Chapter 2

Materials and methods

1. Fish preparation

Bigeye snapper was obtained from a dock to Pattani. The fish were kept in ice with a fish/ice ratio of 1:2 (w/w) and transported to the Department of Food Technology, Prince of Songkla University, Hat Yai within 3 h. Fish meat was then excised for natural actomyosin (NAM) and surimi preparation.

2. Chemicals

Ammonium molybdate, 5-5'-dithio-bis (2-nitrobenzoic acid) (DTNB), adenosine 5'-triphosphate (ATP), β-mercaptoethanol (βME), sodium dodecyl sulfate (SDS) were purchased from Sigma (St. Louise, MO, USA). N,N,N'N'-tetramethylethylenediamine (TEMED), acrylamide, bis-acrylamide, and urea were obtained from Fluka (Buchs, Switzerland).

Cryoprotectants used included trehalose dihydrate (Wako, Tokyo, Japan), sucrose, and sorbitol as crystalline food grade (Merck, Darmstada, Germany).

3. Instruments

Instruments	Model	Company
Electrophoresis apparatus	Mini-Protein II	Bio-Rad, USA
Homogenizer	T25	Ultra turrax, Malaysia
Magnetic stirrer	RO 10 power	KIKAL labortechnik, Germany
pH meter	Denver 15	Fisher Scientific, France
Refrigerated centrifuge	RC-5B plus	Sorvall, USA
Water bath	W 350	Memmert, Germany
Spectrofluorometer	FP-750	Jasco, Japan
Double-beam spectrophotometer	UV-16001	SHIMADZU, Australia
Texture analyzer	TA-XT2	Stable Micro Systems, England
Basket centrifuge	CE21K	Grandimpianti, Italy
Mixer	MK-K77	National, Japan
Scanning Electron Microscope	JSM5800LV	JEOL, Japan

4. Natural actomyosin and surimi preparation

4.1 Natural actomyosin preparation

Natural actomyosin (NAM) was prepared according to the method of Benjakul *et al.* (1997) with a slight modification. Fish muscle was homogenized in chilled 0.6 M KCl pH 7.0 at a ratio of 1:10 (w/v) for 4 min. The container with sample was placed in ice. Each 20 sec of homogenization was followed by a 20 sec rest interval to avoid overheating during extraction. The homogenate was centrifuged at 5,000*xg* for 20 min at 4 ^{0}C. Three volume of chilled water (0-2 ^{0}C) was added to precipitate

NAM. The NAM was collected by centrifuging at 5,000*xg* for 20 min at 4 ^{0}C. The pellet was then dissolved by stirring in an equal volume of chilled 0.6 M KCl, pH 7.0 for 30 min at 0 ^{0}C. Undissolved material was removed by centrifuging at 5,000*xg* for 20 min at 4 ^{0}C. Protein concentration in NAM was calculated using the Biuret method (Copeland *et al.*, 1994)

4.2 Surimi preparation

Bigeye snapper was used as a raw material and surimi was prepared as follows:

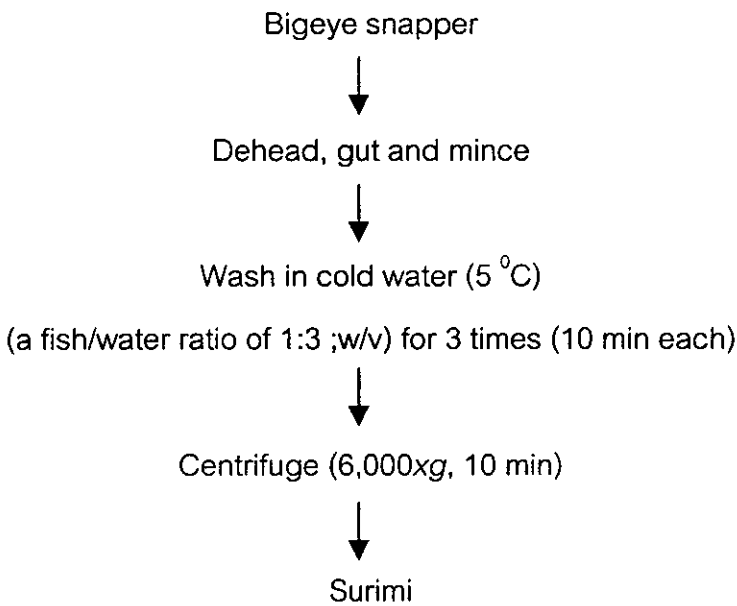


Figure 10. Scheme for surimi preparation.

