CHAPTER 2

METERIALS AND METHODS

2.1 Materials : Chemicals

1. Mungbean starch – Sitthinan Co., Ltd., Thailand : TIS. 948-2533
2. Nimesulide, BP Micronised ($C_{13}H_{12}N_2O_5S$) – Unimark Remedies, Lot No. NMN-093495
3. Carbopol 940 (CP) – Hong huat, Lot No. KK5N4KC478
4. Hydroxypropylmethylcellulose (HPMC) – Colorcon, Lot No. TJ05012N73
5. Methylcellulose (MC) – Colorcon, Lot No. TA19012N22
6. Sodium carboxymethylcellulose (SCMC)
7. Monochloroacetic acid (MCA) – Fluka, Lot No. 1056856 44506410
8. Dichloroacetic acid (DCA), Synthesis grade – Merck, Lot No. 80215241 909
9. Methanol, Analytical grade (MeOH AR) – Labscan, Lot No. 08 08 1136
10. Methanol, Commercial grade (MeOH) – Fisher, Lot No. 0241689
11. Sodium chloride (NaCl) – Labscan, Lot No. 06 01 0135
12. Sodium hydroxide, Analytical grade (NaOH) – Labscan, Lot No. 07 11 0139
13. Glacial acetic acid (CH_3COOH) – Merck, Lot No. K 30626863 220
14. Triethanolamine (TEA) – Unilab, Lot No. AH512191
15. Denatured alcohol (DEB 96) – Hong huat, Lot No. 210993-A
16. Hydrochloric acid (HCl) – Labscan, Lot No. 08 04 0066
17. Sulfuric acid 96% (H_2SO_4) – Labscan, Lot No. 08 07 1184
18. Pure amylose from potato
19. 1%w/v Silver nitrate solution (1%w/v $AgNO_3$)
20. Methyl paraben and Propyl paraben
21. Absolute ethanol (Abs. EtOH) – Merck, Lot No. K 38076483 748
22. Dimethyl sulfoxide (DMSO, C_2H_5OS)
23. Potassium iodide (KI) and Iodine (I_2)

24. Potassiumdihydrogenphosphate / Monobasic Potassium phosphate (KH_2PO_4)
25. *m*-cresol purple TS
26. Propane-1,2-diol (Propylene glycol)
27. Potassium biphthalate ($KHC_6H_4(COO)_2$), Potassium chloride (KCl),
28. Potassium bromide (KBr), FT-IR grade
29. Boric acid (H_3BO_3)
30. Purified water

2.2 Materials : Equipments and Apparatus

1. UV/VIS Spectrophotometer – Shimadzu™ UV–2450, Japan
2. UV/VIS Spectrophotometer – Jasco™ V–530, USA
3. Fourier Transform Infrared Spectrophotometer (FT–IR) – Nicolet Nexus™ 470 FT-IR, USA
4. Scanning Electron Microscopy (SEM) – JEOL™ JSM-5910LV, France
5. X–ray Diffractometer (XRD) – Phillip™ X'Pert MPD X–ray diffractometer, Germany
6. Rotational Viscometer – Brookfield Engineering Laboratories™ R/S-CPS Rheometer : Plate-to-Plate, USA
7. Moisture analyzer – Sartorius™ MA50, Germany
8. Magnetic hot plate stirrer – Heidolph™ MR Hei-Standard, Germany
9. Vortex mixer
10. Analytical balance – Sartorius™ LA Model, Germany
11. Electronic balance – Scout™ Pro SPS402F, USA
12. Hot air oven – Binder™ model ED 240/E2, Germany
13. High temperature stove and Electronic stove
14. Muffle furnace
15. Solvent distillation apparatus
16. Vacuum pump – Medi–pump™ model 1132D
17. pH meter – pHScan™ WP2, Eutech Instruments, USA
18. Universal indicator – Advantec™ Grade Whole Range pH 0–14, Japan, Lot No. 71218012
19. Three–necked round–bottomed flask
20. Bucher funnel and Suction flask 2000 mL.

21. Thermometer 0–100°C
22. Porcelain crucible Desiccator
23. Regenerated cellulose membrane – Spectra/Por™ 7, Spectrum Laboratories Inc., USA (Molecular weight cut off ; MWCO 1000 dalton)
24. Franz diffusion apparatus
25. Titration apparatus – burettes, pipettes, stands and clamps
26. Whatman filter No.1, 150 mm. diameter (Ø) – Whatman™, England
27. Glass tubes and racks, Mortar and pestle
28. Beakers, Erlenmeyer flasks, Volumetric flasks and Cylinders
29. Aluminum trays and plastic spatulas
30. Micropipette 1 mL and 10 mL
31. Water bath
32. Sieves No. 60 and 80
33. Quartz cuvette

2.3 Methods

2.3.1 Amylose content

The amylose content of native MB was determined by the colorimetric method, as described by Juliano (1971). The experiment was calculated as the average of a triplicate determination.

1) Amylose standard curve

An amount of 40.0 mg of pure amylose from potato (amylose standard) was accurately weighed into a 100-mL volumetric flask, then 1.0 mL of 95% ethanol (EtOH) was added and gently swirled to dissolve the amylose standard. The 9.0 mL of 1.0 N sodium hydroxide (NaOH) was added into the amylose standard solution. The solution was boiled in water bath at 100°C for 10 min. When it was cooled down, adjusted to volume with purified water.

