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Characterization of acidic proteolytic enzymes from Monterey sardine (*Sardinops sagax caerulea*) viscera

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Abstract

Total enzyme activity of whole viscera, and partial characterization of acidic proteases from Monterey sardine viscera are presented. Major proteolytic activity in alkali (pH 10) and minor activity in acid (pH 3) were detected. From purified acidic proteases, six fractions with high activity were selected. One fraction (42) showed one band on SDS–PAGE and two bands on isoelectrofocusing, with pI close to 4.0 and 4.5, respectively. The optimal pH for acidic protease activity was 2.5, with high stability in the acid range and marked loss of activity at neutral and alkaline pH. The optimum temperature was 45 °C, and activity was high at 10 °C, whereas denaturation occurred above 55 °C. Activity was inhibited by Pepstatin A but not by SBTI or EDTA. The general characteristics of these enzymes resemble those of the digestive enzymes of other fish. Because Monterey sardine is abundant in Mexico, it is a potential source for biological reagent production.

Keywords: Monterey sardine; Proteases; Viscera; Acidic enzymes; Characterization

1. Introduction

Monterey sardine (*Sardinops sagax caerulea*) is the main fishery product in northwestern Mexico. The annual catch, consisting of 300,000 t, is processed into fish meal (62.5%), canned (30%) and frozen (7.5%) (SAGARPA, 2000). These processes produce wastes, mainly stick-water and viscera, which are environmental pollutants. These contaminants are becoming a serious problem for the sardine industry and it is paramount to find ways to use the wastes and reduce pollution (Doode, 1996), and to generate so-called secondary-material, in contrast to raw material. Fish viscera are a rich source of hydrolytic enzymes, and preparations of such enzymes are biotechnological tools in the food industry (Gildberg, Simpson, & Haard, 2000). Some proteases have been explored as food processing aids and as reducers of stick-water viscosity in fishmeal processing (An & Visessanguan, 2000; Jacobsen & Lykke-Rasmussen, 1984), so the use of sardine viscera for enzyme recovery might be a solution to the sardine industry problem in Mexico.

Digestive enzymes of cold-water fish have been studied most, and information on tropical fish is scarce. Biochemical characteristics of the Monterey sardine have been studied inadequately, in spite of being the main fishery product of the Gulf of California, Mexico. This work was aimed to characterize digestive proteolytic enzymes of Monterey sardine viscera to generate basic information about them. The Monterey sardine, like other small pelagic fish, is susceptible to rapid autolytic degradation of abdominal tissue after capture; this process is caused mainly by proteases from the digestive tract (Martinez & Gildberg, 1988). Studies on fish enzymes have demonstrated that digestive proteases are responsible for rapid abdominal degradation, especially acidic proteases from the stomach and alkaline proteases from the pyloric caeca, pancreas, and intestine (Heu, Kim, & Pyeun, 1995; Pyeun, Kim, & Godber, 1990; Pyeun, Kim, & Heu, 1988). This fact makes fish

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viscera a possible source of proteolytic enzymes for industrial use.

Purification techniques used to isolate enzymes from fish viscera, based on various differences in biological component extracts, include solubility (precipitation with salts, solvents, etc.), size (dialysis, size exclusion chromatography), charge (ion exchange chromatography, electrophoresis), and affinity for specific ligands (affinity chromatography) (Janson & Ryden, 1998). Fish pepsins have been purified mainly by precipitation with ammonium sulphate followed by dialysis, ion exchange chromatography, size exclusion chromatography, and electrophoresis (Simpson, 2000; Gildberg & Raa, 1983; De Vecci & Coppes, 1996).

