CHAPTER 3

MATERIALS AND METHODS

3.1 Raw Materials

1. Black glutinous rice (*Oryza sativa* L.) (The Royal Foundation, Doi Kham, Chiang Mai, Thailand).
2. Distilled water (Polestar demineralized water, Chiang Mai Polestar, Chiang Mai, Thailand).
3. Full fat milk powder (Yok, Chiang Mai, Thailand).

3.2 Starter cultures

Freeze dried cultures of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* (commercial code YC 380), Christian Hansen, Denmark).

3.3 Material and equipment

3.3.1. Materials

(1) Sulfuric acid (Merck, Germany).

(2) Ammonia solution (Merck, Germany).

(3) Ethyl alcohol (VWR International, England).

(4) Diethyl ether (Labscan Asia, Bangkok, Thailand).

(5) Petroleum ether 40-60°C (Labscan Asia, Bangkok, Thailand).

(6) Kjelblet/Coppper for protein analysis (ratio K_2SO_4: $CuSO_4 \cdot 5H_2O$ (9:1)) (Oskon, Bangkok, Thailand).

(7) Boric acid (Merck, Germany).

(8)Mixed indicator or Screen methyl red (0.2(w/v) of methyl red and bromocresol green, 1:1) (methyl red from BDH Laboratory supplies, Poole, UK and bromocresol green from VWR International, England).

(9) Sodium hydroxide (Merck, Germany).

(10) Whatman filters paper no. 1 (Whatman International, England).

(11) 3, 5-Dinitrosalicylic acid (Sigma-aldrich, Switzerland).

(12) Potassium tartrate (VWR International, England).

(13) D-Glucose (Fisher Scientific, UK).

(14) Phenolphthalein (Merck, Germany).

(15) Hydrochloric acid (ACI Labscan, Bangkok, Thailand).

(16) Folin-Ciocalteu-reagent (Merck, Germany).

(17) Sodium carbonate (Merck, Germany).

(18) Gallic acid (Sigma-aldrich, China).

(19) Ferric solution (Fisher Scientific, UK).

(20) 2, 2- bipyridine (Sigma-aldrich, India).

(21) α-amylase powder from *Aspergillus oryzae* (Sigma-aldrich, Switzerland).

(22) Amyloglucosidase (Glucoamylase) from *Aspergillus niger*

(FlukaBioChemika, Switzerland).

(23) Plate Count Agar (PCA) (Himedia Laboratories, India).

(24) Peptone (Himedia Laboratories, India).

(25) Lactose (Himedia Laboratories, India).

(26) MRS (Himedia Laboratories, India).

(27) M-17 broth (Oxoid, England).

(28) Bacteriological Agar (Himedia Laboratories, India).

(29) Acetic acid (Northern Chemical and Glasswares, Chiang Mai, Thailand).

(30) Methanol (Labscan Asia, Bangkok, Thailand).

3.3.2. Equipment

(1) pH meter (Consort® C830T, Belgium).

(2) Shaking water bath (Memmert model SV2945, Germany).

(3) Centrifudge (Hettich Model EBA20, UK).

(4) Heating mantle (PNP).

(5) Hot Air Oven (Memmert model 400, Germany).

(6) Distillation unit for distilled protein (Foss kjeltec™ 8100, Denmark).

(7) Digestion unit for distilled protein (Velp Scientification1007 Digestion, Italy).

(8) Spectrophotometer (Thermo Scientific Genesys model Genesys 10 UV

scanning, USA).

(9) Colorimeter (Minolta Data Processor DP-301 Chroma Meter, Japan).

(10) Viscometer (Cannon, Japan).

(11) Incubator (Memmert, Germany).

(12) Hammer mill (Armfield; series 2000, England)

(13) Autoclave (Hirayama model HA-300MN, Japan)

(14) Sieve of 595 µm (Retsch®, Germany)

3.4 Methods

3.4.1 Preparation of black glutinous rice powder

The rice powder was prepared by grinding the black glutinous rice (*Oryza sativa L.*) sample using a dry miller (Hammer Mill) to get rice powder. The black glutinous rice powders were sieved through a screen with size of 595 µm, packed in polyethylene (PE) bags and kept at 4°C (Iwaki and Kitada, 2007) to be used as a raw material in this research.

3.4.2 Optimization of soaking water and soaking time for enzymatic extraction of black glutinous rice milk

The black glutinous rice powder was soaked with distilled water at a ratio of 1:2.5, 1:5, or 1:10 for rice powder and water, respectively. The soaking times of 30, 60, and 120 min for each soaking ratio were investigated in this study. All soaking conditions were done at room temperature. After soaking, 0.2% of α-amylase (Wongkhalaung and Boonyaratanakornkit, 2000) was added into the rice solution, adjusted the solution pH to

6.5 (Wongkhalaung and Boonyaratanakornkit, 2000) and heated at 90°C for 1 h (α-amylase was used to degrade insoluble starch and starch granules). Following the α-amylase hydrolysis, 0.2% of amyloglucosidase (Wongkhalaung and Boonyaratanakornkit, 2000) was incorporated into the solution, adjusted the pH of the solution to 5.0 (Wongkhalaung and Boonyaratanakornkit, 2000) using acetic acid and heated at 55°C for 6 h (amyloglucosidase was used to produce glucose from a non-reducing end of starch, amylose, amylopectin and amylodextrin chains). The black glutinous rice milk was then be filtered through a sterile white cloth and heated at 90°C for 30 min to inactivate the enzymes. The heated black glutinous rice milk was cooled down and stored at 4°C for further analyses.

Analyses of the rice milk

(1) Physical characteristics

The analysis of physical characteristics was done for viscosity by a viscometer (Steffe, 1996) and color by a colorimeter (Pomeranz and Meloan, 1994).

