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Partial purification and characterization of trypsin-like proteinases in Indian anchovy (*Stolephorus* spp.)

Patcharin Siringan [a], Nongnuch Raksakulthai [b], Jirawat Yongsawatdigul [a,*]

[a] *School of Food Technology, Institute of Agricultural Technology, Suranaree University of Technology, Nakhon Ratchasima 30000, Thailand*
[b] *Department of Fishery Products, Faculty of Fisheries, Kasetsart University, Bangkok 10900, Thailand*

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Abstract

Four fractions (P111, P21, P31, and P4) of proteinases were obtained from various purification steps including heat treatment (60 °C, 10 min), 30–60% ammonium sulfate precipitation, anion exchange, hydrophobic interaction, and gel filtration chromatography. Optimal temperature and pH of all fractions were 50–60 °C and 8.5, respectively. All partially purified proteinases preferably hydrolyzed substrates containing Arg at the P_1 position. All proteinases were inhibited by soybean trypsin inhibitor, leupeptin, and *N*-tosyl-L-lysine chloromethyl ketone. Partially purified proteinases were stable at 35 °C up to 12 h. However, their activity decreased about 40% when incubated at the optimal temperature (50–55 °C) for 2 h. Only P111 was stable at its optimal temperature (60 °C) up to 12 h. Molecular weight (MW) of P111, P21, and P31 was estimated to be 27, 33, 37, 43, 48, 55, 60, and 65 kDa, while MW of P4 was 39 kDa based on activity staining. All partially purified proteinases hydrolyzed washed anchovy mince at 4.0 M NaCl, pH 8.5, at 35 °C and at their optimal temperatures (50–60 °C).

Keywords: Indian anchovy (*Stolephorus* spp.); Trypsin-like proteinase; Partial purification; Characterization

1. Introduction

Indian anchovy (*Stolephorus* spp.) is a pelagic small fish containing high proteinase activity and are popularly used as a raw material for fish sauce production in Southeast Asia. Fish sauce is a traditional condiment prepared by mixing anchovies with 25–30% salt and fermenting at ambient temperature (30–40 °C) for about 12–18 months. Protein solubilization slowly occurs during fermentation by the action of proteolytic activity to produce amino acids and small peptides. Proteinases involved in muscle degradation have been studied. Martinez and Gildberg (1988) reported that proteolytic enzymes from the digestive tract of anchovies (*Engraulis encrasicholus*) degraded abdominal tissue. Heu, Pyeun, Kim, and Godber (1991) reported that two alkaline proteinases isolated from viscera of anchovy (*E. japonica*) were identified to be chymotrypsin-like serine proteinases. Two trypsin-like enzymes isolated from the digestive tract of anchovy were important for muscle degradation (Martinez, Olsen, & Serra, 1988). Two neutral serine proteinases were purified from salted anchovy (*E. japonica*) (Ishida, Sugiyama, Sato, & Nagayama, 1995). However, proteinases from Indian anchovy have never been purified and characterized.

Trypsin-like proteinase activity was observed in the first 2 months of the Philippino fish sauce (patis) fermentation (Orejana & Liston, 1982). In addition, trypsin-like proteinase activities were predominantly found in Indian anchovy (*Stolephorus* spp.). These enzymes hydrolyzed muscle proteins at high salt content (4.0 M NaCl) (Siringan, Raksakulthai, & Yongsawatdigul, 2006a). Thus, these proteinases might play an important role in protein hydrolysis during fish sauce fermentation. Furthermore, activities of these enzymes were detected in fish sauce throughout 12 months of fish sauce fermentation (Siringan, Raksakulthai,

* Corresponding author. Tel.: +66 44 22 4359; fax: +66 44 22 4387.
E-mail address: jirawat@sut.ac.th (J. Yongsawatdigul).

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& Yongsawatdigul, 2006b). Thus, the objective of this study was to partially purify and characterize trypsin-like proteinases in Indian anchovy.

2. Materials and methods

2.1. Materials and chemicals

Indian anchovies (*Stolephorus* spp.) were caught off the Gulf of Thailand, Chonburi province and transported to the laboratory at Suranaree University of Technology within 6 h after catch. Average fish weight was 2.61 ± 0.47 g with the length of 7.59 ± 0.34 cm. Chemicals purchased from Sigma Chemical Co. (St. Louis, MO, USA) were bovine serum albumin (BSA), L-tyrosine, dimethyl sulfoxide (DMSO), Brij 35, butyloxycarbonyl (Boc)-Gln-Ala-Arg-7-amido-4-methylcoumarin (AMC), Boc-Val-Leu-Lys-AMC, and carbobenzoxy (Z)-Arg-Arg-AMC, 2-mercaptoethanol (BME) and proteinase inhibitors. Acrylamide, ammonium persulfate and Tris–base were purchased from Promega Corporation (Madison, WI, USA). Standard molecular weight broad range, sodium dodecylsulfate (SDS), Coomassie blue, and Bis (*N*,*N*′-methylane-bis-acrylamide) were purchased from Bio-Rad Laboratories (Hercules, CA, USA). Boc-Asp(oBzl)-Pro-Arg-AMC, succinyl (Suc)-Ala-Ala-Pro-Phe-AMC and Z-Phe-Arg-AMC were purchased from Bachem A.G. (Bubendorf, Switzerland). Diethylaminoethyl (DEAE)-Sephacel, Sephacryl S-300, and phenyl-Sepharose were purchased from Amersham Biosciences (Uppsala, Sweden). All other chemicals were of analytical grade.

