Materials and Methods

2.1 Bacteria.

Nitrogen-fixing bacterial strains R15 and R17 were *Klebsiella spp* . and isolated from the rhizosphere of wetland rice in Thailand (Harinasut, 1983) and maintained at the Department of Biochemistry, Faculty of Science, Chulalongkorn University. The bacterial cultures used as references were as . follows : *Klebsiella pneumoniae* M5a1, *Klebsiella oxytoca* NG13 (Hirota *et al*, 1978), *Azospirillum lipoferum* FS (Ladha *et al*, 1982), and *Pseudomonas sp.* H8 (Watanabe and Barraquio, 1979; Barraquio *et al*, 1983).

2.2 Rice.

Breeder seeds of *Oryza sativa* L. cultivar RD5 and RD7 were kindly supplied by the Rice Germplasm Bank, Ministry of Agriculture and Cooperative, Thailand. Other references seeds ; IR42, IR58 and Hua-chou-chi-mo-mor (HCCMM) were kindly supplied by the International Rice Research Institute (IRRI), the Philippines.

Foundation seeds of RD7 used in the preparation of rice lectin were obtained from the Department of Rice Research, Ministry of Agriculture and Cooperative, Thailand.

2.3 Radioactive isotopes and chemicals for scintillation counting.

[1-^{14}C]Acetic anhydride in 20% w/w toluene solution of 250 μCi (10-30 mCi.m mol^{-1}) and N-acetyl-D-[1-^{14}C] glucosamine (5.79 mCi.m mol^{-1}) were purchased from Amersham. Chemicals in scintillation cocktail, 2,5-diphenyl oxazole (PPO), 1,4-bis-2-(5-phenyloxazolyl)-benzene (POPOP) and Triton X-100, were obtained from Packard Instrument Co.Inc.

2.4 Chemicals for epi-fluorescence and elctron microscopy.

The following products were from Electron Microscopy Sciences : sodium cacodylate trihydrate, glutaraldehyde (50% biological grade), paraformaldehyde, vinylcyclohe ene dioxide, diglycidyl ether of

polypropyleneglycol, nonenyl succinic anhydride and dimethylaminoethanol. Acridine orange (microscopy grade) and gold trichloride acid trihydrate were supplied by E.Merck. Osmium tetroxide and polyethylene glycol 20,000 were obtained from Sigma.

2.5 Enzymes, sugars and culture media.

Glucosidase, β-neuraminidase, β-N-acetyl-D-glucosaminidase, trypsin, mannosamine, glucose, glucosamine and N-acetyl-D-glucosamine were from Sigma. Bacto-agar, tryptone and yeast extracts were products from Difco Laboratories.

2.6 Materials for gas chromatography.

Stainless steel column (2 m x 1/8 in) of Porapak N (80-100 mesh) was from Water Associates, Inc. USA. Compressed air and hydrogen gas were obtained from the Military Science Department. Standard ethylene gas and oxygen-free nitrogen were purchased from Thai Industrial Gases Ltd. Acetylene was from Ratanachoke Engineering Ltd.

2.7 Materials for column-chromatography.

Sources of gels were as follows : epoxy-activated Sepharose 6B, Sephadex G-25 and Sephadex G-100 from Pharmacia Fine Chemicals; immobilized N-acetyl-D-glucosamine-Sepharose 6B (Selectin 1) from Pierce Chemicals Co ; chitin from Sigma.

2.8 Materials for polyacrylamide gel electrophoresis.

Acrylamide, N,N'-methylene bisacrylamide, tris-(hydroxymethyl) aminomethane and glycine were products of Sigma. TEMED was from BDH Chemical Ltd. Sodium dodecyl sulfate was from Bio-Rad Ltd. EDTA and 2-mercaptoethanol were purchased from Fluka A.G.. Coomassie brilliant blue R-250 was from Serva. Acetic acid and methanol were from J.T. Baker Chemicals Co.

Chemicals for isoelectrofocusing gel electrophoresis were purchased from the following sources; agarose from Sigma, Ampholine from LKB, Nonidet P-40 from Shell Chemicals, urea from Schwarz and Mann, orthophosphoric acid from J.T. Baker Chemicals, and sodium hydroxide from EKA AB. Sweden.

Standard molecular weight protein markers for electrophoresis which include bovine serum albumin, ovalbumin, phosphorylase b, trypsinogen and myoglobin were purchased from Sigma.

2.9 Preparation of culture media.

2.9.1 Rich medium (Luria *et al*, 1960). The composition of rich medium (RM) is as follows :

tryptone	10	$g.l^{-1}$
yeast extract	5	$g.l^{-1}$
sodium chloride	10	$g.l^{-1}$
glucose	2	$g.l^{-1}$

All ingredients were dissolved in distilled water, then the pH was adjusted to 7.0 with 1 N NaOH. For solid medium, Bacto-agar was added 15 $g.l^{-1}$. Sterilization was performed by autoclaving at 121^{o}C for 15 min.

2.9.2 Nitrogen-free medium (Dobereiner, 1977). The composition of nitrogen-free medium (NF) were as follows:

dipotassium hydrogen phosphate	0.05	$g.l^{-1}$
potassuim dihydrogen phosphate	0.15	$g.l^{-1}$
calcium chloride	0.01	$g.l^{-1}$
magnesium sulfate	0.002	$g.l^{-1}$
sodium molybdate	0.002	$g.l^{-1}$
ferric chloride	0.01	$g.l^{-1}$
sodium bicarbonate	0.01	$g.l^{-1}$
glucose	20	$g.l^{-1}$

Each ingredient was dissolved in enough distilled water and autoclaved separately for 15 min. These solutions were then mixed aseptically before used.

