CHAPTER III
MATERIALS AND METHODS

3.1 Materials

Two cultivars of brown rice, 'Chainat 1' (short grain Indica high amylose rice) and Khaw Dawk Mali 105; KDML 105 (long grain Indica low amylose rice) were obtained from Thai Agri-Business Co.,Ltd. They were harvested in 2010, and packed in plastic bags, vacuum-sealed and stored at 20 °C until used for study.

3.2 Chemicals

1. Ethanol
2. Methanol
3. Folin-Ciocalteu reagent
4. Sodium carbonate
5. Gallic acid
6. 1,1-Diphenyl-2-picrylhydrazyl (DPPH)
7. L-ascorbic acid (Vitamin C)
8. 2,2'-Azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS)
9. Potassium persulfate
10. Trolox
11. Sulfuric acid
12. Hydrochloric acid
13. Boric acid
14. Sodium hydroxide
15. Petroleum ether
16. Ammonium chloride
17. Ammonia monohydrate
18. Triton X-100
19. Phenol red

20. Sodium hypochlorite
21. Phenol

3.3 Equipments

1. Cyclone sample mill (IKA, A11, Germany)
2. Test sieve (Aperture size 300 Mic., Endecotts Limited, England)
3. Single screw extruder
4. Spray dryer (Buchi, B-290, Switzerland)
5. Spouted bed dryer
6. Hot air oven (Memmert GmbH&Co., model 400, Germany)
7. Muffle furnace (Gallenkamp, model Tactical 308, U.K.)
8. Kjeldahl apparatus (Buchi, K-324, Switzerland)
9. Soxhlet apparatus (Gerhardt, EV6AII/16, U.K.)
10. Micropipette (Metler Toledo, Switzerland)
11. pH meter (Cyberscan, model 500 pH, Singapore)
12. Centrifuge (Hettich, rotorfix 32, U.K.)
13. Vortex (Vortex-Genie 2, C560E, USA)
14. Hot plate (IKA, C-MAG HP 7, Germany)
15. Rapid visco analyzer (RVA) (Newport Scientific Instrument & Engineering, model RVA 4, Australia)
16. UV-Visible Spectrophotometer (Shimadzu, UV-1601, Japan)
17. Rheometer (Anton-Paar, MCR-301, Austria)
18. Differential scanning calorimetry (DSC) (Instrument specialist, Inc., Q-800, USA)
19. Scanning microscope (Carl Zeiss, model LSM5 PASCAL, Germany)

3.4 Methodology

3.4.1 Preparation of germinated brown rice flour

Chainat 1 and Khao Dawk Mali 105 (KDML 105) brown rice was germinated according to the method of Khampang et. al. (53) with a slight modification. Brown rice was soaked in water at room temperature until root

protuberance was 0.5-1 mm. Germinated brown rice was dried at 50 °C for 10 hours and ground to flour in a grinder and sieved through a 300 μm (50 mesh) screen. Germinated brown rice flour was packed in plastic bags, vacuum-sealed and stored at 20 °C until used.

3.4.2 Raw material analysis

3.4.2.1 Proximate composition

3.4.2.1.1 Moisture content

Two grams of sample was weighed in a silica dish, which was previously dried and weighed. The sample was then dried in an oven at 100 °C for 12 hours, cooled in a desiccator and weighed. The drying and weighing continued until a constant weight was achieved (144). The moisture content was calculated using the following equation;

$$\% \text{ Moisture} = \frac{(W_3 - W_2) \times 100}{W_1}$$

Where,

W_1 = g of sample weight

W_2 = g of silica dish and sample after drying

W_3 = g of silica dish and sample before drying

3.4.2.1.2 Protein content

Sample (0.5-1 g) was placed in a Kjeldahl digestion flask. Then 5 g of catalyst consisting of copper (II) sulfate and potassium sulfate (1:9 w/w) was added with a few glass beads. After that 20 ml of sulfuric acid (97% w/v) was added. The solution was heated at 180 °C until it was clear and then allowed to cool. Before distillation, 20 ml of distilled water and 60 ml of sodium hydroxide (40% w/v) were added. The digestion flask was connected to the distillation unit where a receiving flask containing 60 ml of 2% boric acid and 2-3 drops of mixed indicator (0.1 g bromocresol green and 0.1 g of methyl red in 100 ml of ethanol) was placed. The distillation continued for 7 minutes. The solution in the receiving flask

was titrated with 0.1 N sulfuric acid until the color changed from green to colorless (144). % Nitrogen was calculated using the following equation:

$$\% \text{ Nitrogen} = \frac{(S - B) \times N \times 1.401 \times 100}{W}$$

Where,

S = ml sulfuric acid titration of sample titer

B = ml sulfuric acid titration of blank titer

N = Normality of sulfuric acid

W = g of sample weight

% Protein = % Nitrogen × 5.95 (conversion factor for rice protein)

