

Full Length Research Paper

Partial purification and characterization of alkaline proteases from the Black Sea anchovy (*Engraulis encrasicolus*) digestive tract

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Alkaline proteases from the digestive tract of anchovy were partially purified by ammonium sulfate fractionation, dialysis and Sephadex G-75 gel filtration. The purification fold and yield were 6.23 and 4.49%, respectively. The optimum activities of partially purified alkaline proteases were observed at 60°C and at pH 11.0. The alkaline proteases were stable within the temperature range of 40 to 50°C and pH range of 9.0 to 11.0. They were inhibited by the serine-protease inhibitor phenylmethylsulfonyl fluoride (PMSF) and trypsin specific inhibitor benzamidine, but were not inhibited by the β -mercaptoethanol. The enzymes were slightly activated by metal ions such as Na^+ and Ba^{2+} and inhibited by Cu^{2+} , Zn^{2+} , K^+ and Mn^{2+} at different degrees. The molecular weight of the partially purified enzyme was 24 kDa by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Key words: Alkaline proteases, *Engraulis encrasicolus*, purification, characterization, digestive tract.

INTRODUCTION

Anchovy (*Engraulis encrasicolus*) is a small marine fish, approximately 12 cm in length and it has the highest catch rate among the sea fishes in Turkey (Ustun and Turhan, 1997). According to Turkish Statistical Institute 2009 findings, anchovy originated products constituted 204699 tons of the total fishery products harvest, which was 623191 tons (Sea Products Statistics, 2010). Caught anchovies are used for human consumption in diverse forms such as fresh, frozen fillets, canned and salted. Anchovy processing generates a vast quantity of residues composed of the fish head and viscera which have been traditionally converted to low value by-products such as fish flour powder used as animal feed ingredient or discarded directly to the environment causing pollution problem. Consumable anchovy meat amount is proportionately 57 to 61% (Ustun and Turhan, 1997), and so 80000 to 88000 tons of by-products are generated.

Viscera, one of the most important by-products of

fishing industry, are recognized as potential source of digestive enzymes, especially proteases with high activity over a wide range of pH and temperature conditions (Castillo-Yanez et al., 2006; Klomkiao et al., 2006a; Bougatef et al., 2007; Khaled et al., 2008). Proteases constitute the most important group of industrial enzymes used in the world today (Bougatef et al., 2007; El-Beltagy et al., 2004; Souza et al., 2007), accounting for about 50% of the total industrial enzyme market (Bougatef et al., 2007; Souza et al., 2007). There are several potential uses of proteases for industrial applications involving detergent, food processing, agrochemical and pharmaceutical industries (Hau and Benjakul, 2006; Temiz et al., 2007). Alkaline proteases are mostly used as enzyme-containing detergent powders, besides their minor uses in food processing are for the childproofing of beer and production of protein hydrolysate (Temiz et al., 2007; Moreira et al., 2001).

Various studies have been conducted on new sources of proteolytic enzymes. Martinez et al. (1988) have studied about the purification and characterization of two trypsin-like enzymes from the digestive tract of anchovy (*E. encrasicolus*) caught in the Bay of Biscay. Heu et al.

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(1997) purified and characterized cathepsin L-like enzyme from anchovy (*Encraulis japonica*) muscle. Siringan et al. (2007) partially purified and biochemically characterized trypsin-like proteinases from Indian anchovy (*Stolephorus* spp.). However, there is no valid information regarding purification and characterization of enzymes from the viscera of Black Sea anchovy. Marine animal enzymes tend to exhibit differences in biochemical structure due to the varying marine habitat conditions (Shi et al., 2007).

Today, there is an increasing demand for fish proteolytic enzymes in food processing. Utilization of anchovy viscera in proteolytic enzyme production can be a solution to meet this demand. Thus, in the present study, we described the partial purification and characterization of proteases from the Black Sea anchovy viscera and provide basic information on the storage conditions of food products containing these enzymes and also on basic information on the enzyme characteristics that might contribute to the commercial application of these enzymes to various foods.

