Chapter 2

Materials and Methods

1. Chemicals

Sodium caseinate, ethylenediaminetetraacetic acid (EDTA), ethyleneglycol-bis (β-aminoethyl ether) N,N,N'N'-tetraacetic acid (EGTA), pepstatin A, 1,10-phenanthroline, 1-(L-trans-epoxysuccinyl-leucylamino)-4-guanidinobutane (E-64), 2-mercaptoethanol (β-ME) and bovine serum albumin were purchased from Sigma Chemical Co. (St. Louis, MO, USA.). Soybean trypsin inhibitor, iodoacetic acid and phenylmethanesulfonyl fluoride (PMSF) were obtained from Fluka (Messerschmittstr, Switzerland). Lactacystin, *t*-Butyloxycarbonyl-L-Phenylalanyl-L-Seryl-L-Arginine 4-Methyl-Coumaryl-7-Amide (Boc-Phe-Ser-Arg-MCA), Carbobenzoxy-L-Phenylalanyl-L-Arginine 4-Methyl-Coumaryl-7-Amide (Z-Phe-Arg-MCA) and Carbobenzoxy-L-Arginine-L-Arginine 4-Methyl-Coumaryl-7-Amide (Z-Arg-Arg-MCA) were purchased from the peptide Institute, Inc (Osaka, Japan). Trichloroacetic acid, tris (hydooxymethyl) aminomethane and Folin-Ciocalteu's phenol reagent were obtained from Merck (Darmstadt, Germany). Sodium dodecyl sulfate (SDS) and N,N,N'N'-tetramethyl ethylene diamine (TEMED) were purchased from Bio-Rad Laboratories (Hercules, CA, USA).

2. Instruments

Instruments	Model	Company
Electrophoresis apparatus	Mini-Protean II	Bio-Rad, USA
Double-beam Spectrophotometer	UV-16001	SHIMADZU, Australia
pH meter	pH 500	CyberScan, Singapore
Refrigerated centrifuge	RC-5B plus	Solvall, USA
Water bath	W 350	Memmert, Germany
Magnetic stirrer	RO 10 power	KIKAL labortechnik, Germany
Homoginizer	IKA	labortechnik, Malaysia
Balance	AB 204	METTLER TOLEDO, Switzerland
Spectrofluorometer	RF-1501	SHIMADZU, Japan
Freeze –Dryer	Dura-Top^{TM} µp	FTS system, USA
Eppendorf Micro Centrifuge	5415C	Brinkmann, USA
Purifier	BioLogic LP	Bio-Rad, USA

Methods

3. Fish sample preparation

Bigeye snapper, *Priacanthus macracanthus* (Figure 5) and *Priacanthus tayenus* (Figure 6), were caught from Songkhla-Pattani coast, stored in ice and off-loaded approximately 24-36 h after capture. Fish were transported in ice to Department of Food Technology, Prince of Songkla University, Hat Yai within 2 h. Fish were then filleted, vacuum-packed and kept at –20°C until used.

