CHAPTER II

RESEARCH METHODOLOGY

Material and Equipment

Material

Reagent	Molecular weight	Manufacturers
Acetic acid	60.05	Merck
Acetic anhydride	102.09	Merck
Acetone	58.08	Merck
Acrylamide	71.08	Merck
Ammonium sulfate	132.14	Merck
Ammonium persulfate	228.7	Merck
Bovine serum albumin	67,000	Sigma
Bromophenol blue	670.02	Sigma
Commassie brilliant blue R-250	854.0	Sigma
Ethanol	46.07	Merck
Fluorescent brightener 28	960.9	Sigma
Glycine	75.05	Sigma
Methanol	32.04	Carlo Erba
4-Methylumbelliferone	198.15	Sigma
4-Methylumbelliferyl-*N*-acetyl-β-D-glucosaminide	379.36	Sigma
4-Methylumbelliferyl-*N-N'-N''*-acetyl-β-D-glucosaminide	785.80	Sigma
N,N-methylene bisacrylamide	154.2	Merck

N,N,N,N-tetramethylenediamine	116.2	Merck
Reagent	Molecular weight	Manufacturers
Sephadex G-75 16	-	Sigma
Sodium acetate	136.08	Merck
Sodium chloride (NaCl)	58.44	BDH
Sodium dodecyl sulfate (SDS)	288.4	Merck
Sodium hydroxide (NaOH)	40.0	BDH
Sulfuric acid	98.1	Lab Scan
Tricine	179.18	Fluka
Tris (hydroxymethyl) aminomethane	121.1	Merck
Triton X-100	-	Sigma

Equipment

1. Bio-Rad minigel IEF apparatus
2. Centrifuge J2-21 operation, Beckamn (USA)
3. Microcentrifuge 5804 R Eppendorf (Germany)
4. pH meter Cyberscan 1000 (Singapore)
5. Power supply model 1000/500, Biorad (USA)
6. Electoporesis appartatus, ATTA Cooperation (Japan)
7. Shimazu UV-Vis recording spectrophotometer model UV160A (Japan)
8. Spectrofluorophotometer RF-1501 Shimadzu (Japan)
9. Freeze dryer และ Deep-freeze refrigerator, Sanyo (Japan)

Method

1. Separation of fresh latex by ultracentrifugation

Freshly tapped latex was collected in an ice-chilled beaker tapped trees of RRIM600 clone or as specified. The latex was fractionated by ultracentrifugation (59,000 *g*, 45 min, 4°C) to give four distinct layers. The upper most cream layer contained virtually all rubber particles which lied above the orange-colored band formed by the Frey-Wyssling particle. The middle layer was transparent serum (C-serum) fraction and a large pellet called bottom (lutoid) fraction. The bottom fraction membrane (BFM) was obtained by repeated freezing and thawing of the bottom fraction of centrifuged latex. BFM from three rubber clones (RRIM600, RRIT251, PB311 and BPM-24) were used for studying chitinases activity.

2. Glycol chitin synthesis

Glycol chitin was obtained by reacetylation of glycol chitosan. Five gram of glycol chitosan was dissolved in 100 ml of 10% of acetic acid with continuous stirring. The viscous solution was allowed to stand overnight at room temperature in order to complete dissolution of the polysaccharide. The next day, 450 ml of methanol was slowly added with mixing and the clouding solution is filtered through glass wool on Buchner funnel. The filtrate was transferred into a beaker and 7.5 ml of acetic anhydride was added with magnetic stirring. After about 1 min, the mixture gels and the magnet stopped stirring. The gel was allowed to stand for about 30 min at room temperature and then cut into small pieces. The liquid extruding from the gel pieces was discarded. Gel pieces were transferred to a blender, cover with methanol, and homogenized for 4 min. This suspension was centrifuged at 10,000 rpm for 15 min at 4°C in JA-14 rotor. The gelatinous pellet was resuspended in about 1 volume of methanol, homogenized and centrifuged as in the proceeding step. The pellet was resuspended in 1 L of distilled water contained 0.02% (w/v) stock solution of glycol chitin.

