CHAPTER 3

MATERIALS AND METHODS

3.1. MATERIALS

3.1.1. Source of lectin: Fresh fruiting bodies of the edible mushroom *Lentinus cladopus* Lév. (locally known as '*tek-tek paan*' in Manipuri), were collected from in and around Kakching, Thoubal District, Manipur, India as the source of lectin. This mushroom, shown in **Fig.1**, belongs to the family Polyporaceae of the order Agaricomycetes. The mushroom, which grows on decaying stumps and root of trees, was collected during late August to October for use in the present investigation. The pileus of the mushroom is centrally depressed or funnel shaped. The scales are larger at the centre and smaller towards the margin. The colour is usually white to creamish white. In the State of Manipur, India, it is distributed mainly in the districts of Imphal East, Imphal West, Bishenpur and Thoubal (Sing *et al*., 2002).

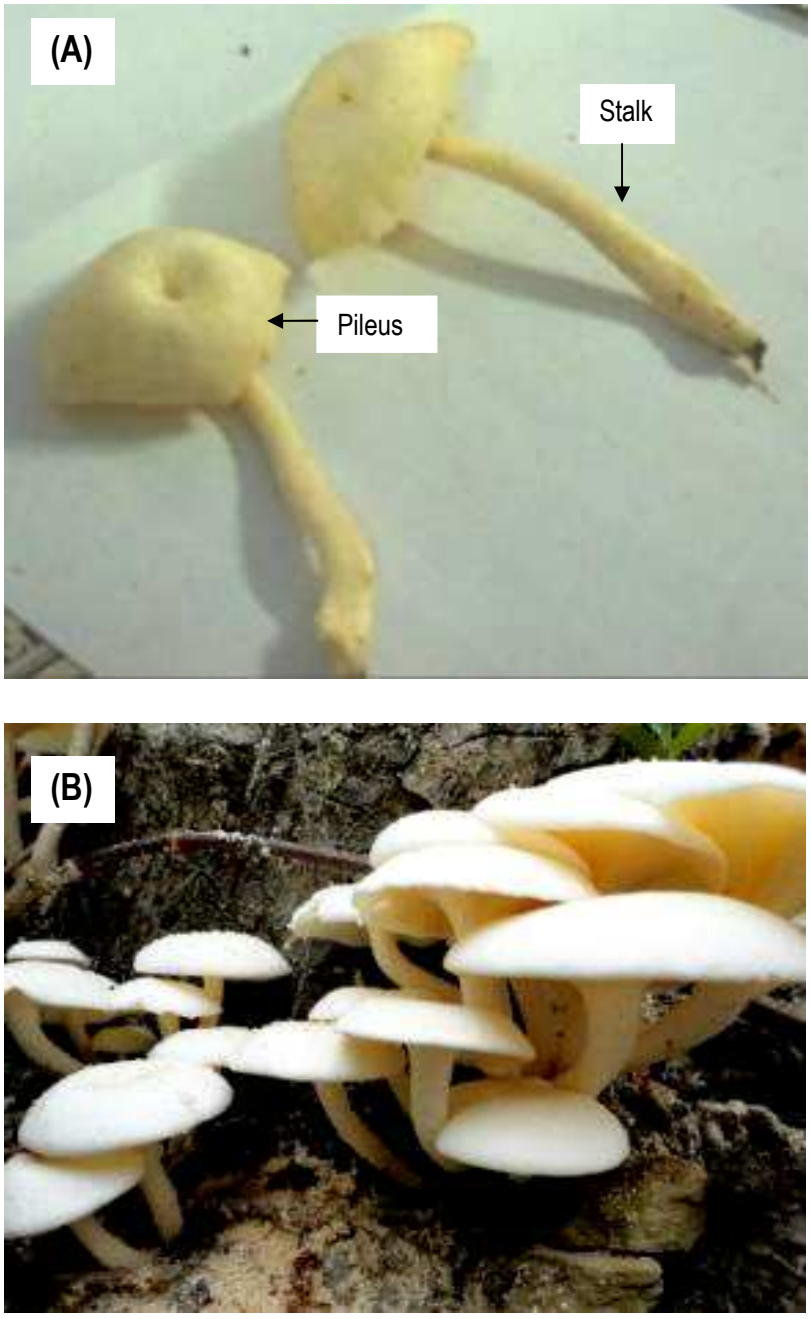

Fig.1: (A) The fruiting body of *Lentinus cladopus* Lév. comprising of the umbrella part (the pileus) and the elongated part (stem/stalk), **(B)** The mushroom in its natural habitat growing on the bark of a mango tree.