MATERIALS AND METHODS

Reagents

Sephadex G-75, benzamidine, sodium phosphate, γ -globulin, azocasein, ammonium sulfate, sodium dodecyl sulphate (SDS), acrylamide, N,N,N,N-tetramethylethylenediamine, phenylmethylsulfonyl fluoride (PMSF), bovine albumin, egg albumin, glyceraldehydes-3-phosphate dehydrogenase, bovine carbonic anhydrase, bovine pancreas trypsinogen and soybean trypsin inhibitor were purchased from Sigma-Aldrich Chemical Co. (St Louis, MO, USA). Tris (hydroxymethyl aminomethane), ethylenediaminetetraacetic acid (EDTA) and β -mercaptoethanol were obtained from Merck Chemical Co. (Darmstadt, Germany). Trichloroacetic acid (TCA) was procured from J. Y. Baker Chemical Co. (Deventer, The Netherlands). All other reagents were of analytical grade.

Anchovy digestive tract

Anchovy (*E. encrasicholus*) was purchased from the fish market at Samsun, Turkey. It was subjected to water wash and digestive tracts were separated and stored in sealed plastic bags at -20°C until used for enzyme extraction.

Enzyme extraction

Enzyme extraction was carried out using the methods of Temiz et al. (2007, 2008) with a slight modification. The stored digestive tracts (100 g) were partially thawed in a refrigerator at 4°C , mixed with 200 mL of 10% NaCl, extracted by a blender (Warring blender 8010 32BL79, CT, USA) for 5 min at 4°C , and subsequently allowed to rest for 2 h at 4°C . The mixture was centrifuged (Sigma, Model 3K30, Osterodeam Harz, Germany) at $26\ 000 \times g$ for 15 min at 4°C . The pellet was discarded and the supernatant was collected. The pH of the supernatant was adjusted to 5.5 with 4 M HCl, rested for 15 min at 4°C , and then centrifuged at $26\ 000 \times g$ for 15 min at 4°C . The precipitate was discarded and the supernatant was collected in a fresh vial and used as crude extract.

Enzyme purification

Step 1

Ammonium sulphate precipitation: The crude extract was subjected to ammonium sulphate fractionation (0 to 20, 20 to 40 and 40 to 60%, w/v saturation) and rested for 30 min at 4°C , and then centrifuged at $15\ 000 \times g$ for 10 min at 4°C . The precipitate was dissolved with 10 mM sodium phosphate buffer 1:10 (v/v), pH 6.5.

Step 2

Dialysis: The 20 to 40% (w/v) ammonium sulphate precipitate obtained from the previous step was dialyzed against the same buffer (10 mM sodium phosphate of pH 6.5) for 12 h at 4°C .

Step 3

Gel Filtration, The enzyme dialysate was subjected to gel filtration using Sephadex G-75 resin. Three grams of resin was swollen overnight in 0.01 M sodium phosphate buffer at pH 6.5. A column (1.6 x 40 cm) was packed and equilibrated by eluting the same buffer at a flow rate of 2 mL min with a computerized controlled peristaltic pump. One milliliter (1 mL) of precipitate was loaded onto the column and eluted with buffer solution, and 2 mL fractions were collected. The absorbance values of the fractions were measured with flow cell (Quartz, 1 mL) by using a ultra-violet (UV)-visible spectrophotometer detector (Helios Gamma UV-Visible Spectrophotometers, Termo Spectronic, Cambridge, UK) at 280 nm. The collection procedure was repeated five times (Temiz et al., 2007; 2008).

Protein content

The protein contents of the crude extract and fractions were determined by the method of Bradford (1976) using γ -globulin as a standard.

