CHAPTER 3

EXPERIMENTAL

3.1 Chemicals and Equipment

3.1.1 Chemicals

Methacrylic acid (MA) was provided by Siam Resin & Chemical Co., Ltd (Thailand). It was distilled under a reduced pressure before use.

Every chemical shown below, which was of analytical grade, was used without further purification.

Pluronic PE 8100 was suppiled by BASF (Thai) Ltd. (Thailand).

Acrylamide (AM), ammonium persulfate (APS), potassium dihydrogen phosphate, dipotassium hydrogen phosphate, sodium carbonate and sodium bicarbonate were purchased from Merck A.G. (Germany).

Tris-(hydroxymethyl)-aminomethane, trichloroacetic acid (TCA), N,N′-methylene-bis-acrylamide (MBA), paraffin wax, imidazole, coomassie brillant blue R-250 were obtained from Fluka A.G. (Switzerland).

Standard alkaline protease (P5380, Type VIII), Bovine serum albumin, L-tyrosine were perchased from Sigma Chemical Co., Ltd. (U.S.A.).

N,N,N′,N′-tetraethylmethylenediamine (TEMED) and casein Hammarsten were purchased from BDH Laboratory Chemical Division (England).

3.1.2 Equipment

The equipment that was used in the current experiments was shown below:

UV-Visible Spectrophotometer, Model DU 650, Backman, U.S.A..

Scanning Electron Microscope (SEM), Model JSM-35 CF, JEOL, Japan.

Fourier Transform Infrared Spectrophotometer (FT-IR), Model 1460X, Perkin Elmer, U.S.A..

High Performance Liquid Chromatography (HPLC), Model LC-3A Shimadzu, Japan.

Lyophilizer, Model Lyph-lock 1L, Labconco Corporation, U.S.A..

Rotatory Evaporator, Model RE 47, Yamoto Scientific Co., Ltd., Japan.

Stirring Motor, Model EURO-STB , Ika Labortechnik, Germany

Freezer (-70°C), Model REVCO, Bara Laboratories Co., Ltd., Thailand.

pH meter, Model SA720, Orion Research Inc., U.S.A..

pH meter Model PHM 83, Radiometer, Copenhagen, Denmark.

Centifuge, Model H-103N Series, Kokusan, Japan.

Incubator, Model Heraeus Type B 5050 E, Heraeus, Germany.

Magnetic stirrer, Model MS-90, Farga Instrument Ltd., Taiwan.

Vortex, Model K 550-GE, Scientific Industries Inc., U.S.A..

Oven, Model DG 82, Yamato Scientific Co., Ltd., Tokyo, Japan

Analytical Balance, Model AE-200S, Metter Instrument A.G., Switzerland.

3.2 Procedure

3.2.1 Homopolymerization and Immobilization of Alkaline Protease

The acrylamide monomer and N,N′-methylene-bis-acrylamide crosslinker were dissolved in 35 cm^3 of carbonate-bicarbonate buffer solution of pH 10.5 and this solution was then poured into 200 cm^3 paraffin wax, which contains Pluronic PE 8100 surfactant. The mixture was purged with nitrogen gas to remove oxygen. After purging, 5 cm^3 of ammonium persulfate and 5 cm^3 of alkaline protease were added and mixed rapidly. This solution was also purged with nitrogen gas. After stable droplets of the monomer and crosslinker in the aqueous phase had formed, N,N,N′,N′-tetraethylmethylenediamine (TEMED) was then injected into the paraffin phase to initiate the polymerization. The reaction flask was purged with nitrogen gas and stirred vigorously during polymerization. After the polymerization reaction had been completed, the mixture was transferred to a beaker and an excess amount of buffer solution was added to separate the system into two phases. The beads were washed four times with the buffer solution and then were freeze dried and stored in a refrigerator. The polymerization scheme describing the reaction sequences was shown in the Figure 3.1. The important reaction parameters described in Sections 3.2.1.1 through 3.2.1.9 were carried out. The dried samples were investigated for the

enzyme protease activity, its percentage immobilization and the percentage conversion of the monomer.

