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Characterization of endogenous proteases from lizardfish *(Saurida Wanieso)* viscera and associated salt-dependent properties

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Abstract

To effectively utilize the lizardfish viscera generated as a by-product of *surimi* manufacturing, the properties of endogenous proteases in the viscera were investigated. Two protease activity groups were identified from lizardfish viscera, and their optimal temperature and pH were 55°C and 7.0, and 45°C and 11.0, respectively. The results of protease inhibitory and fluorogenic substrate specificity assays showed that both dominant proteases belong to the serine protease family. Also, the effects of NaCl on the catalytic activity and thermal stability of proteases from viscera were clarified. The catalytic activity of protease would increase after incubation in NaCl solutions (5-25%) for 30 days and the thermal stability of proteases was also enhanced markedly in 15% NaCl solution. All the results demonstrated the salt-dependent behavior of proteases in NaCl solution and that NaCl-dependent changes in catalytic activity and thermal stability have potential applications for the production of fermented food.

Introduction

Lizardfish *(Saurida wanieso)* is a member of the *Synodontidae* family, which inhabits the Atlantic, Pacific and Indian Oceans. In Southeast Asia, the lizardfish is considered to be of low market value due to its appearance and susceptibility to spoilage (Benjakul *et al.*, 2003). However, the muscle of lizardfish caught in Japan exhibits high gel-forming ability, especially in fresh fish. Thus, lizardfish is a potential raw material for high-grade *surimi* in Japan (Morrissey e*t al.*, 1993). To date, research has mainly focused on the gel-forming ability of lizardfish muscle and its application to *surimi* production. The by-products of fish processing are generally regarded as low-value resources and are typically discarded in the *surimi* industry (Gehring *et al.*, 2011). Furthermore, little information on lizardfish waste, especially the viscera, has been reported.

Utilization of fish waste from the food industry has increasingly gained interest over the past 10 years. The waste has typically been limited to the preparation of fishmeal and silage as a result of poor functional properties (Arvanitoyannis and Kassaveti, 2008). In recent years, a number of studies have been designed to investigate proteases from fish viscera including pepsin (Wu *et al*., 2009), trypsin (Cai *et al.*, 2011), chymotrypsin (Yang *et al*., 2009), elastase (Ketnawa *et al*., 2013) and so on, providing theoretical guidance for the utilization of fish digestive and hydrolytic enzyme in the fish industry. Fish viscera have recently been found to have wide biotechnological potential as an enzyme source, especially of proteases with unique properties for industrial applications. Therefore, the recovery of proteases from fish viscera has played an increasingly important role in the food industry and is garnering much attention.

Among traditional enzyme-aided fermented fish products, fish sauce is the most well-known product, and represents a traditional practice of preserving and producing value-added products from underutilized fish species (Klomklao *et al*., 2006). Fish sauce has recently gained popularity in Europe, North America and other countries (Gildberg, 2004). However, the consumption of fish sauce has been limited due to its high salt content. Further, the high NaCl content in fish sauces is thought to negatively affect both flavor and nutrient value (Xu *et al.*, 2008). Until now, reports have mainly focused on food safety and health issues, such as insufficient salt content leading to the spoilage of fish sauce and the effect of excessive salt consumption on the development of hypertension (Mervaala *et al.*, 1994).

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However, little information regarding the role of salt in enzyme-aided fermentation has been reported. For the effective utilization of endogenous proteases from fish viscera, research on the relationship between salt and fish-derived proteases is necessary. Therefore, in the present study, pH and temperature profiles of endogenous proteases from lizardfish viscera were generated. The major types of proteases were analyzed using protease inhibitors and fluorogenic substrates. Furthermore, the effects of salt on the catalytic activity and thermal stability of proteases were also studied to provide the theoretical reference and instruction for optimum utilization of lizardfish viscera in food processing, especially for fermented foods.

Material and Methods

Materials

Fresh lizardfish *(Saurida wanieso)* was purchased at Nagasaki Fish market, Nagasaki, Japan, placed in an ice box with a fish to ice ratio of 1:2 (w/w) and transported to the laboratory. Fresh lizardfish viscera were collected and washed with cold water to remove blood and scales from the surface. Then, the stomach and the intestine with pancreas were excised from the whole fish viscera, while the remaining viscera were collected together. Subsequently, each section of viscera (intestine + pancreas, stomach, remaining viscera) was weighed and relative proportion to the whole viscera was calculated. Viscera from lizardfish were used for experiments or stored at -20°C until use.

