CHAPTER IV
MATERIALS AND METHODS

4.1 Sample collection and preparation

Okra (*Abelmoschus esculentus* (L.) Moench) was purchased from local markets in Nakorn Pathom, Thailand during April 2010 to April 2011. The immature okra fruits harvested after 40 days were obtained. They were stored at 5 ºC until being used.

4.2 Okra gum extraction

The seeds were removed from okra pod and then the okra pods were cut into small pieces. The pieces of okra pod were weighed in a beaker on an analytical balance and de-ionized water was added at a ratio of okra pod to water equaled 1:2. The okra pods were heated with de-ionized water at neutral pH (pH = 6.5) and varying temperature at 70, 80 and 90 ºC. The time period for the extraction experiments was 10, 30 and 60 min. The mixture was stirred every 5 min. After extraction, the slurry was filtered through cheesecloth to remove insoluble portion and dust. The filtered gum was poured into a tray covered with a plastic (polypropylene) sheet. Then it was dried at a temperature of 60 ºC for 18 h using a hot air oven. The dried gum was removed from the tray, packed and sealed in air tight plastic bags. It was stored overnight at -20 ºC. Then it was ground into okra gum powder by blender. The okra gum powder was weighed and the yield was calculated. The powder was stored at -20 ºC until used. Extractions were performed in triplicates for each experimental condition.

Experimental Design

For determining the optimum condition of okra gum extraction, the experimental design was a factorial design. There were two main factors which were temperature (70, 80, 90 °C) and period of extraction (10, 30, 60 min). The extraction was conducted in triplicates. The experiment consisted of 27 experimental units.

Variable

Independent variable: % yield of okra gum powder

Dependent variable: Temperature and period of extraction

Control treatment: Maturity and variety of okra, okra to water ratio, drying condition and pH

4.3 Okra pectin preparation

The okra gum obtained from the selected extraction condition was precipitated with 95% (v/v) ethanol at a ratio of 1:2 (filtrate: ethanol). After mixing, the mixture was left overnight (12 h) until pectin precipitation occurred. Then, the precipitate was again washed with 95% ethanol at a ratio of 1:1 (v/v) and dried in a hot air oven at 60 ºC for 2 h or until dried. The dried precipitate was then finely ground in a blender to obtain okra pectin powder. The pectin powder was stored at -20 ºC until used.

4.4 Quality determination

4.4.1 Physical properties

a) %Yield

Weight of dried okra pod, okra gum powder and pectin powder was used to calculate percentage yield.

$$\text{Yield}\,(\%) = \frac{\text{Weight of okra gum powder or okra pectin powder (g)}}{\text{Weight of dried okra pod (g)}} \times 100$$

b) Moisture content

Moisture content of fresh okra fruit, okra gum powder and okra pectin powder was determined by drying the sample in a hot air oven at 105 ±3 ºC. All values were calculated on a dry-weight basis, presented as **Appendix A** (77**).**

c) Viscosity

Viscosity was determined using a Brookfield RVTDV-II digital viscometer (Brookfield Engineering Laboratories Inc, Stoughton, MA, USA) with a UL adapter (accessory for measuring liquid of low viscosity) and a small sample adapter with shear rate of 12-14 s^{-1}. Citrus pectin was used as a reference. The okra gum solution (1, 2 and 3% w/v) and okra pectin solution (0.25%, 0.50%, 1.00% and 1.50% w/v) was prepared by dissolving the sample in de-ionized water. Viscosity was then measured at 25 ºC.

d) Color measurement

Color value of okra gum solution was measured three times using a specto-colorimeter (JUKI® Spectro Colorimeter Model JS555, Tokyo, Japan). Each okra gum sample was prepared into a solution at a concentration of 3% w/v using de-ionized water. The value was expressed as L*, a*, b*. The L* value represents lightness, a* and b* values represent redness and yellowness, respectively. Tungsten halogen lamp was used as a light source.

e) pH

The pH of okra gum and pectin solution was measured by using a pH meter (Mettler® Model Delta 340, Switzerland). Prior to measurements, the instrument was calibrated using pH buffer solutions at pH 4.01, 7.00 and 10.01 at 25°C.

f) Water activity (A_w)

The water activity was measured by using a water activity meter (Novasina IC-500 A_w-Lab, Axair Ltd., Pfäffikon, Switzerland) at a controlled temperature (25±1 °C). The sample was packed in a plastic sample container and placed inside the equilibrium chamber until the constant water activity value was obtained.

