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Purification and characterization of trypsin from the viscera of sardine (*Sardina pilchardus*)

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Abstract

Trypsin from the viscera of *Sardina pilchardus* was purified by fractionation with ammonium sulphate, heat treatment and Sephadex G-100 gel filtration with a ninefold increase in specific activity and 9% recovery. The molecular weight of the enzyme was estimated to be 25,000 Da on SDS–PAGE. This enzyme showed esterase-specific activity on *N*α-benzoyl-L-arginine ethyl ester (BAEE). The purified enzyme was inhibited by benzamidine, a synthetic trypsin inhibitor, and phenylmethylsulphonyl fluoride (PMSF) a serine-protease inhibitor, but was not inhibited by the β-mercaptoethanol. The optimum pH and temperature for the enzyme activity were pH 8.0 and 60 °C, respectively. The relative activity at pH 9.0 was 95.5% and the enzyme showed pH stability between 6.0 and 9.0. The N-terminal amino acid sequence of the first 12 amino acids of the purified trypsin was IVGGYECQKYSQ. *S. pilchardus* trypsin, which showed high homology to other fish trypsins, had a charged Lys residue at position 9, where Pro or Ala are common in fish trypsins. The enzyme was strongly inhibited by Zn^{2+} and Cu^{2+}.

Keywords: Trypsin; Purification; Biochemical characterization; Viscera; *Sardina pilchardus*

1. Introduction

Proteases constitute the most important group of industrial enzymes used in the world today, accounting for about 50% of the total industrial enzyme market (Rao, Tanksala, Ghatge, & Deshpande, 1998). They have diverse applications in a wide variety of industries such as detergent, food, agrochemical and pharmaceutical (Gupta, Beg, & Lorenz, 2002; Zukowski, 1992). Proteases are mainly derived from animal, plant and microbial sources.

Today, there is an increasing demand for fish proteolytic enzymes in food processing. Viscera, one of the most important by-products of fishing industry, are recognised 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; Shahidi & Janak Kamil, 2001). A variety of digestive proteolytic enzymes have been isolated from the internal organs of fish. The most important proteolytic enzymes from fish viscera are the aspartic protease pepsin, and serine proteases trypsin, chymotrypsin and elastase. Acidic proteases from fish stomach display high activity between pH 2.0 and 4.0, while alkaline digestive proteases, such as trypsin, are most active between pH 8.0 and 10.0. Trypsin has many industrial applications, and it is a very important enzyme in the food industry. Trypsin has been extracted, purified and characterized in several fish species including true sardine (*Sardinops melanostictus*) and arabesque greenling (*Pleuroprammus azonus*) (Kishimura, Hayashi, Miyashita, & Nonami, 2006a), jacopever (*Sebastes schlegelii*) and elkhorn sculpin (*Alcichthys alcicornis*) (Kishimura et al., 2006b), yellowfin tuna (*Thunnus albacores*) (Klomklao et al., 2006a), skipjack tuna (*Katsuwonus pelamis*) (Klomklao, Benjakul, Visessanguan, Kishimura, & Simpson, 2006b) and Monterey sardine (*Sardinops sagax*

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caerulea) (Castillo-Yanez, Pacheco-Aguilar, Garcıa-Carreno, & Navarrete-Del Toro, 2005).

In Tunisia *Sardina pilchardus* catches were about 12,465 tonnes in 2002 (FAO, 2004). It has been exploited as a raw material for canning industries. During processing, large quantities of waste, including heads and viscera, are generated and discarded. They can represent about 30% of the original raw material. These wastes, which represent an environmental problem to the fishing industry, constitute an important source of proteolytic enzymes and protein. Traditionally, these materials have been converted to powdered fish flour used as animal feed (Strom & Eggum, 1981), but the abundance of proteolytic enzymes and proteins in sardine viscera open the possibility of further utilisation.

One alternative to convert the sardine processing wastes into more marketable and acceptable products is to produce fish powders or fish protein hydrolysates that may be used as carbon and/or nitrogen source for biomass and metabolite production. In a previous paper, we have shown that protease synthesis was strongly induced when *Bacillus subtilis* strain was grown in media containing only sardinella (*Sardinella aurita*) heads and viscera powder (Ellouz, Bayoudh, Kammoun, Gharsallah, & Nasri, 2001). Moreover, several studies revealed that fish protein hydrolysates performed effectively as a nitrogenous source in microbial growth and enzyme production (Clausen, Gildberg, & Raa, 1985; Dufossé, De la Broise, & Guérard, 2001; Ghorbel et al., 2005; Gildberg, Batista, & Ström, 1989; Triki-Ellouz, Ghorbel, Souissi, Kammoun, & Nasri, 2003).

An interesting alternative is to isolate and purify proteolytic enzymes which can be used in the food industry or in fish protein hydrolysate preparation. The recovery of proteases from sardine viscera might be a solution to the pollution problem generated by the sardine processing industries. This paper describes the purification procedure and some biochemical characterization of trypsin from *S. pilchardus* viscera.