3.4.2.1.3 Fat content

Two grams of sample was put in a filter paper and placed in a soxhlet extractor. Then, 90 ml of petroleum ether was added into a round bottom flask. The soxhlet extractor and the round bottom flask were connected together. After that, they were put over an electric heating mantle. The solvent in the flask was heated until it was boiled. Then, the temperature of the heater was adjusted to make the solvent from the soxhlet extractor drip into the flask at the rate of 6 drops per second. Extraction continued for 6 hours. After extraction, the flask was placed on a hot plate for petroleum ether evaporation and put in a hot air oven at 100 °C for 1 hour. The flask was then cooled in a dessicator and weighed (144). The fat content was calculated using the following equation;

$$\% \text{ Fat} = \frac{(W_3 - W_2) \times 100}{W_1}$$

Where,

W_1 = g of sample weight

W_2 = g of round bottom flask

W_3 = g of round bottom flask and fat

3.4.2.1.4 Ash content

One gram of sample was put in a crucible which was pre-heated at 550 °C for 2 hours, cooled and weighed. Then, the crucible with the

sample was heated gently on a hot plate until the sample was thoroughly carbonized. After that, it was placed in a muffle furnace at 550 °C for 2 hours or until ash was free from carbon (white color) (144). The ash content was calculated using the following equation;

$$\% \text{ Ash} = \frac{(W_2 - W_0) \times 100}{(W_1 - W_0)}$$

Where,

W_0 = g of crucible

W_1 = g of crucible and sample before heating

W_2 = g of crucible and sample after heating

3.4.2.1.5 Crude fiber content

Fat-free sample was transferred into a flask and 200 ml of pre-heated 1.25% sulfuric acid was added. The solution was gently boiled for 30 minutes. After that, the boiled acid sample mixture was filtered hot through the Buchner flask funnel fitted with Whatman filter under sufficient suction. The residue was then washed several times with boiling water (until the residue was neutral to litmus paper) and transferred back into a beaker. Then 200 ml of pre-heated 1.25% sodium sulfate was added and boiled for another 30 minutes. After boiling, the mixture was filtered under suction and washed thoroughly, once with hot water and twice with ethanol. The residue was dried at 65 °C for 24 hours and weighed. Then it was transferred into a crucible and placed in a muffle furnace at 550 °C for 4 hours, cooled in a desiccator and weighed (144). The crude fiber content was calculated using the following equation;

$$\%\text{Crude fibre} = \frac{(W_2 - W_3) \times 100}{W_1}$$

Where,

W_1 = g of sample weight

W_2 = g of dry residue before ashing

W_3 = g of dry residue after ashing

3.4.2.1.6 Carbohydrates content

Carbohydrates content was calculated by difference using the following equation;

%Carbohydrates = 100 – (%moisture + %protein + %fat + %ash + %crude fiber)

3.4.2.1.7 GABA content

GABA content was determined by the method of Komatsuzaki et al. (145). Three grams of sample was extracted with 30 ml of 70% ethanol solution. After 1 minute, the mixture was centrifuged at 6000 rpm for 15 minutes. The same volume of 70% ethanol solution was then added to the precipitate as described above and the extraction was repeated. The supernatant was evaporated. Three ml of water was added to the dried supernatant. Then, a 0.1 ml of dissolved supernatant was mixed with 0.2 ml of pH 9 borate buffer and 1 ml of 6% phenol reagent. After mixing for 1 minute in a cooling bath, 0.4 ml of 7.5% sodium hypochlorite reagent was added. The mixture was put in a boiling water bath for 10 minutes. After that the mixture was immediately put in ice bath for 5 minutes then measured colorimetrically with a spectrophotometer at the wavelength of 630 nm.

3.4.2.1.8 Vitamin B1 content

The procedure of Liu et al. (146) was followed. Flour (0.1 g) was extracted with 10 ml of distilled water and filtered through filter paper. A 0.3 ml of the filtrate was adjusted to 10 ml with distilled water. One milliliter of the extract was mixed with 1.5 ml of ammonium buffer and 1 ml of 1% Triton X-100 and adjusted to 10 ml with distilled water. Two to three drops of 0.05% phenol red was added then mixed for 10 minutes. The mixture was measured colorimetrically with a spectrophotometer at the wavelength of 427 nm.