4.4.2 Chemical properties

a) Galacturonic acid (GalA) content

The GalA content of okra gum and pectin were determined by using the method described by Apasara Arkarapanthu *et al*. ,2005 (78). Okra gum and pectin was dissolved in DI water to obtain 0.05% and 0.025% (w/v) solution, respectively. The sample solution was mixed with 0.0125 M (w/v) solution of sodium tetraborate in concentrated H_2SO_4 (95-97%) and was heated at 95 ºC for 5 min in a water bath. After immediately cooling in an ice bath for 10 min, the sample was combined with 100 µl of 0.15% (w/v) *m*-hydroxydiphenyl (Sigma-Aldrich, St Louis, MO, USA) in 0.5% (w/v) NaOH and then vortexed the mixture until mixed well. The mixture was determined for GalA content in a spectrophotometer (UV-1601, Shimadzu, Kyoto, Japan) at a wavelength of 520 nm within 5 to 10 min. D-galacturonic acid in concentration of 1-10 mg/ml was used as the standard curve (r^2= 0.9987), presented as **Appendix B**.

b) Fourier Transform Infrared Spectroscopy analysis

Determination of DM by Fourier Transform Infrared Spectroscopy (FTIR) used the method described by Ravin Gnanasambandam *et al*., 1999 (79). Okra gum and okra pectin powder were desiccated in a dessiccator before analysis by FTIR. The powder was put to pressed surface. FTIR spectra of pectins were obtained by co-adding 64 scans at a resolution of 4.0 cm^{-1} using a Nicolet 6700 FTIR instrument (Thermo Fisher Scientific, Madison, USA) and FTIR–diamond ATR Nicolet 6700. The scanning range was the mid-infrared region (4000 to 400 cm^{-1}). The peak area of esterified and non-esterified carboxyl groups was obtained using OMNIC 8.1 software. An atmosphere spectrum was collected as a blank. The blank spectrum was subtracted from each sample spectra to compensate for absorption due to CO_2 and moisture in the air. Each sample was recorded triplicate spectra. The DM is a percentage of ratios between the area of esterified carboxylic groups and area of total carboxylic groups. It is implied that the ratio of the band area at 1730 - 1760cm^{-1} (corresponding to the area of esterified carboxylic groups (C=O)) and between 1640-1620 cm^{-1} (corresponding to the area of carboxylic groups (COO^-)) should be proportional to the DM, (63, 80). The FTIR spectra of pectin standards absorbance

were used as a calibration curve to get a linear relationship between %DM and the absorbance area at 1730 and 1600 cm^{-1} following this equation (81):

$$\%\text{DM} = 87.609\left(\frac{Area_1}{Area_1 + Area_2}\right) + 25.768$$

Where $Area_1$ = Area of the peak appeared between 1760-1745 cm^{-1}

$Area_2$ = Area of the peak appeared between 1640-1620 cm^{-1}

c) NMR spectroscopy

The DM and DAc was analyzed by NMR to characterize the pectin samples. The modified method was adapted from Bédouet *et al*. (82). A Bruker Avance 500 spectrometer (Billerica, MA, U.S.A.) was used to record ^1H NMR while operating at 500 MHz. Dry samples were dissolved in D_2O (Aldrich, 99.9% D) (10 mg/mL). The pectin solution was analyzed at 80 °C. Determination of proton chemical shifts was examined by the conventional proton pulse sequence provided by Bruker with 30° impulsion. The spectral window was 3000 Hz for 8 k data points with a pulse of 7 µs, an acquisition time of 1.36 s and a relaxation delay of 1 s. NMR spectra were recorded for 256 scans with pre-saturation of the residual water signal. The pulse width and intensity was optimized to achieve maximum water suppression with a minimum of disturbance of any other signal. DM was determined by the ratio of the signal at δ 4.40 ppm from H–4 resonance of esterified galacturonic acid residue to that at δ 3.76 ppm from *O*-methyl residues. DAc of pectin was calculated from the ratio of the isolated signal at δ 4.40 ppm of H–4 resonance of 4-linked galacturonic acid residues to the chemical shift in the region between δ 2.04–2.11 ppm of protons in O-acetyl residues.

