Available online at www.sciencedirect.com
ScienceDirect
Food Chemistry 107 (2008) 213–220
Food Chemistry
www.elsevier.com/locate/foodchem

Endogenous proteinases in true sardine (*Sardinops melanostictus*)

Sappasith Klomklao [a,*], Hideki Kishimura [b], Soottawat Benjakul [c]

[a] *Department of Food Science and Technology, Faculty of Technology and Community Development, Thaksin University, Phattalung Campus, Phattalung 93110, Thailand*
[b] *Laboratory of Marine Products and Food Science, Research Faculty of Fisheries Sciences, Hokkaido University, Hakodate, Hokkaido 041-8611, Japan*
[c] *Department of Food Technology, Faculty of Agro-Industry, Prince of Songkla University, Hat Yai, Songkhla 90112, Thailand*

Received 26 March 2007; received in revised form 24 June 2007; accepted 1 August 2007

Abstract

Characterisation of the autolytic profile of true sardine (*Sardinops melanostictus*) indicated the involvement of heat-activated proteinases, active at both acidic and alkaline pH values. Autolytic activity decreased with increasing NaCl concentration (0–30%). When crude proteolytic activity in true sardine was studied, two activity peaks were observed, at pH 3.5 and 9.0. Crude proteinase extracts exhibited the highest activity at 55 °C and 60 °C when assayed at pH 3.5 and 9.0, respectively. The pH 3.5 peak activity was effectively inhibited by pepstatin A, while the pH 9.0 peak activity was mostly inhibited by soybean trypsin inhibitor, PMSF and TLCK, suggesting that the various proteinases were present in true sardine. The enzymes were stable for up to 8 h at 55 °C. The activities were also stable at a pH range of 2.0–4.0 and still retained high activity toward hemoglobin after incubation at pH 3.5 for 8 h. Activities of the crude extract continuously decreased as NaCl concentration increased. ATP showed no effect on enzyme activity, while $CaCl_2$ and $MgCl_2$ activated the proteinase activity. The results implied that pepsin is a predominant enzyme responsible for autolysis in true sardine during fish sauce fermentation.

Keywords: Protease; Proteolysis; Degradation; Fish; Sardine; Autolysis

1. Introduction

Fish sauce is a clear brown liquid hydrolysate from salted fish and is commonly used as a flavour enhancer or salt replacement in various food preparations (Klomklao, Benjakul, Visessanguan, Kishimura, & Simpson, 2006a). Fish sauce is generally made from small pelagic species such as anchovies and sardines (Amano, 1962; Gildberg, 2001; Saisithi, 1994). Traditionally, fish sauce is produced by mixing one part salt with two or three parts fish and fermenting at a temperature range of 35–40 °C (Klomklao, Benjakul, Visessanguan, Kishimura, & Simpson, 2006; Lopetcharat, Choi, & Daeschel, 2001). During fermentation, proteins are hydrolysed, mainly as a result of autolytic action by endogenous proteinases in fish muscle and digestive tract as well as proteinases produced by halophilic bacteria (Gildberg & Thongthai, 2001; Orejana & Liston, 1982). The fermentation process normally takes a long time to ensure the solubilisation, as well as the flavour and colour development, of fish sauce which is the major limitation for fish sauce production. Understanding the biochemical properties of endogenous proteinases may provide information for the production of fish sauce and would lead to a means for fish sauce acceleration.

True sardine (*Sardinops melanostictus*) is one of the popular species commonly used for fish sauce production in Japan, owing to its wide availability and high proteinase activity. Sardine, like other small pelagic fish, is susceptible to rapid autolytic degradation of abdominal tissue after capture, this process is caused mainly by proteinases from the digestive tract (Martinez & Gildberg, 1988). The presence of several types of proteolytic enzymes in sardine

* Corresponding author. Tel./fax: +66 7469 3996.
E-mail address: sappasith@tsu.ac.th (S. Klomklao).

0308-8146/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2007.08.007

has been widely reported. Acid, neutral and alkaline proteinases have been found in Monterey sardine (*Sardinop sagax caerulea*) muscle (Lugo-Sanchez, Pacheco-Aguilar, & Yepiz-Plascencia, 1997). Castillo-Yanez, Pacheco-Aguilar, Garcia-Carreno, and Toro (2005) isolated trypsin from pyloric caeca of Monterey sardine (*Sardinops sagax caerulea*) and found that the enzyme had an optimal activity at pH 8.0 and at 50 °C. Two acid proteinases were purified in sardine (*Sardina pilchardus*) stomach (Noda & Murakami, 1981). Acid proteinase purified from Monterey sardine (*Sardinops sagax caerulea*) viscera had an optimal pH of 2.5 (Castillo-Yanez, Pacheco-Aguilar, Garcia-Carreno, & Toro, 2004). Recently, Kishimura, Hayashi, Miyashita, and Nonami (2006) purified trypsin with optimal activity at pH 8.0 and at 60 °C, from true sardine (*S. melanostictus*) viscera. However, no information regarding the biochemical properties of endogenous proteinases in true sardine, which might contribute to protein hydrolysis during high salt fermentation, has been reported. The objective of this work was to study the proteolysis and to characterise the endogenous proteinases in true sardine.

2. Materials and methods

2.1. Chemicals

Sodium caseinate, bovine hemoglobin, ethylenediaminetetraacetic acid (EDTA), pepstatin A, soybean trypsin inhibitor, iodoacetic acid, *N*-*p*-tosyl-L-lysine chloromethyl ketone (TLCK), *N*-tosyl-L-phenyl-alanine chloromethyl ketone (TPCK), 1-(L-trans-epoxysuccinyl-leucylamino)-4-guanidinobutane (E-64), *N*-ethylmaleimide, phenylmethanesulfonyl fluoride (PMSF), L-tyrosine and bovine serum albumin were purchased from Sigma Chemical Co. (St. Louis, MO, USA.). Trichloroacetic acid, sodium chloride, tris (hydroxymethyl) aminomethane and Folin-Ciocalteu's phenol reagent were obtained from Merck (Darmstadt, Germany).