2.2. Purification of proteinases

Crude extract was prepared by homogenizing whole anchovy with ice-cold 20 mM phosphate buffer, pH 7.0, at a ratio of 1:2 using a homogenizer (Nihonseiki Kaisha Ltd., Tokyo, Japan). The homogenate was centrifuged at 17,500*g* for 20 min at 4 °C. The supernatant was filtered through four layers of cheesecloth and Whatman paper No. 4 and used as a crude extract. The crude extract was precipitated at 30–60% saturation of ammonium sulfate. The precipitates were dissolved in 50 mM Tris–HCl, pH 8.0 and subsequently dialyzed against 100 volumes of the same buffer for 24 h using SnakeSkin™ pleated dialysis tubing with 10,000 molecular weight cutoff (MWCO) (Pierce Chemical Co., Rockford, IL, USA). The dialysate was centrifuged at 5000*g* for 5 min. The supernatant was loaded onto a DEAE-Sephacel chromatography column (1.5×30 cm) equilibrated with 50 mM Tris–HCl, pH 8.0 and eluted using a linear gradient of 0–1.0 M NaCl, 50 mM Tris–HCl, pH 8.0 at a flow rate of 0.5 ml/min. Fractions of 5 ml were collected and those containing proteinase activity were pooled and applied onto a phenyl-Sepharose column (1.5×30 cm) equilibrated with 50 mM Tris–HCl, pH 8.0, containing 1.5 M ammonium sulfate. After the column was washed with 3 bed volumes of 50 mM Tris–HCl, pH 8.0, containing 1.5 M ammonium sulfate, a linear gradient from 1.1 to 0 M ammonium sulfate was performed at a flow rate of 0.5 ml/min, and a fraction of 5 ml was collected. Fractions containing proteinase activity were pooled and concentrated using 30,000 MWCO ultrafiltration membrane (Vivascience A.G., Hannover, Germany). Concentrated sample was applied to Sephacryl S-300 column (1.5×80 cm) equilibrated with 50 mM Tris–HCl, pH 8.0. The elution was carried out with the same buffer at a flow rate of 0.5 ml/min. The profile of protein elution was measured at 280 nm and proteinase activities were determined as described below. Protein content was measured according to Lowry, Rosenbrough, Farr, and Randall (1951).

2.3. Proteinase activity assay

Proteolytic activity was determined according to the method of Barrett and Kirschke (1981) and Ishida et al. (1995). The reaction mixture containing 0.8 ml of 0.2 M Tris–HCl (pH 8.5), 0.1 ml of 10 μM of Boc-Asp(oBzl)-Pro-Arg-AMC and 0.1 ml of diluted enzyme solution with 0.1% Brij 35 in a total of 1 ml, was incubated at 60 °C for 5 min. The reaction was stopped by adding 1.5 ml of the stopping reagent (methanol:*n*-butanol:distilled deionized water = 35:30:35 (v/v/v)) followed by heating at 95 °C for 3 min. Fluorescence intensity of the liberated 7-amino-4-methylcoumarin (AMC) was measured with a spectrofluorophotometer RF-1501 (Shimadzu Co., Kyoto, Japan) at an excitation wavelength of 380 nm and an emission wavelength of 460 nm. Unit activity was defined as 1 μmole of AMC released/min. Specific activity was expressed as the amount of unit activity per mg protein.

2.4. Temperature and pH optimum

Activities of partially purified proteinases were determined at various temperatures (30–65 °C) at pH 8.5. The activity was also assayed at the optimal temperature and various pHs ranging from 5.5 to 11: pH 5.5–7.0, using McIlvaine's buffer (0.1 M sodium citrate and 0.2 M sodium phosphate); pH 8.0–9.0, using 0.2 M Tris–HCl; pH 9.5–11, using 0.1 M glycine–NaOH.

2.5. Substrate specificity

Various synthetic substrates, including Boc-Gln-Ala-Arg-AMC, Boc-Val-Leu-Lys-AMC, Boc-Asp(oBzl)-Pro-Arg-AMC, Suc-Ala-Ala-Pro-Phe-AMC, Z-Arg-Arg-AMC, and Z-Phe-Arg-AMC were used for determining substrate specificity of partially purified proteinases. Proteolytic activity was determined at the optimal pH and temperature.

2.6. Thermal stability

Partially purified proteinases were incubated at 35 °C and at their respective optimal temperatures for up to 12 h. The remaining activity was determined at respective

optimal temperature and pH at each time interval. Remaining activity was expressed as the percentage of activity remained after incubation at each time interval considering the activity measured in the sample without incubation as 100%.