d) Determination of the degree of acetylation (DAc)

The colorimetric determination was described by McComb and McCready (83). Five ml of okra gum and pectin solution (0.5, 0.1% w/v) was mixed with 1 ml of 9.4% (w/v) sodium hydroxide and 1 ml of 3.75% (w/v) hydroxylamine solution in a 25-ml volumetric flask. The mixture was left to stand for at least 5 min, added with 5 ml of 7.04% (v/v) acidified methanol solution and mixed thoroughly and then made to volume with the ferric perchlorate solution which was prepared by dissolving 1.93 g of ferric chloride hexahydrate in 5 ml of 37% hydrochloric acid and 5 ml of 70% perchloric acid and making up the volume to 100 ml by DI water. After 5

min, the sediment of pectin hydroxamic acid ferric complex was removed by filtering the solution through a Whatman No.1 filter paper into a colorimeter tube. The intensity of the color was determined at a wavelength of 520 nm. The sample blank was prepared by adding 5 ml of sample in a 25-ml volumetric flask. After that 1 ml of 9.4% (w/v) sodium hydroxide solution was added and mixed thoroughly and then placed at room temperature for 2-3 min. Then 1 ml of 3.75% (w/v) hydroxylamine hydrochloride solution was added and then made to volume with the ferric perchlorate solution. The amount of acetyl in the sample was determined by using the standard curve (60-420 µg acetyl/ml, r^2 = 0.9997) from glucose pentaacetate (98% purity), presented as **Appendix C**.

e) Intrinsic viscosity

A Cannon Fenske-type capillary viscometer ASTM n.100 (Carlo Erba, Italy) was used to determine intrinsic viscosity. The viscometer was placed in a thermostatic water bath with controlled temperature at 25±1 °C. The samples were dissolved in 0.1 M NaCl solution. The solution of okra gum and okra pectin was prepared at a concentration ranging from 0.125% to 0.1% (g/ml). The sample solution was filtered and then it was allowed to equilibrate at the bath temperature before starting the experiment. Flow times were recorded with a stopwatch with reproducibility ± 0.01 s. The flow time was measured in triplicates (84). The viscosity of solution was determined against relative viscosity with the equation (85);

$$\eta_r = \eta/\eta_s$$

Where η_r is the relative viscosity,η is the viscosity of pectin solution (Pa s), η_s is the viscosity of the solvent.

Specific viscosity (η_{sp}) was calculated from relative viscosity values using the equation (86);

$$\eta_{sp} = \eta_r - 1$$

Where the solution concentration approaches zero, the reduced viscosity (η_{sp}/C) approaches the intrinsic viscosity. The plot of reduced viscosity and concentration defined intrinsic viscosity. The intrinsic viscosity was obtained by extrapolation of reduced viscosity to zero concentration using the Staudinger equation (87).

$$\eta_i = \lim_{C \to 0} (\eta sp/C)$$

Where C is the concentration of pectin solution (g/ml) and η_i is the reduced viscosity (cm^3/g).

If the relationship between specific viscosity and concentration presents a linear trend, the Huggins and Kramer equation was used to calculate the intrinsic viscosity as the following equation;

$\frac{\eta sp}{C} = [\eta] + [\eta]^2 K_H C$ and $\frac{In\ (\eta r)}{C} = [\eta] + [\eta]^2 K_K C$, respectively.