2.2. Fish sample

Fresh true sardine (*S. melanostictus*), with an average body weight of 55–60 g, was purchased from the local fish market in Hakodate, Japan. The fish were transported in ice, with a fish/ice ratio of 1:2 (w/w), to the Laboratory of Marine Products and Food Science, Research Faculty of Fisheries Sciences, Hokkaido University, Japan. The fish samples were kept at −20 °C until required.

2.3. Autolytic activity assay

Autolytic activity assay was measured according to the method of Morrissey, Wu, Lin, and An (1993) and Visessanguan, Menino, Kim, and An (2001), with a slight modification. Whole true sardine was ground using a meat grinder with a 5 mm plate. Three grams of ground true sardine was incubated for 40 min in a water bath, at various temperatures (30–80 °C), and the autolytic reaction was stopped by adding 27 ml of ice-cold 5% (w/v) trichloroacetic acid (TCA). The concentration of the soluble peptides released was measured using the Lowry method (Lowry, Rosebrough, Fan, & Randall, 1951).

To construct a pH profile, 3 g of ground fish was added with 12 ml of buffer (McIlvain's buffer consisting of 0.2 M sodium phosphate and 0.1 M sodium citrate was used for the pH range of 2.0–7.0, and 0.1 M glycine–NaOH was used for the pH range of 8.0–11) and incubated for 40 min, at 60 °C, in a water bath. The autolytic reaction was terminated by addition of 15 ml of ice-cold 9% (w/v) TCA. Soluble peptides were determined as describe above.

The effect of NaCl on autolytic activity was also studied. NaCl was added into the ground sample to obtain the final concentrations [0, 5, 10, 15, 20, 25 and 30% (w/w)]. After incubation of the mixture at 60 °C for 40 min, 27 ml of ice-cold 5% (w/v) TCA was added and TCA-soluble peptides were determined. Blanks were prepared by adding TCA before incubation at 60 °C. Autolytic activity was expressed as μ mole of tyrosine released/min/g mince.

2.4. Preparation of crude extract

Frozen true sardines were thawed using running water (26–28 °C), until the core temperature reached −2 to 0 °C. The samples were cut into pieces with a thickness of 1–1.5 cm and homogenised in three volumes of acetone at −20 °C for 30 min, according to the method of Klomklao et al. (2006b). The homogenate was filtrated *in vacuo* on Whatman No. 4 filter paper. The residue obtained was then homogenised in two volumes of acetone at −20 °C, for 30 min, and then the residue was air-dried at room temperature until dry and free of acetone odour.

To prepare the crude extract, the powder was suspended in 20 mM phosphate buffer, pH 7.0 at a ratio of 1:20 (w/v) and stirred continuously at 4 °C for 3 h. The suspension was centrifuged for 15 min at 4 °C, at 20,000×*g* (H-200, Kokusan, Tokyo, Japan), to remove the tissue debris and then the supernatant was lyophilised. Before used, the lyophilized sample (10 g) was dissolved with 30 ml of cold distilled water (4 °C) and referred to as "crude extract".

2.5. Enzyme assay

Proteinase activity of the crude extract was assayed using hemoglobin and casein as substrates, according to the method of Klomklao, Benjakul, and Visessanguan (2004). Activity was determined according to the TCA-Lowry assay. Fish extract (200 μl) was added into assay mixtures containing 2 mg of substrate, 200 μl of distilled water and 625 μl of reaction buffer. The mixture was incubated at the test pH and temperature for precisely 20 min. Enzymatic reaction was terminated by adding 200 μl of 50% (w/v) TCA. Unhydrolysed protein substrate was allowed to precipitate for 1 h at 4 °C, followed by centri-

fuging at 10,000×*g* for 10 min. The oligopeptide content in the supernatant was determined by the Lowry assay (Lowry et al., 1951), using tyrosine as a standard. One unit of activity was defined as that releasing 1 μmol of tyrosine per min (μmol/Tyr/min). A blank was run in the same manner, except the enzyme was added after addition of 50% TCA (w/v).

2.6. pH and temperature profile

Proteolytic activity was assayed over the pH range of 2.0–11.0 (McIlvaine's buffer for pH 2.0–7.5 and 0.1 M glycine–NaOH for pH 8.0–11.0) at 60 °C for 20 min. For the temperature profile study, the activity was assayed at different temperatures (20, 30, 40, 50, 55, 60, 65, 70 and 80 °C) for 20 min at pH 3.5 and 9.0.

2.7. pH and thermal stability

The effect of pH on enzyme stability was evaluated by measuring the residual activity after incubation at various pHs, at room temperature for 30 min. Different buffers used were mentioned above. The 200 μl of crude extract was also incubated with McIlvaine's buffer, pH 3.5 at a ratio of 1:1 (v/v), at room temperature, for various times (0, 10, 20, 30, 60, 120, 240, 360, and 540 min). The remaining activity was determined using hemoglobin as a substrate at pH 3.5 and at 55 °C for 20 min.

For thermal stability, the enzyme solution was subjected to heating at different temperatures (20, 30, 40, 50, 60, 70 and 80 °C) for 15 min, in a temperature-controlled water bath (Memmert, Germany). Thereafter, the treated samples were suddenly cooled in iced and then the activity was determined. The crude extract was also incubated at 55 °C for up to 8 h. The heat-treated samples were immediately cooled in iced water and tested for the remaining activity.