Chemicals

Antipain, aprotinin, chymostatin, leupeptin, pepstatin A and phenylmethanesulfonyl fluoride (PMSF) were obtained from Sigma-Aldrich Corporation (St. Louis, MO, USA). Ethylenediaminetetraacetate dihydrate (EDTA), ethylene glycol-bis (beta-amino ethyl ether)-N,N'-tetra acetic acid (EGTA), bovine serum albumin (BSA) and Bio-Rad protein assay kit for protein concentration determination were products of Bio-Rad (Hercules, CA, USA). 4-(2-Aminoethyl)-benzenesulfonyl fluoride hydrochloride (AEBSF; trade name Pefabloc SC), 1, 10-phenanthroline monohydrate and L-3-carboxy-trans-2, 3-epoxypropionyl-L-leucine-4-guanidinobutylamide (E-64) were from Amresco (Solon, OH, USA). Synthetic fluorogenic peptide substrates (MCA substrates) were from Peptide Institute (Osaka, Japan). Casein and tyrosine were from Wako Pure Chemicals (Osaka, Japan). All other reagents were of analytical grade.

Measurement of protease activity

The protease activity of lizardfish viscera toward casein was examined according to the trichloroacetic acid (TCA) assay following the method of Klomklao *et al.* (2008) with modification. The viscera samples (10 g) were mixed fully with 20 mM phosphate buffer (pH 7.0), homogenized, and centrifuged; the supernatant was collected and used as the protease solution. Five hundred microliters of 2% (w/v) casein solution and 100 µL of protease solution were mixed completely and incubated in 100 µL of 200 mM phosphate buffer (pH 7.0) at 55°C or sodium carbonate buffer (pH 11.0) at 45°C. After 30 min, the reaction was terminated by adding 200 µL of ice cold 30% (w/v) TCA. The mixture was allowed to precipitate unhydrolyzed proteins at 4°C for 15 min and then centrifuged at 8,000 × g for 10 min to collect the supernatant. A control test was performed under the same condition, while 200 µL of 30% (w/v) TCA was added before the addition of protease solution. The TCA soluble peptide content was determined by the Lowry method (1951) using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and tyrosine as a standard. In the present study, protease activity was expressed as the amount to release TCA peptide per minute by 1 mg of protein (µmol/min/mg protein).

pH and temperature profiles

The assay of pH profile was determined by incubating the protease solution with 20 mM sodium acetate-hydrochloric acid buffer (pH 1.0-3.0), sodium acetate-acetic acid (pH 4.0-5.0), phosphate buffer (pH 6.0-7.0), Tris-HCl buffer (pH 8.0-9.0) and sodium carbonate buffer (pH 10.0-13.0) at 40°C. For the optimum temperature assay, protease activity of the protease solution was measured from 20 to 70°C at 5°C intervals in 20 mM phosphate buffer (pH 7.0) or sodium carbonate buffer (pH 11.0).

Effect of protease inhibitors

To investigate the types of proteases from intestine + pancreas, the protease solution was incubated with various protease inhibitors at different final concentrations in 0.1 M phosphate buffer (pH 7.0) or sodium carbonate buffer (pH 11.0) at room temperature for 30 min, and residual activity was measured at 55°C (incubated at pH 7.0) or 45°C (incubated at pH 11.0). Control tests were performed without addition of any protease inhibitor and considered as 100% relative activity. The relative activity was calculated as follows:

Assay of protease activity toward fluorogenic substrates

Fluorogenic substrate hydrolyzing activity was measured according to the method described by Zhong *et al*. (2012). In brief, 50 µL of protease solution and 900 µL of 0.1 M phosphate buffer (pH 7.0) or sodium carbonate buffer (pH 11.0) were mixed, and the reaction was initiated by adding 50 µL of 10 µM fluorogenic substrate (MCA-substrate) at 55°C or 45°C for 10 min. After that, the reaction was immediately terminated by addition of 1.5 mL stopping agent (methyl alcohol/isopropyl alcohol/ distilled water = 35:30:35, v/v). The fluorescence intensity of liberated 7-amino-4-methylcoumarin (AMC) was detected by using a fluorescence spectrophotometer (FP-6200; Jasco, Tokyo, Japan) at an excitation wavelength of 380 nm and an emission wavelength of 450 nm. Control tests were performed without addition of any fluorogenic substrate and considered as 100% relative activity. The relative activity was calculated as stated above.

The effect of NaCl on catalytic activity

To evaluate the effect of NaCl on the catalytic activity assay, protease activity was measured in the presence of final NaCl concentrations from 0 to 25%, at intervals of 5%. To further investigate the salt tolerance, samples were diluted 50x with ion exchange water to decrease the effect of residual NaCl on protease activity after incubation with different final NaCl concentrations (0 to 25%) at 4ºC for 30 days. Protease activities were evaluated by measuring the residual protease activity according to the method described above. Moreover, the residual protease activity of diluted proteases was also measured using buffer with the corresponding concentration of NaCl (the same concentration as under incubation).