3.1.2. Source of blood: Rabbit was used as the experimental animal for obtaining blood for preparation of RBC during the course of the present investigation. The rabbit (*Oryctolagus cuniculus*), belonging to order Lagomorpha, was procured from a local farm and reared under standard protocol. Blood was withdrawn from the animal and RBC was prepared as per the procedure described under **METHODS.** All the procedures involving the experimental animal were in accordance with the *Guide for the Care and Use of Laboratory Animals* (National Research Council, 1996)

3.1.3. Chemicals:

(i) For preparation of buffers: Sodium dihydrogen orthophosphate dihydride, tris (hydroxymethyl) aminomethane (Tris) and sodium chloride were obtained from HiMedia Laboratories Pvt. Ltd., Mumbai; Citric acid from Loba Chemie, Mumbai; Hydrochloric acid from Glaxo India Ltd., Mumbai; Glycine from Merck, India.

(ii) For protein estimation: Copper sulphate was from Merck, India; Sodium carbonate and sodium hydroxide from HiMedia Laboratories Limited, Mumbai; Rochelle salt (sodium potassium tartarate) was obtained from Qualigens, Mumbai; Folin-Ciocalteau reagent was obtained from Sisco Research Laboratories, Mumbai.

(**iii) For lectin purification:** Ammonium sulphate was purchased from HiMedia Laboratories Limited, Mumbai; Q-Sepharose fast flow, DEAE-Cellulose and Sephadex G-100 were obtained from Sigma Chemical Company, St. Louis, MO, USA; Activated charcoal was procured from Merck, India

(iv) For gel electrophoresis: Acrylamide, N,N'-methylene-bis-acrylamide, N,N,N',N'-tetramethylethylenediamine, Tris (hydroxymethyl) aminomethane (Tris) were procured from HiMedia Laboratories Pvt. Ltd., Mumbai; Formaldehyde, sodium dodecyl sulphate (SDS) and glycine were from Merck, India; Silver nitrate and sodium thiosulphate were from Sisco Research Laboratories, Mumbai; 2-mercaptoethanol were from Loba Chemie, Mumbai; Ammonium persulphate, acetic acid, ethanol and methanol from Qualigens Fine Chemicals (A Division of Glaxo India Limited), Mumbai.

(v) For sub-unit molecular weight determination: Acrylamide, N,N'-methylene-bis-acrylamide, N,N,N',N'-tetramethylethylenediamine and tris (hydroxymethyl) aminomethane (Tris) were procured from HiMedia Laboratories Pvt. Ltd., Mumbai; Sodium dodecyl sulphate (SDS), formaldehyde, glycine and medium molecular weight (MMW) marker proteins *viz*. phosphorylase b (M_r 97.4kDa), bovine serum albumin (M_r 66kDa), egg white ovalbumin (M_r 43kDa), carbonic anhydrase (M_r 29kDa), lactoglobin (M_r 18.4kDa), and aprotinin (M_r 6.5kDa) were purchased from Merck, India; Silver nitrate and sodium thiosulphate were from Sisco Research Laboratories, Mumbai; 2-mercaptoethanol was from Loba Chemie, Mumbai; Ammonium persulphate, acetic acid, ethanol and methanol from Qualigens Fine Chemicals (A Division of Glaxo India Limited), Mumbai;.