5. Cryoprotective study in NAM

5.1 Cryoprotective effect of trehalose, sucrose and sorbitol alone at different concentrations.

NAM solution in 0.6 M KCl was added with different cryoprotectants at level of 0, 2, 4, 6, and 8% (w/v). The final concentration of protein in the mixture was 2.5 mg/ml. The mixtures were placed in 50-ml polypropylene centrifuge tubes and

frozen stored at –18 ^{0}C for 24 h. Frozen samples were then subjected to freeze-thawing for 1 and 2 cycles. Thawing was performed using running tap water (25 ^{0}C) for 5 min. Thawed samples were homogenized at speed 1 for 30 sec (IKA homogenizer) prior to analysis. The homogenate was kept in ice during analysis.

Denaturation of NAM was monitored by the following analysis:

1). Ca^{2+}ATPase activity according to Benjakul *et al.* (1997)

2). Total sulfhydryl content according to Benjakul *et al.* (1997)

3). Disulfide bond content according to Thannhauser *et al.* (1987)

4). Surface hydrophobicity according to Benjakul *et al.* (1997)

5). Solubility in 0.6 M KCl according to Jiang *et al.* (1988)

5.2 Cryoprotective effect of trehalose, sucrose or sorbitol alone and blends

Mixture design (Scheffe' Simplex-Centroid Design) (Gacula, 1993) was used to formulate the blends under conditon that total amount of cryoprotectant was 8% (Table 3). Cryoprotective effects of all treatments were compared with the control (without cryoprotectant).

Table 3. Cryoprotectant formulae used in bigeye snapper NAM

Treatment	Cryoprotectants (%, w/v)			
	Trehalose	Sucrose	Sorbitol	Total
1	8	0	0	8
2	0	8	0	8
3	0	0	8	8
4	4	4	0	8
5	0	4	4	8
6	4	0	4	8
7	2.67	2.67	2.67	8
8	5.34	1.33	1.33	8
9	1.33	5.34	1.33	8
10	1.33	1.33	5.34	8

Sample preparation and analyses was carried out in the same manner with section 5.1 Two cryoprotectant formulae showing the highest cryoprotective properties were selected for further study.

6. Effect of cryoprotectant on the aggregation of NAM

Cryoprotectants (two formulae; section 5) were added into NAM prior to freezing (final NAM concentration of 8 mg/ml). The cryoprotective efficacy was compared with commercial cryoprotectant and the control (without cryoprotectant). The mixtures were placed in 50-ml polypropylene centrifuge tubes and frozen stored at –18 ^{0}C for 24 h. Frozen samples were subjected to freeze-thawing for 2 and 4 cycles. Freeze-thawed mixtures were centrifuged at 15,000*xg* for 30 min at 4 ^{0}C. To the precipitate, 5 ml of different denaturating solutions were added (Chawla *et. al.*, 1996). The mixtures were stirred for 4 h at 25 ^{0}C and centrifuged at 15,000*xg* for 30 min at 25 ^{0}C. The solutions used included:

- Solution I (1%SDS in 20 mM Tris-HCl pH 8.0)
- Solution II (1%SDS + 8 M Urea in 20 mM Tris-HCl pH 8.0)
- Solution III (1%SDS + 8 M Urea + 2%β-ME in 20 mM Tris-HCl pH 8.0)
- 0.5 N NaOH

Protein in the supernatant of each denaturating solution was analyzed as follows

1). Protein in supernatant was precipitated using 50%TCA. The precipitated protein was recovered by centrifuging at 15,000*xg* for 30 min. The pellet was then redissolved using 0.5 N NaOH and the protein concentration was measured using the Biuret test (Copeland *et al.*, 1994). Percent protein solubility was expressed in term of solubility of protein in each solution compared to protein solubility in 0.5 N NaOH.

2). Protein pattern of aggregate dissolved in three different denaturating solutions was determined under both reducing and non-reducing conditions using SDS-PAGE (10%runing gel and 4% stacking gel) according to the method of Laemmli (1970).

7. Cryoprotective study in surimi

7.1 Cryoprotective effect on surimi with different freeze-thaw cycles

Cryoprotectants were added into surimi prior to freezing and frozen storage at -18 ^{0}C. The cryoprotective efficacy was compared with commercial cryoprotectant and the control (without cryoprotectants). Surimi samples were subjected to freeze-thawing at 0, 1, 2, 4, and 6 cycles in the same manner with section 5. The samples were then analyzed as follows:

7.1.1 Physicochemical properties and composition of protein

1). ATPase activity including, Ca^{2+}-ATPase activity, Mg^{2+}-ATPase activity, Mg^{2+}-Ca^{2+}-ATPase activity, and Mg^{2+}-EGTA-ATPase activity (Benjakul *et al.*, 1997).

