CHAPTER II

MATERIALS AND METHODS

2.1 Materials

2.1.1 Equipments

Autoclave Model HA-3D, Hirayama Manufacturing Corporation Japan.

Centrifuge Model H-103 N Series, Kokusan : Japan.

Electrophoresis unit 2050 MIDGET, Pharmacia LKB : Sweden.

Fraction collector Model 2211, Pharmacia LKB : Sweden.

Laminar Flow Model BVT-124, International Scientific Supply Co.,Ltd. : Thailand.

Lyophilizer Flexi-Dry, Stone Ridge, New York : U.S.A.

Peristaltic pump, Pharmacia LKB : Sweden.

pH meter Model PHM83, Radiometer Copenhagen : Denmark.

Refrigerated Centrifuge Model J2-21, Beckman Instrument Inc. : USA.

Spectrophotometer Model Spectronic 20 D, Milton Roy Company.

Spectrophotometer Model DU 650, Beckman : USA.

Waring blender

Water bath Buchi 461 : Switzerland.

2.1.2 Biological materials

Angled loofah fruits, bean seeds and all plant samples were purchased form local market.

2.1.3 Chemicals

2.1.3.1 Chromatographic materials

DEAE-cellulose, Sephadex G-25, G-100 and standard molecular weight marker for gel filtration were purchased from Sigma, USA. Resin (mixed-bed) AG 501-X8 from Bio-Rad, USA.

2.1.3.2 Chemicals for electrophoresis and isoelectric focusing

Acrylamide and *N,N'*-methylene-bis-acrylamide were products of Sigma and Bio-Rad, USA. Ammonium persulfate, Coomassie Brilliant Blue R-250 and methylene blue were from Bio-Rad, USA. Glycerol, sodium dodesyl sulfate (SDS), sucrose and TEMED were obtained from Sigma, USA. Bromophenol blue and β-mercaptoethanol were from BDH Laboratory Chemical Division, England. Acetic acid and methanol were from E.Merck AG. Darmstadt, Germany. For staining chitinase activity, calcofluor white M2R from Sigma, USA and Triton X-100 was obtained from Packard instrument company INC. Standard molecular weight markers for SDS-

polyacrylamide gel electrophoresis and pI calibration standards were obtained from Bio-Rad, USA. Ampholine (pH 5-7) was from Pharmacia Fine Chemicals, Sweden.

2.1.3.3 Chemicals for agar plate assay and colorimetric method for chitinase activity

Bacto agar was purchased from Difco Laboratories, USA. *p*-dimethylaminobenzaldehyde (DMAB) from Riedel-De Haen AG. Seelze-Hannover. Snail gut enzyme (cytohelicase) was product of Sigma, USA.

2.1.3.4 Other chemicals

Chitinase (from *Serratia marcescens*), Chitin, purified chitin, glycol chitosan and *N*-acetylglucosamine were products from Sigma, USA. Disodium hydrogenphosphate, monosodium dihydrogenphosphate, sodium chloride, trisodium citrate, sodium azide, sodium acetate trihydrate and dipotassium tetraborate were purchased from BDH Laboratory Chemical Division, England. Tris-[hydroxy (methylaminomethane)] and ammonium sulfate were from Carlo erba Reagenti.

2.2 Methods

2.2.1 Extraction of chitinase from fruit pericarp

The extraction followed the procedure described by Pongdontri (1993). The pericarp from 1 kg of washed loofah fruits was weighed and homogenized in 0.02 M sodium phosphate buffer pH 7.4 containing 0.9% NaCl (PBS) (1:1 w/v) with waring blender, filtered through cheesecloth and reextracted with equal amount of the same buffer. The extracts was combined and centrifuged at 7,800 x g for 30 minutes. The supernatant was collected while the pellet was discarded. The fraction was called crude extract from fruit pericarp.