2.7. Effect of proteinase inhibitors

The effect of various inhibitors, including phenylmethylsulfonyl fluoride (PMSF), leupeptin, bestatin, 1-(L-trans-epoxysuccinyl-leucylamino)-4-guanidinobutane (E-64), *N*-tosyl-L-lysine chloromethyl ketone (TLCK), ethylenediaminetetraacetic acid (EDTA), iodoacetic acid, soybean trypsin inhibitor (SBTI), SDS, dithiothreitol (DTT) was determined.

2.8. Activity staining

Molecular weight was estimated using activity staining according to the method of Garcia-Carreno, Dimes, and Haard (1993). Partially purified proteinases were separated on a 10% polyacrylamide gel (Laemmli, 1970). Gels were soaked in 2% casein, 50 mM Tris–HCl (pH 7.5) and shaken for 1 h on ice. Caseinolytic activity was initiated by transferring gels to 0.2 M Tris–HCl (pH 8.5) buffer containing 4.0 M NaCl, and incubated at 60 °C for 10 min. Subsequently, gels were stained in 0.125% Coomassie brilliant blue R-250 in 40% methanol and 10% acetic acid for 2 h. Destaining was carried out using 25% methanol and 10% acetic acid solution. Clear zone on the blue background indicated the presence of proteinase.

2.9. Hydrolysis of washed anchovy muscle at 4.0 M NaCl

Dorsal muscle of Indian anchovy was homogenized in 50 mM NaCl, 10 mM phosphate buffer, pH 7.0 at a ratio of meat to buffer of 1:5. Subsequently, the homogenate was centrifuged at 10,000*g* for 5 min at 4 °C. Precipitates were dissolved in the same buffer and then centrifuged at 10,000*g* for 5 min, 4 °C twice. The precipitates were designated as washed mince. Washed mince (6 g) was homogenized in 100 ml of 4.0 M NaCl in 0.2 M Tris–HCl, pH 8.5 and subsequently centrifuged at 5000*g* for 10 min at 4 °C. The supernatant was used as a substrate. Protein content of soluble washed mince protein was estimated to be 1 mg/ml by the Lowry method using BSA as a standard.

Hydrolysis of washed mince solution at 4.0 M NaCl, pH 8.5 was determined. Reaction mixture contained 1.1 ml of washed mince solution and 0.1 ml of partially purified proteinases (500 Unit/ml). The mixture was incubated either at a respective optimal temperature for 1 h or at 35 °C for 3 h. Reaction was stopped by adding 0.8 ml of 12.5% trichloroacetic acid (TCA). Oligopeptide contents were determined by Lowry method using tyrosine as a standard. Blanks were added 0.8 ml of 12.5% TCA before adding the enzyme. The control was carried out in the absence of proteinases.

3. Results and discussion

3.1. Purification of proteinases

Proteinases in whole Indian anchovy were separated to four peaks by DEAE Sephacel (Fig. 1). Each peak (P1–P3) exhibited several clear bands on SDS-PAGE activity staining (data not shown). There were several proteinases in each fraction with different MW. Only one distinct clear band was observed in P4. All fractions except for P4 were collected for further purification. P1, P2, and P3 fractions were applied onto hydrophobic interaction chromatography (Fig. 2a–c). Fraction P1 was separated to two peaks, P11 and P12 (Fig. 2a). P11 was eluted during washing the column, suggesting that P11 weakly interacted with hydrophobic media. Only one peak showing protolytic activity was obtained from each of P2 and P3, designated as P21 and P31, respectively (Fig. 2b and c). Hydrophobic interaction chromatography (HIC) appeared to effectively separate proteinases from other protein components. The purification fold of all fractions increased after HIC (Table 1). P11 and P12 fractions were further applied to a Sephacryl S-300 column (Fig. 3). Only one peak (P111) with high activity was obtained from P11. Separation of P12 resulted in fractions with similar characteristics (molecular weight, optimal pH and temperature) to P21 and a fraction with minimal activity at high salt (4 M NaCl). These fractions were not included for further studies. Only P111, P21, P31, and P4 were collected for further characterization.

3.2. Temperature and pH optimum

Activity of P111 sharply increased at 60 °C (Fig. 4a). Optimal temperature of P31 and P4 was 55 °C, while that of P21 was 50 °C (Fig. 4b–d). P21 and P31 exhibited the highest activity among partially purified proteinases obtained. Optimal temperature of these enzymes was similar to that of crude proteinase and autolytic activity of whole fish (Siringan et al., 2006a). Optimal pH of all fractions was 8.5 (Fig. 5). It should be noted that activities of these enzymes were minimal at pH 5.5–6.0 (Fig. 5). Therefore, it could be hypothesized that protein hydrolysis by these enzymes during fish sauce fermentation would be min-

