CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

Chemicals

1. Native rice starch (Thai Flour Industry Company Ltd., Bangkok, Thailand).
2. Standard amylose (Sigma-aldrich chemie, England).
3. Sodium hydroxide (NaOH) (RCI Labscan Ltd., Bangkok, Thailand)
4. Glacial acetic acid (CH_3COOH) (RCI Labscan Ltd., Bangkok, Thailand)
5. Methanol (AR grade) (RCI Labscan Ltd., Bangkok, Thailand)
6. 2-propanol (RCI Labscan Ltd., Bangkok, Thailand)
7. Monochloroacetic acid ($ClCH_2COOH$) (Merck Schuchardt OHG, Hohenbrunn, Germany)
8. Hydrochloric acid (HCl) (Merck Schuchardt OHG, Hohenbrunn, Germany)
9. Sodium chloride (NaCl) (Merck Schuchardt OHG, Hohenbrunn, Germany)
10. Epichlorohydrin (C_3H_5ClO) from Sigma-aldrich (Steinhiem, Germany).
11. m-cresol purple (Aldrich, USA)
12. Distilled water
13. Aspirin
14. Sodium starch glycolate (Explotab®)
15. Croscarmellose sodium (Ac-di-sol®)
16. Silicified microcrystalline cellulose (Prosolv SMCC® 90) (JRS Pharma LP, New York, USA)
17. Silver nitrate solution 1%

18. Magnesium stearate
19. Talcum
20. Sulfuric acid 96% (H_2SO_4) (Labscan, Ireland)
21. Iodine
22. Potassium

Apparatus

1. UV-Visible spectrophotometer (Shimadzu UV-2450, Japan)
2. Fourier Transform Infrared Spectrophotometer (Nicolet Nexus 470FT-IR)
3. Scanning electron microscope (JSM-5910LV, JEOL, USA)
4. X-ray diffractometer (X'Pert MPD, Philips, Germany)
5. Disintegration tester (Pharma Test, PTZ, Germany)
6. Jolting volumeter (J. Engelsmann A.-G., Thailand)
7. Hot air oven (Binder ED240/E2, Germany)
8. Hot plate stirrer (Heidolph MR-Hei standard, Germany)
9. Thermometer
10. Water bath
11. Analytical balance (Scaltec type SBC 31, Germany)
12. Electronic balance (Sartorius type LA 230s, Germany)
13. Whatman filter no. 1, 150 mm diameter (Whatman®, Germany)
14. Waterproof pH meter (Eutech Instruments, USA)
15. Sieves no. 40, 60
16. Sinter glass filter por. 3 (ROBU®, Germany)
17. Three-neck round bottom flask
18. Burette
19. Stand and clamp
20. Petri disk
21. Pipette and micropipette
22. Reflux condenser
23. Buchner funnel and vacuum pump
24. Crucibles
25. Volumetric flasks
26. Beakers

27. Aluminum can
28. Desiccators
29. Cylinders
30. Hydraulic hand press
31. Single-punch press (Super line, Mitsubishi Electric Automation Company LTD., Thailand)

2.2 Methods

2.2.1 Amylose content determination

The amylose content of native rice starch was determined using the iodine spectrophotometric method according to Juliano (1971). Starch (0.05 g) was placed in a beaker, and 1 ml of ethanol was added. The beaker was shaken and 9 ml of 1M NaOH was introduced into suspension. The suspension was heated in a boiling water bath for 10 min. The solution was transferred to a 100 ml volumetric flask and mark with distilled water with careful rinsing of the beaker. An aliquot of the test starch solution (5 ml) was pipetted into 100 ml volumetric flask. Then, 1.0 ml of 1M acetic acid was added followed by 2 ml of iodine reagent. The solution was diluted to 100 ml with distilled water and shaken. The absorbance was read at 620 nm using a UV-Visible spectrophotometer (Shimadzu UV-2450) after the solution was left at room temperature for 20 min. The measurement was carried out in triplicates.