Thermal stability

To investigate the thermal stability of proteases from lizardfish intestine + pancreas as well as the effects of NaCl, the samples were thoroughly mixed with 15% NaCl solution at pH 7.0 or 11.0, and subjected to incubation at different temperatures (20, 30, 40, 50 and 60°C) for 180 min. After incubation, the treated samples were immediately cooled in ice water and then the activities were determined as described above. The control test was performed using ion exchange water instead of NaCl solution.

Statistical analysis

All experiments were performed in triplicate. Analysis was performed by using SPSS software (SPSS 11.5 for windows). Differences between variables were evaluated using Duncan's multiple range testing. The data were expressed as mean ± standard deviation.

Result and Discussion

pH and temperature profiles of proteases in lizardfish viscera

To elucidate the properties and distribution of proteases in lizardfish viscera, viscera were divided into three parts: intestine + pancreas, stomach, and remaining viscera. The relative proportions to whole viscera were: 39.65%, 32.68% and 27.67%, respectively, indicating intestine + pancreas and stomach accounted for the greatest proportion of whole lizardfish viscera (more than 70%). Figure 1a shows the pH dependent catalytic activities of proteases in intestine + pancreas and stomach. Compared to these two parts tissues, the activity was hardly detected in remaining viscera (data not shown). And, the protease activity from stomach was only found around at pH 1.0 and it was inhibited absolutely by pepstatin A (data not shown), suggesting the presence of a pepsin-like protease (Wu *et al.*, 2009). Furthermore, in the intestine + pancreas sample, the activity was widely observed at the range from pH 5.0 to 13.0, indicating that the dominant protease is an alkaline protease. A report of the digestive tract of Symphysodon aequifasciata revealed similar protease pH dependency. An acidic protease with the highest activity at pH 2.0 was found in the stomach region and an alkaline protease with optimal activity at pH 5.5 to 13.0 was isolated from the intestines (Chong *et al.*, 2002). The activity from lizardfish stomach at pH 1.0 showed about 40% relative activity of sample from intestine + pancreas at pH 11.0, however, the protein is easier to suffer from the denaturation under the extreme acidic condition.

The temperature profile of proteases from intestine + pancreas at the range from pH 4.0 to 13.0 is shown in Figure 1b. The highest activity is observed at pH 7.0 and 60°C (6.89 µmol/min/mg protein) and pH 11.0 and 40°C (5.69 µmol/min/mg protein) among all the temperatures and pH values. (Figure 1b); And, as shown in Figure 1c, more detail of temperature profile of them revealed that the optimal temperatures were 55°C and 45°C, respectively. The results indicated that there are two types of proteases in lizardfish intestine + pancreas (P1: activated at 55°C and pH 7.0; P2: activated at 45°C and pH 11.0) in term of the temperature and pH characteristic. Additionally, the highest activity of P2 at 45°C (6.98 µmol/min/mg protein) was more than 80% of the maximal activity of P1 at 55°C (7.70 µmol/min/mg protein). Compared

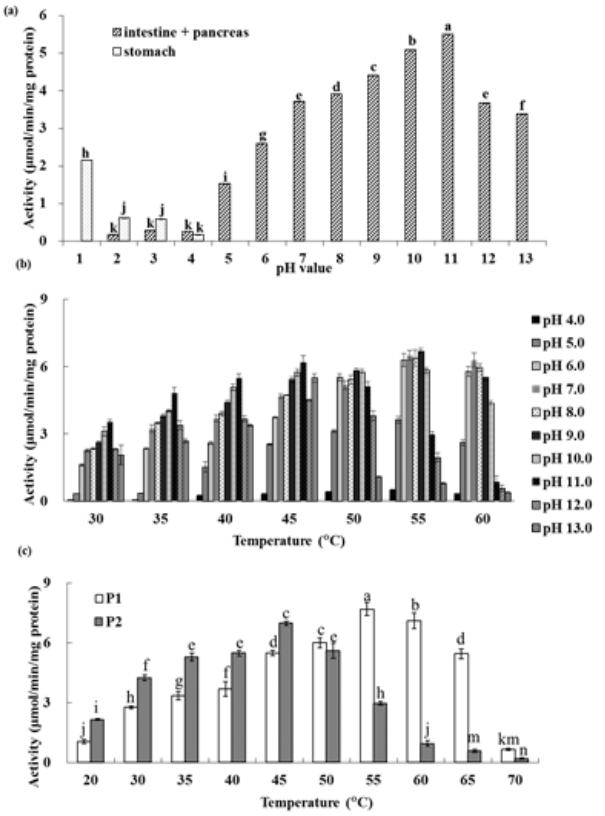

Figure 1 pH and temperature profile of proteases from lizardfish viscera. (a) pH profile of proteases at 40°C; (b) Temperature profile of protease from lizardfish viscera (intestine + pancreas) at different pH (from 4.0 to 13.0) from 30°C to 60°C; (c) Temperature profile of proteases (P1 and P2) from 20°C to 70°C. Data are presented as the means of n = 4 experiments, each performed in triplicate. Different letters indicate statistically significant differences ($p<0.05$)

