CHAPTER II

MATERIALS AND METHODS

MATERIALS

1. Biological materials

1.1 Rice bran

Bran of rice was perchased locally and stored in the cold room at $4^{o}C$ until used.

1.2 Erythrocytes

Human erythrocytes of the blood group A, B, AB and O were obtained from Thai Red Cross.

Rabbit erythrocytes were bleeded from the ear vein of normal adult rabbit reared in the animal quarter of the Department of Biochemistry.

1.3 Nitrogen-fixing bacteria

The five strains of rice rhizospheric nitrogen-fixing bacteria No 5, 6, 15, 17 and 18 were isolated from the rice rhizosphere root as described by Harinasut (14).

2. Chemicals

2.1 General chemicals

Chemicals and solvents used in the present investigation were of analytical or reagent grade, except phenol reagent (Folin-Cilcalteu reagent) was prepared in our laboratory by the methods of

Ciocalteu (15).

2.2 Chemicals for the assay of rice bran lectin activity

Trypsin was bought from BDH Chemicals Ltd.

Wheat germ agglutinin was bought from BMY Chemicals Ltd.

2.3 Chemicals for chromatography

Sephadex G-25, Sephadex G-75, Sephadex G-100, DEAE-Sephadex-A-50, Sepharose 4 B and blue dextran 2000 were purchased from Phamacia Fine Chemicals.

Potassium chromate was bought from E. Merck.

BSA, α-chymotrypsinogen A and cytochrome C were bought from BDH Chemicals Ltd.

N-acety-D-glucosamine was bought from Sigma Chemical Company.

Divinyl sulfone was bought from Fluka Chemical Company.

2.4 Chemicals for gel electrophoresis

Acrylamide, N, N-bis-methylene acrylamide and coomassie brilliant blue were bought from Sigma Chemical Company.

Ammonium persulphate, SDS, N, N, N, N-tetramethylene-diamine (TEMED) and bromophenol blue were bought from BDH Chemicals Ltd.

3. Bacterial media

3.1 Nitrogen-free medium (16)

		Grade	Company
Dipotassium hydrogen phosphate	(0.05 g/l)	Lab	M&B
Potassium dihydrogen phosphate	(0.15 g/l)	Lab	M&B
Calcium chloride	(0.01 g/l)	Analar	BDH
Magnesium sulphate	(0.2 g/l)	Lab	BDH

Sodium molybdate	(0.002 g/l)	Lab	BDH
Ferric chloride	(0.01 g/l)	Analar	BDH
Sodium bicarbonate	(0.01 g/l)	Lab	M&B
Glucose	(20 g/l)	Analar	Fluka

3.2 Rich medium (17)

Tryptone	(10 g/l)	-	Difco
Yeast extract	(5 g/l)	-	BBL
Sodium chloride	(10 g/l)	Analar	Mallinckrodt
Glucose	(0.2 %)	Analar	Fluka

4. Instruments

Beckman model J-21 C centrifuge (Spinco Division of Beckmen Instruments Inc., Palo Alto, California, U.S.A.)

Beckman model 25 spectrophotometer (Beckman Instruments Inc., Fullerton, California, U.S.A.)

HSI Disc electrophoresis chamber (Hoefer Scientific Instruments, San Francisco, California, U.S.A.)

Microtiter U-plates, microdilutor and micropipette (Cooke Engineering Co., U.S.A.)

pH meter (Radiometer, Copenhagen, Sweden)

Spectronic 20 (Bausch & Lomb Inc., Ltd, U.S.A.)

Ultrorack model 2070 fraction collector (LKB Productor AB Bromma, Sweden)

Virtis freeze dryer model 25 SRC (Virtis Co.)

METHODS

1. General methods

1.1 Protein determination

Total protein concentration was determined by the method of Lowry et al.(18) using bovine serum albumin (Sigma) as standard reference protein. Occasionally spectrophotometric method was used to measure the absorbance of protein solutions at 280 nm in a Beckman model 25 spectrophotometer.

