CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Biological materials

Angled loofah seeds and fruits were purchased from local market.

Strains of fungal pathogen ; *Tricoderma viride*, *Aspergillus flavus*, *Curvularia lunata*, *Cercospora kikuchii*, *Fusarium oxysporum*, were provided from the Plant Pathology, Microbiology Division, Department of Agriculture, Kasetsart University and Microbiological Resources Center for Southeast Asia (Bangkok , MERCEN).

Rabbits were obtained from the Faculty of Veterinary Science, Chulalongkorn University.

2.1.2 Chemicals

2.1.2.1 Chromatographic materials

Chitin, triacetyl-chitotriose on agarose bead,DEAE-Cellulose were purchased from Sigma. Sephadex G-25, Polybuffer exchanger 94, polybuffer 74, Sepharose 6B and blue dextran were products of Pharmacia Fine Chemicals. DNP-lysine and standard molecular weight markers for gel filtration were purchased from Sigma.

2.1.2.2 Chemicals for electrophoresis and isoelectricfocusing

Acrylamide and N,N'-methylene-bis-acrylamide were products of Sigma and Biorad. Ammonium persulfate and Coomassie Brilliant Blue R-250 were from Biorad. Glycerol and sodium dodesyl sulfate (SDS) were obtained from Sigma. Bromophenol blue and β-mercaptoethanol were from BDH chemicals Ltd. Acetic acid and methanol were from Merck. Standard molecular weight markers for SDS-polyacrylamide gel electrophoresis and pI calibration standards were obtained from Biorad. Phamalyte (pH 5-7) was from Pharmacia Fine Chemicals.

2.1.2.3 Other chemicals

Disodium hydrogenphosphate, monosodium dihydrogenphosphate, sodium chloride, trisodium citrate, formaline and riboflavin were from BDH Chemicals Ltd. Ammonium sulfate and Tris-[hydroxy (methylaminomethane)] were purchased from Fluka. Trypsin,xanthine, xanthine oxidase, cytochrome c, nitroblue tetrazolium (NBT) and anthrone were products of Sigma. Sulfuric acid was from Merck.

2.1.2.4 Carbohydrates

Monosaccharides : glucose, galactose, fructose, manitol and sorbitol were purchased from Fluka. Fucose, xylose, mannose, arabinose, manosamine, galactosamine, ribose, 2-deoxyglucose, D-galacturonic acid, N-acetyl-D-galactosamine, and N-acetyl-D-glucosamine were obtained from Sigma.

Disaccharides : Sucrose, lactose and moltose were products of Sigma.

Polysaccharides : chitin, chitotriose, starch and glycogen were purchased from Sigma and chitosan were provided by Srinakarinwirot Prasanmitr University.

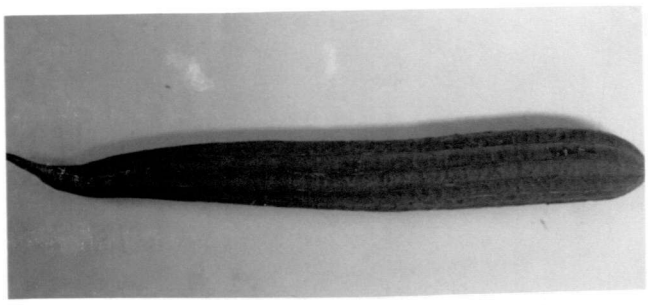
2.2 METHODS

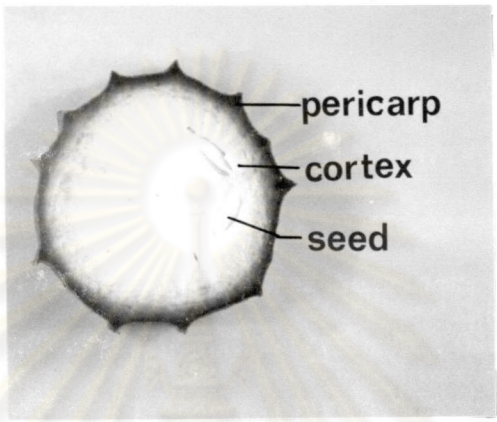
2.2.1 Extraction of lectin from fruit pericarp

The pericarp from 1 kg of washed loofah fruits was weighed and homogenized in 0.02 M 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 were combined and centrifuged at 7,800 g for 30 minutes. The supernatant was collected while the pellet was discarded. The fraction was called crude extract.