2.8. Effect of inhibitors on proteinase activity

The effect of inhibitors on proteinase activity was determined according to the method of Klomklao, Benjakul, Visessanguan, Kishimura, and Simpson (2007), by incubating enzyme solution with an equal volume of proteinase inhibitor solution to obtain the final concentration designated (0.1 mM E-64, 1 mM *N*-ethylmaleimide, 1 mM iodoacetic acid, 1 mM PMSF, 1.0 g/l soybean trypsin inhibitor, 5 mM TLCK, 5 mM TPCK, 1 mM pepstatin A and 2 mM EDTA). The mixture was allowed to stand at room temperature (26–28 °C) for 30 min. Thereafter, the remaining activity was measured and percent inhibition was calculated.

2.9. Effect of some chemicals on proteinase activity

Different chemicals were mixed with the crude extract to obtain the concentration designated (0, 0.1, 0.5 and 1 mM for ATP; 0, 1, 5 and 10 mM for $CaCl_2$ and $MgCl_2$). The mixtures were kept at room temperature for 30 min and residual activity was determined by the hemoglobin–TCA Lowry method (Klomklao et al., 2004).

2.10. Effect of NaCl

The effect of NaCl on crude proteinase activity was studied. NaCl was added into the standard reaction assay to obtain the final concentrations of 0, 5, 10, 15, 20, 25 and 30% (w/v). The residual activity was determined at 55 °C and at pH 3.5 for 20 min using hemoglobin as the substrate.

2.11. Protein determination

Protein concentration was measured by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

2.12. Statistical analysis

A completely randomised design was used throughout this study and the experiments were done in duplicate. Data were subjected to analysis of variance (ANOVA) and mean comparison was carried out using Duncan's multiple range test (Steel & Torrie, 1980). Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS for Windows; SPSS Inc.).

3. Results and discussion

3.1. Autolysis in true sardine

Autolytic activity of true sardine was characterised by temperature and pH profiles, as shown in Fig. 1a and b, respectively. The temperature profile analysed at the natural pH of fish, without adjustment, indicated that autolytic activity increased markedly from 30 °C to the highest peak at 60 °C, before becoming rapidly inactivated at higher temperatures. High level of autolytic activity at relative high temperature indicated the presence of heat stable proteinases. For the pH profile, optimum pH values of autolytic activity, analysed at 60 °C, were found at 3.5 and 8.0. The results indicated that two major groups of proteases were localised in true sardine. Visessanguan et al. (2001) found that autolysis of arrowtooth flounder is due to a heat-activated proteinase active at both acidic and alkaline pH values. Maximum autolytic activity of Indian anchovy was found at 60 °C (Siringan, Raksakulthai, & Yongsawatdigul, 2006). Benjakul, Leelapongwattana, and Visessanguan (2003) reported that the highest autolysis was observed at 65 and 60 °C for mince and washed mince from lizardfish muscle. From the results, autolysis of true sardine was thought to be mediated by heat-activated proteinases that are optimally active at acidic and alkaline pH values.

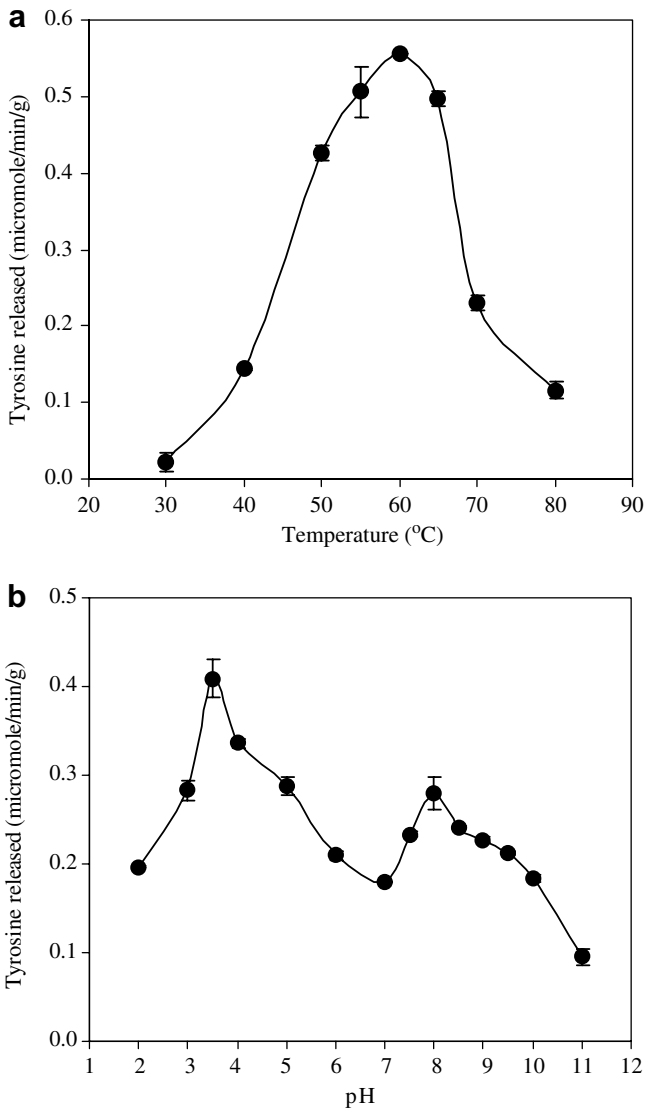

Fig. 1. Temperature (a) and pH (b) profile of true sardine autolytic activity. Autolytic activity was determined by incubating true sardine mince at various temperatures. For pH profile, autolytic activity was determined by incubating true sardine mince at 60 °C at various pH values. Autolytic activity was expressed as μmol of tyrosine released/min/g mince. Bars represent the standard deviation from duplicate determination.