2. Materials and methods

2.1. Sardine viscera

Sardine (*S. pilchardus*) was purchased from the local market at Sfax City, Tunisia. It was washed twice with water and viscera were separated, and then stored in sealed plastic bags at −20 °C until used for enzyme extraction.

2.2. Preparation of enzyme extract

Viscera from *S. pilchardus* (250 g) were homogenized for 30 s with 500 ml extraction buffer A (10 mM Tris–HCl pH 8.0, 10 mM $CaCl_2$). The mixture was centrifuged at 10,000*g* for 15 min at 4 °C. The pellet was discarded and the supernatant was collected and used as crude protease extract.

2.3. Enzyme purification

2.3.1. Ammonium sulphate precipitation

The crude enzyme extract was subjected to ammonium sulphate fractionation and the precipitate in the 30–60% saturation range was collected by centrifugation 15 min at 10,000*g*. The precipitate was suspended in buffer A and dialyzed 24 h at 4 °C against repeated changes in the same buffer.

2.3.2. Heat treatment

The dialyzed precipitate between 30% and 60% saturation was heat-treated in a water bath at 50 °C for 30 min with continuous stirring, followed by immediate cooling in ice-water. The resulting precipitate was discarded after centrifugation at 10,000*g* for 10 min at 4 °C.

2.3.3. Gel filtration

The heat-treated enzyme was then subjected to gel filtration on a Sephadex G-100 column (2.6 × 100 cm) pre-equilibrated with buffer B (25 mM Tris–HCl buffer pH 8.0 containing 0.5‰ Triton X-100). Enzyme fractions of 5 ml were eluted at a flow rate of 28 ml/h with the same buffer. Protein content (Abs 280 nm) and protease activity were measured. Fractions showing protease activities were collected and stored at −20 °C for further analysis. All the purification steps were conducted at temperatures not exceeding 4 °C.

2.3.4. Polyacrylamide gel electrophoresis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS–PAGE) was carried out for the determination of purity and molecular weight of the enzyme as described by Laemmli (1970), using a 5% (w/v) stacking gel and a 15% (w/v) separating gel. Samples were prepared by mixing the purified enzyme at 1:5 (v/v) ratio with distilled water containing 10 mM Tris–HCl pH 8.0, 2.5% SDS, 10% glycerol, 5% β-mercaptoethanol and 0.002% bromophenol blue. Samples were heated at 100 °C for 5 min before electrophoresis. Gels were stained with 0.25% Coomassie Brilliant Blue R250 in 45% ethanol–10% acetic acid and destained with 5% ethanol–7.5% acetic acid. The molecular weight of the enzyme was estimated using a low molecular weight calibration kit as markers (Sigma). The molecular mass markers used are: Bovine serum albumin (66,000 Da); ovalbumin (45,000 Da); carbonic anhydrase (29,000 Da); trypsin inhibitor (20,100 Da) and α-lactoalbumin (14,200 Da).

2.3.5. Protein concentration

Protein concentration was determined by the method of Bradford (1976) using bovine serum albumin (BSA) as a standard.

2.4. Determination of protease activity

Protease activity was measured by the method of Kembhavi, Kulkarni, and Pant (1993) using casein as a

substrate. A 0.5 ml aliquot of the enzyme, suitably diluted, was mixed with 0.5 ml 100 mM Tris–HCl (pH 8.0) containing 1% casein, and incubated for 15 min at 60 °C. The reaction was stopped by addition of 0.5 ml 20% trichloroacetic acid. The mixture was allowed to stand at room temperature for 15 min and then centrifuged at 10,000*g* for 15 min to remove the precipitate. The absorbance was measured at 280 nm. A standard curve was generated using solutions of 0–50 mg/l tyrosine. One unit of protease activity was defined as the amount of enzyme required to liberate 1 μg tyrosine/millilitre in 1 min under the experimental conditions used.

2.5. Biochemical properties

2.5.1. Specific activity

Esterase activity was evaluated according to Blanco and Guisan (1988) using BAEE as substrate. Two hundred and fifty microlitres of enzyme solution were added to 2.5 ml 0.5 mM BAEE in 50 mM phosphate buffer, pH 7.6 and the increase in absorbance at 253 nm was measured every 30 s for 10 min. One BAEE unit was calculated as the amount of enzyme that hydrolyses 1 μmol of BAEE/min under the conditions described.

2.5.2. N-terminal amino acid sequence of the enzyme

The purified enzyme was electrophoretically transferred from SDS–PAGE to a polyvinylidene difluoride membrane (PVDF). The region containing the protease band on the PVDF was excised and the protein N-terminal amino acid sequence was determined by the Edman degradation method on an ABI Procise 494 protein sequencer (Applied Biosystems).

2.5.3. Effect of pH on activity and stability of trypsin

The effect of pH was determined with casein as a substrate. Protease activity was studied in the pH range of 6.0–11.0 at the optimal temperature (60 °C). For the measurement of pH stability, the enzyme was incubated at 40 °C for 1 h in different buffers and then the residual proteolytic activity was determined under standard assay conditions. The following buffer systems were used: 100 mM sodium acetate, pH 6.0; phosphate buffer, pH 7.0; Tris–HCl buffer, pH 8.0; glycine-NaOH buffer, pH 9.0–11.0.

