CHAPTER III

MATERIALS AND METHODS

Materials

Ornate threadfin bream *N. hexodon*, Japanese threadfin bream *N. japonicus*, Fork-tailed threadfin bream *N. fergosus*, Lattice monocle bream *Scolopsis taeniopterus*, Purple-spotted bigeye *Priacanthus tayenus*, Tiger-toothed croaker *Otolithes ruber*, Brushtooth lizardfish *Saurida undosquamis*, Nile tilapia *Oreochromis niloticus*, Ruby tilapia *O. niloticus* x *O. placidus* were used in this experiment. All of fresh fishes used in SDS-PAGE analysis were bought from Sathon seafood distribution market, Bangkok, Thailand. Ornate threadfin bream for extraction of sarcoplasmic proteins was caught from Indo-West Pacific region, frozen-on-board (-40°C) and kept at -18°C by Thai fishing ship. It was transferred by ship to Thailand in 30-45 days, and shored at fish market, Amphur Muang, Samut Sakhon Province.

Cod *Gadus morhua* was perchased from supermarket at State College, PA, USA.

Chemicals

- Unless otherwise stated, chemicals were obtained from Sigma-Aldrich Chemical, USA. A list of other chemicals and suppliers is as given below;
- Acrylamide-bisacrylamide Ready solution DNA/PAGE (40:5): 40% w/v acrylamide monomer solution containing 5% w/w *N, N'*-methylene-bisacrylamide. (Amersham Bioscience, USA)
- Beta-lactoglobulin (BioPURE® Davisco Foods international, Inc., USA)
- Sodium caseinate (EP® Euro Proteins, USA)

Equipments

- 5-place balance (Perkin Elmer, USA)
- Centrifuge, Sorvall® model RC-5C Plus (Du Pont, USA)
- DSC instrument, model Q100 (TA Instruments Ltd, USA).
- Freeze-dryer, Heto Drywinner (Heto Holten A/S, Denmark)
- Gel filtration system:
  - XK 16/100 column (Amersham Bioscience, USA)
  - Pharmacia peristaltic pump model P-1 (Amersham Bioscience, USA)
  - Fraction collector systems, RediFrac (Phamacia Biotech, Sweden)
- Microcentrifuge, Eppendorf model 5415 C (Eppendorf, Germany)
- Oscillatory dynamic stress rheometer (Ares III model, TA Instruments Ltd, USA)
- pH meter, Cyberscan pH 1000 (Eutech Instruments, Singapore)
- Polyacrylamide gel electrophoresis system:
  - Electrophoresis apparatus, Hoefer® SE600 series (Hoefer Scientific Instruments, USA)
  - Power supply, Bio-Rad, 3000Xi (Bio-Rad, USA)
- Spectrofluorometer, Model 206 (Perkin Elmer, USA)
- Spectrophotometer, Helios α (Thermo Spectronic, USA)
- Temperature controlled unit, Environ-shaker model 3597 (Lab-Line Instruments, Inc., USA)
- Ultra Turrax® T25 basic IKA Labortechnik homogenizer (IKA® Works Inc., USA; Homogenizer 115V, 50/60 Hz; Dispersing tool: S25NK-19G, Fine, 19 mm. dia.)
- Waring blender, model 51BL30 with MC2 mini stainless steel container (Waring Commercial, USA)

3.1 Physicochemical properties of TBSP

3.1.1 Sarcoplasmic proteins preparation

Fish was thawed by running tap water. Fish fillets were prepared and muscle was cut into small pieces. Forty grams of flesh fish was homogenized, with 5 volumes of cold 0.1 M phosphate buffer pH 7, by using a Waring blender at lower speed for 30 sec and high speed for 30 sec. The homogenates was then centrifuged at 12,000 x *g* for 15 min at 4°C (Morioka et al., 1997). The supernatants were freeze-dried. Protein powders were pooled, packed in plastic bags and kept at 4°C until used. These protein powders were used for further study.

3.1.2 Proximate analysis and amino acid profile

The proximate analysis and amino acid profile of TBSP powder (wet basis) were determined by Medallion Laboratories, 9000 Plymouth Avenue North, Minneapolis MN 55427 (www.medallionlabs.com) using AOAC method (AOAC, 1997).

- Protein was determined by Dumas method: AOAC 968.06 Protein (crude) in animal feed, Dumas Method (F = 6.25).
- Fat was determined by acid hydrolysis: AOAC 952.06 Fat in various food stuffs.
- Moisture was determined by AOAC 930.15 Moisture in various foods.
- Ash was determined using AOAC 923.03 Ash of flour.
- Carbohydrate content was calculated by adding the percentages of protein, moisture, fat & ash and subtracting the result from 100%.
- Amino acid profile was analyzed by internal method of Medallion Laboratories, 9000 Plymouth Avenue North, Minneapolis MN 55427 (www.medallionlabs.com). The peptide linkages in the food sample's protein are broken by acid hydrolysis, by heating with 6 M HCl for 24 h. After the acid is removed, the sample is diluted with pH 2.2 buffer solution and centrifuged. The sample is then injected in the HPLC. Responses are compared to a standard.

