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Autolytic activity and biochemical characteristics of endogenous proteinases in Indian anchovy (*Stolephorus indicus*)

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

Maximum autolytic activity of Indian anchovy (*Stolephorus indicus*) was found at 60 °C. Autolytic activity decreased with increased NaCl concentration. Remaining autolytic activity at 25% NaCl (w/w) was 52%. Crude proteinase extracts exhibited the highest activity at 60 °C, using either casein or acid-denatured hemoglobin (dHb) as a substrate. Optimal pH of crude extracts was found at 8.5 for casein and 9.5 for dHb. Activity of crude extract decreased >50% when NaCl concentration was greater than 0.1 M. Crude extract was stable for up to 8 h at 4, 30, and 60 °C. Crude proteinase hydrolyzed several synthetic substrates of trypsin, including Boc-Asp(oBzl)-Pro-Arg-MCA, Boc-Val-Leu-Lys-MCA, and Boc-Gln-Ala-Arg-MCA. Soybean trypsin inhibitor (SBTI), leupeptin, phenylmethanesulfonyl fluoride (PMSF), and *N*-tosyl-L-lysine chloromethyl ketone (TLCK) inhibited activities of proteinase, indicating trypsin-like characteristics. Molecular weight of proteinases exhibiting caseinolytic activity at 4.0 M NaCl were estimated to be 63, 53, 46, 40, 35, and 31 kDa, using electrophoresis activity staining.

Keywords: Indian anchovy (*Stolephorus indicus*); Autolytic activity; Endogenous proteinase; Trypsin-like proteinase

1. Introduction

Fish sauce is a clear amber liquid containing free amino acids and oligopeptides with distinct aroma and flavour. Indian anchovy (*Stolephorus indicus*) is widely used as a raw material for fish sauce manufacturing in Southeast Asia. Fish is typically mixed with 20–30% solar salt and left in a concrete tank at ambient temperature for 8-12 months (Saisithi, 1994). The major limitation of fish sauce production is the long fermentation time. Fish endogenous proteinases and microbial proteinases could play an important role in protein hydrolysis during fish sauce fermentation. Understanding biochemical characteristics of endogenous proteinases would lead to a means for fish sauce acceleration.

Anchovy (*Engraulis encrasicholus*), a raw material used for salted fish in Europe, is susceptible to proteolytic degradation by digestive enzymes from pyloric caeca and intestine (Martinez & Gildberg, 1988). Two trypsin-like enzymes purified from pyloric caeca and intestine had pH optimum at 8-9.5 (Martinez, Olsen, & Serra, 1988). Major proteinases in pyloric caeca and intestine were trypsin, chymotrypsin, elastase and aminopeptidase (Martinez & Serra, 1989). Furthermore, alkaline proteinase was predominant in fish muscle and brine during the ripening of salted anchovy (*E. encrasicholus*) (Hernandez-Herrero, Roig-Sagues, Lopez-Sabater, Rodriguoz-Jerez, & Mora-Ventura, 1999). According to Heu, Pyeun, Kim, and Godber (1991), proteolytic activity in intestine of anchovy (*E. japonica*) resulted from alkaline proteinases. These enzymes were later purified and identified as trypsin and chymotrypsin, exhibiting an optimal pH between

* Corresponding author. Tel.: +66 44 22 4359; fax: +66 44 22 4150.
E-mail address: jirawat@sut.ac.th (J. Yongsawatdigul).

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8 and 9 (Heu, Kim, & Pyeun, 1995). Ishida, Sugiyama, Sato, and Nagayama (1995) reported that neutral serine proteinase, found in muscles of anchovy (*E. japonica*), exhibited optimum catalytic activity at pH 7.5. Activity of these proteinases decreased when NaCl concentration was increased above 10% NaCl. Heu, Kim, Cho, Godber, and Pyeun (1997) purified a cathepsin L-like, with optimal pH 6, from muscles of the same anchovy. Furthermore, activities of trypsin, chymotrypsin and cathepsin L purified from anchovy (*E. japonica*) decreased with increasing NaCl concentration (Choi, Heu, Kim, & Pyeun, 2004). Based on previous studies, many different proteinases were present in fish and might contribute to protein hydrolysis during high salt fermentation. However, endogenous proteinases in Indian anchovy (*S. indicus*) have not been studied and characterized. Our objective was to investigate autolytic activity in Indian anchovy (*S. indicus*). Additionally, biochemical characteristics of predominant endogenous proteinases were characterized.