Total proteolytic activity

Total proteolytic activity was measured according to the spectrophotometric method based on azocasein described by Garcia-Carreno and Haard (1993) with a slight modification. Fifty microliter (50 μL) of the enzyme solution was mixed with 0.5 mL of 1.0% azocasein in 10 mM sodium phosphate buffer, pH 6.5, containing 1 mM CaCl_2 , and incubated for 30 min at 30°C . The reaction was stopped by adding of 0.5 mL 20% trichloroacetic acid. The mixture was allowed to stand at 4°C for 30 min and then centrifuged at $12\ 000 \times g$ for 10 min at 4°C to remove the precipitate. The absorbance of the supernatant was measured at 440 nm. One unit of total proteolytic activity was defined as the amount of enzyme that produces an increase in the absorbance of 1.0 in 1 h at 440 nm. Specific activity was expressed as the amount of unit activity per mg of protein.

Effect of pH on activity and stability

Activities of partially purified proteases were assayed over the pH range of 5.0-13.0 at 30°C for 30 min, using 1.0% azocasein as a substrate. The effect of pH on the enzyme stability was evaluated by measuring the residual enzyme activity after incubation at various pHs for 30 min at 30°C . The following buffer systems were

Table 1. Summary of purification of alkaline proteases from digestive tract of anchovy (n=3)*.

Purification step	Protein (mg mL ⁻¹)	Total proteolytic activity (U mL ⁻¹)	Specific activity (U mg ⁻¹)	Yield (%)	Purification factor
Crude extract	4.910	11.38	2.318	100	1.0
Ammonium sulphate precipitation					
0-20%	0.48	1.31	2.75	11.53	1.19
20-40%	1.71	6.56	3.86	57.61	1.66
40-60%	1.25	2.99	2.39	26.28	1.03
Dialysis	1.10	5.21	4.75	45.79	2.05
Gel filtration	0.037	0.51	14.51	4.49	6.23

*All operations were carried out at 4°C. Only precipitate formed between 20 to 40% (w/v) saturation with ammonium sulphate was subjected to dialysis and gel filtration on Sephadex G-75.

used: 100 mM citrate phosphate buffer, pH 5.0; 100 mM Tris-HCl buffer, pH 7.0 to 12.0; 100 mM NaOH-KCl buffer of pH 13.0.

Effect of temperature on activity and stability

To investigate the effect of temperature, the activities of partially purified proteases were tested at different temperatures ranging from 30 to 70°C, using azocasein as a substrate for 30 min at pH 6.5. For thermal stability, the enzymes were incubated at various temperatures (40, 50 and 60°C) for 15, 30, 45 and 60 min, then cooled rapidly in an ice bath for 5 min and residual activity was assayed.

Effects of inhibitors

The effects of inhibitors on activities of partially purified proteases were studied using phenylmethylsulfonyl fluoride (PMSF), benzamidine, β-mercaptoethanol and EDTA. After were pre-incubated with inhibitors for 30 min at 30°C, the activities of remaining enzymes were measured and then the percentage inhibition was calculated.

Effects of metal ions

The effects of various metal ions (5 mM) on activity of partially purified proteases were investigated using Mn²⁺, Zn²⁺, Na⁺, K⁺, Cu²⁺, Ba²⁺ and Mg²⁺.

Determination of molecular weight

The molecular weight was estimated with sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) by the method of Laemmli (1970) with bovine albumin (66 kDa), egg albumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), bovine carbonic anhydrase (29 kDa), bovine pancreas trypsinogen (24 kDa) and soybean trypsin inhibitor (20.10 kDa) as standard proteins.

RESULTS AND DISCUSSION

Partial purification of alkaline proteases

The purification steps, protein concentration, specific

activity and yield of alkaline proteases are shown in Table 1. In the first purification step, the 20 to 40% ammonium sulfate precipitate showed higher specific activity (3.86 U/mg) than 0 to 20% and 40 to 60% ammonium sulphate precipitate. 1.66-fold increase in purity was obtained by the 20 to 40% ammonium sulfate precipitate. Ammonium sulphate precipitation was a simple method and generally introduced as an initial step. The saturated solution selectively precipitates proteins by the salt out mechanism from the crude extract (Klomkiao et al., 2006b; Khantaphant and Benjakul, 2010; Wang et al., 2010). Martinez et al. (1988) found that ammonium sulphate precipitation (20-70%) of trypsin-like enzyme from anchovy digestive tract resulted in a 2.7-fold increase in purity.