2.10 Maintenance of bacterial cultures.

2.10.1 Short-term maintenance. All bacterial strains were maintained on RM agar medium and subcultured every week.

2.10.2 Long-term maintenance. The bacterial cultures were maintained for years by 2 methods ; a) freeze-drying under aseptic condition and b) kept under sterile glycerol 15% v/v at - 70 ^{o}C.

2.11 Cultivation of nitrogen-fixing bacteria.

A single colony from RM agar was inoculated into a 5 ml RM broth, incubated in a rotary shaking water-bath at 37 ^{o}C overnight and used as starter. A 1% inoculum from starter was inoculated into 200 ml NF broth in 500 ml Erlenmeyer flask, incubated in a rotary shaking water-bath at 37 ^{o}C for 20-24 h. Cells were collected and washed with sterilized PBS pH 7.4 in a MSE bench-top centrifuge by spinning at full speed for 5 min.

2.12 Preparation of rice seedlings.

Healthy rice seeds were soaked in tap water for 2 h,surfaced sterilized in 95% ethanol for exactly 1 min, followed by thoroughly rinse with sterile distilled water, and soaked 20 min each in 30 %, 20% and 10% solution of Clorox respectively (3-5 drops of Tween 20 was added in the solution of Clorox), then extensively washed with sterile distilled water for at least 3 times. These surface sterilized seeds were germinated in moist petridishes for 2-3 days in the dark. Three germinated seeds were transferred to each sterilized test tube (30x200 mm) containing 5 ml distilled water

capped with cotton plug. Growth of rice seedlings were maintained aseptically in a cabinet at 28 ± 1°C and 7,500 lux from fluorescent lamps (12 h photoperiod). After 7 days, bacterial contamination was checked by taking one loop of solution in the hydroponic culture and streaking on an RM agar. Only contamination-free rice seedlings were used for further studies (in 2.13 and 2.14).

2.13 Preparation of root exudate (Bhuvaneswari and Bauer, 1978).

Hydroponic culture solution from 35 tubes (each bearing 3 rice seedlings) were pooled, centrifuged at 20,000 g for 15 min and filtered through a Millipore membrane (0.45 u pore size) to remove small particles of plant debris, and freeze-dried in a Virtis Model 25SRC.

2.14 Bacterial inoculation for microscopic studies.

All the steps were performed in aseptic condition. Bacteria produced in NF broth were collected and washed with sterile PBS pH 7.4 and adjusted to 10^9 cells.ml^{-1} (OD_{600} = 6.0) in PBS. Cells were divided

into 2 equal portions, one portion was stained in acridine orange (1 : 10,000) for 1 second, washed 4 times with PBS, then added back to the remaining portion. A 0.1 ml aliquot of stained bacterial mixture (10^9 cells.ml^{-1} PBS) was inoculated into each tube containing 3 rice seedlings (day 7 after germination) and incubated as described previously. At various time after inoculation (0-48h), root samples from these seedlings were washed and examined under a fluorescence microscope study.

2.15 Observation by epi-fluorescence microscopy.

The roots of rice seedlings taken from one tube were extensively washed with PBS, cut into small pieces (3-6 mm), placed on a glass slide, covered with a coverslip, then viewed under the glycerol immersed 100 x objective lens of an epi-fluorescence microscope (Nikon, Optiphot) equipped with B filter cassette (excitation filter wavelength 420-485 nm and absorption filter of 520-580 nm). Photomicrographs were made on Kodak 400 ASA color slide film.

2.16 Preparation of root samples for electron microscopy.

2.16.1 Fixation. The small pieces (1-2 mm) of extensively washed root were fixed in a mixture of 2.5% glutaraldehyde and 2.0% paraformaldehyde in 0.1 M sodium cacodylate buffer pH 7.4 at 0 ^{o}C, overnight, washed 3 times with the same buffer. Post-fixation was performed in 2% osmium tetroxide in cacodylate buffer for 2 h, then washed another 3 times with excessive buffer.

2.16.2 Dehydration. The washed post-fixed root samples were dehydrated in the following ethanol series : 35% (v/v) ethanol, 30 min; 50% (v/v) ethanol, 30 min; 70% (v/v) ethanol, 30 min; then 95% (v/v) ethanol, 1 h; and absolute ethanol for 1 h twice.

2.16.3 Freeze-fracture. The dehydrated root samples in absolute ethanol were wrapped in parafilm, frozen in liquid nitrogen, and then fractured with a cold blade under liquid nitrogen. The frozen-fractured root samples were thawed in absolute ethanol at room temperature.

2.16.4 Drying and coating for scanning electron microscopy (SEM). The dehydrated samples were dried in a critical point dryer (Samdri-780, Tousimis Research Corporation) using liquid carbondioxide as transitional liquid. The dried samples were fixed on stub with silver paint, sputter-coated with a very thin layer of gold (0.01-0.1 μ) in a Fine Coat, Ion Sputter JFC-1100-Jeol, and examined under a scanning electron microscope (Jeol JSM-20) operated at 5-25 kV.

