Chapter 2

Materials and methods

1. Materials

Raw materials

Skipjack tuna *(Kastsuwonus pelamis*) internal organs, including spleen, stomach, intestine, bile sac, liver, and pancrease were obtained from Chotiwat Industrial Co. Ltd., Songkhla, Thailand. Tuna internal organs were transported in ice with the sample/ice ratio 1:2 (w/w) to the Department of Food Technology, Prince of Songkla University, Hat Yai within 30 min.

Chemical reagents

Potassium sulfate, copper sulfate, sodium hydroxide, trichloroacetic acid, hydrochloric acid, sulfuric acid, magnesium oxide, potassium chloride, sodium chloride, ferrous chloride, Magnesium sulfate, Manganese chloride, formaldehyde, peptone, yeast extract, and standard plate count agar (PCA) were obtained from Merck (Darmany, German). Histamine dihydrochloride, *O*-phthaldialdehyde (OPT), sodium caseinate, bovine haemoglobin and bovine serum albumin were purchased from Sigma Chemical Co. (St. Louis, MO, USA.). Trichloroacetic acid, sodium chloride, trisodium citrate, tris (hydroxymethyl) aminomethane and Folin-Ciocalteu's phenol reagent were obtained from Merck (Darmstadt, Germany). Casamino acid was obtained from Oxold (Hampshire, England).

2. Instruments

Instrument	Model	Company/Country
pH meter	Cyberscan 500	Singapore
Magnetic stirrer	BIG SQUID	IKA LABORTECHNIK, Germany
Water bath	W350	Memmert, Germany
Microcentrifuge	MIKRO20	Hettich ZENTRIFUGAN, Germany
Refrigerated centrifuge	RC-5B plus	Sorvall, USA
Colorimeter	ColorFlex	HunterLab Reston, USA
Double–beam Spectrophotometer	UV - 16001	SHIMADZU, Japan
Spectrofluorophotometer	RF - 1501	SHIMADZU, Japan
Homogenizer	T25 basic	IKA LABORTECHNIK, Malaysia

3. Characterization of proteolytic activity in different internal organs

3.1 Preparation of enzyme extracts

Frozen internal organs including spleen, stomach, intestine, liver, pancreas, bile sac and pool organs were thawed using a running water. The samples were cut into pieces. Samples were finely ground in liquid nitrogen to a powder form according to Simpson and Haard (1984). The powder was suspended in distilled water and stirred continuously at 4°C for 30 min. The suspension was centrifuged for 30 min at 4ºC at 5,000 xg. The supernatant was collected and referred to as “crude

proteinases". The protein content in the extract was measured according to the method of Lowry *et al*. (1951).

3.2 Temperature profile

Preteolytic activity was evaluated with 0.2 M McIlvaine's buffer at pH 3.0, 5.0 and 7.0 and using 0.1 M glycine-NaOH at pH 9.0 according to An *et al*. (1994) using substrate -TCA-Lowry assay at various temperatures (25, 30, 35, 40, 45, 50, 55, 60 and 65 ^{O}C). Haemoglobin (pHs 3, 5) and casein (pHs 5, 7, 9) were used as the substrates.

3.3 pH profile

Optimum pH was evaluated by measuring the activity of enzyme extracts using substrate-TCA-Lowry assay at optimal temperature of each organs in the pH range of 2.0-12.0 (0.2 M McIlvaine's buffer for pH 2.0-7.5 and 0.1 M glycine-NaOH for pH 8.0-12.0). Haemoglobin (pHs 3, 4, 5) and casein (pHs 5, 6, 7, 8, 9, 10, 11 and 12) were used as the substrates.

3.4 Effect of NaCl on Proteinase Activity

Effect of NaCl at different concentrations on proteinase activity was studied. NaCl was added into the standard reaction assay to obtain the final concentration of 0, 5, 10, 15, 20, 25 and 30% (w/v). The residual activity was determined at the optimal pH and temperature of each organs for 20 min using haemoglobin (pH 3.0-5.0) and casein (pH 5.0-12.0) as substrates.

