CHAPTER 2

MATERIALS AND METHODS

1. Materials

1.1 Fish samples

White shrimps (*Penaeus vannamei*) with an average size of 55-60 shrimp/kg were obtained from a farm in Songkhla province. Bigeye snapper (*Priacanthus teyanus*) and lizardfish (*Saurida undosquamis*), off-loaded approximately 24-36 h after capture, were purchased from the dock in Songkhla province. All samples were kept in ice using the fish ice ratio of 1:2 (w/w) and transported to the Department of Food Technology, Prince of Songkhla University, Hat Yai within 1 h. Upon the arrival, shrimps were washed with clean water, immediately deheaded, peeled and deveined. Fish were washed and filleted. The flesh was minced using a mincer with a hole diameter of 5 mm. The mince was placed in polyethylene bag and kept on ice during preparation.

1.2 Chemicals

All chemicals for gel preparation and analyses were of analytical grade. Sodium chloride, trichloroacetic acid and Coomassie blue R-250 were obtained from Merck (Darmstadt, Germany). Sodium dodecyl sulfate (SDS), calcium choride, β-mercaptoethanol (βME), glycerol, high molecular weight marker, adenosine 5′-tripolyphosphate (ATP), ammonium molybdate, 5-5′-dithio-bis (2-nitrobenzoic acid) (DTNB), 1-anilinonaphthalene-8-sulphonic acid (ANS), glutaraldehyde and trypsin were purchased from Sigma (St Louis, MO, USA). Microbial transglutaminase (MTGase) was obtained from Ajinomoto Co.,Ltd. (Kawasaki, Japan). *N,N,N′,N′*-tetramethylethylenediamine (TEMED), acrylamide and bis-acrylamide were procured from Fluka (Buchs, Switzerland). Potassium chloride (KCl) and urea were obtained from Ajax Finechem (Wellington, Auckland, New Zealand).

2. Instruments

Instruments	Model	Company/City/Country
pH meter	CG 842	Schott, Mainz, Germany
Magnetic stirrer	BIG SQUID	IKA labortechnik, Stanfen, Germany
Homogenizer	T25 basic	IKA labortechnik, Selangor, Malysia
Oil bath	B-490	Buchi, Flawil, Switzerland
Water bath	W350	Memmert, Schwabach, Germany
Microcentrifuge	MIKR 020	Zentrifugan, Hettich, Germany
Refrigerated centrifuge	RC-5B plus	Sorvall, Norwalk CT, USA
Double-beam Spectrophometer	UV-16001	Shimadzu, Kyoto, Japan
Colorimeter	ColorFlex	HunterLab Reston, VA, USA
Electrophoresis apparatus	Mini-Protein II	Bio-Rad, CA, USA
Spectrofluorometer	RF-1501	Shimadzu, Kyoto, Japan
Texture analyzer	TA-XT2	Stable Micro Systems, Surrey, UK
Mixer	MK-K77	National, Tokyo, Japan
Differential scanning calorimeter	DSC 7	Perkin Elmer, Michigan, USA
Scanning Electron Microscope	JSM 5800LV	JEOL, Akishima, Japan

3. Methods

3.1 Determination of chemical composition and property of white shrimp, bigeye snapper and lizardfish mince

3.1.1 Proximate analysis

Shrimp and fish flesh were determined for moisture, protein, ash and fat contents according to AOAC method (AOAC, 1999). The values were expressed as % (wet weight basis).

3.1.2 Determination of nitrogenous constituent

Fractionation of muscle was carried out according to the methods of Hashimoto *et al*. (1979) (Appendix). Each fraction containing different composition, e.g. non-protein nitrogen, sarcoplasmic protein, myofibrillar protein, alkaline-soluble protein and stroma was subjected to nitrogen determinination using Kjeldahl method (AOAC, 1999). Protein patterns of different

fractions were determined using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 10% running gel and 4% stacking gel as described by Laemmli (1970).