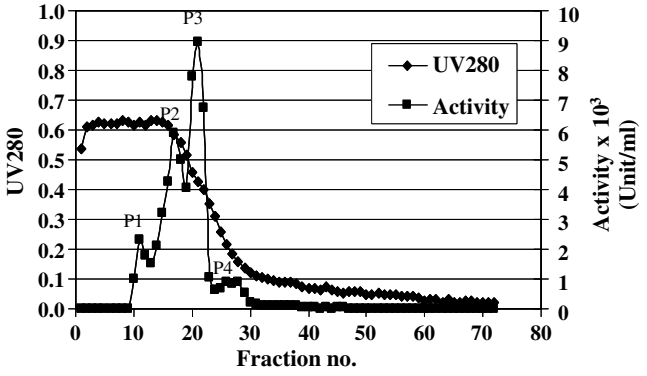

Fig. 1. Chromatogram of proteinases obtained from DEAE-Sephacel.

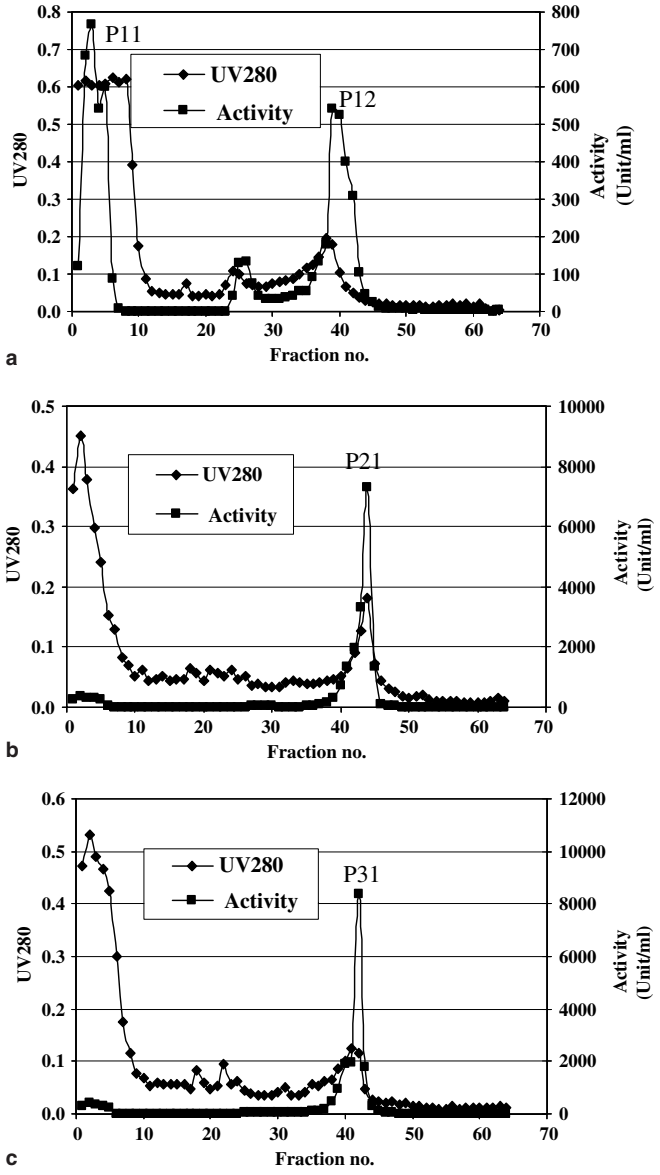

Fig. 2. Chromatogram of proteinases P1 (a), P2 (b), and P3 (c) obtained from phenyl-Sepharose.

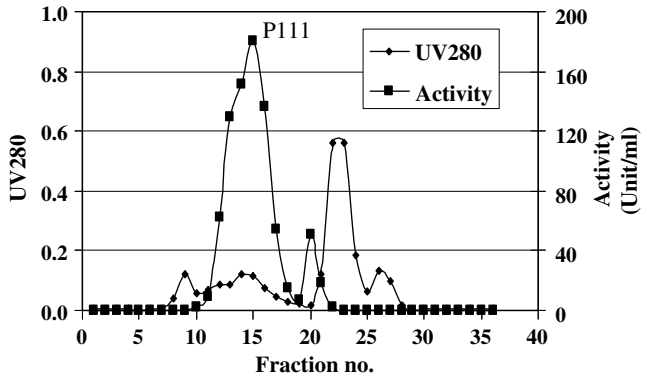

Fig. 3. Chromatogram of proteinases P11 obtained from gel filtration (Sephacryl S-300).

imal. This might explain a long fermentation period of fish sauce. Based on these results, all partially purified proteinases exhibited maximum activity at high temperature and alkaline pH. Alkaline proteinase in anchovy (*E. encrasicholus*) which was a predominant enzyme in salted anchovy showed the optimal temperature at 50 °C and pH 8.0 (Hernandez-Herrero, Roig-Sagues, Lopez-Sabater, Rodriguoz-Jerez, & Mora-Ventura, 1999). Two trypsin-like proteinases purified from the digestive tract of anchovy (*E. encrasicholus*) exhibited pH optimal at 8.0–9.0. Trypsin-like proteinases predominantly found in whole anchovy extract, were detected throughout 12 months of fish sauce fermentation, indicating that these enzymes were stable at high salt content (≈4.5 M NaCl) (Siringan et al., 2006b). Therefore, acceleration of protein hydrolysis by fish endogenous proteinase could be possible if optimal temperature and pH of proteinases are attained. Gildberg (2001) reported that rate of protein hydrolysis of fish sauce from male capelin and cod intestine increased when pH of fermentation was initially adjusted to pH 8.0, an optimal pH of cod trypsin.

