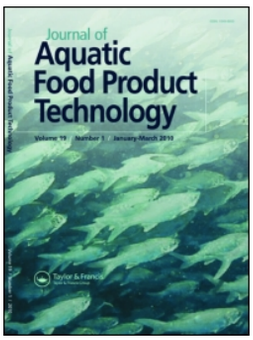

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Autolysis and Characterization of Sarcoplasmic and Myofibril Associated Proteinases of Oxeye Scad (*Selar boops*) Muscle

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ABSTRACT

The autolytic profile of oxeye scad mince was characterized. Mince showed higher proteolytic activity than washed mince. The highest autolysis was observed at 65 and 60°C for mince and washed mince, respectively. Both mince and washed mince showed the optimum pH for autolysis at pH 9.0, and their activities decreased with increasing NaCl concentration (0–3.5%). Autolysis of washed mince was markedly inhibited by soybean trypsin inhibitor (SBTI), suggesting that myofibril-associated proteinase was serine proteinase. Sarcoplasmic proteinase was characterized to be heat-activated alkaline proteinase having the optimal pH and temperature of 9.0 and 60°C, respectively. The activities were stable at pH range of 8.0–11.0 at 20–40°C. The crude proteinase was inhibited by *N-p*-tosyl-L-lysine chloromethyl ketone, SBTI, and phenylmethanesulfonyl fluoride, suggesting the predominance of serine proteinases, especially trypsin. NaCl suppressed the activity while β-mercaptoethanol, dithiothreitol, and $CaCl_2$ activated the activity. Therefore, trypsin-like proteinase is a major endogenous proteinase responsible for autolysis in oxeye scad muscle. The present results can be used as scientific guidelines to predict the gel strength of surimi made from oxeye scad muscle.

KEYWORDS

Proteinase; proteolysis; degradation; fish; oxeye scad; autolysis

Introduction

Postmortem muscle softening in fish is a serious problem during storage and processing. Proteolytic degradation of myofibrillar proteins has an adverse effect on gel-forming properties of surimi. The breakdown of myofibrillar proteins inhibits the development of three-dimensional gel network (Benjakul et al., 1996). In general, weakening of surimi gels occurs at temperatures above 50–70°C (modori). This phenomenon is induced by endogenous thermal stable proteinases, which can degrade myosin. During the water-minced flesh wash, approximately 60% of the muscle proteinases responsible for softening can be eliminated (Chang-Lee et al., 1989); however, the remaining activity is sufficient to degrade myofibrillar proteins resulting in gel weakening (Benjakul et al., 1996, 2003a, 2003b).

Alkaline proteinases are also implicated in some fish species. Benjakul et al. (2003a) reported that heat-activated alkaline proteinase in bigeye snapper (*Priacanthus macracanthus*) played an important role in degradation of muscle protein, especially at 60–65°C. The sarcoplasmic proteinase that contributes to poor gel forming ability of lizardfish (*Saurida tumbil*) muscle is also a heat-activated alkaline proteinase with an optimum pH and temperature of 8.0 and 65°C, respectively (Benjakul

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et al., 2003c, 2003d). Alkaline proteinase from white croaker (*Sciaena schlegeli*) had an optimum activity at 60°C and pH 8.0 (Makinodan et al., 1987). Alkaline proteinase with the optimum pH of 8 and temperature of 62°C was found in herring (*Clupea harengus*) muscle (Stoknes et al., 1993). Choi et al. (1999) reported two alkaline proteinases with optimum pH and temperature of 8.0 and 55°C, respectively, in Atlantic menhaden muscle.

Serine proteases were found to be responsible for textural breakdown of threadfin bream (*Nemipterus bathybius*; Toyohara and Shimizu, 1988), oval-filefish (Toyohara et al., 1990), and lizard-fish (*Saurida undosquamis, Saurida wanieso, Saurida elongata*; Suwansakornkul et al., 1993). These serine proteinases can be roughly subclassified into two groups based on their solubility in muscle homogenates. One group is tightly bound to myofibrils, which is called myofibril-bound serine protease (MBSP; Osatomi et al., 1997). MBSP degrades myofibrillar proteins, especially myosin heavy chain (MHC) effectively at around 55°C (Osatomi et al., 1997). The other, called sarcoplasmic serine proteinase (SSP), was detected in the soluble fraction of fish muscle homogenates. SSP and MBSP have been reported from the muscle of different fish species (Wu et al., 2010). Fish mince contained both sarcoplasmic proteases and myofibril-associated proteases (Yarnpakdee et al., 2009). Washing the mince with 50 mM NaCl could remove sarcoplasmic proteases to some degree. Nevertheless, the protease associated with the myofibrils still remained as indicated by the protein degradation, which was found in washed mince (Kinoshita et al., 1990; Toyohara et al., 1990). Benjakul et al. (2003b) reported that the maximum autolysis of mince and washed mince from two species of bigeye snapper (*P. macracanthus* and *P. tayenus*) was found at 60°C. Myofibril-bound serine proteases in carp (*C. carpio*), with an optimal temperature of 55°C, were responsible for the degradation of myosin heavy chain and other myofibrillar proteins such as α-actinin, β-actin, and tropomyosin (Jiang et al., 2006). Cao et al. (1999) reported that myofibril-bound serine protease from carp (*C. carpio*) muscle hydrolyzed MHC at 55–60°C effectively, whereas α-actinin and actin were not degraded.

