally optimum for digestive enzyme activity of fish. Optimum pH between 8 and 10 has been reported for enzyme activities of species related to Monterey sardine, such as anchovy *E. encrasicholus* and *E. japonica* and sardine *S. melanostica*, showing a general profile of alkaline activity (Heu et al., 1995; Martinez et al., 1988; Murakami and Noda, 1981).

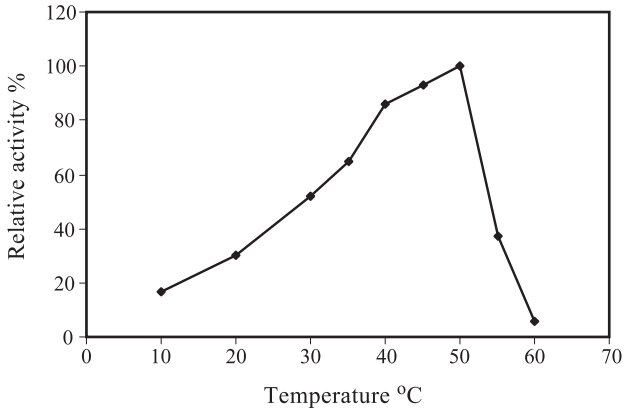

Fig. 7. Optimum temperature. Activity at pH 8 (50 mM Tris·HCl pH 8.0+$CaCl_2$ 10 mM) was evaluated using 1 mM BAPNA as substrate and changing temperature from 10 to 60 °C. Percentage of enzyme activity was estimated based on the highest activity detected in this assay as 100%.

3.2.6. pH stability

Sardine trypsin was stable in the pH range from 7 to 8, with approximately 50% loss of activity at pH 4 and 11 (Fig. 6). In general, trypsin stability is related to protein net charge at a particular pH. Accordingly, fish trypsins are unstable at acid or extremely alkaline pH (Murakami and Noda, 1981).

3.2.7. Optimum temperature

The optimum temperature for Monterey sardine trypsin activity under experimental conditions was 50 °C (Fig. 7), which is higher than the optimum temperature for trypsins from other fish species such as cod *Gadus ogac*, capelin *M. villosus*, anchovy *E. encrasicholus* and *E. japonica*, and sardine *S. melanostica*, which have optimum activity at temperatures between 40 and 45 °C (Simpson and Haard, 1984; Martinez et al., 1988; Murakami and Noda, 1981).

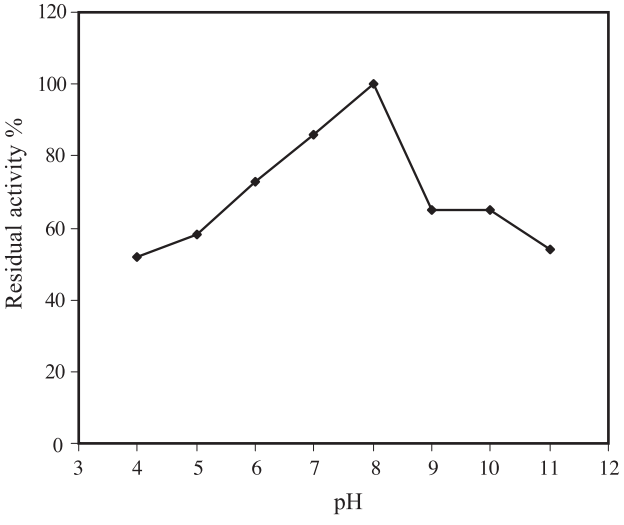

Fig. 6. pH stability. Residual activity was measured after incubation of enzyme extracts, with substrate solution pH varying from 4 to 11 for 60 min at 25 °C; 100% of enzyme activity is the activity of enzyme without incubation.

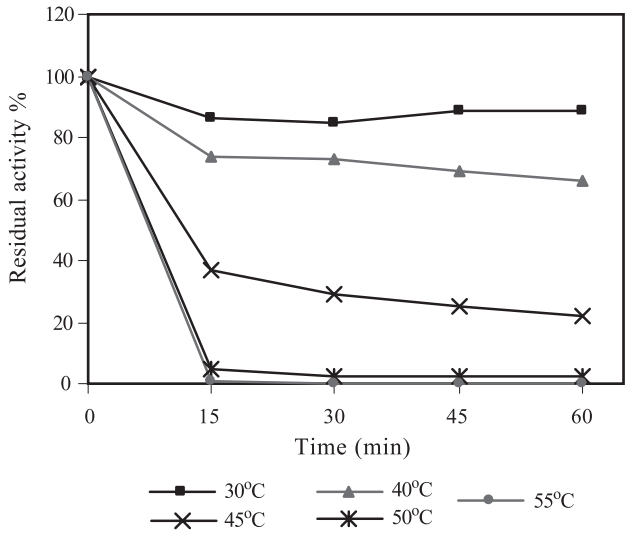

Fig. 8. Temperature stability. Residual activity at pH 8 after incubation of enzyme extract with 1 mM BAPNA for 60 min at temperatures from 30 to 55 °C. Percentage of enzyme activity is based on the activity of enzyme without incubation as 100%.