The most important proteolytic enzymes from fish viscera are the aspartic protease pepsin, and serine proteases trypsin, chymotrypsin, and elastase (Gildberg, 1988; Martinez & Serra, 1989). Acidic proteases from fish stomachs display high activity between pH 2 and 4, while alkaline digestive proteases are most active between pH 8 and 10 (Simpson, 2000). Usually, fish synthesize two pepsins: Pepsin I and Pepsin II. Pepsin I is reported most abundant in species like sardine (*Sardinops melanostica*) and capelin (*Mallotus villosus*) (Noda & Murakami, 1981; Gildberg & Raa, 1983), while pepsin II seems to be most abundant in species like cod (*Gadus morhua*) and salmon (*Oncorhynchus keta*) (Gildberg, 1988; Sanchez-Chiang, Cisternas & Ponce, 1987). Fish pepsins have very low activity on small peptide substrates, and like other aspartic proteases, exhibit high activity on hemoglobin (Sanchez-Chiang et al., 1987; Squires, Haard & Feltham, 1986). Pepsin I hydrolyzes hemoglobin maximally between pH 3 and 4, and pepsin II between pH 2 and 3. Pepsins of cold and temperate water fish have maximal stability between pH 2 and 5, while pepsins from warm water species are stable even at pH 7 (Kubota & Ohnuma, 1970). The optimal temperature for these enzymes is between 37 and 55 °C, and they have between 40 and 60% activity from 5 to 10 °C, but lower thermostability than mammalian pepsins. Active pepsins have molecular weights close to 35 kDa (Gildberg, 1988; Gildberg & Raa, 1983). Isoelectric points (pI) of pepsin I and pepsin II are from 6.5 to 7, and 4 to 4.5, respectively. Generally, the pI's of mammalian pepsins are lower than those of fish pepsins, maybe because of the higher content of basic amino acids in fish pepsins (Herriot, 1962). Pepsins are susceptible to inhibition by Pepstatin A (Simpson, 2000), which is highly specific for this kind of enzyme (Zollner 1993). On the other hand, the aspartic proteases are unaffected by most serine, thiol, and metallo-protease inhibitors (Gildberg, 1988). This study intended to evaluate total proteolytic activity of sardine viscera, and to purify and characterize acidic proteolytic enzymes of the stomach.

2. Materials and methods

2.1. Reagents

Hemoglobin, casein, pepstatin A, soybean trypsim inhibitor (SBTI), EDTA, glycin, citric acid, tris buffer, trichloroacetic acid (TCA), ammonium sulphate and SDS–polyacrylamide gel electrophoresis (PAGE) markers for molecular weights 14,000–66,000 were purchased to Sigma Chemical Co. (St. Louis MO, USA). Sodiumdodecilsulphate (SDS), acrylamide, ammonium persulphate (APS), tetramethylethylenediamine (TEMED), Coomassie blue, from Bio-Rad Laboratories (Mexico). Sulphopropyl-sepharose (SP-Sepharose) fast flow and dialysis tubing were from Amersham Biosciences (Uppsala, Sweden). All other reagents were of analytical grade.

2.2. Sample

Samples of Monterey sardine were obtained from Productos Pesqueros de Guaymas S. A. at Guaymas, Sonora, Mexico. Fish were sexually mature, of average weight 88.4 g. Sardines were transported in ice to the laboratory within 18 h of capture and eviscerated immediately. Viscera were frozen at −20 °C until use.

2.3. Preparation of enzyme extracts

Enzyme extracts were prepared from whole viscera (100 g samples) by homogenizing for 1 min with 200 ml ice-cold distilled water, using tissue homogenizer Tizzumizer Tekman model SDT 1810 (Tecman Co. West Germany). The homogenate was centrifuged at 26,000 × *g* for 20 min at 2–4 °C. The supernatant was the enzyme extract. Extract for purification of acidic proteolytic enzymes was obtained by the same process, using 100 g of stomachs.

2.4. Total proteolytic activity

Activity was evaluated according to Garcìa-Carreño and Haard (1993) and Simpson and Haard (1987), with slight modifications as follows: Proteolytic activity was assayed at pH between 2 and 12, by using universal buffer (Stauffer, 1989, chap. 4). Hemoglobin (2%) was used as the substrate in acid pH (2–6) and casein (2%) in neutral and alkaline pH (7–12). Enzyme extract from whole viscera (20 μl) was mixed with 0.5 ml of corresponding buffer and 0.5 ml of corresponding substrate at 25 °C. The reaction was stopped 30 min later by adding 0.5 ml of 20% TCA, and centrifuged in Eppendorf test tubes for 5 min at 6500 × *g*. The supernatant was separated from the undigested substrate and absorbance at 280 nm was recorded. Assays were done in triplicate and appropriate blanks were included.

Proteolytic activity was expressed as Δ absorbance (280 nm) in 30 min per mg protein in enzyme extract under assay conditions.