In 2010, the catch of pelagic fish in the Gulf of Thailand was approximately 418,520 tons (Department of Fisheries, 2010). Among all dark-fleshed fish species, oxeye scad (*Selar boops*) is an abundant species commonly caught in southern Thailand, especially in the Thasala coast of Nakhon Si Thammarat. It has been reported that scad muscle can be used as a raw material for the production of surimi (Benjakul et al., 2008) and antioxidant peptides (Jiang et al., 2014; Thiansilakul et al., 2007a, 2007b). Therefore, the use of these pelagic fish for surimi production is a major challenge to transform the underutilized fish protein resources into human foods, particularly protein gel-based products. However, poor gelling characteristic was observed in the fish kept in ice for a long time, which was associated with degradation and denaturation of myofibrillar proteins (Wongwichian et al., 2013). The proteolytic activity, distribution, and type of proteinase of fish varied, depending on species and seasons (Suwansakornkul et al., 1993). So far, no information on proteolysis of oxeye scad, which is associated with gel quality, has been reported. The objective of this investigation was to study the autolysis and to characterize the sarcoplasmic and myofibril associated proteinases of oxeye scad (*S. boops*).

Materials and methods

Chemicals

Sodium caseinate, bovine hemoglobin, ethylenediaminetetraacetic acid (EDTA), pepstatin A, soybean trypsin inhibitor (SBTI), iodoacetic acid, *N-p*-tosyl-L-lysine chloromethyl ketone (TLCK), *N*-tosyl-L-phenyl-alanine chloromethyl ketone (TPCK), E-64, 1-(L-trans-epoxysuc cinyl-leucylamino)-4-guanidinobutane, *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) amino-methane, and Folin-Ciocalteu's phenol reagent were obtained from Merck (Darmstadt, Germany).

Fish sample

Oxeye scad (*S. boops*) with an average weight of 110–120 g was obtained from the fishing port in Thasala along the coast of the Gulf of Thailand. The fish, off-loaded approximately 12 h after capture, were placed in ice with a fish/ice ratio of 1:2 (w/w) and transported to the School of Agricultural Technology, Walailak University, Thasala Nakhon Si Thammarat, within 30 min. The fish samples were kept on ice for 30 min prior to preparation of mince.

Preparation of mince and washed mince

To prepare fish mince, the fish samples were immediately washed, gutted, filleted, skinned, and then ground through a 4 mm plate mincer. Washed mince was prepared according to the method of Toyohara et al. (1990) with a slight modification. Washing process was used to prepare the myofibril associated proteinases by removing water soluble proteins. Therefore, the sarcoplasmic proteinases were removed. Also, the remaining proteinase after leaching was referred to as myofibril associated protease. The comminuted fish meat was homogenized with 5 vol of 50 mM NaCl for 2 min, using an IKA Labortechnik homogenizer (Selangor, Malaysia). The homogenate was centrifuged at 10,000 × *g* at 4°C for 10 min, using a Biofuge primo centrifuge (Sorvall, Hanau, Germany). The washing process was repeated twice. The final precipitate is designated as "washed mince." To minimize the denaturation, the preparation was conducted at 1–4°C. Mince and washed mince were subjected to analysis for moisture content. The moisture content of both minces were adjusted to be equal.

Autolysis of mince and washed mince

Autolytic activity assay was measured according to the method of Visessanguan et al. (2001) with a slight modification. Mince or washed mince (3 g) was incubated for 60 min in a water bath, at various temperatures (30–80°C), and the autolytic reaction was terminated by addition of 27 mL of cold 5% (w/v) trichloroacetic acid (TCA). The mixture was homogenized for 1 min at 10,000 rpm. The homogenate was centrifuged at 8,000 × *g* for 10 min using a microcentrifuge. TCA-soluble peptide content in the supernatant was measured using the Lowry method (Lowry et al., 1951), with L-tyrosine as a standard and expressed as μmole tyrosine/g sample.

To construct a pH profile, 3 g of mince or washed mince were homogenized at 11,000 rpm for 1 min with 12 mL of different buffers having various pH (McIlvain's buffer consisting of 0.2 M sodium phosphate and 0.1 M sodium citrate was used for the pH ranges of 2.0–7.0, and 0.1 M glycine-NaOH was used for the pH ranges of 8.0–11) and incubated for 60 min at optimum temperature. The autolytic reaction was stopped by addition of 15 mL of cold 9% TCA. Soluble peptides were determined as described above.

To determine the type of proteinase causing the autolysis of mince and washed mince, various proteinase inhibitors were added into mince and washed mince to obtain the final concentration designated (2 mM EDTA, 0.01 mM SBTI, 0.01 mM E-64, and 10 mM pepstatin A; Klomklao et al., 2004). The mixtures were allowed to stand in ice for 2 h, followed by incubation at the optimal temperature for 60 min. Autolysis was terminated, and TCA-soluble peptide content was determined.

The effect of NaCl on autolytic activity was also studied. Mince or washed mince (3 g) was mixed with NaCl at different concentrations (0, 2, 2.5, 3, and 3.5% w/w). The mixtures were incubated at the optimal temperature for 60 min. Autolysis was terminated by addition of 27 mL of 5% TCA. TCA-soluble peptide content was determined. Blanks were prepared by adding TCA before incubation at the optimal temperature. Autolytic activity was expressed as μmole of tyrosine released/g sample.

Preparation of crude sarcoplasmic proteinase

Frozen fillets were partially thawed with running water (25°C), finely comminuted, and centrifuged at 5,000 × *g* for 30 min at 4°C. The fluid obtained, referred to as "crude sarcoplasmic proteinase" was kept in ice during activity assay.

Enzyme assay

Proteinase activity of the crude sarcoplasmic fluid was assayed using hemoglobin and casein as substrates, according to the method of Klomklao et al. (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 30 min. Enzymatic reaction was terminated by adding 200 µL of 50% (w/v) TCA. Unhydrolyzed protein substrate was allowed to precipitate for 1 h at 4°C, followed by centrifuging 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).