3.3. Substrate specificity

All fractions efficiently hydrolyzed Boc-Asp(oBzl)-Pro-Arg-AMC and Boc-Gln-Ala-Arg-AMC (Table 2), which

Table 1
Purification table of partially purified proteinase from Indian anchovy

Steps	Total activity ($\times 10^3$ Units)	Specific activity ($\times 10^3$ Units/mg)	Purity (fold)	Yield (%)
Crude extract	1108	0.08	1.00	100
Heat treatment (60 °C, 10 min)	1653	0.24	2.86	149
30–60% $(NH_4)_2SO_4$ precipitation	1262	2.12	25.1	114
DEAE Sephacel (P1)	32.8	0.44	5.21	2.96
Phenyl Sepharose (P11)	7.23	0.33	3.91	0.65
Sephacryl S-300 (P111)	2.48	1.31	15.5	0.22
Phenyl Sepharose (P12)	33.2	24.6	292	2.99
Sephacryl S-300 (P121)	0.04	0.42	4.97	0.00
Sephacryl S-300 (P122)	1.45	11.2	132	0.13
Sephacryl S-300 (P123)	2.00	7.59	90.1	0.18
DEAE Sephacel (P2)	126	5.91	70.1	11.4
Phenyl Sepharose (P21)	253	188	2227	22.9
DEAE Sephacel (P3)	217	21.9	260	19.6
Phenyl Sepharose (P31)	359	218	2583	32.4
DEAE Sephacel 4 (P4)	53.1	15.4	183	4.80

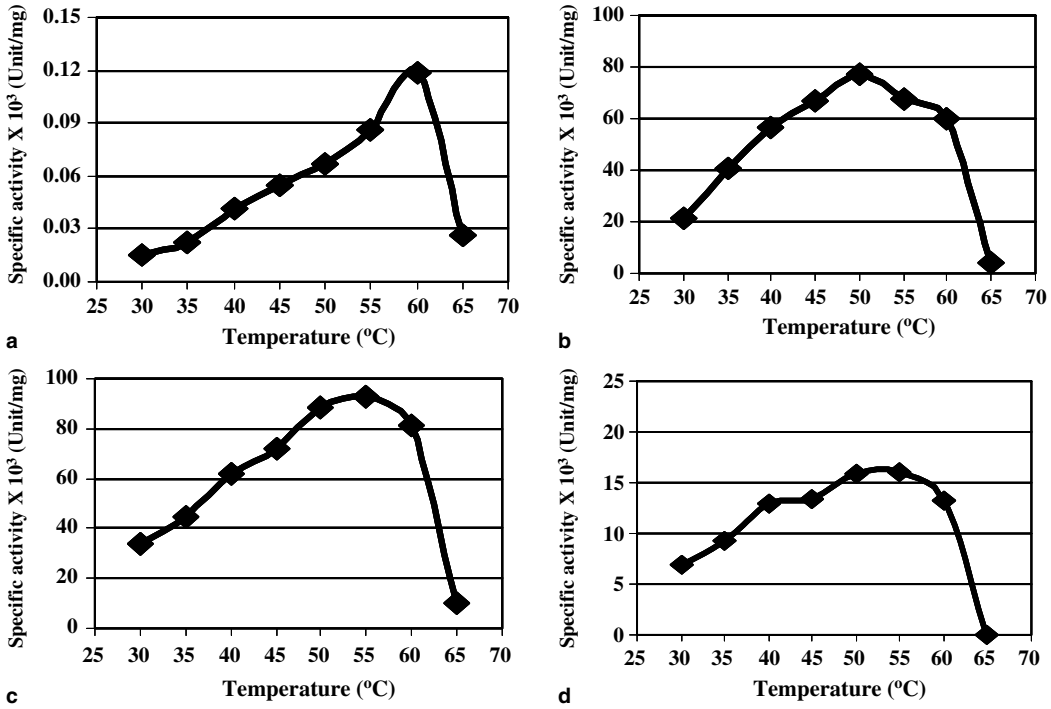

Fig. 4. Temperature profiles of partially purified proteinases, using Boc-Asp(oBzl)-Pro-Arg-AMC as a substrate assayed at pH 8.5, P111 (a), P21 (b), P31 (c), and P4 (d).