2). Ca^{2+}sensitivity (Seki and Narita, 1980)

$$Ca^{2+}\text{sensitivity} = \left(1 - \frac{(Mg^{2+}\text{-EGTA-ATPase}) \times 100}{Mg^{2+}\text{-}Ca^{2+}\text{-ATPase}}\right)$$

3). Total sulfhydryl content (Benjakul *et al.*, 1997)

4). Disulfide bond content (Thannhauser *et al.*, 1987)

5). Surface hydrophobicity (Benjakul *et al.*, 1997)

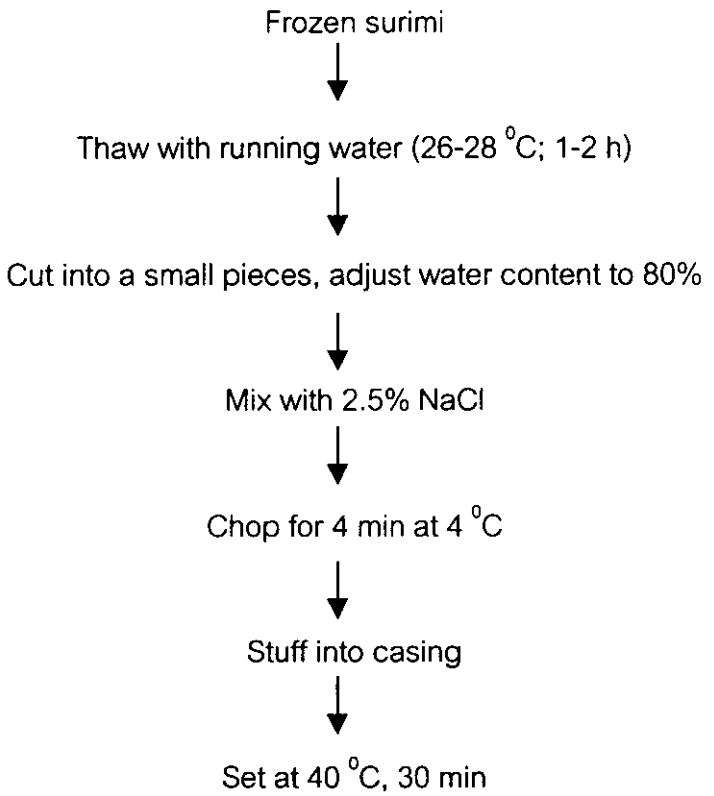
6). Solubility in 0.6 M KCl (Jiang *et al.*, 1988)

7). pH (Benjakul *et al.*, 1997)

8). SDS-PAGE (10% running gel and 4% stacking gel) (Laemmli, 1970)

7.1.2 Gel forming ability

Surimi gels were prepared as described in Figure 11



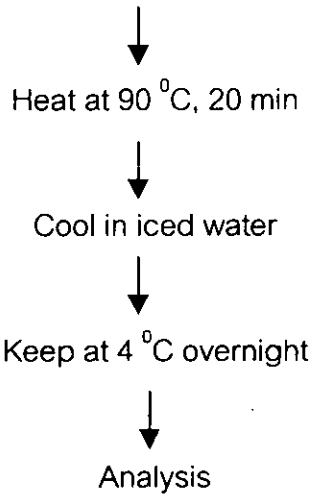


Figure 11. Scheme for surimi gel preparation.

Surimi gels were determined as follows:

1). Textural properties

Textural properties of gel were determined by measuring breaking force and deformation using punch test according to the method of Benjakul *et al.* (2001)

2). Whiteness

Whiteness of gel was determined as described by Auh *et al.* (1999)

$$\text{Whiteness} = 100 - [(100 - L^*)^2 + a^{*2} + b^{*2}]^{1/2}$$

3). Expressible moisture

Expressible moisture was analyzed according to the method of Benjakul *et al.* (2001)

7.2 Cryoprotective effect of surimi during extended frozen storage

Cryoprotectants were added into surimi and mixed thoroughly prior to freezing, followed by frozen storage at -18 °C. The cryoprotective efficacy was compared with commercial cryoprotectant and the control (without cryoprotectants). Samples were taken for analysis after 0, 1, 2, 4, 6, 8, 10, and 12 weeks of storage.

Sample analyses were carried out in the same manner with section 7.1 and microstructure study of surimi gel was performed using scanning electron microscopy (SEM) after 0, 4, 8 and 12 weeks of storage.

Microstructure study

Surimi gels (0.5x0.5x0.3 cm) was fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2 for 2 h at room temperature. Fixed specimens were dehydrated in graded ethanol solution with serial concentrations of 50, 60, 70, 80, 90 and 100%. Dehydrated samples were transferred to 100% acetone for increasing strength and critical point dried (Balzersnod. CPD 030, Balzers Process Systems, Liechtenstein) using CO_2 at transition fluid. The prepared samples were mounted on copper specimen holders, sputter-coated with gold (Balzer mod. SCD 004) and examined on a JSM 5800LV (JEOL, Ltd., Akishima, Japan)

8. Statistical analysis

Data were subjected to analysis of variance (ANOVA). Comparison of means was carried out by 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.)