3.1.3 Thermal denaturation

Thermal denaturation of muscle proteins was studied using Differential Scanning Colorimetry (DSC). The samples (15-20 mg) were accurately weighed into aluminum pans and sealed. The samples were scanned at 10 °C/min over the range of 20-100 °C using ice water as the cooling medium. The empty pan was used as the reference. Total denaturation enthalpy (ΔH) was calculated by measuring the area in the DSC thermogram. The maximum transition temperature (T_{max}) was estimated from the thermogram.

3.2 Study on the effect of setting condition and MTGase on gel forming ability of some marine fish and shrimp meats

3.2.1 Determination of MTGase activity

MTGase acivity was measured by the hydroxamate method (Folk, 1970). Freshly prepared substrate mixture containing 350 μl of 0.1 mol kg^{-1} Tris-acetate, pH 6.0, 25 μl of 2.0 M hydroxylamine and 75 μl of 0.1 M *N*-α-CBZ-L-glutamidyglycine was added with 50 μl of enzyme. The mixture was incubated at 37 °C for 10 min. The reaction was terminated by adding 500 μl of 15% TCA containing 5% $FeCl_3$. The resulting suspension was centrifuged at 9,000xg for 5 min and the absorbance was measured at 525 nm using a spectrophotometer. The calibration was performed using L-glutamic acid-γ-monohydroxamic acid as a standard. One unit of MTGase was defined as the amount of enzyme required to catalyze the formation of 1 μmol hydroxamic acid min^{-1} at pH 6.0 and 37 °C (Ho *et al*., 2000).

MTGase powder from *Streptoverticilium mobaraense* (TG-K) was measured. It found that the powder contained 45.8 units/g. Composition of products is maltodextrin 99% amd MTGase 1%.

3.2.2 Effect of setting condition and MTGase on gel forming ability

To study the effect of various MTGase on gel properties of mince samples, MTGase at different levels (0, 0.2, 0.4, 0.6 and 0.8 units/g sample) was added into the mince of white shrimp, bigeye snapper and lizardfish. Gels were prepared as depicted in Figure 6.