The pH and temperature profile

The pH and temperature profile on proteinase activity was determined according to the method of Klomklao et al. (2008). Proteolytic activity was assayed over the pH ranges 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 30 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 30 min under optimum pH.

The pH and thermal stability

The effect of pH on enzyme stability was determined according to the method of Klomklao et al. (2008), by measuring the residual activity after incubation at various pHs, at room temperature for 30 min. Different buffers were used as mentioned above. The remaining activity was determined.

For thermal stability, the enzyme solution was subjected to heating at different temperatures (20, 30, 40, 50, 60, 70, and 80°C) for 30 min in a temperature-controlled water bath. The heat-treated samples were immediately cooled in iced water and tested for the remaining activity.

Effect of inhibitors on sarcoplasmic proteinase activity

The effect of inhibitors on sarcoplasmic proteinase activity was determined according to the method of Klomklao et al. (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 SBTI, 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.

Effect of some chemicals on sarcoplasmic proteinase activity

The effect of some chemicals on sarcoplasmic proteinase activity was determined according to the method of Klomklao et al. (2007). Different chemicals were mixed with the sarcoplasmic fluid to obtain the concentration designated (0, 1, 5, and 10 mM for βME and DTT; and 0, 50, 100, and 200 mM for NaCl and $CaCl_2$). The mixture was allowed to stand at room temperature (26–28°C) for 30 min. Thereafter, the residual activity was measured.

Statistical analysis

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

Results and discussion

Effect of temperature on autolysis of oxeye scad mince and washed mince

Autolytic activity of oxeye scad mince and washed mince incubated at different temperatures was monitored and expressed in term of TCA-soluble peptide, as shown in Figure 1A. Highest TCA-soluble peptides in mince and washed mince were found when it was incubated at 65 and 60°C, respectively (Figure 1A). A high level of autolytic activity at relative high temperature indicated the presence of heat stable proteinases. Considering the autolytic activity between mince and washed mince, higher activity was found in mince at all temperatures employed. The result suggested that the mince may be composed of both sarcoplasmic and myofibril-associated proteases. Washing the mince with 50 mM NaCl could remove sarcoplasmic proteases to some degree. Thus, the remaining protease was lower in washed mince. Proteolysis of fish muscle caused by endogenous proteases has been widely reported. The highest autolysis of goatfish (*Mulloidichthys martinicus*) mince and washed mince was observed at 60°C (Yarnpakdee et al., 2009). Maximum autolytic activity of Indian anchovy was found at 60°C (Siringan et al., 2006). Benjakul et al. (2003d) reported that the highest autolysis was observed at 65 and 60°C for mince and washed mince from lizardfish (*Saurida tumbil*) muscle.

Effect of pH on autolysis of mince and washed mince

The pH profiles for the autolysis of mince and washed mince conducted at the optimal temperature (65 and 60°C, respectively) for 60 min are shown in Figure 1B. For both mince and washed mince, the major activity peak was found at pH 9. Therefore, alkaline proteases were the major proteases. Alkaline proteinase from white croaker had an optimum activity at 60°C and pH 8.0 (Makinodan et al., 1987). Alkaline proteinase with an optimum pH of 8 and temperature of 62°C was found in herring muscle (Stoknes et al., 1993). Choi et al. (1999) reported two alkaline proteinases with optimum pH and temperature of 8.0 and 55°C, respectively, in Atlantic menhaden muscle.

Effect of various protease inhibitors on autolysis of mince and washed mince

The effect of various protease inhibitors on autolysis of mince and washed mince is shown in Figure 2A. The decrease in TCA-soluble peptides was observed in the presence of proteinase inhibitors ($p < 0.05$; Figure 2A). SBTI was the most effective protease inhibitor for mince and washed mince. The autolysis was partially inhibited in presence of EDTA and E-64. However, pepstatin A did not exhibit the inhibitory activity against autolysis of oxeye scad mince and washed

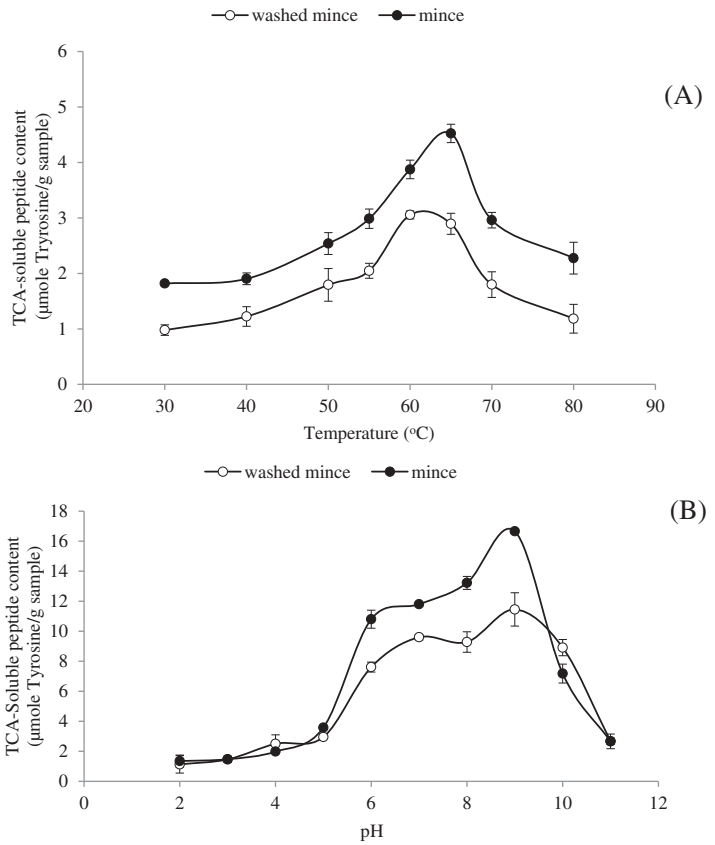

Figure 1. TCA-soluble peptide content of oxeye scad mince and washed mince incubated at different temperatures (A) and TCA-soluble peptide content of oxeye scad mince (65°C) and washed mince (60°C) incubated at various pHs (B). Autolysis was conducted for 60 min. Bars represent the standard deviation of triplicate determinations.