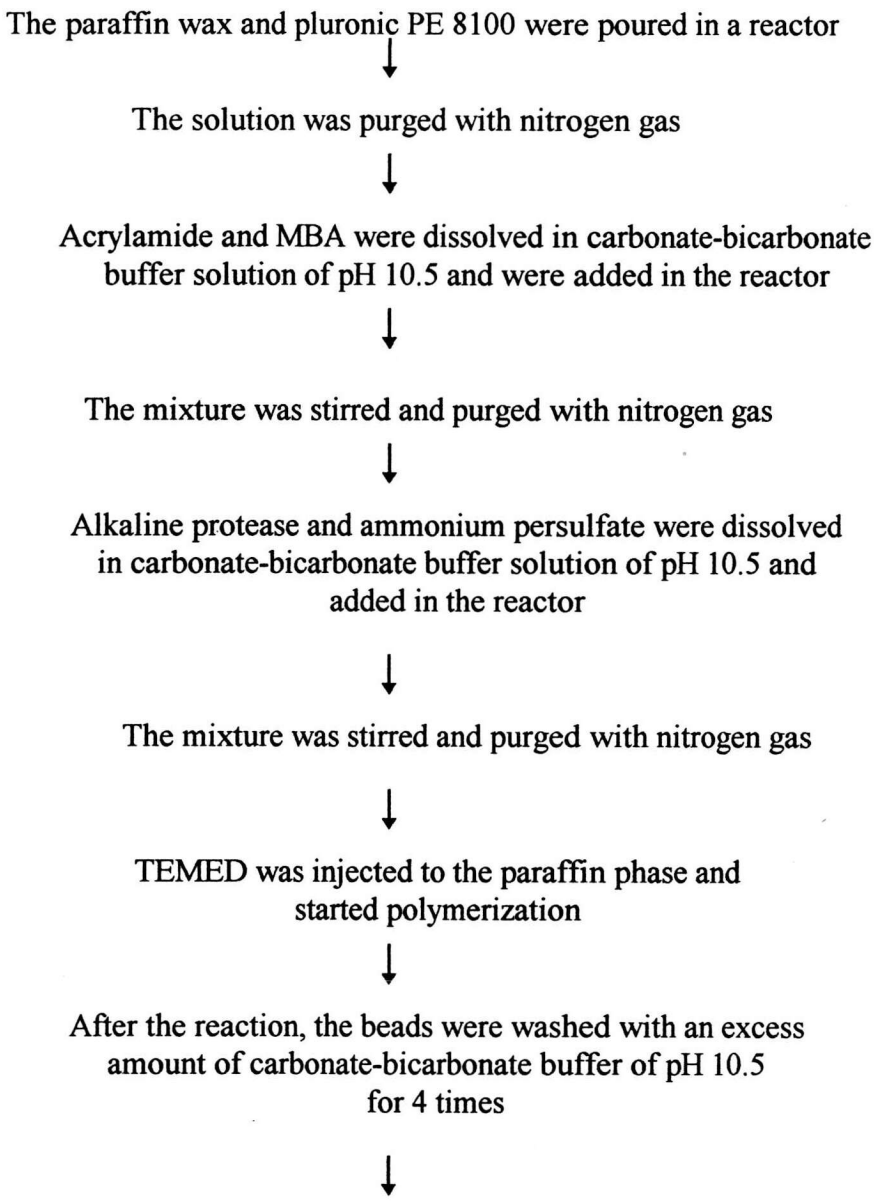

Figure 3.1 Reaction scheme for homopolymerization and immobilization of alkaline protease

3.2.1.1 Effect of Monomer Concentrations on the Enzymatic Activity

Acrylamide concentrations of 3.14, 4.57, 6.28, 9.14 mM in 35 cm^3 carbonate-bicarbonate buffer solution (pH 10.5) were carried out by following the procedure as mentioned above. The dried samples were determined for the protease activity, percentage immobilization of enzyme, and percentage conversion of monomer. The optimum protease activity was used as a constant for the next reaction parameter.

3.2.1.2 Effect of Enzyme Concentrations on the Enzymatic Activity

The alkaline protease enzyme used in all experiments was secreted from *Bacillus lichenifromis*. Various amounts of alkaline protease of 0.25, 0.5, 1.5, 2.5, 5.0 mg/5 cm^3 were investigated for the effects of enzyme concentration. The reaction steps were carried out as mentioned above. The dried samples were determined for the enzyme protease activity, percentage immobilization of enzyme, and percentage conversion of the monomer. The optimum protease activity was used as a constant for the next reaction parameter.