2.5.4. Optimum temperature and thermal stability

The effect of temperature on trypsin activity was studied from 40 to 70 °C for 15 min at pH 8.0. Thermal stability of the purified trypsin was determined by incubating the enzyme 240 min at 40, 50, 60 and 70 °C at pH 8.0. Aliquots were withdrawn at desired time intervals to test the remaining activity at standard conditions. The non-heated enzyme was considered as control (100%).

2.5.5. Effects of enzyme inhibitors

The effects of inhibitors on protease activity were studied using phenylmethylsulfonyl fluoride (PMSF), benzamidine, β-mercaptoethanol and ethylenediaminetetraacetic acid (EDTA). The purified enzyme was preincubated with inhibitors for 30 min at 20 °C and then the remaining enzyme activity was estimated using casein as a substrate.

2.5.6. Effect of metal ions

The effects of various metal ions (2 mM) on enzyme activity were investigated using Ca^{2+}, Mn^{2+}, Zn^{2+}, Na^{+}, K^{+}, Cu^{2+}, Ba^{2+} and Mg^{2+}.

3. Results and discussion

3.1. Purification of trypsin

Trypsin from viscera of *S. pilchardus* was purified by the three-step procedure described in Section 2. In the first step, the crude enzyme extract was fractionated with ammonium sulphate. The fraction F2 (30–60% saturation) showed higher specific activity (3627 U/mg of protein) than F1 (0–30% saturation; 1147 U/mg of protein). No activity was detected in final supernatant. The 30–60% ammonium sulphate precipitate, which gave the highest specific activity (Table 1), was then heat treated at 50 °C for 30 min. This step resulted in a considerable increase in the specific activity. Heat treatment has proven to be an important strategy for purification of thermostable enzymes. Heat treatment denatures thermolabile proteins in the crude extract (Bez-

Table 1
A summary of the purification of trypsin from sardine (*S. pilchardus*)

Purification steps	Total activity (U)	Total protein (mg)	Specific activity (U/mg protein)	Recovery (%)	Purification fold
Crude extract	335,636	331.7	1011.86	100	1
Step 1: Ammonium sulphate precipitation					
F1 $(NH_4)_2SO_4$ (0–30%)	134,254.4	117	1147.47	40	1.12
F2 $(NH_4)_2SO_4$ (30–60%)	139,020.43	38.33	3626.93	41.42	3.58
Step 2: Heat treatment					
Heated 30–60% precipitate	68,872.50	13.28	5186.18	20.52	5.12
Step 3: gel filtration					
Chromatography Sephadex G-100	31,147	3.31	9409.97	9.28	9.29

All operations were carried out at 4 °C. Only precipitate formed between 30% and 60% saturation with ammonium sulphate was subjected to gel-filtration on Sephadex G-100.

erra et al., 2001). Moreover, it is responsible for a significant breakdown of other undesired thermostable proteins. The heated enzyme was then subjected to gel filtration on a Sephadex G-100 column. The elution profiles of trypsin activity and proteins from Sephadex G-100 are shown in Fig. 1. This procedure yielded a single peak of protease activity.

The results of the purification procedure are summarised in Table 1. After the final purification step, the enzyme was purified ninefold with a recovery of 9% and a specific activity of 9410 U/mg protein.

3.2. N-terminal amino acid sequence of S. pilchardus trypsin

The N-terminal amino acid sequence of the purified trypsin was determined by the automated Edman method after SDS–PAGE and electroblotting. The 12 N-terminal amino acid sequence was IVGGYECQKYSQ. The N-terminal amino acid sequence of *S. pilchardus* trypsin was aligned with the sequences of other fish trypsins (Gudmundsdottir et al., 1993; Kishimura, Hayashi, Miyashita, & Nonami, 2005; Kishimura et al., 2006a, 2006b; Klomklao et al., 2006a, 2006b; Toyota et al., 2002) (Fig. 2). The N-terminal seven amino acid sequence of *S. pilchardus* trypsin (IVGGYEC) was identical with those of fish trypsins. *S. pilchardus* trypsin had a charged Lys residue at position 9, where Pro or Ala are common in fish trypsin. The highest homology was observed with trypsin TR-II from Japanese anchovy *Engraulis japonica* (Kishimura et al., 2005). *S. pilchardus* trypsin differs from that of Japanese anchovy with only one amino acid in the first 12 amino acids. The Lys-9 in *S. pilchardus* trypsin was replaced by Pro-9 in Japanese anchovy trypsin.

