

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

Bambara groundnut was purchased from a local market, Songkhla, Thailand. The lipase enzyme from *chromobacterium viscosum* (437707, 100KU) was purchased from Merck. Pancreatic Alpha-amylase (EC 3.2.1.1, type VI-B from porcine pancreas, A3176, 25 U/mg), amyloglucosidase (EC 3.2.1.3 from *Aspergillus niger*, A7095, 300 U/mL), PGO enzyme kits (P1179) and o-dianisidine (D3252) were purchased from Sigma Aldrich Chemical Co.,Ltd, Other chemicals were of analytical grade.

3.2 Sample preparation

3.2.1 Flour preparation

Bambara groundnuts seeds were dried in a tray dryer at 60°C overnight. The seed coat was manually removed. Then, the seed was dried in tray dryer at 60°C and milled using a blender to obtain the flour.

3.2.2 Defatted flour preparation

Bambara groundnuts flour was soaked in hexane (1:15 W/V) for 60 mins with stirring. Then, it was centrifuged at 2,000 g for 10 min. Pellet was dried and milled using a blender to obtain Bambara groundnut defatted flour. For supernatant, it was dried at room temperature for evaporation of hexane to obtain Bambara groundnut oil.

3.2.3 Starch isolation

The Bambara groundnut flour (200 g) was mixed with 2 L of distilled water and it was covered with aluminum foil. The mixture was blended for 1 hour and incubated at 40°C for 5 h. It was centrifuged at 10,000 g for 30 min to collect the residue. The two L of 2% NaCl was added in the residue and blended for 10 min. The suspension was incubated at 4°C for 12 h, then centrifuged at 10,000 g for 30 min.

The residue was collected and added with 2 L of 0.03M NaOH. The suspension was blended for 10 min and incubated for 12 h at 4°C. The supernatant was discarded by centrifugation at 10,000xg for 30 min. For the residue, it was mixed with water to prepare a slurry and fiber was removed by a 75- μ m screen. Then, it was centrifuged at 10,000 g for 30 min to discard the supernatant. The residue starch was air dried at 30°C (Adebowale & Lawal, 2002).

3.3 Chemical composition

The proximate analysis of Bambara groundnut flour and starch were determined by AOAC (2000) method.

3.3.1 Determination of amylose

Amylose content was determined according to Juliano (2012). The 100 mg of sample was weighed in a tube and 1 mL of 95% ethanol and 9 mL of 1M NaOH were added. The mixture was stirred until the solution is clear, then the volume was adjusted to 100 mL in volumetric flask. The 0.5 mL of diluted sample was mixed with 9.2 mL of distilled water. The 0.1 mL of 0.9 N Ammonium chloride and 0.2 mL of 0.2% iodine were added in the mixture and incubated at room temperature for 30 min. Absorbance was measured at 620 nm using a spectrophotometer (Thermo scientific SPN-1).

3.3.2 Free fatty acid (FFA) analysis

The BG oil was converted to the methyl ester by using the method described by Akintayo and Bayer, (2002). Gas chromatography (Agilent Technologies, 7000B) was used. A flame ionization detector (Agilent Technologies, 7890a FID-Detector) was used as detector and Helium was used as the carrier gas. The column initial temperature was set at 70°C and increased the rate of at 5°C/min to a final temperature of 240°C while the injection port was maintained at 240°C. The peaks were identified by comparison with standard fatty acid methyl esters (Akintayo, 2002; Aremu, Mamman, & Adebisi, 2013).

3.4 Hydrolysis of triglyceride (TG)

TG of Bambara Groundnut oil was hydrolysed by lipase to obtain FFA. BG oil (5 g.) was mixed with 10 ml of 0.1M Tris-HCl buffer pH 8 and 2 ml of Tween-20. Then, lipase (500 unit per g of oil) was prepared and added in the solution, then incubated at 37°C in a water bath. The samples were collected at 0, 24, 48, 72, 96 h to determined free fatty acids by titration method. The inactivated enzyme was done by heated in boiling water.