2.2.2 Preparation of cross-linked carboxymethyl rice starch (MRS)

The cross-linked carboxymethyl rice starch (MRS, chemical structure is shown in Figure 2.1) was prepared from native rice starch (RS) *via* dual-reaction with monochloroacetic acid (MCA) and epichlorohydrin (ECH) as carboxymethylating and cross-linking agents, respectively. The reaction was preceded under alkaline condition with the presence of sodium hydroxide. The different types of organic solvent, methanol and 2-propanol, were utilized as reaction mediums, and reaction yielded the 2 groups of MRSs (MRS-M and MRS-I, respectively) (shown in Table 2.1).

The MRS-M was synthesized according to the procedure described by Kittipongpatana et al. (2006) with slight modifications. Monochloroacetic acid (40 g)

was dissolved in 290 ml of methanol in a three-neck round bottom flask. Then, while stirring, 138 g of RS was added into the solution. The solution of sodium hydroxide was introduced into the mixture, followed by the various amounts of ECH (0, 0.5, 1, 3, 10, and 15 g per 100 g starch), respectively. The mixture was heated to 70°C and continuously stirred at this temperature for 1 h. At the end of reaction, the pH of mixture was neutralized by glacial acetic acid. The neutralized mixture was filtered using Buchner funnel to remove the supernatant. The powder product was washed several times with 70-90% double-distilled methanol to remove undesirable by products. The final product was finally washed with 100% methanol and dried in an oven at 50°C for 18 h. The obtained modified starch was passed through sieve no.60 and kept in a tightly close container.

The MRS-I was prepared according to the method described by Filbert and Woodbury (1950). First, in the three-neck round bottom flask, 29.5 g of MCA was dissolved in 437.5 g of 2-propanol. The RS (115.5 g) was introduced while the solution was stirring. The sodium hydroxide solution was added into the mixture. Then, the several amounts of ECH (0, 0.1, 1, 3, 7.5, and 15 g per 100 g starch) was added following. The mixture was heated to 81°C and constantly agitated for 30 min. The further step of procedure was similar as synthesis of MRS-M detailed above.