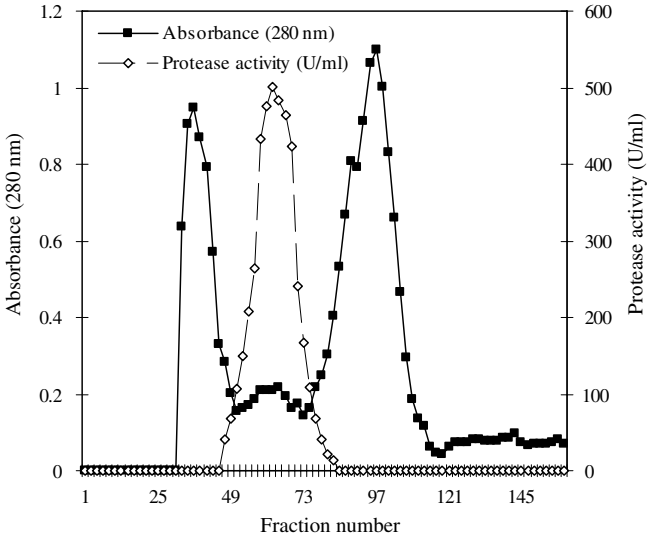

Fig. 1. Purification profile of trypsin from sardine (*S. pilchardus*) by gel filtration on Sephadex G-100 column. The enzyme preparation (30–60% saturation with ammonium sulphate) was applied to a 2.6×100 cm column, equilibrated and eluted with buffer B. Fractions (5 ml) collected from the column were assayed for proteins content at 280 nm and protease activity as described in Section 2. Flow rate = 28 ml h^{-1}.

Sardine: *S. pilchardus*	I	V	G	G	Y	E	C	Q	K	Y	S	Q
Cod	I	V	G	G	Y	E	C	T	K	H	S	Q
Chum salmon	I	V	G	G	Y	E	C	K	A	Y	S	Q
Japanese anchovy (TR- I)	I	V	G	G	Y	E	C	Q	A	H	S	Q
Japanese anchovy (TR- II)	I	V	G	G	Y	E	C	Q	P	Y	S	Q
True sardine (TR-S)	I	V	G	G	Y	E	C	K	A	Y	S	Q
Arabesque greenling (TR-P)	I	V	G	G	Y	E	C	T	P	H	T	Q
Jacopever (TR-J)	I	V	G	G	Y	E	C	K	P	Y	S	Q
Elkhorn sculpin (TR-E)	I	V	G	G	Y	E	C	T	P	H	S	Q
Skipjack tuna (A, B & C)	I	V	G	G	Y	E	C	Q	A	H	S	Q
Yellowfin tuna (TR-A)	I	V	G	G	Y	E	C	Q	A	H	S	Q

Fig. 2. Comparison of N-terminal amino acid sequence of the purified trypsin from sardine (*S. pilchardus*) with other enzymes: cod (Gudmundsdottir et al., 1993), chum salmon (Toyota et al., 2002), Japanese anchovy (TR-I and TR-II) (Kishimura et al., 2005), True sardine (TR-S) and arabesque greenling (TR-P) (Kishimura et al., 2006a), jacopever (TR-J) and elkhorn scuplin (TR-E) (Kishimura et al., 2006b), skipjack tuna (A, B and C) (Klomklao et al., 2006a) and yellowfin tuna (TR-A) (Klomklao et al., 2006b).

3.3. Biochemical characterization

3.3.1. Molecular weight

The molecular weight of the purified enzyme was estimated by SDS–PAGE as 25,000 Da (Fig. 3) which is in the range of 22,000–28,000 Da for trypsins purified from other fish species (Simpson, 2000). The molecular weight of *S. pilchardus* trypsin was similar to those from other fish species such as Monterey sardine (*S. sagax caerulea*) (Castillo-Yanez et al., 2005), true sardine (*S. melanostictus*) and arabesque greenling (*P. azonus*) (Kishimura et al., 2006a), jacopever (*S. schlegelii*) and elkhorn sculpin (*A. alcicornis*) (Kishimura et al., 2006b), yellowfin tuna (*T. albacores*) (Klomklao et al., 2006a), skipjack tuna (*K. pelamis*) (Klomklao et al., 2006b).

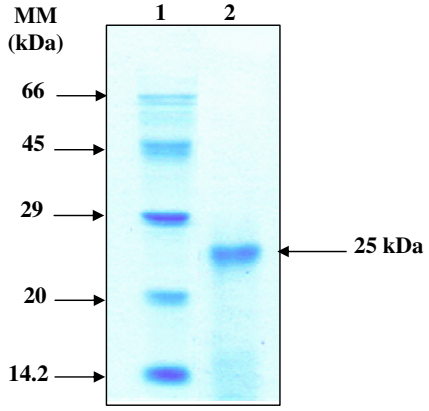

Fig. 3. SDS–polyacrylamide gel electrophoresis of the purified trypsin from viscera of sardine (*S. pilchardus*). Lane 1: standard protein markers of different molecular weights; lane 2: purified trypsin.

3.3.2. Specific activity

Esterase (BAEE)-specific activity in the pure enzyme was evaluated. *S. pilchardus* trypsin showed a specific activity of 5.88 U/mg enzyme on the ester substrate benzoyl-L-arginine ethyl ester.