to the maximal activity of P1, the protease activity of P1 was more than 70% at 65°C (5.46 μmol/min/mg protein) and showed about 50% at 40°C (3.70 μmol/min/mg protein). In contrast, P2 was temperature-sensitive proteases with only 35% of activity at 55°C (2.96 μmol/min/mg protein). From the above, P1 and P2 in intestine + pancreas have obvious differences in temperature and pH requirements, P1 still showed high activity when the temperature was higher than 60°C (more than 70%) and P2 also have high proteolytic activity when the pH was higher than pH 12 in spite of it is weak to resistant thermal change, suggesting it has potential application for protein hydrolysis.

Protease characterization of lizardfish intestine + pancreas using protease inhibitor and substrate specificity assays

As shown in Table 1, two protease groups (activated at pH 7.0 or 11.0) showed similar trends with respect to protease inhibitors, although they showed obvious difference in temperature-dependency. P1 and P2 were almost completely inhibited by 10 mM AEBSF (relative activity of 8.78% and 0.00%, respectively). At 1 mM AEBSF, the relative activity of P1 and P2 was 45.26% and 22.57%, respectively. Furthermore, the other serine protease inhibitors such as PMSF, aprotinin and chymostatin also inhibited protease activity, compared to other types of inhibitors. The relative activity of P1 and P2 was inhibited to 19.57% and 49.81% by 10 mM PMSF, 29.96% and 7.16% by 0.003 mM aprotinin, and 48.56% and 12.56% by 0.1 mM chymostatin, respectively, suggesting the dominant proteases in lizardfish intestine + pancreas belong to the serine protease family. However, apart from AEBSF, which showed the highest inhibitory effects on both types of proteases, PMSF and aprotinin showed the greatest inhibition of P1 and P2, respectively, indicating that these are different serine protease types.

On the other hand, the activities of both proteases were inhibited only about 20% by metalloprotease inhibitors such as 1 mM 1, 10-phenanthroline, 1 mM EGTA and 1 mM EDTA (Table 1). These results indicate that minor metalloproteases exist in the lizardfish intestine + pancreas. In the presence of E-64, a cysteine protease inhibitor, the activity of both proteases was more than 90% at a concentration of 0.1 mM. For the aspartic protease inhibitor pepstatin (0.1 mM), the relative activities of P1 and P2 were 99.58% and 99.59%, respectively. From the above, it is suggested that compared to serine proteases the other proteases are minor proteases in lizardfish intestine + pancreas.

Boc-Asp-Pro-Arg-MCA, Boc-Val-Pro-Arg MCA, Boc-Gln-Arg-Arg-MCA and Boc-Leu-Arg-Arg-MCA, which are substrates of serine proteases, were susceptible to hydrolysis by the proteases in intestine + pancreas (Table 2). However, the specific hydrolytic sites were notably different between P1 and P2. P1 preferred to cleave peptides having an Arg residue on position 1 and a Pro on position 2. However, P2 preferred to cleave peptides containing Arg in both positions 1 and 2. The relative hydrolytic activities of P1 toward Boc-Asp-Pro-Arg-MCA and Boc-Val-Pro-Arg-MCA were 100.00% and 68.86%, respectively, whereas those of P2 were only 58.70% and 65.45%, respectively. P2 hydrolyzed Boc-Gln-Arg-Arg-MCA and Boc-Leu-Arg-Arg-MCA at the relative activities of 100.0% and 95.59%, respectively, whereas P1 showed low hydrolytic activities of 20.12% and 19.90%, respectively.

Furthermore, the hydrolysis of Z-Arg-Arg-MCA containing Arg-Arg residue was only weakly detected with P2, as it is always reported as a cysteine protease

Table 1. Effect of different protease inhibitors on the activity of proteases (P1 and P2)