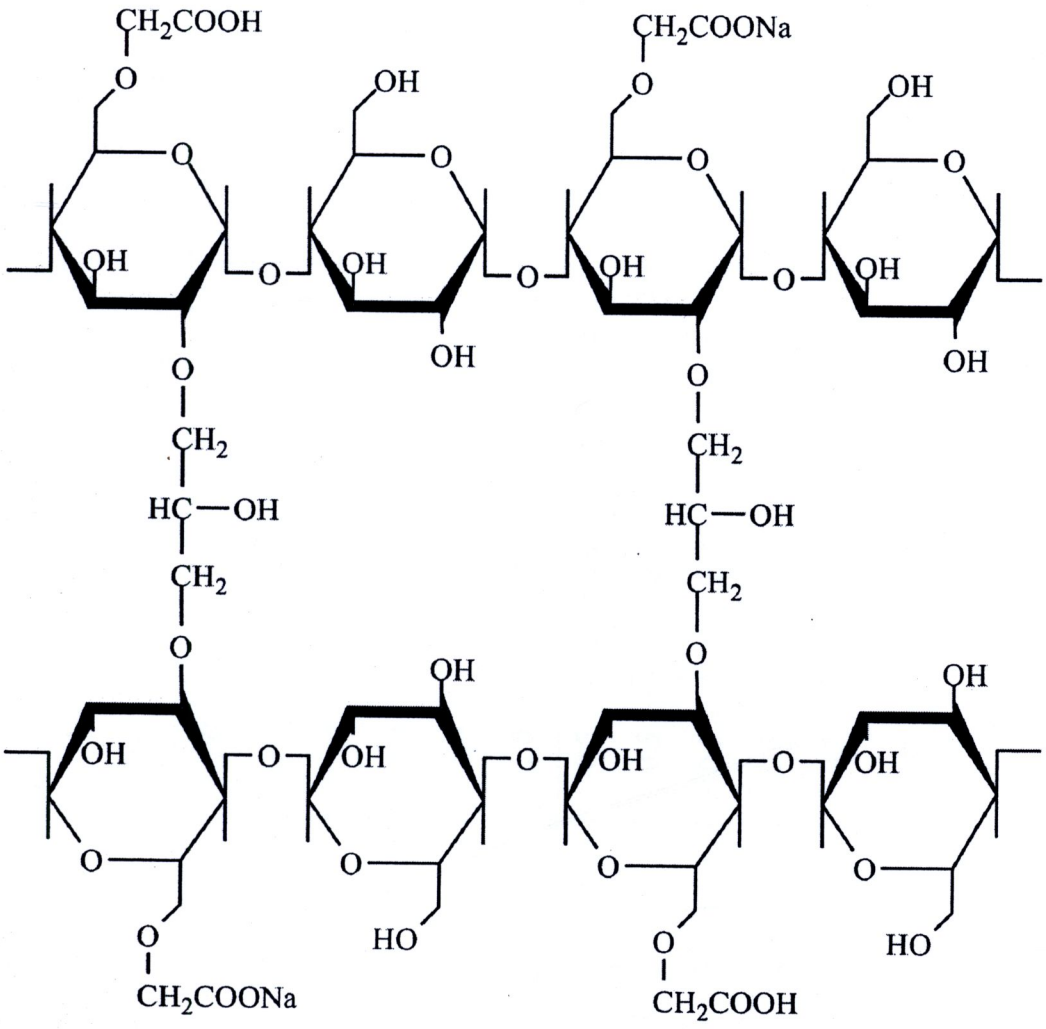

Figure 2.1 Chemical structure of cross-linked carboxymethyl rice starch (MRS)

Table 2.1 The compilation of reaction conditions used for synthesis of cross-linked carboxymethyl rice starch

Sample	Type of solvent	Solvent content (g)	Starch (g)	T (°C)	Time (h)	ECH (g / 100 g starch)	n_{NaOH}/ $n_{AGU(s)}$	n_{MCA}/ $n_{AGU(s)}$
MRS-M-01	Methanol	290.0	138.0	70	1.0	0.0	1.17	0.40
MRS-M-02	Methanol	290.0	138.0	70	1.0	0.5	1.17	0.40
MRS-M-03	Methanol	290.0	138.0	70	1.0	1.0	1.17	0.40
MRS-M-04	Methanol	290.0	138.0	70	1.0	3.0	1.17	0.40
MRS-M-05	Methanol	290.0	138.0	70	1.0	10.0	1.17	0.40
MRS-M-06	Methanol	290.0	138.0	70	1.0	15.0	1.17	0.40
MRS-I-01	2-Propanol	437.5	115.5	81	0.5	0.0	1.04	0.36
MRS-I-02	2-Propanol	437.5	115.5	81	0.5	0.1	1.04	0.36
MRS-I-03	2-Propanol	437.5	115.5	81	0.5	1.0	1.04	0.36
MRS-I-04	2-Propanol	437.5	115.5	81	0.5	3.0	1.04	0.36
MRS-I-05	2-Propanol	437.5	115.5	81	0.5	7.5	1.04	0.36
MRS-I-06	2-Propanol	437.5	115.5	81	0.5	15.0	1.04	0.36

2.2.3 Determination of degree of substitution (DS)

The degree of substitution (DS) of modified starch is the average number of hydroxyl group substituted with carboxymethyl and sodium carboxymethyl group in starch structure at C6. The degree of substitution was determined using the USP XXXII method described for croscarmellose sodium. The method contained two steps; titration and residue on ignition. The DS can be calculated by Equation (2.1):

$$DS = A + S \tag{2.1}$$

The degree of acid carboxymethyl substitution (A) can be calculated using Equation (2.2):

$$A = 1150M / (7120 - 412M - 80\,C) \tag{2.2}$$

The degree of sodium carboxymethyl substitution (S) can be calculated using Equation (2.3):

$$S = (162 + 58A)\,C / (7120 - 80\,C) \tag{2.3}$$

Where M is the net number of milliequivalent of base required for the neutralization of 1 g carboxymethyl starch (CMS) as determine in titration testing; C is the percentage of residue on ignition of CMS as determine in residue on ignition testing.