Pipette 1, 2, 3, 4 and 5 mL aliquots of the amylose standard solution was transferred into five 100-mL volumetric flasks. Then 0.2, 0.4, 0.6, 0.8 and 1.0 mL of 1.0 N acetic acid (CH_3COOH) was added into the aliquot portions, respectively. Afterwards, 2.0 mL of the iodine and potassium iodide (KI/I_2) TS2 solution was added and adjusted to volume with purified water. The amylose standard solution

was allowed to stand at room temperature for 20 min. Remark : KI/I_2 TS2 solution (USP XXXII) – Dissolved 12.7 g of iodine (I_2) and 20 g of potassium iodide (KI) in purified water, and diluted to 1000.0 mL with purified water. Pipette 10.0 mL of the KI/I_2 stock solution transferred to 100-mL volumetric flask, added 0.6 g of KI and diluted with purified water to volume. The KI/I_2 TS2 solution was prepared immediately before use.

Absorbance (A) of the amylose standard solution was detected by UV/VIS spectrophotometer (Shimadzu™ UV-2450, Japan) at 620 nm against purified water (Blank solution). The amylose standard curve was exhibited the relation between the amylose standard concentration at 2, 4, 8, 12, 16 and 20 μg/mL and absorbance of amylose standard. Thereupon, the percent apparent amylose content of native MB was provided.

2) Sample determination

The procedure of sample determination was followed on above by replacing the pure amylose from potato with 0.1 g of native MB powder. The determination was carried out in triplicate.

2.3.2 Chemical starch modifications

The native mungbean starch was modified with MCA and/or DCA. The carboxymethyl group (-O-CH_2-CO-R) was subsequently submitted with MCA via the carboxymethylation reaction and DCA was a cross-linking agent.

1) Carboxymethyl modified mungbean starch modification

Carboxymethyl modified mungbean starch (CMMS or C-MB-100) was prepared using the methodology of Kittipongpatana et. al. (2006). MCA (40.0 mg) was dissolved in 1000-mL three-necked round-bottomed flask with 260.0 mL of MeOH and placed in the water bath to keep the temperature. Then, 138.0 g of native MB was dispersed in the MCA solution to be a suspension. Afterwards, the NaOH solution (50%w/w NaOH - 40 g of NaOH on 40 mL of purified water) was added slowly into the mixture. The slurry was stirred continuously, warmed up to 70±2°C with reflux condition, and held at this temperature for 60 min. At the end of reaction, the slurry was neutralized to pH 7.0 with glacial acetic acid in order to stop the reaction. The modified starch preparation was collected by suction filtration with whatman No.1 filter paper, washed with 70-90% double distilled MeOH to remove

the salts and by products. The positive test was non precipitate that detected in the filtrate with silver nitrate ($AgNO_3$) TS (0.1 N) solution, and then the modified MB was washed with MeOH AR grade in the last time. The CMMS was dried at 50°C for 24 h in hot air oven, sieved through mesh #80. The finally CMMS was colleted in an air tight container. Remark : 0.1 N of $AgNO_3$ TS solution (USP XXXII) – Dissolved about 17.5 g of $AgNO_3$ in purified water, and then diluted to 1000.0 mL with purified water.

2) Cross-linked modified mungbean starch modification

Cross-linked modified mungbean starch (L-MB-100 or Cross-linked MB) was prepared by replacing MCA with dichloroacetic acid (DCA) with the procedure described by Christoph et. al. (2007). Native MB (138.0 g) was dispersed in methanol (200 mL) in 1000-mL three-necked round-bottomed flask. The slurry was stirred continuously, placed in the water bath to keep the temperature and mixed for 30 min to be suspension. Afterwards, a NaOH solution (50%w/w NaOH - 40 g of NaOH on 40 mL of purified water) was added slowly into the mixture for 30 min. DCA (54.58 g) was added into the slurry, rinsed with 60.0 mL of MeOH. The slurry was stirred continuously, warmed up to 70±2°C with reflux condition, and held at this temperature for 60 min. At the end of reaction, the slurry was neutralized to pH 7.0 with glacial acetic acid in order to stop the reaction. The modified starch preparation was collected by suction filtration with whatman No.1 filter paper, washed with 70-90% double distilled MeOH to remove the salts and by products. The positive test was non precipitate that detected in the filtrate with $AgNO_3$ TS solution (0.1 N), and then the modified MB was washed with MeOH AR grade in the last time. The cross-linked MB was dried at 50°C for 24 h in hot air oven, sieved through mesh #80. The finally cross-linked MB was colleted in an air tight container. Remark : 0.1 N of $AgNO_3$ TS solution (USP XXXII) – Dissolved about 17.5 g of $AgNO_3$ in purified water, and then diluted to 1000.0 mL with purified water.