2. Materials and methods

2.1. Materials and chemicals

Fresh Indian anchovies (*S. indicus*) caught off the Gulf of Thailand, Chonburi province, were transported to the Suranaree University of Technology laboratory within 6 h after catch. Fish were kept frozen at −20 °C and used throughout the experiment. Average fish weights were 2.61 ± 0.47 g with the length of 7.59 ± 0.34 cm. Casein, hemoglobin (Hb), 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, carbobenzoxy (Z)-Arg-Arg-AMC, phenylmethanesulfonyl fluoride (PMSF), leupeptin, 1-(L-*trans*-epoxysuccinyl-leucylamino)-4-guanidinobutane (E-64), bestatin, pepstatin A, *N*-tosyl-L-lysine chloromethyl ketone (TLCK), *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK) and ethylenediaminetetraacetic acid(EDTA) were purchased from Sigma Chemicals Co. (St. Louis, Mo., 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). All other chemicals were of analytical grade.

2.2. Autolysis assay

Autolysis activity was measured according to the method of Greene and Babbitt (1990). Whole anchovy was ground and incubated at various temperatures (30–90 °C). Oligopeptide contents were determined according to the method of Lowry, Rosenbrough, Farr, and Randall (1951). The effect of NaCl concentration (0–25% w/w) on autolytic activity of anchovy was also investigated at the optimal temperature. Blanks were prepared by adding 5% trichloroacetic acid (TCA) before incubation at 60 °C for 20 min. Autolytic activity was expressed as μmole of tyrosine released/min/g mince.

2.3. Preparation of crude extracts

Crude extracts were prepared by homogenizing whole anchovy with ice-cold 20 mM phosphate buffer, pH 7.0 at a ratio 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.

2.4. Proteinase activity assays

Proteinase activity was determined, using either 1% casein or 1% acid-denatured hemoglobin (dHb) as substrates (An, Seymour, Wu, & Morrissey, 1994). The reaction was carried out at 60 °C for 20 min, a linear range determined from the time course of study. TCA-soluble oligopeptides content was determined by the Lowry method, using tyrosine as a standard (Lowry et al., 1951). Activity was defined as nmole of tyrosine released/min/ml of crude extract. Specific activity was expressed as the amount of activity per mg protein.

2.5. Effects of temperature, pH and NaCl on proteinase activity

The activity of crude extract was determined at various temperatures (30–90 °C), at pH 7.0, as described above. The activity was also assayed at optimal temperature and various pHs ranging from 3 to 12: pH 3.0–8.0, using McIlvaine's Buffer (0.1 M sodium citrate and 0.2 M sodium phosphate); pH 8.5–9.0, using 0.2 M Tris–HCl; pH 9.5–10, using 0.1 M glycine–NaOH; pH 12, using 0.1 M $NaHCO_3$–Na_2CO_3.

To study thermal stability, crude extract was incubated at 4, 30, and 60 °C for up to 8 h. The remaining activity was determined at 60 °C in 0.1 M glycine–NaOH buffer (pH 9.5) using 1% dHb as a substrate.

The effect of NaCl on proteolytic activities was determined at various NaCl concentrations (0–2.5 M) in 0.2 M Tris–HCl (pH 8.5) and 0.1 M glycine–NaOH (pH 9.5) when casein and dHb were used as substrates, respectively.

2.6. Synthetic substrate specificity

Proteolytic activity was determined using various synthetic substrates, including Boc-Gln-Ala-Arg-AMC, BocVal-Leu-Lys-AMC, Boc-Asp(oBzl)-Pro-Arg-AMC

Suc-Ala-Ala-Pro-Phe-AMC, Z-Arg-Arg-AMC and Z-Phe-Arg-AMC according to the method of Barrett and Kirschke (1981) and Ishida et al. (1995). All synthetic substrates were prepared at either 1 or 10 mM in DMSO. The reaction mixture containing 0.8 ml of 0.2 M Tris–HCl (pH 8.5), 0.1 ml of 10 μM of substrate solution and 0.1 ml of diluted crude extracts 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. The fluorescence intensity of the liberated 7-amino-4-methylcoumarin (AMC) was measured by 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 one μmole of AMC released/min/ml of crude extract. Specific activity was expressed as the amount of unit activity per mg of protein.