Protease inhibitors[b]	Concentration (mM)	Relative activity (%)	
		P1	P2
Control		100.00 ± 0.24 a[a]	100.00 ± 1.98 a
AEBSF	1	45.26 ± 0.96 k	22.57 ± 0.72 j
	10	8.78 ± 0.29 o	0.00 ± 0.79 n
PMSF	1	54.45 ± 0.47 i	62.64 ± 2.49 fg
	10	19.57 ± 0.84 n	49.81 ± 0.96 i
Aprotinin	0.0003	39.65 ± 1.36 l	19.38 ± 0.32 j
	0.003	29.96 ± 1.32 m	7.16 ± 0.18 m
Chymostatin	0.01	61.09 ± 0.54 h	14.47 ± 0.72 k
	0.1	48.56 ± 1.99 j	12.56 ± 0.31 k
Antipain	0.01	73.84 ± 0.74 fg	63.88 ± 2.59 f
	0.001	72.16 ± 3.07 g	59.03 ± 2.20 g
1,10-phenanthroline	1	75.02 ± 2.51 f	74.07 ± 3.61 cd
	10	53.43 ± 2.37 i	71.98 ± 0.58 de
EGTA	1	80.04 ± 2.34 e	84.29 ± 2.34 b
	10	74.74 ± 0.72 fg	55.07 ± 1.34 h
EDTA	1	85.08 ± 0.93 d	77.65 ± 3.28 c
	10	79.86 ± 3.27 e	76.65 ± 1.60 c
Leupeptin	0.01	86.26 ± 1.99 d	68.81 ± 1.76 e
	0.1	78.90 ± 0.96 e	62.99 ± 3.08 f
E-64	0.01	93.75 ± 0.55 b	99.31 ± 3.57 a
	0.1	90.30 ± 1.52 c	99.19 ± 3.51 a
Pepstatin A	0.001	99.81 ± 0.86 a	99.71 ± 2.13 b

[a]Different letters in the same row indicated statistical difference ($p<0.05$). Data are shown as mean ± standard deviation (n=4).

[b]AEBSF: 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride; PMSF: Phenyl methane sulfonyl fluoride; EGTA: Ethylene glycol-bis (beta-amino ethyl ether)-N,N'-tetra acetic acid; EDTA: Ethylenediaminetetraacetic acid; E-64:L-3-Carboxy-trans-2,3-epoxypropionyl-L-leucine-4-guanidinobutylamide.

substrate (Zhong *et al*., 2012). The hydrolytic activity of P1 and P2 toward Suc-Ala-Ala-Pro-Phe-MCA and Suc-Leu-Leu-Val-Tyr-MCA, which are known as chymotrypsin-like serine protease substrates (Yang *et al*., 2009), were scarcely detected, suggesting that the chymotrypsin-like serine protease was not the dominant serine protease in lizardfish intestine + pancreas.

The effect of NaCl on the catalytic activity of proteases in lizardfish intestine + pancreas

In general, the consumption of fish and shellfish sauces, which are high in salt, should be limited due to their health effects. However, during the fermentation of fish sauce, the addition of NaCl is necessary to inhibit the growth of harmful microorganisms. Recently, research has also shown that high salt concentrations are useful for limiting the formation of histamine during the fermentation of tuna *(Thunnus albacares)* viscera (Besas and Dizon, 2012); > 17% salt concentration in the fermentation broth could minimize histamine formation. Therefore, salt concentration is important for fermented foods in terms of microbiological and biochemical changes during fermentation.

In order to optimize the application of proteases from lizardfish viscera, it is necessary to consider the relationship between NaCl concentration, protease activity and thermal stability during fermentation. Generally, NaCl concentration has a dramatic effect on enzyme catalysis (Park and Raines, 2001). As shown in Figure 2 a, with increasing NaCl concentration, the suppressive effect of NaCl on the protease activities of intestine + pancreas was obviously observed. At low salt concentrations such as 5%, the activity of P1 and P2 was 6.76 μmol/min/mg protein and 5.94 μmol/min/mg protein, respectively. Compared to the activity of sample un-incubated in NaCl solution (7.70 μmol/min/mg protein of P1 and 6.98 μmol/min/mg protein of P2, respectively), the relative activities of P1 and P2 were not markedly decreased (87.79% and 85.10%, respectively). However, higher salt concentrations markedly inhibited the protease activities, especially at > 20%. The relative activity of P1 and P2 was only 13.57% (1.04 μmol/min/mg protein) and 9.51% (0.66 μmol/min/mg protein) at 25% NaCl, respectively. Previous research reported that the proteolytic activities of acid and alkaline

Table 2. Substrate specificity of proteases (P1 and P2) on synthetic fluorogenic substrates

Synthetic fluorogenic substrates (0.5 μM)[b]	Relative activity (%)	
	P1	P2
Boc-Asp(OBzL)-Pro-Arg-MCA	100.00 ± 0.46 a[a]	58.70 ± 1.41 f
Boc-Val-Pro-Arg-MCA	68.86 ± 0.30 b	65.45 ± 0.94 e
Boc-Gln-Arg-Arg-MCA	21.12 ± 1.43 c	100.00 ± 1.20 a
Boc-Leu-Arg-Arg-MCA	19.90 ± 0.34 d	95.59 ± 0.07 b
Boc-Gln-Gly-Arg-MCA	17.04 ± 0.20 e	74.81 ± 2.10 c
Boc-Phe-Ser-Arg-MCA	14.23 ± 0.09 f	73.14 ± 3.34 d
Boc-Gly-Lys-Arg-MCA	10.19 ± 0.06 g	66.65 ± 0.53 e
Suc-Ala-Ala-Pro-Phe-MCA	6.53 ± 0.07 h	1.83 ± 0.12 i
Suc-Leu-Leu-Val-Tyr-MCA	5.01 ± 0.06 i	0.55 ± 0.03 i
Z-Phe-Arg-MCA	4.89 ± 0.03 i	22.46 ± 0.47 h
Z-Arg-Arg-MCA	2.00 ± 0.04 j	52.79 ± 1.15 g
Arg-MCA	0.72 ± 0.02 k	2.12 ± 0.24 i