mince. SBTI is a serine proteinase inhibitor, while E-64 is considered to be cysteine proteinase inhibitor (Umezawa, 1976). The pepstatin A can inhibit most of the aspartic proteinases, and EDTA acts as a metalloproteinase inhibitor (Jiang et al., 1993). From the result, serine proteinase was the dominant proteinase in oxeye scad muscle. Myofibril-bound protease in lizardfish (*Saurida wanieso*) was classified as a trypsin-like protease, which showed optimal activity at 50–60°C and pH at 7–8, and it was also inhibited by SBTI, leupeptin, and TLCK (Cao et al., 1999). Yarnpakdee et al. (2009) found that cysteine proteases and serine proteases were most likely responsible for protein degradation of goatfish (*Mulloidichthys martinicus*). All tested inhibitors showed inhibition towards autolysis with varying degrees, suggesting that oxeye scad contained a variety of proteases with different characteristics.

Effect of sodium chloride on autolysis of mince and washed mince

Proteolytic activity of mince and washed mince slightly decreased with increasing NaCl concentration (Figure 2B). The decrease in activity might be due to the salt-induced denaturation of enzymes. The "salting out" effect was postulated to cause the enzyme denaturation. The water molecule is drawn from the enzyme molecule by salt, leading to the aggregation of those enzymes (Klomklao et al., 2004, 2007). However, serine proteinase in threadfin bream muscle was activated in the presence of 2–4% NaCl (Kinoshita et al., 1990). Benjakul et al. (2003d) reported that

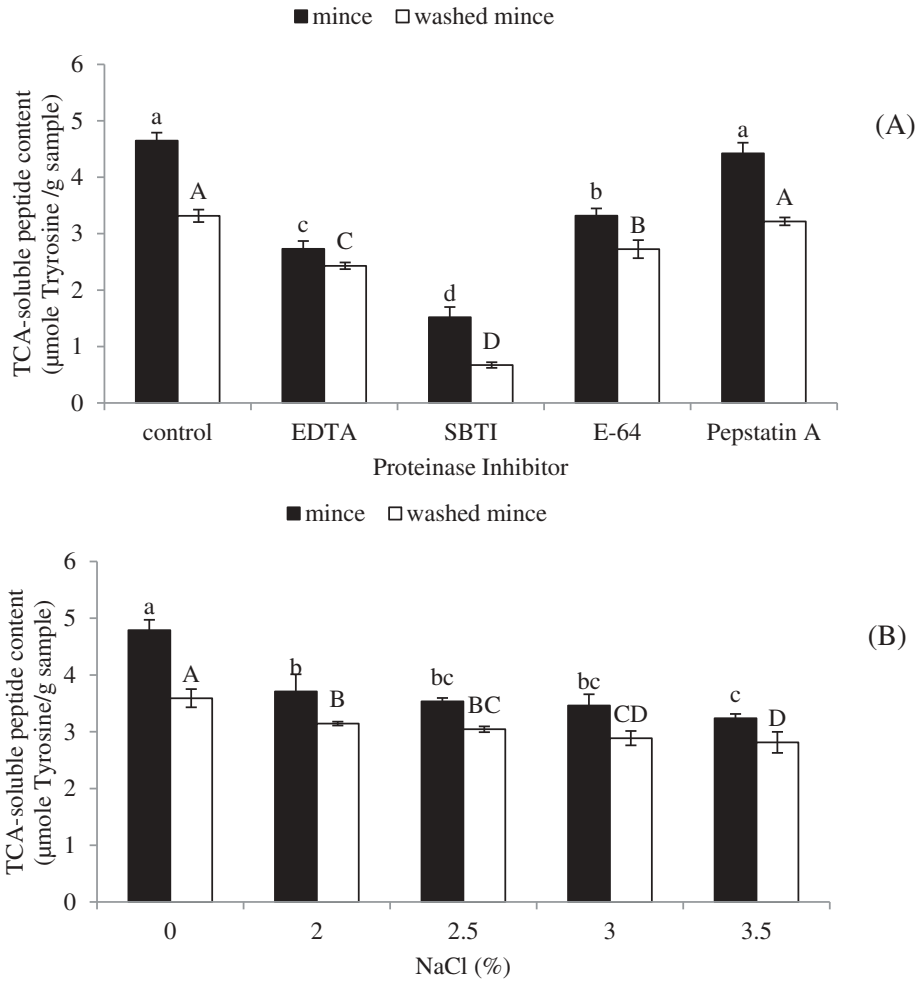

Figure 2. Effect of various proteinase inhibitors (A) and NaCl (B) on autolysis of oxeye scad mince and washed mince. Different letters on the bars indicate significant differences ($p < 0.05$).

myofibril associated proteinases from lizardfish (*Saurida tumbil*) were still active in the presence of NaCl at a level of 2–3%, which is commonly used in surimi processing. This may result in the degradation of protein matrix formed during thermal gelation process. From the result, proteinases from oxeye scad muscle can be partially inhibited by adding 2–3.5% NaCl.