3.3.3. Effect of pH on activity and stability

The effect of pH on enzyme activity was determined over a pH range of 6.0–11.0. The purified enzyme was active between pH 6.0 and 10.0, with an optimum around pH 8.0–9.0 (Fig. 4a). The relative activities at pH 7.0 and 9.0 were about 58.5% and 95.5%, respectively, of that at pH 8.0. As seen in Fig. 4a, protease activity decreased significantly above pH 9.0 and was 37.6% of the maximum activity at pH 10.0. Optimum pH between 8.0 and 10.0 has been reported for enzyme activities of fish species such as Monterey sardine (Castillo-Yanez et al., 2005), anchovy (*E. japonica*) (Heu, Kim, & Pyeun, 1995) and sardine (*Sardinops melanostica*) (Murakami & Noda, 1981).

For investigation of the pH stability, the enzyme was incubated in different buffers of varying pH values for 1 h at 40 °C and the residual activities were determined at 60 °C and pH 8.0. Trypsin was very stable in a broad pH range, maintaining over 91% of its original activity between pH 6.0 and 9.0, and more than 50% of its activity was

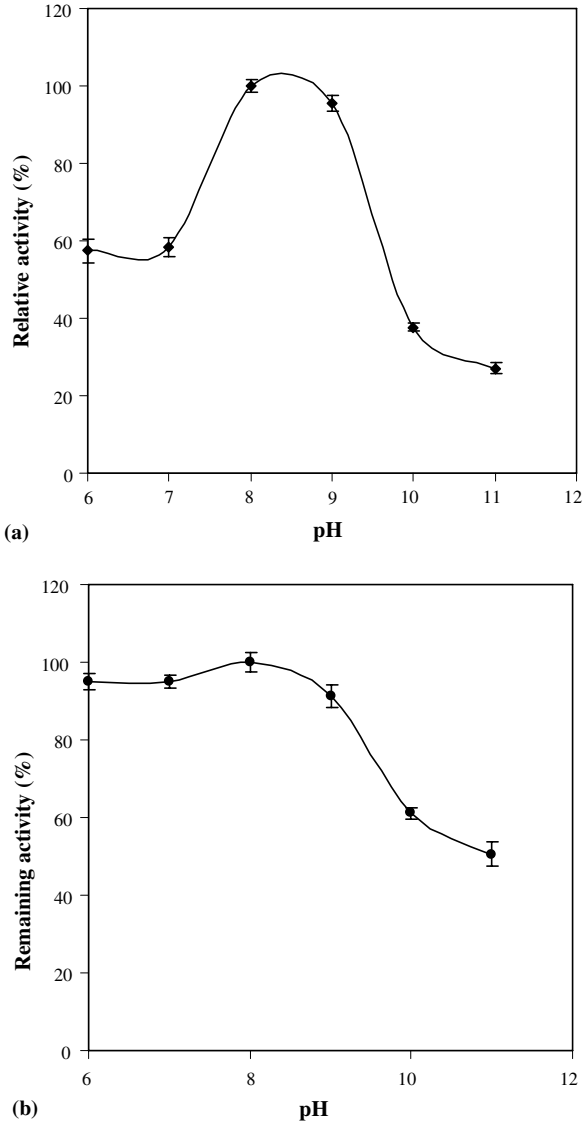

Fig. 4. Effect of pH on activity (a) and stability (b) of the purified trypsin. The protease was assayed in the pH range of 6.0–11.0 using buffers of different pH values at 60 °C. The maximum activity obtained at pH 8.0 was considered as 100% activity. The pH stability of the enzyme was determined by incubating the enzyme in different buffers for 1 h at 40 °C and the residual activity was measured at pH 8.0 and 60 °C. The activity of the enzyme before incubation was taken as 100%. Buffer solutions used for pH activity and stability are presented in Section 2.