3.5 Proteolytic assay

Proteolytic activity of crude proteinases from individual internal organs was determined using haemoglobin (pH 3.0-5.0) and casein (pH 5.0-12.0) as substrates according to the method of An *et al.* (1994). To initiate the reaction, 200 µl of crude proteinase was added into assay mixtures containing 2 mg of substrate, 200 µ l of distilled water and 625 µl of reaction buffer. After 20 min, enzymatic reaction was stopped by adding 200 µl of 50% (w/v) trichloroacetic acid (TCA). Unhydrolyzed protein substrate was allowed to precipitate for 15 min at 4ºC, followed by centrifuging at 7,000 xg for 10 min. The oligopeptide content in the supernatant was determined by the Lowry assay (Lowry *et al.* 1951) using tyrosine as a standard. Activity was expressed as tyrosine equivalents in TCA-supernatant. One unit of activity was defined as that releasing 1 mmole of tyrosine per min (mmol/Tyr/min). A blank was run in the same manner, except the enzyme was added after addition of 50 %TCA (w/v).

3.6 Protein determination

Protein concentration was measured by the method of Lowry *et al*. (1951) using bovine serum albumin as a standard.

3.7 Changes in proteolytic activity of internal organs during fermentation

Each internal organs including spleen, stomach, intestine, liver, pancreas, bile sac and pooled organs cut into pieces (90 g) were mixed with solar salt (30 g) and transferred to the glass bottles and covered with polyethylene film. The

containers were placed outdoor with temperature ranging from 27 to 35 ^{o}C. The sample was taken at month 0.25, 0.5, 1, 2, 3, 4, 5, 6, 8, 10 and 12 for proteolytic assay under the optimum condition for each organ.

4. Effect of storage condition on chemical and microbiological changes of tuna internal organs during storage.

4.1 Raw material preparation

Skipjack tuna *(Kastsuwonus pelamis*) internal organs, including spleen, stomach, intestine, bile sac, liver, and pancrease were divided into 2 lots. One lot was immediately iced with sample/ice ratio of 1:2 (w/w) and placed in an insulated container with a drain valve. Another lot was allowed to stand at room temperature. Samples of each lot were taken at the different storage times (0, 4, and 8 h) for analyses and fish sauce fermentation.

4.2 Chemical analyses

4.2.1 Determination of TCA-soluble peptides

TCA-soluble peptides were determined according to the method described by Morrissey *et al*. (1993). Sample (3 g) was homogenized with 27 ml of 5% TCA (w/v) for 1 min at room temperature using an IKA Labotechnik homogenizer (Selangor, Malaysia). The homogenate was kept in ice for 1 h and centrifuged at 7,500xg for 5 min. Soluble peptides in the supernatant were measured and expressed as μmole/g.

4.2.2 Determination of pH

The pH of tuna internal organs was measured as described by Benjakul *et al* (1997). Sample was homogenized using an IKA Labortechnik homogenizer (Selangor, Malaysia) with 10 volumes of deionized water (w/v) and the pH was measured using a pH meter (Cyberscan 500, Singapore).

4.2.3 Determination of total volatile bases (TVB) and trimethylamine (TMA) contents

TVB and TMA contents were determined using the Conway micro diffusion assay according to the method of Conway and Byrne (1936). Sample (4 g) was extracted with 16 ml of 4% tricloroacetic acid (TCA). The mixtures were filtered using Whatman No.41 filter paper and the filtrate was used for analysis. To determine the 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.

4.2.4 Determination of histamine.

Histamine was analyzed by the standard fluorometric method (AOAC, 2000). Sample (5 g) was homogenized in 25 ml of methanol for 2 min and the mixture was incubated in a water bath at 60 ^{o}C for 15 min. After cooling to 25 ^{o}C, the volume was adjusted to 50 ml with methanol and filtered through Whatman No. 1 filter paper. The methanol filtrate was collected and loaded onto an ion exchange column (200×7 mm) packed with Dowex 1-x8 (Sigma Chemical Co., St. Louis, MO, USA), which was converted to hydroxide form by 2 N NaOH. The sample eluents and the standard

solutions were derivatized with *O*-phthaldialdehyde (OPT). The fluorescence intensity of the derivatized products was then measured using a RF-1501 spectrofluorophotometer (Shimadzu, Kyoto, Japan) at an excitation wavelength of 350 nm and emission wavelength of 444 nm. The histamine standard solutions ranging from 0.1 to 0.3 ppm was used to prepare a standard curve. Histamine contents in the samples were calculated from the standard curve.