[a]Different letters in the same column indicate significant differences ($p < 0.05$). Data are shown as mean ± standard deviation (n=4).

[b]Ala: L-alanyl; Arg: L-arginine; Asp(OBzl): (2S)-2-amino-3-(benzyloxycarbonyl) propionyl; Boc: *t*-butyloxycarbonyl; Gln: L-glutaminyl; Leu: L-leucyl; Lys: L-lysyl; MCA: 4-methylcoumaryl-7-amide; Phe: L-phenylalanyl; Pro: L-prolyl; Ser: L-seryl; Suc: Succinyl; Tyr: L-tyrosine; Val: L-valyl; Z: benzyloxycarbonyl.

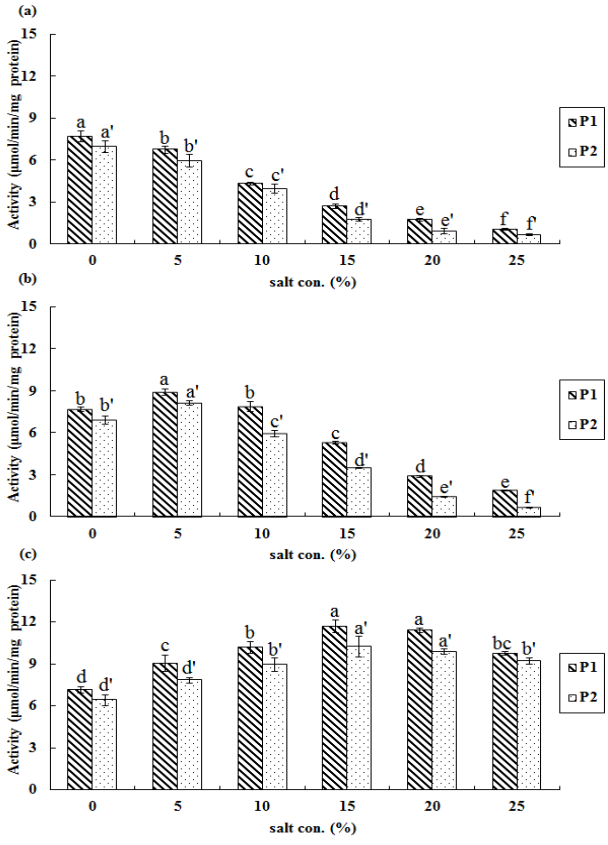

Figure 2. Salt-dependent catalytic activity of proteases from lizardfish viscera (intestine + pancreas) (a) at various NaCl concentration (from 5% to 25%), (b) after incubation in various NaCl solutions (from 5% to 25%) for 30 days, (c) after 50X reduction in salt concentration with exchange water after incubation in various concentration of NaCl solution (from 5% to 25%) for 30 days. Data are presented as the means of n = 4 experiments, each performed in triplicate. Different letters indicate statistically significant differences ($p<0.05$)

proteases were strongly inhibited by high NaCl concentrations (Klomklao *et al*., 2008). The loss of activity in salt solutions may be caused by enzyme denaturation. Klomklao *et al*. (2006) presented the "salting out" effect of NaCl on enzyme denaturation. In this salt system, water molecules are easily drawn out from protease molecules, promoting the aggregation of enzymes (Klomklao *et al*., 2004). Therefore, increased ionic strength caused by high salt concentration results in increased denaturation and loss of protease activity (Tungkawachara *et al*., 2003).

However, after incubation for 30 days in NaCl solution, the loss of protease activity from intestine + pancreas was decreased instead of increased (Figure 2b). To avoid the factor of bacteria, the CFU (Colony-Forming Units) of sample incubated in different concentration NaCl solution were measured and the rare colony were found in sample during the period of 30 days (data not shown). For the protease incubated in 15% NaCl solution, the activities were from 2.70 μmol/min/mg protein to 5.28 μmol/min/mg protein (P1) and from 1.75 μmol/min/mg protein to 3.50 μmol/min/mg protein (P2), indicating that the residual protease activities were enhanced markedly after incubation in high NaCl concentrations. A similar protective effect was observed in the presence of 20% and 25% NaCl (Figure 2b). In addition, Figure 2b also showed improved catalytic activities at 5% and 10% NaCl. In the presence of 5% NaCl, the relative activities of P1 and P2 increased to 115.13% and 114.60%, respectively. Also, the relative activities of P1 and P2 increased from 4.33 μmol/min/mg protein