2.2.2 Extraction of chitinase from leaves

Extraction from leaves was performed according to the method described by Boller *et al.* (1988). Leaves were washed, weighed and homogenized in 100 mM sodium citrate buffer pH 5.0 (1:2 w/v) with waring blender. The extracts was centrifuged at 15,000 x g for 10 minutes. The supernatant was collected as crude extract from leaves.

2.2.3 Induction and extraction of chitinase from bean seeds

Bean seeds were steeped for 30 minutes in water and then grown on water trays containing 0.5, 1 and 2 % chitin, harvested seedling at 0, 6, 12, 18, 24, 30 and 36 hours. The extraction from bean seeds

followed the procedure described by Powning and Irzykiewicz (1965). Bean seeds from chitin inductions were homogenized in waring blender with 0.05 M citrate buffer pH 4.5 (1:2 w/v). The extraction was centrifuged 10,000 x g for 20 minutes and the supernatant was collected for chitinase assays.

2.2.4 Preparation of colloidal chitin

Colloidal chitin was prepared from commercial chitin by the method of Shimahara and Takiguchi (1988). Chitin powder (20 g) is added slowly into 800 ml of concentrated hydrochloric acid below 5°C with vigorous stirring. After homogeneous dispersion of chitin powder the mixture is heated gently up to 37°C with moderate stirring, filtered through glass wool and collected in 8 liters of deionized water below 5°C with stirring. After 30 minutes, the suspension was kept overnight below 5°C. The supernatant was decanted and the pellet washed with distilled water by centrifugation at 5,000 x g for 15 minutes until the washing become neutral. The acid-free residue was suspended in 500 ml of deionized water with vigorous stirring. The colloidal chitin solution was stored in a dark place below 5°C.

2.2.5 Preparation of glycol chitin

Glycol chitin was obtianed by acetylation of glylcol chitosan by a modication of the method of Trudel and Asselin (1988). Glycol chitosan (5 g) was dissolved in 100 ml of 10% acetic acid by grinding in a mortar.

The viscous solution was allowed to stand overnight at 22°C. Methanol (450 ml) was slowly added and the solution was vacuum filtered through a Whatman No. 4 filter paper. The filtrate was transfered into a beaker and 7.5 ml of acetic anhydride was added with magnetic stirring. The resulting gel was allowed to stand for 30 min at room temperature and then cut into small pieces. The liqiud extruding from the gel pieces was discarded. Gel pieces were transferred to a waring blender, covered with methanol, and homogenized for 4 min at top speed. The suspension was centrifuged at 27,000 xg for 15 min at 4°C. The gelatinous pellet was resuspended in 1 volume of methanol, homogenized, and centrifuged as in the preceding step. The pellet was resuspended in distilled water (500 ml) containing 0.02 % (w/v) sodium azide and homogenized for 4 min. This was the final 1% (w/v) stock solution of glycol chitin.

2.3 Detection and determination of chitinase activity

2.3.1 Dectection of chitinase on agar plate

Lytic activity was estimated by a modification of the plate method described by Roberts and Selitrennikoff (1988). The assay medium contained 0.3 % colloidal chitin, 2 % (w/v) agar and 0.02 % sodium azide in 50 mM potassium phosphate buffer pH 6.1. The medium was autoclaved, and poured into petri dish plate. After the agar had solidified, 6 mm diameter wells were made on the agar. Fifty microlitres of the chitinase samples or commercial enzyme was added to each well,

the plate was incubated at 37°C for 48 hr. Chitin hydrolysis was indicated by the appearance of clear zone.