3.2. Effect of sodium chloride on autolysis

The effect of NaCl at different concentrations on autolysis in true sardine, incubated at 60 °C for 40 min, is shown in Fig. 2. TCA-soluble oligopeptides decreased with increasing NaCl concentration. Remaining autolytic activity at 30% NaCl (w/w) was 49%. The results suggested that endogenous proteinases in true sardine could hydrolyse muscle proteins, even at high salt concentration, but to a lesser extent than in the absence of salt. Thermostable proteinase in salted anchovy muscle may still be active and able to degrade myofibrillar protein in commercial salted fillets containing 16–17% NaCl (Ishida, Niizelei, & Nagayama, 1994). The activity of acid proteinases from sardine was reduced with the addition of 3.42 M NaCl (Noda & Murakami, 1981). Siringan et al. (2006) reported that the autolytic activity in Indian anchovy decreased with increasing NaCl concentration and the autolytic activity at 25% NaCl was about 52% of the control. From the results, more than 50% of autolytic activity remained in the presence of a high concentration of NaCl (10–25%), indicating that endogenous proteinases in true sardine were still active in the presence of NaCl, at a high level of NaCl concentration (25%), which is commonly used in fish sauce production. This may result in the degradation and hydrolysis of protein during fermentation.

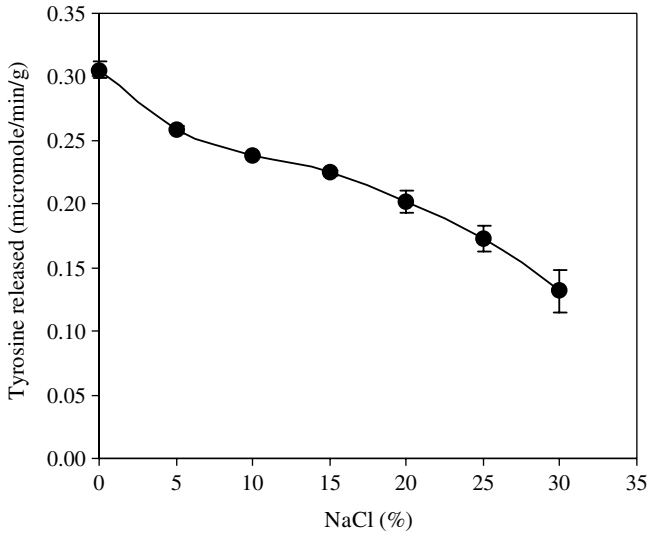

Fig. 2. Effect of NaCl on autolytic activity of true sardine. NaCl was added into the true sardine mince to obtain the final concentrations (0–30%, w/w). The mixture was incubated at 60 °C for 40 min. Autolytic activity was expressed as μmol of tyrosine released/min/g mince. Bars represent the standard deviation from duplicate determination.

3.3. pH and temperature profile

The pH activity curves of crude proteinases from true sardine are shown in Fig. 3a. Optimum peaks appeared at pH 3.5 and 9.0. The results indicated that at least two major proteinases were present in true sardine. The major peak was presumed to be due to acid proteinase, while the other peak was postulated to be due to alkaline proteinase, especially trypsin. Moreover, chymotrypsin, serine proteinase, was also presumed to exhibit the activity peak at pH 9.0. The results were coincidental with the high inhibitory activity of PMSF, soybean trypsin inhibitor, TLCK and TPCK at pH 9.0 against crude proteinases in true sardine extract (see Table 1). Siringan et al. (2006) reported that both acid and alkaline proteinases were predominant in Indian anchovy. Major proteolytic activity in alkaline (pH 10) and minor activity in acid (pH 3) were detected from Monterey sardine viscera (Castillo-Yanez et al., 2004). Optimum pH values of