3.5 Effect of ethanol as a solvent on complex formation

3.5.1 Sample preparation in ethanol-water solution

To study the effect of ethanol on complex formation. Defatted BG flour or starch were gelatinized with DI water (1% W/V) at 99°C for 60 min. After that, the hydrolyzed oil (from 3.4) that mixed with ethanol was add for 0, 2, 4, 6, 8, 10% g starch and obtain a final concentration of ethanol at 25% and 50%. The suspension was cool down to room temperature then was dried and milled to obtain the complexed Bambara groundnut flour.

3.5.2 Determination of complex index value (Kaur & Singh, 2000)

The complex formation between amylose and lipids was determined by the complex index (CI) value, according to Kaur & Singh (2000). The free amylose before and after amylose-lipid complex formation were measured by the reduction of iodine binding capacity with amylose. The amylosed-lipid sample (5 g) was mixed with 25 ml of distilled water. The solution was vortexed for 2 min. After that, it was centrifuged at 3,000 rpm (for 15 min). The 500 μ l of supernatant and distilled water (15 ml) were added to 2 ml of iodine (2.0% KI and 1.3% of iodine in distilled water). The absorbance was measured at 690 nm., The amount of amylose before lipid complex formation was used as the reference and CI was evaluated using equation as to follow.

$$CI = \frac{ABS \text{ of reference} - ABS \text{ of sample}}{ABS \text{ of reference}} \times 100 \quad (1)$$

Where ABS = absorbance

Sample = Bambara groundnut flour after lipid complex formation

Reference = The amount of amylose before lipid complex formation

3.5.3 Determination of crystalline structure

Wide-Angle X-ray Scattering (WAXS) was used to determine the crystalline structure, using beam line 1.3: SAXS/WAXS at Synchrotron Light Research Institute, Thailand. The sample containing 17-20% moisture content was put on Kapton tape and placed on the metal frame with square aperture of 10x10 mm. The sample was expanded to X-ray beam with the energy of 9 keV for 300 min. The result was interpreted by SAXSIT program.

3.5.4 Determination of thermal properties

Differential Scanning Calorimetry (DSC) (DSC 1 STARe System, Mettler-Toledo Ltd., Thailand) was used to determine a gelatinization property. An approximately 7 mg of sample was weighted into a 60 μ L stainless steel pan and distilled water was added to obtain a starch-water in ratio of 1:3 (w/w). A stainless steel pan without sample was used as the reference and indium was used as the standard. The DSC run was heated from 25 to 150°C at a heating rate of 3°C/ min. The thermal transitions of starch sample were defined as onset temperature (T_o), peak of gelatinization temperature (T_p), conclusion temperature (T_c) and the enthalpy of gelatinization (ΔH).

3.6 Modification of Bambara groundnut flour

3.6.1 Preparation of modified Bambara groundnut flour

To study the effect of protein in flour and the amount of lipids added, 1% defatted BG flour or starch were mixed with DI water and hydrolyzed oil, with 0, 2.5, 5, 10, 15% (per g flour), then gelatinized at 99°C for 60 min in the stirring system. The suspension was cooled to room temperature, dried and milled then sieved to obtain a particle size of 90-125 μ m.