2.5. Purification of acidic proteolytic enzymes

Stomach enzyme extract was mixed with ammonium sulphate, and precipitate of 20–70% saturation was collected and dissolved in 50 ml of 10 mM citrate/HCl buffer pH 3.0. After dialysis for 24 h and four changes of 3 l of the same buffer, the sample was adjusted to pH 3.0 by addition of 0.1 M HCl, and centrifuged at 20,000×*g* for 20 min. Then, it was applied to a cation-exchange column (Gildberg, Olsen, & Bjarnason, 1990). Ion exchange chromatography was performed on a prepacked column of SP-Sepharose fast flow (Amersham Pharmacia Biotech) previously equilibrated with 10 mM citrate/HCl pH 3.0 buffer (equilibration buffer). After loading the sample, the column was washed with equilibration buffer. Protein was eluted in two steps: by stepwise increments of 0.5 pH units up to pH 5.0, then by stepwise increments in NaCl concentration from 0 to 1 M. The elution rate was 0.4 ml/min, and 0.5 ml fractions were collected (Fig. 2).

2.6. Characterization of acidic proteolytic enzymes

2.6.1. Electrophoresis

To investigate the purity of acidic enzymes, 12% poliacrylamide gels with 0.1% SDS (SDS–PAGE) at pH 7.5 were used (Laemmli, 1970). Electrophoresis assays were run at pH 8.3 and 2–5 °C. Substrate electrophoresis was also used to evaluate gel activity (Garcia-Carreño, Dimes, & Haard, 1993). Molecular weight markers used were proteins from 14,000 to 66,000 Da, as follows: Bovine serum albumin 66,000 Da; ovoalbumin 45,000 Da; gliceraldehyde-3-phosphate dehydrogenase 36,000 Da; carbonic anhidrase 29,000 Da; trypsinogen 24,000 Da; trypsin inhibitor 20,000 Da and α-lactoalbumin 14,200 Da.

2.6.2. Acidic proteolytic activity

Protease activity was evaluated according to Diaz-Lopez, Moyano-López, Alarcón-López, García-Carreño, and Navarrete-Del Toro (1998) using hemoglobin (Hb) as the substrate, as follows: 1 ml of 0.5% Hb in 0.1 M glycine/HCl pH 3.0 buffer was mixed with 20 μl of enzyme extract, and the reaction mixture was incubated for 20 min at 25 °C. The reaction was stopped by adding 0.5 ml of 20% TCA. The TCA precipitate was chilled for 30 min at 4 °C, and then the absorbance of TCA soluble peptides at 280 nm was recorded in a UV–vis spectrophotometer. One unit of activity was defined as the amount of enzyme catalyzing the formation of 1 μg of tyrosine from hemoglobin per minute under assay conditions calculated as $\Delta Abs._{280/min} \times 1000 \times 1.52$ divided by 0.051 (where 1.52 is the total reaction volume and 0.051 is the extinction coefficient of tyrosine). Porcine pepsin was used as control.

2.6.3. Effect of inhibitors

Inhibitor susceptibility was measured according to Garcia-Carreño and Haard (1993). Enzyme extracts were incubated with different specific protease inhibitors such as the aspartic protease inhibitor (Pepstatin A), serine-protease inhibitor (soybean trypsin inhibitor, SBTI), and metallo-protease deactivator (EDTA). Five microliters of inhibitor solution was mixed with 10 μl of enzyme extract and incubated for 60 min at 25 °C, and then 1 ml substrate solution (0.5% Hb in Gly–HCl buffer) was added and remaining activity was measured. Appropriate blanks and inhibitor solvents were used as controls; percentage activity in inhibition assays was reported, considering 100% activity in the absence of inhibitor.

2.6.4. Electrofocusing

Isoelectric points of isolated enzymes were evaluated by analytical electrofocusing in thin layer polyacrylamide flat gel (LKB ampholyne PAG plate) containing ampholyne over pH range 3.5–9.5. An isoelectric focusing calibration kit (Amersham Pharmacia Biotech), containing 11 proteins of known isoelectric points was used as a reference, and proteins were stained with coomassie brilliant blue as described by Gildberg et al. (1990).

2.6.5. Optimum pH

Optimum pH was evaluated by measuring the activity of enzyme extracts using Hb 0.5% as substrate at 25 °C, and varying pH from 1.5 to 7.0 in universal buffer (Stauffer, 1989, chap. 4), following the recommendations of Asgeirsson and Bjarnason (1991) as described above. Percentage of enzyme activity was estimated considering 100% the highest activity detected in the assay.

