Chapter 3

Methodology

3.1 Materials

Instruments

1. Centrifuge (5804 R, Eppendrof)
2. Micropipettes (Boeco, Germany)
3. Hotplate and Stirrer (Sterillin, France)
4. Freeze dryer (Telstar, Spain)
5. Hot air oven (Memmert, Germany)
6. Spectrophotometer (Shimadzu, Japan)
7. Sonicator (Elma, Germany)
8. ELISA plate reader (BioTek, USA)
9. Electrical balance (Boeco, Germany)
10. Accu-Check pens and reader (Roche, Thailand)

Chemicals

1. Amyloglucosidase from *Aspergillus niger* (Sigma)
1. Alpha-amylase heat stable from *Bacillus licheniformis* (Sigma)
2. Glucose oxidase kit (Linear, Spain)
3. Rat insulin ELISA (Mercodia; Sweden)
4. CHO testing kit (Linear, Spain)
5. Triglyceride testing kit (Linear, Spain)
6. Distilled water (Milford, USA)
7. Ethanol, Analytical grade (Merck, Germany)
8. 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Fluka, USA)
9. Butylate hydroxyl toluene (BHT) (Merck, Germany)
10. Glucose ($C_6H_{12}O_6$) (Merck, Germany)
11. D-glucose ($C_6H_{12}O_6$) (Amersham Biosciences, USA)
12. Fructose ($C_2H_2O_6$) (Fluka, USA)

13. Ribose ($C_5H_{10}O_5$) (Fluka, USA)
14. Sucrose ($C_{12}H_{22}O_{11}$) (Fluka, USA)
15. Tyrosine ($C_9H_{11}NO3$) (Fluka, USA)
17. Distilled water (Milford, USA)

3.2 Rice bran stabilization "SRB"

Rice bran of Khao-Dawk-Mali 105 was purchased from the local mill in Yasothorn, Thailand. Rice was grown in organic farm in Tambol Barg-Ruer where the farm was approved by the Organic Agriculture Certification of Department of Agricultural Extension. Freshly milled rice bran was stabilized (lipase inactivation) as soon as possible by heating rice bran in hot air oven at 130-140 ˚C for 3 minutes (Qureshi et al., 2002).

3.3 Digestive enzymes

Ten milligrams of Apha-amylase was dispersed in 6.5 mL distilled water and stirred on magnetic stirrer plate until it was dissolved. Then, an aliqoute of 5 μL of α-amyloglucosidase solution (No. 9913, Sigma-Aldrich) was added.

3.4 Extraction and digestion of stabilized rice bran

A 120 g of stabilized rice bran was dispersed in 480 mL. warm-distilled water. The solution was kept at 60-70 ˚C while stirring on magnetic hot-plate stirrer for 1 hour. After cooling to ambient temperature, the suspension was centrifuged at 10,000 rpm (11,485 x g), 25 ˚C for 10 min. The supernatant was transferred to a beaker and 7 mL of digestive enzymes solution was added. The mixture was stirred on the magnetic-stirring hot-plate where the temperature was kept at 37 ˚C throughout the stirring period of 30 min. The mixture was centrifuged at 10,000 rpm, 25 ˚C for 10 min before a clear supernatant was transferred to a flask and freeze-dried (Freezone 18, Labconco Ltd., Kansas City, MO). Approximately 12.79 gm of crude

dried extract was obtained. Aliquots of the extract were subjected to tests for antioxidant and other biochemical properties.