The effects of various inhibitors (PMSF, TLCK, TPCK, SBTI, bestatin, pepsatatin A, leupeptin and EDTA) and $CaCl_2$ concentration (0–100 mM) on crude proteinase activity were determined using Boc-Asp (oBzl)-Pro-Arg-AMC as a substrate.

2.7. Activity staining

Activity staining was performed according to the method of Garcia-Carreno, Dimes, and Haard (1993). Crude proteinases were separated on 10% polyacrylamide gel (Laemmli, 1970). Subsequently, gel was immersed in 2% casein, 50 mM Tris–HCl (pH 7.5) for 30 min on ice. Caseinolytic activity was initiated by transferring gels to either 0.2 M Tris–HCl (pH 8.5) buffer containing 4.0 M NaCl or without NaCl, and incubated at 60 °C for 15 min. Subsequently, gels were stained in 0.125% Coomassie brilliant blue R-250 in 40% methanol and 10% acetic acid for 3 h. Destaining was carried out using 25% methanol and 10% acetic acid solution. A clear zone on the blue background indicated the presence of proteinase.

3. Results and discussion

3.1. Effect of temperature and salt on autolytic activity

TCA-soluble oligopeptides increased with temperature and reached a maximum at 60 °C (Fig. 1). High level of autolytic activity at relatively high temperature indicated the presence of heat stable proteinases. Ishida, Niizeki, and Nagayama (1994) showed that the serine proteinase isolated from salted anchovy muscle exhibited maximal activity at 55 °C. Moreover, proteinases found in threadfin bream (Kinoshita, Toyohara, & Shimizu, 1990), anchovy (*E. japonica*) (Heu et al., 1995) and

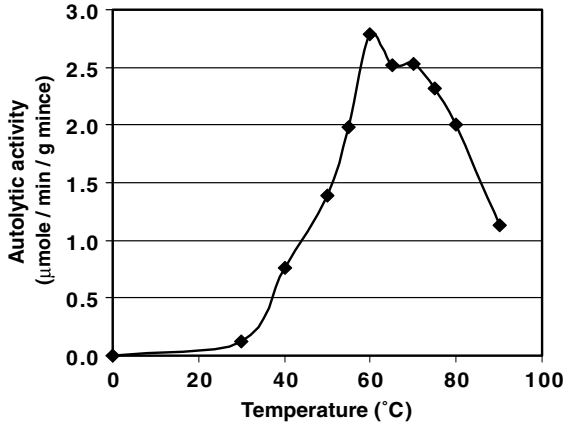

Fig. 1. Effects of temperature on autolytic activity of Indian anchovy.

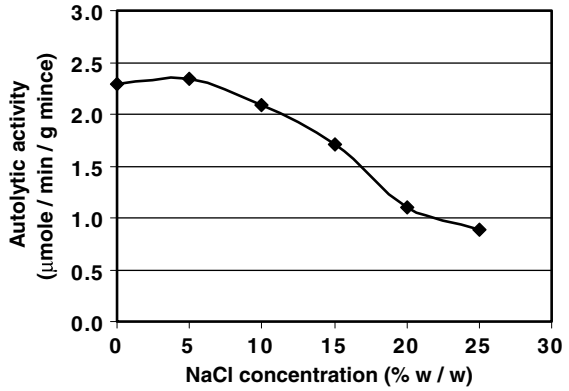

Fig. 2. Effects of NaCl on autolytic activity of Indian anchovy.

Atlantic menhaden (Boye & Lanier, 1988) showed maximal activity at around 45–65 °C.

TCA-soluble oligopeptides decreased with increasing NaCl concentration (Fig. 2). The autolytic activity at 25% NaCl was about 52% that of the control (no NaCl), suggesting that endogenous proteinases in Indian anchovy could hydrolyze muscle proteins, even at high salt concentration, but to a lesser extent than in the absence of salt. Trypsin-like proteinase activity was observed during the first month of anchovy (*Stolephorus* spp.) fermentation in 22-28% NaCl (Orejana & Liston, 1982). Gildberg and Shi (1994) reported the presence of trypsin, chymotrypsin and elastase activity in fish sauce made from cod viscera mixed with 20–25% NaCl. Our results indicated that endogenous proteinases in Indian anchovy hydrolyzed anchovy proteins to a certain extent at high salt content (25–30% NaCl) when incubated at the optimum temperature (60 °C).