2.6.6. pH stability

The effect of pH on stability of sardine acidic enzymes was estimated by incubating enzyme extracts and varying pH from 3.0 to 8.0 (universal buffer) with buffer solution over 60 min at 25 °C and measuring residual activity (Stauffer, 1989, chap. 4). The 100% of the enzyme activity is the activity of enzyme without incubation.

2.6.7. Optimum temperature and thermostability

Activity was evaluated with 0.1 M Gly-HCl at pH 3.0 according to Gildberg and Raa (1983) using Hb 0.5% as substrate over temperature range from 10 to 70 °C. Percentage of enzyme activity was estimated considering 100% the highest activity detected in the assay.

Thermostability was measured by evaluating residual activity at pH 3.0 after incubation of enzyme extract for 60 min at various temperatures from 30 to 70 °C. The 100% of the enzyme activity is the activity of enzyme without incubation.

2.6.8. Protein evaluation

Protein contents were evaluated according to Bradford (1976) using bovine serum albumin as a standard (1 mg/ml).

3. Results and discussion

Total proteolytic activity of Monterey sardine viscera extract is shown in Fig. 1. Two peaks in proteolytic enzyme activity maxima were found at pH 3.0 and 10. Alkaline activity was 60% higher than acidic. This viscera enzyme activity pattern was similar to that of related species such as sardine (*Sardinops melanostica*) and anchovy (*Engraulis encrasicholus*) (Martinez & Serra, 1989; Murakami & Noda, 1981).

3.1. Purification

Cation-exchange chromatography of the 45 eluted fractions yielded two main peaks (Fig. 2). The first peak was in fractions eluted between pH 3.5 and 5.0, and the second with NaCl at concentrations between 0 and 1 M. Table 1 shows protein content and activity of the main fractions from chromatography. Fraction 42 from chromatography was purified 23 times with a yield in total activity of 0.2%.

Fraction 42 revealed one band during SDS–PAGE (Fig. 3), indicating an acidic proteolytic enzyme with specific activity 1010 U/mg. Fraction 41, eluted by pH increment from 3 to 3.5, showed low acidic proteolytic activity and several proteins considered as contaminants, because of their low acidic activity. Fraction 43, eluted with pH increment from 4 to 4.5, showed the same band as fraction 42 but with very low specific activity. Protein in fractions 78 and 79 do not match with the one present in fraction 42, so the acidic activity in these fractions should be due to another acidic enzyme, maybe Pepsin I. However, no effort to isolate it was attained. The main band in fraction 80 (MW ≈31 kDa) is a different band than that in fraction 42 (MW

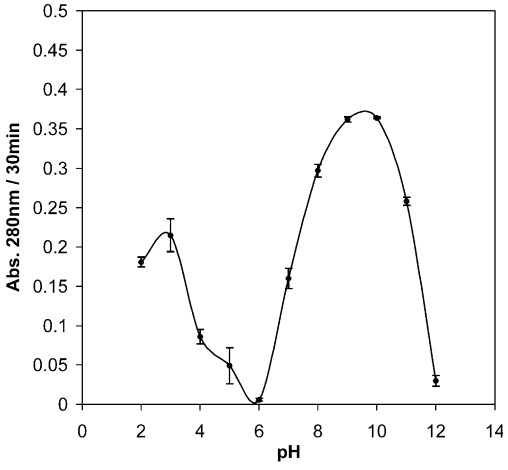

Fig. 1. Overall proteolytic activity of enzymes from whole Monterey sardine viscera. Hemoglobin (2%) was used as the substrate in acid pH (2–6) and casein (2%) in neutral and alkaline pH (7–12). Activity is expressed in terms of ΔAbs 280 nm/30 min.

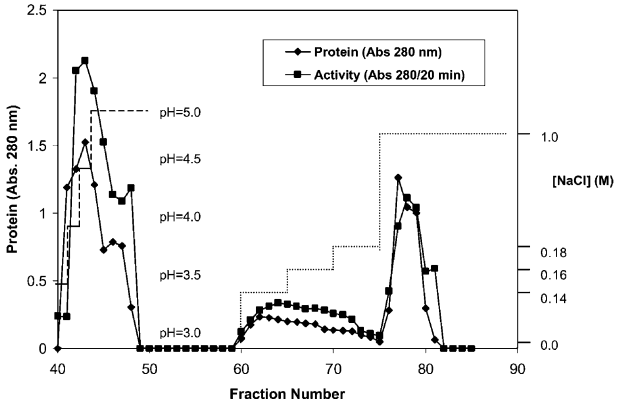

Fig. 2. Ion exchange chromatography. Elution with stepwise pH increments from 3.0 to 5.0 (fractions 40–56), and NaCl concentration increments from 0 to 1 M (fractions 57–85). Protein content is expressed in Abs 280 nm and activity in terms of ΔAbs 280 nm/20 min.