3.1.3 Determination of sulfhydryl group

Sulfhydryl group (-SH) of TBSP was determined by the method of Koka, Milolajcik and Grould (1968) with slight modification using Ellman's reagent (5,5' dithio-bis-2-nitrobenzoic acid, DTNB). Exactly 0.1 g of TBSP was added directly to 3 mL urea (8 M in phosphate buffer, pH 6.8) containing 0.1 mL DTNB (40 mM, pH 6.6), which was then diluted to 10 mL using water, followed by absorbance reading at the wavelength of 412 nm with spectrophotometer. All the experiments were conducted at room temperature. Sulfhydryl content was calculated from the relation,

$$\text{SH group } (\mu\text{moles/g}) = \frac{OD_{412} \times \text{Volume of sample (mL)}}{1.36 \times 10^4 \text{ (L mole}^{-1}\text{)} \times 1000 \text{ (mL L}^{-1}\text{)} \times \text{weight of sample (g)}}$$

where, volume of sample prepared is 10 mL and weight of sample is 0.1 g

3.1.4 Determination of surface hydrophobicity

Surface hydrophobicity of proteins was determined using the hydrophobic fluorescence probe, l-anilino-8-naphthalene sulfonate (ANS). The measurement was performed according to the method of Kato and Nakai (1980) and Wicker et al. (1986) with slight modification. Proteins solution (1 mg/mL in 0.1 M phosphate buffer, pH 7.0) was serially diluted with 0.1 M phosphate buffer (pH 7.0) to obtain protein amounts of 20, 40, 60, 80 and 100 μL in a total volume of 2.8-3.00 mL (0.1 M phosphate buffer pH 7.0). In all cases, 0.1 mL ANS (1.0 mM in 0.1 M phosphate buffer pH 7.0; final concentration is 32 μM in the test tube) was added. After keeping at room temperature for 15 min, fluorescence intensity (RFU) of ANS was measured with a spectrofluorometer using an excitation wavelength of 380 nm and emission wavelength of 475 nm. Since the volume was slightly different (protein solution was added in an incremental fashion in the same cuvette) the following corrections were applied:

Correction of 20 μL = (2.92/3.00) x RFU

40 μL = (2.94/3.00) x RFU

$$60\ \mu L = (2.96/3.00) \times RFU$$

$$80\ \mu L = (2.98/3.00) \times RFU$$

$$100\ \mu L = (3.00/3.00) \times RFU$$

The initial slope (S_o) of the fluorescence intensity (corrected for RFU) plotted versus protein concentration (%) plot was calculated by linear regression analysis. S_o was used as an index of protein hydrophobicity.

3.1.5 Protein determination by modified Lowry's method

The modified Lowry's method (Peterson, 1977) was used as an alternative method of protein determination whenever appropriate.

Stock reagents

1. Copper-tartrate-carbonate (CTC). A solution of about 20% sodium carbonate was added slowly while stirring to a solution of copper sulfate-tartrate to give final concentrations of 0.1% copper sulfate (pentahydrate), 0.2% potassium tartrate, 10% sodium carbonate.
2. 10% sodium dodecyl sulfate (SDS)
3. 0.8 N Sodium hydroxide (NaOH)
4. 2 N Folin-Ciocalteu phenol reagent

Working solutions

1. 0.15% Sodium deoxycholate (DOC)
2. 72% Trichloroacetic acid (TCA)
3. Bovine serum albumin (BSA) (1 mg/mL)
4. Reagent A: A mixture of one part of stock CTC, SDS, NaOH and distilled water.
5. Reagent B: A mixture of one part of Folin-Ciocalteu phenol reagent and five parts of distilled water.

The amount of 0.1 mL of 0.15% DOC was added to 1.0 mL of fish protein sample (containing $\leq 200\ \mu g$ of protein) in 1.5-microcentrifuged tube. After mixing, the sample was allowed to stand for 10 minutes. Then, 0.1 mL of 72% TCA solution was added, mixed and allowed to stand for 10 minutes. The precipitate was centrifuged at 12,000 x *g* for 10 minutes using microcentrifuge.

The tubes were inverted over an absorbent paper to remove supernatant. The pellet was suspended with 1.0 mL of Reagent A and mixed vigorously. After 10 minutes, 0.5 mL of Reagent B was added and mixed thoroughly. Absorbance readings were recorded at 750 nm (A750) after 30 minutes standing. Assays were carried out in triplicates and blanks were prepared the same as sample but used distilled water as sample. The absorbance was used in calculation for protein concentration with equation from the standard curve. The procedure for modified Lowry's assay is summarized by a flow chart as shown in Figure 3.1. A standard curve was obtained using BSA (10-100 μg/mL). A linear plot of BSA concentration versus A750 was carried out.