1.2 Carbohydrate determination

The carbohydrate content was determined by anthrone reaction (19). Glucose was used as standard reference carbohydrate.

1.3 0.1M phosphate buffer pH 7.4 in 0.85% NaCl (PBS)

0.1 M Na_2HPO_4 (14.2g/l) 900 ml was adjusted to 7.4 with 0.1 M NaH_2PO_4 (12.0 g/l).

Add NaCl 8.5 g and make up to 1 lit with above buffer.

2. Purification of rice bran lectin

All operations were carried out at 4°C unless otherwise indicate.

2.1 Preparation of the defatted rice bran

Each lot of rice bran (approximately 1,000 g) was suspended in 3 volume (7,200 ml) of acetone and stirred for 4 hrs at room temperature. The suspension was then filtered and the residue was quickly air dried.

2.2 Preparation of the crude PBS extract

Each lot of the defatted rice bran (800 g) from step 2.1 was suspended in 2 volume (3,840 ml) of PBS and then stirred for 15 hrs. The clear supernatant solution (2,500 ml) was obtained by centrifugation at 8,000 x g for 10 min. A small aliquot (5 ml) of the crude PBS extract was saved for assays of specific hemagglutination activity and of total protein concentration.

2.3 Ammonium sulphate fractionation

The clear superantant solution from step 2.2 (2,500 ml) was brought to 60% ammonium sulphate saturation by adding gradually solid ammonium sulphate (36.1 g/100 ml). The suspension was stirred for 1 hr and centrifuged at 8,000 x g for 15 min. The protein precipitate was suspended in 150 ml of PBS and dialyzed against 5 changes of 2 liters of the same buffer. A small aliquot (5 ml) of the dialysate was saved for assays of specific hemagglutination activity and total protein concentration.

2.4 Column chromatography

The dialysate from step 2.3 was furthur purified by the 2 schemes of chromatographic methods.

2.4.1 Chromatography on Sephadex G-25 and DEAE-Sephadex-A-50 columns and then Sephadex G-100 column

2.4.2 Affinity chromatography on N-acetyl-D-glucosamine-Sepharose 4 B column

2.4.1 Purification by ion exchange chromatography

a. Sephadex G-25 gel filtration

1 ml of dialysate (17 mg total protein) from step 2.3 was applied to a 1.8 x 25 cm column of Sephadex G-25 previously equilibrated with 0.1 M Tris-HCl buffer, pH 7.4 until pH of the eluent was equal to that of the buffer. The sample was eluted with the same buffer at the flow rate 0.5 ml/min. Three ml fractions were collected. The protein in each fraction was determined by measuring the absorbance at 280 nm and determined for the specific hemagglutination activity. The fraction showing positive specific hemagglutination activity were combined and redetermined the specific hemagglutination activity of the pooled fractions.

b. DEAE-Sephadex A-50 column

The combined protein solution (about 150 mg protein in 180 ml) from step a. was applied to a 2.2 x 40 cm column of DEAE-Sephadex A-50, previously equilibrated with 0.1 M Tris-HCl buffer, pH 7.4 until pH of the eluent was equal to that of the buffer. The flow rate from the reservior (500 ml of 1.5 M NaCl in 0.1 M Tris-HCl buffer, pH 7.4) to the mixing chamber (500 ml of 0.1 M Tris-HCl buffer, pH 7.4) and the flow rate from the mixing chamber to the column were 0.33 ml/min and 0.66 ml/min respectively. The column was eluted with a linear salt gradient of 0-1.0 M NaCl. Fractions of 3 ml each were collected, determined for protein concentration by measuring the absorbance at 280 nm, and determined for the specific hemagglutination activity. The fractions showing positive specific hemagglutination activity were combined and redetermined the specific hemagglutination activity of the pooled fractions.

c. Sephadex G-100 gel filtration

Pooled protein solution (6.62 mg protein in 7 ml) from step b. was applied to a 2.2 x 40 cm column of Sephadex G-100, previously equilibrated with 0.1 M Tris-HCl buffer, pH 7.4 until pH of the eluent was equal to that of the buffer. The sample was eluted with the same buffer at the flow rate 0.5 ml/min. Fractions of 3 ml each were collected, determined for protein concentration by measuring the absorbance at 280 nm and determined for the specific hemagglutination activity. The fractions showing positive specific hemagglutination activity were pooled and redetermined the specific hemagglutination activity of the combined fractions.