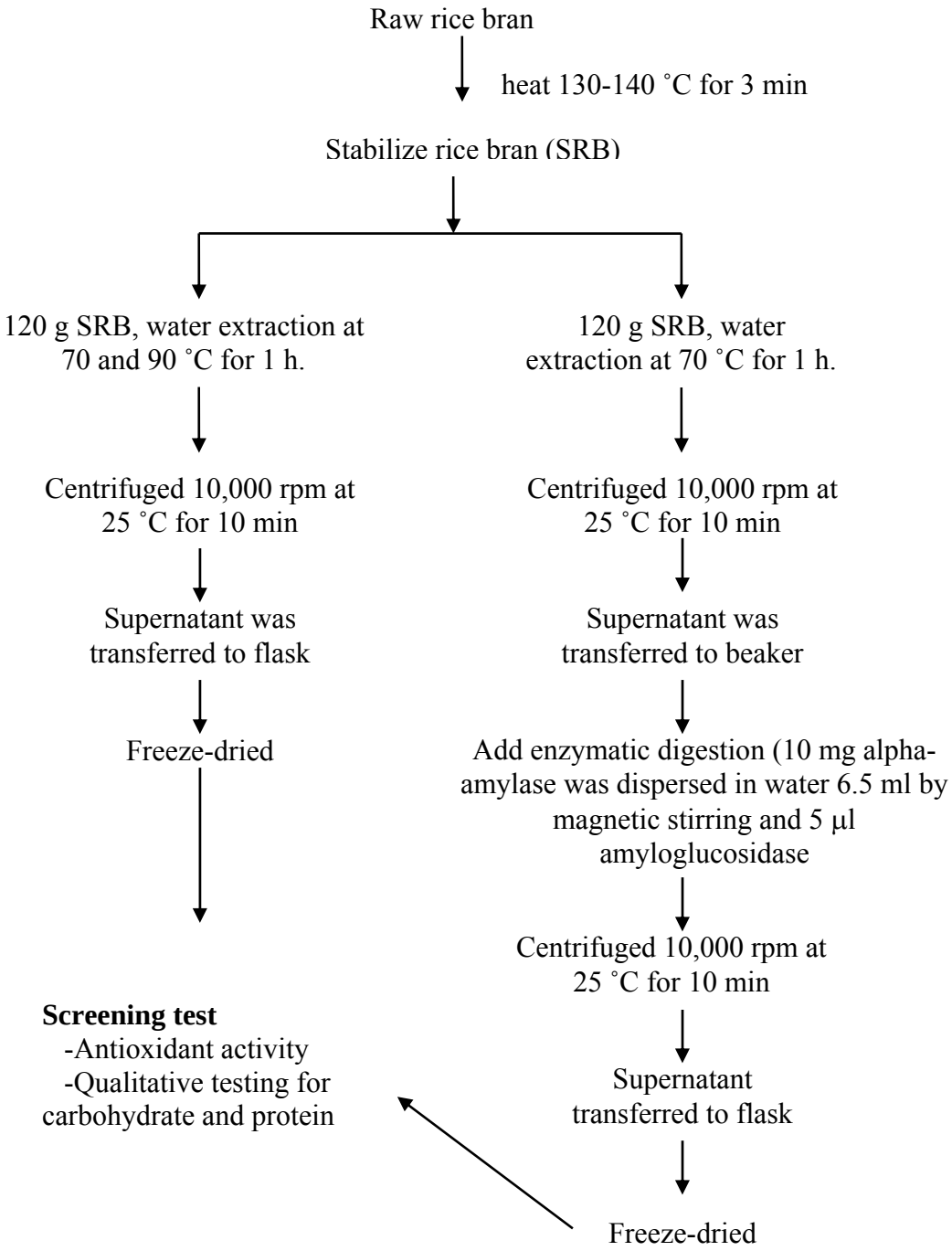

Figure 3.1

Procedure for rice bran extract from raw rice bran

3.5 Test substances administration

Rice bran extract was suspended in distilled water and administered orally in a dose of either 22.05, 220.5 or 2205 mg/kg/day, everyday for 30 days. Each dose was so prepared that a constant volume of 0.1 mL/100 g of body weight will be administered to each rat. The control animals received only distilled water in the same volume and through the same route. In this study**,** dose of 22.05 mg/kg/day was modified from the experiment in diabetic patients who received a sachet of 10 gram of stabilized rice bran in 90 ˚C hot water per day. The dosage was then, calculated for rats using a program provided by Food and Drug Administration, USA. (http://www/fda.gov/cder/cancer/animalframe.htm).