3.2.1.3 Effect of Polymerization Stirring Rate on the Enzymatic Activity

The polymerization stirring rate was studied at 100, 200, 300, 400 rpm. The reaction steps were carried out as mentioned above. The dried samples were determined for the enzyme protease activity, percentage immobilization of enzyme

and percentage conversion of the monomer. The optimum protease activity was used as a constant for the next reaction parameter.

3.2.1.4 Effect of Polymerization Time on the Enzymatic Activity

Polymerization time was investigated at 1, 2, 3, 4 h for the enzyme activity. The reaction steps were carried out as mentioned above. The dried samples were determined for the enzyme protease activity, percentage immobilization of enzyme and percentage conversion of the monomer. The optimum protease activity was used as a constant for the next reaction parameter.

3.2.1.5 Effect of Polymerization Temperature on the Enzymatic Activity

The optimum polymerization temperature was studied at 0, 10, 20, 30, 40°C. The reaction steps were carried out as mentioned above. The dried samples were determined for the enzyme protease activity, percentage immobilization of enzyme and percentage conversion of the monomer. The optimum protease activity was used for studying the subsequent effect.

3.2.1.6 Effect of Initiator Concentrations on the Enzymatic Activity

The concentrations of ammonium persulfate (APS) that were used as initiator containing 3.13, 6.56, 9.39, 12.52 mM in the medium were investigated. The APS was dissolved in 5 cm^3 of carbonate-bicarbonate buffer solution of pH 10.5. The reaction steps were carried out as mentioned above. The dried samples were

determined for the enzyme protease activity, percentage immobilization of enzyme and percentage conversion of the monomer. The optimum protease activity was used for further study of reaction parameters.

3.2.1.7 Effect of Accelerator Concentrations on the Enzymatic Activity

N,N,N′,N′-tetraethylmethylenediamine (TEMED) was used as an accelerator. Various concentrations of TEMED of 47.75, 95.50, 143.25, 191.05 mM were prepared for carrying out the polymerization. The reaction steps were followed as mentioned above. The dried samples were determined for the enzyme protease activity, percentage immobilization of enzyme, and percentage conversion of the monomer. The optimum protease activity was used for the other reaction factors.

3.2.1.8 Effect of Crosslinker Concentrations on the Enzymatic Activity

N,N′-methylene-bis-acrylamide (MBA) was used as a crosslinker, which was dissolved in 35 cm^3 of carbonate-bicarbonate buffer solution of pH 10.5. The MBA concentrations were prepared to have 15, 30, 60, 90, 120 mM in the medium. The reaction steps were followed as mentioned above. The dried samples were determined for the enzyme protease activity, percentage immobilization of enzyme and percentage conversion of the monomer. The optimum protease activity was used to investigate the surfactant concentration effect.

3.2.1.9 Effect of Surfactant Concentrations on the Enzymatic Activity

The effect of surfactant was evalulated by varying the amounts of Pluronic PE 8100 concentrations of 5.3, 10.6, 15.9, 21.2 mM in the medium. The reaction steps were carried out as mentioned above. The dried samples were determined for the enzyme protease activity, percentage immobilization of enzyme and percentage conversion of the monomer. The optimum protease activity was used to study the effect of monomer ratio.

3.2.1.10 Effect of Acrylamide/Methacrylic Acid Ratios on Enzymatic Activity

The reaction conditions from Section 3.2.1.9 showing an optimum actitivity was chosen for a copolymerization between acrylamide and methacrylic acid. The ratios of acrylamide to methacrylic acid of 100/0, 97.5/2.5, 95/5, 90/10% W/W were investigated for its enzyme activity.

All reaction products were carried out for their protease activity, water absorption. IR analyses, scanning electron micrography and protein staining were made to identify its functional groups, morphology and existence of the enzyme. The optimum reaction conditions for homopolymerization and copolymerization were chosen for investigation of effects of pH and temperature on activity, protein digestion, and storage stability on activity. Durability of immobilized beads was also analyzed.

3.2.2 FT-IR Identification

The acrymide homopolymer and acrylamide-methacrylic crosslinked copolymer were identified for functional groups by an FT-IR spectrophotometer. Standard FT-IR spectra are used as a standard for comparing with the reaction products.

3.2.3 Thermal Analysis

Polyacrylamide and poly(acrylamide-co-methacrylic acid) were analysed for glass transition temperature by the Differential Scanning Calorimetric (DSC) technique.