2.16.5 Embedding. For observation under a transmission electron microscope (TEM), a dehydrated sample in absolute ethanol was embedded in Spurr's resin (Spurr 1969). The embedding medium was freshly prepared before used by gently mixing together 10 g vinylcyclohexene dioxide, 6 g diglycidyl ether of polypropyleneglycol, 26 g nonenyl succinic anhydride and 0.4 g dimethylaminoethanol. Starting with the dehydrated samples in one volume of absolute ethanol, an equal volume of embedding medium was added, swirled and left standing for 1 h, then added another 2 volume of embedding medium, gently mixed and left for another 1 h.

These embedded samples were then transferred to the second change of embedding medium for 2-3 h, and left overnight in the third change of embedding medium. In the next morning, each of the resin-infiltrated sample was transferred to a capsule containing embedding medium and left for plastic polymerization in an oven at 70°C for at least 8 h.

2.16.6 Microtomy and staining for TEM. Ultra-thin sections of an embedded sample (0.01-0.15 μ) as shown by the gray to gold interference color were cut on an ultramicrotome (LKB 2088 Ultrotome V) equipped with a diamond knife. They were placed on a copper grid, air-dried, and stained with LKB commercial reagent of uranyl acetate and lead citrate in a stainer (LKB 2168 Ultrastainer, Carlsberg System). Sections were viewed and photographed on a Jeol Tokyo, Japan, 200 CX electron microscope operated at 80 kV.

Thick sections for light microscopy were cut with glass knives and stained with 0.05 % toluidine blue for 1 min.

2.17 Preparation of bacterial samples for electron microscopy.

The washed bacteria in 0.1 M sodium cadodylate buffer pH 7.4 were fixed in 1% glutaraldehyde at 0 ^{o}C for 1 h, washed 3 times with cacodylate buffer. The packed cells were resuspended in 1-2 drops of warm 25 mg.ml^{-1} agar solution. The agar block containing bacteria was cut into small cubes of 1-2 mm^{3} and postfixed in 2% osmium tetroxide in cacodylate buffer for 1 h, then processed further for electron microscopy using the same method as described in the preparation of root samples.

2.18 Enzyme treatment of bacterial associated rice root.

Bacterial associated rice roots were prepared as in 2.14. These seedling roots were cut into small pieces (5mm), placed in a test tube containing 1 ml enzyme solution in PBS of either a) β-N-acetyl glucosaminidase 1 enzyme unit or b) neuraminidase 0.2 enzyme unit or c) β-glucosidase 10 mg or d) trypsin 10 mg or e) PBS as control, then incubated at 25 ^{o}C for 20 h and processed further for microscopic studies.

2.19 Acetylene Reduction Activity (ARA) Assay.

Nitrogen-fixing activity of rice plant in association with bacteria was assayed in hydroponic culture tubes, in which 3 rice plants were growing with and without bacterial inoculation. The cotton plugs were replaced with sterilized rubber stopper fitted with Suba Seal serum stopper. Three replicate tubes were performed for each set of experimental condition. For each tube, 10% gas-phase volume was replaced with acetylene. At zero time, the base line concentration of C_2H_2 and C_2H_4 in the head space volume was determined by taking 100 µl gas-phase sampling with a sterile disposable syringe and injected to a Porapak N column (90°C) of a Varian 3700 gas chromatograph, equipped with a flame-ionization detector (FID) using 30 $ml.min^{-1}$ of oxygen-free nitrogen as carrier gas. Ethylene production in each tube was followed everyday (0-4 d) and calculated in μ $mol.tube^{-1}.d^{-1}$ by comparing the peak height with known amount of standard C_2H_4 at the same retention time.

2.20 Protein determination.

Protein concentration in root exudate, and lectin fractionated from rice were assayed according to Lowry (1951). Using 100 ul of samples to mix with 3 ml freshly prepared alkaline copper reagent which contained 100 ml 2% Na_2CO_3 in 0.1 N NaOH, 1 ml 0.5% $CuSO_4.5H_2O$ and 1 ml 1% potassium sodium tartrate. The mixture was incubated at room temperature for 10 min prior to addition of 0.3 ml 1.0 M Folin phenol reagent, then mixed and left at room temperature for 30 min before measuring the absorbance at 650 nm in a Spectronic 21. The protein concentration was calculated from a standard curve constructed with bovine serum albumin (20-100 ug) treated in the same manner as the samples.

2.21 Hemagglutination assay.

Hemagglutination activity of lectin in rice root exudate and bran, embryo and root extract were assayed with trypsinized rabbit erythrocytes (Lis and Sharon, 1973b).

2.21.1 Preparation of trypsinized rabbit erythrocytes. Blood sample (5 ml) was drawn from rabbit's ear vein and transfered into a test tube containing 1.25 ml anticoagulant (trisodium citrate 1.32 g%, citric acid 0.48 g% and dextrose 1.47 g%). The plasma was removed by centrifugation at 500 g for 5 min. Rabbit erythrocytes were washed 3 times with PBS and suspended in 1 ml PBS. Equal volume of trypsin (1 $mg.ml^{-1}$) was added, mixed and incubated at 37 ^{o}C for 30 min. After incubation, the trypsinized rabbit erythrocytes were washed at least 4 times with excess PBS and made up to 2% suspension in PBS.