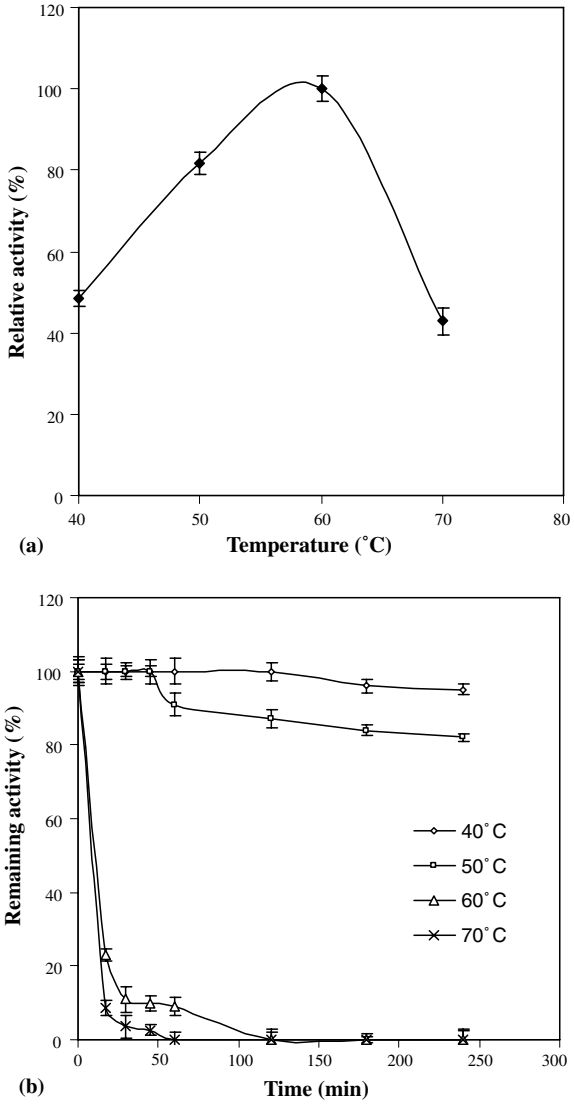

Fig. 5. Effect of temperature on activity (a) and stability (b) of the purified trypsin from sardine (*S. pilchardus*). The temperature profile was determined by assaying protease activity at temperatures between 40 and 70 °C. The activity of the enzyme at 60 °C was taken as 100%. The temperature stability was determined by incubating purified enzyme at 40, 50, 60 and 70 °C from 0 to 240 min at pH 8.0 and residual enzyme activities were estimated at regular intervals under standard conditions. The non-heated enzyme was considered as control (100%).

retained at pH 11.0 (Fig. 4b). The pH stability of *S. pilchardus* trypsin is higher than Monterey sardine trypsin, which was stable in the pH range from 7.0 to 9.0 (Castillo-Yanez et al., 2005).

3.3.4. Effect of temperature on the activity and stability of the enzyme

The effect of temperature on activity was determined by assaying enzyme activity at different temperatures (Fig. 5a). Trypsin from *S. pilchardus* was active at temperatures from 40 to 70 °C and had an optimum at 60 °C. The relative activities at 50 and 70 °C were about 81.6% and 42.8%, respectively, of that at 60 °C. The optimum temperature for *S. pilchardus* trypsin was similar to that of true sardine (*S. melanostictus*) (Kishimura et al., 2006a) and was higher than those from other fish species such as mullet (*Mugil cephalus*) (55 °C) (Guizani, Rolle, Marshall, & Wie, 1991) and arabesque greenling (*P. azonus*) (TR-P) (Kishimura et al., 2006a), elkhorn sculpin (*A. alcicornis*) (TR-E) (Kishimura et al., 2006b) which have optimum temperatures at 50 °C.

The thermal stability profile of the purified enzyme showed that the enzyme is highly stable at temperatures below 40 °C but was inactivated at higher temperatures (Fig. 5b). The enzyme at 40 °C remains fully active even after 240 min of incubation, indicating that this protease might be used under mild heating conditions. The enzyme retained more than 80% of its initial activity after 4 h incubation at 50 °C, while 11% and 3.5% of the maximal activity remained within 30 min of incubation at 60 and 70 °C, respectively. The thermal stability of *S. pilchardus* trypsin is higher than Monterey sardine (*S. sagax caerulea*) trypsin, which retained less than 5% of its original activity after 15 min incubation at 50 °C (Castillo-Yanez et al., 2005).

3.3.5. Effect of enzyme inhibitors on protease activity

The effect of a variety of enzyme inhibitors, such as chelating agent and a group specific agent on the activity was investigated (Table 2). The enzyme was inhibited by the serine-protease inhibitor PMSF and trypsin specific inhibitor benzamidine (10 mM) (approximately 42%). These data indicate that the protease belonged to the serine proteases. The enzyme was not affected by β-mercaptoethanol. Metalloprotease inhibitor EDTA (10 mM) inhibited the enzyme activity by 80%, indicating the importance of Ca^{2+} in enzyme stabilization.

Table 2
Effect of inhibitors on sardine (*S. pilchardus*) trypsin activity

Inhibitors	Concentration (mM)	Residual enzyme activity (%)
None		100
PMSF	10	35
	20	0
Benzamidine	5	70
	10	58
β-mercaptoethanol	5	100
EDTA	10	20

Enzyme activity measured in the absence of any inhibitor was taken as 100%. The remaining protease activity was measured after preincubation of enzyme with each inhibitor at 20 °C for 15 min.
PMSF: phenyl-methyl-sulfonyl fluoride; EDTA: ethylenediaminetetraacetic acid.

Table 3
Effect of various metal ions (2 mM) on trypsin activity

Ion (2 mM)	Relative activity (%)
None	100
Ca^{2+}	107
Mg^{2+}	98.5
Zn^{2+}	48.9
Mn^{2+}	68.3
Cu^{2+}	37.5
Ba^{2+}	91.8

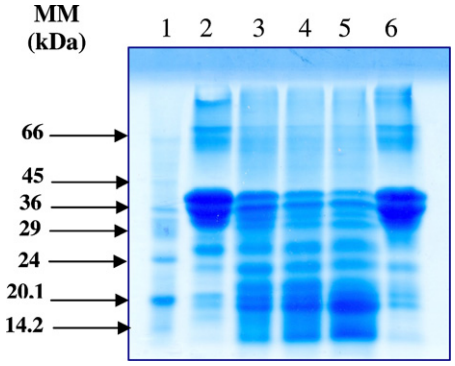

Fig. 6. Degradation of casein by the purified trypsin. 0.5 ml of 1% casein was incubated with trypsin for 60 min at various temperatures. Lane 1, standard proteins; lane 2, native casein; lanes 3–6 enzyme was incubated 60 min at 40, 50, 60 and 70 °C, respectively. Bovine serum albumin (BSA; 66,000 Da), egg white ovalbumin (45,000 Da), glyceraldehydes-3-P dehydrogenase (36,000 Da), bovine carbonic anhydrase (29,000 Da), bovine trypsinogen (24,000 Da), soybean trypsin inhibitor (20,100 Da) and α-lactoalbumin (14,200 Da) were used as molecular weight markers.