3. Fluorimetric assay of endo- and exochitinase activity

Due to their sensitivity both 4-Methylumbelliferyl-*N*-*N'*-*N''*-acetyl-β-D-glucosaminide (4-MU-β-$(GlcNAc)_3$) and 4-Methylumbelliferyl-*N*-acetyl-β-D-glucosaminide (4-MU-β-GlcNAc) were used as substrates for assaying endo-and exochitinase, respectively (O'Brien and Colwell, 1987). The enzyme reaction was performed by incubating 5 µl of 1 mM substrate with 5 µl of enzyme solution in 200 µl of 50 mM Acetate buffer, pH 5. The mixture was incubated for 20 min at 37°C and the reaction was stopped by adding 1 ml of 1 M glycine buffer (pH 11). The fluorescence was measured using spectrofluorometer (excitation 355 nm, emission 460 nm). One unit of enzyme activity was reported as nmol of 4-Methylumbelliferone (4-MU) liberated per minute. Specific activity was expressed as units per mg of protein.

4. Protein determination

Protein content was estimated by the method of Bradford (1976) using Bio-Rad protein dye reagent concentrate (Bio-Rad). Bovine serum albumin was used as the standard.

The sample (20 µl) was added with 1 ml of dye reagent (1 part dye reagent concentrate with 4 parts deionized water) to each eppendorf and vortex. After incubation at room temperature for 5 min, protein concentration were monitored by measuring at absorbance at 595 nm in glass or polystyrene cuvettes using a standard curve of 0.2-0.9 mg/ml of bovine serum albumin (BSA) for calibration.

5. Gel electropheresis

5.1 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Denaturing gel electrophoresis was performed according to the method of Laemmli (1970). The slab gel contained a lower separating gel containing 12% acrylamide (15x10x0.1 cm) prepared from a stock solution of 30% (w/v) of acrylamide and 0.8% (w/v) of

N,*N*'-bismethyleneacrylamide. The gels contained acrylamide in 1.5 M Tris-HCl pH 8.9 and 0.1% SDS and were polymerized by the adding stock of tetramethyl-ethylenediamine (TEMED) and 1% (w/v) of ammonium persulfate. An upper stacking gel of 3% acrylamide (15x5x0.1 cm) in 0.5 M Tris-HCl pH 6.8 and 0.1% SDS was polymerized as the same manner. The electrode buffer (pH 8.3) contained 0.025 M Tris-HCl, 0.192 M glycine and 0.1% SDS. The protein samples were treated with solubilizing buffer with final concentration of 0.0625 M Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol and 0.001% bromophenol blue. Before apply the sample, the proteins were dissociated by immersing the samples for 10 min in boiling water bath. Electrophoresis was performed at constant current of 20 mA for 1 hr. Protein bands on the gel were fixed and stained overnight at room temperature with 0.2% Coomassie blue R-250 in 50% methanol and 10% acetic acid. The gel was destained with two changes of 50% methanol and 10% acetic acid until the background was clear. The relative molecular weights of proteins band were estimated by using standard molecular weight markers, which included phosphorylase (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa) and ∝-lactalbumin (14 kDa).

5.2 Polyacrylamide isoelectrofocusing

Isoelectric focusing was performed in Bio-Rad minigel IEF apparatus using the method described by Bio-Rad company. The polyacrylamide slab gel (5 ml)contained 1 ml monomer concentrated stock solution, composed of 24% (w/v) acrylamide and 0.075% bisacrylamide, 5% (v/v) glycerol and 2% Biolyte 3/10. The gel mixture was initially polymerized by adding 0.02% ammonium persulfate and 5 μl of TEMED, and immediately pores into the casting tray. The gel bond for polyacrylamide attached to the glass plate was allowed to spread uniformly underneath the glass plate. The gel was polymerized for 1 hr at room temperature. After the gel had set completely, the casting tray was removed. Sample (2.5 μl) were applied directly to the gel surface and left to diffuse into the gel for 15 min. Focusing was carried out at constant voltage in a stepped fashion at 100 V for 15 min, 200 V for 15 min and finally 450 V for 60 min. After focusing was complete, the glass plate was removed from the gel supporting film. In addition, protein bands in IEF gel were also fixed in a solution containing 30% (v/v) methanol,

5% (w/v) trichloroacetic acid and 3.5% sulfosalicylic acid for 15 min and stained with 0.025% Coomassie blue G 250 in 3.5% perchloric acid for 1 hr, followed by addition of 7% (v/v) acetic acid for intensification and preservative.