The 20 to 40% ammonium sulphate precipitate, which gave the highest specific activity was then dissolved in 10 mM sodium phosphate buffer of pH 6.5 and dialyzed for 12 h at 4°C. The dialysis step increased the specific activity from 3.86 to 4.75 and purification factor 1.66 to 2.05 (Table 1). These results show that the dialysis procedure after ammonium sulphate precipitation increases the purification factor and the specific activity. El-Beltagy et al. (2004) and Olivás-Burrola et al. (2001) reported that dialysis of 40 to 60% ammonium sulphate precipitate obtained from the viscera of boliti fish and the digestive tract of tropical sierra increased the purification factor from 1.5 to 8.9 and from 4.16 to 4.33, respectively.

When the dialyzed enzymes were subjected to gel filtration on a Sephadex G-75 column, only one active peak was observed as shown in Figure 1, in which the elution profiles of proteolytic activity and proteins from Sephadex G-75 are shown. This active peak has a high specific activity (14.51 U mg⁻¹) and purification fold (6.23), as shown in Table 1. These results suggest that anchovy viscera, the most important by-product of fishing industry might be a good source of proteases production. El-Beltagy et al. (2004) purified an acidic protease from viscera of boliti fish (*Tilapia nilotica*), using ammonium sulphate, dialysis and gel filtration. Purity of 18.3-fold and specific activity of 1.10 U mg⁻¹ were achieved for acidic

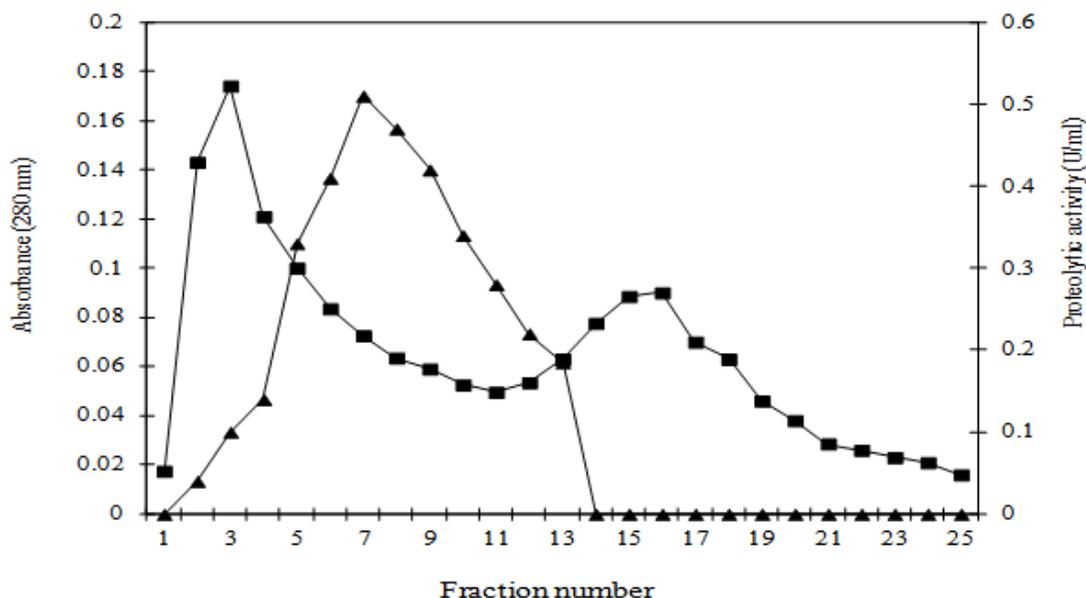


Figure 1. Partial purification profile of alkaline proteases from the digestive tract of anchovy by gel filtration on sephadex G-75 column. ■, absorbance at 280 nm; ▲, proteolytic activity (n=3).