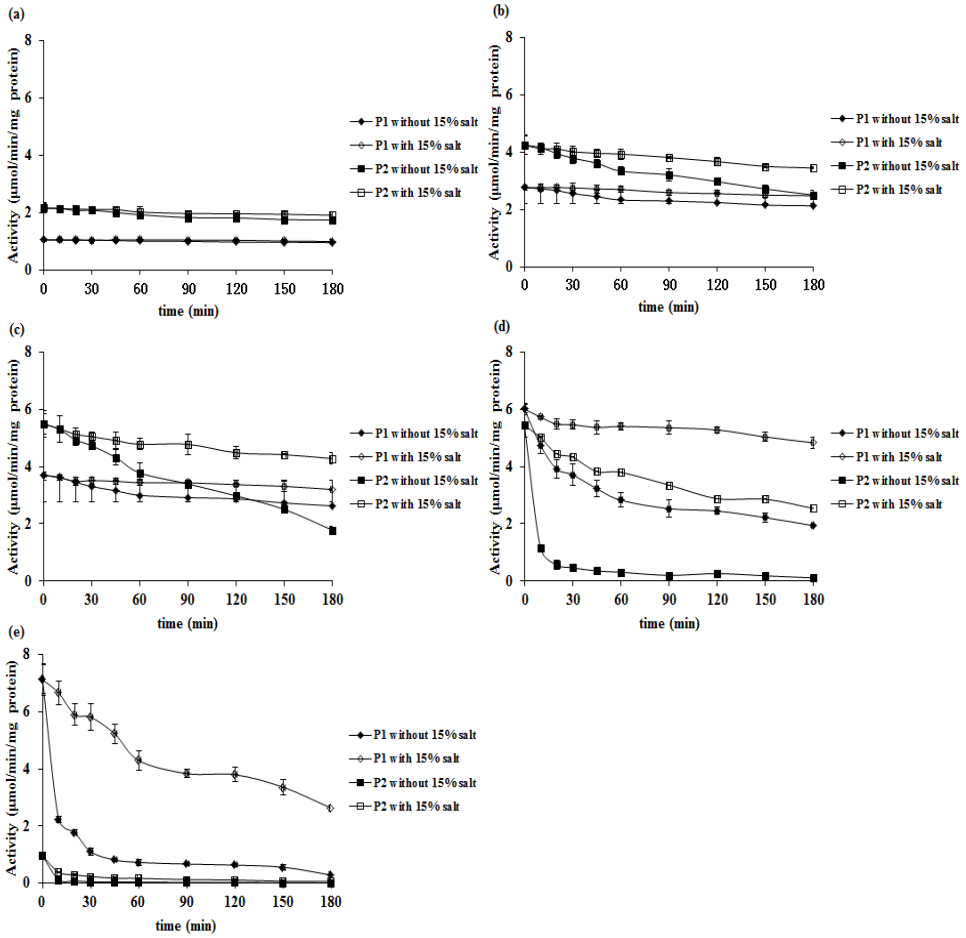

Figure 3. Thermal stability of protease (P1 and P2) at (a) 20°C, (b) 30°C, (c) 40°C, (d) 50°C, (e) 60°C. Data are presented as the means of n = 4 experiments, each performed in triplicate

to 7.87 μmol/min/mg protein, and 3.96 μmol/min/mg protein to 5.93 μmol/min/mg protein in the presence of 10 % NaCl, respectively.

Protease activity is generally expressed in a complex manner, and is not only related to the active site but also controlled by surface electrostatic interaction (Inouye *et al*., 1997). In salted sardine food, only one alkaline protease (a trypsin-like protease) and one acid protease (a pepsin-like protease) were observed to be stable for 3 months in the presence of 15-20% NaCl (Noda *et al*., 1982). They suggested that the fish proteins were resistant to conformational change at a high NaCl concentration. Furthermore, the specific interaction between salt ions and charged groups on the surface of proteases may be involved in enzyme activation and stability. In the presence of 4 M NaCl, the activity of a thermostable neutral metalloprotease from a bacterial broth culture was 13-15 times higher than in the absence of NaCl (Inouye *et al*., 1998). Therefore, the presence of salt ions is thought to be involved in conformational changes and thereby influence protease activity.