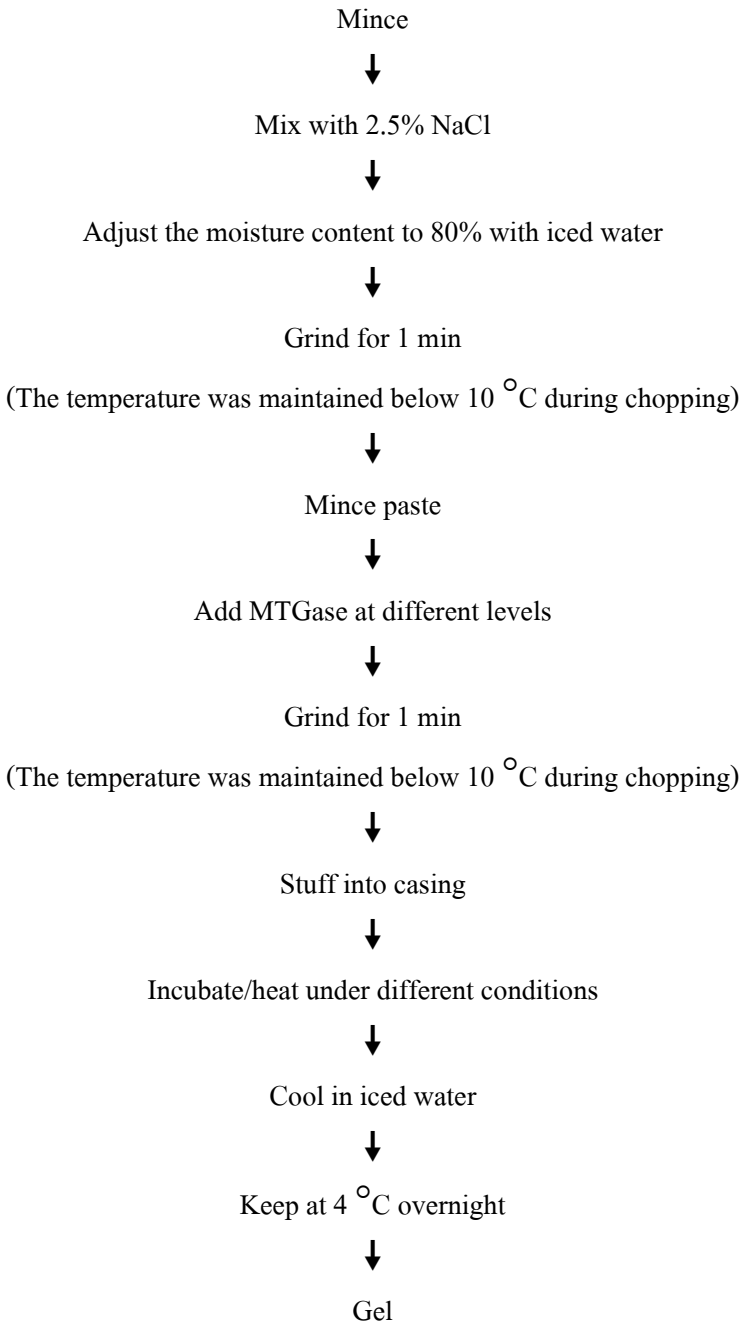

Figure 6 Scheme for gel preparation

After the mince paste containing different MTGase levels was stuffed into polyvinylidine casing with a diameter of 2.5 cm, both ends of the casing were sealed tightly. The sol was subjected to heating under different conditions to obtain the different gels as follows:

- Setting at 25 °C for 2 h, followed by heating at 90 °C for 20 min.
- Setting at 40 °C for 30 min, followed by heating at 90 °C for 20 min.

The gel were cooled in iced water and stored overnight at 4 °C prior to analyses as follows:

1. Breaking force and deformation

Gels were equilibrated and evaluated at room temperature (28–30 °C). Seven cylinder-shaped samples with a length of 2.5 cm were prepared and subjected to determination. Breaking force (gel strength) and deformation (elasticity/deformability) were measured using the texture analyzer equipped with a spherical plunger (diameter 5 mm; depression speed 60 mm min^{-1}).

2. Whiteness

L* (lightness), a* (redness/greenness) and b* (yellowness/blueness) – values of gels were measured using a Colorfex colorimeter (HunterLab Reston, VA, USA). Whiteness of gel was determined as described by Park (1994) using the following equation:

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

3. Protein patterns

Protein patterns of gels were determined using SDS-PAGE according to method of Laemmli (1970) with 10% running gel and 4% stacking gel. To prepare the protein sample, 27 ml of 5% (w/v) SDS solution heated to 85 °C were added to the sample (3 g). The mixture was then homogenized for 2 min using an IKA labortechnik homogenizer. The homogenate was incubated at 85 °C for 1 h to dissolve total proteins. The samples were centrifuged at 3500xg for 20 min to remove undissolved debris. Protein concentration was determined by the Biuret method (Robinson and Hodgen, 1940), using bovine serum albumin as standard. Sample with the protein content of 20 mg was loaded onto the gel. Electrophoresis was conducted at 15 mA/plate. After separation by SDS–PAGE made of 4% stacking gel and 10% separating gel, proteins were fixed and stained for 3 h in 0.125% Coomassie Brilliant Blue R-250 in 40% methanol and 10% glacial acetic acid. Gels were destained for 15 min with destaining

solution I (50% methanol and 7.5 % glacial acetic acid) and with the destaining solution II (5% methanol and 7.5 % glacial acetic acid) for 3 h.