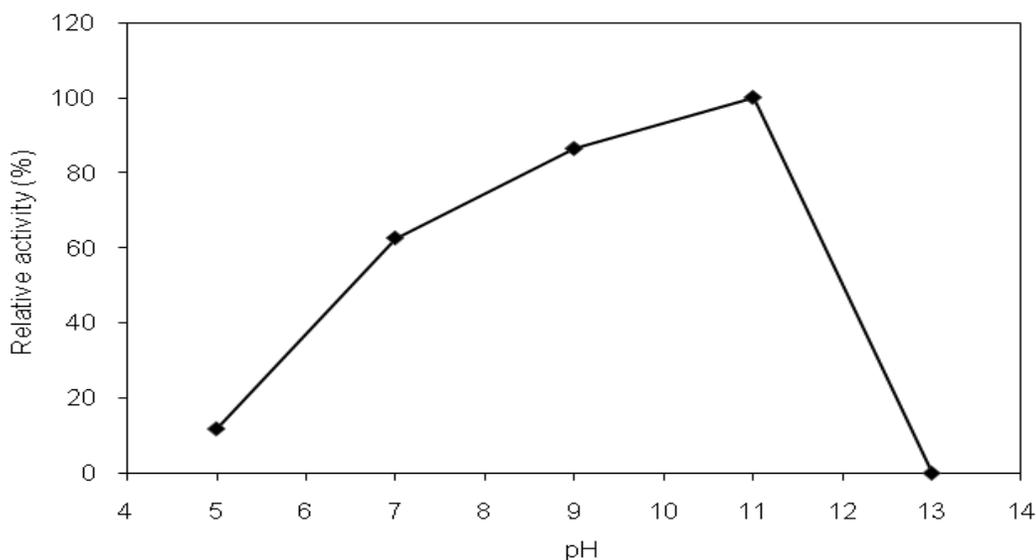


Figure 2. Effect of pH on activity of alkaline proteases. The activity was evaluated using azocasein as a substrate in the pH range of 5.0 to 13.0 at 30°C. The maximum activity obtained at pH=11.0 was considered as 100 % (n=3).

protease.

Trypsin-like enzyme from intestine and pyloric caeca of spotted goatfish (*Pseudupeneus maculatus*) was purified to homogeneity with 96.0 and 57.7-fold purity, using heat treatment, ammonium sulphate precipitation and gel filtration, respectively (Souza et al., 2007).

Effect of pH on activity and stability

The effect of pH on enzyme activity was determined over a pH range of 5.0 to 13.0. The partially purified enzyme was active between pH 7.0 and 11.0, with an optimum pH 11.0 when azocasein was used as substrate (Figure 2). The relative activity at pH 7.0 was about 63% of that at

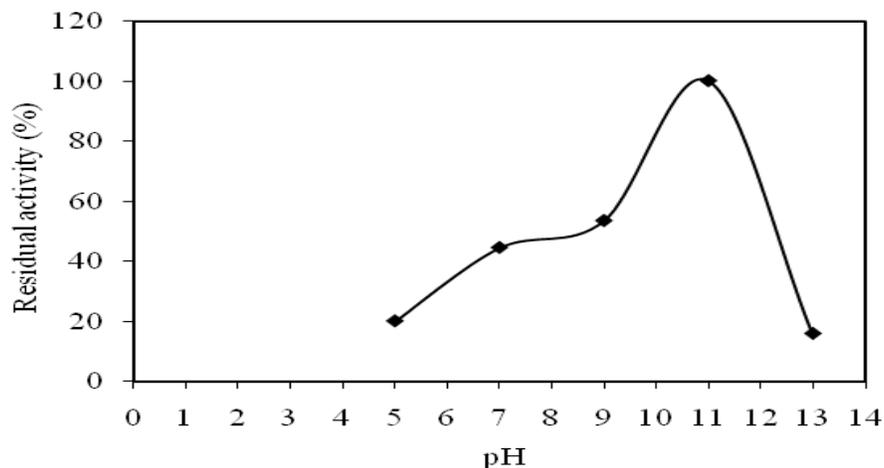


Figure 3. Effect of pH on stability of alkaline proteases. The pH stability of the enzyme was determined by incubating the enzyme in different buffers for 30 min at 30°C and the residual activity was measured at pH=11.0. The activity of the enzyme before incubation was taken as 100% (n=3).