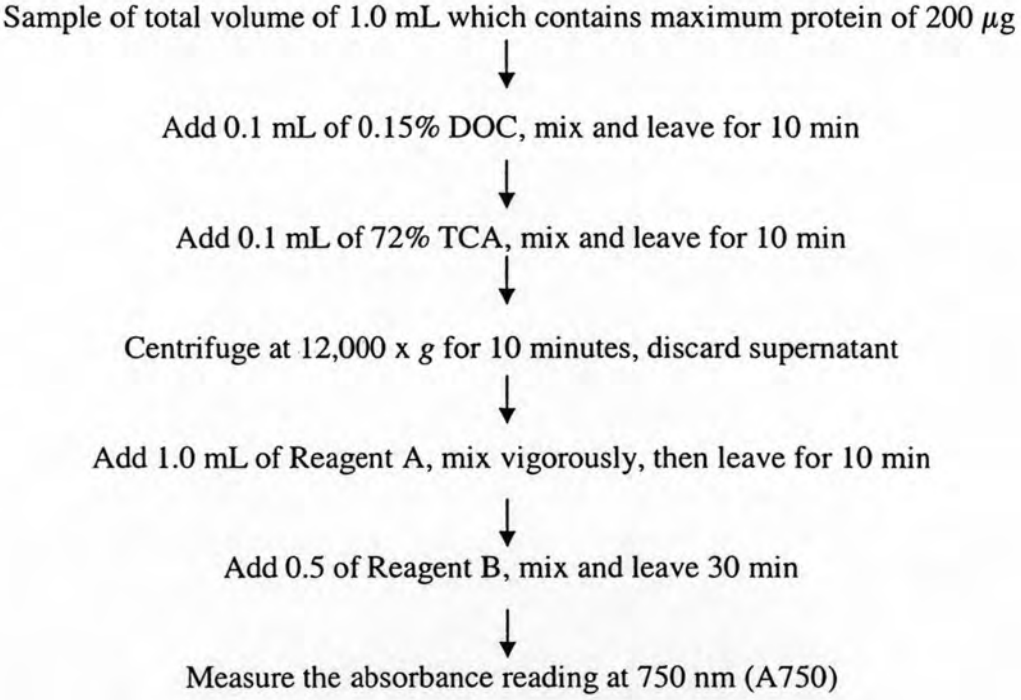

Figure 3.1 The flow chart of the modified Lowry's assay

3.1.6 Determination of amino group content by trinitrobenzenesulfonic acid

The amino group content was determined by TNBS method. The TNBS method is based on the reaction of primary amino groups with 2, 4, 6-trinitrobenzenesulfonic acid (TNBS) reagent (Adler-Nissen, 1979). Twenty microliters of protein solutions (1 mg/mL in phosphate buffer pH 7) were added to 980 μL of 0.10 M sodium tetraborate buffer (pH 9.3) followed by 25 μL of 0.03 M TNBS in a 1.5 mL-plastic cuvette. The mixture was agitated to ensure complete mixing, and allowed to stand for 30 min (for 60 min in study an effect of inhibitors on crude protease of TBSP; section 4.2.4) at room temperature. The reagent blank consisted of 25 μL of 0.03 M TNBS in 1 mL of 0.10 M sodium tetraborate. Absorbance was read at 420 nm and α-amino groups were expressed in terms of amino group. The amount of free amino gruoup in TBSP samples is calculated from the equation below:

$$\text{Amino group (moles/g protein)} = \frac{\text{A420 x 0.001025 (L)}}{\text{20300(L mole}^{-1}\text{cm}^{-1}\text{ x 1 cm x W (g)}}$$

where W (g) is the amount of protein present in 0.025 mL of the TBSP (1% w/w) solution added to each cuvette for the TNBS assay. It can be shown that;

W (g) = 0.025 mL x 0.01 g/mL x Kjeldahl protein (%)

3.1.7 Determination of molecular weight by SDS-PAGE

SDS-PAGE (12.5% gel) was performed according to the Laemmli system (Laemmli, 1970) to estimate the molecular weight of fish sarcoplasmic proteins. The following stock solutions were prepared.

Stock Solutions

- Acrylamide-bisacrylamide Ready solution DNA/PAGE (40:5): 40% (w/v) acrylamide monomer solution containing 5% (w/w) *N, N'*-methylene-bisacrylamide
- Resolving gel buffer (1.5 M Tris-HCl, pH 8.8)
- Stacking gel buffer (0.5 M Tris-HCl, pH 6.8)

- Sodium dodecyl sulphate solution (SDS) (10% SDS)
- Ammonium persulphate, 10% w/v, freshly prepare prior to use.
- TEMED (*N, N, N', N'*- tetramethylenediamine)
- Reducing sample buffer (0.06 M Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.025% bromophenol blue and 5% 2-mercaptoethanol).

Separating gel and stacking gel were prepared in a gel sandwiches plate (120x100x1 mm) according to the formula shown in Table 3.1

Table 3.1 Separating gel and stacking gel formula

Stock solutions (mL)	Separating gel, 12.5%	Stacking gel, 4%
1. Acrylamide-bisacrylamide ready solution (40:5)	4.68	0.5
2. Separating gel buffer, pH 8.8	3.75	-
Stacking gel buffer, pH 6.8	-	1.25
3. Deionized water	6.34	3.17
4. 10% SDS	0.15	0.05
5. 10% Ammonium persulphate	0.075	0.025
6. TEMED	0.005	0.0025
Total volume	15.0	5.0

Modified from Amersham Pharmacia Biotech (1999)

- Staining solution: Coomassie Brilliant Blue R-250 0.1% (w/v) in 40% methanol (v/v) and 10% glacial acetic acid.
- Destaining solution : 40% methanol (v/v) and 10% glacial acetic acid (v/v)
- Electrode buffer (Tank buffer): 0.025 M Tris, 0.192 M glycine, 0.1% SDS, pH 8.3.

Electrophoresis gels were prepared as shown in Table 3.1. The Hoefer® electrophoresis apparatus was set according to the manufacture's instruction. Fish sarcoplasmic proteins were extracted as described in section 3.1.1, and the supernatants before freeze-dried were used as protein samples for applying to lane.

The protein samples were prepared by adjusting fish protein concentration to 1 mg/mL with sample buffer and heated for 1 minute in a boiling water bath. Protein concentration was determined by the modified Lowry's method (Peterson, 1977). Fish sarcoplasmic proteins samples containing 6 μg of protein were applied to each lane. The molecular weight markers were also loaded for the MW estimation. The electrophoresis was performed on the Bio-Rad 3000Xi power supply with a constant current 15 mA for the part of stacking gel. The constant current was increased to 20 mA per gel plate when the sample lines start to enter the resolving gel.