2.21.2 Hemagglutination of trypsinized erythrocytes. A serial (2 fold) dilution of lectin solution (25 ul) with PBS was performed in a microtiter U-plate (Cooke Engineering Co.), one blank well and one positive control well for each row were filled with 25 ul of PBS and WGA (0.1 $mg.ml^{-1}$) respectively. Equal volume of 2% trypsinized rabbit erythrocytes suspension was added into each well. The plate was shaken vigorously clockwise and left at room temperature for about 30 min. Agglutinated cells formed a fuzzy mat on the bottom of the well, whereas non-agglutinated cells

settled into a cleary circumscribed red dot. The agglutination end point was taken as the last dilution of lectin in which erythrocytes formed a fuzzy mat on the bottom of the well. Agglutination activity (titer or HU) was defined as the reciprocal of this end-point dilution. In comparing the purity of lectin preparation, the specific titer was expressed as HU per mg protein.

2.22 Determination of bound lectin on root surface by competitive binding assay.

The bound lectin on root surface of rice seedling was determined by use of ^{14}C-N-acetyl-D-glucosamine (^{14}C-GlcNAc) which is the sugar known to bind specifically to rice lectin followed by competition of total ^{14}C-GlcNAc incorporation with non-labelled sugar competitors which are specific and non-specific hapten of rice lectin.

2.22.1 Radiolabelling of bound lectin on root surface by ^{14}C-GlcNAc. The incubation tube contained small pieces (5 mm) of root from 3 seedlings (7-day-old) and 500 µl solution of 0.5 µCi ^{14}C-GlcNAc (5.79 mCi.mmol^{-1}) in PBS. Five sets, with triplicate tubes in

each set, of the above incubation tubes were prepared. For the control set, roots were immediately washed with PBS on a filter paper No.1 until no radioactivity was detected in the washing ; and for the rest of another 4 sets, tubes were incubated at 25°C for 2 h prior to extensive washing of roots in PBS. The incorporated ^{14}C-GlcNAc remained with the root samples at this step included ; a) the specific binding of ^{14}C-GlcNAc to the bound lectin on root surface, b) the non-specific binding of the radioactive sugar on the root surface, and c) the uptake of radioactive sugar into the root cells.

2.22.2 Determination of specific binding between ^{14}C-GlcNAc and bound lectin by competitive binding assay. In order to determine the specific binding of ^{14}C-GlcNAc to the bound lectin on the root surface, the washed ^{14}C-GlcNAc radiolabelled roots from 2.22.1 were incubated either in 1 ml PBS for control or in 1 ml of another 4 different sugar solutions of GlcNAc, mannosamine (ManN), glucose (Glc) and glucosamine (GlcN) (20 mg.ml^{-1}) which are competitors at 25°C for 1 h. The radioactivity of ^{14}C-GlcNAc chased out from the root into these sugar solutions were counted by taking a 500

μl aliquot from each tube and put into a scintillation vial, then evaporated in a dessicator connected to a suction pump until dry. Six millilitre of Triton X-100 Toluene scintillation fluid (PPO 7.3 $g.l^{-1}$, POPOP 167 $mg.l^{-1}$, Triton X-100 250 $ml.l^{-1}$ and toluene 750 $mg.l^{-1}$) was added to each vial and counted in a Packard PL-Tricarb, PRIAS, liquid scintillation counter. The specific binding of ^{14}C-GlcNAc to the bound lectin on root surface was determined by subtracting the radioactivity of ^{14}C-GlcNAc chased out by GlcNAc in $dpm.mg^{-1}$ DNA with the radioactivity of ^{14}C-GlcNAc chased out by other non-specific sugar-competitors.

The uptake of ^{14}C-GlcNAc into the root cells was determined by washing the roots 3 times with distilled water and homogenized in 1 ml distilled water in a hand-homogenizer, then 100 μl aliquot was taken for radioactive counting in 6 ml Triton X-100-Toluene scintillation fluid and another 500 μl aliquot for DNA determination (Giles and Myers, 1965). Root homogenate was mixed with 500 μl distilled water and 1 ml 20% PCA and heated at 70°C for 20 min. Freshly prepared 4% diphenylamine in glacial acetic acid (2 ml) was added, followed by 0.1 ml of 1.6% aqueous acetaldehyde. The

mixture was incubated at 30°C, overnight and the absorbancy difference at 595 and 700 nm was read in a Spectronic 21. The amount of DNA was calculated from the standard curve constructed from calf thymus DNA treated in the same manner.

Statistical analysis of F test and Tukey's HSD (Honestly Significant Difference) test (Wayne W.D., 1978) were performed with all the data obtained as described in Appendix A.

2.23 Isolation of rice lectin from bran, embryo and root.

Bran was prepared from rice seeds with a home-made rice mill at the Institute of Rice Research, Ministry of Agriculture and Cooperative. Embryo was dissected from dehusked seed with blade and roots were cut from seedling grown as described in 2.12.

All the following steps were performed at 4°C unless otherwise specified. Bran and embryos were defatted twic by extraction in 4 volume of acetone and stirred for 4 h at room temperature, then air dried. Either defatted embryos or small pieces of root were homogenized in PBS using a Waring blender at full speed

for 3 min. Homogenized embryos, roots and defatted bran were then suspended in PBS and stirred overnight to extract soluble proteins and centrifuges in a Bechman J-21C at 7,020 g for 20 min to remove cells debris and the supernatant fraction (SF-8) was collected. Ammonium sulfate was added in the supernatant fraction to 60% saturation with constant stirring. The precipitate was collected by spinning at 13,000 g for 30 min, dissolved in PBS, and dialyzed against PBS to remove ammonium sulfate. Any precipitate formed during dialysis was later removed by centrifugation at 13,000 g for 30 min. The final clear supernatant so-called AS-60 was kept at - 20°C for further purification.