3) Cross-linked carboxymethyl modified mungbean starch modification

Seven condition of cross-linked carboxymethyl modified mungbean starches (CL-MBs) were prepared as a single step reactions, as described by Kittipongpatana et. al. (2006) and Christoph et. al. (2007). Carboxymethylation and

cross-linking reaction were carried out through a reaction between native MB and MCA, with the addition of 1-10% DCA as a cross-linking agent under alkaline condition (Figure 2.1). Native MB (138.0 g) was dispersed in MeOH (200 mL) in 1000-mL three-necked round-bottomed flask. The slurry was stirred continuously, placed in the water bath to keep the temperature and mixed for 30 min to be suspension. Afterwards, a NaOH solution (50%w/w NaOH - 40 g of NaOH on 40 mL of purified water) was added slowly into the mixture for 30 min. MCA (40 g) was dissolved in 30 mL of MeOH to be the solution. The MCA solution and DCA (1-10% of weight of native MB) was added into the slurry, rinsed with 30.0 mL of MeOH. The slurry was stirred continuously, warmed up to 70±2°C with reflux condition, and held at this temperature for 60 min. At the end of reaction, the slurry was neutralized to pH 7.0 with glacial acetic acid in order to stop the reaction. The modified starch preparation was collected by suction filtration with whatman No.1 filter paper, washed with 70-90% double distilled MeOH to remove the salts and by products. The positive test was non precipitate that detected in the filtrate with $AgNO_3$ TS solution (0.1 N), and then the modified MB was washed with MeOH AR grade in the last time. The cross-linked MB was dried at 50°C for 24 h in hot air oven, sieved through mesh #80. The finally CL-MBs was colleted in an air tight container. Remark : 0.1 N of $AgNO_3$ TS solution (USP XXXII) – Dissolved about 17.5 g of $AgNO_3$ in purified water, and then diluted to 1000.0 mL with purified water.

The synthetic condition of starch modification and starch modification apparatus diagram were shown in Table 2.1 and Figure 2.2, respectively.

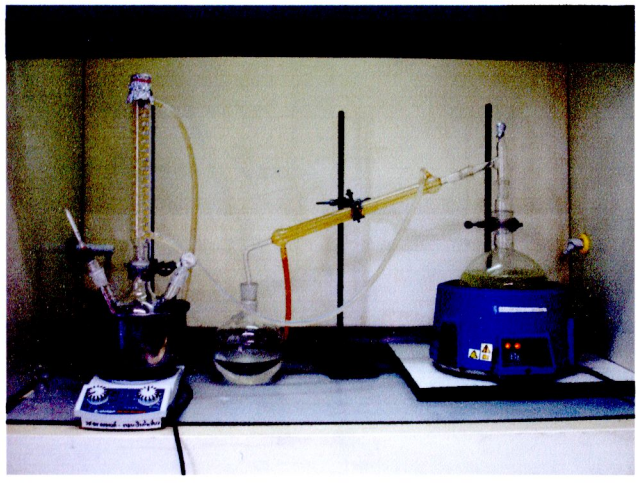

Figure 2.1 Methanol distillation apparatus and Starch modification apparatus

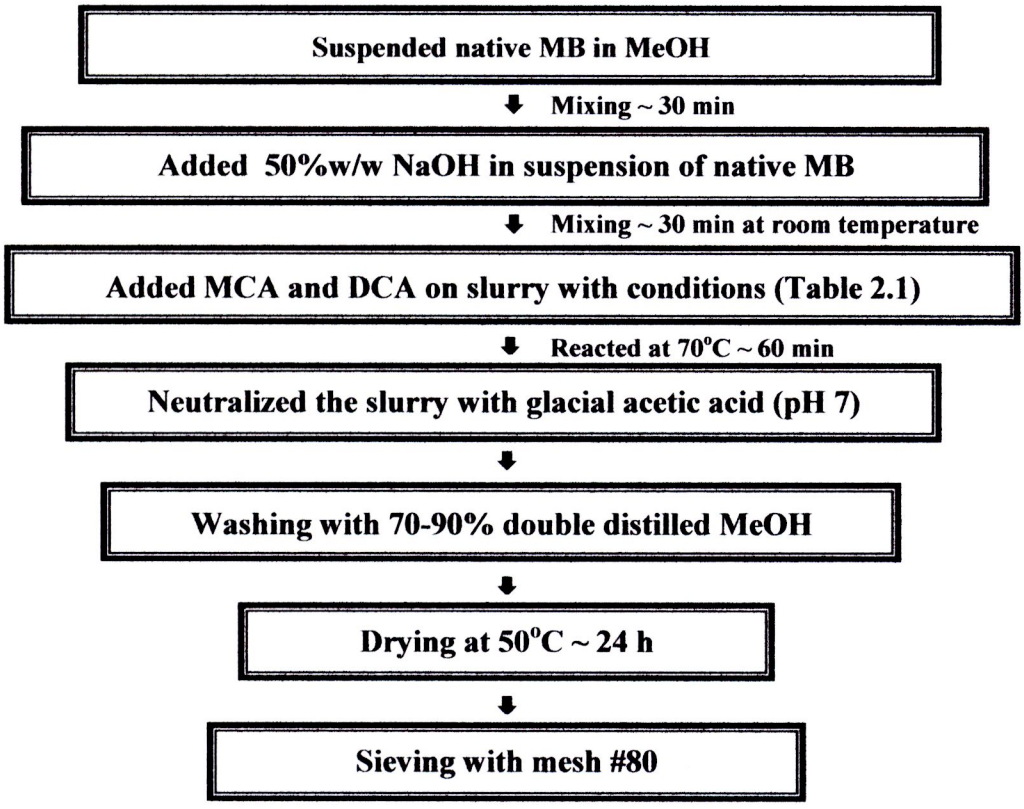

Figure 2.2 Cross-linked carboxymethyl modified mungbean starch modification diagram