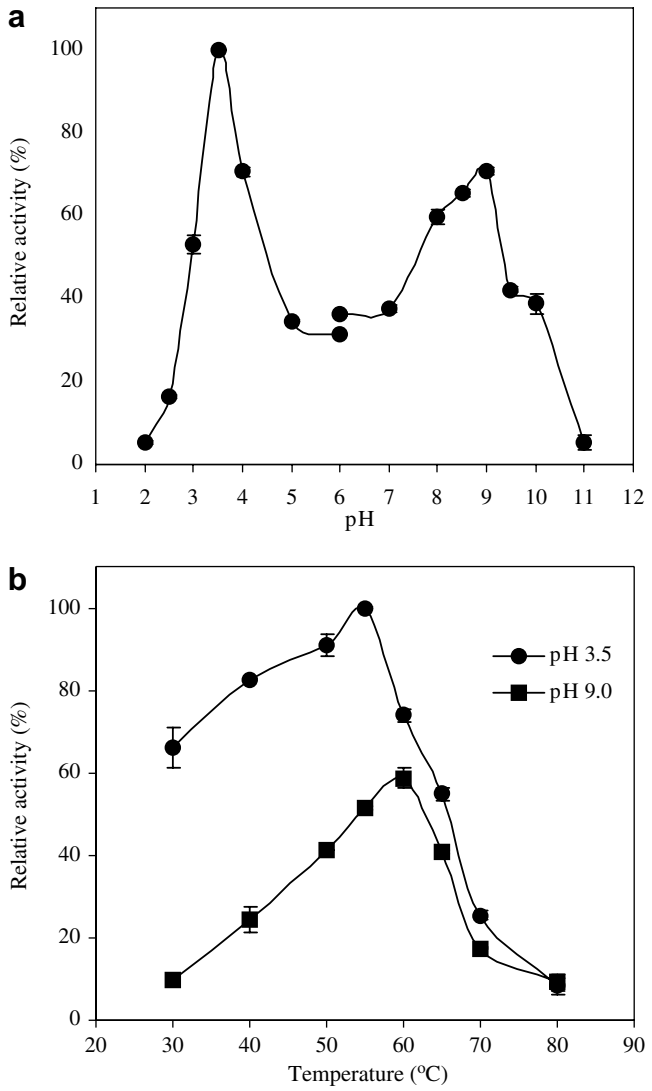

Fig. 3. pH (a) and temperature (b) profile of crude proteinases from true sardine. Proteolytic activity was assayed at various pH values at 60 °C. For temperature profile, the activities were run at different temperatures at pH 3.5 and 9.0. One unit of activity was defined as that releasing 1 μmol of tyrosine/min. Bars represent the standard deviation from duplicate determination.

bigeye snapper (*Priacanthus tayenus*) sarcoplasmic proteinases were found at pH 5.0 and 8.5 (Benjakul et al., 2003).

The temperature profiles of crude proteinase are presented in Fig. 3b. Maximum hydrolytic activity of the crude enzyme was at 55 and 60 °C when assayed at pH 3.5 and 9.0, respectively. The activity was higher at pH 3.5 than at other pH values (Fig. 3a and b). This result was in agreement with the higher rate of autolysis from ground true sardine at pH 3.5. The results suggested that the major proteinases in true sardine were possibly heat-stable acidic proteinase. Generally, 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 from true sardine. This would partly explain the limited rate of protein hydrolysis during fish sauce fermentation.

Table 1
Effect of various inhibitors on crude proteinase activity[a]

Inhibitors	Concentration	% Inhibition[b]	
		pH 3.5	pH 9.0
Control		0	0
E-64	0.1 mM	11.6 ± 0.12	13.3 ± 1.02
N-ethylmaleimide	1 mM	5.78 ± 0.74	6.37 ± 2.72
Iodoacetic acid	1 mM	5.97 ± 0.33	6.06 ± 0.19
PMSF	1 mM	10.3 ± 0.60	82.5 ± 4.50
Soybean trypsin inhibitor	1.0 g/l	14.2 ± 2.86	72.3 ± 2.25
TLCK	5 mM	26.7 ± 5.71	74.9 ± 0.45
TPCK	5 mM	22.2 ± 3.16	36.1 ± 0.45
Pepstatin A	0.01 mM	88.0 ± 1.57	14.9 ± 0.67
EDTA	2 mM	31.2 ± 0.60	29.4 ± 0.55

[a] Crude extract (200 μl) was mixed with 200 μl of single protease inhibitor to obtain the final concentration shown. The residual proteolytic activity was measured using casein and hemoglobin as substrates.
[b] Values are mean ± standard deviation from duplicate determinations.

3.4. Effect of inhibitors on proteinase activity

The effect of various inhibitors on crude proteinases of true sardine is shown in Table 1. Pepstatin A showed the highest inhibition (88%) when tested at pH 3.5, while various inhibitors, including PMSF, soybean trypsin inhibitor, TLCK and TPCK, rendered high inhibition when assayed at pH 9.0. High inhibition by pepstatin A, at pH 3.5, indicated the presence of aspartic proteinase such as cathepsin D or pepsin in true sardine. E-64, *N*-ethylmaleimide and iodoacetic acid are considered to be cysteine protease inhibitors (Klomklao et al., 2004). Soybean trypsin inhibitor and PMSF are serine protease inhibitors (Benjakul et al., 2003). TLCK and TPCK are specific inhibitor of trypsin and chymotrypsin, respectively. Pepstatin A can inhibit most of the aspartic proteases. EDTA is metalloprotease inhibitors (Klomklao et al., 2007). From the results it was noted that EDTA showed partial inhibitory activity (approximately 29–31%). EDTA is a chelator for many ions. This indicated the presence of proteases which require metal ions for their activity. Those enzymes include calpain, which needs calcium ions for activation and metalloproteinases. The high inhibition of PMSF, soybean trypsin inhibitor and TLCK towards the proteinases from true sardine indicated the presence of serine proteinases, especially trypsin, in this fish species. Benjakul et al. (2003) reported that sarcoplasmic proteinases from bigeye snapper (*Priacanthus tayenus*) were effectively inhibited by pepstatin A, at pH 5.0, while at pH 8.5 the activity was inhibited by several inhibitors. Siringan et al. (2006) found that PMSF, leupeptin, soybean trypsin inhibitor and TLCK effectively inhibited crude proteinases from Indian anchovy. From the inhibitory studies it can be inferred that aspartic proteinase was the major crude proteinase and trypsin-like serine proteinase was the minor proteinase in true sardine.

Table 2
Effect of some chemicals on crude proteinase activity[a]

Chemicals	Concentration (mM)	Relative activity (%)[b]
ATP	0	100
	0.1	99.2 ± 0.2
	0.5	100 ± 0.9
	1	99.0 ± 1.2
$CaCl_2$	0	100
	1	124 ± 0.6
	5	139 ± 1.9
	10	142 ± 2.5
$MgCl_2$	0	100
	1	116 ± 3.1
	5	129 ± 2.3
	10	132 ± 2.6

[a] Each enzyme solution was incubated with the same volume of chemical at 25 °C for 30 min and the residual activity was determined using hemoglobin as substrate for 20 min at pH 3.5 and 55 °C.
[b] Values are mean ± standard deviation from duplicate determinations.