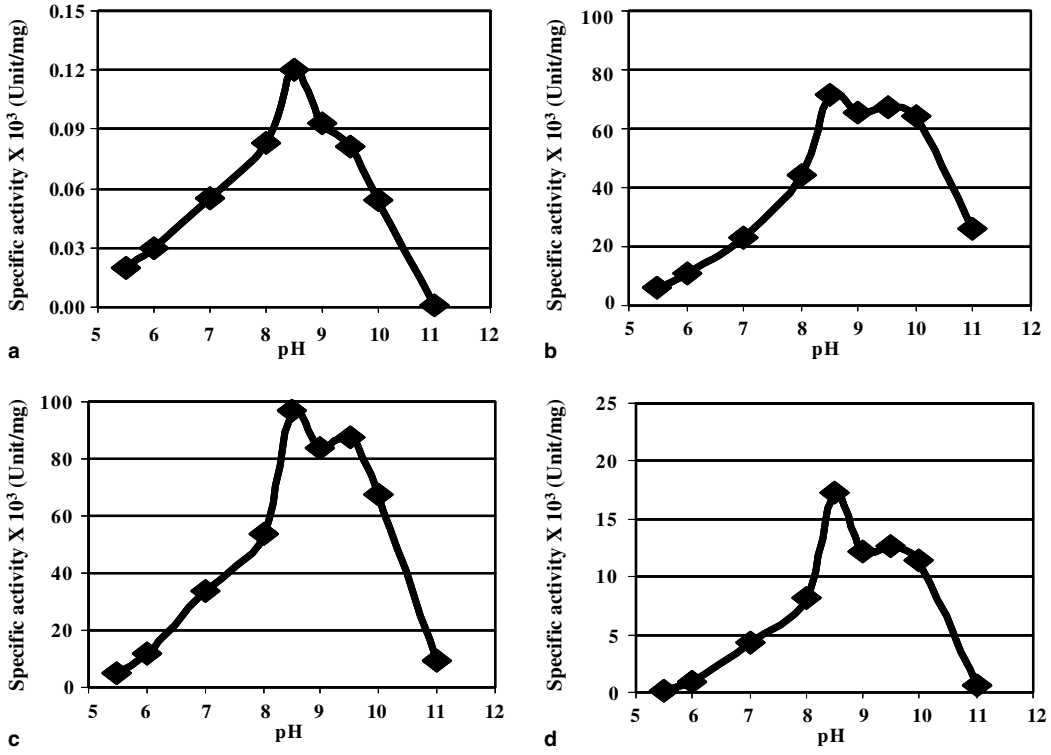

Fig. 5. pH profiles of partially purified proteinases, using Boc-Asp(oBzl)-Pro-Arg-AMC as a substrate assayed at each respective optimal temperature, P111 (a), P21 (b), P31 (c), and P4 (d).

are substrates of trypsin, while Boc-Val-Leu-Lys-AMC and Z-Phe-Arg-AMC were slightly hydrolyzed. Suc-Ala-Ala-Pro-Phe-AMC and Z-Arg-Arg-AMC, which are substrates of chymotrypsin and cathepsin B, respectively, were hardly hydrolyzed. These results indicated that partially purified proteinases were trypsin-like due to the preferential cleav-

Table 2
Hydrolytic activity towards various synthetic substrates of partially purified proteinases at its optimal temperature and pH

Substrates	Specific activity (Unit/mg)			
	P111	P21	P31	P4
Boc-Asp(oBzl)-Pro-Arg-AMC	113	65,146	87,979	19,603
Boc-Val-Leu-Lys-AMC	4.20	4466	1505	749
Boc-Gln-Ala-Arg-AMC	39.81	31,754	33,115	8405
Suc-Ala-Ala-Pro-Phe-AMC	0	865	0	0
Z-Arg-Arg-AMC	0	864	0	0
Z-Phe-Arg-AMC	4.05	4708	1236	142

age of Arg at P_1 position. Trypsin-like proteinases purified from pyloric caeca of menhaden and rainbow trout hydrolyzed the synthetic substrate of trypsin, which contained Arg at P_1 position (Kristjansson, 1991; Pavlisko, Rial, & Coppes, 1999). Ishida et al. (1995) found that neutral serine proteinases type I and II from muscle of salted anchovy (*E. japonica*) exhibited the highest hydrolytic activity towards Boc-Asp(oBzl)-Pro-Arg-AMC and Boc-Gln-Ala-Arg-AMC, respectively. It should be mentioned that proteolytic activities of P21 and P31 were higher than others. P21 and P31 appeared to be predominant proteinases purified from Indian anchovy.

3.4. Thermal stability

All fractions were stable at 35 °C, an average temperature of fish sauce fermentation (Fig. 6). At their optimal temperature, activity of P21, P31, and P4 decreased about 40% after 2 h incubation and remained stable afterward throughout 12 h (Fig. 6b–d). Only P111 was stable throughout 12 h at its optimal temperature (Fig. 6a). Autolytic degradation of Indian anchovy was typically induced at 60 °C. However, our results demonstrated that incubating whole anchovy at 55–60 °C for longer than 2 h was likely to decrease degree of hydrolysis due to the instability of trypsin-like proteinases.