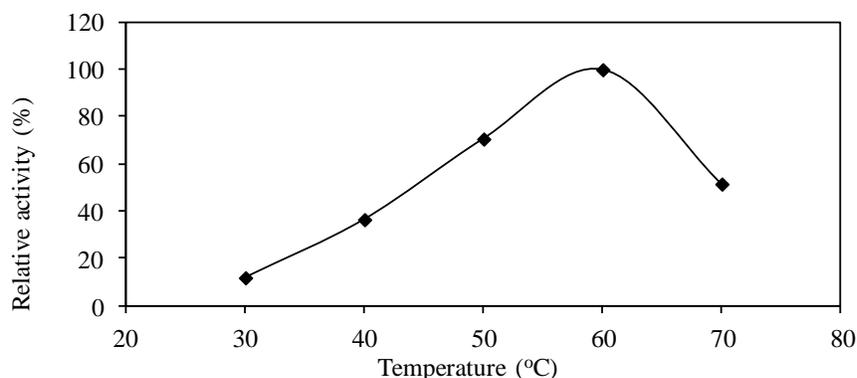


Figure 4. Effect of temperature on activity of alkaline proteases. Activity was evaluated using azocasein as a substrate. The temperature profile was determined by assaying proteolytic activity at temperatures between 30 and 70°C. The activity of the proteases at 60°C was taken as 100% (n=3).

pH 11.0. Very acidic pH conditions caused loss of activity while there was no activity at pH 13.0. This is probably because of enzyme denaturation at very acidic and very high alkaline pH values. Similar values have been reported for spotted goatfish *P. maculatus* (Souza et al., 2007) and hybrid tilapia *O. niloticus* x *O. aureus* (Wang et al., 2010).

The pH stability of proteases from anchovy is shown in Figure 3. The enzyme was very stable in a narrow pH range, maintaining 100% of its original activity at pH 11.0 and 55% of its activity at pH 9.0. Various other authors including Martinez et al. (1988), Siringan et al. (2007) and Souza et al. (2007) concluded that fish trypsin-like enzymes were stable in the alkaline pH range. However, anchovy proteases were unstable below pH 7.0 and at

pH 13.0. This finding is in agreement with that of trypsin from sardinelle (Khalend et al., 2008). According to our results, it is clear that the proteases from anchovy digestive tract could be a potential candidate to be added to commercial laundry detergents having a pH range generally between 9.0-11.0 (Jellouli et al., 2009).

Effect of temperature on activity and stability

The effect of temperature on activity was determined by assaying enzyme activity at different temperatures, using azocasein as a substrate (Figure 4). The partially purified proteases from anchovy were active at temperatures from 40 to 70°C and had an optimum at 60°C. The