2.2.3.1 Titration

About 1 g of CMS, accurately weighed, was added to a 500-ml Erlenmeyer flask. Then, 300 ml of sodium chloride solution (10% w/v) was introduced. The stopper was inserted and the mixture was left for 5 min with intermittent shaking. Five drops of m-cresol purple TS and 15 ml of 0.1 N hydrochloric solution were added into the mixture, and then the mixture was shaken. When the mixture was still purple, 0.1 N hydrochloric solution in 1 ml portions was added until the solution became yellow. Then, the mixture was back titrated with 0.1 N NaOH solution until the mixture turn to purple at the endpoint. The net number of milliequivalent of base required for the neutralization of 1 g CMS (M) was calculated using Equation (2.4):

$$M\,(mEq) = mmole \times Valence \tag{2.4}$$

Where m is 10^{-3}; mole is mass in grams per molecular weight of sodium hydroxide and valence of sodium hydroxide is 1.

2.2.3.2 Residue on ignition

The ceramic crucible was placed in the oven at 100 °C for 1 h and kept in desiccator for constant weight. About 1 g of CMS was added into a weighed crucible. The crucible containing CMS was ignited at 400 °C for 1 h to obtain the black residue. About 1 ml of sulfuric acid was quietly dropped into the entire black residue. The moisten residue was heated until the complete of volatile of white fumes. The crucible containing residue was ignited at 800±25 °C for 3.5 h to obtain the white residue. Thereafter, the crucible was placed in desiccator for 30 min and accurately weighed. Percentage of residue on ignition was calculated using Equation (2.5):

$$C = (\text{weight of residue} / \text{weight of CMS})\ 100 \qquad (2.5)$$

2.2.4 Solubility in water, swellability and degree of cross-linking (DC)

The solubility and swellability of RS and MRSs in unheated water was determined. Sample (0.2 g) was dispersed in 20 ml of water (1% w/v) and left at room temperature for 24 h. The solubilization and/or swelling of samples were achieved.

In literatures, degree of cross-linking (DC) of cross-linked starch with ECH could be calculated from un-reacted ECH in reaction filtrate (Hamerstrand et al., 1960), and unbound glycerol and glycerol monoether (De Miguel et al., 1999). However, in this paper, the methods listed above were not suitable to measure DC due to there was two competitive reactions (carboxymethylation and cross-linking) in CMRS's manufacture. Chatakanonda et al. (2000) had studied cross-linking of rice starch and they investigated DC from change of viscosity. Therefore, in this research, DC of CMRS was measured according to Chatakanonda et al. (2000) with some modifications. Change of swelling volume of CMRS was used to calculate DC because swelling power was one of the properties that affected by starch cross-linking. Increasing of cross-linking level resulted in decreasing of swelling (Kittipongpatana et al., 2010; Silva et al., 2006). The Equation was provided below.

$$DC = \frac{S_0 - S_{MRS}}{S_0} \qquad (2.6)$$

Where S_0 is the volume of 1% w/v the control sample (carboxymethyl starch without

cross-linking, 0% ECH) and S_{MRS} is swelling volume of the cross-linked carboxymethyl starch.

2.2.5 pH of solution and clarity of paste

RS and MRSs solutions (1% w/v) were prepared to determine the pH of solution using a waterproof pH meter (Eutech Instruments, USA). Then, these solutions were heat up to 90°C for 30 min and cooled to 25°C for 1 h. Percent light transmission (%T) of solution was measured at λ=650 nm in a UV-Visible spectrophotometer (Shimadzu UV-2450).

2.2.6 Infrared spectroscopy (IR)

Infrared (IR) spectra were run on Fourier-Transformed Infrared Spectrophotometer (Nicolet Nexus 470FT-IR) using KBr disc technique. Pellets were made from native and modified rice starch samples (about 2 mg) with KBr. Transmission was measured at the wavenumber range of 4000–400 cm^{-1}. The substitution reaction was confirmed by the presence of the carbonyl (COO–) group at the peak of wavenumber 1600 to 1700 cm^{-1} in the IR spectra.