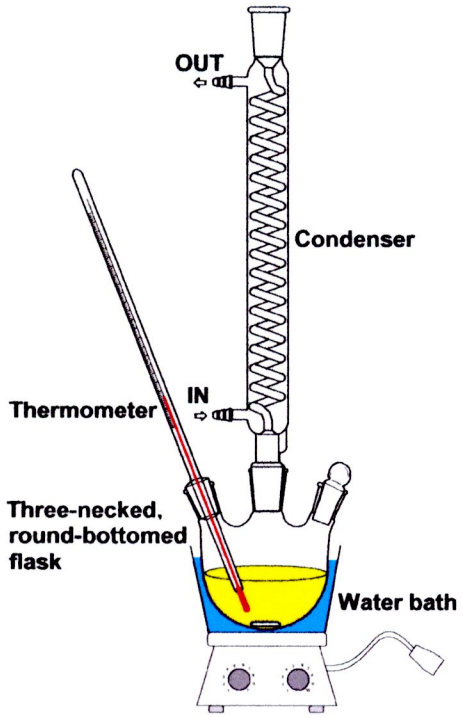

Figure 2.3 Starch modification apparatus diagram

Table 2.1 Condition for carboxymethylation and/or cross-linking of mungbean starch

Type of modified starches	Native MB (g)	MeOH (mL)	50%w/w NaOH (g)	MCA (g)	DCA (g)
C-MB-100	138	260	80	40	-
L-MB-100	138	260	80	-	54.58
CL-MB-1	138	260	80	40	1.38
CL-MB-4	138	260	80	40	5.52
CL-MB-5	138	260	80	40	6.90
CL-MB-7	138	260	80	40	9.66
CL-MB-8	138	260	80	40	11.04
CL-MB-9	138	260	80	40	12.42
CL-MB-10	138	260	80	40	13.80

2.3.3 Modified starch identifications

1) Determination of the degree of substitution

The degree of substitution (DS) is the amount of carboxymethyl groups (-O-CH_2-CO-R), contained in the carboxymethyl starch network that substituted hydroxyl groups (-OH). The DS of modified starches were determined using a USP XXXII method, as described for sodium croscarmellose. The method consisted of titration, with a residue left on ignition. The DS value was calculated as the averaged of a triplicate determination, following a equation 1.

$$DS = A + S \qquad \text{——— ❶}$$

Calculate the degree of acid carboxymethyl substitution (A) by the formula 2

$$A = \frac{1150M}{7102 - 412M - 80C} \qquad \text{——— ❷}$$

Calculate the degree of sodium carboxymethyl substitution (S), by the formula 3

$$S = \frac{(162 + 58A)C}{7102 - 80C} \qquad \text{——— ❸}$$

in which *M* is the net number of milliequivalents (mEq) of base required for the neutralization of 1 g of carboxymethyl starch, on the dried basis. *C* is the percentage of residue on ignition of carboxymethyl starch.

1.1) Titration step

An amount of 1.0 mg of modified starch was accurately weighed into a 500-mL erlenmeyer flask, added 300.0 mL of 10%w/v NaCl solution was added. Afterwards, the 25.0 mL of NaOH (0.1 N) was added into the mixture. The flask was enclosed by the stopper and allowed to stand for 5 min with intermittent shaking, and 5 drops of *m*-cresol purple TS was added. Then, the 15.0 mL of HCl (0.1 N) was added from a burette, enclosed the flask by the stopper and shaken after each addition. A yellow color was produced after the HCl was added. If the solution was violet, added 1-mL portions of HCl (0.1 N) until the solution became yellow. The yellow solution of sample was titrated with NaOH (0.1 N), mixing the solution by shaking until a violet color endpoint was produced (Figure 2.4). Calculate the net number of milliequivalents (*M*) of base required for the neutralization of 1 g of carboxymethyl starch, on the dried basis.

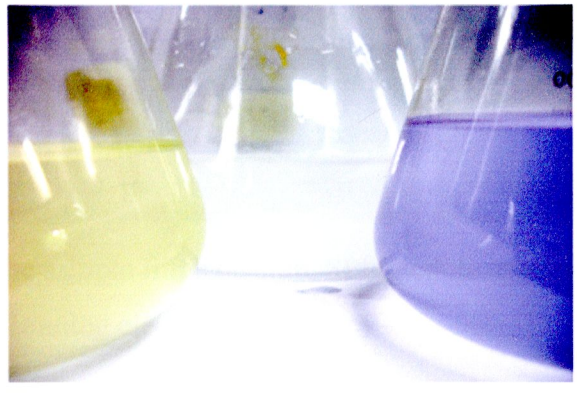

Figure 2.4 Titration step with a violet color endpoint

1.2) Residue on ignition

The porcelain crucible was cleaned and ignited at 180±25°C for 2 h, cooled down the crucible in a desiccator that silica gel was used as desiccant, and weighted it accurately. An amount of 1.0 g of modified starch was weighed into the crucible which accurately weigh. The modified starch became a black residue after it was ignited in a muffle furnace at 300±25°C for 1 h. The residue was moistened with a small amount (1.0 mL) of sulfuric acid (H_2SO_4), and heated gently on stove in a

well-ventilated hood until white fume were no longer evolved. The residue was repeated the moistening with 1-mL portions of H_2SO_4 if the white fume were evolved. These was ignited again at 800±25°C for 1 h until it was completely incinerated with a white residue, cooled down the crucible in a desiccator, and weighed it accurately. Calculate the percentage of residue on ignition of carboxymethyl starch (*C*). From these two steps, the DS value is the sum of $A + S$, followed the equation 1.