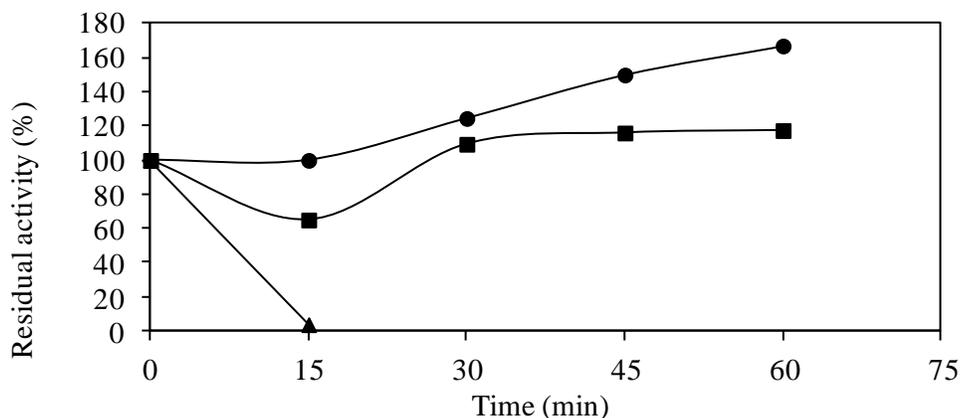


Figure 5. Effect of temperature on stability of alkaline proteases. The temperature stability was determined by incubating the alkaline proteases at different temperatures (●, 40°C; ■, 50°C; ▲, 60°C) for 60 min at pH=6.5. Aliquots were withdrawn at 15 min intervals and residual enzyme activities were determined under standard conditions. The non-heated enzyme was considered as the control (100 %) (n=3).

Table 2. Effect of inhibitors on the activity of alkaline proteases from digestive tract of anchovy (n=3).

Inhibitor	Concentration (mM)	Inhibition (%)
Control	-	0
PMSF	5	71
Benzamidine	5	49
β-mercaptoethanol	5	0
EDTA	2	19

relative activities at 40, 50 and 70°C were about 37, 71 and 52%, respectively, of that at 60°C. Generally, these data shows parallelism with those reported by Kurtovic et al. (2006), Bougatef et al. (2007), Khantaphant and Benjakul (2010) and Wang et al. (2010). The optimal temperature range of trypsin-like proteinases in Indian anchovy was from 50 to 60°C (Siringanet et al., 2007). Trypsin-like enzyme from spotted goatfish has the optimal temperature of 52°C (Souza et al., 2007). The differences in optimal temperatures can be associated with the different living temperatures.

The effect of temperature on stability of partially purified proteases is shown in Figure 5. As can be seen from the figure, during incubation at 40°C, the activity of the enzyme increased after first 15 min, and it remained stable after 30 min of incubation at 50°C. In resemblance with the results of our study, Bougatef et al. (2007) asserted that the trypsin from the viscera of sardine at 40 and 50°C remained significantly active even after 250 min of incubation. In addition, Castillo-Yanez et al. (2005), Shi et al. (2007) and Khaled et al. (2008) reported that good thermal stability was observed in fish trypsins incubated at 40°C for 15-45 min, while the enzyme lost all its activity

when heated at 60°C for 15 min. The trypsin from hoki fish (Shi et al., 2007) and *Sardinella viscera* (Khaled et al., 2008) were found to be inactive when heated at 60°C for 30 min. According to Lu et al. (2008), trypsin of mandarin fish lost its activity when heated at 50°C for 30 min. Thermal stabilities of fish proteolytic enzymes vary with species and substrate differences, in addition to incubation conditions.

Effect of enzyme inhibitors

The effect of several inhibitors on anchovy proteases activity were determined and the results are summarized in Table 2. As seen, the enzyme was inhibited by the serine-protease inhibitor PMSF (71%) and trypsin specific inhibitor benzamidine (49%). These results show that the partially purified enzyme from anchovy digestive tract exhibit protease characteristics. Our results are similar with other data in the study of Martinez et al. (1988), Kristjansson (1991), Bougatef et al. (2007), Shi et al. (2007) and Lu et al. (2008) since they reported the inhibition of fish trypsins by PMSF and benzamidine. The

Table 3. Effect of metal ions (5 mM) on the activity of alkaline proteases from digestive tract of anchovy (n=3).

Ion	Relative activity (%)
Control	100
Mn ²⁺	84
Zn ²⁺	24
Na ⁺	108
K ⁺	76
Cu ²⁺	3
Ba ²⁺	108
Mg ²⁺	97

enzyme was not affected by β -mercaptoethanol. Based on the results, metallo-protease inhibitor EDTA, which chelates the metals ions required for the enzyme, partially inhibited enzyme activity by 19%, indicating the importance of some metal ions in enzyme stabilization. It was suggested that metal ions most likely were also the factors for enzyme activity.