The electrophoresis was run until the tracking dye reached the bottom of the gel. The gel was removed from the sandwiches, stained with Coomassie Blue R-250 gel stain solution (1 g Coomassie Blue R-250 in 450 ml methanol, 450 ml deionized water and 100 ml glacial acetic acid) and destained few times with 200 ml of Coomasie gel destain solution (the mixture of 100 ml methanol, 800 mldeionized water and 100 ml glacial acetic acid) until the gel was clear (Amersham Pharmacia Biotech, 1999). After destaining, gels were stored in water for further analysis. The protein molecular weight marker (wide range, Sigma) was used: Aprotinin, bovine lung (6.5 kDa); α-Lactalbumin, bovine milk (14.2 kDa); Trypsin inhibitor, soybean (20 kDa); Trypsinogen, bovine pancreas (24 kDa); Carbonic anhydrase, bovine erythrocytes (29 kDa); Glyceraldehyde-3-phosphate dehydrogenase, rabbit muscle (36 kDa); Ovalbumin, chicken egg (45 kDa); Glutamic dehydrogenase, bovine liver (55 kDa); Albumin, bovine serum (66 kDa); Fructose-6-phosphatekinase, rabbit muscle (84 kDa); Phosphorylase b, rabbit muscle (97 kDa); β-Galactosidase, *E. coli* (116 kDa); Myosin, rabbit muscle (205 kDa).

The gels bands were recorded by photography using a digital camera. Gel band intensity from the pictures was transferred to digital data using QuantiScan software version 2.1 for Windows (Biosoft, UK). Molecular weight of proteins was calculated by the method described in the manufacture's manual (Amersham Pharmacia Biotech, 1999). A linear plot of ln MW (kDa) of standard proteins (markers) against the migration distance was obtained. Relative mobility is the ration of a protein and tracking dye migration from the top to the bottom.

3.1.8 Determination of molecular weight by gel filtration

TBSP (1% w/w) was dissolved in 0.1 M phosphate buffer, pH 7 and stirred for 1 h followed by centrifugation at 12,000 x *g* for 15 min. The supernatant was retained. The molecular weight of fish sarcoplasmic proteins was determined by gel filtration using a Sephacryl S-200 HR column (16/70) with flow rate 0.5 mL/min, fraction volume = 2.5 mL/tube. The column (16/70) had a void volume (V_o) = 60 cm^3. Sample elution was performed by 0.05 M phosphate buffer, pH 7.0, containing 0.15 M NaCl in refrigerator (4°C). Absorbance readings were determined spectrophotometrically at 280 nm with 1 cm path length cuvettes. The column was calibrated with the following standard proteins: beta-amylase from sweet potato (200 kDa), alcohol dehydrogenase from yeast (150 kDa), bovine serum albumin (66 kDa), carbonic anhydrase from bovine erythrocytes (29 kDa) and Cytochrome C from horse heart (12.4 kDa).

3.1.9 Differential scanning calorimetry

DSC was performed using a DSC instrument as described in Owusu-Apenten, Chee and Hwee (2003). TBSP (0.5 g) was added to 0.1 M phosphate buffer pH 7, stirred 1 h at room temperature, and then centrifuged at 12,000 x *g* for 15 min. Beta-lactoglobulin and casein (10 mg) were each dissolved with 100 μL buffer (50 mM potassium phosphate buffer, pH 6.8) and then microcentrifuged at 16,000 x *g* for 15 min. The supernatant was retained for DSC. Twenty milligrams of (~10% w/w) of TBSP, beta-lactoglobulin and casein supernatants were placed in a pre-weighed aluminum pan. The total weight of sample was determined by differences using a 5-place balance. DSC scans were run from 40°C to 110°C at a rate of 5°C/min using a Q100 DSC instrument. The protein content for test samples were then determined using the Lowry's method (section 3.1.5).

3.2 Biochemical characteristics of proteases from TBSP

3.2.1 Effect of substrates choice on crude protease of TBSP

Preparation of TBSP crude enzymes for protease study

TBSP protein solution (1% w/v) was prepared by dissolving 0.25 g of TBSP powder with ~25 g of distilled water and adjusting to pH 7 with 0.1 M HCl or NaOH. The final solution was then adjusted to exactly 25 mL giving 1% (w/w) TBSP solution. The solution was stirred by magnetic stirrer for 1 h, and then centrifuged at 12,000 x *g* for 10 minutes, the supernatant was collected and used as crude enzymes.

The effect of substrates choice on crude protease of TBSP was studied according to slight modified enzymatic specific activity (ESA) method of Munilla-Moran and Stark (1989). The substrates were azocasein, sodium caseinate and endogenous protein substrate.

3.2.1.1 Protease assay with azocasein substrate

TBSP solution (0.25 mL) was added to 0.75 mL of 0.2% azocasein in 1.5 mL-microcentrifuge tube and incubated at 55°C for 1 h. Then, 0.5 mL of cold 75% TCA solution was added to the tube to stop the activity of enzyme and to precipitate undigested substrate. After standing in the refrigerator for 10 minutes, the assay mixtures were centrifuged at 12,000 x *g* for 10 minutes. Absorbance readings TCA-soluble proteins were diluted for appropriate which were determined spectrophotometrically at 450 nm with 1 cm path length 1.5 mL-disposable plastic cuvettes. Assays were carried out in triplicates and blanks were prepared by adding the 0.75 mL of 0.2% azocasein in 1.5 mL-microcentrifuge tube then following by 0.5 mL of cold 75% TCA solution. The solution was mixed. Two hundred and fifty microliters of TBSP solution was added to the tube and incubated at 55°C for 1 h.