The pH and temperature profiles of sarcoplasmic proteinases

The pH activity curves of crude proteinases from oxeye scad muscle are shown in Figure 3A. Optimal activity appeared at pH 9. The temperature profiles of crude proteinases are presented in Figure 3B. Maximum hydrolytic activity of the crude enzyme was found at 60°C when assayed at pH 9.0. This was in agreement with the higher rate of autolysis from ground oxeye scad at pH 9.0. Due to the high activity at alkaline pH and increased activity at higher temperature tested, it was presumed that heat-activated alkaline proteinases were predominant. Thus, the major proteinases in oxeye scad muscle were possibly heat stable alkaline proteinases. Alkaline proteinase with the optimum pH of 8 and temperature of 62°C was found in herring muscle (Stoknes et al., 1993). Choi et al. (1999) reported two alkaline proteinases with optimum pH and temperature of 8.0 and

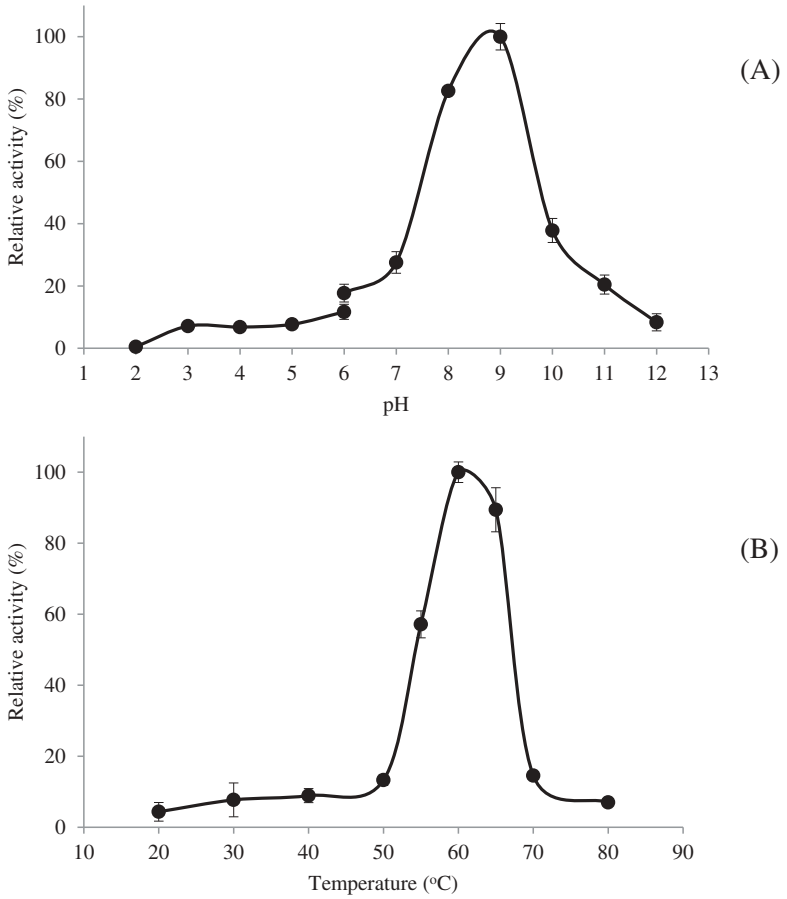

Figure 3. The pH (A) and temperature (B) profile of crude proteinases from oxeye scad muscle. Bars represent the standard deviation of triplicate determinations.

55°C, respectively, in Atlantic menhaden muscle. Optimum pH values of bigeye snapper (*Priacanthus macracanthus*) sarcoplasmic proteinases were found at pH 6.5 and 8.5 with an optimum temperature of 60°C (Benjakul et al., 2003a). Optimal activity of lizardfish protenases was at pH 8.0 at 55°C (Benjakul et al., 2003d). Therefore, sarcoplasmic proteinase can involve degradation of fish muscle, but it can be partially prevented by proper washing process.

The pH and thermal stability of sarcoplasmic proteinases

Crude proteinases from oxeye scad muscle were stable in the pH ranging from 8.0 to 11.0 (Fig. 4A), with the remaining activity above 95%. However, the activity was slightly decreased at pH below 7.0. The stability of trypsin at particular pH might be related to the net charge of the enzyme at that pH (Klomklao et al., 2006). At extreme pH, strong intramolecular electrostatic repulsion caused by high net charge results in swelling and unfolding of the protein molecules (Benjakul et al., 2000; Klomklao et al., 2007). Inactivation of enzyme activity at acidic pH was also reported for the anionic trypsins from capelin (Hjelmeland and Raa, 1982), true sardine (Kishimura et al., 2006), Atlantic white croaker (Pavlisko et al., 1997), yellowfin tuna (Klomklao et al., 2006), and skipjack tuna (Klomklao et al., 2009b). For thermal stability, crude proteinases from oxeye scad muscle was stable at temperature below 40°C, but the activity sharply decreased