4.2.5 Determination of biogenic amine

Biogenic amines in commercial tuna internal organs were extracted according to the procedure of Koutsoumanis *et al.* (1999) with a slight modification.

Sample (4 g) was added with 10 ml of 10% TCA solution and homogenized at 13,500 rpm using an Ultra Turrax homogeniser (IKA Labortechnik, Selangor, Malasia) for 2 min. The homogenate was centrifuged at 12,000 *xg* for 10 min at 4^{o}C using a Sorvall Model RC-5B Plus centrifuge (Newtown, CT, USA). The pellet was extracted with 10 ml of 10% TCA solution and the supernatants were combined and made up to 25 ml with 10% TCA solution.

Derivertization of extracted sample was performed according to the method of Şenöz *et al.,* (2000). The extract (0.5 ml) was added with 50 µl of 1, 7 diaminoheptane (3.0 mg/ml) as the internal standard. The solution was then mixed with 100 µl of 2 N NaOH and 150 µl of saturated sodium bicarbonate. One ml of dansyl chloride (10mg/ml) was added to each sample, mixed very well, and then incubated for 45 min at 40^{o}C. Residual dansyl chloride was removed by adding 50 µl of 25% ammonia and centrifuged at 2,500 *xg* for 30 min and the supernatant was filtered (0.45 µm). A 20 µl of filtrate was then injected onto the HPLC (600E Waters,

Massachusetts, USA). Quantitative analysis of amines was carried out on Waters HPLC system (Water Associates, Miliford, MA, USA). Separation was achieved using a column Hypersil BDS C18 (ThermoHypersil, Hercules, USA). A gradient elution program was used with the mobile phase of 100% methanol (solvent A) and nanopure distilled water (solvent B), starting with 55% solvent A and 45% solvent B and finishing with 100% solvent A and 0% solvent B after 45 min. The flow rate was 1.5 ml/min. Data were processed and calculated with a CSW32 program-based integrator. A stock standard solution was prepared in 3mg/ml by adding an accurately weighed amount of each amine standard (tryptamine, phenylethyamine, putrescine, cadaverine, histamine and tyramine) and diluting to volume with water. Stock solution were stored at 4 ^{o}C for 1 months. Five working standard solutions were stored for 1 week at 4 ^{o}C.

4.3 Microbiological analyses

Tuna internal organs (15g) were transferred aseptically to a stomacher bag containing 135 ml of 0.1 %(w/v) peptone solution having 10% (w/v) NaCl. The mixture was mixed vigorously for 1 min using a stomacher. The prepared samples were then subjected to the following analyes:

4.3.1 Total aerobic bacterial counts

Total aerobic bacterial counts were determined using a standard plate count agar containing 10% (w/v) NaCl with the incubation for 48 h at 37 ^{o}C (Tanasupawat *et al.,* 1992).

4.3.2 Determination of halophilic bacteria

Halophilic bacteria were determined using JCM media (pH 7.0-7.2) (Namwong *et al.,* 2005). The medium (1 L) consisted of 5 g of casamino acid, 5 g of yeast extract, 1 g of sodium glutamate, 3 g of trisodium citrate, 20 g of $MgSO_4 \cdot 7\ H_2O$, 2 g of KCl, 20 g of NaCl, 0.036 g of $FeCl_2 \cdot 4H_2O$, 0.00036 g of $MnCl_2 \cdot 4\ H_2O$ and 20 g of agar. Diluted sample (0.1 ml) was spread on the surface of media and incubated at 37 ^oC for 96 h.