2.24 Affinity chromatography.

2.24.1 Chromatography on Selectin 1. Selectin 1, a commercial immobilized GlcNAc-Sepharose 6B, was washed with PBS and packed into a column (1.2 x 6 cm). The gel was equilibrated with PBS. Loading of AS-60 (15 ml) was delivered by a peristaltic pump (Periplex, LKB) at a constant flow rate of 15 $ml.h^{-1}$. Unbound proteins were completely washed out with PBS as monitored by A_{280}. Lectin, the affinity-bound protein

was then eluted with 0.2 M GlcNAc in PBS and collected in 2.5 ml fractions. The protein profile was measured by absorbancy at 280 nm. Protein fraction was passed through a Sephadex G-25 column packed in 5 ml syringe to separate sugar hapten, the protein peak at void volume was collected and assayed for lectin by hemagglutination test. The lectin fractions were pooled and stored at -20 ^{o}C for further study.

2.24.2 Chromatography on immobilized GlcNAc-Sepharose 6B. Immobilized GlcNAc-Sepharose 6B gel was prepared by the method of Vretbald (1976). Epoxy-activated Sepharose 6B was swollen and washed with water on a glass filter for 1 h. Wet gel (5 ml) was washed with 0.1 M NaOH, 5 ml solution of GlcNAc at various concentrations (50,100,160,180 mg.ml^{-1} in 0.1 M NaOH) was added. The mixture was incubated at 45^{o}C for 15 h on a rotary shaking water-bath at 100 rpm. The product was washed extensively with water followed by 0.05 M Tris buffer in normal saline pH 8.0 (TBS) and 0.05 M sodium formate buffer in normal saline pH 4.0. It was finally transferred to a column (1.2 x 6 cm) and equilibrated with PBS. Further steps for sample application and elution were the same as in 2.24.1

2.24.3 Chromatography on Chitin. Chitin column was prepared according to Bloch and Burger (1974). Chitin of uniform particle size (80-100 mesh) was extensively washed with distilled water, 0.05 N HCl, 1% Na_2CO_3 and ethanol respectively until the A_{280} of washed solution was lower than 0.05. It was then packed into a column of 2x30 cm, and equilibrated with PBS. Loading of AS-60 (20 ml) was maintained at the flow rate of 15 $ml.h^{-1}$. The unbound proteins were completely washed out with PBS and followed by 0.1 M phosphate buffer pH 7.4 to remove sodium chloride. Finally, the column was eluted with 0.05 N HCl or chitin hydrolysate 1% w/v as described by Rupley (1964). Protein fraction eluted by chitin hydrolysate was passed through a Sephadex G-25 column packed in 5 ml syringe to discard oligosaccharide hapten. The fraction was then assayed for lectin activity by hemagglutination test. The protein fractions eluted by acid were immediately assayed for lectin activity. The positive fractions were pooled, neutralized with 0.1 N NaOH, then dialyzed against PBS.

2.24.4 Binding capacity of affinity gel. The maximal lectin-binding capacities of the affinity gel was determined. AS-60 from bran was applied at increasing concentration to the tested affinity column until the hemagglutination activity was detected with unbound protein fractions. The column was washed and the lectin was desorbed as described above.

2.25 Chromatography on Sephadex G-100.

To determine the molecular weight of lectin, gel filtration by Sephadex G-100 was employed. A Sephadex G-100 column (1.6x80 cm) was equilibrated with PBS pH 7.4 and calibrated with standard molecular weight markers of bovine serum albumin (68,000), ovalbumin (43,000), chymotrypsinogen A (25,000) and lysozyme (13,500). The concentrated protein sample, in a total volume not exceeding 1% bed volume, was applied and chromatography was carried out at a flow rate of 10 $ml.h^{-1}$. Fractions of 3 ml were collected, protein profile was monitored by measurement of absorbance at 280 nm on every other fractions. The molecular weight of the unknown protein was estimated from its K_{av} (K_{av} = (Ve-Vd)/Vi) using a standard curve constructed from the

K_{av} values of standard proteins. Vo and Vi were the elution volume of blue dextran and potassium dichromate respectively.

2.26 Polyacrylamide gel electrophoresis (PAGE).

Electrophoresis was performed according to Reisfeld (1962). Using a slab gel consisting of 2 sections (i) a small pore separating gel (15x12x0.15 cm) of 10% w/v polyacrylamide, 0.27% N,N'-methylene bisacrylamide, 67 mg% ammonium persulfate and 0.67% v/v of TEMED in 0.8 M potassium hydroxide-acetic acid buffer pH 4.3, and ii) a large-pore stacking gel (15x4x0.15 cm) of 2.5% w/v polyacrylamide, 0.63% N,N'-methylene bisacrylamide, 0.55 mg% riboflavin-5-phosphate and 0.06% (v/v) of TEMED in 0.06 M potassium hydroxide-acetic acid buffer pH 6.7. The electrode buffer was 1.6 M β-alanine-acetic acid pH 4.5. The samples were diluted with sample buffer (5:1) (sample buffer composed of electrode buffer and trace of sucrose and trace of bromphenol blue). The electrophoretic migration proceeded from the anode towards the cathode with constant current of 25 mA/plate at 15° C in a LKB 2001 Vertical Electrophoresis apparates connected to a

thermostat water-bath of LKB 2209 Multi Temp.