3.2.4 Determination of Protease Activity

3.2.4.1 Preparation of Buffer Solution and Calibration Curve of L-Tyrosine

a) 0.1 M Phosphate Buffer Solution of pH 7.5 and 8.0

Disodium hydrogen phosphate stock solution: 53.65 g of disodium hydrogen phosphate was dissolved in 1000 cm^3 of water.

Sodium dihydrogen phosphate stock solution: 27.8 g of sodium dihydrogen phosphate was dissolved in 1000 cm^3 of water.

The phosphate buffer solutions of pH 7.5 and 8.0 were prepared by measuring the stock solutions as shown below and the final volume of 1000 cm^3 was made up with distilled water.

pH	$NaH_2PO_4(cm^3)$	$Na_2HPO_4(cm^3)$
7.5	80.0	420.0
8.0	26.5	473.5

b) 0.1 M Tris-Hydrochloride Buffer Solution of pH 8.5 and 9.0

121.2 g of Tris(hydroxy-methyl)aminomethane was dissolved in distilled water first, and was adjusted the pH to 8.5 and 9.0 with 0.1 M hydrochloric acid and, diluted finally to 1000 cm^3 with distilled water.

c) 0.1 M Carbonate-Bicarbonate Buffer Solution for pH 9.5, 10.0 and 10.5

Sodium carbonate stock solution: 21.2 g of sodium carbonate was dissolved with 1000 cm^3 of water.

Sodium bicarbonate stock solution: 16.8 g of sodium bicarbonate was dissolved with 1000 cm^3 of water.

Aliquots of the stock solution were measured and mixed to give the appropriate pHs as follows:

pH	$Na_2CO_3(cm^3)$	$NaHCO_3(cm^3)$
9.5	65.0	185.0
10.0	137.0	112.5
10.5	202.5	47.5

The mixed solutions were adjusted with 5-N sodium hydroxide solution or 2-N hydrochloric acid to the desired pH values and they were then made up to 1000 cm^3 with distilled water.

d) 0.1 M Carbonate Buffer Solution of pH 11.0

Sodium carbonate (10.6 g) was dissolved in distilled water, and the solution was adjusted to pH 11 with 5-N sodium hydroxide. The adjusted pH solution was made up to 1000 cm^3 with distilled water.

e) 0.5% W/V Casein Solution

Hammersten-type casein (0.5 g) was dissolved in a carbonate-bicarbonate buffer solution of pH 10.5 by placing the solution on a hot plate at 50°C with occasional stirrings. It was then cooled to room temperature and the solution was checked for its pH value. If nesessary, the solution pH should be adjusted with 5-N sodium hydroxide solution. The adjusted pH solution was diluted with the distilled water to the volume of 100 cm^3.

f) 0.5% W/V Bovine Serum Albumin Solution (BSA)

Bovine serum albumin (0.5 g) was dissolved in a buffer solution of pH 10.5. If necessary, the BSA solution was adjusted for the pH value with the buffer solution to pH 10.5. It was then diluted with the distilled water to 100 cm^3.

g) 0.5% W/V Gelatin Solution

Gelatin (0.5 g) was dissolved in a buffer solution of pH 10.5. It was heated on a hot plate with additional stirrings. It was cooled to room temperature, was adjusted to pH 10.5, and was then diluted to give 100 cm^3.

h) 10% W/V Trichloroacetic Acid Solution (TCA)

Trichloroacetic acid (10 g) was dissolved in distilled and it was made up to 100 cm^3.

i) Calibration Curve of L-Tyrosine

Tyrosine stock solution: L-tyrosine (0.01g) was weighed accurately and dissolved in 50 cm^3 volumetric flask with carbonate-bicarbonate buffer solution of pH 10.5 which was used for checking the enzymatic activity.

Five aliquots of the tyrosine stock solution as shown below were diluted with a buffer solution to make up to the volume of 5 cm^3. The solution

Tyrosine stock solution (cm^3)	Buffer (cm^3)	Concentration ($\mu g/cm^3$)
0.0	5.0	0
0.5	4.5	20
1.0	4.0	40
1.5	3.5	60
2.0	3.0	80
2.5	2.5	100

were put in a UV spectrophotometer. Their respective concentrations were obtained from the absorbane measured at 280 nm, against a blank buffer. A calibration curve was built to give a linear relationship concentration absorbance.