One unit of enzyme activity is defined as the amount of enzyme required to cause a unit increase in absorbance at 450 nm across a 1 cm path length, per hour, under the conditions of the assay. Enzymatic specific activity was expressed as a unit of enzyme activity per mg protein (Unit/mg). The protein content was determined by modified Lowry's method in 3.1.5. Enzyme specific activity was determined using the equations below,

$$\text{Enzymatic Specific Activity} = \frac{\text{Enzyme Activity}}{\text{Protein concentration}}$$

$$\text{Enzyme Activity} = A450_{(t1)} - A450_{(t0)} / \text{time (h)}$$

3.2.1.2 Protease assay with sodium caseinate substrate

Protease activity was monitored by sodium caseinate digestion method. The assay was the same as 3.2.1.1 but using sodium caseinate as substrate and hydrolysis of proteins was detected using a spectrophotometer at absorbance 280 nm with 1 cm path length UV-Visible 1.5 mL-disposable plastic cuvette.

Enzyme specific activity was determined using the equations as shown in section 3.2.1.1 with absorbance reading at 280 nm.

3.2.1.3 Protease assay with endogenous substrate

Protease activity was monitored by endogenous substratre digestion method. The assay was the same as 3.2.1.1 but using endogenous substratre (TBSP solution was pipetted 0.5 mL) as substrate and hydrolysis of proteins was detected using a spectrophotometer at absorbance 280 nm with 1 cm path length UV-Visible 1.5 mL-disposable plastic cuvette.

Enzyme specific activity was determined using the equations as show in section 3.2.1.1.

3.2.2 Determination of the pH-activity profile for crude protease of TBSP

The pH-activity profile for crude TBSP protease was determined. TBSP solution was prepared from TBSP powder by dissolving TBSP (1% w/w) in deionized water then adjusted to pH 2, 3, 4, 5, 6, 7, 8 and 9 with 0.1 M HCl or NaOH followed by stirring at room temperature for 1 h (rechecked pH every 15 min). Proteolytic activity was assayed at temperatures of 55°C for 30 min by using the endogenous

substrate assay (3.2.1.3). The absorbance reading was taken at 280. Proteolytic activity was expressed as OD change per hour per mg of protein.

3.2.3 Determination of the temperature-activity profile for crude protease of TBSP

The assay temperature profile for crude protease of TBSP was determined. TBSP solution was prepared from TBSP powder by dissolving TBSP (1% w/w) in deionized water, adjusting to pH 7 with 0.1 M HCl or NaOH and stirring at room temperature for 1 h. Proteolytic activity of 1% (w/w) TBSP solution was assayed at temperatures of 25, 30, 35, 40, 45, 50, 55, 60, 65 and 70°C for 30 min. The activities of enzyme at different temperature were studied by endogenous substrate assay (3.2.1.3). The absorbance reading was taken at 280 nm. Proteolytic activity was expressed as OD change per hour per mg of protein.

3.2.4 Effect of inhibitors on crude protease of TBSP

Stock solution of iodoacetic acid (IAA, inhibitor for cysteine proteases, 10 mM) was prepared in distilled water. Stock solutions of phenyl methyl sulfonyl fluoride (PMSF, inhibitor for serine proteases, 10 mM), 1,10-phenanthroline (inhibitor for metallo proteases, 10 mM) and pepstatin A (inhibitor for aspartic acid proteases, 10 μM) were prepared in methanol. The list of inhibitors for proteases was in the book of Beynon and Salvesen (1989).

TBSP solution was prepared from TBSP powder by dissolving TBSP (1% w/w) in distilled water and adjusting to pH 7 with 0.1 M HCl and NaOH. Before incubation with inhibitors, the TBSP solution was preheated in 60°C for 3 min and centrifuged to remove large protein components that can interfere with the results. TBSP solution was mixed with inhibitor stock solutions to give final concentration of IAA (1 mM), PMSF (1 mM), 1,10-phenanthroline (1 mM) and pepstatin A (1 μM). The inhibitors were incubated with enzyme for 30 minutes at 55°C. Then 0.1 mL of the resulting mixtures were assayed for enzyme activity using sodium caseinate substrate and endogenous substrate activity assays (section 3.2.1.2 and section 3.2.1.3,

respectively). The extent of protein hydrolysis was determined by the TNBS method described in section 3.1.6, with absorbance at 420 nm. The activity was determined against a control (distilled water for IAA or methanol for PMSF, 1,10-phenanthroline and pepstatin A). Proteolytic activity was expressed as OD change per minute per mg of protein as Enzymatic Specific Activity (ESA, A_{420}/min/mg) as shown in section 3.2.1.2 and 3.2.1.3. The ESA was used for % inhibition calculation by equation below:

$$\% \text{ Inhibition} = 100 \times \frac{(ESA_{control} - ESA_{inhibitor})}{ESA_{control}}$$

3.2.5 Effect of activators on crude protease of TBSP

The effect of metal ions (chloride salts) on endogenous substrate activity assay were investigated. Activators: LiCl, NaCl, KCl, $MgCl_2$, $CaCl_2$, $MnCl_2$, $FeCl_2$, $ZnCl_2$, $FeCl_3$ and $AlCl_3$ were prepared in water to make the stock solutions (5 mM).