(vi) For native molecular weight determination: Sephadex G-100, bovine serum albumin (M_r 66kDa), chicken egg ovalbumin (M_r 44kDa), α-chymotrypsin (M_r 29kDa), lysozyme (M_r 18.4kDa), and blue dextran (M_r 2000kDa) were obtained from Sigma Chemical Company, St. Louis, MO, USA.

(vii) For characterization of purified lectin: D-Glucose, D-galactose, D-mannose, D-arabinose, L-arabinose, D-lactose, D-fructose, L-sorbose, L-

rhamnose,D-fucose, L-fucose, D-ribose, D-cellobiose, D-mellibiose, D-galactonolactone, D-galacturonic acid, D-glucosamine, D-galactosamine, N-acetyl-D-glucosamine, N-acetyl D-galactosamine, N-acetyl-D-mannosamine, methyl-α-D-mannopyranoside, methyl-β-D-glucopyranoside, methyl-β-D-galactopyranoside, 2-acetamido-D-deoxy-galactonolactone, 4-methyl-D-galactoside, D- raffinose, N-acetyl-D-lactosamine, thyroglobulin, ovalbumin, peroxidase were purchased from Sigma Chemical Company, St. Louis, MO, USA; Heparin, mannan, dextran, gum tragacanth, gum ghatti were procured from HiMedia Laboratories Pvt. Ltd., Mumbai; Magnesium chloride, calcium chloride and manganese chloride were obtained from Qualigens Fine Chemicals (A Division of Glaxo India Limited), Mumbai. EDTA was procured from Central Drug House, New Delhi.

(viii) Other chemicals: All other chemicals were of analytical reagent grade.

(ix) Water: TKA Smart2Pure UV/UF (Niederelbert, Germany) purified deionized water was used for the experiments.

(x) Dialysis membrane: Dialysis membrane-70, a cellulosic membrane having a diameter of 24.26mm and a capacity of holding 1.16mL volume per cm of the bag, was procured from HiMedia Laboratories Pvt. Limited, Mumbai.

3.1.4. Reagents and buffers:

(i) Phosphate buffered saline (PBS): 50mM Phosphate buffer pH 7.4 containing 0.9% NaCl

(ii) Tris buffered saline (TBS): 50mM Tris buffer pH 7.4 containing 0.9% NaCl

(ii) Elution buffers: 50mM Phosphate buffer pH 7.4 containing 20mM sodium chloride for DEAE-Cellulose ion exchange chromatography. 20mM Tris buffer containing 25mM sodium chloride for Q-Sepharose ion exchange chromatography.

(iii) Reagents and other buffers: Reagents**,** other buffers and their compositions are mentioned under **METHODS.**

3.1.5. Packed column for DEAE-cellulose chromatography: The DEAE-cellulose powder (resin) used in the ion-exchange chromatography for purification of lectin was pre-treated following the method described in the product information provided by Sigma Chemical Co., USA. The anion exchanger DEAE-cellulose was pre-treated by a procedure involving successive steps of (i) suspending 3g of DEAE-cellulose dry resin in 15mL of distilled water with constant stirring for 30 min, (ii) filtration and re-suspension in 20mL of 0.1N NaOH containing 0.5M NaCl for 10min, (iii) washing with 20mL of 0.5M NaCl solution, (iv) re-suspension of the resin in 20mL of 0.1M HCl containing 0.5M NaCl, (v) thorough washing with distilled water until the eluent pH was 5 or greater. The resin was suspended in 20mL of 1M NaCl and the pH of the slurry was adjusted to 7-8 with NaOH solution. The slurry was then filtered and about 50mL of distilled water was passed through the column. The pre-treated resin was then re-suspended to equilibrate with 50mM phosphate buffer, pH 7.4 containing 20mM NaCl. The resulting slurry was poured into a column in a single stroke to ensure even packing and to avoid trapping of air bubbles. The final packed column of desired bed volume (4.2mL, dimension 4x1cm) was obtained by allowing more of the buffer to flow through the column at an appropriate flow rate by connecting to a peristaltic pump (Ravel Hiteks Pvt. Ltd., Chennai, India).