Metformin is an insulin-sensitizing and antihyperglycemic agent used in the treatment of non–insulindependent diabetes mellitus (NIDDM). Metformin, treatment leads to significant weight loss and decrease in body fat in hyperinsulinemic obese adolescents (Kay et al., 2001). In this study, metformin was suspended in distilled water and administered orally in a dose of 9.55 mg/kg BW once daily for three day in two week. It was increased to 1700 mg (850 mg twice daily) for fifteen day according to modified of Kay et al. (2001). The dosage was calculated for rats using a program provided by Food and Drug Administration, USA. (http://www/fda.gov/cder/cancer/animalframe.htm).

3.6 Determination of total antioxidant activity

Antioxidant activity of stabilized rice bran was determined by scavenging effect on 2, 2-diphenyl-1-picryhydrazyl radical ($DPPH^{\bullet}$) generated by the chemical method according to a protocol by Yu et al.,2002. Rice bran extract and rice bran oil was diluted in distilled water and ethanol, respectively, to final concentration of 10, 50, 100, 250 and 500 μg/mL. An aliquote of 500 μl of sample solution was mixed with 500 μl of $6x10^{-5}$ M DPPH in ethanol. The absorbance of each sample was measured at 517 nm by a UV–visible spectrophotometer (Shimadzu, Japan) after the reaction mixture was allowed to stand for 30 min at room temperature. Compare

DPPH radical scavenging capacity of each sample with butylate hydroxytoluene (BHT). BHT was used as reference standard tested in the same system. All tests were conducted in triplicate. The results were expressed as the inhibition percentage determined from the difference in absorbance (*A*) of DPPH between the control and samples.

$$\text{Inhibition } (\%) = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \text{ X } 100$$

The half effective concentration (EC_{50}) value of sample against DPPH radicals is the concentration required to scavenge 50% DPPH free radicals in the reaction mixture. The linear regression analysis was plotted between % radical scavenging and the concentration, and EC_{50} value was calculated by prism program.

3.7 Tests for biochemical property of rice bran extraction

3.7.1. Qualitative Tests for Carbohydrates

Carbohydrate can be identified using condensation reaction, dehydrating acids and reducing sugar according to the procedure of Schreck and Loffredo modular laboratory in chemistry (http://www.cerlabs.com/experiments/10875404464.pdf) which react with the carbohydrates to products highly colored products. In our experimental procedure we tested of carbohydrates using the molisch, iodine, Selwinoff's, Bial's, Barfoed's and Benedict's test.

Molisch test

2 mL of water, galactose, diluted bran extract were pipetted into a test tube (T1,T2 and T3, respectively). 2 drop of Molisch reagent were added to each of the sugar solutions. After mixing, 1 mL of concentrated sulfuric acid was added to each of the test tubes. Using a Pasteur pipette drops of sulfuric acid was carefully added down the inner sides of each tube. Any color change was then observed and recorded.

Iodine test

2 mL of water, glucose, starch and diluted bran extract were pipetted into a test tube (T1,T2,T3 and T4, respectively). A drop of iodine solution was added to each of the test tubes. If starch is present a dark blue or purple color will be noted.

Benedict's test

2 mL of water, sucrose, galactose and diluted bran extract were pipetted into a test tube (T1,T2,T3 and T4, respectively). 2 mL of Benedict's solution was added to each of the test tubes. Place the four test tubs into the hot water bath for at least five minutes. Any color change was then observed and recorded.

Barfoed's test

2 mL of water, sucrose, glucose and diluted bran extract are pipetted into a test tube (T1,T2,T3 and T4, respectively). 2 mL of Barfoed's reagent was added to each of the test tubes. Place the four test tubes into the hot water bath for at least five minutes. Any color change was then observed and recorded.