The effect in activation of TBSP was determined. One hundred milligram of TBSP (2%) was dissolved with 5 mM concentrations of various metal ions stock solutions, adjusted pH 7 and stirred at room temperature (25°C) for 1 h. These solutions were incubated at 55°C for 1 h. The remaining activities of the resulting samples are measured according to the endogenous substrate activity assay as shown before (section 3.2.1.3). The relative activity was expressed as the percentage ratio of the enzymatic specific activity of samples with metals and without metal.

3.2.6 Effect of frozen storage and freeze-drying on crude protease of TBSP

During this research, some samples of ornate threadfin bream were stored in a freezer at -18°C for about 1.5 years. During the study also, sarcoplasmic protein extracts were freeze-dried to produce TBSP powder. The purpose of the study described here was to evaluate the effect of 1.5-year frozen storage as well as freeze-drying on the crude protease activity TBSP.

The effects of processing method (form of storage and freeze-drying) on crude protease activity in threadfin bream were studied. Crude proteases extract was prepared in two samples using ornate threadfin bream samples purchased freshly (fresh frozen fish) from a market in Thailand or stored frozen for 1.5-years (frozen storage fish). Fresh solution of enzyme extract was prepared directly from fish tissue by homogenization with a Waring blender (before freeze-drying) as described in section 3.1.1, and the freeze-dried crude protease from TBSP was prepared by freeze-drying fresh solution which treatments are described below:

- Fresh frozen threadfin bream was extracted. The supernatant was obtained as sample 1 (S1).
- Fresh frozen threadfin bream was extracted. The supernatant was freeze-dried and reconstituted with water as sample 2 (S2).
- Frozen storage threadfin bream (1.5 years) was extracted. The supernatant was obtained as sample 3 (S3).
- Frozen storage threadfin bream (1.5 years) was extracted. The supernatant was freeze-dried and reconstituted with water as sample 4 (S4).
- Fresh frozen cod was extracted. The supernatant was obtained as sample 5 (S5).
- Fresh frozen cod was extracted. The supernatant was freeze-dried and reconstituted with water as sample 6 (S6).

These samples were assayed for protease activity using endogenous substrate activity assay (section 3.2.1.3).

3.2.7 Determination of transglutaminase in TBSP

The supernatant of TBSP was used to determine residual transglutaminase activity with the colorimetric hydroxamate assay method (Folk and Cole, 1966) as described by the enzyme manufacturer (Sigma, USA) and detail was described in Appendix A.

3.3 Functional properties test

3.3.1 Solubility

Solubility of proteins was determined by the method of Wahyuni, Ishizaki, and Tanaka (1999) with slight modification. TBSP powder (40 mg) was suspended in 4 mL (w/w) of deionized water adjusted to pH 2.0 to 9.0 using 0.1 M HCl or NaOH and stirred by a magnetic stirrer for 60 min. Each sample was centrifuged at 12,000 x *g* for 10 min, and the protein concentration of the supernatant was determined using the modified Lowry's method (section 3.1.5). The protein solubility was expressed as percent of protein extracted in the supernatant with respect to that in the unreacted sample. The protein content of unreacted sample was determined by the Kjeldahl method.

The solubility property was calculated using equation (Vojdani, 1996):

$$\% \text{ solubility} = \frac{\text{amount of soluble protein}}{\text{total amount of protein}} \times 100$$

3.3.2 Water and oil holding capacity

Water holding capacity and oil holding capacity (WHC and OHC) was determined by the method of Were, Hettiarachchy, and Kalapthy (1997) with slight modification. Protein samples (0.5 g) were weighed into 15-mL centrifuge tubes. Distilled water or corn oil (Sigma) (2.5 mL) was added, and mixed to form a homogeneous paste. The tubes were centrifuged for 10 min at 12,000 x *g* by centrifuge. The small amount of supernatant was carefully removed by decanting, and the tubes were weighed to determine the weight of the hydrated pellet. The WHC and OHC were recorded as the difference between the weight of the hydrated pellet and the original product per weight of dry product.

3.3.3 Foaming properties

3.3.3.1 Foam capacity and stability methods

The studies were conducted with the following proteins: sodium caseinate, beta-lactoglobulin and TBSP. Standard proteins and TBSP (5% w/v) were dissolved in solvent (distilled water or salts solution, followed by stirring with magnetic stirrer for 2 h. and adjusted to different pH values using 0.1 M HCl or NaOH.

The foaming properties were studied using the method described by Phillips et al. (1990) with slight modification. Foams were formed by whipping the protein solutions in a blender at ambient temperature (23±1°C). Foam capacity (overrun) and foam stability measurements were made at 10 min intervals for a total of 30 min. Whipping was measured using initial 40 mL volumes of sodium caseinate, betalactoglobulin or TBSP.

The protein dispersions (40 mL, initial volume) were whipped in a laboratory Waring blender with mini stainless steel container (Waring Commercial, USA) at speed No.1 (18,000 rpm) for 5 min. The whipped sample was immediately transferred into a cylinder; the top of cylinder was covered with paraffin film and allowed to stand at 25°C in the temperature controlled unit. The volume of the initial, total and liquid drainage volume from the foam phase was measured at 0, 10, 20 and 30 min.