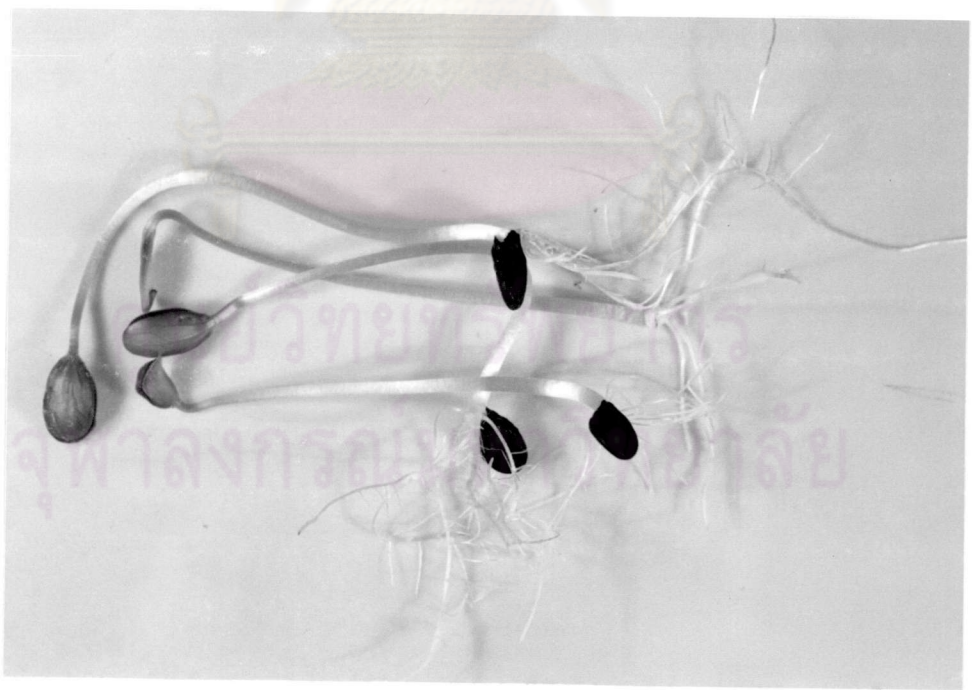
2.2.2 Extraction of lectin from loofah seedling

The extraction followed the procedure described by Skubatz and Kessler (1984). Loofah seeds were sterilized with 40% chlorox for 15 minutes and washed with sterile water. Sterilized seeds were sown on top of the moistened-autoclaved paper in steriled plastic boxes. The seedlings were grown at 25°C in an environment-controlled chamber under a 12-hr photoperiod. Standard grown-light lamp (1200 lux by Digicon Lux meter LX-50) served as light source. Seedlings were collected at the age of 0-11 days. Each seedlings were divided into three parts; cotyledon, hypocotyl and root. Each part was extracted for surface materials with sterile distilled water (1:5 w/v) by gentle agitation for 91 minutes. The solution was





A. Angled loofah fruit & its X-section.



B. 9 day-old angled loofah seedlings.

Figure 1 Angled loofah fruit and seedlings.

filtered through cheesecloth, lyophylized and redissolved in PBS (2:1 w/v). The solution obtained was called surface extract.

After subjected to surface washing, the remaining cotyledons, hypocotyls and roots were ground in a cold mortar with steriled distilled water (1:1 w/v). The suspension was filtered through cheesecloth, lyophylized and dissolved in PBS (2:1 w/v). These fractions were the homogenates of each part of the seedlings.