2) <u>Scanning electron microscopy (SEM)</u>

The morphology of the starch powder granules, and the surface and shape of the CL-MBs were analyzed using SEM-JEOL™ JSM-5910LV, France, to compare against with CMMS, cross-linked MB and native MB. The granule of samples were sprinkled over a double-sided glued tape of carbon (a 20 nm of thick coal layer) and mounted on a plastic stub. Afterwards, the sample films were coated with a 40-50 nm thickness gold layer under a vacuum state by SPI-Module sputter coater in order to provide conductivity (Figure 2.5). These were analyzed using an accelerating voltage of 15 kV under a high vacuum mode at a 750X magnification.

Figure 2.5 The sample films on stub, SPI-Module sputter coater and Scanning electron microscopy

3) <u>Fourier-transformed infrared (FT-IR) spectroscopy</u>

The FT-IR measurements were performed in solid state with a Nicolet Nexus™ 470 FT-IR Spectrophotometer, USA by using KBr disc technique. The spectra were obtained at the resolution of 4 cm^{-1}. The 256 scans were averaged for each measurement. The measurement was acquired against a KBr disc cell as background in the region of 4000-400 cm^{-1}. In the FT-IR spectra, the substitution reaction of carbonyl group (-C=O-) was confirmed by a strong sharp peak at wave number 1600–1700 cm^{-1}.

For the KBr disc technique, all starch powders and potassium bromide (KBr), FT-IR grade were dried at 105°C for 24 h before tableting. The starch powder (1 mg) was grinded to be fine powder with approximately 200 mg dry KBr, FT-IR grade with an agate mortar and pestle until the starch sample was well dispersed and the mixture had the consistency of fine flour and must be homogenously dispersed. (theoretically < 2 microns). The mixture powder was transferred to a suitable die and press. The die was attached to a vacuum pump, allowed to stand for 1 min under vacuum state and compressed the mixture powder at 10 tons/inch^2 for 3-5 min. Afterwards, the pressure was released and the starch sample disc was extracted for insertion into the cell holder of the FT-IR spectrophotometer.

4) X-ray diffraction (XRD)

The X-ray diffractograms (XRD) of the starch powders were obtained with Phillip™ X'Pert MPD X-ray diffractometer, Germany. The XRD was analyzed at a Bragg angle (2θ) of 5 to 60° and a scan rate of 5.0°/min.

5) Cold-water solubility

The verification that CMMS, Cross-linked MB and CL-MBs were soluble in cold-water, were carried out by adding 0.1 g of modified starch powder into 10 mL of distillated water (1%w/v). Afterwards, these were mixed with vortex mixer and the solubility of modified starch powders were observed by comparing it with native mungbean starch.

2.3.4 Physicochemical properties study

The physicochemical properties consisted of moisture content, pH, free swelling capacity (FSC), clarity and viscosity of seven condition of cross-linked carboxymethyl modified mungbean starch (CL-MBs) were studied. The experiments was measured against CMMS, cross-linked MB and four commercial polymers as carbopol 940 (CP), hydroxypropylmethylcellulose (HPMC), methylcellulose (MC) and sodium carboxymethylcellulose (SCMC).

1) Moisture content

The moisture content was determined by loss on drying with moisture analyzer (Sartorius™ MA50, Germany) The method was modified from <731> loss on drying (USP XXXII). An amount of 1-2 g of finely starch powder was accurately weighed into an aluminum tray. The starch powder was equated to 5 mm thickness

and heated at 105±2°C. The experiment was calculated as the average of a triplicate determination.

2) Free swelling capacity (FSC)

Free swelling capacity (FSC) was determined by using the teabag method, as described by Christoph et.al. (2007). An amount of 0.2-0.4 g of dry starch powder was accurately weighed (m_s) into a dry teabag that was exactly weighed (m_{tb}). The bag was locked with a thread and put into a beaker that the excess of water at room temperature. The bag was taken out of the water after 15, 30, 60, 120, 180 and 300 min. These was hanged by a thread for 30 min in order to release the superfluous water. Afterwards, the total mass of the teabag with hydrogel was weighed (m_w). The FSC value was calculated as the averaged of a triplicate determination, following a equation 4.

$$q_{FSC} = \frac{m_t - m_{tb} - m_w}{m_s} \qquad \text{———} \ ④$$

with m_t = the total mass of the teabag with hydrogel.

m_{tb} = the mass of the empty, dry teabag.

m_w = the mass of the water, absorbed by the empty, wet teabag.

m_s = the mass of the dry starch or polymer sample.

Remark : The m_{tb}, m_w and m_s valves were determined before starting the swelling procedure.

3) pH of solution

The modified starch powder was dissolved in water at 1, 3, 5%w/v. These solutions were determined by using a pH meter-pHScan™ WP2, Eutech Instruments, USA. The pH value was calculated as the averaged of a triplicate determination.