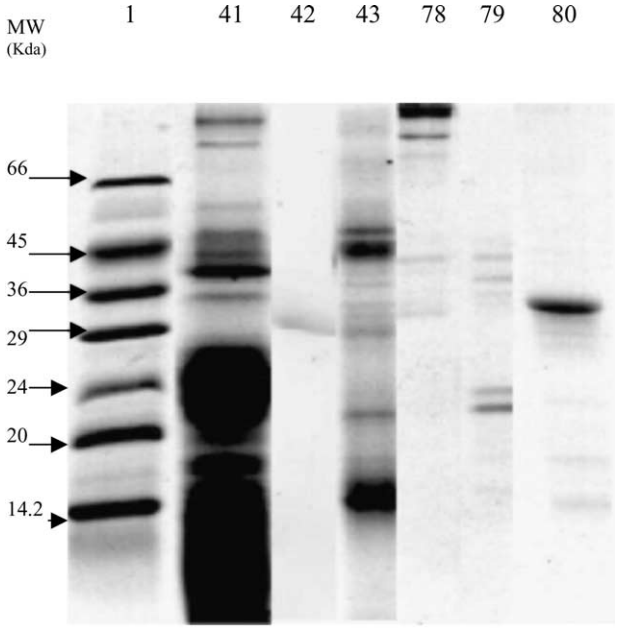

Fig. 3. SDS–polyacrylamide gel electrphoresis (PAGE). Line 1 MWM, next lanes fractions 41,42, 43, 78, 79 and 80 from ion exchange chromatography.

Table 1
Protein and activity of crude extract and fractions

Fraction	Protein (mg/ml)	Activity (U[a])	Specific activity (U/mg)	Recovery (%)	Purific. fold
Crude extract	1.06	0.46	43.4	100.0	1
(NH4)2SO4 Frac.	0.66	1.70	258.5	66.0	6.0
42	0.10	2.10	1009.6	0.2	23.3
43	4.23	2.30	54.4	5.7	1.2
77	1.09	1.55	141.7	1.0	3.3
78	1.09	2.57	235.1	1.6	5.4
79	1.26	2.57	203.6	2.0	4.7
80	1.83	1.96	107.0	2.1	2.5

[a] 1 U = 1 μg of tyrosine equivalent released from hemoglobin per min.

≈29 kDa). This opinion is supported by the specific activity values in both fractions (1010 U/mg for fraction 42 and 107 U/mg for fraction 80). Fractions 42 and 43 were selected for further characterization. Because of the small volume and protein concentration of fraction 42, it was used only for isoelectric point and inhibition studies, whereas fraction 43 was used to characterize optimum temperature, thermostability, optimum pH, and pH stability.

3.2. Isoelectric point (pI)

Isoelectrofocusing for fraction 42 yielded two bands of pI 4.0 and 4.5 (line 2) (Fig. 4). These values were close to those reported for pepsin IIa of Atlantic cod (*Gadus morhua*) (4.0), and pepsin II of capelin (*Mallotus villosus*) (3.5) (Gildberg et al. 1990; Gildberg & Raa, 1983). As for the pepsin IIa, acidic proteases from fraction 42 were eluted with 10 mM citrate–HCl buffer pH 4 without salt, and showed similar electrical mobility in ion exchange chromatography.

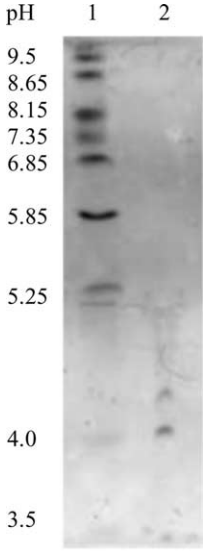

Fig. 4. Isoelectric point: Lane 1, protein markers; lane 2, fraction 42.