3.5. Effect of proteinase inhibitors

Activity of all fractions was greatly suppressed by SBTI, leupeptin, and SDS (Table 3). TLCK and PMSF moderately inhibited proteolytic activity of all fractions. E-64 and iodoacetic acid which are cysteine proteinase inhibitors, slightly inhibited proteinase activity. P111 was moderately inhibited by EDTA, while P21, P31, and P4 were slightly inhibited. Bestatin, a metallo proteinase inhibitor specific for aminopeptidase, slightly inhibited activities of all proteinases. These enzymes were unlikely to be either metallo proteinase or aminopeptidase. DTT inhibited activity of all partially purified proteinases to a varied degree. This maybe because DTT disrupted disulfide bonds which stabilized the conformations of native enzymes. Based on inhibitor studies, partially purified proteinases from Indian anchovy exhibited trypsin-like characteristics.

3.6. Activity staining

Proteinases with various molecular masses that exhibited activity at 4.0 M NaCl were purified from our study.

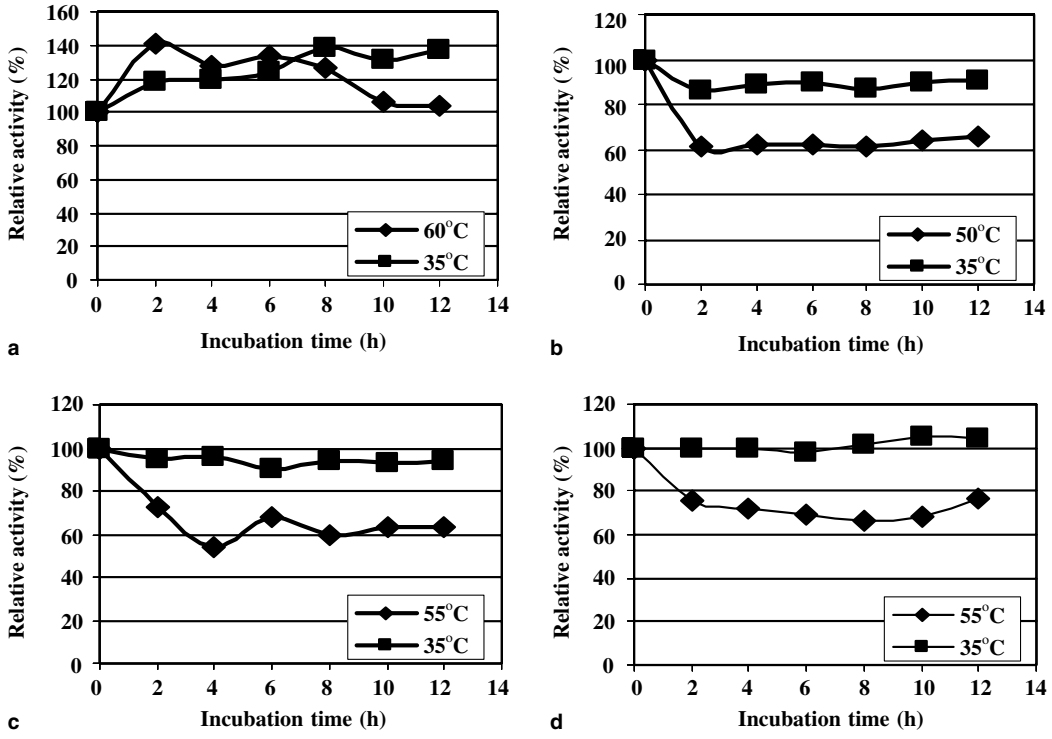

Fig. 6. Thermal stability of partially purified proteinases at 35 °C and each respective optimal temperature, P111 (a), P21 (b), P31 (c), and P4 (d).

Table 3
Effect of proteinase inhibitors on partially purified proteinase activities

Inhibitors	Classification	Final concentration	Inhibition (%)			
			P111	P21	P31	P4
EDTA	Metallo proteinase	10 mM	43.0	10.6	23.4	25.4
PMSF	Serine proteinase	10 mM	42.7	53.8	43.8	50.9
Bestatin	Metallo proteinase	10 μM	0.00	18.0	2.72	0.26
Iodoacetic acid	Cysteine proteinase	1 mM	0.00	13.9	7.49	0.00
E-64	Cysteine proteinase	10 μM	24.6	63.8	55.4	41.4
Leupeptin	Serine and cysteine proteinase	100 μM	102	99.6	104	103
TLCK	Serine proteinase	100 μM	15.5	88.0	51.9	69.3
SBTI	Serine proteinase	0.02 mg/ml	101	98.7	103	103
SDS	Detergent	2% (w/v)	102	99.3	104	103
DTT	Reducing reagent	10 mM	79.4	75.5	71.5	55.9