2.3.2 Determination of chitinase activity by colorimetric method

Colorimetric asay for plant endochitinase was determined by measuring monomeric N-acetylglucosamine (NAG) from enzymatic hydrolysis of the principle products of plant endochitinase using colloidal chitin as substrate. The assay mixture consisted of 10 µl of 1 M sodium acetate buffer (pH 3.5) and 0.4 ml enzyme solution in 10 mM sodium acetate buffer (pH 5.0), the mixture was incubated in shaking water bath at 37°C, and reaction started by adding 0.1 ml of 12 mg/ml colloidal chitin. After 2 hr the reaction was stopped by centrifugation at 1,000 xg for 3 min, 0.3 ml supernatant and 30 µl of 1 M potassium phosphate pH 7.1 was pipetted into test tube and incubated with 20 µl snail gut enzyme. After incubation at 37°C for 1 hr, 100 µl of 0.8 M sodiumborate buffer (pH 9.8) was added. The mixture was incubated in boiling water bath for exactly 3 min, and then rapidly cooled in an ice-water bath. After addition of 3 ml of DMAB reagent the mixture was incubated for 20 min at 37°C and A_{585} is measured in immediately.

For each enzyme preparation (Enz) measured, an enzyme blank (EB) and an enzyme blank with internal standard (EI) are carried through the procedure. The enzyme blank contains 0.1 ml water instead of the colloidal chitin. The enzyme blank with internal standard contains 0.1 ml of 2 mM NAG instead of the colloidal chitin.

For each series of measurements, a substrate blank (SB), a reagent blank (RB), and reagent blank with internal standard (RI) are carried through the procedure. The substrate blank contains 0.4 ml of 10 mM sodium acetate buffer, pH 5.0, instead of the enzyme preparation, The reagent blank contains 0.4 ml of the same buffer and 0.1 ml water instead of colloidal chitin and reagent blank with internal standard also contains 0.4 ml of the same buffer and 0.1 ml of NAG instead of colloidal chitin. The amount of NAG equivalents released by the enzyme is calculated as followed :

$$\left[\frac{A_{585}\,(\text{Enz}) - A_{585}\,(\text{EB})}{A_{585}\,(\text{EI}) - A_{585}\,(\text{EB})} - \frac{A_{585}\,(\text{SB}) - A_{585}\,(\text{RB})}{A_{585}\,(\text{RI}) - A_{585}\,(\text{RB})} \right] 200 \text{ nmol}$$

One unit of chitinase is defined as the amount that catalyzes the release of soluble chitooligosaccharides containing 1μmol of NAG in 1 min at infinite dilution. The initial slope of the standard curve is used to calculate the unit.

2.3.3 Detection of chitinase activity in polyacrylamide gel electrophoresis (PAGE)

Detection of chitinase activity after polyacrylamide gel electrophoresis under native or denaturing conditions were performed by modification of mothods described by Trudel and Asselin (1989). Native and SDS-PAGE for detecting chitinase activity were performed at

pH 8.9 according to Davis (1964) using 12% (w/v) polyacrylamide resolving gels containing 0.01% glycol chitin and 5 % (w/v) polyacrylamide stacking gels.

2.3.3.1 Native PAGE

Samples for native PAGE contained 15 % (w/v) sucrose and 0.01 % (w/v) bromophenol blue. Samples were loaded in two identical sets and after electrophoresis, the gel was divided into two part for proteins and activity staining. For staining chitinase activity, the gel were stained in 0.01 % (w/v) Calcofluor white M2R in 500 mM Tris-HCl pH 8.9 (freshly prepared). After 5 min, the gel were destained by incubated for about 1 hr at room temperature in distilled water. Lytic zone of chitinase activity were visualized by placing the gels on UV box and photographed.

2.3.3.2 SDS-PAGE

For SDS-PAGE (Appendix B), samples was incubated at 50°C for 30 min with 15 % (w/v) sucrose and 2.5 % (w/v) SDS in 125 mM Tri-HCl (pH 6.7) with-or without 2% β-mercaptoethanol. Electrophoresis was run at constant current of 20 mA at 4°C for 65 min. Samples were loaded in two identical sets and after electrophoresis, the gel was divided into two part for proteins and activity staining. For chitinase activity stained, the gel were incubated for 2 h at 37°C in 100 mM sodium acetate buffer (pH 5.0) containing 1% (v/v) Triton

X-100 purified through a mixed-bed resin deionizing column (AG 501-X8) with reciprocal shaking. After incubation, the gel were stained and destained for lytic zone as for native gels.