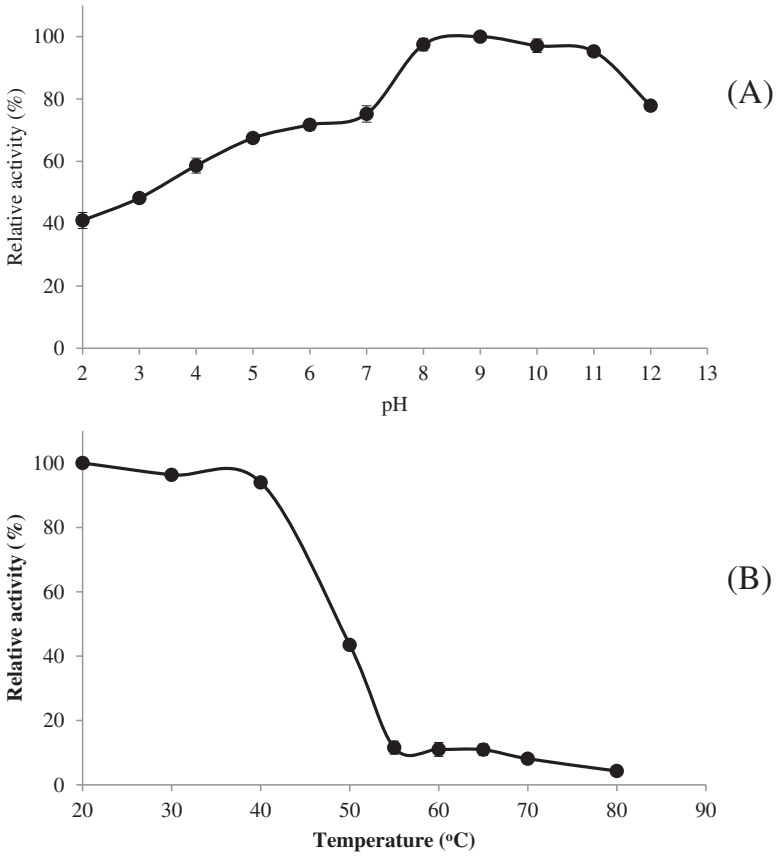

Figure 4. Effect of pH (A) and heating temperature (B) on the stability of crude proteinases from oxeye scad muscle. Bars represent the standard deviation of triplicate determinations.

above 50°C (Fig. 4B). Crude proteinases from oxeye scad muscle were almost completely inactivated at 80°C. At high temperatures, enzymes most likely underwent denaturation and lost their activity. Similar results were noticeable for the thermal stability of trypsin obtained from Atlantic blue carp (Dendinger and O'Connor, 1990), starfish (Kishimura and Hayashi, 2002), and true sardine (Kishimura et al., 2006). In general, enzymes are inactivated at high temperature due to the partial unfolding of the enzyme molecule. The mechanism for increasing thermal stability of proteins appears to be due to strengthening of hydrophobic interactions and disulfide bonding in the interior of the protein molecule (Kim et al., 1992). Higher thermostability was associated with a higher number of intramolecular disulfide bonds in protease. Disulfide bonds may stabilize a folded conformation of enzymes (Creighton, 1983).

Effect of various inhibitors and some chemical on crude proteinase activity

The effect of various inhibitors on crude proteinases of oxeye scad muscle is shown in Table 1. TLCK showed the highest inhibition (83%). SBTI, PMSF, and TPCK partially inhibited the activity. SBTI and PMSF are serine protease inhibitors (Benjakul et al., 2003b). TLCK and TPCK are specific inhibitors of trypsin and chymotrypsin, respectively. The inhibitor for aspartic protease involving pepstatin A did not show inhibitory effects toward trypsin activity. High inhibition of TLCK, SBTI, and PMSF toward the proteinases from oxeye scad muscle indicated the presence of serine proteinases, especially trypsin, in this fish species. *N*-ethylmaleimide, E-64, and iodoacetic acid also

Table 1. Effect of various inhibitors on proteinase activity of crude extracts from oxeye scad muscle.

Inhibitors	*Concentration*	*% Inhibition*[#]
Control	0	0a*
E-64	0.1 mM	14.95 ± 1.05c
N-ethylmaleimide	1 mM	32.52 ± 0.96e
Iodoacetic acid	1 mM	13.18 ± 1.09c
PMSF	1 mM	56.03 ± 0.92g
SBTI	1.0 g/L	62.49 ± 1.05h
TLCK	5 mM	83.07 ± 0.34i
TPCK	5 mM	36.49 ± 2.16f
EDTA	0.01 mM	27.73 ± 0.93d
Pepstatin A	2 mM	9.87 ± 1.17b

[#]Different letters in the same column indicate significant differences ($p < 0.05$).
*Mean ± *SD* from triplicate determination.

showed some inhibitory activity. Therefore, it can be inferred that the minor sarcoplasmic proteinase was cysteine proteinase. EDTA is a metalloprotease inhibitor (Klomklao et al., 2007). From the result, it was noted that EDTA showed partial inhibitory activity (approximately 27%), since EDTA is a chelator for many ions. From this point of view, oxeye scad muscle would be composed of proteinases that require metal ions for their activity.

Activity was increased in the presence of reducing agents (βME and DTT), especially when the concentration increased (Table 2). The result was in agreement with Iwata et al. (1974), who found that reducing agents markedly increased the activity of alkaline proteinase from carp muscle. It has been reported that lizardfish sarcoplasmic proteinase had the sulfhydryl group in the active site and reduced state was required for full activity. Iwata et al. (1974) found that alkaline proteinase activity in carp muscle was activated by reducing agent but inhibited by the sulfhydryl blocking agents. White croaker muscle contained alkaline proteases including protease I, which was trypsin-like enzyme, and protease II, which was SH-enzyme (Busconi et al., 1984). Activity was enhanced in the presence of $CaCl_2$, especially when the concentration used increased (Table 2). Calcium ions promoted the formation of active trypsin from trypsinogen and stabilized trypsin against autolysis (Klomklao et al., 2011). Calcium ions not only protected trypsin against self-digestion but also

Table 2. Effect of some chemicals on proteinase activity of crude extracts from oxeye scad muscle.