4. Expressible moisture content

Expressible moisture content was measured according to the method of Ng (1978). Gel samples were cut into thickness of 0.5 cm, weighed (X) and placed between two pieces of Whatman filter paper No. 1 at the top and two pieces of the same filter paper at the bottom. The standard weight (5 kg) was placed on the top of the sample and maintained for 2 min. The sample was then removed from the papers and weighed again (Y). Expressible moisture content was calculated and expressed as the percentage of sample weight with the following equation:

$$\text{Expressible moisture content (\%)} = 100 \times [(X-Y)/X]$$

5. Microstructure

Microstructure of the gels from each species added with MTGase at the level rendering the highest breaking force for both setting conditions was studied using scanning electron microscopy, in comparison with the control gel (without MTGase addition). Gel samples (0.25x0.25x0.25 cm) were fixed with 2.5% glutaraldehyde in 0.2 M phosphate buffer, pH 7.2 for 2 h at room temperature (Hayat, 1981). Fixed specimens were dehydrated in graded ethanol solution with serial concentrations of 50, 70, 80, 90 and 100% and critical point dried (Balzers mod. CPD 030, Blazers Process Systems, Liechtenstein) using CO_2 as transition fluid. The prepared samples were mounted on copper specimen holders, sputter-coated with gold (Balzers mod. SCD 004) and examined on a JSM 5200 scanning electron microscope (JEOL, Ltd., Akishima, Japan).

3.3 Effect of post-mortem storage on crossing and gel enhancing ability of MTGase on mince from some marine fish and shrimp

3.3.1 Preparation of ice-stored some marine fish and shrimp

White shrimp, bigeye snapper and lizardfish were kept in the styrene foam box containing crushed ice with a fish/ice ratio of 1:2 (w/w). The fish were placed between the layers of ice. The box was kept at room temperature (28-30°C). To maintain the ice content, molten ice

was removed and replaced with an equal amount of ice every 2 days. At the time designated (0, 5 and 10 days), fish were removed for analyses and gel preparation.

3.3.2 Analyses

1. TCA-soluble peptide content

TCA-soluble peptide contents were determined according to the method of Morrissey *et al*. (1993). Ground sample (3 g) was homogenized with 27 ml of 5% (w/v) TCA using an IKA labortechnik homogenizer. The homogenate was kept in ice for 30 min and centrifuged at 5000xg for 20 min using a RC-5B plus centrifuge (Sorvall, Norwalk, CT, USA). Soluble peptides in the supernatant were measured according to the Lowry method (Lowry *et al.,* 1951) and expressed as µmol tyrosine/g muscle.

2. Thiobarbituric acid reactive substances (TBARS)

TBARS was determined according to the method of Buege and Aust (1978). Ground sample (0.5 g) was dispersed in 10 ml of TBARS (0.375 g of thiobarbituric acid, 15 g of trichloroacetic acid (TCA), and 0.875 ml of hydrochloric acid in 100 ml of distilled water). The mixture was heated in boiling water for 10 min, followed by cooling in running tap water. The mixture was centrifuged at 3600xg for 20 min and the absorbance was measured at 532 nm. A standard curve was prepared with malonaldehyde bis (dimethyl acetal) (MDA) at concentration ranging from 0 to 10 ppm. TBARS was calculated and expressed as mg malondialdehyde/kg.

3. pH

pH of samples was determined as described by Benjakul *et al.* (1997). Ground sample was homogenized with 10 volumes of deionized water (w/v) using an IKA labortechnik homogenizer and the pH was measured using a pH meter (Schott, Mainz, Germany)

4. Total volatide bases (TVB) and trimethylamine (TMA) contents

TVB and TMA contents were determined using the Conway microdiffusion assay according to the method of Conway and Byrne (1936). Sample (2 g) was mixed with 8 ml of 4% TCA. The mixtures were homogenized at 6,500 rpm using an IKA Labortechnik homogenizer for 1 min. The homogenate was filtered using Whatman No. 41 filter paper and the filtrate was used for analysis. To determine TMA content, formaldehyde was added to the filtrate to fix ammonia present in the sample. TVB and TMA were released after addition of saturated

K_2CO_3 and diffused into the boric acid solution. The titration of solution was performed and the amount of TVB or TMA was calculated.

