III. RESEARCH METHODOLOGY

3.1 MATERIALS & INSTRUMENTS

3.1.1 Materials

Pea flour (Yunnan Xing Yi Foodstuff Co. Ltd., China), rice flour (Thai Better Foods Co. Ltd., Thailand), and sticky rice flour (Thai Better Foods Co. Ltd., Thailand), were obtained from local market. Potato amylose standard (Sigma Chemical Co., St. Louis) was used for apparent amylose content analysis, while for resistant starch analysis, Resistant Starch Assay Kits Megazyme International Ireland, Ltd. (pancreatic α-amylase, amyloglucosidase, GOPOD reagent buffer and enzymes, D-Glucose standard solution, resistant starch control) were used.

3.1.2 Instruments

The main instruments were a scanning electron microscope (SEM, LeO 1450 VP, England), a differential scanning calorimeter (DSC, Mettler Toledo, TGA/SDTA 851e, Switzerland) a rapid visco analyzer (RVA, Model 4D, Newport Scientific, Australia), a texture analyzer (TA-XT2, Stable Micro System, Texture Technologies Corp., USA), a spectrophotometer (UV Vis. Biochrom/Libra S22, England), and color analyzer (ColorQuest XE HunterLab, Hunter Associates Laboratory Inc., Virginia-USA).

3.2 EXPERIMENTAL DESIGN

This research was divided into three parts. The preliminary research was investigation on the properties of raw materials (rice flour, sticky rice flour, and pea flour) involved chemical composition, apparent amylose content, granular morphology, thermal and pasting properties. In experiment I (Figure 3), the effect of mixing rice flour with sticky rice flour at various ratios (100:0, 97.5:2.5, 95:5, 92.5:7.5, 90:10) on apparent amylose content, granular morphology, thermal and pasting properties was also investigated. In experiment II (Figure 4), trial was conducted to determine the solid content, proper cooking time and temperature to obtain rice cake. Similar trial was conducted for pea cake. Cakes resulted from various ratios of rice flour and sticky rice flour were then analyzed in terms of starch digestibility, textural and color properties. Commercially prepared pea cake and rice cake were also analyzed for comparison.

In experiment III (Figure 5) various cold setting conditions were conducted on pea cake and the extreme ratios of rice cake (100:0 and 90:10). First condition was 6 hours at room temperature / ± 25 °C which represented vendors that make cakes in the morning, place them in room temperature then sell them in the afternoon or evening. Second condition was 6 hours at refrigeration temperature (4 °C) which represented some vendors that place the cakes in iced box during time between after cooking to selling (approximately 6 hours). Third condition was 24 hours at 4 °C which came from theoretical point of view that RS content will increase after storage for 24 hours or longer at refrigeration temperature for some starches. Experiment for 12 or 24 hours at room temperature was impossible to be conducted as spoilage occurred on the cakes. Clearer experimental steps can be seen on the following figures.
1. Add water to obtain 20% solid content
2. Cooking 95°C for 25 min with continue stirring
3. Gel forming (6 h, room T)
4. Analysis:
   1. Textural properties
   2. Color properties
   3. In vitro starch digestibility

Figure 3. Flowchart of experiment I

Figure 4. Flowchart of experiment II
3.3 METHOD OF ANALYSIS

3.3.1 Moisture Content (AOAC, 1995)

Empty moisture cans were pre-dried in 105 °C oven for 15 min and cooled in desiccator. Then cans and 1-2 g of sample were weighed and dried in 105 °C oven for 3 hrs. After cooling in desiccators, and cans containing sample were weighed until constant weight is obtained (Δ weight less than 0.0005 g).

\[ \% \text{ moisture} = \frac{w - (w_1 - w_2)}{w} \times 100 \]

- \( w \) = original sample weight
- \( w_1 \) = sample weight + can after drying
- \( w_2 \) = weight of dried empty can

3.3.2 Ash Content (AOAC, 1995)

Sample of 5-10 g was weighed into a tared crucible. Very moist sample should be pre-dried first. Crucibles were placed in cool muffle furnace. Tongs, gloves, and protective eyeware should be used if the muffle furnace was warm. Sample was then ignited 12-18 hrs (or overnight) at about 550 °C. Muffle furnace was turned off and wait to open it until the temperature has dropped to at least 250 °C, preferably lower. Door must be opened carefully to avoid losing ash that may be fluffy. Crucibles were transferred to a desiccator with a porcelain plate and desicant then weighed after cooling.