2.2.3 Procedure of surface washing and homogenization of the washed roots

Sample of roots were extracted four times in distilled water at ambient temperature (Skubatz & Kessler,1984), each time at the ratio of 1:10 (w/v). Fraction I was firstly obtained from immersing the root in water for 1 minute. Three following washes (fraction II-IV) were obtained by succesive immersion at 30-minute interval in three changes of water. Concurrently, another sample of root with the same surface area was immersed in water for a single 91 minutes period (fraction V). Each fraction (fractions I - V) was filtered and lyophylized to dryness. The dried material was dissolved in PBS at the ratio 2:1 (w/v) to yeild the crude surface extracts and tested for hemagglutinating activity. After the forth wash, the roots were homogenized in distilled water (1:1 w/v) with mortar (fraction VI). The homogenate was filtered, lyophylized and dissolved in PBS (2:1 w/v) and tested for residual hemagglutinating activity.

2.2.4 Preparation of trypsinized rabbit erythrocytes

Blood sample (5 ml) was drawn from rabbit ear's vein and

transferred into a test tube containing 1.25 ml anticoaggulant (1.32g% trisodium citrate). The plasma was removed by centrifugation at 500 g for 5 minutes. The rabbit erythrocytes were washed three times with PBS and suspended in 1 ml PBS. Equal volume of trypsin (1 mg/ml) was added, mixed and incubated at 37°C for 90 minutes. After incubation, the trypsinized rabbit erythrocytes were washed at least four times with excess PBS and made up to 2% suspension in the same buffer.

2.2.5 Hemagglutination assay (HA)

HA was determined by the method of Lis & Sharon (1973). Two fold serial dilution of 25 µl of lectin solution was prepared in a microtiter plate with PBS as diluent. To each well, 25 µl of 2% suspension of trypsinized rabbit erythrocytes in PBS was added and mixed well by slight agitation. After incubating for 30 minutes at room temperature, agglutination patterns were visually determined in comparison with a control reaction containing PBS and 2% trypsinized rabbit erythrocytes.

Hemagglutinating activity (HA) was the highest titer value of lectin showing agglutination. Specific HA was the highest titer per mg protein where the titer value was the reciprocal of dilution factor.

2.2.6 Sugar inhibition test

Two fold dilutions of 25 µl of 500 mM sugar solutions were prepared on a microtiter plate with PBS as diluent. Equal volume of lectin solution (25 µl) adjusted to a titer of 4 was added to each well, mixed with slight agitation and left for 30 minutes. Then 25 µl of 2 % trypsinized rabbit erythrocytes was added and the agglutina-

tion pattern observed.

Incubations of PBS with 25 μl each of either lectin or sugar solution and 2% trypsinized rabbit erythrocytes were carried out as control. The minimal concentration of the sugar which can inhibit the hemagglutination activity was recorded.

2.2.7 Ammonium sulfate fractionations

Crude extract of loofah pericarp was subjected to sequential fractionations with ammonium sulfate (0-30%, 30-50% and 50-70%) by addition of appropriate amount of solid ammonium sulfate with gentle mixing. The precipitate in each step of fractionation was collected by centrifugation at 7,800 g for 15 minutes at 4°C, redissolved in PBS and dialysed against excess amount of PBS at 4°C overnight. Each fraction was tested for hemagglutinating activity with 2% trypsinized rabbit erythrocytes, and the protein content was determined.

2.2.8 Column chromatography

Several techniques of column chromatography were employed in the attempt to purify the chitin specific lectin.

2.2.8.1 Chitin affinity column

Chitin of uniform particle size (100 mesh size) prepared according to section 2.2.11 was packed into a 1.4 X 5 cm column and equilibrated with 0.02 M phosphate buffer pH 7.4 (PB). Twelve micrograms of protein from 30-50% ammonium sulfate fraction

of pericarp extract or 0.86 mg protein of root surface extract was loaded to the column and washed with equilibrating buffer at the flow rate of 10 ml/hr. After the unbound protein were completely washed out, the column was eluted with 100 mg/ml chitin (prepared according to section 2.2.11) in the same buffer. The chitin eluted fraction was then passed through a sephadex G-25 column by spinning or filtered through millipore (ultrafree-PF low binding cellulose membrane) to remove oligosaccharide hepten, chitin. The fractions were then assayed for hemagglutinating activity. The HA possitive fractions were pooled and stored at -20°C for further study.