3.6.2 Determination of complex index value

Following the section 3.5.2

3.6.3 Determination of crystalline structure

Following the section 3.5.3

3.6.4 Determination of thermal properties

Following the section 3.5.4

3.6.5 Properties of modified Bambara groundnut flour

3.6.5.1 In vitro digestibility

The modified method of AOAC method (2002) was used to determine starch fraction, rapidly digestible starch (RDS), slowly digestible starch (SDS), resistant starch (RS). The 100 mg of samples were weighed into a centrifuge tube and the 4 ml of sodium maleate buffer pH 6 that contained pancreatic α -amylase (No.P1750, Sigma–Aldrich) and amyloglucosidase (3 U/mL) was added in the sample. The solution was incubated in a shaking water bath at 37°C for 20 min and 16 h. Then, the 4 mL of 99% ethanol was added and the solution was centrifuged to collect supernatant. The 8 mL of 50% ethanol was added in the pellet, the mixture was centrifuged for collect supernatant, this step was repeated. The pellet of digested starch at 16 h was re-suspended in 2 mL of 2M KOH and kept in 4°C until digested starch completed soluble. Next, the 8 mL of 1.2M sodium acetate buffer pH 3.8 and 0.1 mL of amyloglucosidase (3,300 U/mL) were added in the solution and incubated in water bath at 50°C for 30 min. The solution was adjusted to 100 mL with DI water before determine the free glucose by PGO enzyme reagent. For the supernatant of the sample at 20 min, the supernatants obtained from the 2 steps was adjust the volume to 100 ml with 100 mM sodium maleate buffer pH 4.5 and 0.1 ml of diluted sample was incubated with 10 μ g of AMG solution (300 U/ml) in 100 mM sodium maleate buffer pH 6.0 at 50°C for 20 min.

To determine the free glucose by PGO enzyme reagent, the 2.5 mL of PGO enzyme reagent was added in 0.05 mL of diluted sample and mixed with 0.2 mL of distilled water, then incubated at 37°C for 30 min. The absorbance of the solution was measured at 440 nm. The percentages of RDS, SDS, and RS were calculated as follows:

$$\text{RDS (\%)} = \text{glucose released at 20 min} \times 0.9 \times 100$$

$$\text{SDS (\%)} = (\text{glucose released at 16 h} - \text{glucose released at 20 min}) \times 0.9 \times 100$$

RS (%) = glucose released in undigestible starch at 16 h x 0.9x
100

3.6.5.2 Dynamic Rheology

The dynamic viscosity of complexed Bambara groundnut flour was carried out in a stress-controlled rheometer MCR 52 with a controlled temperature of 37°C. A parallel plate was used. The sample was mixed with DI water for 15% (W/V) and it was dropped on the parallel plate. First, a strain sweep test at strain range of 0.1-100% a constant frequency of 1 rad/s and 37°C was conducted to set the upper limit of the linear viscoelastic region (LVR). Second, a frequency sweep test with a frequency range of 0.6-100 rad/s at 37°C was performed at the 1% strain, where it was within LVR for all samples. Viscoelastic parameters, which are storage or elastic modulus (G'), loss or viscous modulus (G''), loss tangent ($\tan \delta = G''/G'$), and complex viscosity ($|\eta^*|$) were measured.

3.7 Statistical analysis

The experiments were conducted in duplicate. Analysis of variance (ANOVA) was performed using SPSS version 16.0. Comparison of mean was executed using Duncan's Multiple Range tests.

3.8 References

- Adebowale, K. O., & Lawal, O. S. (2002). Effect of annealing and heat moisture conditioning on the physicochemical characteristics of Bambara groundnut (*Voandzeia subterranea*) starch. *Nahrung*, 46(5), 311-316. doi:10.1002/1521-3803(20020901)46:5<311::Aid-food311>3.0.Co;2-z
- Akintayo, E. (2002). Akintayo E. T., and Bayer E. (2002). Identification of oils by NMR Spectroscopy. *Rivista Italiana Delle Sostanze Grasse*. 79, 207-210. *Rivista Italiana Delle Sostanze Grasse*, 79, 207-210.
- Aremu, M., Mamman, S., & Adebisi, O. (2013). Evaluation of fatty acids and physicochemical characteristics of six varieties of bambara groundnut [*Vigna subterranea* L. Verde) seed oils. *Rivista Italiana delle Sostanze Grasse*, 90, 107-113.

Kaur, K., & Singh, N. (2000). Amylose-lipid complex formation during cooking of rice flour. *Food Chemistry*, 71(4), 511-517. doi:[https://doi.org/10.1016/S0308-8146\(00\)00202-8](https://doi.org/10.1016/S0308-8146(00)00202-8)