CHAPTER III

EXPERIMENTAL

3.1 Chemicals, Equipment, Glassware and Analytical Instruments

3.1.1 Chemicals

Acrylamide (AM, Siam Chemicals, Thailand) and the anionic comonomer, itaconic acid (Merck, Hohenbrunn, Germany) were used as monomers. N,N′-methylenebisacrylamide (N-MBA, Fluka, Buchs, Switzerland) was used as a crosslinker. Ammonium persulfate (APS, Merck, Hohenbrunn, Germany) and N,N,N′,N′-tetramethylenediamine (TMED, Fluka, Buchs, Switzerland) were used as the redox initiator pair. For a graft polymer of cassava starch-*g*-poly[acrylamide-*co*-(itaconic acid)], cassava starch was supplied from Thai Wah Co., Ltd, Thailand. It was the product from tapioca cultivated in summer. It contained 13.5% moisture, 0.20% ash, pH range of 4.0-7.0 and viscosity of 550 B.U. The foam formation reagents sodium bicarbonate ($NaHCO_3$, Sigma-Aldrich, St. Louis, U.S.A) and 0.05%V^{-1} Lutrol F® 127 (BASF, Ludwigshafen, Germany), a triblock copolymer of polyoxyethylene/polyoxypropylene/polyoxyethylene, with a molecular weight range 9800-14600 g mol^{-1} which was used as a constant ingredient for the whole experiment.

∝-Amylase (EC 3.2.1.1) from *Bacillus* species with a catalytic activity of 1600 IU/mg protein, was supplied by Sigma-Aldrich (St. Louis, U.S.A). One unit is defined as the amount of enzyme liberating 1.0 mg of maltose from starch in 3 min at pH 6.9 at 20°C.

Other chemicals of analytical grade, as shown in Table 3.1, were used without further purification.

Table 3.1 Chemicals

Chemicals	Source
Hydrochloric acid, HCl	J.T.Baker Inc, Phillipsburg, USA
Sodium hydroxide, NaOH	Merck, Darmstadt, Germany
Sodium chloride, NaCl	Merck, Darmstadt, Germany
Magnesium chloride, $MgCl_2.6H_2O$	Carlo Erba, Milan, Italy
Calcium chloride, $CaCl_2$	Fluka, Buchs, Switzerland
Boric acid, H_3BO_3	Fluka, Buchs, Switzerland
Citric acid monohydrate, $H_3C_6H_5O_7.H_2O$	Merck, Hohenbrunn, Germany
Tri-sodium phosphate, $Na_3PO_4.12H_2O$	Fluka, Buchs, Switzerland
Tri-sodium citrate, $Na_3C_6H_5O_7.2H_2O$	Fluka, Buchs, Switzerland
Cupric sulfate, $CuSO_4.5H_2O$	Merck, Darmstadt, Germany
Potassium hydrogen phosphate, KH_2PO_4	Merck, Darmstadt, Germany
Di- potassium hydrogen phosphate, K_2HPO_4	Merck, Darmstadt, Germany
Glucose, $C_6H_{12}O_6$	Carlo Erba, Milan, Italy
3,5-dinitrosalicylic acid, $C_7H_4N_2O_7$	Sigma-Aldrich, St. Louis, U.S.A
Sodium potassium tartrate, $COOK(CHOH)_2COONa.4H_2O$	Carlo Erba, Milan, Italy

3.1.2 Equipment and Glassware

a. Mechanical stirrer : Ika-Ruhrwerke RW20, Staufen, Germany

b. Water bath circulator : WBU 45 memmert, Schwabach, W-Germany

c. Magnetic stirrer : Snijders, Tilburg, Holland

d. Analytical balance : Mettler AE260, Switzerland

e. Oven : Hotpack, U.S.A.

f. pH meter : Hach, Model EC20, Colorado, U.S.A.

g. Autoclave : Sanyo, Model MLS-3020, Japan

h. Centrifuge : Hitachi, Model Himac CR 5B2, Japan

i. Incubator : Gallenkamp, England

j. Other general laboratory glassware and equipment.

-Flat bottomed flask

-Steel wire filtering with 100-mesh aluminium screen

-Four-necked round bottomed flask

-Reduced pressure filtering system

-Condenser

-Thermometer

3.1.3 Analytical Instruments

Fourier Transform Infrared Spectroscopy, Model Nicolet Impact 410, U.S.A.