Chemicals	*Concentration (mM)*	*Relative activity (%)*[#]
βME	0	100.00 ± 0.00a*
	1	117.77 ± 0.68b
	5	121.02 ± 0.19c
	10	125.47 ± 0.56d
DTT	0	100.00 ± 0.00a
	1	104.03 ± 1.78b
	5	110.57 ± 0.75c
	10	138.12 ± 1.65d
NaCl	0	100.00 ± 0.00a
	50	94.27 ± 1.59b
	100	73.26 ± 0.25c
	200	61.57 ± 1.27d
$CaCl_2$	0	100.00 ± 0.00a
	50	109.54 ± 0.42b
	100	114.73 ± 1.1c
	200	126.12 ± 0.74d

[#]Different letters in the same chemicals indicate significant differences ($p < 0.05$). *Mean ± *SD* from triplicate determination.

slightly increased its activity (Klomklao et al., 2009a). These results confirmed that the predominant sarcoplasmic proteinases in oxeye scad muscle were trypsin-like enzymes. For the effect of NaCl, the proteinase activity was inhibited to a high extent in the presence of NaCl, particularly as the concentration increased. NaCl inhibited carp muscle alkaline proteinase activity (Iwata et al., 1974).

Conclusion

Autolysis of oxeye scad muscle was caused by both sarcoplasmic and myofibril associated proteinases. Trypsin-like proteinase, with optimal activity at pH 9.0 and 60°C, was the predominant proteinases in the crude extract. Activity decreased at low pH and high temperature with increasing NaCl concentrations, whereas it increased with increasing $CaCl_2$ and reducing agent concentrations. Therefore, these endogenous proteinases may be associated with the muscle softening and gel weakening of this species. However, the addition of appropriate food additives with proper heating is one of the possible ways to strengthen the surimi gel produced from oxeye scad muscle.

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References

Benjakul, S., Artharn, A., and Prodpran, T. 2008. Properties of protein-based film from round scad (*Decapterus maruadsi*) muscle as influenced by fish quality. LWT-Food Sci. Tech. 41: 753–763.

Benjakul, S., Seymour, T. A., Morrissey, M. T., and An, H. 1996. Proteinase in Pacific whiting surimi wash water: Identification and characterization. J. Food. Sci. 61: 1165–1170.

Benjakul, S., Visessanguan, W., and Leelapongwattana, K. 2003a. Purification and characterization of heat-stable alkaline proteinase from bigeye snapper (*Priacanthus macracanthus*) muscle. Comp. Biochem. Physiol. B 134: 579–591.

Benjakul, S., Visessanguan, W., and Leelapongwattana, K. 2003b. Comparative study on proteolysis of two species of bigeye snapper, *Priacanthus macracanthus* and *Priacanthus tayenus*. J. Sci. Food Agric. 83: 871–879.

Benjakul, S., Visessanguan, W., and Thummaratwasik, P. 2000. Isolation and characterization of trypsin inhibitors from some Thai legume seeds. J. Food Biochem. 24: 107–127.

Benjakul, S., Visessanguan, W., and Tueksuban, J. 2003c. Changes in physico-chemical properties and gel-forming ability of lizardfish (*Saurida tumbil*) during postmortem storage in ice. Food Chem. 80: 535–544.

Benjakul, S., Visessanguan, W., and Tueksuban, J. 2003d. Heat-activated proteolysis in lizardfish (*Saurida tumbil*) muscle. Food Res. Int. 36: 1021–1028.

Busconi, L., Folco, E. J., Martone, C., Trucco, R. E., and Sanchez, J. J. 1984. Identification of two alkaline proteases and a trypsin inhibitor from muscle of white croaker (*Micropogon opercularis*). FEBS Lett. 176: 211–214.

Cao, M.-J., Hara, K., Osatomi, K., Tachibana, K., Izumi, T., and Ishihara, T. 1999. Myofibril-bound serine proteinase (MBP) and its degradation of myofibrillar proteins. J. Food Sci. 64: 644–647.

Chang-Lee, M. V., Pacheco-Aguilar, R., Crawford, D. L., and Lampila, L. E. 1989. Proteolytic activity of surimi from Pacific whiting (*Merluccius productus*) and heat-set gel texture. J. Food Sci. 54: 1116–1119.

Choi, Y. J., Lanier, T. C., Leem, H. G., and Cho, Y. J. 1999. Purification and characterization of alkaline proteinase from Atlantic menhaden muscle. J. Food Sci. 64: 768–771.

Creighton, T. E. 1983. Proteins: Structures and Molecular Properties. New York, NY: W H Freeman.

Dendinger, J. E., and O'Connor, K. L. 1990. Purification and characterization of a trypsin-like enzyme from the midgut gland of the Atlantic blue crab, *Callinectes sapidus*. Comp. Biochem. Physiol. B 95: 525–530.

Department of Fisheries. 2010. Fishery Statistics Capture Product Yearbook 2010. Bangkok, Thailand: Author.

Hjelmeland, K., and Raa, J. 1982. Characteristics of two trypsin type isozymes isolated from the Arctic fish capelin (*Mallotus villosus*). Comp. Biochem. Physiol. B 71: 557–562.

Iwata, K., Kobashi, K., and Hase, J. 1974. Studies on muscle alkaline protease-II. Some enzymatic properties of carp muscular alkaline protease. Bull. Jap. Soc. Sci. Fish. 40: 189–200.

Jiang, H., Tong, T., Sun, J., Xu, Y., Zhao, Z., and Liao, D. 2014. Purification and characterization of antioxidative peptides from round scad (*Decapterus maruadsi*) muscle protein hydrolysate. Food Chem. 154: 158–163.

Jiang, S., Her, Y., Lee, J., and Wang, J. 1993. Comparison of the cathepsin D from mackerel (*Scomber australasicus*) and milkfish (*Chanos chanos*) muscle. Biosci. Biotechnol. Biochem. 57: 571–577.

Jiang, X. J., Zhang, Z. J., Cai, H. N., Hara, K., Su, W. J., and Cao, M. J. 2006. The effect of soybean trypsin inhibitor on the degradation of myofibrillar proteins by an endogenous serine proteinase of crucian carp. Food Chem. 94: 498–503.