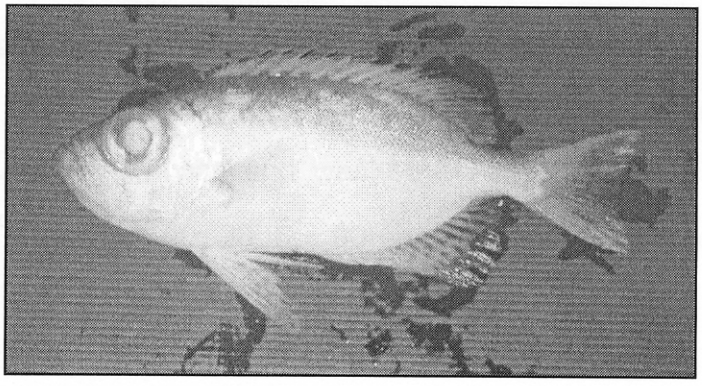

Figure 5 *Priacanthus macracanthus*

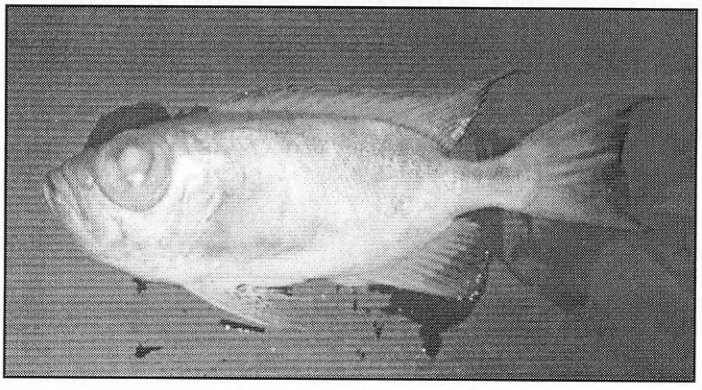

Figure 6 *Priacanthus tayenus*

4. Study on autolysis of mince and washed mince from bigeye sanpper

4.1 Preparation of washed mince

Washed mince was prepared according to the method of Toyohara *et al.* (1990c) with a slight modification. The comminuted flesh was homogenized with 5 volumes of 50 mM NaCl for 2 min using IKA Labortechnik homogenizer followed by centrifuging at 9,600 xg at 4°C for 10 min using a Sorvall Model RC-B Plus centrifuge. The washing process was repeated twice. To minimize the denaturation of enzymes, the preparation was conducted at temperature below 4°C.

4.2 Autolysis of mince and washed mince

Autolytic activity assay was performed according to the method of Morrissey *et al.* (1993). Mince and washed mince (3 g) were incubated at either 50 or 60°C for 10, 20, 30, 60, 90 and 120 min. The autolytic reaction was terminated by addition of 27 ml of cold 5% TCA. The mixture was homogenized for 2 min using a homogenizer and kept on ice for 1 h. The mixture was centrifuged at 8,500 rpm for 10 min (Eppendorf Micro Centrifuge) to collect the TCA-soluble supernatant. The soluble peptides released were measured using Lowry method (Lowry *et al.*, 1951) and autolytic activity was expressed as micromole of tyrosine released per minute (μmole Tyr/min). To determine the autolytic pattern, the reaction was terminated by adding preheated 5%(w/v) sodium dodecyl sulfate (SDS) (95°C), followed by SDS-PAGE analysis.

To determined the autolytic pattern, the reaction was terminated and proteins were solubilized with 5% (w/v) SDS, instead of 5% TCA. Twenty-seven ml of preheated 5%(w/v) sodium dodecyl sulfate (SDS) (95°C) was added to 3 g sample preincubated at different temperatures and times. The mixture was then homogenized for 1 min at speed 2. The homogenate was

incubated in an 85°C water bath for 1 h to dissolve completely all proteins. Insoluble residues were centrifuged at 8,500 rpm for 10 min (Eppendorf Micro Centrifuge) at room temperature. The supernatant was used for electrophoretic analysis and protein assay by Lowry method (Lowry *et al.*, 1951) using bovine serum albumin as standard.

4.3 Sodium dodecyl sulfate-gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed according to the method of Laemmli (1970). Protein solutions were mixed at 1:1 (v/v) ratio with the SDS-PAGE sample treatment buffer (0.125 M Tris-Cl pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol) and boiled for 3 min. The sample (20 μg) were loaded on the gel made of 4% stacking and 10% separating gels and subjected to electrophoresis at a constant current of 15 mA per gel using a Mini-Protean II Cell apparatus . After electrophoresis, the gels were stained with 0.2 % Coomassie brilliant blue R-250 in 45% methanol and 10% acetic acid and destained with 30% methanol and 10% acetic acid.

5. Characterization of sarcoplasmic proteinase from bigeye snapper

5.1 Preparation of sarcoplasmic fluid

Frozen fillets were partially thawed with running water (25°C), finely comminuted and centrifuged at 5,000 xg for 30 min at 4°C using a Sorvall Model RC-B Plus centrifuge. The fluid obtained was placed in ice during activity assay.