(2) Chemical characteristics

The chemical analyses for rice milk were performed according to the method published in AOAC (2000) no. 955.04 for moisture content and AACC (2000) for proximate analysis, including protein contents (method no 991.20), fat contents (method no 905.02), crude fiber (method no 985.29) and ash contents (method no 945.46). Measurement of pH was conducted by a pH meter. Total soluble solid contents were determined using a refractometer. Reducing sugar, total sugar, total phenolic content, phytic acid, and anthocyanin content were carried out according to a modified method of Rattanapanone (2011), James (1995), Tananuwong and Tewaruth (2010), Kong and Lee (2010) and Sompong et al. (2011), respectively.

(3) Statistical analysis

Results in this section were analyzed using a Randomized Complete Block Design and was carried out by a SPSS program (version 16.0). Differences between treatments were analyzed by Duncan's New Multiple Range Test. The soaking time and ratio that produced rice milk with the highest reducing sugar and antioxidant properties were selected for the next experimental section.

3.4.3 Optimum condition of α-amylase extraction to produce black glutinous rice milk

This section used one ratio of soaking water and soaking time based on the result of the section 3.4.2. After soaking the rice powder, 0.2% of α-amylase was added into the rice solution, adjusted the solution pH to 6.5 using acetic acid and heated at different conditions of 60, 80, or 90°C for either 30, 60, or 120 min (Wongkhalaung and Boonyaratanakornkit, 2000; Park et al., 2005; Apar and Ozbek, 2005). The solution was also treated with 0.2% of amyloglucosidase, adjusted the pH of the solution to 5.0 using acetic acid and heated at 55°C for 6 h. The black glutinous rice milk was collected after filtration through a sterile white cloth. The milk was heated at 90°C for 30 min, cooled down and stored at 4°C for further analyses.

Analyses of the rice milk

(1) Physical characteristics

Physical characteristics of rice milk were determined by following the methods stated in section 3.4.2.

(2) Chemical characteristics

The chemical properties of the rice milk were examined for pH, moisture content, total sugar, total soluble solid, reducing sugar, total phenolic content, phytic acid and anthocyanin content based on the procedures in the section 3.4.2.

(3) Statistical analysis

Results in this section were analyzed using a Randomized Complete Block Design, which was run in a SPSS program (version 16.0). Differences between treatments were analyzed by Duncan's New Multiple Range Test. The extraction condition of α-amylase that produced the rice milk with the highest reducing sugar and antioxidant properties was chosen to be further investigated in the next section.

3.4.4 Optimum condition of amyloglucosidase extraction to produce black glutinous rice milk

The results of the sections 3.4.2 and 3.4.3 for soaking ratio, soaking time and extraction condition of α-amylase enzyme were utilized in this section. After α-amylase extraction, the rice solution was added with 0.2% of amyloglucosidase, adjusted the solution pH to 5.0 using acetic acid and extracted at temperatures of 50, 55, or 60°C for 90, 180, or 360 min (Wongkhalaung and Boonyaratanakornkit, 2000; Park et al., 2005; Apar and Ozbek, 2005). At the end of the extraction period, the rice milk was filtered through a sterile white cloth, heated at 90°C for 30 min, cooled down and kept at 4°C for physicochemical and microbial determination.

Analysis of the milk

(1) Physical characteristics

Physical characteristics of rice milk in this section were determined for

viscosity and color using the methods stated in section 3.4.2.

(2) Chemical characteristics

Chemical properties of the rice milk were established by following the methods stated in the section 3.4.3.

(3) Microbial analysis

Microbial analyses, including total microbial count and lactic acid bacteria, were carried out according to a modified method of Wohlsen et al. (2006) and Marshall (2006), respectively.

(4) Statistical analysis

Statistical analysis was done following the methods stated in the section 3.4.3.

3.4.5 Production of fermented rice from black glutinous rice milk

The extraction conditions of α-amylase and amyloglucosidase that produced rice milk with the highest reducing sugar and antioxidant capacities were continued to be used in this section.

To make a fermented rice product, black glutinous rice milk was supplemented with either 0, 1 or 2% (w/w) full fat milk powder. The addition of the milk powder was carried out to understand whether the growth of yoghurt cultures would be better in the presence of milk protein. A previous study showed that an addition of yoghurt cultures in black glutinous rice milk only caused an incubation time of 12 h at 42-43°C (unpublished data). After thoroughly mixed, the milk was pasteurised at 90°C for 30 min, cooled down to a temperature around 42°C, inoculated with 0.02% (w/w) a freeze dried cultures that composed of *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* (Krzeminski et al.,

2011) and incubated at 43±1°C over a period of 14 h to reach pH 4.4 - 4.2. At the end of the incubation time, the fermented rice was cooled down to 4°C and analyzed.

Analysis of yoghurt

(1) Physical characteristics

The physical analyses included viscosity by a viscometer (Steffe, 1996), color by a colorimeter, syneresis (Isanga and Zhang, 2009) and water holding capacity (Isanga and Zhang, 2009).

(2) Chemical characteristics

Several chemical properties of the fermented rice product that were determined were pH, moisture content (AOAC no. 955.04, 2000), total soluble solid by a refractometer, total sugar (James, 1995) reducing sugar (Rattanapanone, 2011), total acidity (Park et al., 2005) and antioxidant properties according to the method in the section 3.4.2.

(3) Microbial analysis

Determination of yoghurt cultures was done using M-17 medium for *S. thermophilus* and MRS medium acidified to 5.4 using acetic acid for *L. bulgaricus* (Ashraf and Shah, 2011).

(4) Statistical analysis

Statistical analysis was carried out following the methods stated in the section 3.4.3.