Bial's test

2 mL of water, glucose, ribose and diluted bran extract were pipetted into a test tube (T1,T2,T3 and T4, respectively). 2 mL of Bial's reagent was added to each of the test tubes. Place the four test tubes into the hot water bath for at three minutes. Any color change was then observed and recorded.

Seliwanoff's test

2 mL of water, glucose, fructose and diluted bran extract were pipetted into a test tube (T1,T2,T3 and T4, respectively). 2 mL of Seliwanoff's reagent was added to each of the test tubes. Place the four test tubes into the hot water bath for at five minutes. Any color change was then observed and recorded.

3.7.2. Qualitative Tests for Protein

Physical and chemical properties of amino acids and proteins were classified different functional groups presence were identified using various chemical reactions (Lowry et al., 1951).

Ninhydrin reaction

1 mL of distill water, tyrosine and diluted bran extract were pipetted into a test tube (T1, T2 and T3, respectively). 2 drop of ninhydrin reagent was added to each of the test tubes. Place the test tubes into the hot water bath for at ten minutes. Any color change was then observed and recorded.

Biuret test

1 mL of distill water, albumin and diluted bran extract were pipetted into a test tube (T1, T2 and T3, respectively). 3 mL of 10 % of NaOH (sodium hydroxide) was added to each of the test tubes. And then a few drops of 0.5 % of $CuSO_4$ (copper (II) sulfate) to the sample solution. Any color change was then observed and recorded.

Xanthoproteic test

1 mL of distill water, arginine, tryptophan and diluted bran extract were pipetted into a test tube (T1, T2, T3 and T4, respectively). 1 mL of nitric acid was added to each of the test tubes. And then 40% sodium hydroxide until the solution tests slightly basic using litmus or pH paper. Any color change was then observed and recorded.

Millon’s test

1 mL of distill water,albumin, tyrosine and diluted bran extract were pipetted into a test tube (T1,T2,T3 and T4, respectively). A drop of 1% $NaNO_2$ (sodium nitrite) was added to each of the test tubes. Any color change was then observed and recorded.

Hopkins-Cole Reaction

1 mL of distill water, arginine, tryptophan and diluted bran extract were pipetted into a test tube (T1,T2,T3 and T4, respectively). 2 mL of Hopkins-Cole reagent was added to each of all solutions. After mixing, 1 mL of concentrated sulfuric acid is added to each of the test tubes. Using a Pasteur pipette sulfuric acid was carefully added down the inner sides of each tubes. Any color change was then observed and recorded.

3.8 Animals

Male Sprague Dawley rats age 5-6 weeks of age, were purchased from the National Laboratory Animal Centre, Mahidol University at Salaya, Nakhon Pathom, Thailand. Animals were maintained under controlled temperature (24˚C ± 1˚C) with 60% humidity and a 12-h light and 12-h dark cycle. The approval of the ethic committee was obtained before the study onset by the Animal Committee at the Faculty of Medical Science, Thammasat University.

3.9 Diets

Rats were fed *ad libitum* with standard chow diet (CP, Samutpragran, Thailand) and water for seven days prior to the experiment. The standard chow consisted of 13, 55 and 31 percent of energy derived from fat, carbohydrate and protein, respectively. The obesity-induced diet was the diet that modified from obesity-induced diet from Claret et al. (2004). The obesity-induced diet consisted of high percentage of energy derived from fat (65, 23 and 11 percent of energy derived fat, carbohydrate and protein, reapectively) and rich in saturated fatty acid and cholesterol. The diet was prepared in the kitchen of the Nutrition Unit, Thammasat-chalermprakiet Hospital, Thammasat University. The ingredients were shown in the table 3.1.

Table 3.1

Compositions of high-fat diet as was estimated by Nutri Survey Program from Nutrition Divisions, Department of Health, Ministry of Public Health.