3.5. Effect of some chemicals on proteinase activity

The effect of ATP, $CaCl_2$ and $MgCl_2$ on the crude proteinase activity was determined (Table 2). ATP showed no influence on proteinase activity in a concentration dependent manner. On the other hand, $CaCl_2$ and $MgCl_2$ activated the crude proteinase activity. With increasing concentrations of $CaCl_2$ and $MgCl_2$, an increase in activity was observed (Table 2). ATP is known as the activating agent for cathepsin D but not for pepsin (Gildberg, Olsen, & Bjarnason, 1990; Pillai & Zull, 1985). The stimulating influence of divalent ions revealed a difference between the pepsins and cathepsin D (Gildberg et al., 1990). Xu, Wong, Rogers, and Fletcher (1996) found that acidic proteinases from the stomach of the deepwater finfish orange roughy (*Hoplostethus atlanticus*) were activated with Ca^{2+} and Cu^{2+}, while ATP (100 μM) did not increase the enzyme activity. Gildberg et al. (1990) also reported that ATP had no effect on the activities of cod pepsins whereas divalent cations such as Ca^{2+} and Cu^{2+} stimulated the activity of cod pepsins, but had no effect on cathepsin D. From the results, it can be concluded that the major endogenous proteinases in true sardine were pepsin.

3.6. pH and thermal stability

Crude proteinases from true sardine were stable at pHs ranging from 2.0 to 5.0 (Fig. 4a), with the remaining activity above 90%. However, the activity was slightly decreased in the neutral and alkaline pH ranges. The stability of the enzyme at a particular pH might be related to the net charge of the enzyme at that pH (Klomklao et al., 2006b). At extreme pHs, strong intramolecular electrostatic repulsion caused by high net charge, results in swelling and unfolding of the protein molecules (Damodaran, 1996; Klomklao et al., 2007). Inactivation of enzyme activity at neutral and alkaline pH was also reported for the acidic

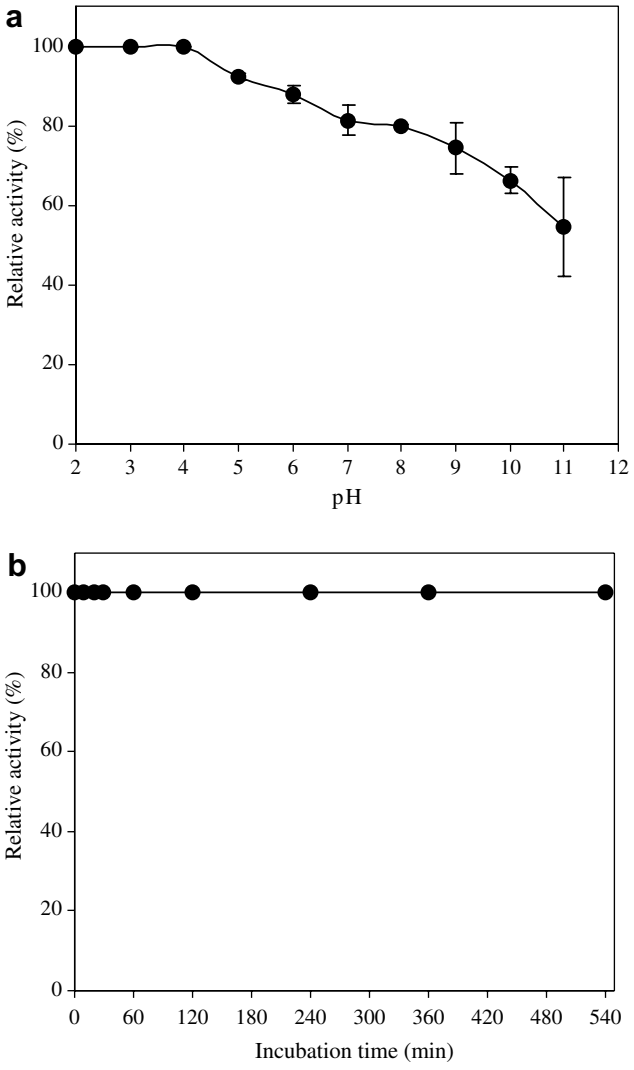

Fig. 4. Effect of pH (a) and incubation time (b) on the stability of crude proteinase from true sardine. The crude enzymes were incubated at various pHs for 30 min. To study the effect of incubation time, the enzyme was incubated at pH 3.5 for different times. Residual activity was determined using hemoglobin as substrate.

proteinases from Monterey sardine (Castillo-Yanez et al., 2004) and sardine (*S. melanostica*) (Noda & Murakami, 1981).

When the crude extract was incubated with McIlvaine's buffer, pH 3.5 at room temperature for various times (0–540 min), a relative activity of 100% was observed after incubated for up to 8 h (Fig. 4b). The results indicated that crude proteinases were very stable for up to 8 h at pH 3.5, which is the optimum pH of the enzyme. Thus, high pH stability of endogenous proteinases at pH 3.5 would be advantageous for accelerating fish sauce fermentation. Adjusting the pH of the fish mixture to optimum pH (pH 3.5) would maximise autolytic activity without lessening the enzyme activity.