3.2.4.2 Determination of Protease Activity

Protease activity was assayed by the modified method of Richardson and Tewhaiti [81]. The activities of free protease and immobilized protease were determined by using casein as a substrate. The reaction mixture consisted of 1.9 cm^3 of 0.1 M carbonate-bicarbonate buffer of pH 10.5, 0.1 cm^3 of free enzyme solution or 0.05-0.1 g of immobilized enzyme suspension in buffer which contained 1.0 cm^3 of 0.5% W/V casein solution. This reaction mixture was incubated in a water bath at 45°C for 20 min, followed by stopping the reaction with 10% W/V of trichloroacetic acid (TCA). Unhydrolyzed casein was removed by centrifugation at 3500 rpm for 20 min. The absorbance of the solution or the supernatant of free tyrosine libereated was measured at 280 nm of the UV spectrophotometer in a 1-cm quartz cuvette. The experiment was consisted of the blank tube, control tube, and sample tube. Blank tube was prepared for the zero adjustment of a measurement of tyrosine absorption without enzyme (free or immobilized enzyme). The blank tube was added with the buffer solution (2.0 cm^3) instead. The control tube was prepared for substracting the interference of the other proteins. The control tube was consisted the same mixture, i.e. chemicals, as the sample tube with different additions of the reagents. The sample tube was added with the casein solution and followed by the stopping reaction with TCA solution while the control tube was different. The

enzyme activity was calculated as the amount of enzyme that liberated 1 microgram of tyrosine from casein per minute per gram of enzyme (free or immobilized) under the above condition. The concentrations could be obtained from a calibration curve by measuring the absorbance of tyrosine samples at 280 nm. The enzymatic activity could be calculated by eq. 2.1.

3.2.4.2.1 Effect of pH on the Enzymatic Activity

The effect of pH on the activity of the immobilized and free protease was studied in serveral buffer solutions. The pH was adjusted with the buffering systems: phosphate buffer (pH 7.5 and 8.0), tris-hydrochloride buffer (pH 8.5-9.0) and carbonate-bicarbonate buffer (pH 9.5-11.0). The activity was checked with the same procedure as mentioned above in the Section 3.2.4.2. The casein solution was prepared in the same buffering pH that was used for checking the enzymatic activity.

3.2.4.2.2 Effect of Thermal Stability on the Enzymatic Activity

The optimum temperatures on the activity of immobilized and free protease were determined by varying incubated temperatures at 25, 37, 45, and 60°C. The same experimental procedure as described in Section 3.2.4.2 was then carried out.

3.2.4.2.3 Effect of Enzymatic Activity on Protein Digestion

The casein, bovine serum albumin (BSA), gelatin , animal hairing and blood were used as substrates for checking the enzymatic activity. The same procedure as described in Section 3.2.4.2 was carried out to assess the enzymatic properties. The ability of protein digestion by both the free and immobilized enzyme on activity were compared.

3.2.4.2.4 Effect of Storage Stability on the Enzymatic Activity

The free enzyme and immobilized enzyme were stored at the various temperatures of -20, 4, 25, 37, 45 and 60°C and the residual enzymatic activity was checked every week for one months.

3.2.5 Investigation of Number Washings for Removing Paraffin Wax and Free Enzyme

After polymerization, the immobilized beads were washed with 0.1 M carbonate-bicarbonate buffer solution, pH 10.5, several times to remove the residual paraffin wax and free enzyme that was not immobilized on the beads. The volume of washing solution of buffer solution of pH 10.5 must be beyond the amount of solution needs for beads swelling, i.e. about 10 cm^3. The number of washings was evaluated by washing the wet beads for 1-5 times. Paraffin wax floated on to the surface of washing solution which was removed by scrapping. The remaining washing solutions were evaluated for the residual wax and enzymatic activities. The washed samples were freeze dried and were assayed for their enzymatic activity.

3.2.6 Determination of Amount of Leakage of the Enzyme Molecules on the Beads under Washings

The dried beads of known enzymatic activity were separated into four segments. The four samples were washed with carbonate-bicarbonate buffer of pH 10.5 for one, two, three, and four times, respectively. The washed samples were freeze dried and their enzymatic activities were then determined.

3.2.7 Determination of Percentage Conversion

3.2.7.1 Assessment by the Weights of Polymer Obtained

The experimental procedure for polymerizations described in Section 3.2.1 was carried out. The percentage conversion was defined as the weight of the total amount of polymer obtained from the weight of monomer charged.