Composition	Amount (g)	Carbohydrate (g)	Protein (g)	Fat (g)	Energy (kcal)
Sugar	58	57.7	-	-	223.5
Standard chow	100	41.8	24	4.5	304
Wheat flour	150	114.5	15.5	1.5	546.5
Pork liver	100	2.4	19.9	4	126
Egg white, hen	33	-	3.3	0.4	16.8
Pork belly	100	2.8	13.9	33.5	368.1
Margarine	200	0	0.8	172.6	1558.8
Egg yolk, hen	200	4	28.6	60.2	672.1
Total	941	223.2	106	276.7	3816
		% Kcal			
CHO		23.39			
Fat		65.26			
Protein		11.11			

Note; 941 g of composition mixture gave a result of 745g cookie.

3.10 Experimental design

After seven days of acclimation, 48 rats were randomly divided into 6 groups as follows:

Group 1: rats received standard chow with distilled water

Group 2: rats received high-fat diet with distilled water

Group 3: rats received high-fat diet with rice bran extract at the dose of 22.05 mg/kg BW.

Group 4: rats received high-fat diet with rice bran extract at the dose of 220.5 mg/kg BW.

Group 5: rats received high-fat diet with rice bran extract at the dose of 2205 mg/kg BW.

Group 6: rats received high fat diet with metformin at the dose of 9.55 mg/kg BW once daily for three day in two week. It was increased to 1700 mg (850 mg twice daily) for fifteen day.

Amounts of food intake and body weight were measured every consecutive days throughout the study period. At the end of the forth week, animals were fasted for 16 hour before blood collection for biochemical measurements and glucose tolerance test. Animals were sacrificed within four days after finishing the protocol and organs were weighted and kept in 10 % neutral buffered formalin for further histological examination. The animals were anesthetize and sacrificed for blood collection from a common carotid artery. Blood will be allowed to coagulate before being centrifuged and the serum will be separated. The serum will be assayed for biochemical examination.

3.11 Biochemical measurements

3.11.1 Glucose determination

The levels of glucose in blood were determined using the glucosemeter Accu-Check monitors (Roche, Thailand). Rats were weighed and the tip of the tail was clipped to obtain blood for glucose measurement immediately prior to glucose

administration and at 30, 60, 90, 120 and 150 minutes after glucose administration (2g/kg body weight). The glucose dehydrogenase enzyme in test strip, in the presence of the coenzyme, converts glucose in the blood sample to gluconolactone. A series of hourly plasma glucose of each rat were analyzed and glucose concentrations were plotted against the time of blood withdrawals. Area under the curve was calculated using trapezoidal rule (Wolever et al., 1991).

3.11.2 Determination of triglyceride

The level of triglyceride in plasma was determined using the enzymatic method described by Wahlefeld et al. (1974). Using a lipoprotein lipase from microorganisms for the rapid and complete hydrolysis of triglyceride to glycerol followed by oxidation to dihydroxyacetone phosphate (DAP) and hydrogen peroxide. The hydrogen peroxide produced then reacts with 4-aminophenazone and 4-chlorophenol under the catalytic action of peroxidase to form a red dyestuff.

$$\text{Triglyceride} \xrightarrow{\text{lipoprotein lipase}} \text{Glycerol + fatty acid}$$

$$\text{Glycerol + ATP} \xrightarrow{\text{glycerol kinase}} \text{Glycerol-3-P + ADP}$$

$$\text{Glycerol-3-P + } O_2 \xrightarrow{\text{glycerol-3-oxidase}} \text{DAP + } H_2O_2$$

$$H_2O_2 + \text{4- aminoantipyrine + p-chlorophenol} \xrightarrow{\text{peroxidase}} \text{4- (p-benzoquinone-monoimino)-phenazone +2 } H_2O + HCL$$

The concentration was quantitated by spectrophotometer at the wavelength of 546 nm. Triglyceride concentration was calculated according to the following equation:

$$\text{Triglyceride concentration} = \frac{A \times 200}{As}$$

Where A and As are the absorbance of the sample reaction tube and 200 mg per deciliter standard triglyceride in standard reaction tube, respectively. The amount of triglyceride concentration was expresses as mg/dL.