2.4.2 Purification by affinity chromatography

a. Preparation of N-acetyl-D-glucosamine-Sepharose 4 B (20)

One hundred ml of sedimented Sepharose 4 B gel was washed with 0.5 M sodium carbonate buffer, pH 11. To the drained gel were added 100 ml of buffer and 10 ml divinylsulfone. The mixture was stored at 25°C with stirring for 70 min, and then washed on a Buchner funnel. The drained gel was mixed with 10% (w/v) solution of N-acetyl-D-glucosamine in sodium carbonate buffer. The reaction was allowed to proceed for 15 hrs with stirring. The gel was finally washed with buffer, excess amount of water and PBS.

b. N-acetyl-D-glucosamine Sepharose 4 B column

2.5 ml of partially purified rice bran lectin (42.5 mg) from step 2.3 was applied to a 2.2 x 25 cm affinity column of N-acetyl-D-glucosamine Sepharose 4 B. After loading, flow rate of 0.5 ml/min of PBS was applied until no absorbance at 280 nm was detected in the

effluent. The lectin was then eluted from the column upon application of a solution of 0.1 M N-acetyl-D-glucosamine in PBS, and collected in 3 ml fractions. The absorbance at 280 nm of every fraction was determined, and the fractions corresponding to the protein peak were pooled and extensively dialyzed against large volumes of PBS. After no trace of N-acetyl-D-glucosamine was detected in the PBS, the specific hemagglutination activity of the nondialyzable lectin fraction was assayed.

The volume of purified rice bran lectin from this step was usually concentrated by freeze drying in a Virtis model 25 SRC freeze dryer or Aquacide 1-A.

3. Characterization of rice bran lectin

3.1 Preparation of trypsinized erythrocytes for agglutination test (21)

Erythrocytes of human A, B, AB, O and rabbit were washed 3 times in PBS, centrifuged at 1,000 x g for 10 min between washing. The packed erythrocytes were treated with equal volume of trypsin solution (1 mg/ml PBS) at 37°C for 30 min. After treatment, the erythrocytes were washed at least 4 times in the same manner as above. The last packed erythrocytes were suspended in PBS to give a final concentration of 2% (v/v).

3.2 Agglutination test of rice bran lectin (22)

Assay were performed in duplicate in a microtiter U-plates (Cooke Engineering) at room temperature (27 °C). Solutions containing lectin were serially 2-fold diluted in PBS. To each well of the U-plate were added, in the following order: 25 µl of rice bran lectin

dilution and 25 µl of trypsinized erythrocytes suspension. Control wells contained trypsinized erythrocytes suspension without rice bran lectin and trypsinized erythrocytes suspension to which wheat germ agglutinin was added in stead of rice bran lectin. The plates were shaken vigorously, then allowed to sit for about 30 min before agglutination was evaluated. Agglutinated cells formed a fuzzy mat on the bottom of the well, whereas unagglutinated 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) was defined as the reciprocal of this end-point dilution, and the specific agglutination activity as titer per mg of protein, measured by the method of Lowry et al. (18).

3.3 Inhibition assay of agglutination activity with trypsinized erythrocytes

For assay of inhibition of agglutination by various sugars, 25 µl of 2 fold serial dilutions of test sugar solution (50 mg/ml) were mixed with an equal volume of lectin solution (0.1 mg/ml). After 30 min, 25 µl of trypsinized erythrocytes suspension was added and the mixtures were allowed to sit for another 30 min. Then the agglutination was examined and the minimal sugar concentration showing hemagglutination inhibition was determined.