\[ \% \text{ ash} = \frac{\text{weight after ashing} - \text{tare weight of crucible}}{\text{original sample weight}} \times 100 \]
3.3.3 Protein Content (Kjeldahl Method, AOAC 1995)

Sample of 100-250 mg was put into Kjeldahl flask. Then 1±0.1 g of K₂SO₄, 40±10 mg of H₂O₂, and 2±0.1 ml of H₂SO₄ were added. Boiling stones around 2-3 items were then put and boil the solution for 1-1.5 hrs. At distillation stage, a little amount of water is transferred step by step through flask wall and shaken carefully to reform the crystal. Solution was then transferred to the distillation ware, rinsed 5-6 times with 1-2 distilled water, followed by adding rinsing water to distillation ware and 8-10 ml of 60% NaOH-5% Na₂S₂O₃. Erlenmeyer was placed under the condenser with 5 ml H₃BO₃ and 2-4 drops red-methylene blue added. End of condenser must be soaked in H₃BO₃ solution. At titration stage, sample solution was diluted into 50 ml, and then titrated with 0.02 N standardized HCl until grey color appears. Volume of HCl for titration was then reported.

\[
% \text{N} = \frac{N \text{ HCl} \times \text{corrected acid volume} \times 14 \text{ g/mole N} \times 100}{\text{g of sample}}
\]

\[
% \text{N} \times 6.25 = \% \text{protein}
\]

3.3.4 Crude Fat (AOAC, 1995)

Total fat content was analyzed by soxhlet method. All the glassware (round bottom flask) was rinsed with hexane and dried in an oven at 102 °C for 30 min and cooled in a desiccator. Accurately 1-2 g of sample was weighed and covered with filtered paper. Sample was put into soxhlet extractor and condenser was set above the round bottom flask. Hexane solvent was poured into round bottom flask adequately. Sample and solvent was heated or refluxed above 5 hours or until solvent have dropped clearly to the flask. Sample was taken out of extractor then the solvent was distilled until there was almost no solvent in flask. The round flask with extracted oil was stored in oven at 105 °C, cooled to desiccator and weighed until it has constant weight. Total fat content (\%) = (X - Y) / W x 100%, where X = weight of empty flask and extracted fat, Y = weight of empty flask, and W = weight of sample.

3.3.5 Apparent Amylose Content (Juliano, 1971)

Sample (12% MC) of 0.1 g was weighed into 100 ml volumetric flask in duplicate. Then 1 ml of 95% ethanol and 9 ml of 1 M NaOH were added. The flasks were then boiled in waterbath for 10 min to gelatinize the sample. After cooling, distilled water was added to make the volume exactly 100 ml. After mixing, sample were kept standing overnight at room temperature. Blank solution was prepared following the previous steps except taking sample to the volumetric flask. After 24 hrs, flask was thoroughly mixed and 5 ml of sample solution was then transferred to an empty 100 ml volumetric flask. About 70 ml distilled water then added, followed by 1 ml of glacial acetic acid and 2 ml of iodine solution. The volume was adjusted to exactly 100 ml with distilled water. After mixing, let it be kept standing for 20 min to develop dark purple color. The absorbance was measured at 620 nm after setting zero with the blank solution. The value of the absorbance was calculated into the apparent amylose content using standard calibration curve developed from potato amylose standard.

3.3.6 In Vitro Starch Digestibility (Megazyme International Ireland Inc., 2008)

The samples were incubated in a shaking water bath with pancreatic α-amylase and amyloglucosidase for 16 h at 37 °C to hydrolyzed digestible starch to glucose. The reaction was terminated with 4 ml ethanol and the indigested resistant starch (RS) was recovered by centrifugation.
(5000g, 10 min). The supernatant was then decanted and washed with 50% ethanol twice to remove the digested starch. The sediment was solubillized in 2 ml of 2 M KOH in an ice bath, neutralized with 8 ml sodium acetate (1.2 M) and the RS was hydrolyzed to glucose with of amylglucosidase (0.1 ml, 3300 U/ml) for 20 min. The glucose oxidase / peroxidase reaction was used to measure glucose released from the digested and resistant starches. Absorbance was read at 510 nm after a 20 min incubation period at 50 °C. Starch fractions were calculated as follows:

\[
\text{Resistant starch} = \Delta E \times F/W \times 10.3/0.1 \times 1/1000 \times 100/W \times 162/180
\]

\[
= \Delta E \times F \times 1000 \times 100/W \times 162/180
\]

\[
\text{Non-resistant starch} = \Delta E \times F/W \times 9.27
\]

\[
= \Delta E \times F \times 100/0.1 \times 1/1000 \times 100/W \times 162/180
\]

\[
= \Delta E \times F/W \times 90
\]

\[
\text{Total starch} = \text{Resistant starch} + \text{Non-resistant starch}
\]

Where,

\[
\Delta E = \text{absorbance (reaction) read against the reagent blank}
\]

\[
F = \text{conversion from absorbance to microgram (the absorbance obtained for 100 μg of D-glucose in the GOPOD reaction is determined, and } F = 100 \text{ (μg of D-glucose) divided by the GOPOD absorbance for this 100 μg of D-glucose.}}
\]

\[
100/0.1 = \text{volume correction (0.1 ml taken from 100 ml)}
\]

\[
1/1000 = \text{conversion from micrograms to milligrams}
\]

\[
W = \text{dry weight of sample analyzed}
\]

\[
= \text{“as is” weight x [(100-moisture content)/100]}
\]

\[
100/W = \text{factor to present RS as a percentage of sample weight}
\]

\[
162/180 = \text{factor to convert from free D-glucose, as determined, to anhydro-D-glucose as occurs in starch}
\]

\[
10.3/0.1 = \text{volume correction (0.1 ml taken from 10.3 ml) for samples containing 0-10 % RS where the incubation solution is not diluted and the final volume is } \sim 10.3 \text{ ml}
\]

3.3.7 Granular Morphology

Samples were magnified from 500 to 5000 X under SEM. Detector SE1 was used with acceleration potential of 5.00 kV during micrography using SEM.

3.3.8 Thermal Properties

Samples of approximately 3 gram were weighed into aluminium pans followed by addition of 6 μl of water. The pans containing mixtures were then hermetically sealed to prevent moisture loss and equilibrated at ambient temperature for 2 h. Samples which have been equilibrated were heated from 10 °C to 120 °C at a rate of 10 °C/min using differential scanning calorimeter (DSC). The onset temperature (T_o), peak temperature (T_p), conclusion temperature (T_c), the gelatinization enthalpy change (ΔH) were also calculated and expressed as joule per gram sample (J/g).

3.3.9 Pasting Properties

Paste viscosity was determined by using rapid visco analyzer (RVA). A programmed heating and cooling cycle was used where the samples were held at 50 °C for 1 min, heated to 95 °C at 6 °C/min, and held at 95 °C for 2.7 min, prior to cooling from 95 to 50 °C at 6 °C/min and holding at 50
°C for 2 min. Parameters recorded were pasting temperature, peak viscosity, final viscosity (viscosity at 50 °C), breakdown viscosity (peak-trough viscosity), and setback viscosity (final-trough viscosity).

### 3.3.10 Textural Properties

Textural properties were analyzed by using texture analyzer (TA). Sample was sliced to smaller size (6x6x1) cm. Texture profile analysis was conducted under condition of 100 mm/min pretest speed, 50 mm/min test speed, 100 mm/min post test speed, 20% strain, P/36R probe, with weight calibration as follows; return distance of 30 mm and return speed of 10 mm/sec.

### 3.3.11 Color Properties

The ColorQuest (XE HunterLab, Hunter Associates Laboratory Inc., Virginia-USA) based on CIE system ($L^*$, $a^*$, $b^*$) was used to investigate the color of rice cake and pea cake. The instrument was calibrated each time before its use using area view of 0.375 RSIN/RSEX, light tab reflectant and white tile reflectant.

### 3.3.12 Statistical Analysis

Results were expressed as mean of values ± standard deviation of independent determinations. Analysis of variance and comparison of means using Duncan’s test ($p \leq 0.05$) were performed using the statistical software SPSS 16.0 for windows, SPSS Inc., Chicago, USA.