2.2.7 Morphology

The scanning electron microscopy (SEM) was studied to analyze the morphology of RS and its derivatives. Morphological investigation of sample was performed using a scanning electron microscope (JSM-5910LV, JEOL, USA). The sample was placed on copper stub tabbed with carbon tape and coated with gold. The sample observed using an accelerating voltage of 15 kV with 3000x.

2.2.8 X-ray diffraction (XRD)

X-ray diffraction patterns of RS and its derivatives were recorded with an X-ray diffractometer (X'Pert MPD, Philips, Germany). The samples were scanned at the angle (2θ) of 5 to 60° with a scanning rate of 2° min^{-1}.

2.2.9 Differential scanning calorimetry (DSC)

Thermal properties of RS and MRSs were studied by Perkin Elmer differential scanning calorimeter (DSC). The sample was weighted about 2.5-5.0 mg and placed in a sealed type aluminum pan. Distilled water (10 µl) was added with a

micropipette to sample in the pan. The pan was sealed, reweighed and allowed to stand overnight at room temperature before DSC analysis. Three replicates were heated from 30 to 120° C at a heating rate of 10° C min^{-1}. After measurement, peak temperature or gelatinization (T_p), onset temperature (T_o) and enthalpy of gelatinization (ΔH) in DSC thermograph were determined.

2.2.10 Water uptake

The water uptake of RS and MRSs was measured in triplicate using Nogami's apparatus with slight modification (Anutrakulchai, 2010; Nogami et al., 1969). First, about 0.5 g of sample was placed in a stainless steel tube sample holder which was supported with filter paper at the bottom side. Then, the tube holder containing sample was placed on a sinter glass filter which attached to a pipette by silicone tube filled with distilled water. The volume of sample water uptake was recorded suddenly at 0, 10, 20, 30, 40, 50, 60, 120, 150, 180, 210, 240, 270, 300 sec and 6, 7, 8, 9, 10, 15, 20, 25, 30, 40, 50, 60 min. Finally, the water uptake profiles of sample were plotted to determine their water absorption behaviors, when X axis was time and Y axis was volume of water uptake of sample.

2.2.11 Flowability

Angle of repose

Angle of repose (θ) is identified as the angle between the horizontal and the slope of a mound of granular material lowered from some marked elevation (Carr, 1965). The angle of repose of a bulk solid can be described with the Equation 2.7:

$$\text{Tan } \theta = h/r \qquad (2.7)$$

Where θ is repose angle (degree); h is height of a mound of samples; r is a radius of base of the mound. The lower repose angle of a material is meaning the more flowable the material. Higher angles (i.e., 50–60°) point material with hard flow, while a lower angle (i.e., 30–40°), indicates a material with relatively simple flow.

Bulk and tapped density determination

Sample (50 ml) was filled into a 100 ml-graduated cylinder. The cylinder was lift and dropped at 2-second intermission onto a hard surface for three times from

a height of 1 inch (Carr, 1965). The sample was measured in triplicates. The bulk density was obtained following Equation (2.8):

$$\text{Bulk density} = M/V_b \quad (2.8)$$

Where M is weight of sample (g); V_b is volume of the sample after experiment described above.

The cylinder containing sample was placed on the jolting volumeter (J. Engelsmann A.-G., Thailand). The tapping cycle was set at 500 times. Each sample was measured in triplication. When the process had done, the volume of sample (V_t) was recorded and also utilized to calculate the tapped density by Equation (2.9):

$$\text{Tapped density} = M/Vt \quad (2.9)$$

2.2.12 Pressure hardness profile

RS and MRSs tablets (flat, round, 13 mm diameter, 500 mg weight) were prepared using a hydraulic hand press by compressing for 3 s. Magnesium stearate suspension (2% w/v in an acetone) was used as a lubricant before each compression. For each condition of sample, tablets were compressed at different pressure levels (0.5, 1.0, 2.0 and 3.0 T). All tablets were stored in a desiccator for 24 h before hardness measurement.

2.2.13 Tablet preparation

To study the MRSs as tablet disintegrant, silicified microcrystalline cellulose (SMCC) tablets and aspirin tablets were prepared by direct compression method using single-punch press. Aspirin was chosen as a model drug because of its poor solubility. Weight of each SMCC tablet was 705.71 mg while there was 712.60 mg for aspirin tablet. In all condition, diameter of flat and round tablet was 13.0 mm. Several types of disintegrant used in this study were RS, swellable MRSs, and two commercial grade super-disintegrants; sodium starch glycolate (SSG) and croscarmellose sodium (CCS).