When electrophoresis was completed, the gels were immediately removed from the glass plates, fixed and stained in 0.1% Coomassie blue R in 50% methanol and 10% acetic acid for at least 2 h, Destaining was carried out in a solution of 5% methanol and 7% acetic acid.

2.27 SDS-Polyacrylamide gel electrophoresis (SDS-PAGE).

The SDS-PAGE was performed on 1.5 mm slab gels using Tris-glycine buffer system first described by Laemmli (1970). The resolving gel consisted of 10-15% and 0.27-0.4% gradient of acrylamide and N,N'-methylene bisacrylamide respectively, 0.02% ammonium persulfate, 0.07% v/v TEMED and 0.1% SDS as separating gel (15x12x0.15 cm) in 0.38 M Tris-HCl buffer pH 8.8. The stacking gel (15x4x0.15 cm) was consisted of 3% acrylamide, 0.08% N,N'-methylene bisacrylamide, 0.2% ammonium persulfate, 0.07% v/v TEMED, 0.1% SDS and 2 mM EDTA in 0.12 M Tris-HCl buffer pH 6.8. Samples to be analyzed were treated with solubilizing medium containing 1% SDS, 4% v/v glycerol, 1% v/v 2-mercaptoethanol in 0.062 M Tris-HCl pH 6.8 and incubated for 2 min in a boiling-water bath prior to

application. In non-reducing condition 2-mercaptoethanol was omitted from the solubilizing medium. The electrode buffer was 0.1% SDS in 0.025 M Tris-0.192 M Glycine pH 8.3. Electrophoresis was performed at constant current of 30 mA until the tracking dye (bromphenol blue) moved into the separating gel, the current was then changed to 25 mA and the electrophoresis was continued until the tracking dye reached the bottom of the gel. The gels removed were stained and destained as previously described in PAGE.

2.28 Isoelectric focusing (IEF) gel electrophoresis.

Isoelectric focusing gels were moulded in glass tubes (100 x 0.2 and 0.5 mm, internal bore) by sealing one end with parafilm and hold in vertical position. The preparation of electrofocusing gel was performed according to O'Farrell (1975), but the protein sample (250-500 µg per gel tube) was dissolved in the polyacrylamide mixture, which contained 3% polyacrylamide, 0.16% bisacrylamide, 0.55 $g.ml^{-1}$ urea, 2% (v/v) Nonidet P-40 , 35 $\mu l.ml^{-1}$ of Ampholine pH 3-10 and 15 $\mu l.ml^{-1}$ of Ampholine pH 4-6. The complete gel mixture was then carefully loaded into the gel tube with

a hypodermic needle fitted with a long polyethylene tubing to avoid air-trapping in the gel tube. The gel was overlayed with water and allowed to polymerize (about 1 h). The polymerized gel surface was washed with distilled water and overlayed with 6M urea. The parafilm was removed and the gel tube was fitted into the LKB 2001 Vertical Electrophoresis connected to a thermostat water-bath of LKB 2209 Multi Temp at 15°C. The upper cathode chamber was filled with 20 mM NaOH whereas the lower anode chamber was filled with 10 mM H_3PO_4. Electrophoresis was carried out at 200 V for 30 min, 300 V for 30 min, 400 V for 12-16 h and finally 800 V for 1 h. At the end of the run, all gel tubes were immediately removed and placed on ice. The gels removed from 2x100 mm glass tubes were subjected to the second dimension electrophoresis on SDS slab gel, and those of 5 x 100 mm were for staining.

2.28.1 pH measurement of IEF gel. Measurement of pH along the gel length was performed by using a calibrated mixture of pH markers; phosphorylase b, bovine serum albumin, ovalbumin, trypsinogen and myoglobin, of which their pI at 25°C 6.38, 5.18, 4.7, 8.7 and 7.35 to 7.45 respectively. The pI standard

calibration curve of these protein markers was constructed, and the pI of the protein samples were read from standard curve.

2.28.2 Protein staining of IEF gel. The cylindrical IEF gel was stained according to Gorg (1978). The gel was immediately placed into 20 g% TCA for 1 h for fixing, then stained for 1 h in a solution of 0.15 g% Coomassie Blue-G 250, 45% methanol, 10% acetic acid and 0.5 g% copper sulfate, and finally destained in a solution of 25% methanol, 10% acetic acid and 1 g% copper sulfate.

2.29 IEF-SDS two dimensional electrophoresis.

Electrophocusing in the first dimension was performed in a cylindrical tube gel 2 x 100 mm). The IEF gel was equilibrated in the solubilzing medium for SDS-PAGE for 30 min at room temperature, then placed on top of the slab gel and fixed in place with warm 1% (w/v) agarose solution in solubilizing medium. The standard molecular weight markers was electrophoresed in separate well paralled to the second dimension electrophoresis of IEF gel on the same plate. Electrophoresis was carried out as described in 2.26.