The thermal stability of the enzyme was examined for 15 min in the temperature range of 20–80 °C and depicted in Fig. 5a. The enzyme activity was stable up to 50 °C and

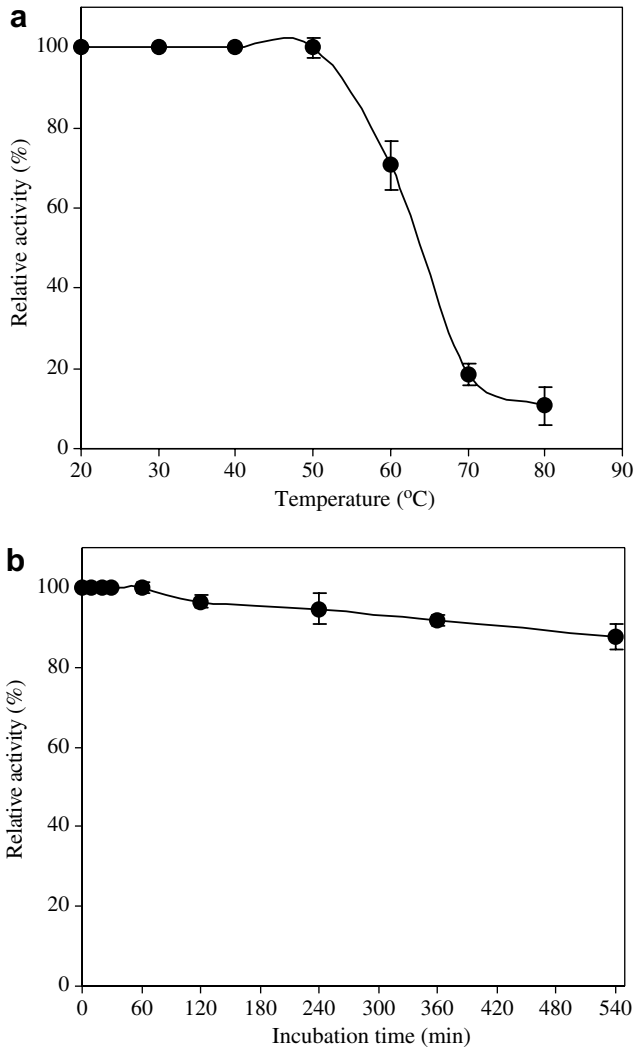

Fig. 5. Effect of heating temperature (a) and heating time (b) on the stability of crude proteinase from true sardine. The crude enzymes were heated at different temperatures for 15 min. To study the effect of heating time, the enzyme was heated at 55 °C for different times. Residual activity was determined using hemoglobin as substrate.

lost its stability slightly at 60 °C. The crude proteinases were not stable at higher temperature. Approximately 10% of activity was retained after heating at 80 °C. At high temperature, the enzyme most likely underwent denaturation and lost its activity. The result was in accordance with Castillo-Yanez et al. (2004) who reported that denaturation of acidic proteinase from Monterey sardine viscera occurred above 55 °C.

Thermal stability of crude proteinases was also tested at 55 °C at various times (Fig. 5b). No marked changes in relative activity were observed after heating for up to 1 h. However, a slight decrease in relative activity was found when the time was increased. More than 90% activity remained when heated for 8 h, suggesting that the enzyme was very heat stable for up to 8 h at 55 °C. Therefore, incubating whole fish at the optimum temperature (55 °C) could be an effective way for acceleration of fish sauce production.

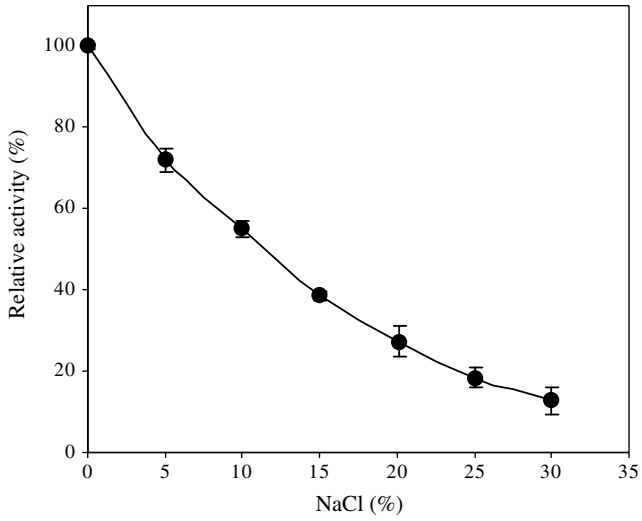

Fig. 6. Effect of NaCl on proteinase activity of crude extracts from true sardine. NaCl was added in the activity assay buffer to final concentrations of 0–30% (w/w). Residual activity was determined using hemoglobin as substrate.

3.7. Effect of NaCl on proteinase activity

Proteolytic activity of crude extracts decreased gradually with increasing NaCl (Fig. 6). The activity at 30% NaCl was about 15% that of control (no NaCl). The decrease in activity might be due to the denaturation of enzymes. The 'salting out' effect was postulated to cause the enzyme denaturation. The water molecule is drawn from the trypsin molecule by salt, leading to the aggregation of those enzymes (Klomklao et al., 2004; Klomklao et al., 2007). From the result, proteolytic activity of the crude extract appeared to be more sensitive to NaCl than was autolytic activity (Fig. 2). In autolysis, enzymes were associated with muscle proteins and present in the cell matrix, which tended to minimise structural and conformational changes caused by heat and high ionic strength environment. For this reason, proteinase activities were less affected by high salt content in the autolysis study. Therefore, lowering salt content would be a means to increase the rate of protein hydrolysis. Klomklao et al. (2006a) found that reduction of salt content in fish sauce made from true sardine accelerated the hydrolysis of fish protein and retarded loss proteolytic activity during fermentation.

4. Conclusion

Autolysis of true sardine was caused by both heat-acidic and alkaline protienases. Pepsin, with optimal activity at pH 3.5 and 55 °C, was the predominant proteinases in the crude extract. Activity decreased with increasing NaCl concentration. Therefore, incubation of true sardine at optimal activity combined with the reduction of NaCl concentration could be an effective way to accelerate the fish sauce fermentation.

References

Amano, K. (1962). Influence of fermentation of the nutritive value on fish with special reference to fermented fish products of Southeast Asia. In E. Heen & R. Kreuzer (Eds.), *Fish in nutrition* (pp. 180–200). London: Fishing News.