5.2 Enzyme assay

Proteinase activity was determined using casein-TCA-Lowry assay according to the method of An *et al.* (1994a). Enzyme solution (200 μl) was added to the preincubated reaction mixture containing 2 mg of casein and 625

μl reaction buffer (0.2 M McIlvaines's buffer, pH 2.0-7.5 and 0.1 M NaH_2PO_4-0.05 M $Na_2B_4O_7$, pH 8.0-10.5). The mixture was incubated at pH and temperature specified for precisely 40 min. Enzymatic reaction was terminated by adding 200 μl of 50% (w/v) trichloroacetic acid (TCA). After addition of TCA solution, unhydrolyzed proteins were allowed to precipitate for 15 min at 4°C and centrifuged at 8,500 rpm for 10 min. The oligopeptide content in the supernatant was determined by Lowry assay (Lowry *et al.*, 1951). Activity was expressed as tyrosine equivalents in TCA-supernatant. One unit of activity was defined as that releasing 1 nmole of tyrosine per min (nmol Tyr/min). A blank was run in the same manner, except the enzyme was added after addition of 50% (w/v) trichloroacetic acid (TCA).

5.3 pH and temperature profile

Activity assay was carried out at pH 3.0, 5.5 and 8.5 using 0.2 M McIlvaines's buffer (pH 2.0-7.5) and 0.1 M NaH_2PO_4-0.05 M $Na_2B_4O_7$ (pH 8.0-10.5) at various temperatures (25, 30, 37, 40, 45, 50, 55, 60, 65 and 70°C). Opimum pH was then determined over the pH range of 2.5 to 10.5 under optimum temperature.

3.4 Inhibitor study

Sarcoplasmic fluid was incubated with an equal volume of stock solutions of various proteinase inhibitors to obtain the final concentration designated (1 μM pepstatin A, 10 mM EDTA, 10 mM EGTA, 1 mM iodoacetic acid, 0.01 mM soybean trypsin inhibitor, 1 mM PMSF, 10 μM E-64, 1 mM 1,10-phenanthroline, 10 μM lactalcystin) at room temperature (26-28°C) for 10 min. The residual activity was assayed by casein-TCA-Lowry method.

Washed mince was also mixed with different proteinase inhibitors (0.01 and 0.1 mM soybean trypsin inhibitor, 0.01 mM E-64, 1 mM iodoacetic acid).

Mixtures were incubated at 60°C for 120 min. Reaction was terminated by addition of preheated 5% (w/v) sodium dodecyl sulfate (SDS) (95°C) and supernatant was subjected to SDS-PAGE analysis.

6. Purification of sarcoplasmic proteinase of *P. macracanthus*

6.1 Preparation of crude enzyme

Frozen bigeye snapper fillets were partially thawed with running water (25°C) until the temperature reached 0-2°C. Thawed samples were finely comminuted and centrifuged at 5,000 xg for 30 min at 4°C using a Sorvall Model RC-B Plus centrifuge. The obtained fish fluid was used as crude enzyme. All preparation procedures were carried out at 4°C.

6.2 Heat treatment

Fish fluid was diluted with an equal volume of 20 mM Tris-HCl, pH 7.5 , which was referred to as starting buffer (SB). The mixture was heated at 60 °C for 6 min in a water bath with continuous stirring, followed by immediate cooling in ice-water. The resulting precipitate was discarded after centrifugation at 13,800 xg at 4°C for 15 min. The supernatant was collected and filtered through Whatman paper No.4 and subjected to further purification.

6.3 Phenyl-Sepharose 6 Fast Flow chromatography

The supernatant was added with ammonium sulfate to obtain a final concentration of 1 M prior to loading to Phenyl-Sepharose 6 Fast Flow (high sub) colomn (1.6 x 19.5 cm), which was equilibrated with 1 M ammonium sulfate in SB. Sample was loaded to column at a flow rate of 1 ml/min at room temperature. Column was then washed with 1 M ammonium sulfate in SB until absorbance at 280 nm (A_{280}) was less than 0.05, then eluted with a linear gradient of 1 to 0 M ammonium sulfate in SB at a flow rate of 1 ml/min.