5. Formaldehyde content

Formaldehyde content was analyzed according to the method of Amano *et al.* (1963). Sample (5 g) was mixed with 20 ml of 5% TCA and homogenized with an IKA Labortechnik homogenizer at a speed of 19,000 rpm for 4 min. The homogenate was filtered with whatman No. 41 filter paper. The residue was treated with 10 ml of 5 % TCA, homogenized and filtered. The filtrates were combined and pH was adjusted to 6.0-6.5 using 0.1 N KOH or 1 N KOH. The resulting filtrate was made up to final volume of 50 ml with distilled water. An aliquot (3 ml) of filtrate was mixed with 30 ml of acetylacetone reagent thoroughly and the mixture was incubated at 60 °C for 15 min and cooled with running water. The absorbance was measured at 412 nm and the formaldehyde content was calculated from a stardard curve.

6. Protein patterns

Protein patterns were analyzed using SDS-PAGE (Laemmli, 1970) as described previously. To prepare the protein sample, 27 ml of 5% (w/v) SDS solution heated to 85 °C were added to the sample (3 g). The mixture was then homogenized for 2 min using an IKA Labortechnik homogenizer. The homogenate was incubated at 85 °C for 1 h to dissolve total proteins. The samples were centrifuged at 3500xg for 20 min to remove undissolved debris. Protein concentration was determined by the Biuret method (Robinson and Hodgen, 1940), using bovine serum albumin as standard. Sample with the protein content of 20 mg was loaded onto the gel. Electrophoresis was conducted as mentioned previously. Protein bands were stained and destained as described above.

7. ATPase activity

ATPase activity was assayed according to the method of Benjakul *et al.* (1997). Natural actomysin (NAM) prepared as described by Benjakul *et al.* (1997) was diluted to 3–5 mg/ml with 0.6 M KCl, pH 7.0. Diluted NAM solution (0.5 ml) was added with one of the following solution for each ATPase activity assay to total volume of 9.5 ml: 10mM $CaCl_2$ for Ca^{2+}-ATPase; 2 mM $MgCl_2$ for Mg^{2+}-ATPase; 0.1 mM EGTA for Mg^{2+}-EGTA-ATPase. To each assay solution, 0.5 ml of 20 mM ATP was added to initiate the reaction. The reaction was conducted for 10 min at 25 °C and terminated by adding 5 ml of chilled 15% (w/v)

trichloroacetic acid. The reaction mixture was centrifuged at 3500xg for 5 min and the inorganic phosphate liberated in the supernatant was measured by the method of Fiske and Subbarow (1925). Specific activity was expressed as μmol inorganic phosphate released/mg protein/min. A blank solution was prepared by adding chilled trichloroacetic acid prior to addition of ATP.

3.3.3 Effect of MTGase on gel-forming ability of some marine fish and shrimp stored in ice for different times

Samples kept in ice for different times (section 3.3.1) were washed with tap water. The flesh was removed manually and minced to uniformity. The mince obtained was used to prepare gel containing MTGase at a level showing the highest breaking force. Gel preparation and analyses were performed as described in section 3.2.2.

3.4 Effect of protein substrate denaturatuion on cross-linking activity of MTGase

3.4.1 Preparation of natural actomyosin (NAM)

Natural actomyosin (NAM) was prepared according to the method of Benjakul *et al*. (1997) with a slight modification. Fish muscle (10 g) was homogenized in 100 ml of chilled 0.6 M KCl, pH 7.0 for 4 min using an IKA Labortechnik homogenizer. 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 centrifuge at 5,000xg for 30 min at 4 °C. Three volumes of chilled water (0-2 °C) were added to precipitate NAM. The mixture was collected by centrifuging at 5,000xg for 20 min at 4 °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 4 °C.