3.3.6. Effect of metal ions

The effect of some divalent cations (2 mM) on the activity of *S. pilchardus* trypsin was studied at pH 8.0 and 60 °C by the addition of the respective cation to the assay mixture (Table 3). Ca^{2+} slightly increased protease activity. Mg^{2+} and Ba^{2+} showed no influence on the enzyme activity. However, Mn^{2+}, Zn^{2+} and Cu^{2+} inhibited the enzyme activity by 31.7%, 51.1% and 62.2%, respectively. The enzyme activity was not affected by Na^{+} and K^{+}.

3.3.7. Hydrolysis of casein

The degradation of the casein by the purified trypsin at various temperatures was investigated by SDS–PAGE and shown in Fig. 6. Hydrolysis of casein was maximal at approximately 60 °C. At 70 °C the profile of casein is similar to that of native casein, indicating that the protease was labile at this temperature.

4. Conclusions

Trypsin could be isolated at low cost from viscera produced as waste in *S. pilchardus* industrial processing. The purification to homogeneity of the trypsin was achieved by ammonium sulphate precipitation (30–60% saturation), heat treatment and gel filtration through Sephadex G-100. After the final purification step, the enzyme was purified ninefold with a specific activity of 9410 U/mg and 9% recovery. The purified enzyme preparation was homogenous on SDS–PAGE and its molecular weight was estimated to be 25,000 Da. Based on SDS–PAGE, specific activity for BAEE and N-terminal sequencing, the alkaline protease isolated from viscera of *S. pilchardus* was trypsin. The enzyme showed an optimum temperature at 60 °C and optimum pH of 8.0. The enzyme was stable at a pH range of 6.0–9.0 and at temperatures in the range 40–50 °C.

Further research is needed to determine properties of *S. pilchardus* trypsin as a possible biotechnological tool in the fish processing and food industries.

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References

Bezerra, R. S., Santos, J. F., Paiva, P. M. G., Correia, M. T. S., Coelho, L. C. B. B., Vieira, V. L. A., et al. (2001). Partial purification and characterization of a thermostable trypsin from pyloric caeca of tambaqui (*Colossoma macropomum*). *Journal of Food Biochemistry, 25*, 199–210.

Blanco, R. M., & Guisan, J. M. (1988). Protecting effects of competitive inhibitors during very intense insolubilized enzyme-activated support multipoint attachments: trypsin (amine)-agarose (aldehyde) system. *Enzyme and Microbial Technology, 10*, 227–232.

Bradford, M. (1976). A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein dye binding. *Analytical Biochemistry, 72*, 248–254.

Cancre, I., Ravallec, R., Van Wormhoudt, A. V., Stenberg, E., Gildberg, A., & Le Gal, Y. (1999). Secretagogues and growth factors in fish and crustacean protein hydrolysates. *Marine Biotechnology, 1*, 489–494.

Castillo-Yanez, F. J., Pacheco-Aguilar, R., Garcıa-Carreno, F. L., & Navarrete-Del Toro, M. A. (2005). Isolation and characterization of trypsin from pyloric caeca of Monterey Sardine *Sardinops sagax caerulea*. *Comparative Biochemistry and Physiology B, 140*, 91–98.

Clausen, E., Gildberg, A., & Raa, J. (1985). Preparation and testing of an autolysate of fish viscera as growth substrate for bacteria. *Applied and Environmental Microbiology, 50*, 1556–1557.

Dufossé, L., De la Broise, D., & Guérard, F. (2001). Evaluation of nitrogenous substrates such as peptones from fish: A new method based on Gompertz modeling of microbial growth. *Current Microbiology, 42*, 32–38.

Ellouz, Y., Bayoudh, A., Kammoun, S., Gharsallah, N., & Nasri, M. (2001). Production of protease by *Bacillus subtilis* grown on sardinelle heads and viscera flour. *Bioresource Technology, 80*, 49–51.

FAO. (2004). Food and Agriculture Organisation. Fishery statistics, Rome, Italy.

Ghorbel, S., Souissi, N., Triki-Ellouz, Y., Dufossé, L., Guérard, F., & Nasri, M. (2005). Preparation and testing of sardinella protein hydrolysates as nitrogen source for extracellular lipase production by *Rhizopus oryzae*. *World Journal of Microbiology and Biotechnology, 21*, 33–38.

Gildberg, A. (1992). Recovery of proteinase and protein hydrolysate from fish viscera. *Bioresource Technology, 39*, 271–276.