3.2.7.2 Assessment by the Residual Monomer at Different Polymerization Times

Conversion of monomer was carried out indirectly by sampling 4 cm^3 of the residual acrylamide monomer in the polymeric system in 5, 10, 15, 20, 25, 30, 45, 60, 90, and 120 min. The dispersed polymer solution was diluted with 5 cm^3 of carbonate-bicarbonate buffer solution pH 10.5. The unreacted acrylamide was determined by High Performance Liquid Chromatography (HPLC). This method was referred by the method of Husser, E. R., Stehl, R. H., Price, D. R., and Delap, R. A. [65].

The mobile phase consisted of 15% methanol, 85% methylene chloride (V/V). The flow rate of mobile phase was 1 cm^3/min at 50 kg/cm^2 pressure. The analytical column was a Zorbax-N (250 x 4.6 mm) available from Dupont, Inc. It is a prepacked, 10-micron particle column containing a permanently bonded cyano-stationary phase on a porous silica support. The spectrophotometer was set at 240 nm.

The standard solutions were prepard by dissolved 0.05 g of acrylamide in 50 cm^3 methanol. It was diluted to give the standards of 0.2, 0.25, 0.5,1.0,5 μg of acrylamide per 5 x 10^{-3} cm^3 of solvent. A peak area against the amount of acrylamide solution injected was plotted as a result.

The residual acrylamide monomer was extracted by adding 8 cm^3 of methanol to 2.0 cm^3 of the dispersed polymer solution. The mixture was stirred for 30 min with a magnetic stirrer. It was then centrifuged for 15-20 min to isolate the polymer from the solution. A 5 x 10^{-3} cm^3 aliquot of the supernatant was injected directly onto the column. The area of the acrylamide peak was measured and the equivalent amount of acrylamide from the calibration graph was then read off. The percentage conversion of acrylamide can be calculated by eq. 2.3.

3.2.8 Determination of Percentage of Enzymatic Immobilization

The enzymatic activities of free and immobilized enzyme were carried out as mention above in Section 3.2.4.2. The total enzymatic activity of free enzyme was calcutated by multiplying the enzymatic activity (units) with the weight of free enzyme added in the reaction. The total of enzymatic activity of immobilized enzyme

was calculated also by muliplying the enzymatic activity of immobilized enzyme with the total weight of polymer obtained in the reaction. The percentage of enzymatic immobilization was calculated by dividing the total enzymatic activity of immobilized enzyme with the total enzymatic activity of free enzyme.

3.2.9 Determination of Water Absorption

3.2.9.1 In Deionized Water

Water absorption of the polyacrymide and poly(acrylamide-co-methacrylic acid) beads were measured gravimetrically at room temperture. Dry beads samples were weighed (0.1 g) and equilibrated in 100 cm^3 of deionized water for 30 min. Gels were poured in the filtering system and were left for 2 h to render a full swelling before recording the gel weight. Water absorption ratio was defined as the difference weights of beads before and being equilibrated in deionized water (in gram per gram of the dry beads).

3.2.9.2 In Salt Solutions

The same experimental procedure as described in Section 3.2.9.1 was carried out, except a series of salt solutions of 0.9% W/V of sodium chloride, potassium chloride, magnesium chloride and calcium chloride were used instead of deionized water.

3.2.9.3 Effect of Temperature on Water Absorption

The procedure as described in Section 3.2.9.1 was carried out for investigating the water absorption of polyacrylamide and poly(acrylamide-co-methacrylic acid) at various temperatures of 25, 30, 35, 40, 45°C.

3.2.10 Morphology of Polyacrylamide and Poly(acrylamide-co-methacrylic acid) Beads

Polyacrymide and poly(acrylamide-co-methacrylic acid) beads were evaluated using a scanning electron microscope (SEM) for the surface and interior morphologies. The size of the beads on the photograph was also measured.

3.2.11 Protein Staining

This method was adapted from an electrophoretic analysis method for protein purification methods [82]. The staining solution was prepared by dissolved 1.0 g of Coomassie blue R-250 in 450 cm^3 of methanol, 450 cm^3 of distilled water and 100 cm^3 of glacial acetic acid. The destaining solution was prepared by mixing 100 cm^3 of methanol, 100 cm^3 of glacial acetic acid and 800 cm^3 of distilled water.

The free enzyme and enzyme immobilized beads were stained with the staining solution for 5-10 min in a small container and the staining solution was then poured. The destaining solution was slowly added with occasional shakings. The procedure could take up to 24 h but it could be accelerated by using several changes of the destaining solution. The protein would be visualized as an intense blue color on the beads.