Effect of metal ions

The effects of some metal ions (5 mM) on the activity of alkaline proteases from digestive tract of anchovy are shown in Table 3. According to the results, Na⁺ and Ba²⁺ are responsible for a slight increase in the enzyme activity. Mg²⁺ showed no influence on the enzyme activity. However, Cu²⁺ and Zn²⁺ strongly decreased the enzyme activity. Our results share similarity with the study of Bezerra et al. (2005) and Souza et al. (2007) concluding that Cu²⁺ and Zn²⁺ inhibited the activity of trypsin-like enzymes and alkaline proteases strongly. The inhibition effects by Mn²⁺ and K⁺ on anchovy alkaline proteases were less intensive than those displayed by the Cu²⁺ and Zn²⁺ ions.

Molecular weight of enzyme

The SDS-PAGE corresponding to the different stages of the purification process is shown in Figure 6. The last step (gel filtration fraction) showed a single band on SDS-PAGE. The molecular weight of this band was estimated to be 24 kDa which is similar with the value that is reported for trypsin from the spleen of yellowfin tuna (*Thunnus albacores*) (Klomklao et al., 2006a) and tongol tuna (*Thunnus tonggol*) (Klomklao et al., 2006b). Generally, fish trypsins have been reported to have molecular weights in the range of 23-28 kDa (Jellouli et al., 2009).

Conclusion

In the present study, proteolytic enzymes from anchovy

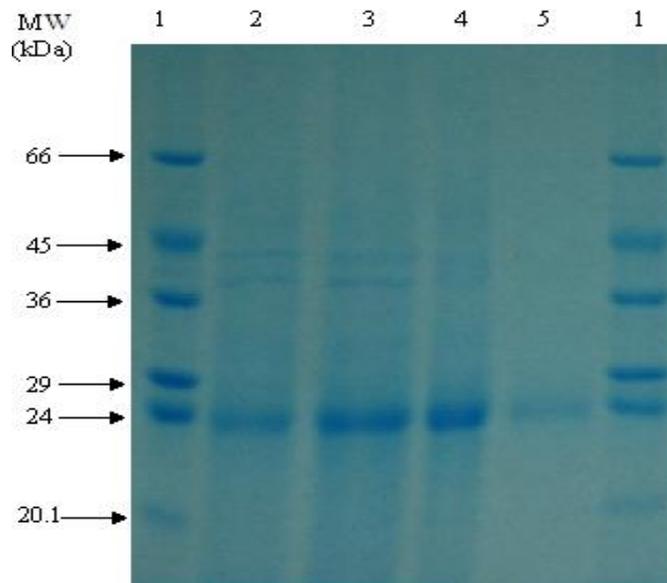


Figure 6. SDS-PAGE of the different purification steps for alkaline proteases from digestive tract of anchovy. Line 1, standard proteins; line 2, crude extract; line 3, 20-40% ammonium sulphate precipitation; line 4, 20-40% dialysis; line 5, gel filtration (n=3).

harvested in Turkey were partially purified and identified based on the molecular weight, inhibitor and metal ion sensitivity, optimal pH and temperature, and pH and temperature stability. They were inhibited by PMSF and benzamidine, slightly activated by metal ions such as Na⁺ and Ba²⁺ and inhibited by Cu²⁺, Zn²⁺, K⁺ and Mn²⁺. The enzyme showed an optimum temperature at 60°C and optimum pH of 11.0. The enzyme activity was stable at pH range of 9.0 to 11 and at temperatures in the range of 40 to 50°C. Molecular weight of the partially purified enzymes was estimated to be 24 kDa. Further purification and characterization should be applied for better results. This alkaline protease may have utility for food and detergent industries.

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