Scanning Electron Microscope, Model JSM-6400, JEOL, Japan

Thermal Gravimetric Analysis, Model Netzsch STA 409 C, Netzsch Gerätebau GmbH, Germany

Spectrophotometer, Spectronic-20, Model Genesys, Thermo Electron Corp., USA

3.2 Procedures

3.2.1 Gelatinization of Cassava Starch

Cassava starch 4.0 g was mixed with 30 cm^3 of distilled water in a 500 cm^3 4-necked round bottomed flask. The system was stirred mechanically at 250 rpm under heating within the temperature range of 80±2 °C for 30 minutes under the nitrogen atmosphere to form a slurry-paste.

3.2.2 Graft Copolymerization of Acrylamide and Itaconic Acid onto the Gelatinized Cassava Starch

The gelatinized starch was then cooled to 45 °C. A mixture of 100 cm^3 water containing; 0.90 mol of acrylamide, 0.10 mol of itaconic acid, 1.0% wt monomer of N-MBA, 1.0% wt monomer of ammonium persulfate, was added into the reaction flask. The ingredients were stirred by a small-bladed propeller for 5 minutes while heating at the desired temperature. After the set temperature of 45°C was reached, 2.0 g of sodium bicarbonate powder was added. Lutrol F® 127 of 0.05 g, and 0.20 cm^3 of TMED were then added sequentially. The reaction between itaconic acid and sodium bicarbonate took place immediately to produce carbon dioxide bubbles, which floated upwards to the solution surface. Foam formation and solution polymerization continued for 30 minutes, after which the bubble production ceased and the reaction mixture became milky white. A fine powder then precipitated. The resulting polymer was filtered, washed several times with distilled water, dewatered with methanol, dried, and cut into small pieces, which were dried again at 65°C for 24 hours in a vacuum oven until reaching a constant weight and then it was milled.

A product with the highest water absorption capacity could be obtained by investigation of the influential effects on graft copolymerization. Various reaction parameters of grafting characteristics and water absorption were investigated as shown in Table 3.2.

Table 3.2 Parameters for Graft Copolymerization of Cassava Starch and Acrylamide/Itaconic Acid

Parameters	Condition
Mole ratio of the monomer, AM:IA	100:0-85:15
Weight ratio of starch-to-monomer	1:2-3.5:2
Ammonium persulfate, APS (%wt of the monomers)	0.5-2.0
Crosslinking agent, NMBA (%wt of the monomers)	0.5-2.5
Reaction temperature, °C	35-65
Agitation rate, rpm	150-300

3.2.2.1 Effect of Mole Percent of Acrylamide-to-Itaconic Acid Ratio on Graft Copolymerization

The amount of starch was remained constant for each experiment while the of AM-to-IA ratios (mole percent) were varied as 100:0, 98:2, 96:4, 94:6, 92:8, 90:10, and 85:15.

3.2.2.2 Effect of Starch-to-Monomer Ratio on Graft Copolymerization

The same reaction procedure as described above was carried out. The amount of starch remained constant for each experiment while one of the monomer mixture was varied. The different starch-to-monomer ratios to be investigated were 1:2, 1.5:2, 2:2, 2.5:2, 3.0:2, and 3.5:2. Each batch of the reaction mixture was conducted at the optimum acrylamide-to-itaconic acid ratio.

3.2.2.3 Effect of Ammonium Persulfate on Graft Copolymerization

From the experiment data gained, the optimum ratios of the AM-to-IA (Section 3.2.2.1) and starch-to-monomer (Section 3.2.2.2) were fixed. Concentrations of ammonium persulfate of 0.5, 1.0, 1.5 and 2.0% weight based on the monomer mixture were added to each batch of the gelatinized starch.

3.2.2.4 Effect of the Crosslinking Agent, N-MBA on Graft Copolymerization

The crosslinking agent was added in each batch of the gelatinized starch-monomer mixture with the optimum ratios of the AM-to-IA (Section 3.2.2.1) and starch-to-monomer (Section 3.2.2.2), and ammonium persulfate (Section 3.2.2.3) were fixed. The concentration of crosslinking agent was varied at 0.5, 1.0, 1.5, 2.0, and 2.5 % weight based on the monomer mixture.

3.2.2.5 Effect of Reaction Temperature on Graft Copolymerization

The same reaction procedure as described above was carried out, except for the optimum ratios of the AM-to-IA (Section 3.2.2.1) and starch-to-monomer (Section 3.2.2.2), ammonium persulfate (Section 3.2.2.3), and crosslinking

agent (Section 3.2.2.4) were fixed. The reaction temperature was varied at 35, 45, 55, and 65°C.