Fractions of 3 ml were collected. Fractions with caseinolytic activity were pooled and further purified by anion exchanger, Source 15 Q chromatography.

6.4 First Source 15Q chromatography

After fractions with caseinolytic activity on Phenyl-Sepharose 6 Fast Flow chromatography were collected and dialyzed against SB for 10-12 h at 4°C. Sample was then chromatographed on anion exchanger, Source 15Q column (1.6 x 5 cm) previously equilibrated with SB. Sample was loaded to column at a flow rate of 0.5 ml/min at room temperature. The column was washed with SB until absorbance at 280 nm (A_{280}) was less than 0.05 and then eluted with a linear gradient of 0 to 0.5 M NaCl in SB at a flow rate of 1 ml/min. Fractions of 2 ml were collected. Fractions with caseinolytic activity was pooled and further purified by second anion exchanger, Source 15Q chromatography.

6.5 Second Source 15Q chromatography

Pooled fractions of first Source 15Q chromatography were dialyzed against SB for 10-12 h at 4°C. Sample was then chromatographed on second anion exchanger, Source 15Q column (1.6 x 5 cm) previously equilibrated with SB. Sample was loaded to column at a flow rate of 0.5 ml/min at room temperature. The column was washed with SB until absorbance at 280 nm (A_{280}) was less than 0.05 and then eluted with a linear gradient of 0 to 0.5 M NaCl in SB at a flow rate of 1 ml/min. Fractions of 2 ml were collected. Fractions containing the caseinolytic activity were pooled and further purified by SEC-HPLC chromatography.

6.6 Size exclusion chromatography

Pooled fractions with caseinolytic activity obtained from second Source 15 Q chromatography were dialyzed against SB for 10-12 h at 4°C. The dialysate was lyophilized and then dissolved in distilled water prior to size exclusion chromatography. The sample was chromatographed on Superose 12 HR 10/30 column, which was equilibrated with approximately 2 bed volumes of SB. Sample was loaded to column at a flow rate of 0.2 ml/min at room temperature and then eluted with the same buffer at a flow rate of 0.2 ml/min. Fractions of 1 ml were collected and fractions with caseinolytic activity were pooled and used for further study. During purification, protein concentration was measured at 280 nm, and proteinase activity was measured using casein as a substrate at pH 8.5 and 60°C.

7. Characterization of purified sarcoplasmic proteinase from *P. macracanthus*

7.1 Determination of molecular weight

The molecular weight of purified proteinase were determined using a size exclusion chromatography on Superose 12 HR 10/30 column. The proteinase separated on size exclusion chromatography was estimated for its molecular weight by plotting available partition coefficient (K_{av}) against the logarithm of molecular weight of the protein standards. The elution volume (V_e) was measured for each protein standard and the proteinase. Void volume (V_0) was estimated by the elution volume of blue dextran (M_r 2,000,000). The standards used included aprotinin (M_r 6,500), carbonic anhydrase (M_r 29,000), bovine serum albumin (M_r 66,000) and alcohol dehydrogenase (M_r 150,000).

7.2 Optimum pH and temperature

The optimum pH of caseinolytic activity was measured using casein-TCA-Lowry method at 60°C over the pH range of 5 to 10 (0.2 M McIlvaines's buffer for pH 5.0-7.0 and 0.1 M Na_2PO_4-0.05 M $Na_2B_4O_7$ for pH 8.0-10.0). The optimum temperature was determined at pH 8.5 over a temperature range of 40 to 70°C.

7.3 Effect of inhibitors on proteinase activity

The effect of inhibitors on caseinolytic activity was determined by incubating proteinase with an equal volume of proteinase inhibitor solution to obtain the final concentration designated (1 μM pepstatin A, 10 μM E-64, 0.01 mM soybean trypsin inhibitor, 10 μM lactacystin, and 10 mM EDTA). After incubation the mixture at room temperature (26-28°C) for 10 min, remaining activity was measured by casein-TCA Lowry method (An *et al.*, 1994a).