2.30 Determination of the carbohydrate content in lectin.

The sugar content in lectin was determined by Phenol-Sulfuric acid Method as described by Dubois (1956). The sample solution (2 ml) was mixed with 0.05 ml 80% w/w phenol, then 5 ml of concentrated H_2SO_4 was added rapidly and directly against liquid surface. The tubes were allowed to stand for 10 min, then shaken and incubated at 25°C for 10 to 20 min. Reading of the absorbancy was carried out at 490 nm for hexose, and 480 nm for pentose and uronic acid. The amount of carbohydrate was determined by reference to a standard curve constructed from D-glucose for hexose and L-arabinose for pentose. The glycoproteins, fetuin and horse-radish peroxidase, were used as positive control, whereas a nonglycoprotein, wheat germ agglutinin, was used as negative control.

2.31 Amino acids composition of lectin.

To accurately determine the amount of cysteine and methionine, lyophilized protein samples were first oxidized with a mixture of 0.5 ml 30% hydrogen peroxide

and 4.5 ml 89% (w/w) performic acid, at - 10°C for 16 h (Moore , 1963). The oxidation was terminated by adding 0.8 g sodium pyrosulphite. The formic acid was liberated in a rotary evaporator at the temperature not exceed 50° C. The dried oxidized sample was then hydrolysed in a vacuum with 6 M HCl at 110° C for 24 h. Analysis of amino acids were performed according to the general principle described by Spackman *et al* (1958) with an Amino Acid Analyzer Hitachi 835-50. Glutathione and wheat germ agglutinin were determined for amino acids composition by similar methods as the control for recovery and correction during the oxidation and hydrolization steps.

2.32 Stability test for lectin.

2.32.1 Heat stability. The purified rice lectin was tested for heat stability by incubating the lectin sample dissolved in PBS pH 7.4 at various temperatures from 0 to 100° C for 2 h. The lectin activity before and after treatment were determined by hemagglutination assay.

2.32.2 pH stability. The pH stability of rice lectin was performed by dissolving lyophilized lectin samples in various pH buffers ranging from 2 to 12 and left standing at room temperature for 24 h. Then they were dialyzed against PBS and their lectin activity was assayed at pH 7.4 by hemagglutination test. The remaining activity of lectin after treatment in various pH for 24 h was expressed as per cent of the specific activity before treatment.

2.33 Radiolabelling of rice lectin.

Rice embryo lectin was radiolabelled with ^{14}C-acetic anhydride according to Miller and Great (1972). One hundred microlitre benzene was added to 1-^{14}C-acetic anhydride solution (250 μCi in 8.3 μl toluene), and slowly applied to the lyophilized embryo lectin (50 mg) in order to maintain a uniform acetylation through the solid material. After complete wetting, the material was stored for 6 days at 4^{o}C. The solvent was then removed by freeze drying and the residue dissolved in PBS pH 7.4. Excess ^{14}C-acetic acid was removed from the labeled sample by low speed centrifugation through

the syringe-packed Sephadex G-25 twice. The radioactivity of labelled lectin was then measured in 6 ml Triton X-100-Toluene scintillation fluid in a Packard PL Tricarb liquid scintillation spectrometer with 85% efficiency, counted for 5-10 min. The specific radioactivity of ^{14}C-labeled embryo lectin obtained was 7.0×10^3 dpm.μg^{-1} protein.

2.34 Bacterial agglutination test.

Washed bacterial suspension in PBS 20 μl (10^8 cells.ml^{-1}) was mixed with 20 μl of rice lectin (1 mg.ml^{-1}) in the presence or absence of 20 μl GlcNAc (1 mg.ml^{-1}). After standing for 15 minutes, the agglutination formed was observed under a Nikon Optiphot microscope at 1000 x magnification.

2.35 Binding activity of radiolabelled rice lectin.

From the bacterial suspension of 10^8 cells.ml^{-1}, a 150 μl aliquot was transferred into a series of 100 μl of ^{14}C-labelled embryo lectin at various concentrations (3-100 $\mu g.ml^{-1}$), in the presence and absence of 100-fold

concentration of non-labelled embryo lectin or root lectin or 0.9 M GlcNAc. After incubation with hand shaking from time to time at 0 ^{o}C for 30 min, the bacteria were pelleted by centrifugation at 1000 g in a microcentrifuge, Tomy Seiko Co, Model MC-15A for 2 min and washed twice with PBS, then counted for radioactivity of bacterial bound lectin in 6 ml of Triton X-100-Toluene in scintillation fluid in a Packard PL-Tricarb liquid scintillation counter at 85% efficiency. The specific binding of ^{14}C-embryo lectin on the bacterial cells was determined by subtracting non-specific binding of ^{14}C-embryo lectin bound to the bacteria in the presence of 100-fold nonlabelled lectin or 0.9 M N-acetyl-D-glucosamine from the total binding.

Characterization of lectin binding sites on the bacterial cells was performed by graphical analysis according to Scatchard (1949). Nonlinear Scatchard plot was analysed by weighted nonlinear least-squares curve fitting system of Thakur (1980) to estimate the affinity of lectin receptor (K) and the concentration of receptor sites (R).

2.36 Localization of lectin receptors utilizing colloidal gold as marker.

2.36.1 Preparation of colloidal gold. Monodisperse gold sols can be prepared in a variety of sizes from gold chloride by varying the type of reducing agents and the amount of a reducing agent as described by Horisberger (1985).