Where K_H is Huggins' constant and K_K is Kraemer's constant. The intrinsic viscosity of samples was defined as the intercept of both equations.

f) Determination of molecular weight

Intrinsic viscosity was determined as a characteristic of macromolecules related to both of flow behavior and molecular size and shape. The intrinsic viscosity is related to molecular weight distribution as the term of average molecular weight value (M_w) through the Mark-Houwink-Sakurada equation;

$$[\eta] = K(M_w)^{\alpha}$$

Where K and α are temperature-depending parameters, solute and solvent characteristics. For pectin the constants, K and α, are 0.0436 and 0.78, respectively (88).

g) Determination of sugars content

The reducing and non-reducing sugar of sample was determined using the phenol-sulfuric acid assay (89). This methods based on the absorbance at 490 nm of a colored aromatic complex formed between phenol and the carbohydrate. Okra gum and pectin solution (50 µl, 0.1% w/v) was mixed with 500 µl of 4% (w/v) phenol followed by 2.5 ml 96% sulfuric acid. The solution was transferred from test tubes to the cuvettes and measured the absorbance at 490 nm using a UV-visible spectrophotometer (UV-1601, Shimadzu, Kyoto, Japan). Blank sample was prepared using 500 µl of 4% (w/v) phenol and by 2.5 ml 96% sulfuric acid. The concentration of sugar in the samples was determined by using the standard curve from glucose (5-50 µg of sugar/ml, $r^2 = 0.9882$), presented as **Appendix D**.

h) Soluble and insoluble dietary fiber

Determination of soluble and insoluble dietary fiber was carried by the Food Chemistry Laboratory, Institute of Nutrition, Mahidol University using an enzymatic-gravimetric method of AOAC, presented as **Appendix E** (90).

4.5 Application of okra gum and pectin in food products

Okra gum and pectin were tested as a thickening agent or stabilizer in food products. They were prepared at a concentration of 0.15% (w/v) and 0.075% (w/v), respectively due to the preliminary showed that those viscosity provided the suitable consistency to beverage products.

4.5.1 Non-fat pasteurized chocolate milk

Okra gum and pectin were used to improve consistency and mouthfeel compared with control. The formulation is shown in **Appendix F**. Non-fat pasteurized was heated to 80 °C and then okra gum/okra pectin mixed with sugar and cocoa powder was added into the hot milk. The milk was stirred until the dry ingredients were completely dissolved and then passed though a homogenizer until became homogenous. The hot milk was cooled to 8 °C and stored in a refrigerator.

4.5.2 Orange-flavored beverage

Okra gum and pectin were mixed with sugar and citric acid. The mixture was added into water and stirred until completely dissolved. The solution was added with food colorings (sunset yellow and tartrazine) and then added with an orange flavor. The beverage was kept in a refrigerator at 8 °C. The formulation is shown in **Appendix F**.

4.5.3 Sensory evaluation of the food products added with okra gum and pectin

The acceptability of non fat pasteurized chocolate milk and orange-flavored beverage adding with okra gum and pectin was determined by sensory evaluation. In this experiment, fifteen panelists from department of Food and Nutrition

for development evaluated the acceptability of food products adding with okra gum and pectin. Both non-fat pasteurized chocolate milk and orange-flavored beverage were prepared on the experiment day and stored at 8 °C in a refrigerator until served to the panelists. All samples were served in a white paper cup labeled with three-digit number codes selected from a random number table. The order of sample presentation to each panelist was randomized.

Sensory evaluation consisted of color, odor, mouthfeel and overall acceptability using nine-point hedonic scales (9=like extremely, 5= neither like nor dislike, 1= dislike extremely). Five-point just-about-right scales were used in sample viscosity (The score ranged from 1 to 5 where 5=much too thick, 3=just-about-right, 1=much too thin). The questionnaire is presented in **Appendix G**.

4.5.4 Stability test

The non-fat pasteurized chocolate milk was studied in the stability test. The sample was kept in a container under refrigeration (8 °C) for 3 days. Phase separation which is obviously separation of 2 layers of solution was determined by visual observation. The precipitation was calculated from weight of precipitated cocoa powder.

4.6 Statistical Analysis

All data were presented as mean values of three determinations ± standard deviation (SD). Differences between mean values were analyzed by analysis of variance (ANOVA) using Duncan's Multiple-Range Test with a confidence interval of 95%.