2.4 Determination of protein

Protein concentration was determined by the coomassie blue method according to Bradford (1976), using bovine serum albumin as standard. One hundred microlitres of sample was mixed with 1 ml protein reagent (see Appendix A) and left for 5 min before recording the absorbance at 595 nm.

2.5 Purification of chitinase

2.5.1 Ammonium sulfate fractionations

Crude extract of loofah pericarp was subjected to sequential fractionations with ammonium sulfate (0-30, 30-50, 50-70, 70-100 %) by slow addition of solid ammonium sulfate with gentle mixing. The protein precipitate was obtained by centrifugation at 7,800 x g for 15 minutes at 4°C , redissolved in 0.02 M sodium phosphate buffer pH 7.4 and dialysed against excess amount of the same buffer at 4°C overnight. Each fraction was tested for chitinase activity and the protein content was determined.

2.5.2 Column chromatography

2.5.2.1 DEAE-cellulose chromatography

DEAE-cellulose (10 g) was swelled in 1 liter of distilled water and then washed several times at room temperature to remove the fine particles. The resin was activated by washing sequentially with excess volume of 0.5 M HCl for 30 min followed by distilled water until the pH was 7.0. The activated resin was equilibrated with 0.02 M sodium phosphate buffer pH 7.4 overnight. The prepared DEAE-cellulose was packed into a column (2.5 x 20 cm) at the height of 16 cm. The column was equilibrated with 0.02 M sodium phosphate buffer pH 7.4 and then the 30-70% ammonium sulfate fraction of pericarp extract was dialysed against excess volumn of 0.02 M phosphate buffer pH 7.4 at 4°C overnight and loaded onto DEAE-cellulose column at flow rate 30 ml/hr. The column was washed with equilibrated buffer until A_{280} was negligible, then the column was subjected to 300 ml of 0-0.6 M NaCl linear gradient elution. Fractions of 5 ml were collected for measurement of A_{280} and chitinase activity.

2.5.2.2 Gel permeation chromatography

Sephadex G-100 was swellen in boiling distilled water for 5hr. The swollen gel was degassed before packing in column (1.8 x 50 cm). The column was equilibrated with 0.02 M sodium phosphate buffer (pH 7.4) at constant flow rate of 12 ml/hr using a

peristaltic pump. Concentrated enzyme (1.0 ml) was applied to the column and eluted with equilibrated buffer. Fractions of 2 ml were collected. The absorbance at 280 nm was measured.

2.6 Polyacrylamide Gel Electrophoresis (PAGE)

After electrophoresis as described in 2.3.3 both nondenaturing and denaturing PAGE were stained for proteins in the staining solution (0.2 % Coomassie brillaint blue R-250, 50 % methanol and 10 % acetic acid) for at least 2 hr. The gel was then destained with destaining solution containing 50% methanol and 10% acetic acid (see Appendix B) until the background was cleared.

2.7 Determination of molecular weight of chitinase

2.7.1 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was carried out as described by Bollag and Edelslein (1991). Three volumes of sample were boiled with one volume of sample buffer, containing 1% (w/v) SDS, 4% glycerol, 1% β-mercaptoethanol and trace amount of bromophenol blue in 62 mM Tris-HCl pH 6.8 for 5 minutes. Twenty micrograms sample was loaded on discontinuous SDS-PAGE with 12% separating gel and a 5% stacking gel prepared as described in appendix B. The electrode buffer used was 25 mM Tris in 192 mM glycine, pH 8.3 with 0.1% SDS. The electrophoresis was performed with constant current 20 mA/slab gel in

Bio-Rad Mini Protein II Dual Slab cell until bromophenol blue reached the bottom of the gel.

The protein bands on the gel were fixed and stained with 0.2% Coomassie Brilliant Blue R-250 in 50% methanol and 10% acetic acid until the background was clear.