6. Chitinase activity staining

Chitinolytic enzyme activities were also examined after polyacrylamide gel electrophoresis. A chitinase assay was carried out as described by Trudel and Asselin (1989) with some modifications. To visualize the active enzyme, 0.01% glycol chitin was incorporated into 12% (w/v) polyacrylamide gels. After electrophoresis, the gel was incubated in 0.2% Triton X-100 in 50 mM Acetate buffer, pH 5 at 37°C for 16 hr. The gel was transferred in a fresh solution of the 0.01% calcofluor white M2R, specific fluorescent chitin stain, in 0.5 M Tris-HCl pH 9. After 5 min, the gel was removed and washed several times in distilled water for 1 hr. Chitinase isozymes were visualized as cleared zones by placing the overlay gel on UV transluminator and were photographed.

7. Characterization of endo- and exochitinase

7.1 Effect of pH on endo- and exoochitinase activity

Enzyme activity, a function of pH, was determined by using 50 mM buffer at pH ranging from 3 to 9 using 1 mM of 4-MU-β-$(GlcNAc)_3$ and 4-MU-β-GlcNAc as substrates for endo- and exochitinase, respectively. pH stability was determined by pre-incubating the enzyme in 50 mM buffer (pH 3-9) for 20 min at 37°C. Enzyme activity was assayed as described above. Acetate, Phosphate and Tris-HCl buffers (50 mM) were used for the ranges of 3-5, 6 and 7-9, respectively.

7.2 Effect of temperature on endo- and exochitinase activity

The temperature optimum of the enzyme was screened at various temperatures ranging from 30°C to 70°C. The thermal stability of the enzyme was tested at various temperatures ranging from 37°C to 75°C. The enzyme was pre-incubated at the indicate temperature for 20 min and adjusted back to 4°C before assaying using 1 mM of 4-MU-β-$(GlcNAc)_3$ and 4-MU-β GlcNAc as substrates for endo- and exochitinase, respectively.

7.3 Km and Vmax value determination

Michaelis-Menten constant (Km) of endo- and exochitinase were studied using 4-MU-β-$(GlcNAc)_3$ and 4-MU-β-GlcNAc as substrates for endo- and exochitinase, respectively. The Km values were obtained from the reciprocal plot of enzyme activity and substrate concentration. The Vmax values also obtained from the same plot.

8. Protein microsequencing

Purified endo- and exochitinase solution from IEF preparation were checked N-terminal amino acid sequencing using the Edman-degradation method as performed by Scientific Equipment Center (Prince of Songkla university).

9. Purification of endo- and exochitinase

Bottom fraction membrane (BFM) was prepared from isolated bottom fraction by repetitive (4-5 times) freeze-thawing at -20 and 37°C. The pellet or BFM was obtained after centrifugation at 10,000 *g* for 20 min (4°C). BFM was extracted with 0.2% Triton X-100 in 50 mM Acetate buffer (pH 5) for overnight and then centrifuged. After centrifugation, the obtained soluble fraction was called BFM-X. SM2 was added into BFM-X fraction to remove Triton X-100. After complete preparation of sample, it was further purified in the next steps.

CM-Sepharose column (2.5 x 12 cm) was pre-equilibrated with 50 mM Acetate buffer pH 5 at flow rate of 20 ml/h at 4°C. After loading BFM-X solution, the column was washed with same buffer until the absorbance at 280 nm was nearly zero. The column was eluted with linear gradient between 0-0.5 M NaCl in the same buffer. The fractions containing high endo- and exochitinase activity were pooled, desalted and concentrated for further purification.

To further purification, the concentrated enzyme from ion-exchange chromatography was loaded onto a Sephadex G-75 column (1.5 x 110 cm), which was equilibrated with the same buffer. The flow rate 10 ml/h and fractions were collected and their absorbance at 280 nm and exo- and endochithinase activity measured.

The concentrated enzyme from Sephadex G-75 column was applied to isoelectric focusing (IEF) for isolated both enzymes. After focusing was completed, supporting gel was cut into two parts. The first part of gel was placed on the plastic box that contained with 50 mM Acetate buffer pH 5.0, while the second part of gel was fixed by fixing solution (10% TCA) and following stained for protein using Coomassie blue G 250. Then, comparing the situation of each banding protein obtained from the second part with the first part which did not stain. The situation of gel in the first part (not stain) that coincided with protein band (second part) was sliced from the gel, following extracted with the same buffer and then centrifuged to obtain the soluble protein. Finally, each fraction derived from IEF preparation was assayed for identifying enzyme activity (endochitinase or exochitinase) by base on the specific substrate in assaying.