To further investigate the effect of NaCl on enzyme activity after incubation in NaCl solution, the relative activities of proteases incubated at 4°C for 30 days were measured after 50 x dilution with ion exchange water to reduce the effect of NaCl (Figure 2c). The activities were obviously enhanced after incubation in NaCl solution from 5% to 25%, especially in 15% NaCl. After incubation in 15% NaCl solution, the activities of P1 and P2 increased to 11.69 μmol/min/mg protein and 10.23 μmol/min/mg protein, respectively. Furthermore, after incubation in 25% NaCl solution, the activities of P1 and P2 increased to 9.76 μmol/min/mg protein and 9.23 μmol/min/mg protein, respectively. The recovery of protease activity indicated that the aggregation of protease caused by the presence of salt may be mitigated by the reduction of salt concentrations, especially the salt concentration was lower than 15%.

Effect of NaCl on the thermal stability of proteases in lizardfish intestine + pancreas

Figure 3 showed the thermal stability of proteases from intestine + pancreas in the absence of NaCl. P1 showed well thermal stability at 20°C, 30°C and 40°C (Figure 3a, b and c); the activity was 2.63 μmol/min/mg protein after incubation at 40°C for 180 min and

there were still 71.0% compared to the activity of un-incubated sample at the same temperature (3.70 μmol/min/mg protein). However, after incubation at 50°C and 60°C for 60 min, the activities were only 2.83 μmol/min/mg protein (47.09%) and 0.74 μmol/min/mg protein (10.39%), respectively (Figure 3d and F). On the other hand, the thermal stability of P2 was lower than that of P1 (Figure 3). The activity was 3.78 μmol/min/mg protein (68.85%) after incubation at 40°C for 60 min and 1.78 μmol/min/mg protein (32.42%) after 180 min. Furthermore, the activities were strongly decreased at 50°C and 60°C and they were only 1.16 μmol/min/mg protein and 0.13 μmol/min/mg protein, respectively, with 10 min incubation. After 60 min, minimal activity was observed at 50°C and 60°C (0.31 μmol/min/mg protein and 0.05 μmol/min/mg protein, respectively).

It is noteworthy that the thermal stability of proteases was markedly enhanced in the presence of 15% NaCl solution, especially at 50°C and 60°C (Figure 3d and e). The activity of P1 was 5.40 μmol/min/mg protein and 4.83 μmol/min/mg protein after incubation for 60 min and 180 min at 50°C (Figure 3d), respectively, and was higher than the same condition in the absence of NaCl (2.83 μmol/min/mg protein and 1.94 μmol/min/mg protein, respectively). After incubation at 60°C, the activity of P1 was 4.29 μmol/min/mg protein at 60 min and 2.62 μmol/min/mg protein at 180 min in the presence of NaCl; in comparison, the activity was 0.74 μmol/min/mg protein and 0.29 μmol/min/mg protein, respectively, when incubated under the same conditions without NaCl (Figure 3e).

Similarly, the activities of P2 were 4.78 μmol/min/mg protein at 40°C, 3.80 μmol/min/mg protein at 50°C and 0.18 μmol/min/mg protein at 60°C after incubation for 60 min in the presence of 15% NaCl (Figure 3c, d and e). Even at 180 min incubation, the activity was still 2.54 μmol/min/mg protein at 50°C (Figure 3d). In contrast, the activities for incubation under the same conditions in the absence of NaCl were only 3.78 μmol/min/mg protein, 0.31 μmol/min/mg protein and 0.05 μmol/min/mg protein, respectively (Figure 3c, d and e).

To date, the salt-dependency of fish protein has not been reported. However, research on a bacterial neutral metalloprotease showed that thermal stability was enhanced in the presence of 0.5-4.0 M NaCl, especially at a concentration of 0.5-1.5 M (Inouye *et al.*, 1998). In fact, the degree of activation by NaCl was affected by the degree of chemical modification (Inouye *et al*., 1998). They speculated the salt-dependent behavior of protease activity at a high salt concentration is related to the electrostatic properties, and that the acidic amino acids on the surface of enzymes, which react with hydrated salt cations, might enhance stability (Madern *et al*., 1995; Szeltner and Polgár, 1996).

Conclusion

Two types of serine proteases (P1 and P2) were observed in lizardfish intestine + pancreas, and showed high catalytic activity at 55ºC and pH 7.0 and 45ºC and pH 11.0, respectively. The resistance to high temperatures and extreme alkalinity of the protease is advantageous, suggesting the potential utilization of proteases from *surimi* manufacturing by-products. Furthermore, improvements of the catalytic activity and thermal stability of lizardfish proteases in the presence of NaCl were reported. After incubation in different NaCl solutions (from 5% to 25%) for 30 days, the relative catalytic activities of protease were increased, especially at 5% and 10% NaCl. In addition, in the presence of 15% NaCl, the thermal stability of proteases was obviously improved at various temperatures (20°C to 60°C), particularly at 50°C and 60°C, revealing the special role of salt ions in protease activity. The results of our study strongly indicate the potential application of proteases from lizardfish viscera to enzyme-aided industries.

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