4) Clarity

The samples were prepared in the same fashion as those in pH of the solution test. The clarity of the gels was determined using Jasco™ V-530, USA, in 620 nm of a distilled water blank. The clarity value was calculated as the averaged of a triplicate determination.

5) Viscosity

The samples were prepared in the same fashion as those in the clarity test. The viscosity of gels was determined using a Brookfield Engineering Laboratories™ R/S-CPS rheometer, USA, with a plate-to-plate format. The measuring system was SP25 with the controlled shear rate mode (CSR) at set at 25±1°C. The viscosity of the gels was taken from the average obtained after a triplicate determination. Brookfield Rheo 2000 software was used to analyze all data and viscosity was reported with Pa.s. The viscosity of gels was measured by 3-step method:

Step 1 :	Step time	60 sec
	#MP	10
	Start value	0 D[1/s]
	End value	100 D[1/s]
Step 2 :	Step time	60 sec
	#MP	15
	Start value	100 D[1/s]
	End value	100 D[1/s]
Step 3 :	Step time	60 sec
	#MP	10
	Start value	100 D[1/s]
	End value	0 D[1/s]

The viscosity was analyzed at shear rate 100 s^{-1}.

The samples were dissolved in distillated water at 1, 3 and 5%w/v and dissolved in the buffer solution at pH 2-10 at 1%w/v.

Remark : The buffer solution (pH 2-14) was described by USP XXXII.

0.2 M Potassium dihydrogenphosphate solution (KH_2PO_4) : 27.22 g of KH_2PO_4 was dissolved in distillated water and adjusted to 1000 mL.

0.2 M Potassium biphthalate ($KHC_6H_4(COO)_2$) solution :

40.85 g of $KHC_6H_4(COO)_2$ was dissolved in distillated water and adjusted to 1000 mL.

0.2 M the mixture of Potassium chloride (KCl) and Boric acid (H_3BO_3) solution : 12.37 g of H_3BO_3 and 14.91 g of KCl were dissolved in distillated water and adjusted to 1000 mL.

0.2 M Potassium chloride (KCl) solution : 14.91 g of KCl was dissolved in distillated water and adjusted to 1000 mL.

1. *Acid Phthalate Buffer*

Pipette 50.0 mL of $KHC_6H_4(COO)_2$ (0.2 M) solution was transferred into 200-mL volumetric flask. Then, the specified volume of HCl (0.2 M) was added on and adjusted to volume with purified water.

pH = 2.4	0.2 M of HCl	=	42.2 mL
= 3.4		=	10.4 mL

2. *Neutralized Phthalate Buffer*

Pipette 50.0 mL of $KHC_6H_4(COO)_2$ (0.2 M) solution was transferred into 200-mL volumetric flask. Then, the specified volume of NaOH (0.2 M) was added on and adjusted to volume with purified water.

pH = 4.4	0.2 M of NaOH	=	6.6 mL
= 5.4		=	34.1 mL

3. *Phosphate Buffer*

Pipette 50.0 mL of KH_2PO_4 (0.2 M) solution was transferred into 200-mL volumetric flask. Then, the specified volume of NaOH (0.2 M) was added on and adjusted to volume with purified water.

pH = 6.4	0.2 M of NaOH	=	11.6 mL
= 7.0		=	29.1 mL
= 7.4		=	39.1 mL
= 8.0		=	46.1 mL

4. *Alkaline Borate Buffer*

Pipette 50.0 mL of 0.2 M the mixture of KCl and H_3BO_3 solution was transferred into 200-mL volumetric flask. Then, the specified volume of NaOH (0.2 M) was added on and adjusted to volume with purified water.

pH = 9.4	0.2 M of NaOH	=	32.1 mL
= 10.0		=	43.7 mL

6) Stability test

The revolution of viscosity, pH and clarity was studied at the stability conditions. The conditions were quoted from British Pharmaceutical codex (1996). All polymer samples were experimented at accelerating condition, included 8°C and 45°C for 3 months, and underwent a heating-cooling (HC) stability (8 cycles–each cycle consisted of 2 days at 8°C and another 2 days at 45°C). The facts were compared with the data before stability test (freshly preparation).

The gel degeneration was duplicated in the stability test with accelerating condition, described by British Pharmaceutical codex (1996):

- Low temperature : the gel segregation or precipitation could been taken place at the low temperature. In this study, the gel was stored at 8°C for 3 months.
- High temperature : the gel segregation or precipitation could been taken place at the high temperature. In this study, the gel was stored at 45°C for 3 months.
- High and low temperature : the HC cycle was a stress to the gels. In this study, the gel was stored at high and low temperature (8°C and 45°C) for 8 cycles (8 cycles–each cycle consisted of 2 days at 8°C and another 2 days at 45°C).

2.3.5 Alcohol tolerability

The study of alcohol tolerability was studied the changing of physico-chemical properties, included pH, clarity and viscosity of CL-MBs. The modified starch powder was dissolved in 3 media: EtOH:H_2O mix of ratio 0:100, 30:70 and 50:50, at 1, 3 and 5%w/v. The methods of study referred above mentioned. The experiments was determined against CMMS, cross-linked MB and four commercial polymers as CP, HPMC, MC and SCMC. The gels was stored at 8°C and 45°C for 3 months, and underwent a HC stability (8 cycles–each cycle consisted of 2 days at 8°C and another 2 days at 45°C). The facts were compared with the data before stability test (freshly preparation).