For preparation of colloidal gold at the diameter of 5 nm (Au_5). A 3 ml aliquot of 1% $HAuCl_4$ solution was added to 240 ml double-distilled water in a clean flask and the solution was neutralized with 5.4 ml of 0.2 N K_2CO_3. Then 2 ml of ether solution of white phosphorus was added. This ether solution consisted of 4 parts diethyl ether and 1 part phosphorous saturated ether. The mixture was hand shaken for 1 min and allowed to stand at room temperature for 15 min. The solution gradually developed a purple-brown color. It was then rapidly heated on an open flame with handshaking, boiled for 5-10 min until ruby color developed, and cooled.

For the colloidal gold of diameter 12 nm (Au_{12}), a mixture of 10 ml 0.1% $HAuCl_4$ neutralized with

1.5 ml 0.1 M K_2CO_3 was added as rapidly as possible at room temperature to 10 ml of 0.07% sodium ascorbate, which was vigorously stirred with a clean magnetic bar. The colloid formed almost immediately. Double-distilled water of 78.5 ml was then added to adjust the volume to 100 ml. The colloid had an orange-red color.

2.36.2 Conjugation of proteins with colloidal gold. Root lectin and ovomucoid are proteins used for conjugation with colloidal gold because ovomucoid has binding affinity to rice lectin and is normally used for lectin localization. The adsorption isotherms were prepared for each protein in order to determine the minimum amount of protein and optimum pH conditions required for stabilization of the colloidal gold (Horisberger, 1985). A serial dilution of protein solutions (started from 1 $mg.ml^{-1}$ solution) were made to a volume of 100 μl each. Colloidal gold sols was adjusted with 0.2 M K_2CO_3 or 1 M H_3PO_4 to pH of 5.5 when used for conjugation to ovomucoid, and to pH 7 for root lectin. One ml of pH adjusted colloidal gold sols were added to each protein solution tube and mixed rapidly. After 5 min, a 0.1 ml 10% NaCl solution was added to coagulate the unstabilized particles. Coagulation was

estimated spectrophotometrically at the λmax of gold sols (520nm).

The pH variable adsorption isotherms were prepared in the same manner except that an optimum quantity of the protein, as determined above, was added to a series of gold sols ranging in pH from 3 to 10. The pH of the colloidal gold was adjusted beforehand by addition of either 0.2 M K_2CO_3 or 1 M H_3PO_4, and two drops of 1% PEG 20,000 were added to 10 ml of colloidal gold before the pH electrode was inserted for measurement.

Once the optimum pH and protein concentration to stabilize colloidal gold had been determined, the preparation of protein-colloidal gold marker was achieved on a large scale of 100 ml. The lectin solution in PBS was filtered through a millipore filter (0.4 μ pore size) and placed in an ultraclean plastic bottle. The colloid was then added with stirring, after 5 min, a 5 ml of 1% PEG 20,000 was added, and centrifuged at 31,300 g in a Beckman J-21C at 4^{o}C for 30 min. The supernatant fluid was discarded and the precipitate was then resuspended in 0.02 M Tris buffer in normal saline (TBS) and 0.5 mg.ml^{-1} PEG 20,000.

2.36.3 Validity test of protein-gold complexes.

a) RL-Au complex. The complex of RL-Au was confirmed by using chitin pellet as the testing material. A few chitin pellets were washed with TBS, incubated with RL-Au for 30 min (or with colloidal gold as control), and washed 3 times, then observed for pink to dark red color on chitin pellets. If RL was successfully conjugated with colloidal gold to be RL-Au complex, the more pink color on chitin pellet was easily visualized comparing to the control preparation.

b) OV-Au complex. The same method was applied as in a) but wheat germ pellets were used instead of chitin pellets.

2.36.4 Direct method for localization of lectin receptors. Lectin receptors on bacterial surface and root epidermis were locallized directly with root lectin-gold complex (RL-Au) (Horisberger, 1985). Washed bacteria, 10^8 cells or 3-4 pieces of rice root about 1-2 mm long were incubated in 1 ml of root lectin-Au_{12} (A_{520}=2.0) at room temperature for 1 h with handshaking from time to time. Then the samples were washed three times in TBS containing 0.5 $mg.ml^{-1}$ PEG 20,000 (TBSP) to remove unbound gold complex, and further processed for

TEM. For bacterial samples, they were suspended in agar blocks prior to TEM processing. Control for non-specific binding must be done parallel to the samples by adding 44.2 mg GlcNAc (0.2 M) in 1 ml RL-Au_{12} solution before incubation.

2.36.5 Indirect method for locallization of lectin receptors. Ovomucoid (OV) which is composed of GlcNAc residues at the end of carbohydrate part of molecule, was chosen to conjugate with colloidal gold and used as specific probe. The specimens used for localization of lectin receptors by this method were bacteria and small pieces of rice root (1-2 mm long).

The bacteria 10^8 cells or 3-4 pieces of root samples (1-2 mm) were incubated in 1 ml rice root lectin (2 $mg.ml^{-1}$) at room temperature for 1 h, then washed 3 times with TBSP to remove excess lectin, and further incubated with 1 ml OV-Au_{12} (A_{520} = 2.0) for another 1 h with handshaking from time to time. The samples were washed 3 times with TBSP and further processed for TEM.

Controls must be performed in 2 conditions, firstly for specific binding, GlcNAc 44.2 mg was added together with 1 ml root lectin solution during incubation, and secondly for self-conjugation of OV-Au by omitting the incubation step with root lectin.