Relative molecular weight of the protein was estimated from standard curve plotted on semilog scale between the molecular weight of protein markers included phosphorylase b (97,400), BSA (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), trypsin inhibitor (21,500) and lysozyme (14,000).

2.7.2 Gel filtration on Sephadex G-100

Gel filtration was performed at room temperature on colum of Sephadex G-100 (50 x 1.8 cm) with flow rate of 12 ml/hr and fractions of 2 ml were collected. The molecular weight calibration curve was produced from acid phosphatase (95,000), BSA (68,000), ovalbumin (43,000), chymotrypsinogen (23,240), cytochrome c (12,380). Protein in each fraction was monitored with A280. Phosphate buffer saline (0.2 M) was used in equilibration of the column during the run of standard proteins.

2.8 Isoelectric focusing polyacrylamide gel

Isoelectric focusing polyacrylamide gel was performed on Bio-Rad mini IEF system, using a sheet of gel support film for acrylamide

(Bio-Rad) attached to the glass plate. A mixture of 5.4 % acrylamide, 0.2% bisacrylamide, 11.86% sucrose and 1.98% ampholine (pH range 5-7), 0.06 % (v/v) TEMED and 0.1 mM ammoniumpersulfate (see Appendix C) was pipetted through the space between the glass plates and the gel support film was assembled on casting tray with precaution to avoid air bubble. The gel was left to polymerize for 1 hr at room temperature, then lifted from the casting tray with a flat spatula. Salt free purified samples were applied by micropipette on a 0.5 x 1.0 cm filter paper which were laid individually in row at the middle of the gel. The samples were allowed to diffuse into the gel for 15 minutes. The gel was then turned upside down and placed directly on top of the graphite electrodes of the Mini IEF Cell (Bio-Rad Model III). Electrofocusing was carried out under constant voltage in a stepwise increase of 100 volt for 15 minutes, 200 volt for 15 minutes and 450 volt for 1 hr. A mixture of pI calibration standards composed of Phycocyanin (4.65), β-lactalbumin B (5.10), bovine carbonic anhydrase (6.00), human carbonic anhydrase (6.50), Equine myoglobin (7.00), human hemoglobin A (7.10), human hemoglobin C (7.50), lentin lectin (7.80, 8.00, 8.20) and cytochrome c (9.60) was included in the electrophoretic run.

For protein staining, the gel on the gel support film was placed into staining solution (0.5 % $CuSO_4$, 0.04 % Coomassie Brilliant Blue R-250, 10 % acetic acid and 27 % ethanol) overnight. The gel was then washed with destaining solution (12 % ethanol, 7 % acetic acid and 0.5 % $CuSO_4$) until the background was cleared (see Appendix C). Isoelectric pH (pI) of the protein was estimated from the standard curve

plotted between the pI and migration distance from the cathode of the standard proteins.

2.9 Optimum pH of chitinase

The partial purified enzyme was used to study the effect of pH on the enzyme activity. The chitinase activity was measured at various pH by colorimetric method as described in 2.3.2 , using colloidal chitin as a substrate. The enzyme was incubated in 1 M citrate buffer at pH 3, 3.5, 1 M acetate buffer at pH 3.5, 4, 4.5, 5 , 1 M phosphate buffer pH 5, 6, 7 and 1 M Tris-HCl at pH 7, 8 and 9.

2.10 Optimum temperature of chitinase

The partial purified enzyme was used to study the effect of temperature on the chitinase activity. The enzyme was incubated in 1 M citrate buffer pH 3.5 at different temperatures at 25, 30, 35, 37, 40, 50 and 60 °C. The reaction was followed by measuring chitinase activity with colorimetric method as described in 2.3.2, using colloidal chitin as a substrate.

2.11 Substrate specificity of chitinase

The partial purified enzyme was used to study substrate specificity for chitinase activity. The enzyme was incubated in buffer at 37 °C for 2 hr, using colloidal chitin, purified chitin, glycol chitin

and glycol chitosan as substrate. The reaction was followed by colorimetric method as described in 2.3.2.