Gildberg, A., Batista, I., & Ström, E. (1989). Preparation and characterization of peptones obtained by two-step enzymatic hydrolysis of whole fish. *Biotechnology and Applied Biochemistry, 11*, 413–423.

Gudmundsdottir, A., Gudmundsdottir, E., Oskarsson, S., Bjarnason, J. B., Eakin, A. K., & Craik, C. S. (1993). Isolation and characterization of cDNAs from Atlantic cod encoding two different forms of trypsinogen. *European Journal of Biochemistry, 217*, 1091–1097.

Guizani, N., Rolle, R. S., Marshall, M. R., & Wie, C. I. (1991). Isolation, purification and characterization of a trypsin from the pyloric caeca of mullet (*Mugil cephalus*). *Comparative Biochemistry and Physiology B, 98*, 517–521.

Gupta, R., Beg, Q. K., & Lorenz, P. (2002). Bacterial alkaline proteases: molecular approaches and industrial applications. *Applied Microbiology and Biotechnology, 59*, 13–32.

Heu, M. S., Kim, H. R., & Pyeun, J. H. (1995). Comparison of trypsin and chymotrypsin from the viscera of anchovy (*Engraulis japonica*). *Comparative Biochemistry and Physiology B, 112*, 557–567.

Kembhavi, A. A., Kulkarni, A., & Pant, A. (1993). Salt-tolerant and thermostable alkaline protease from *Bacillus subtilis* NCIM No. 64. *Applied Biochemistry and Biotechnology, 38*, 83–92.

Kishimura, H., Hayashi, K., Miyashita, Y., & Nonami, Y. (2005). Characteristics of two trypsin isozymes from the viscera of Japanese anchovy (*Engraulis japonica*). *Journal of Food Biochemistry, 29*, 459–469.

Kishimura, H., Hayashi, K., Miyashita, Y., & Nonami, Y. (2006a). Characteristics of trypsins from the viscera of true sardine (*Sardinops melanostictus*) and the pyloric ceca of arabesque greenling (*Pleuroprammus azonus*). *Food Chemistry, 97*, 65–70.

Kishimura, H., Tokuda, Y., Yabe, M., Klomklao, S., Benjakul, S., & Ando, S. (2006b). Trypsins from the pyloric ceca of jacopever (*Sebastes schlegelii*) and elkhorn sculpin (*Alcichthys alcicornis*): Isolation and characterization. *Food Chemistry*, in press, doi:10.1016/j.foodchem.2005.11.040.

Klomklao, S., Benjakul, S., Visessanguan, W., Kishimura, H., Simpson, B. K., & Saeki, H. (2006a). Trypsins from yellowfin tuna (*Thunnus albacores*) spleen: Purification and characterization. *Comparative Biochemistry and Physiology B, 144*, 47–56.

Klomklao, S., Benjakul, S., Visessanguan, W., Kishimura, H., & Simpson, B.K. (2006b). Purification and characterisation of trypsins from the spleen of skipjack tuna (*Katsuwonus pelamis*). *Food Chemistry*, in press.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, 227*, 680–685.

Murakami, K., & Noda, M. (1981). Studies on proteinases from the digestive organs of Sardine-purification and characterization of three alkaline proteinases from the pyloric caeca. *Biochimica and Biophysica Acta B, 65*, 17–26.

Rao, M. B., Tanksala, A. M., Ghatge, M. S., & Deshpande, V. V. (1998). Molecular and biotechnological aspects of microbial proteases. *Microbiology and Molecular Biology Reviews, 62*, 597–635.

Shahidi, F., & Janak Kamil, Y. V. A. (2001). Enzymes from fish and aquatic invertebrates and their application in the food industry. *Trends in Food Science and Technology, 12*, 435–464.

Simpson, B. K. (2000). Digestive proteases from marine animals. In N. F. Haard & B. K. Simpson (Eds.), *Seafood enzymes* (pp. 191–213). New York, NY: Marcel Dekker.

Strom, T., & Eggum, B. O. (1981). Nutritional value of fish viscera silage. *Journal of the Science of Food and Agriculture, 32*, 115–120.

Triki-Ellouz, Y., Ghorbel, B., Souissi, N., Kammoun, S., & Nasri, M. (2003). Biosynthesis of protease by *Pseudomonas aeruginosa* MN7 grown on fish substrate. *World Journal of Microbiology and Biotechnology, 19*, 41–45.

Toyota, E., Ng, K. K. S., Kuninaga, S., Sekizaki, H., Itoh, K., Tanizawa, K., et al. (2002). Crystal structure and nucleotide sequence of an

anionic trypsin from chum salmon (*Oncorhynchus keta*) in comparison with Atlantic salmon (*Salmo salar*) and bovine trypsin. *Journal of Molecular Biology, 324*, 391–397.

Zukowski, M. M. (1992). Production of commercially valuable products. In R. H. Doi & M. Mc. Gloughlin (Eds.), *Biology of bacilli: application to industry* (pp. 311–337). London: Butterworth-Heinemann.