3.2.2.6 Effect of Agitation Rate on Graft Copolymerization

The same reaction procedure as described above was carried out, except for the optimum ratios of the AM-to-IA (Section 3.2.2.1) and starch-to-monomer (Section 3.2.2.2), ammonium persulfate (Section 3.2.2.3), and crosslinking agent (Section 3.2.2.4) were fixed. The agitation rate was varied from 150-300 rpm

3.2.3 Removal of Free Polymers

The dried product derived from the above sections that was ground previously into a powder form was stirred in distilled water (about 1 g in 300 cm^3 of distilled water) at room temperature for 24 hours. The mixture was centrifuged to separate the graft copolymer. The graft copolymer was washed with distilled water and centrifuged to allow another separation of grafted copolymer. Then it was dehydrated with methanol to give fine precipitate, dried in the oven at 65°C for 24 hours and it was weighed to examine the amount of the free polymers.

3.3 Characterization of the Copolymer

The synthesized copolymers were characterized as follows:

3.3.1 Functional Groups by FTIR Spectroscopy

The functional groups of the copolymers were examined by a Fourier Transform Infrared Spectroscopy (FTIR), Nicolet Impact 410 using a KBr pellet.

Nicolet Omnic Interface Software was connected to the Nicolet FTIR in a data acquisition system.

3.3.2 Determination of Percentage Free Polymers

The weights of polymer after a removal of a free polymer from Section 3.2.3. were calculated for the amounts of free polyacrylamide, poly(itaconic acid), and poly[acrylamide-*co*-(itaconic acid)] produced as by products.

3.3.3 Determination of Percentage Add-on

In a 125-cm^3 Erlenmeyer flask mounted with a condenser, 0.5 g of the grafted copolymer was refluxed in 50 cm^3 of 1 M HCl for 2 hours. The polymer was filtered and washed with distilled water until pH 7 was reached and then it was dried. The residual starch (substrate) after acid hydrolysis was checked with iodine solution to observe the completion of reaction.

The weight percentages of polyacrylamide, poly[acrylamide-*co*-(itaconic acid)] in the graft copolymer or the so called "percentage add-on" were computed from the weight difference between the graft copolymer and soluble starch, which had been removed by the acid hydrolysis.

3.3.4 Determination of Percentage Grafting Ratio

The experimental procedure of Section 3.3.3 also gave the weights of the polymer in grafts and the substrate (starch), which was considered as the percentage grafting ratio as computed in Section 2.4.

3.3.5 Determination of Percentage Grafting Efficiency

The experimental procedures of Sections 3.2.3 and 3.3.3 were carried out with the grafted product. The percentage of the total synthetic polymer formed that had been grafted to starch or the so called "percentage grafting efficiency" was computed.

3.3.6 Determination of Surface Morphology of the Copolymer

The surface morphology of the copolymers was investigated using a scanning electron microscope (SEM), JEOL, model JSM-6400, Japan.

3.3.7 Determination of Grafting Characteristics of Graft Copolymer by Thermal Gravimetric Analysis (TGA)

The grafting characteristics of graft copolymer was investigated using thermal gravimetric analysis (TGA), Netzsch STA 409 C, in nitrogen atmosphere at 20 cm^3/min, was used in this study in a temperature range from ambient to 600°C at a heating rate of 10°C/min.

3.4 Water Absorption/Retention Capacities of the Copolymers

3.4.1 In Distilled Water

The dried graft copolymer of 0.10 g was soaked in 200 cm^3 of distilled water for 1 hour. The swollen copolymer was filtered through a preweighed 100-mesh aluminium screen and allowed to drain for 30 minutes. Then it was weighed to determine the weight of the water-swollen gel.

The water absorption capacity was calculated in g g^{-1} of the dried polymer as Equation 3.1 as follows:

$$\text{Water Absorption } (g\ g^{-1}) = \frac{W_s - W_d}{W_d} \quad (3.1)$$

Where W_s is the weight of the water swollen gel (g)

W_d is the weight of the dry polymer (g)

3.4.2 In Salt Solutions

The same experimental procedure as described in Section 3.4.1 was carried out by replacing the distilled water with a series of sodium chloride, magnesium chloride, and calcium chloride solution of 0.9 % w v^{-1} in order to measure the extent.