The foam capacity (FC, % overrun) was calculated by the following equation:

$$\% \text{ overrun} = \frac{(\text{total volume-drainage volume}) \times 100}{\text{initial volume}}$$

The foam stability (FS) was calculated by the following equation:

$$\% \text{ foam stability} = \frac{(\text{initial volume-drainage volume}) \times 100}{\text{initial volume}}$$

3.3.3.2 Effect of pH

The effect of pH on foaming properties was studied. Proteins were dissolved in distilled water and adjusted pH to 2, 5, 7 and 9 with 0.1 M HCl or NaOH. The procedures of FC and FS were described in section 3.3.3.1.

3.3.3.3 Effect of salts

3.3.3.3.1 Effect of cation salts

To test the effect of different cations sodium caseinate, beta-lactoglobulin and TBSP (5% w/w) were dissolved in 0.2 M NaCl, NH_4Cl, $MgCl_2$ and $CaCl_2$ solution, adjusted to pH 7 and stirred for 1 h. Foaming characteristics were then determined as described in section 3.3.3.1.

3.3.3.3.2 Effect of anion salts

To test the effect of different anions sodium caseinate, beta-lactoglobulin and TBSP (5% w/w) were dissolved in NaSCN, NaCl and Na_2SO_4 solution (0.2 M), adjusted to pH 7 and stirred for 1 h. Foaming characteristics were then determined as described in section 3.3.3.1.

3.3.4 Emulsifying properties

The study was conducted with beta-lactoglobulin and TBSP. The proteins solution (0.5% w/v) were dissolved in distilled water, adjusted pH 7 with 0.1 M HCl or NaOH and stirred for 2 h.

Emulsifying activity index (EAI) and emulsion stability index (ESI) were determined by the turbidimetric technique of Pearce and Kinsella (1978). A measured amount of pure corn oil and aqueous protein solution (10 and 30 mL, respectively) were homogenized by homogenizer at speed 22,000 rpm for 1 min at ambient temperature (23±1°C). Triplicate preparations were made for each study.

3.3.4.1 Determination of emulsifying activity index

Emulsifying activity index (EAI) was determined by the turbidimetric technique of Pearce and Kinsella (1978). Aliquots (1 mL) of the emulsion were diluted serially with 0.1% SDS (sodium dodecyl sulfate) solution to give final dilutions in the range 1/50 to 1/500. The absorbance of the diluted emulsion was then determined in a 1-cm path length disposable plastic cuvette at a wavelength of 500 nm using spectrophotometer. Absorbance of duplicate aliquots of each emulsion was measured in each case. The EAI was calculated using,

$$\text{EAI } (m^2/g \text{ sample}) = \frac{2T}{10000 \times \Phi C}$$

$$T = \frac{2.303 \text{ (A x dilution factor)}}{l}$$

where A is the observed absorbance, dilution factor (50-500), l is the path length of the cuvette (1 cm), Φ is the oil volume fraction of the emulsion (oil volume (10 mL) divided by total solution (40 mL)) and C is the initial protein concentration (0.5 g/100 mL).

3.3.4.2 Determination of emulsion stability index

From the 100-mL beaker containing the emulsion, a 10 mL aliquot of the emulsion was pipetted into a 10.0-mL cylinder immediately. Periodically, aliquots of the emulsion at 0 and 10 min were taken for dilution and turbidity measurement as described in EAI. The ESI was calculated using equation below:

$$\text{ESI (min)} = \frac{T \times \Delta t}{\Delta T}$$

where ΔT is the change in turbidity, T occurring during the time interval Δt (10 min).

3.3.5 Gelation

3.3.5.1 Oscillatory dynamic rheology

Gelation of proteins was determined by the method of Yongsawatdigul and Park (2003) with slight modification.TBSP (5% w/w) was prepared by dissolving the protein powder in distilled water, followed by stirring 1 h and pH was adjusted to 7. The gelation properties of TBSP were evaluated using an oscillatory dynamic stress rheometer. The storage modulus (G'), loss modulus (G") and tan delta were measured with temperature sweep (20-90°C) scan at a rate of 1°C/min (Ramp rate) and using dynamic temperature ramp testing (parallel plate diameter 50 mm, Gap = 1.000 mm,

strain = 1%, and frequency = 0.1 rad/s). Mineral oil was used to prevent moisture evaporation during measurement.

3.3.5.2 Effect of salts

The effect of salts (10, 100, 200 mM NaCl, 5 mM $CaCl_2$ and combination of NaCl and $CaCl_2$) on the gelation of beta-lactoglobulin and TBSP were studied similarly to the approach described in section 3.3.5.1 except that different salts were added to the protein solution before heating. For each salt, gelation extent was measured from the maximum G' value at 90°C.

3.3.5.3 Effect of pH

The effect of pH on the gelation of beta-lactoglobulin and TBSP were studied similarly to the approach described in section 3.3.5.1 with adjusted pH (5, 7 and 8.5) when stirring the protein solution before heating. For each pH, gelation extent was measured from the maximum G' value at 90°C.

3.3.5.4 Effect of enzyme inhibitors

Stock solution of iodoacetic acid (IAA, 10 mM) was prepared in distilled water. Stock solutions of phenyl methyl sulfonyl fluoride (PMSF, 10 mM), 1,10-phenanthroline (10 mM) and pepstatin A (10μM) were prepared in methanol. The list of compounds includes inhibitors for sulfhydryl proteases, serine proteases, Zn-metallo proteases and aspartic acid protease (Beynon and Salvesen, 1989).