Benjakul, S., Leelapongwattana, K., & Visessanguan, W. (2003). Comparative study on proteolysis of two species of bigeye snapper, *Priacanthus macracanthus* and *Priacanthus tayenus*. *Journal of the Science of Food and Agriculture, 83*, 871–879.

Castillo-Yanez, F. J., Pacheco-Aguilar, R., Garcia-Carreno, F. L., & Toro, M. A. N. (2004). Characterization of acidic proteolytic enzymes from Monterey sardine (*Sardinops sagax caerulea*) viscera. *Food Chemistry, 85*, 343–350.

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

Damodaran, S. (1996). Amino acids, peptides, and proteins. In O. R. Fennema (Ed.), *Food chemistry quality* (pp. 322–429). New York: Marcel Dekker.

Gildberg, A. (2001). Utilization of male Arctic capelin and Atlantic cod intestines for fish sauce production-evaluation of fermentation conditions. *Bioresource Technology, 76*, 119–123.

Gildberg, A., Olsen, R., & Bjarnason, J. B. (1990). Catalytic properties and chemical composition of pepsins from Atlantic cod (*Gadus morhua*). *Comparative Biochemistry and Physiology Part B, 96*, 323–330.

Gildberg, A., & Thongthai, C. (2001). The effect of reduced salt content and addition of halophilic lactic bacteria on quality and composition of fish sauce made from sprat. *Journal of Aquatic Food Product Technology, 10*, 77–88.

Ishida, M., Niizelei, S., & Nagayama, F. (1994). Thermostable proteinase in salted anchovy muscle. *Journal of Food Science, 59*, 781–785.

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

Klomklao, S., Benjakul, S., & Visessanguan, W. (2004). Comparative studies on proteolytic activity of spleen extracts from three tuna species commonly used in Thailand. *Journal of Food Biochemistry, 28*, 355–372.

Klomklao, S., Benjakul, S., Visessanguan, W., Kishimura, H., & Simpson, B. K. (2006a). Proteolytic degradation of sardine (*Sardinella gibbosa*) proteins by trypsin from skipjack tuna (*Katsuwonus pelamis*) spleen. *Food Chemistry, 98*, 14–22.

Klomklao, S., Benjakul, S., Visessanguan, W., Kishimura, H., & Simpson, B. K. (2006). Effects of the addition of spleen of skipjack tuna (*Katsuwonus pelamis*) on the liquefaction and characteristics of fish sauce made from sardine (*Sardinella gibbosa*). *Food Chemistry, 98*, 440–452.

Klomklao, S., Benjakul, S., Visessanguan, W., Kishimura, H., & Simpson, B. K. (2007). Purification and characterization trypsins from the spleen of skipjack tuna (*Katsuwonus pelamis*). *Food Chemistry, 100*, 1580–1589.

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

Lopetcharat, K., Choi, Y. J., & Daeschel, M. A. (2001). Fish sauce products and manufacturing: A review. *Food Research International, 17*, 65–68.

Lowry, O. H., Rosebrough, N. J., Fan, A. L., & Randall, R. J. (1951). Protein measurement with Folin phenol reagent. *Journal of Biological Chemistry, 193*, 256–275.

Lugo-Sanchez, M. E., Pacheco-Aguilar, R., & Yepiz-Plascencia, G. (1997). Catalytic activities of crude enzyme fractions from Monterey sardine. *Journal of Food Science, 62*, 976–979.

Martinez, A., & Gildberg, A. (1988). Autolytic degradation of belly tissue in anchovy (*Engraulis encrasicholus*). *International Journal of Food Science and Technology, 23*, 185–194.

Morrissey, M. T., Wu, J. W., Lin, D., & An, H. (1993). Protease inhibitor effects on torsion measurements and autolysis of Pacific whiting surimi. *Journal of Food Science, 58*, 1050–1054.

Noda, M., & Murakami, K. (1981). Studies on proteinases from the digestive organs of sardine-II, Purification and characterization of two acid proteinases from the stomach. *Biochimica et Biophysica Acta, 658*, 27–34.

Orejana, F. M., & Liston, J. (1982). Agents of proteolysis and its inhibition in patis (fish sauce) fermentation. *Journal of Food Science, 47*, 198–203, 20.

Pillai, S., & Zull, J. E. (1985). Effects of ATP, vanadate and molybdate on cathepsin D-catalyzed proteolysis. *Journal of Biological Chemistry, 260*, 8384–8389.

Saisithi, P. (1994). Traditional fermented fish: Fish sauce production. In A. M. Martin (Ed.), *Fisheries processing biotechnology application* (pp. 111–131). London: Chapman and Hall.

Siringan, P., Raksakulthai, N., & Yongsawatdigul, J. (2006). Autolytic activity and biochemical characteristics of endogenous proteinases in Indian anchovy (*Stolephorus indicus*). *Food Chemistry, 98*, 678–684.

Steel, R. G. J., & Torrie, J. H. (1980). *Principles and procedures of statistics: A biometrical approach* (second ed.). New York: McGraw-Hill (p. 633).

Visessanguan, W., Menino, A. R., Kim, S. M., & An, H. (2001). Cathepsin L: A predominant heat-activated proteinase in arrowtooth flounder muscle. *Journal of Agricultural and Food Chemistry, 49*, 2633–2640.

Xu, R. A., Wong, M. L., Rogers, M. L., & Fletcher, G. C. (1996). Purification and characterization of acidic proteases from the stomach of the deep water finfish orange roughy (*Hoplostethus atlanticus*). *Journal of Food Biochemistry, 20*, 31–48.