4.3.3 Proteolytic bacteria

Proteolytic bacteria were determined using a standard plate count agar containing 10% (w/v) NaCl and 1% (w/v) casein with the incubation at 37 ^oC for 72 h (Tanasupawat *et al.,* 1992).

Microbiological data were transformed into logarithms of the number of colony forming units (CFU/g.).

5. Effect of storage condition on chemical, physical and microbiological changes of tuna internal organs during fermentation.

5.1 Fish sauce fermentation

Tuna internal organs kept in ice or at room temperature for different times were subjected to fermentation. Tuna internal organs (27 kg) were mixed with solar salt (9 kg) and transferred to the earthen jar (50 liters). The earthen jars were covered with black plastic bag and aluminum lid to prevent the contamination of foreign materials as well as insects. The earthen jars containing samples were placed outdoor with temperature ranging from 27 to 35 ^oC. The liquid formed was taken at month 0.25, 0.5, 1, 2, 3, 4, 5, 6, 8, 10 and 12. Liquid formed at time designated was

filtered using Whatman filter paper No. 1. The filtrate was subjected to chemical and physical analysis.

5.2 Chemical analysis

5.2.1 Determination of pH

The pH of fish sauce was determined directly using a pH meter using (Cyberscan 500, Singapore).

5.2.2 Determination of total volatile bases (TVB) and trimethylamine (TMA) contents

TVB and TMA contents were determined using the Conway micro diffusion assay according to the method of Conway and Byrne (1936) as described in section 4.2.3.

5.2.3 Determination of total nitrogen content

Total nitrogen in each sample was determined using Kjeldahl method (AOAC, 2000). Total nitrogen content was expressed as mg nitrogen/ml.

5.2.4 Determination of formol nitrogen content

Formol nitrogen content, a convenient index of the degree of protein hydrolysis, was determined by the titration method as described by Thai Industrial Standard (1983). Sample with an appropriate dilution (10ml) was titrated to pH 7.0 with 0.1 N NaOH and then 10 ml of formalin solution (38%, v/v; pH 9) were added to the nautralized sample. Titration was continued to obtain pH of 9 with 0.1 N NaOH.

Formol nitrogen content was expressed as mg formol nitrogen/ml using the following equation:

Formol nitrogen content (mg N/ml) = ml of NaOH (pH 7-pH 9) ×0.1×14

5.2.5 Determination of ammonia nitrogen content

Sample with an appropriate dilution (50 ml) was placed in 400 ml Kjeldahl flask containing 100 ml of distilled water and 3 g of MgO. The mixture was distilled and the distillate was collected in 50 ml of 4% boric acid consisting of the mixed indicator (methyl red: bromocresol green: methylene blue). The solution was then titrated with 0.05 N H_2SO_4 to reach the end-point. Ammonia nitrogen content was expressed as mg nitrogen/ml using the following equation:

Ammonia nitrogen content (mg N/ml) = 5.6 × 0.05× (ml of H_2SO_4)

5.2.6 Determination of amino nitrogen content

Amino nitrogen was calculated based on the formol and ammonia nitrogen contents as follows: (TISI, 1983)

Amino nitrogen content (mg N/ml) = Formol nitrogen content – Ammonia nitrogen content.

5.2.7 Determination of salt content

Salt content in sample was measured by the method of AOAC (2000). Samples (20 ml) were diluted with 180 ml of distilled water. Diluted sample (1 ml) was added with 10 ml of 0.1 N $AgNO_3$ and 10 ml of conc. HNO_3. The mixture was boiled gently on the hot plate until all samples except $AgCl_2$ were dissolved (usually 10 min). The mixture was then cooled using a running water. Then 50 ml of distilled water and 5 ml of ferric alum indicator were added. The mixture was titrated with standardized 0.1 N KSCN until solution became permanent light brown. The percentage of salt was then calculated.

5.2.8 Determination of histamine.

Histamine was analyzed by the standard fluorometric method (AOAC, 2000) as described in section 4.2.4.

5.2.9 Determination of biogenic amine

Biogenic amine was analyzed by the method of Koutsoumanis *et al* . (1999) as described in section 3.2.5.