3.4.3 In Buffer Solutions

The same experimental procedure as described in Section 3.4.1 was carried out with a series of buffer solution ranging from pH 3 to 11 in place of distilled water. The buffer solution of pH 3 to 11 were prepared by mixing the proper amount of 0.20 M boric acid, 0.05 M citric acid, and 0.10 M tri-sodium phosphate [47].

3.5 Biodegradation Study of the Graft Copolymer

3.5.1 Enzymatic Hydrolysis of the Graft copolymers

In a 125-cm^3 erlenmeyer flask, the dried graft copolymer 0.05 g was soaked in 50 cm^3 phosphate buffer pH 7.0 for 30 minutes, 2.0 cm^3 of enzyme solution was added. The graft copolymer and phosphate buffer pH 7.0 without α-amylase were used as a control. The mixture was incubated and shook at a 70 rpm for 24 hours at 37°C. Then, the samples were taken and investigated using the dinitrosalicylic acid (DNS) method, iodine, and Benedict's test. The buffer and enzyme solution was replenished every 24 hours to ensure that enzymatic activity remained at a desired level throughout the experiment duration and the incubation time was varied at 1, 2, 3, 4, and 5 days For each sample studied, this procedure was repeated three times, and the values reported as an average of those from the three runs.

Finally, the degraded copolymer was heated to destroy the enzyme, cooled at room temperature. The mixture was centrifuged to separate the degraded copolymer. The degraded copolymer was washed with distilled water and centrifuged to allow another separation of the grafted copolymer. Then it was dehydrated with methanol to give fine precipitate, dried in the oven at 65°C for 24 hours.

3.5.1.1 Effect of Enzyme Concentration

The same experiment procedure as described above was carried out, only the α-amylase concentration was varied at 2040, 4080, 6120, 8160, 10200, 20400, and 30600 U cm^{-3}. Each amount of enzyme was conducted at the optimum unit of enzyme.

3.5.1.2 Effects of Mole Percent of AM-to-IA Ratio and Starch-to-Monomer Ratio

The same experiment procedure as described above was carried out, except for α-amylase concentration fixed at 10200 U cm^{-3}. The mole percents of AM-to-IA ratio and starch-to-monomer ratio were varied at 100:0, 95:5, 90:10 and 1:2, 2:2, 3:2, respectively.

3.5.2 Determination of the Amount of Reducing Sugar

To obtain the amount of glucose liberated a standard curve was plotted. This was done by preparing mixtures of standard glucose solution, and distilled water according to the fixed volumes as indicated in Table 3.3. The blank solution comprises 1.0 cm^3 of distilled water and 1.0 cm^3 of DNS solution. The mixture was heated at 100°C for 5 minutes. After cooling to room temperature, 10 cm^3 of distilled water was added.

The absorption of the standard glucose solution of different concentrations was determined with a UV/VIS spectrophotometer (Spectronic-20, model Genesys). Measurement was done at a wavelength of 540 nm. Subsequently, the amount of glucose liberated is obtained by comparison with this curve. The plot should be linear. Use a spreadsheet and linear regression to obtain a standard curve.

The reducing sugars in the degradation solution were quantified by the DNS method [12], 1.0 cm^3 of DNS was added to 1.0 cm^3 of sample in a test tube. The mixture was heated at 100°C for 5 minutes to develop the color and let cool. After cooling to room temperature, 10 cm^3 of distilled water was added and measured the absorbance at 540 nm. In each case three replicas were made. The amount of reducing sugar was considered as the average of all the results by comparison with a standard curve.

Table 3.3 Volume of the standard glucose solution, distilled water used in constructing the standard curve

Standard glucose solution (cm^3)	Distilled water (cm^3)	Glucose concentration ($g\ l^{-1}$)
0	1.0	0
0.2	0.8	0.1
0.4	0.6	0.2
0.6	0.4	0.3
0.8	0.2	0.4
1.0	0	0.5

Concentration of standard glucose solution is 0.5 $g\ l^{-1}$

The amount of reducing sugar was calculated in $g\ l^{-1}$ as follows:

$$\text{Reducing sugar } (g\ l^{-1}) = \frac{\text{Absorbance at 540 nm}}{\text{Slope}} \quad (3.2)$$

3.5.3 Determination of Surface Morphology of the Copolymer

The surface morphology of the degraded copolymers was studied by a scanning electron microscope (SEM), JEOL, model JSM-6400, Japan.