3.2. Effects of temperature, pH and NaCl on proteolytic activity

Maximum hydrolytic activity of crude proteinase towards casein and dHb was at 60 °C (Fig. 3),

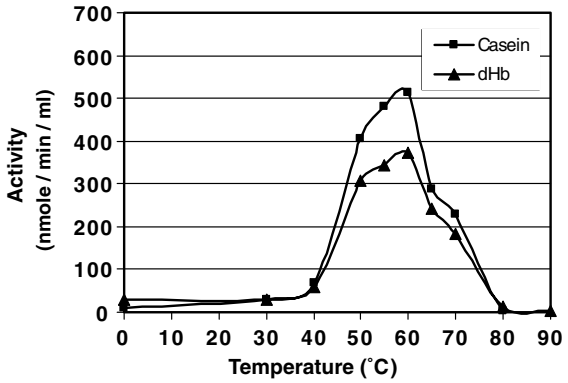

Fig. 3. Temperature profiles of crude proteinases assayed at pH 7.0.

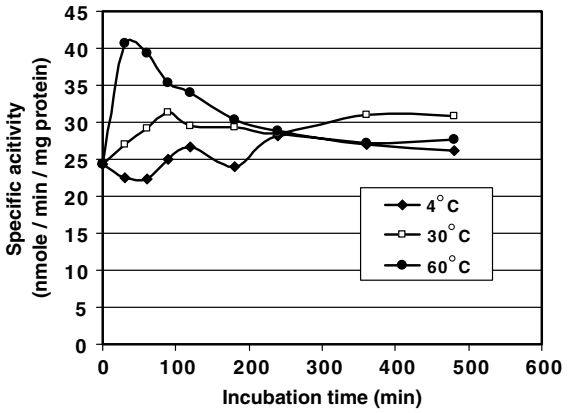

Fig. 5. Thermal stabilities of crude extracts incubated at 4, 30 and 60 °C.

corresponding to the optimal temperature of autolysis. This implied that the majority of proteinases in Indian anchovy were likely to be extracted by low ionic strength buffer. Optimal pH values of crude proteinases from Indian anchovy were 3.0 and 8.5–9.5 (Fig. 4). Both acid and alkaline proteinases were predominant in Indian anchovy. Alkaline proteinase appeared to be stable at low pH in salted ripened anchovy (Hernandez-Herrero et al., 1999). Additionally, alkaline proteinase exhibited the optimal pH in a broad range of 6–10, depending on the substrate (Kolodziejska & Sikorski, 1996). Typically, fish sauce fermentation is carried out outdoors at temperatures of 30–35 °C with pH ranging from 5.3 to 5.8, which was far different from the optimum condition of endogenous proteinases. This would partly explain the limited rate of protein hydrolysis during fish sauce fermentation.

Crude proteinases showed high stability at 4, 30 and 60 °C (Fig. 5). Specific activities of crude proteinases incubated at 60 °C were highest because heat treatment eliminated contaminant proteins. Crude proteinases were stable for up to 8 h at all studied temperatures. High thermal stability of endogenous proteinases at 60 °C would be advantageous for accelerating fish sauce fermentation. Incubating whole fish at the optimal temperature (60 °C) would maximize autolytic activity without lessening the enzyme activity.

Proteolytic activity of crude extracts decreased with increasing NaCl (Fig. 6). Remaining activities at 2.5 M NaCl were 33% and 20% when using dHb and casein as substrates, respectively. Autolytic activity at 15% NaCl (about 2.5 M NaCl) was approximately 77% of that without NaCl (Fig. 2). Proteolytic activity of the crude extract appeared to be more sensitive to NaCl than was autolytic activity. In autolysis, enzymes were associated with muscle proteins and present in the cell matrix, which tended to minimize structural and conformational changes caused by heat and a high ionic strength environment. For this reason, proteinase activities were less affected by high salt content in the autolysis study. High salt content (25–30% NaCl) used in fish sauce fermentation is another factor limiting protein hydrolysis. Our results showed that the remaining autolytic activity of Indian anchovy at 10% NaCl was about 90% (Fig. 2); thus, lowering salt content would be a means to increase the rate of protein hydrolysis.