Kim, H. R., Meyers, S. P., and Godber, J. S. 1992. Purification and characterization of anionic trypsins from the hepatopancreas of crayfish, *Procambarus clarkii*. Comp. Biochem. Physiol. B 103: 391–398.

Kinoshita, M., Toyohara, H., and Shimizu, Y. 1990. Diverse distribution of four distinct types of modori (gel degradation)-inducing proteinases among fish species. Nippon Suisan Gakk. 56: 1485–1492.

Kishimura, H., and Hayashi, K. 2002. Isolation and characteristics of trypsin from pyloric caeca of the starfish *Asterina pectinifera*. Comp. Biochem. Physiol. B 132: 485–490.

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

Klomklao, S., Benjakul, S., Kishimura, H., and Chaijan, M. 2011. 24 kDa trypsin: A predominant protease purified from the viscera of hybrid catfish (*Clarias macrocephalus* × *Clarias gariepinus*). Food Chem. 129: 739–746.

Klomklao, S., Benjakul, S., and Visessanguan, W. 2004. Comparative studies on proteolytic activity of spleen extracts from three tuna species commonly used in Thailand. J. Food Biochem. 28: 355–372.

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

Klomklao, S., Benjakul, S., Visessanguan, W., Kishimura, H., Simpson, B. K., and Saeki, H. 2006. Trypsins from yellowfin tuna (*Thunnus albacores*) spleen: Purification and characterisation. Comp. Biochem. Physiol. B 144: 47–56.

Klomklao, S., Kishimura, H., and Benjakul, S. 2008. Endogenous proteinases in true sardine (*Sardinops melanostictus*). Food Chem. 107: 213–220.

Klomklao, S., Kishimura, H., Benjakul, S., and Simpson, B. K. 2009a. Autolysis and biochemical properties of endogenous proteinases in Japanese sandfish (*Arctoscopus japonicus*). Int. J. Food Sci. Technol. 44:1344–1350.

Klomklao, S., Kishimura, H., Nonami, Y., and Benjakul, S. 2009b. Biochemical properties of two isoforms of trypsin purified from the intestine of skipjack tuna (*Katsuwonus pelamis*). Food Chem 115:155–162.

Lowry, O. H., Rosebrough, N. J., Fan, A. L., and Randall, R. J. 1951. Protein measurement with Folin phenol reagent. J. Biol. Chem. 193: 256–275.

Makinodan, Y., Yokoyama, Y., Kinoshita, M., and Toyohara, H. 1987. Characterization of an alkaline proteinase of fish muscle. Comp. Biochem. Physiol. B 87: 1041–1046.

Osatomi, K., Sasai, H., Cao, M., Hara, K., and Ishihara, T. 1997. Purification and characterization of myofibril-bound serine proteinase from carp (*Cyprinus carpio*) ordinary muscle. Comp. Biochem. Physiol. B 116: 183–190.

Pavlisko, A., Rail, A., and Coppes, Z. 1997. Characterization of trypsin purified from the pyloric caeca of the southwest Atlantic white croaker *Micropogonias furnieri* (Sciaenidae). J. Food Biochem 21: 383–400.

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

Steel, R. G. J., and Torrie, J. H. 1980. Principles and Procedures of Statistics: A Biometrical Approach. New York, NY: McGraw-Hill.

Stoknes, I., Rustad, T., and Mohr, V. 1993. Comparative studies of the proteolytic activity of tissue extracts from cod (*Gadus morhua*) and herring (*Clupea harengus*). Comp. Biochem. Physiol. B 106: 613–619.

Suwansakornkul, P., Itoh, Y., Hara, S., and Obatake, A. 1993. Identification of proteolytic activities of gel-degrading factors in three lizardfish species. Nippon Suisan Gakk. 59: 1039–1045.

Thiansilakul, Y., Benjakul, S., and Shahidi, F. 2007a. Compositions, functional properties and antioxidative activity of protein hydrolysates prepared from round scad (*Decapterus maruadsi*). Food Chem. 103: 1385–1394.

Thiansilakul, Y., Benjakul, S., and Shahidi, F. 2007b. Antioxidative activity of protein hydrolysate from round scad muscle using Alcalase and Flavourzyme. J. Food Biochem. 31: 266–287.

Toyohara, H., Sakata, T., Yamashita, K., Kinoshita, M., and Shimizo, Y. 1990. Degradation of oval-filefish meat gel caused by myofibrillar proteinase(s). J. Food Sci. 55: 364–368.

Toyohara, H., and Shimizu, Y. 1988. Relation between the modori phenomenon and myosin heavy chain breakdown in threadfin bream gel. Agric. Biol. Chem. 52: 255–257.

Umezawa, H. 1976. Structures and activities of protease inhibitors of microbial origin. Meth. Enzymol. 45: 678–695.

Visessanguan, W., Menino, A. R., Kim, S. M., and An, H. 2001. Cathepsin L: A predominant heat-activated proteinase in arrowtooth flounder muscle. J. Agric. Food Chem. 49: 2633–2640.

Wongwichian, C., Chaijan, M., and Klomklao, S. 2013. Physicochemical instability of muscles from two species of scad during iced storage. Chiang Mai J. Sci. 40: 681–688.

Wu, G. P., Chen, S. H., Liu, G. M., Yoshida, A., Zhang, L. J., Su, W. J., and Cao, M. J. 2010. Purification and characterization of a collagenolytic serine proteinase from the skeletal muscle of red sea bream (*Pagrus major*). Comp. Biochem. Physiol. B 155: 281–287.

Yarnpakdee, S., Benjakul. S., Visessanguan, W., and Kijroongrjana, K. 2009. Autolysis of goatfish (*Mulloidichthys martinicus*) mince: Characterisation and effect of washing and skin inclusion. Food Chem. 114: 1339–1344.