The effect of enzyme inhibitors (IAA, PMSF, 1,10-phenathroline and pepstatin A) on the gelation of TBSP was studied similarly to the approach described in section 3.3.5.1, except TBSP solution was mixed with inhibitor stock solutions to give final concentration of IAA (1 mM), PMSF (1 mM), 1,10-phenanthroline (1 mM) and pepstatin A (1 μM). The inhibitors were added to the protein solution before heating. For each inhibitor, gelation extent was measured from the maximum G' value at 90°C.

3.3.5.5 Effect of transglutaminase addition

The effect of transglutaminase addition (0.2% w/w, Ajinomoto: Activa®-TGase) with and without 100 mM NaCl and 5 mM $CaCl_2$ on the gelation of beta-lactoglobulin and TBSP were studied similarly to the approach described in section 3.3.5.1, except that transglutaminase was added to the protein solution before heating. The gelation extent was measured from the maximum G' value at 90°C.

3.4 Modification of TBSP

3.4.1 Chemical and enzymatic modification of TBSP

3.4.1.1 Chemical modification of TBSP by acylation

3.4.1.1.1 Succinylation of TBSP

Succinylation was performed by the procedure of Franzen and Kinsella (1976b). TBSP powder (2.0 g) was dispersed in distilled water (200 mL). Increments of succinic anhydride were added to a total of 2.0 g (100% of protein) with stirring. During succinylation the pH was maintained between 7 and 8 with small additions of 3.5 M NaOH. After the pH stabilized, the reaction mixture was stirred 1 h. The solution was dialyzed against 0.01 M sodium phosphate buffer pH 7.0 at 4°C, 36 h to remove impurities and excess reagents. The chemically modified protein was recovered by lyophilization. The extent of succinylation of TBSP was determined with TNBS.

3.4.1.1.2 Acetylation of TBSP

Acetylation was performed by the procedure of Franzen and Kinsella (1976b). TBSP (2.0 g) was dispersed in deionized water (200 mL). The acetic anhydride was added in 0.2-mL increments to a total of 2.0 mL. During acetylation the pH was maintained between 7 and 8 with 3.5 M NaOH. After the pH stabilized, the reaction mixture was stirred 1 h. The solution was dialyzed against 0.01 M sodium phosphate buffer at 4°C for 36 h, and the acetylated protein was recovered by lyophilization.

3.4.1.1.3 Degree of modification

The degree of modification (MD) of the proteins was calculated from a numerical comparison of free amino group (TNBS assay, section 3.1.6) for the modified and unmodified original protein. The equation of modification is:

$$\% \text{ MD of amino group} = 100 \times \frac{(\text{amino group (initial)} - \text{amino group (final)})}{\text{amino group (initial)}}$$

3.4.1.2 Enzymatic modification

3.4.1.2.1 Modification of TBSP by trypsin hydrolysis

TBSP (12 g) was dispersed in deionized water (120 mL), adjusted to pH 7.0 with 0.1 M HCl or NaOH with strring by a magnetic stirrer. Then 10% TBSP solution was transfer to 500-mL flask to which was added 0.2% trypsin solution (24 mg in 2 mL distilled ionized water, 0.2% (w/w) of TBSP protein). The protein solution was incubated at 55°C for 1, 2, 4, 6, 12, 24, 36, 48, 60 and 70 h in a shaking water bath for studying hydrolysis time. The 1-h and 24-h digested protein samples were chosen for studying properties of modified TBSP (section 4.4.2), then recovered by lyophilization.

3.4.1.2.2 Degree of hydrolysis

The degree of hydrolysis (DH) of the proteins was determined by the slightly modified methods of Adler-Nissen (1979). DH of the proteins was calculated from a numerical comparison of free amino group between the modified and unmodified original protein. The degree of hydrolysis can be determined from relation below.

$$DH\,(\%) = 100\left[\frac{[NH_2]}{M_p \times A}\right]$$

where, $[NH_2]$ = free amine groups determined from TNBS (moles/g),

Mp = mass of protein in the reaction (g),

the constant A = 0.008 moles of peptide/g protein

First, it is necessary to calculate the free amino groups released by hydrolysis as follows.

$$\text{Amino group (moles/g)} = \frac{\text{OD x Vol}}{\varepsilon \text{ x l x Vp x Cp}}$$

where, OD = optical density measurement from TNBS assay (section 3.1.6),

Vol = final volume of cuvette (0.001 L),

ε = molar extinction coeffienct (20300 M^{-1} cm^{-1}),

l = cuvette path length = 1 cm,

Vp = volume of protein (TBSP) added to the cuvette (25 x 10^{-6} L),

Cp = concentration of protein determined by Lowry method (1.8 g/L)

Using a consistent set of values (from excel file) becomes:

$$\text{Amino group (moles/g protein)} = \frac{\text{OD} \times 1\text{L} \times 1000}{20300\,(\text{L moles}^{-1}\,\text{cm}^{-1}) \times 1\text{cm} \times 25\text{L} \times 1.8\text{g/L}}$$

3.4.2 Properties of modified TBSP

- Surface hydrophobicity was tested as described in section 3.1.4

- Solubility was tested as described in section 3.3.1

- Foaming properties was tested as described in section 3.3.3.1

- Emulsifying properties was tested as described in section 3.3.4

- Gelation was tested as described in section 3.3.5.1