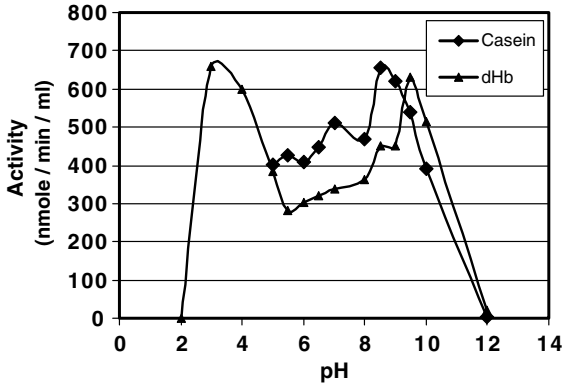

Fig. 4. pH profiles of crude proteinases assayed at 60 °C.

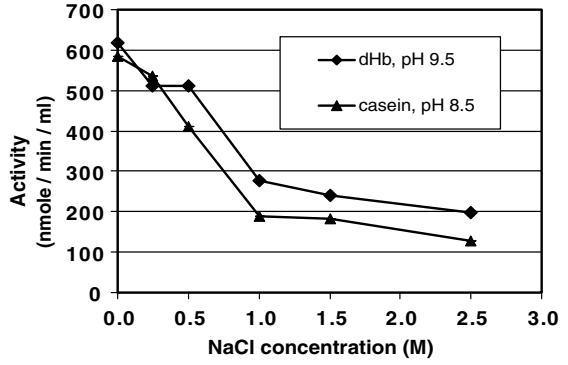

Fig. 6. Effects of NaCl on proteolytic activities of crude extracts assayed at 60 °C and either pH 8.5 using casein or pH 9.5 for dHb.

Table 1
Hydrolytic activity towards synthetic substrates of crude proteinases at 60 °C, pH 8.5

Substrates	Specific activity (units/mg protein)
Suc-Ala-Ala-Pro-Phe-AM	0
Boc-Val-Leu-Lys-AMC	4.94 ± 1.34
Boc-Gln-Ala-Arg-AMC	31.8 ± 12.67
Boc-Asp(oBzl)-Pro-Arg-AMC	85.9 ± 5.39
Z-Arg-Arg-AMC	0
Z-Phe-Arg-AMC	2.24 ± 1.27

Table 2
Effect of inhibitors on crude proteinases activity

Inhibitors	Final concentration	Inhibition (%)
Control	–	0
SBTI	0.023 mg/ml	99.3
TLCK	100 μM	77.7
TPCK	100 μM	3.3
PMSF	1 mM	76.8
Leupeptin	100 μM	97.8
E-64	10 μM	15.8
Pepstain A	10 μM	0
Bestatin	10 μM	5.1
EDTA	10 μM	27.8

3.3. Synthetic substrate specificity

Boc-Asp(oBzl)-Pro-Arg-AMC, a substrate for α-thrombin and trypsin-like proteinase, was strongly hydrolyzed by the crude proteinase from Indian anchovy (Table 1). Boc-Val-Leu-Lys-AMC and Boc-Gln-Ala-Arg-AMC, which are substrates for plasmin and trypsin, respectively, were slightly hydrolyzed (Table 1). In contrast, Suc-Ala-Ala-Pro-Phe-AMC, Z-Arg-Arg-AMC and Z-Phe-Arg-AMC, which are substrates for chymotrypsin, cathepsin B, and cathepsin L, respectively, were hardly hydrolyzed. Barrett, Rawlings, and Woessner (1998) reported that trypsin and trypsin-like enzymes preferred to cleave amide substrates containing Arg and Lys at the P_1 position and Pro at the P_2 position. Thus, predominant proteinases in the crude extract from Indian anchovy showed a trypsin-like characteristic, based on synthetic substrate studies. Ishida et al. (1995) found that neutral serine proteinases, types I and II from muscle of salted anchovy (*E. japonica*), exhibited the highest hydrolytic activities towards Boc-Asp(oBzl)-Pro-Arg-AMC and Boc-Gln-Ala-Arg-AMC, respectively.

3.4. Effect of inhibitors

General serine proteinase inhibitors (PMSF, leupeptin) and specific trypsin inhibitors (SBTI and TLCK) effectively inhibited crude proteinases, while E-64, bestatin, EDTA and TPCK showed a slight inhibition (Table 2). These results confirmed that predominant proteinases exhibited trypsin characteristics. Typically, trypsin requires moderate $CaCl_2$ concentration (20–50 mM) for activation and conformational stabilization (Barrett et al., 1998). However, Ca^{2+}, at concentrations up to 100 mM, had no effect on proteolytic activity of Indian anchovy (data not shown). It could be speculated that the predominant proteinase was unlikely to be trypsin.