2.2.8.2 Triacetylchitotriose column

Triacetylchitotriose immobilized on beaded agarose was packed in 1 cm X 1 cm column. The gel was equilibrated with phosphate buffer pH 7.4. The 30-50% ammonium sulfate fraction of pericarp extract (12 mg) or 0.86 mg of root surface fraction was passed through the column at a constant flow rate of 10 ml/hr. Unbound proteins were completely washed out with equilibrating buffer as monitored by absorbance at 280 nm. The column was then eluted with 100 mg/ml chitin in equilibrating buffer and collected in 1 ml fractions. The protein profile was monitored by measuring absorption at 280 nm. The eluted fractions were treated in the same manner as fractions from chitin column described in 2.2.8.2.

2.2.8.3 DEAE-Cellulose chromatography

The 30-50% ammonium sulfate fraction of pericarp was dialysed against excess volume of 0.02 M phosphate buffer pH 7.4 at

4°C overnight and loaded onto a DEAE-Cellulose column (12 X 1.6 cm) preequilibrated with 0.02 M phosphate buffer pH 7.4 with a flow rate of 40 ml/hr. The column was washed with the same buffer until the absorbance at 280 nm was negligible , then the column was subjected to 100 ml of 0-0.6 M of NaCl gradient. Fractions of 2 ml were collected throughout the chromatographic run. Hemagglutinating activity and A_{280} was determined for every other fractions, the active fractions were pooled.

2.2.8.4 Chromatofocusing column

Sample from the DEAE-Cellulose column was dialysed overnight against excess volume of 25 mM imidazole-HCl buffer, pH 7.4 at 4°C, and applied to a column (14 X 1.0 cm) of polybuffer exchanger 94 (Pharmacia) preequilibrated with 25 mM imidazole-HCl buffer pH 7.4. The column was eluted with fourteen column volumes of polybuffer 74 (dilution 1:8 in distilled water), at a flow rate of 42 ml/hr, 1 ml fractions were collected . After that the column was washed with 25 mM imidazole and 1 M NaCl, respectively to elute the proteins with pI values lower than 4. Absorbance at 280 nm and pH's were measured. To remove the polybuffer, the pooled fractions of each peak was dialysed against saturated ammonium sulfate solution overnight 4°C. The protein precipitates were collected by centrifugation at 7,800 g for 15 minutes, redissolved in PBS and dialysed in excess volume of PBS at 4°C overnight to remove ammonium sulfate. The fractions were stored at -20°C for further analysis.

2.2.9 Non Denaturing Polyacrylamide Gel Electrophoresis

Discontinuous nondenaturing polyacrylamide gel electrophoresis (ND-PAGE) was employed for several purposes in this study.

2.2.9.1 Identification of lectin on gel slices

Discontinuous polyacrylamide gel electrophoresis was performed according to Ornstein and Davis on slab glass plates. The separating gel (15 X 12 X 0.15 cm^3) consists of 7.5 % acrylamide and the stacking gel (15 X3 X 0.15 cm^3) consists of 3% acrylamide prepared as described in Appendix A. The electrode buffer was 25 mM Tris in 192 mM glycine pH 8.3 (Appendix A). Samples were diluted 3:1 with sample buffer (62 mM Tris-HCl pH 6.8, 4 % glycerol and trace of bromophenol blue). The electrophoretic migration proceeded from cathode towards the anode with constant current of 30 mA/plate at 15°C in an LKB 2001 Vertical Electrophoresis apparatus connected to a thermostat water-bath (LKB 2209 Multitemp).

When the electrophoresis was completed, the gel was immediately removed from the glass plates and the identical run of samples were longitudinally cut, one for protein staining and another was transversely cut into 2 mm slices from the top to the end of the separating gel and individually soaked in 0.2 ml PBS (Namjuntra,1985). Hemagglutinating activity of each slice was tested. The active fractions were then tested for chitin specificity.