2.3.6 Cross-linked carboxymethyl modified mungbean choosing

From the physicochemical properties and alcohol tolerability, the three best cross-linked carboxymethyl modified mungbean starch (CL-MBs) were chosen to formulate nimesulide (NM) gel.

2.3.7 Nimesulide gel formulation

1) Definition

- *Polymer gel* : the polymer gel consist of a cross-linked polymer network inflated with a solvent such as water. They have the ability to reversibly swell or shrink without active ingredients and additives.

- *Gel formulation* : the polymer gel consist of a cross-linked polymer network inflated with a solvent such as water. The active ingredients and additives were added on.

- *Gel base* : the polymer gel consist of a cross-linked polymer network inflated with a solvent such as water, but without active ingredients.

- *Freshly prepared* : the gel base and gel formulation were formulate within 24 h.

- *3-month storage at 8°C* : the gel base and gel formulation were preserved in well-tight, light-resistant containers, and stored at 8±1°C for 3 months (British Pharmaceutical codex, 1996).

- *3-month storage at 45°C* : the gel base and gel formulation were preserved in well-tight, light-resistant containers, and stored at 45±1°C for 3 months (British Pharmaceutical codex, 1996).

- *Heating-cooling cycle (HC or FT)* : the gel base and gel formulation were preserved in well-tight, light-resistant containers, and stored at a HC stability for 8 cycles (8 cycles–each cycle consisted of 2 days at 8±1°C and another 2 days at 45±1°C) (British Pharmaceutical codex, 1996).

2) Nimesulide gel preparations

The preliminary batches were formulated by varying the concentration of gelling agent and other excipients. Nimesulide ($C_{13}H_{12}N_2O_5S$) were kept constant at 1%w/w, as an active ingredient. Each material was weighed accurately and added on the order by mixing. Three-chosen CL-MBs and commercial polymers (CP and SCMC) were used as a gelling agent in the formulation. The gelling agent was

dispersed in the distillated water at the concentration in the formula. The suspension was stirred and allowed to stand over night at room temperature in order to be completely swelling.

In the experiment, the gel formulation without active ingredient was called gel base. NM was used as an active ingredient in the gel formulation. Three-chosen and commercial polymers (CP and SCMC) were used as a gelling agent. Dimethyl sulfoxide (DMSO) and denatured ethanol (DEB 96) was used as a solvent. DMSO was a penetrate enhancer. Propylene glycol was used as a humectant and triethanlamine (TEA) as a pH adjuster. Methyl paraben and propyl paraben were added as a preservative.

2.3.8 Physicochemical properties of nimesulide gel formulations

The physicochemical properties of NM gel formulations consisted of color, pH, gel separation, gel splitting and viscosity of all NM gel formulations with three-chosen CL-MBs and commercial polymer. The experiments was measured against the gel base with the same gelling agent. All properties of NM gel formulations from three-chosen. CL-MBs were compared with the NM gel formulations from commercial polymer.

1) Color

NM gel formulations were observed by using color card, against the gel bases at the same polymer (Figure 2.6).

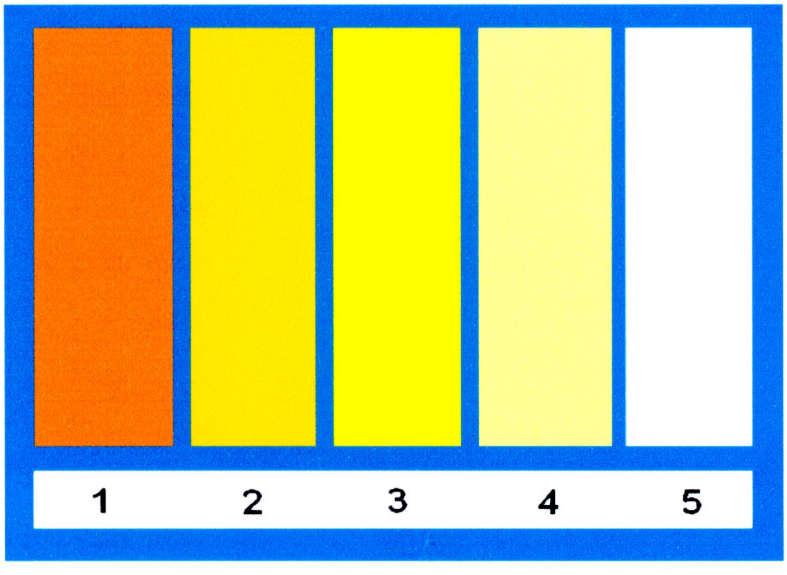

Figure 2.6 Color card

2) Gel separation

NM gel formulations were observed the gel separation when these were preserved in well-tight, light-resistant containers, against the gel bases at the same polymer.

3) Gel splitting

NM gel formulations were spread over the glass slide and covered with a glass cover slit. The gel splitting was observed by using a microscope at a 40X magnification. Their image were compared with the gel base image at the same polymer.