3.4.2.1.9 Total phenolic compounds and antioxidant capacity

1) Sample extraction

Samples were extracted with 95% ethanol, 1:2 (w/v) for 24 hours. The solution was centrifuged at 3000 rpm for 15 minutes. The supernatant was brought out and used as the extract (147).

2) Determination of total phenolic compound (TPC)

The TPC was assayed by the method modified from Ferreira et al. (148). Extract sample 10 µl was added to 790 µl distilled water and mixed with 50 µl of Folin-Denis reagent. Then, a 150 µl of 7.5% (w/v) sodium carbonate was added and incubated for 30 minutes. The absorbance was measured at 725 nm using a spectrophotometer. The results were expressed as milligrams of gallic acid equivalents (GAE) per gram rice sample. All analyses were performed in triplicate.

3) Determination of antioxidant activity

a. DPPH (1,1-Diphenyl-2-picrylhydrazyl)

Extract samples 20 µl were mixed with 180 ml of 167 µM DPPH solution and allowed to react for 5 minutes at room temperature. Then the absorbance was measured using a spectrophotometer at 540 nm using spectrophotometer. L-ascorbic acid (Vitamin C) was used as reference. DPPH radical scavenging assay was expressed as L-ascorbic acid (Vitamin C) equivalent antioxidant capacity (VCEAC) (147).

b. ABTS (2,2-Azinobis-3-ethylbenzothiazoline-6-sulfonic acid)

The working ABTS solution was prepared by mixing 7 mM ABTS and 2.4 mM potassium persulfate in equal quantities and allowed to react for 12 hours at room temperature in the dark condition. The mixed solution was diluted with ethanol to obtain an absorbance of 0.98 ± 0.01 units at 734 nm using a spectrophotometer. Extract sample 70 µl was reacted with 630 µl of ABTS solution. After 30 minutes, the absorbance was taken at 734 nm. Trolox was used as reference.

ABTS radical cation scavenging assay was expressed as trolox equivalent antioxidant capacity (TEAC) (147).

3.4.3 Preparation of pregelatinized germinated brown rice flour for studying an effect of different techniques of pregelatinization process on physico-chemical properties of flour

3.4.3.1 Preparation of pregelatinized germinated brown rice flour from single screw extruder

A 100 g of germinated brown rice flour was mixed with water at the water:flour ratio of 1:3, 1:2 and 1:1. The flour was heated in two zones of barrels at the same barrel temperatures of 60, 80 and 100 °C at the screw speed of 50 rpm (obtained from preliminary test). A 2×3×3 randomized complete block factorial design was employed to investigate the influence of rice cultivars, water:flour ratio and temperature on physico-chemical properties of pregelatinized germinated brown rice flour. The extrudates were collected and dried in a hot air oven at 45 °C for 12 hours. After that they were ground into flour and sieved through a 300 μm (50 mesh) screen. Extruded flour was packed in plastic bags, vacuum-sealed and stored at 20 °C until used for study.

Figure 3.1 Single screw extruder

3.4.3.2 Preparation of pregelatinized germinated brown rice flour from hot air oven

A 100 g of germinated brown rice flour was mixed with water at the water:flour ratio of 1:3, 1:2 and 1:1. The flour was heated at temperatures of 60, 80 and 100 °C until moisture content of flour was below 10%. A 2×3×3 randomized complete block factorial design was employed to investigate the influence of rice cultivars, water:flour ratio and temperature on physico-chemical properties of pregelatinized germinated brown rice flour. The processed flour was packed in plastic bags, vacuum-sealed and stored at 20 °C until used for study.

Figure 3.2 Hot air oven

3.4.3.3 Preparation of pregelatinized germinated brown rice flour from spray dryer

A 100 g of germinated brown rice flour was mixed with water in order to give a 5% concentration (obtained from preliminary test). The flours were heated at the inlet temperature of 100 and 120°C. A 2×2 randomized complete block factorial design was employed to investigate the influence of rice cultivars and temperature on physico-chemical properties of pregelatinized germinated brown rice

flour. After that the spray dried flour was collected. The flour was packed in plastic bags, vacuum-sealed and stored at 20 °C until used for study.

Figure 3.3 Spray dryer

3.4.3.4 Preparation of pregelatinized germinated brown rice flour from spouted bed dryer

A 100 g of germinated brown rice flour was mixed with water in order to give a 5% concentration (obtained from preliminary test). The flours were heated at the temperature of 100 and 120°C. A 2×2 randomized complete block factorial design was employed to investigate the influence of rice cultivars and temperature on physico-chemical properties of pregelatinized germinated brown rice flour. After that the spouted flour were collected. Spouted flour was packed in plastic bags, vacuum-sealed and stored at 20 °C until used for study.