2.2.9.2 Purification by protein elution from gel slice

To purify the pericarp lectin by elution from gel slice,

the electrophoresis was done as described in section 2.2.9.1. The pre-identified band position was cut into 4 mm slice, then cut into small pieces and the protein was eluted by Biorad Electroeluter Model 422 using PBS as eluent. The gel pieces were put into the glass tube with the membrane cap below, then 2 mA constant current per glass tube was applied. After 4 hour of elution, glass tubes were removed and eluted protein in the membrane cap was collected and pooled. Hemagglutinating activity was assayed. This fraction was called purified pericarp lectin.

2.2.9.3 Monitering proteins in purification procedure

The purity of the protein in each purification step was monitored by ND-PAGE in Biorad Mini-Protein II Duel Slab cell electrophoresis apparatus.The separating gel(7 X 6 X 0.05 cm) contains 12.5% acrylamide and the stacking gel (7 X 2 X 0.05 cm) contain 3 % (w/v) acrylamide. The preparations and compositions of which are included in Appendix A. The electrode buffer was 25 mM Tris and 192 mM glycine pH 8.3 (see Appendix A). The samples were diluted with sample buffer (3:1) (sample buffer composed of 62 mM Tris-HCl pH 6.8, 4 % glycerol and trace of bromophenol blue). The electrophoresis was carried out with a constant current for 15 mA per slab gel.

After the electrophoresis , the gel was 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 A).

2.2.10 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was carried out as described by Laemmli (1970). Three volumes of sample were boiled with one volume of solubilizing medium 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 2 minutes. The treated sample was loaded on discontinuous SDS-polyacrylamide slab gel with 12.5 % separating gel and a 3 % stacking gel (7 X 2 X 0.05 cm) prepared as described in Appendix B. The electrophoresis was performed with constant current of 15 mA/slab gel in Biorad Mini Protein II Dual Slab cell until bromophenol blue reached the bottom of the gel. The electrode buffer used was 25 mM Tris in 192 mM glycine, pH 8.3 with 0.1% SDS.

The protein bands on the gel were fixed and stained overnight 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 and the relative electrophoretic mobility. The 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).

2.2.11 Preparation of soluble chitin

Soluble chitin was prepared according to the method of Bloch and Burger (1974). Chitin in the form of ground clam shells was blended with a blender, sieved through a sieve No.100 and washed successively with water, 0.05 M HCl, 1 % Na_2CO_3 and 95 % ethanol by

suction through filter paper (Whatman # 1) in a buchner funnel and allowed to dry in the oven at 60°C. The resulted powder was called chitin powder.

To prepare the soluble chitin, the chitin powder was then hydrolysed with cold concentrated HCl at the ratio of 1:10 (w/v) at 0°c. To avoid browning of the solution, the hydrolysis reaction was maintained at 4°C overnight, after which the pH was brought to 7 with cold 50% NaOH. During the pH adjustment the temperature was kept at 0°C. The precipitate of unhydrolysed chitin and NaCl was removed by filtration and washed with a small volume of water. The filtrate or chitin hydrolysate was lyophylized. The resulting white powder was solubilized in distilled water or buffer and used for sugar specificity test or purification of lectin.

2.2.12 Preparation of formalinized rabbit erythrocytes

Formalinized erythrocytes were prepared as described by Nowak and Barondes (1975). Trypsinized rabbit erythrocytes were prepared according to section 2.2.4. The cells were suspended in PBS at 8% (v/v). Equal volume of formaline (3 % solution in PBS with pH adjusted to 7.4 with 0.1 M NaOH) was added. The mixture was incubated at 37°C for 16 hours with moderate shaking. The cells were then washed 4 times in 5 volumes of PBS per pack cell volume and stored at 4°C as a 10 % suspension in the same buffer. They could be used in the agglutination assays for months after preparation.