4) pH of nimesulide gel formulations

NM gel formulations were dissolved in distillated water at 10%w/v concentration. These solutions were determined by using a pH meter-pHScan™ WP2, Eutech Instruments, USA. The pH value was calculated as the averaged of a triplicate determination, compared with the pH of the gel base at the same polymer.

5) Viscosity of nimesulide gel formulations

NM gel viscosity was determined using a Brookfield Engineering Laboratories™ R/S-CPS rheometer, USA, with a plate-to-plate format. The measuring system was SP25 with the controlled shear rate mode (CSR) at set at 25±1°C. The viscosity of the gels was taken from the average obtained after a triplicate determination. Brookfield Rheo 2000 software was used to analyze all data and viscosity was reported with Pa.s. The viscosity of gels was measured by 3-step method:

Step 1 :	Step time	60 sec
	#MP	10
	Start value	0 D[1/s]
	End value	100 D[1/s]
Step 2 :	Step time	60 sec
	#MP	15
	Start value	100 D[1/s]
	End value	100 D[1/s]
Step 3 :	Step time	60 sec
	#MP	10

Start value	100	D[1/s]
End value	0	D[1/s]

The viscosity was analyzed at shear rate 100 s^{-1}.

2.3.9 Releasing study of nimesulide gel formulations

The releasing study of NM gel formulations were modified from in-vitro drug diffusion study of Kumar et. al. (2010). The method was determined by using Franz diffusion apparatus. The withdrawn sample was detected by UV/VIS spectrophotometer (Shimadzu™ UV-2450, Japan). The experiment was calculated as the average of a triplicate determination.

1) Nimesulide standard curve

NM was a gift of the Bangkok Lab and Cosmetics Co., Ltd. An amount of 10.0 mg of NM (active ingredient) was accurately weighed into a 100-mL volumetric flask, then adjusted to volume with 1.0 N of NaOH. The solution was called the NM standard stock solution at 0.1 mg/mL concentration.

The working standards were prepared by the serial dilution technique. Pipette 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8 and 2.0 mL aliquots of the NM standard stock solution was transferred into 10-mL volumetric flask, respectively and adjusted to volume with 1.0 N of NaOH. Afterwards, the absorbance (A) of the NM working standards were detected by UV/VIS spectrophotometer at 392 nm to estimated NM against 1.0 N of NaOH (Blank solution). The NM standard curve was exhibited the relation between the NM standard concentration (mg/mL) and the absorbance of NM at 392 nm.

2) Nimesulide gel formulation releasing study

The NM gel formulation releasing was ascertained by using Franz diffusion apparatus (Figure 2.7). Regenerated cellulose membrane was used for this study. In the Franz diffusion cell, 1.0 g of NM gel formulation was accurately weighted into the donor chamber. The entire surface of membrane was contacted with the receptor chamber that containing of 0.1 N of NaOH. In the receptor chamber, the media solution was continuously stirred about 100 rpm by using a magnetic stirrer. The temperature of study was maintained 37±1°C. This study was fulfilled for 180 min with the interval of 1, 3, 5, 10, 15, 30, 60, 120 and 180 min. The

solution of sample was withdrawn at predestine period of time and the equal volume at the same temperature was replaced with 0.1 N of the fresh NaOH. The absorbance of withdrawn sample was determined at 392 nm to estimate NM against the gel base (Blank solution) at the same polymer. The NM content at each period of time was calculated from NM standard equation above.

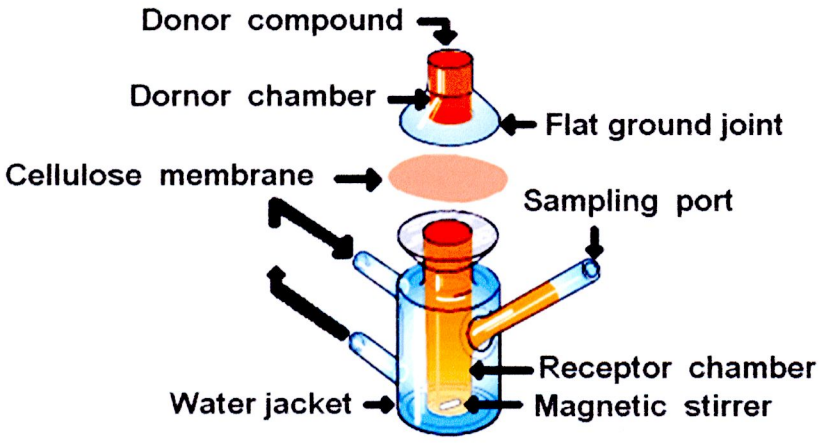

Figure 2.7 Franz diffusion apparatus

2.3.10 Stability test of nimesulide gel formulations

The physiochemical properties and releasing test of NM gel formulations were studied at the stability conditions. The conditions were quoted from British Pharmaceutical codex (1996). All polymer samples were experimented at accelerating condition, included 8°C, room temperature and 45°C for 3 months, and underwent a HC stability (8 cycles–each cycle consisted of 2 days at 8°C and another 2 days at 45°C). The facts were compared with the data before stability test (freshly preparation).

2.3.11 Statistical analysis

Physicochemical properties data was statistically analyzed using a one-way ANOVA test (Duncan's test). The evaluations were determined using an SPSS version 15.0 program. The results were considered significantly different to a 95% confidence level (P-values <0.05).