3.4.2 Thermal stability of NAM

NAM solution (3-5 mg/ml) was incubated at different temperatures (0, 10, 20, 30, 40, 50 and 60 °C). At definite time (0, 5, 10, 30 and 60 min), samples were taken and immediately cooled in iced water. Ca^{2+}-ATPase activity was then measured according to the method of Benjakul *et al.* (1997). The inactivation rate constant (KD) was calculated as described by Arai *et al.* (1973) as follows:

$$KD = (\ln C_0 - C_t)/t$$

where $C_0 = Ca^{2+}$-ATPase activity before incubation

$C_t = Ca^{2+}$-ATPase activity after incubation for time t

t = incubation time (s)

3.4.3 Effect of MTGase on cross-linking and physico-chemical properties of natural actomyosin with different degrees of denaturation

NAM solution (3-5 mg/ml) was pre-incubated at 50 °C for different times (0, 5, 10, 20 and 30 min). The solution was immediately cooled in iced water. After pre-incubation, the sample was added with MTGase at different levels (0, 0.2, 0.4, 0.6 and 0.8 units/g protein) and incubated at 40 °C for 30 min. The sample was determined as follows:

1. Protein solubility

Solubility was determined according to the method of Benjakul and Bauer (2000). To 1 g sample, 20 ml of 0.6 M KCl was added and the mixture was homogenized for 1 min at speed of 12,000 rpm using an IKA Labortechnik homogenizer. The homogenate was stirred at 4°C for 4 h, followed by centrifuging at 8500*xg* for 30 min at 4 °C. To 10 ml of supernatant, cold 50% (w/v) TCA was added to obtain the final concentration of 10%. The precipitate was washed with 10% TCA and solubilized in 0.5 M NaOH. The sample was also directly solubilized by 0.5 M NaOH and used for total protein determination. Protein content was determined using the Biuret method (Robinson and Hodgen, 1940) and expressed as the percentage of total protein in the sample.

2. Surface hydrophobicity

Surface hydrophobicity was determined as described by Benjakul *et al.* (1997) using 1-anilinonaphthalene-8-sulphonic acid (ANS) as a probe. NAM dissolved in 10 mM phosphate buffer, pH 6.0 containing 0.6 M NaCl was diluted to 0.1, 0.2, 0.3, and 0.5% (w/v) protein using the same buffer. The diluted protein solution (2 ml) was added with 10 µl of 8 mM ANS in 0.1 M phosphate buffer, pH 7.0. The fluorescence intensity of ANS-conjugates was measured using a FP-750 spectrofluorometer (Shimadzu, Kyoto, Japan) at an excitation wavelength of 374 nm and an emission wavelength of 485 nm. The initial slope of the plot of fluorescence intensity versus NAM concentration was referred to as SoANS.

3. Total sulfhydryl group content

Total sulfhydryl group content was determined using 5, 5′-dithio-bis (2-nitrobenzoic acid) (DTNB) according to the method of Ellman (1959) as modified by Benjakul *et al.* (1997). To one ml of NAM solution (0.4%), 9 ml of 0.2 M Tris–HCl buffer, pH 6.8,

containing 8 M urea, 2% SDS and 10 mM EDTA, were added. To 4 ml of the mixture, 0.4 ml of 0.1% DTNB in 0.2 M Tris–HCl (pH 8.0) was added and incubated at 40 °C for 25 min. The absorbance at 412 nm was measured using a UV-16001 spectrophotometer (Shimadzu, Kyoto, Japan). A blank was conducted by replacing the sample with 0.6 M KCl. Sulfhydryl content was calculated using the extinction coefficient of 13,900 M^{-1} cm^{-1}.