2.2.13 Purification using formalinized trypsinized rabbit erythrocytes

The purification procedure was modified from Reitherman *et al* (1974). Predetermined amount of sample and formalinized erythrocytes were combined and PBS was added to bring the final volume to 30% (v/v) cell suspension, which was incubated at room temperature for 30 minutes with gentle shaking. The cells were then washed 4 times with 10 pack cell volumes of PBS by suspension and centrifugation at 650g for 5 minutes. The lectin was eluted by agitating the washed cells (30% v/v) in PBS containing 100 mg/ml chitin. After 30 minutes, the supernatant was collected by centrifugation at 1,800 g for 5 minutes and dialysed against large volume of distilled water to remove the chitin. The formalinized erythrocytes could be washed and reused for another purification. The dialysed sample was lyophylized and redissolved in small volume of PBS.

2.2.14 Molecular weight determination by gel filtration on Sepharose 6B column

Gel filtration was performed at room temperature on column of Sepharose 6B (50 X 1.5 cm) with flow rate of 15 ml/hr and fractions of 2 ml were collected. The molecular weight calibration curve was produced from thyroglobulin (MW 669,000), alcohol dehydrogenase (MW 150,000), BSA (MW 68,000), ovalbumin (MW 43,000), chymotrypsinogen (MW 23,240), myoglobin (MW 17,000) and cytochrome c (MW 12,380). Protein in each fraction was monitored with A_{280}. Phosphate buffer saline (0.02 M) was used in equilibration of the column during the run of standard proteins. PBS with 5% chitin was used when lectin

was applied to the column.

2.2.15 Isoelectric focusing polyacrylamide gel

Isoelectric focusing polyacrylamide gel was performed on Biorad Mini IEF System, using a sheet of gel support film for acrylamide (Biorad) 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 assembled on casting tray with precaution of avoiding 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 fiter 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 (Biorad 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), lentil lectin (7.80 , 8.00 , 8.20), 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, 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.

2.2.16 Thermostability test

Equal amount of the lectin was incubated for 20 minutes in a water bath at different temperatures from 0-120°C. Then each sample was rapidly cooled in ice and assayed for hemagglutinating activity in comparison to the lectin which was kept at -20°C. Result was expressed as percentage of hemagglutinating activity of the lectin incubated at room temperature.

2.2.17 pH stability test

Purified lectin was dialysed against distilled water and lyophylized prior to being dissolved in buffer with different pH values from 3-10. The buffer used were 0.02 M citrate buffer (pH 3.0, 5.0), 0.02 M phosphate buffer (pH 6.0,7.0,8.0), 0.02 M Tris-HCl (pH 8.0,9.0) and 0.02 M glycine-NaOH (pH 10.0). After incubation for 1 hr at room temperature, hemagglutinating activity was examined. The result was expressed as percentage of HA in comparison to the control which was dissolved in PBS at pH 7.4.

2.2.18 Inhibition of fungal growth by cellophane-transfer technique

Inhibition effect on the spore germination of fungus can be

tested following the method of Neely & Himelick (1966).

2.2.18.1 Spore preparation

The test fungi; *Trichoderma viride, Aspergillus Flavus Curvularia lunata, Cercospora kikuchii* and *Fusarium oxysporum*, were grown in 9 cm culture plate on potato dextrose agar(PDA) (see Appendix D) at room temperature for 14,21 or 28 days, depending upon the maturation of conidia. The colonies were flooded with steriled-distilled water and agitated with rubber policeman. The spore suspension was filtered through steriled cotton wool into a sterile tube and spore concentration was appropriately adjusted according to the following standards : large spore, 20 per field at 100 x magnification ; moderatly large spore, 5 per field at 400 x magnification ; small spore, 10 per field at 400 x magnification.

2.2.18.2 Fungal growth inhibition test

Cellophane discs were cut from folded sheet of cellophane with a paper punch, placed in distilled water, and filter paper discs were cut from Whatman # 1 into 12.7 mm diameter disc , then sterilized by autoclaving.