5.3 Physical analyses

5.3.1 Determination of fluorescence intensity

Fluorescence intensity of samples with an appropriate dilution was determined as described by Morales and Jimenez-Perez (2001) with a slight modification. The fluorescence intensity was measured at an excitation wavelength of 347 nm and emission wavelength of 415 nm using a RF-1501 spectrofluorophotometer (Shimadzu, Kyoto, Japan).

5.3.2 Determination of UV-absorbance

The UV-absorbance of samples was measured according to the method of Ajandouz *et al* (2001). Appropriate dilution was made using distilled water and the absorbance was measured at 294 nm using a spectrophotometer (UV-1601, Shimadzu, Japan).

5.3.3 Determination of non enzymatic browning

Non enzymatic browning was determined using the method of Hendel *et al* (1950). Prior to analysis, sample (5 ml) was extracted in 50 ml of 50% (w/v) ethanol with a continuous stirring for 1 h. The extracted was filtered using a Whatman No.1 filter paper. The filtrate was then subjected to the absorbance measurement at 420 nm using a spectrophotometer (UV-1601, Shimadzu, Japan).

5.3.4 Determination of color

The color of fish sauce was determined by measuring the L* (black-white component, luminosity), a* (+ red to – green component) and b* (+ yellow to – blue component) values using a colorimeter (Juki Corp, Tokyo, Japan). Samples were pipetted into a 5 cm diameter glass petridish. The sample was illuminated with D65-artificial daylight (10^{o} standard angle). The color variations of each sample were compensated by recording the average of three reading.

5.4 Microbiological analysis

Fish sauce (15 ml) was transferred aseptically to a duran bottle containing 135 ml of 0.1% (w/v) peptone solution comprising 10% (w/v) NaCl and shaken well for 1 min. The prepared samples were subjected to determinations as described in section 4.3.1, 4.3.2 and 4.3.3.

6. Role of proteinases in fish sauce fermentation of skipjack tuna internal organs

6.1. Effect of calcium chloride on proteolytic activity and fish sauce fermentation/aging

6.1.1 Fermentation of fish sauce

Tuna internal organ (9 kg) with pH adjusted to 9 was mixed with solar salt at different levels (15 and 25% w/w). The mixture was then mixed with $CaCl_2$ at different levels (0, 1.5 and 3 % w/w) and transferred to earthen jar and covered with polyethylene film. The containers were placed in an incubator at 37°C. The liquid formed was taken for analysis at 0.25, 0.5, 1, 2, 3, 4, 5 and 6 months. For the treatment added with 15% salt, the salt content was adjusted to 25% after the total nitrogen content reached the constant level. The control was prepared in the same manner without pH adjustment.

6.1.2 Analyses

After incubating for the designated time, the samples were filtered using a Whatman filter paper No. 1. The filtered liquid obtained was used for analysis.

6.1.2.1 Chemical and physical analyses

Chemical and physical analysis of fish sauce during fermentation were carried out as mentioned in section 5.2.1 – 5.2.6 and 5.3.1 – 5.3.4.

6.1.2.2 Measurements of proteinase activity

At the time designated, fish sauce sample was dialyzed with 10 volumes of distilled water at 4°C for 24 h to remove salt prior to the proteolytic activity assay. Proteolytic activity was measured using casein-TCA-Lowry assay at pH 9 and 55°C for 20 min. (Klomklao *et al*., 2004). One unit of activity was defined as that releasing 1 mmole of tyrosine per min (mmol/Tyr/min). A blank was run in the same manner, except the enzyme was added after addition of 50 %TCA (w/v).

6.2 Aging of fish sauce

The fish sauce obtained after 6 months of fermentation was subjected to aging by leaving the filtered fish sauce in the earthen jar outdoor at temperature ranging from 27 to 35 °C for up to 2 months. During aging, samples were taken every 2 weeks for analyses including A_{420}, A_{294}, fluorescence intensity as well as color as described in sections 5.3

7. 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 window version 10: SPSS.