Figure 3.4 Spouted bed dryer

3.4.4 Pregelatinized flour analysis

3.4.4.1 Chemical composition

3.4.4.1.1 Protein content

Protein content was determined using the method of AOAC (144) as described in. 3.4.2.1.2

3.4.4.1.2 GABA content

GABA content was evaluated using the method of Komatsuzaki et al. (145) as described in. 3.4.2.1.7

3.4.4.1.3 Vitamin B1 content

Vitamin B1 content was measured according to the method of Liu et al. (146) as described in. 3.4.2.1.8

3.4.4.2 Physical properties

3.4.4.2.1 Pasting properties

A rapid visco analyser (RVA) was used to measure pasting properties. The moisture content of the flour samples were determined and used to compensate for differences in moisture content between samples by adjusting the quantities of flour and distilled water used to prepare the RVA sample (149). RVA tests were performed with around 3 g of flour samples and approximately 25 g of distilled water. After the canister was fitted to the device, the operations were run based on the approved profile (149). Each sample was held at 50 °C for 1 minute, heated to 95 °C at 12 °C /minute, held for 2.5 minutes, cooled down to 50 °C at 12 °C/minute, and held at 50 °C for 1.5 minutes. Total elapsed time was 12.5 minutes. The RVA measures pasting temperature (PT), peak viscosity (PV), trough viscosity (TV) and final viscosity (FV) based on curve. Breakdown viscosity (BD) and setback viscosity (SB) were calculated as the difference between PV and TV, and FV and TV,

respectively (65). PT was defined as the first point at which the viscosity increases by 10 cP or faster within 0.1 minutes (149). All analyses were performed in triplicate.

3.4.4.2.2 Thermal properties

Differential scanning calorimetry (DSC) was used. For gelatinization studies, flour slurry was prepared by mixing 3-5 mg of flour with water (ratio, flour/water of 30/70 w/w). The slurry was put into an aluminum pan and hermetically sealed to prevent moisture loss then incubated at 4 °C for 12 hours. After equilibrating, the sample pan was heated from 30 °C to 100 °C at a rate at 10 °C/min. For retrogradation studies, gelatinized flour slurry, stored at 5 °C for 14 days, was scanned at 10 °C/min from 5 to 95 °C. The onset temperature (T_o), peak temperature (T_p), conclusion temperature (T_c) and gelatinization enthalpy change (ΔH) were calculated. Triplicate measurements were conducted to obtain the average values (150).

3.4.4.2.3 Rheological properties

Dynamic rheological properties during heating and cooling cycles of the starch suspensions at 10% (w/w) were determined using a Rheometer with parallel-plate geometry of 40-mm diameter and gap geometry 1000 μm. Rheological properties were described in terms of storage (G′), loss moduli (G′′), and tan δ (G′′/G′) and were obtained using the software of analysis program. Flour suspensions were scanned from 30 to 100 °C during heating and from 100 to 5 °C during cooling at a rate of 5 °C/min at a strain and frequency set at 2% and 5 rad/s, respectively (151).

3.4.4.2.4 Swelling and solubility properties

Swelling and solubility of the samples were determined according to the procedure of Schoch (152) as modified by Unnikrishnan and Bhattacharya (153). About 500 mg (dry basis) of sample was cooked in 20 ml of water at various temperatures ranging from 50 to 90 °C for 30 minutes. They were weighed and made equivalent to 25 g by the addition of water. They were centrifuged at 3000 rpm for 15 minutes. Supernatant was decanted carefully, and residue was

weighed for swelling power determination. Ten milliliters of the supernatant was pipetted out to a wide-mouth petri dish and kept on a boiling water bath for evaporation. Afterwards, the dishes were dried at 105 °C for 3 hours, cooled, and weighed. Solubility and swelling power were estimated using the following formulas:

Swelling power = mg of the wet residue/500 - mg of the dried sample (1)

Solubility = mg of the dry residue × 2.5 × 100/mg of the sample (db) (2)

3.4.4.2.5 Morphological properties

Scanning electron micrographs were observed by scanning electron microscopy (SEM). Flour sample was applied on an aluminium stub, and the flour was coated with gold-palladium (60:40). An acceleration potential of 10 kV was used during micrography (154).

3.5 Statistical analysis.

The data were subjected to analysis of varience (ANOVA) using general linear model procedure, SPSS for window version 10.00 (SPSS Inc., USA). Means comparison was performed using Duncan’s multiple range test.