Four sets of triplicated cellophane discs were prepared on top of the moistened filter paper on microscope slide, for a series of test concentrations desired : 2,4,6 μl of test samples and 4 μl of the test sample with 5 μg of chitin were added to each set of cellophane discs, respectively. Approximately 0.4 l of spore suspension of the test fungus was seeded on the cellophane disc by lightly touch the end of the capillary tube with a 0.85 mm inside

diameter to the cellophane and incubated at the room temperature in steriled moistened chamber. The inhibition of fungal growth were determined at the appropriate times after cellophane discs were seeded as follow : *T. viride* for 18 hr, *A. flavus* for 21 hr, *C. lunata* for 8 hr, *C. kikuchii* for 18 hr , and *F. oxysporum* for 24 hr.

After the incubation, each set of cellophane discs was transfered from the filter paper to other microscope slide and stained with lactophenol aniline blue and covered with two 18 mm square covered slip. A drop of water was placed at the margin of each cover slip. Degree of inhibition of fungal growth was determined from the number of nongerminated spore or spore which had germ tubes less than half of the spore length. The result was expressed as percentage of the nongerminated spore to total spore in the same field.

2.2.19 Detection of Superoxide Dismutase (SOD) activity

2.2.19.1 Spectroscopic method by xanthine-xanthine oxidase system

SOD activity was determined based on SOD inhibition of superoxide-mediated cytochrome c reduction (McCord & Fridovich, 1969). A 1 ml assay mixture consists of 41.5 mM phosphate buffer pH 7.8 (equilibrated with oxygen by an air pump at least 1 hr before used), 1μM xanthine, 0.2μM cytochrome c (see Appendix E). Appropriate amount of xanthine oxidase was added to start the reaction which was performed at 25°C. Cytochrome c reduction was measured by the increase in absorbancy at 550 nm. The amount of xanthine oxidase was adjusted to give the rate of 0.02 A_{550}/min. When SOD was to be assessed, it was included in the reaction mixture.

One unit Of SOD activity was expressed as the amount of enzyme that caused 50% inhibition of cytochome c reduction.

2.2.19.2 SOD activity stain on ND-PAGE

ND-PAGE was performed on 7.5% acrylamide gel on a Biorad Mini Protein II Dual Slab cell electrophoresis as described in section 2.2.9.3. Two identical sets of samples were run on the gel. After the electrophoresis was completed , the slab gel was removed and the gel was cut to separate the identical set of samples, one for protein stain with Coomassie brilliant blue and another for SOD activity stain. Position of SOD were visualized by photochemical nitroblue tetrazolium (NBT) stain (Beauchamp & Fridovich,1971). The gel was submerged in 2.45 mM NBT solution at room temperature in a light protected box for 15 minutes. Then, transfered to a reaction mixture consisting of 0.028 M TEMED and 28 μM riboflavin in 0.036 M phosphate buffer pH 7.8 which was preequilibrated with air at least 1 hr before used (see Appendix E). This step was carried out for 20 minutes without light exposure. At the end of incubation period, the gel was placed under a fluorescent lamp. Photoreduction of NBT mediated by oxygen radicals turned the background staining of the gel into dark blue except at the position where SOD was present. SOD scavenged the local oxygen radicals preventing photoreduction of NBT in the area , thus , appeared in the gel as colorless band.

2.2.20 Protein determination

Protein concentration is determined by Coomassie brilliant blue G-250 method (Bradford,1976). Coomassie blue G-250 solution

was prepared by dissolving 100 mg Coomassie brilliant blue G-250 in 50 ml 95 % ethanol following by 100 ml 85 % (w/v) phosphoric acid and made up to 1l with water. Alternatively Biorad protein assay kit II has been used.

An aliquot of 100 μl of protein solution was added to 5 ml of Coomassie brilliant blue G-250 solution for 10-100 μg protein assay or 1 ml for 1-10 μg assay. The absorbance at 595 nm was measured after 2 minutes and before 1 hr of incubation. Bovine serum albumin was used as standard protein.

2.2.21 Determination of carbohydrate by anthrone reaction

Amount of carbohydrates can be quantitated by antrone reaction. Anthrone (0.2 g) was dissolved in 100 ml conc.sulfuric acid and left at room temperature for 30 minutes before use. Sample (0.5 ml) was mixed with 5 ml of anthrone solution and observed the green color at A_{625} within 5 minutes. Sugar concentration was determined by using glucose as standard.