3.3. Effect of Inhibitors

Table 2 summarizes the susceptibility of acidic proteolytic enzymes (Fraction 42) to inhibitors of aspartic-proteases (Pepstatin A), serine-proteases (soybean trypsin inhibitor), and the metallo-proteases (EDTA). Pepstatin A is a very specific inhibitor, with one of the lowest known *Ki* for pepsin (45 pM, Zollner, 1993). The inhibition of acidic proteolytic activity in sardine viscera by pepstatin A confirms the presence of the aspartic protease pepsin. SBTI and EDTA did not inhibit Monterey sardine acidic enzymes.

3.4. Optimum pH

The effect of pH on acidic proteolytic activity in Hb is shown in Fig. 5. The optimum pH was 2.5, with about 90% of maximum activity detected at pH 2, 3, and 3.5. Normally, the activities of mixed pepsins converge in a broad pH optimum at about 3.0 (Moriarty, 1973). The optimum pH for fish pepsins generally falls in the range 2–4, with pepsin I having optimal pH between 3 and 4, and pepsin II between 2 and 3 (Gildberg, 1988).

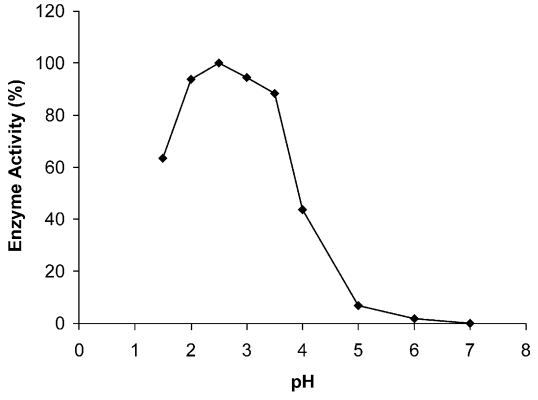

Fig. 5. Optimum pH. Activity was measured in universal buffer using 0.5% Hb as substrate at 25 °C and varying pH from 1.5 to 7.0 (Stauffer, 1989, chap. 4). Percentage of enzyme activity was estimated considering 100% the highest activity detected in this assay.

Table 2
Effect of inhibitors

Inhibitor	Residual enzyme activity (%)[a]
Pepstatin A	4.4
SBTI[b]	110.0
EDTA[c]	124.1

[a] % Residual enzyme activity was the remaining activity after 60 min incubation of enzyme with corresponding inhibitor.
[b] SBTI, soybean trypsin inhibitor.
[c] EDTA, etilen diamino-tetracetic acid.

3.5. pH stability

Acidic proteolytic enzymes of fish are generally stable at low pH, and Monterey sardine acidic enzymes are no exception. In the present study, these enzymes were stable at pH from 3 to 6, and became susceptible at neutral and alkaline pH (Fig. 6). Similar results were reported for *S. melanostica*, in which the enzymems were stable between pH 2 and 6, and showed drastic loss of activity at pH 7 (Noda & Murakami, 1981). Cold-water species like capelin (*Mallotus villosus*) and Atlantic cod (*Gadus morhua*) possess visceral acidic proteolytic enzymes that are stable between pH 2 and 5, whereas enzymes from warm-water species like bonito (*Katsuonus pelamis*) are stable even at neutral pH (Kubota & Ohnuma, 1970).

3.6. Optimum temperature

Fig. 7 shows the effect of temperature on acidic proteolytic enzymes from Monterey sardine viscera. The optimum temperature was 45 °C, with 50% activity at 10 °C. Close results were reported for stomach extracts from the cold-water fish capelin (Gildberg, 1988). On the other hand, purified pepsin from the same fish had optimum temperature 40 °C, suggesting that other compounds in the crude extract help stabilize the enzyme (Gildberg & Raa, 1983). Kubota and Ohnuma (1970), Owen and Wiggs (1971) and Gerard and Le Gal (1987), reported that aspartic protease activity often express a linear function with temperature below the optimum. From the data in Fig. 7, the correlation between enzyme activity and temperature below the optimum showed a linear R^2 of 0.9949.