3.4 Growth of nitrogen-fixing rhizospheric bacteria

The nitrogen-fixing rhizospheric bacteria were maintained in rich medium agar slants in screw-cap tubes. To perform an agglutination test, the bacteria from slants were streaked on NF plates and grown in NF + 10% rich medium respectively.

3.5 Preparation of bacteria for agglutination test with rice bran lectin

The nitrogen-fixing rhizospheric bacteria and Escherichia coli were grown in 250 ml of NF + 10% rich medium, pH 7 at 37°C (14). The bacterial cells were harvested in early stationary phase (about 12 hrs) by centrifugation at 1,000 x g for 10 min. The packed cells were taken and diluted to 1% (v/v) in PBS.

3.6 Agglutination of rice bran lectin with nitrogen-fixing rhizospheric bacteria

The agglutination test was performed in the same manner as in step 3.2 using 25 µl of 1% (v/v) bacterial suspension instead of trypsinized erythrocytes. One row of the test plate was used for each bacterial culture, using the last well as negative control by omitting lectin. Suspension of Escherichia coli was used in the last row in every set of experiment to compare the agglutination activity of lectin between Escherichia coli and N_2-fixing bacteria.

3.7 Inhibition assay of agglutination activity with nitrogen-fixing rhizospheric bacteria

Inhibition of agglutination by various sugars was determined in the same manner as in step 3.3 using 25 µl of 1% (v/v) bacterial suspension instead of trypsinized erythrocytes.

3.8 Polyacrylamide gel electrophoresis

The method was modified from that of Ornstein and Davis (23) using 8% polyacrylamide for separating gel and 2.5% for stacking gel. The electrophoretic buffer contained 6.32 g of Tris and 3.94 g of glycine per 1 liter (pH 8.9). The dimensions of the running and stacking gels were 6 x 90 and 6 x 10 mm, respectively. The volume of the protein sample applied to each gel varied between 50-200 µl, including 2 drops of glycerol and 1 drop Bromphenol blue as a tracking dye. The time of the electrophoretic run was about 2 hrs at a constant current of 3 ma/gel at room temperature. The gels were stained by 0.25% Coomasie brilliant blue as described by Weber and Osborn (24) for at least 2 hrs and then destained overnight in a destaining solution containing 75 ml of glacial acetic acid, 50 ml of methanol, and 875 ml of water. After destaining the gels were stored in 7.5% acetic acid solution.

3.9 Molecular Weight determination

a. SDS-polyacrylamide gel electrophoresis

The electrophoresis procedure was according to Weber and Osborn (24), using 10% polyacrylamide for separating gel, the running buffer was composed of 3.4 g of NaH_2PO_4 , 10.23 g of Na_2HPO_4 and 1 g of SDS/liter. The solution of protein sample containing 50 µg of freezed dried protein in 10 µl buffer (0.34 g of NaH_2PO_4 , 1.02 g of Na_2HPO_4, 1 g of SDS, 1 ml of 2-mercaptoethanol, 0.015 g of Bromphenol blue and 36 g of urea per 100 ml of water) was layered on top of 6 x 90 mm gel. The electrophoretic run was about 5 hrs at a constant current of 8 ma/gel at room temperature. After the run, gels were stained in 0.25% Coomassie

Brilliant blue for at least 2 hrs. The gels were then destained in a destaining solution, containing 75 ml of acetic acid, 50 ml of methanol and 875 ml of water, and finally stored in this destaining solution.

b. Gel filtration

The molecular weight of purified lectin was estimated on a Sephadex G-75 column chromatography (fractionation range 3,000-80,000 dalton) packed in a 2.2 x 51 cm column with the total bed volume of 194 ml. The void volume and the elution volume of the column were determined, using blue dextran 2,000 and potassium chromate as markers. Standard protein markers were bovine serum albumin (67,000 dalton), α-chymotrypsinogen-A (25,000 dalton) and cytochrome C (13,000 dalton). The column was eluted with PBS at the flow rate of 1 ml/min and a volume of 3 ml was collected for each fraction.