3.5. Activity staining

Molecular weights (MWs) of crude proteinases from Indian anchovy were estimated to be 31, 40, 45, 53, and 63 kDa, based on activity staining gel electrophoresis (Fig. 7(a) and (b): lanes 1 and 2). Protein bands with caseinolytic activity in the absence of NaCl (Fig. 7(a)) were more evident than in the presence of 4.0 M NaCl (Fig. 7(b)). Proteolytic activity, observed at 4.0 M NaCl, confirmed that proteinases in Indian anchovy could

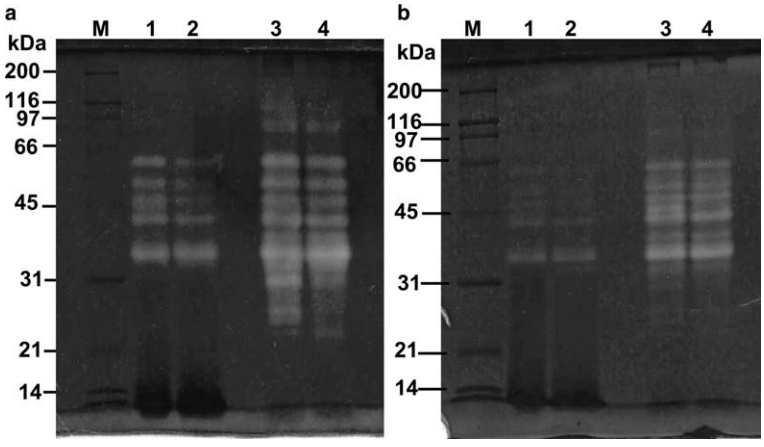

Fig. 7. Activity staining (SDS–PAGE, 10% acrylamide) of crude proteinases found in Indian anchovy incubated at 60 °C, pH 8.5 in the absence (a), and presence (b) of 4 M NaCl. Loaded protein content was 30 μg. M, molecular weight standard; lanes 1 and 2, crude extract; lanes 3 and 4, partially purified proteinases by heat-treatment and precipitation with 30–60% ammonium sulfate. Lanes 1, 3 and lanes 2, 4 were samples without and with 2-mercaptoethanol, respectively.

hydrolyze protein substrates at high salt content (≈4 M NaCl). When crude extracts were partially purified by heat treatment at 60 °C for 10 min and subsequent precipitation with 30–60% ammonium sulfate (Fig. 7(a) and (b): lanes 3 and 4), more clear bands were observed. MWs of these clear bands were estimated to be 18, 20, 25, 28, 31, 35, 40, 45, 53, 63, 80 and 94 kDa in the absence of NaCl. In the presence of 4.0 M NaCl, protein bands with MW < 30 kDa and those with MW > 80 kDa did not show casienolytic activity. These results indicated that proteinases with MWs of 31, 35, 40, 45, 53 and 63 kDa could hydrolyze casein at high salt content. It seems likely that these proteinases might play an important role in protein hydrolysis during fish sauce fermentation. Our study is the first to report the activity of proteinases at high salt content (4.0 M NaCl) in Indian anchovy. It should be noted that 2-mercaptoethanol (BME) did not affect caseinolytic activity, as the pattern of clear bands in the presence of BME was similar to that without BME (Fig. 7). These results implied that proteinases with casinolytic activity contained no subunits linked by disulfide bonds. Neutral serine proteinases extracted from salted anchovy had MWs about 25 and 37 kDa (Ishida et al., 1995). Since MW of trypsin purified from various fish species was 24–29 kDa (Heu et al., 1995; Kristjansson, 1991; Martinez et al., 1988; Pavlisko, Rial, & Coppes, 1999), it was likely that proteinases exhibiting casinolytic activity at high salt content were trypsin-like proteinases. Purification and characterization of these enzymes are under investigation.

4. Conclusions

Autolytic activity of Indian anchovy showed an optimal temperature of 60 °C. Autolytic activity also decreased >5% NaCl. Crude extract exhibited an optimum pH at 8.5–9.5. Trypsin-like proteinases were the predominant proteinases in the crude extract. Several proteinases of different molecular weights were observed by activity staining at 4.0 M NaCl, suggesting that proteinases from Indian anchovy could participate in protein hydrolysis during fish sauce fermentation. Therefore, incubation of Indian anchovy at 60 °C and in 10% NaCl for a period of time before full salting at 25% NaCl could be an effective way to accelerate the fish sauce fermentation process.

Acknowledgements

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