3.7. Temperature stability

Thermostability is closely related to the optimum temperature in most fish digestive enzymes studied (Gildberg, 1988). Acidic enzymes from Monterey sardine viscera were almost 100% active at 30 °C for 60 min. At 45 °C there was a loss of 30% after 15 min, and 50% after 60 min. At 55 °C, activity was reduced 70% after 60 min. Above 55 °C, all activity stopped within 5–10 min (Fig. 8). Similar results were reported for capelin (*M. villosus*) by Gildberg and Raa (1983) and for sardine (*S. melanostica*) by Noda and Murakami (1981). Acidic proteolytic enzymes in Monterey sardine viscera were fully active for at least 60 min at temperatures below 45 °C, and showed a pronounced loss of activity at 55 °C and above. This characteristic is important for potential technological applications of these enzymes, especially in food processing.

4. Conclusions

Data on total proteolytic activity in Monterey sardine viscera showed that alkaline activity was higher than

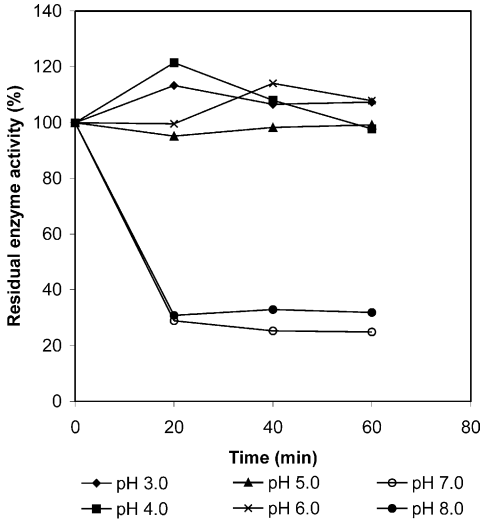

Fig. 6. pH stability. Residual activity was measured after incubation of enzyme extracts, with substrate solution pH varying from 3.0 to 8.0 (universal buffer) for 60 min at 25 °C (Stauffer, 1989, chap. 4). 100% of enzyme activity is the activity of enzyme without incubation.

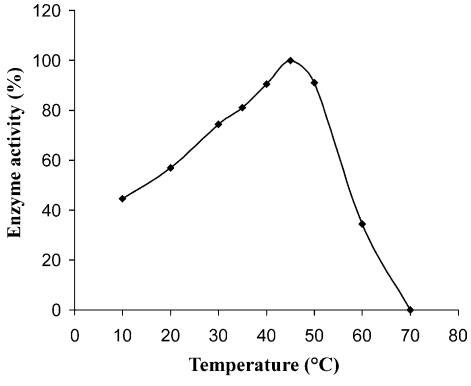

Fig. 7. Optimum temperature. Activity at pH 3.0 (Gly–HCl 0.1 M) was evaluated using 0.5% Hb as substrate and changing temperature from 10 to 70 °C. Percentage of enzyme activity was estimated considering 100% the highest activity detected in this assay.

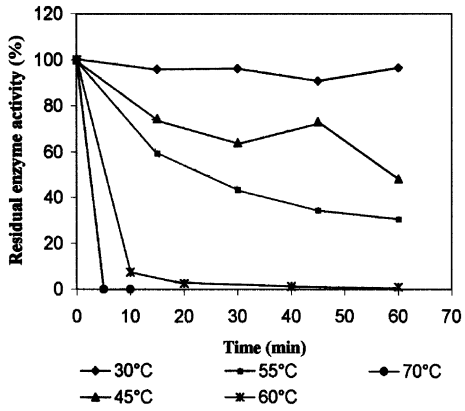

Fig. 8. Temperature stability. Residual activity at pH 3.0 after incubation of enzyme extract with 0.5% Hb for 60 min at temperatures from 30 to 70 °C. 100% of enzyme activity is the activity of enzyme without incubation.

acidic activity, and confirms the importance of alkaline enzymes in the digestive process.

Results indicated that the acidic proteolytic enzymes isolated from Monterey sardine viscera belong to the aspartic protease class and are similar to pepsin II reported for other fish species. The dependence of activity on pH and temperature could make these enzymes a biotechnological alternative for food processing when acid pH and low temperatures are needed like in milk clotting and other emerging processes. Also, these enzymes might aid in the enzymatic treatment of stick-water, in which a reduction in viscosity is required for further processing of effluent. Due to the high activity detected, further research is under way to determine if Monterey sardine byproduct viscera can be used to obtain a high value-added product (proteolytic enzymes) that could be used as a processing aid to reduce the stick-water disposal.

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