Molecular weight (MW) of P111 fraction was estimated to be 27, 38, 47, 58, 60, and 65 kDa, while P21 showed MW of 37, 43, 48, 58, 60, and 65 kDa (Fig. 7). P31 showed MW of 27, 33, 37, 43, and 50 kDa. Only one distinct caseinolytic activity band at 39 kDa was observed in P4 (Fig. 7). Biochemical characteristics including pH and temperature optima, inhibitor, and substrate specificity of fraction P21 appeared to coincide with those of P31. In addition, these two fractions showed the similar binding characteristics to DEAE-Sephacel and phenyl-Sepharose (Figs. 1 and 2). For this reason, both P21 and P31 might share common proteinases, particularly those with MW of 37–50 kDa (Fig. 7). Siringan et al. (2006b) reported the presence of trypsin-like proteinases with MW of 37, 47, and 53 kDa in the fish sauce samples throughout 12 months of fermentation. These proteinases exhibited the similar trypsin-like characteristics and MW to those of P21 and P31 fractions. Hence, it could be speculated that proteinases contained in P21 and P31 fractions were highly stable in >25% NaCl and these enzymes could play a vital role in protein hydrolysis under their optimal conditions. MW of trypsin-like isolated from carp, anchovy, and rainbow trout was estimated to be 25–30 kDa (Cao et al., 2000; Kristjansson, 1991; Martinez et al., 1988). The native-PAGE showed similar patterns to those obtained from SDS-PAGE (data not shown), suggesting that partially purified proteinases might be a single polypeptide without subunit.

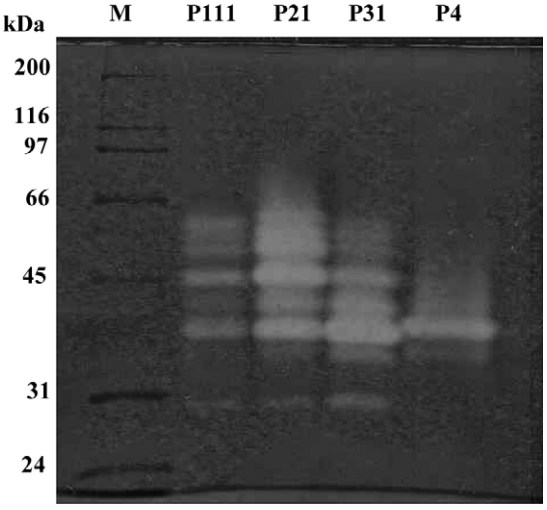

Fig. 7. Activity staining of partially purified proteinases in Indian anchovy incubated at 60 °C, pH 8.5, 4.0 M NaCl. M, Molecular weight marker. Loaded protein was 3 μg. P111, P21, P31, and P4 denoted for proteinase fractions.

3.7. Hydrolysis of washed anchovy muscle at 4.0 M NaCl

All partially purified proteinases hydrolyzed washed anchovy mince at 4.0 M NaCl (pH 8.5) at 35 °C and at an optimal temperature of each fraction (Fig. 8). P111 showed higher activity than others at the optimal temperature due to its higher thermal stability. However, activities of all fractions at 35 °C were lower than those at the optimal temperature. It should be noted that all fractions did not hydrolyze washed anchovy mince at 4.0 M NaCl, pH 5.5 (data not shown). Normally, pH and temperature of fish sauce fermentation ranged from 5.5 to 5.8 and at 25 to 35 °C, respectively. Under such conditions, activities of partially purified proteinases appeared to be minimal. However, trypsin-like proteinases in Indian anchovy could be activated by increasing incubation temperature to about 50–60 °C and adjusting pH to 8.5.

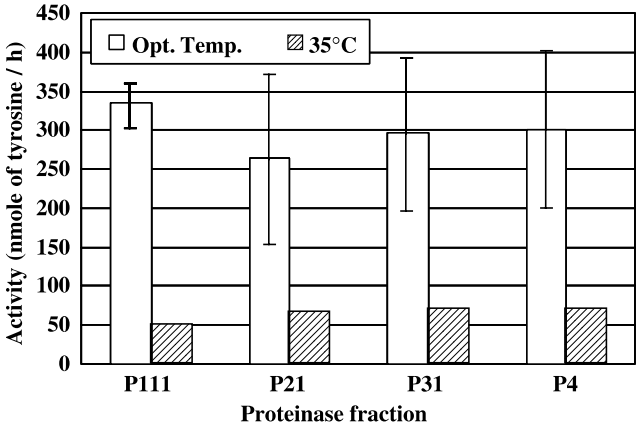

Fig. 8. Proteolytic activity of partially purified proteinases towards washed mince solubilized in 4.0 M NaCl, 0.2 M Tris–HCl, pH 8.5.

4. Conclusions

Partially purified proteinases from Indian anchovy exhibited the optimal temperature and pH at 50–60 °C and 8.5, respectively. Several trypsin-like proteinases were observed in activity staining at high salt content (4.0 M NaCl). All partially purified proteinases also hydrolyzed washed anchovy mince at 4.0 M NaCl, pH 8.5. Thus, adjusting pH and temperature of fish sauce fermentation to optimal conditions of proteinases could be a means to accelerate fish sauce fermentation.

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