Blank tablets containing various types of disintegrant were prepared by compression of mixing powder of SMCC, disintegrant, purified talcum and magnesium stearate into flat and round tablets (shown in Table 2.2). For aspirin tablets, aspirin amount was constant as 500 mg in each tablet and SMCC was utilized as a diluent (shown in Table 2.3). Aspirin, SMCC, several amounts of disintegrants

(2, 3, 4, 5, and 6%), purified talcum and magnesium stearate were mixed and compressed into the tablets (shown in Table 2.4). Each component was screened through a sieve no. 40 before weight, and mixed in a polyethylene (PE) bag with tumbling technique.

Table 2.2 Formulations for blank tablet compressed by direct compression

Composition	Quantity (mg)/Tablet
SMCC	675.00
Disintegrant (2%)	13.50
Talcum (2%)	13.77
Mg. stearate (0.5%)	3.44

Table 2.3 Tablet Formulations for Aspirin with 2% disintegrants by direct compression

Composition	Quantity (mg)/Tablet
Aspirin	500.00
SMCC	175.00
Disintegrant (2%)	13.50
Talcum (3%)	20.66
Mg. stearate (0.5%)	3.44

Table 2.4 Composition of the aspirin tablet formulations with various amounts of disintegrant

Composition	Quantity (mg)/Tablet				
	Disintegrant concentration (%)				
	2	3	4	5	6
Aspirin	500.00	500.00	500.00	500.00	500.00
SMCC	175.00	168.25	161.50	154.75	148.00
Disintegrant	13.50	20.25	27.00	33.75	40.50
Talcum (3%)	20.66	20.66	20.66	20.66	20.66
Mg. stearate (0.5%)	3.44	3.44	3.44	3.44	3.44

2.2.14 Quality control of tablet

Weight variation and thickness

The weights of tablets (20 tablets) from each tablet formulation were measured on a digital balance (Scaltec, Germany). The average values and standard deviation were calculated. The thickness of tablets was measured using a micrometer.

Tablet friability

Tablet friability was determined using a friabilator (Pharma Test, Germany). Twenty tablets was preweighed (Wp) and rotated at 25 rpm for 4 min. Then, fine from tablets was removed and the tablets were reweighed (Wr). The percentage weight loss was calculated by Equation (2.10):

$$\text{Friability (\%)} = 100 * (Wp - Wr) / Wp \qquad (2.10)$$

Hardness

The hardness of tablets was measured using a digital hardness tester (Erweka, Germany).

2.2.15 Disintegration time (DT)

Disintegration time (DT) of tablets was experimented in distilled water as a medium in a disintegration test apparatus (Pharma Test, PTZ) according to USP. The temperature was controlled at 37±1°C during test. The time was run until no component from tablets was left on the mesh. Then, this time was recorded as the DT of experimental tablet. Six determinations of formula were carried out and then the average was calculated.

2.2.16 Wetting time and water absorption ratio (R) of tablet

Four circular tissue papers and a filter paper were placed in a petri-dish with 9 cm diameter. The eosin solution (0.05% w/v) was introduced in the petri dish to identify the complete wetting of the tablet surface. A tablet was placed on the surface of wet filter paper with eosin solution in the petri dish at room temperature (25±2°C). The time for solution wetted the tablets completely, solution reached up to tablet surface, was noted as the wetting time (Bi et al., 1996). The measurements were carried out in triplicates. The weight of tablet after (W_A) and before (W_B) water absorption was measured utilizing a digital balance. Water absorption ratio (R) was calculated according to the following Equation (2.11):

$$R = (W_A - W_B) / W_B \qquad (2.11)$$

2.2.17 Statistical Analysis

Analysis of variance (ANOVA) was used to compare mean differences of the samples. Duncan's multiple range test (DMRT; $p \leq 0.05$) was also used by SPSS software version 11.