7.4 Activity staining

Native polyacrylamide gels were prepared using 10% separation gels and 4% stacking gels by leaving out the SDS and reducing agent from the standard Laemmli protocol (Laemmli, 1970). Purified proteinase was incubated with and without an equal volume of proteinase inhibitor stock solutions to obtain the final concentration designated (1 μM pepstatin A, 10 μ M E-64, 0.01 mM soybean trypsin inhibitor, 0.1 mM soybean trypsin inhibitor, and 10 mM EDTA) for 10 min at room temperature. After incubation, proteinase was mixed with sample treatment buffer (0.125 M Tris-HCl, pH 6.8 containing 20% (v/v) glycerol) at 4:1 (v/v) ratio, and 5 μg of proteins were loaded on the gel. The proteins were subjected to electrophoresis at a constant current of 15 mA per gel by Mini-Protean II Cell apparatus. After

electrophoresis, gels were immersed in 100 ml of 2% (w/v) casein in 50 mM Tris-HCl buffer, pH 7.5 for 1 h with constant agitation at 0°C to allow the substrate to penetrate into the gels. The gels were then transferred to 2% casein (w/v) in 0.1 M Na_2PO_4-0.05 M $Na_2B_4O_7$ buffer, pH 8.5 and incubated at 60 °C for 30 min with constant agitation to develop the activity zone. The gels were fixed and stained with 0.125% Coomassie blue R-250 in 45% ethanol and 10% acetic acid and destained in 30% methanol and 10% acetic acid. Development of clear zones on blue background indicated proteolytic activity.

7.5 Substrate specificity

The hydrolytic activity on various synthetic substrates including Boc-Phe-Ser-Arg-MCA, Z-Phe-Arg-MCA and Z-Arg-Arg-MCA were determined according to the method of Osatomi *et al.* (1997) with a slight modification. The mixture, containing 700 µl of 0.1 M Na_2PO_4-0.05 M $Na_2B_4O_7$ buffer, pH 8.5 and 100 µl of 10 µM substrate solution were preincubated at 60°C for 5 min. The enzymatic reaction was initiated by adding 200 µl of enzyme solution with appropriate dilution to the mixture and then incubated at 60°C for 10 min. The reaction was terminated by adding 1.5 ml of the stopping mixture (methyl alcohol : n-butyl alcohol : distilled water = 35 : 30 : 35, v/v for Boc-Phe-Ser-Arg-MCA and 2 mM iodoacetic acid for Z-Phe-Arg-MCA and Z-Arg-Arg-MCA). Reaction mixture was centrifuged at 10,000 rpm for 5 min. Then fluorescence intensity was measured at an excitation of 380 nm and emission of 460 nm using spectrofluorometer.

8. Hydrolysis of natural actomyosin by purified proteinase

Natural actomyosin (NAM) was prepared according to the method of Benjakul *et al.* (1997). Bigeye snapper muscle (7.5 g) was homogenized in 75 ml chilled (4°C) 0.6 M KCl, pH 7.0 for 4 min using IKA Labortechnik

homogenizer. The extract was centrifuged at 5,000 ×g for 30 min at 0°C. Three volumes of chilled distilled water were added to precipitate NAM. NAM was collected by centrifuging at 5,000 ×g for 20 min at 0°C. Mixture consisting of 1 g NAM (14 mg protein) and purified proteinase (1 unit) were incubated at 60°C for 0, 5, 10, 20, 30 and 60 min. Control was performed by incubating NAM at 60°C for 60 min without the addition of purified proteinase. Reaction was terminated by adding preheated 5%(w/v) SDS (95°C). Supernatnat was subjected to SDS-PAGE analysis.

9. Protein determination

Protein concentration was measured by the method of Lowry *et al.* (1951) using bovine serum albumin as standard. Protein concentration in fractions obtained from ion exchange chromatography and size exclusion chromatography was determined according to the method of Bradford (1976).

10. Statistical analysis

Completely randomized design was used throughout this study. Data was subjected to analysis of variance (ANOVA) and mean comparison was carried out using Duncan's Multiple Range Test (DMRT). Statistical analysis was performed using the statistical Package for Social Sciences (SPSS for windows: SPSS Inc.).