The difference may be related to the cold water areas inhabited by these fish. Monterey sardine is a temperate water species, so its trypsin should be compared to trypsin from mullet *Mugil cephalus*, a tropical warm water species with optimal activity temperature of 60 °C (Guizani et al., 1991). At 25 °C, activity of isolated trypsin was approximately 40% of the maximum, which is substantially greater than the 10% reported for anchovy *E. japonica* (Heu et al., 1995).

3.2.8. Temperature stability

Thermostability of this enzyme is similar to trypsin of other fish. It is thermolabile at ≥50 °C, stable at ≤30 °C and

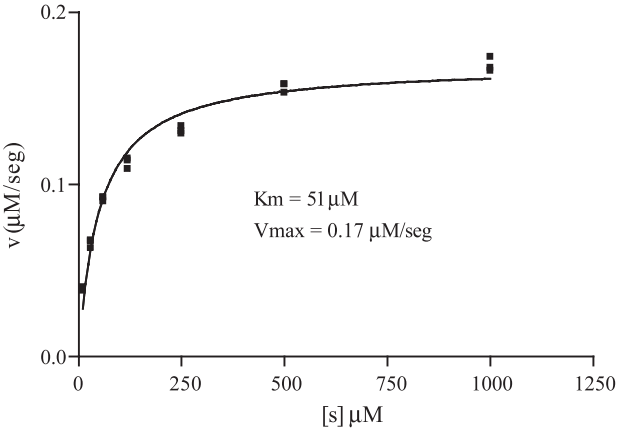

Fig. 9. Michaelis–Menten plot for trypsin kinetics. BAPNA concentrations (1–0.01 mM); enzyme concentration: 0.08 µM; buffer: 50 mM Tris · HCl pH 8.0+$CaCl_2$ 10 mM; 30 °C ($y=V_{max}[x]/K_m+[x]$); R^2=0.97.

Table 3
Kinetic constants from Monterey sardine trypsin and other fish trypsins

Trypsin	K_m (mM)	k_{cat} (seg^{-1})	k_{cat}/K_m (seg^{-1} mM^{-1})
Monterey sardine (*S. sagax c.*)*	0.051	2.12	41.0
Anchovy (*E. japonica*)[a,*]	0.049	1.55	31.0
Salmon (*O. keta*)[b,*]	0.029	2.29	79.0
Carp (*C. carpio*)[c,*]	0.039	3.10	79.5
Mullet (*M. cephalus*)[d,*]	0.490		
Anchovy (*E. encrasicholus*) "A"[e,*]	0.830	1.55	1.86
Anchovy (*E. encrasicholus*) "B"[e,*]	0.660	3.2	4.84

[a] Heu et al. (1995).
[b] Sekizaki et al. (2000).
[c] Cohen et al. (1981).
[d] Guizani et al. (1991).
[e] Martinez et al. (1988)
* Substrate: Nα-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA).

sensitive at 45 °C (Fig. 8). Specifically, Monterey sardine trypsin is similar in this characteristic to those of other cold water fish, that is, it is very stable at temperatures lower than 45 °C and increasingly inhibited above 55 °C (Heu et al., 1995; Martinez et al., 1988; Murakami and Noda, 1981).

3.2.9. Kinetic characteristics

Kinetic constants K_m and k_{cat} for trypsin in Monterey sardine viscera were calculated from a Michaelis–Menten plot (Fig. 9) and shown in Table 3, along with the corresponding values for other fish trypsins. K_m and k_{cat} for the pure trypsin were 0.051 mM and 2.12 s^{-1}, respectively, and were close to those reported for trypsins from anchovy *E. japonica* and salmon *O. keta*. In contrast, these values were far from those reported for mullet *M. cephalus* and Atlantic cod *Gadus morhua* (Arnt and Walther, 1989; Simpson and Haard, 1984; Guizani et al., 1991; Heu et al., 1995; Sekizaki et al., 2000). The catalytic efficiency (k_{cat}/K_m) of Monterey sardine trypsin, 41 s^{-1} mM^{-1}, was close to trypsin from anchovy *E. japonica* (31 s^{-1} mM^{-1}) and lower than trypsin in carp and salmon (Table 3) (Cohen et al., 1981; Heu et al., 1995; Sekizaki et al., 2000). The catalytic efficiency of Monterey sardine trypsin, like those reported in the literature for other fish, is higher than mammalian trypsins (Simpson, 2000).

4. Conclusions

Based on SDS-PAGE and isoelectric focusing analyses, activities for the specific substrates BAPNA and TAME, and its susceptibility to inhibitors, the enzyme isolated from the pyloric caeca of Monterey sardine was trypsin. The enzyme was similar in many biochemical characteristics to trypsin in other fish, but was substantially different from cold water species in its optimal activity temperature. Characteristics of the isolated trypsin are interesting from a technological perspective, especially maximum activity at pH 8.0, high neutral pH activity, optimal activity temper-

ature at 50 °C and low thermostability. These characteristics suggest that the enzyme could be an important biotechnological tool for the fish processing and food industries.

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