4. Disulfide bond content

Disulfide bond in NAM was determined by using 2-nitro-5-thiosulfobenzoate (NTSB) assay according to the method of Thannhauser *et al.* (1987). To 0.5 ml of NAM sample (1 mg/ml), 3.0 ml of freshly prepared NTSB assay solution, pH 9.5, were added. The mixture was incubated in dark at room temperature (25–27 °C) for 25 min. Absorbance at 412 nm was measured. Disulfide bond content was calculated using the extinction coefficient of 13,900 M^{-1} cm^{-1}.

5. Protein patterns

Protein patterns were determined using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 10% running gel and 4% stacking gel as described by Laemmli (1970).

6. Free amino group content

Free amino group content was determined according to the method of Benjakul and Morrissey (1997). Diluted samples (125 μl) were mixed thoroughly with 2.0 ml of 0.2125 M phosphate buffer, pH 8.2, followed by the addition of 1.0 ml of 0.01% TNBS solution. The mixture was then placed in a water bath at 50 °C for 30 min in dark. The reaction was terminated by adding 2.0 ml of 0.1 M sodium sulfite. The mixture was cooled to room temperature for 15 min. The absorbance was measured at 420 nm and free amino group content was expressed in terms of L-leucine.

3.5 Effect of MTGase on cross-linking and physicochemical properties of natural actomyosin added with different levels of formaldehyde

NAM solution (3-5 mg/ml) containing different levels of formaldehyde (0, 10, 20 and 30 μg/g protein) was allowed to stand in ice for 1 h. The sample was then determined as described in section 3.4.3

The sample solution added with different levels of formaldehyde was mixed with MTGase at different levels (0, 0.2, 0.4, 0.6 and 0.8 units/g protein). The mixture was then incubated at 40 °C for 30 min. The sample was then subjected to determination as appeared in section 3.4.3.

3.6 Effect of MTGase on cross-linking and physicochemical properties of natural actomyosin with different degrees of hydrolysis

3.6.1 Preparation of NAM with different degrees of hydrolysis

NAM hydrolysate was prepared as per the methods of Benjakul and Morrissey (1997). To prepare hydrolyaste, NAM (10 mg/ml) in 0.6 M KCl phosphate buffer pH 7.5 was preincubated at 37 °C for 10 min. The enzymatic hydrolysis was started by adding various amounts of trypsin. After hydrolysis for 90 min, the enzymes were inactivated by placing the reaction mixture at 85 °C for 15 min in water bath. The mixture was then centrifuged at 2,000xg at 4 °C for 10 min and the supernatant was collected and used as NAM hydrolysate. DH of NAM hydrolysate was determined as described by Benjakul and Morrissey (1997). To obtain NAM hydrolysate with different DHs (0, 5, 10, 20 and 30%), the enzyme amount calculated from the plot between DH and log (Enzyme concentration) were added (Benjakul and Morrissey, (1997). All samples were subjected to the following determinations.

1. Free amino group content as described by Benjakul and Morrissey (1997).
2. Protein patterns using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 10% running gel and 4% stacking gel as described by Laemmli (1970).
3. Surface hydrophobicity determined as described by Benjakul *et al.* (1997)

3.6.2 Effect of MTGase on cross-linking and physico-chemical properties of natural actomyosin with different degrees of hydrolysis

NAM hydrolysates with different DHs (0, 5, 10, 20 and 30%) were added with MTGase at different levels (0, 0.2, 0.4, 0.6 and 0.8 units/g protein). The mixture was incubated at 40 °C for 30 min. The sample was then determined as described in section 3.4.3.

4. Statistical analysis

All experiments were run in triplicate and CRD (Completely Randomized Design) was used. Analysis of variance (ANOVA) was performed and means comparisons were carried out by Duncan's multiple range tests (Steel and Torrie, 1980). Analyses were conducted using a SPSS package (SPSS 11.0 for windows, SPSS Inc, Chicago, IL).