3.11.3 Determination of cholesterol

The level of cholesterol in plasma was determined using the enzymatic method described by Richmond et al. in 1992. In the presence of the former the mixture of phenol and 4- aminoanantipyrine (4-AA) are condensed by hydrogen peroxide to from a quinoantipyrine dye proportional to the concentration of cholesterol in the sample.

$$\text{Cholesteryl esters} \xrightarrow{\text{Cholesterol esterase}} \text{Cholesterol + fatty acid}$$

$$\text{Cholesterol + } O_2 \xrightarrow{\text{Cholesterol oxidase}} \text{Cholestenone + } H_2O_2$$

$$\text{4- aminoantipyrine + Phenol} \xrightarrow[H_2O_2]{\text{peroxidase}} \text{Quinoneimine + 4 } H_2O$$

The concentration was quantitated by spectrophotometer at the wavelength of 500 nm. Cholesterol concentration was calculated according to the following equation:

$$\text{Cholesterol concentration} = \frac{A \times 200}{As}$$

Where A and As are the absorbance of the sample reaction tube and 200 mg per deciliter standard cholesterol in standard reaction tube, respectively. The amount of cholesterol concentration was expresses as mg/dL.

3.11.4 Other biochemical tests

HDL-cholesterol and LDL-cholesterol were measured by COBAS INTEGRA automate instrument at the department of Chemistry Laboratory, Thammasat-chalermprakiet Hospital, Thammasat University.

3.11.5 Determination of insulin

The animals were fasted for 16 hours and blood samples were collected from carotid artery using tube with EDTA. Tubes were kept on ice until centrifugation at 3000 rpm for 5 min. Plasma was stored at –20 °C before being analyzed. Plasma insulin levels were analyzed by using the commercial ELISA kits from Mercodia AB (Uppsala, Sweden). Mercodia Insulin ELISA is a solid phase two-site enzyme immunoassay. It is based on the direct sandwich technique in which two monoclonal antibodies are directed against separate antigenic determinants on the insulin molecule. During incubation insulin in the sample reacts with peroxidase- conjugated anti-insulin antibodies and anti-insulin antibodies bound to microtitration well. A simple washing step removes unbound enzyme labelled antibody. The bound conjugate is detected by reaction with 3,3',5,5'-tetramethylbenzidine (TMB). The reaction is stopped by adding acid to give a colorimetric endpoint that is read spectrophotometrically.

The concentration of insulin is obtained by computerized data reduction of the absorbance for the Calibrators, except for Calibrator 0, versus the concentration using cubic spline regression by prism program.

3.12 Assessment of insulin resistance and beta-cell function

The homeostasis model assessment of insulin resistance (HOMA-IR) and homeostasis model assessment of beta-cell function (HOMA-β) represent insulin sensitivity and pancreatic beta-cell function, respectively (Matthews et al., 1985; Tripathy et al., 2000). These methods are based on fasting levels of glucose and insulin. HOMA-IR was calculated according the following formula:

$$\text{HOMA-IR} = \frac{\text{FBG (mmol/L)} \times \text{Fasting insulin (}\mu\text{U/mL)}}{22.5}$$

$$\text{HOMA-}\beta = \frac{20 \times \text{Fasting insulin (}\mu\text{U/mL)}}{\text{FBG (mmol/L)} - 3.5}$$

3.13 Statistical analysis

Data were reported as mean±standard error of mean (SEM). The comparison between two groups was analyzed by unpaired two-tailed Student's t-test. Data were analyzed between group using one-way analysis of variance (ANOVA) and post hoc least-significant difference (LSD) test. Statistically significant difference was defined as a P value less than 0.05. The Pearson's correlation was used to correlate between abdominal fat and blood parameters. P values less than 0.05 or 0.01 were considered significant.