3.1.6. Packed column for Q-Sepharose chromatography: The Q-Sepharose fast flow was pre-treated following the method described in product information provided by Sigma Chemical Co., USA. 5mL of the pre-swollenQ-Sepharose resin was equilibrated in 50mL of 20mM Tris buffer pH 7.8 containing 25mM NaCl. The column was mounted vertically on a suitable stand and the slurry was poured into the column in a single stroke to ensure even packing and to avoid trapping of air bubbles. The final packed column having the desired bed volume (4.2mL, dimension 4x1cm) was obtained by allowing more of the buffer to flow through the column with a flow rate of 30mL/h by connecting to a peristaltic pump (Ravel Hiteks Pvt. Ltd., Chennai, India). The resin was regenerated after use by washing the column with 10mL of 1-2M NaCl solution followed by 10mL of 0.1M NaOH in 0.5M NaCl. The resin was rinsed thoroughly with several volumes of distilled water and finally equilibrated with 20mM Tris buffer, pH 7.8 containing 25mM NaCl.

3.1.7. Packed column for gel filtration: A required amount (6g) of dry Sephadex G-75 (fractionation range 1-50kDa) was added to 150mL of 50mM phosphate buffer pH 7.4 containing 0.9% NaCl (PBS) in a beaker and the gel particles were allowed to hydrate for 12h at room temperature. The resulting hydrated gel suspension was transferred to a filter flask after decanting half the supernatant and then degassed for 5-10min with occasional swirling using a vacuum pump. 150mL of already degassed PBS was added with gentle swirling, allowed 90-95% of the gel particles to settle down, and then fine and broken gel particles were removed by decantation. This procedure of removal of fine and broken gel particles was repeated three more times. The resulting slurry was poured into a column in a single smooth movement avoiding splashing of slurry to ensure even packing and to avoid trapping of air

bubbles**.**The final column of the desired bed volume (82 x 0.9cm) was packed by allowing more of the buffer to flow through the column.

3.2. METHODS

3.2.1. Lectin extraction:

Fruiting bodies of *Lentinus cladopus* Lév.were washed thoroughly, air dried, ground to coarse powder, and kept stored in an air-tight plastic container at room temperature until use. 10g of the powder was soaked in 70mL of 20mM Tris-HCl buffer, pH 7.8 containing 25mM NaCl, 0.1mM EDTA and 1mM β-mercaptoethanol under ice-cold condition. After soaking for an hour, it was homogenized in a mortar and pestle under ice-cold condition. The resulting homogenate was filtered through a double layered cotton cloth and then centrifuged at 17,000g for 30min at 4°C. The light brown supernatant obtained was then subjected to treatment with activated charcoal (90 mg/mL supernatant) for 15min in ice-cold condition. After the charcoal treatment, the resulting suspension was centrifuged at 17,000g for 30min at 4°C and then filtered through a funnel plugged with glass wool to obtain a clear and colorless solution as the crude lectin preparation.

3.2.2. Preparation of rabbit RBC suspension: A suitable spot on the posterior side of pinna of rabbit (*Oryctolagus cuniculus*) was first shaved and sterilized using an alcohol swab**.** Blood (1mL) was withdrawn through a vein at the sterilized spot using a fresh heparinized syringe**.** All these procedures were performed in accordance with the *Guide for the Care and Use of Laboratory Animals* (National Research Council, 1996) causing minimal pain

to the experimental animal. The rabbit RBC was first separated from the blood by centrifuging at 1500-2000 rpm for 2min using an ordinary tabletop centrifuge. The resulting pellet of RBC was re-suspended in 5mL of normal saline (0.9% NaCl) with gentle mixing and centrifuged at 1500-2000 rpm for 2min. This procedure of re-suspension and subsequent centrifugation was repeated four times to obtain a suitably washed pellet of the RBC. 0.2mL of the pellet of the washed RBC was mixed with 9.8mL of Normal saline (0.9% NaCl) to obtain 2% RBC suspension (v/v).

3.2.3. Lectin assay: Lectin assay was performed following the method of Devi *et al*. (2009) involving a standard serial dilution technique. A 2% suspension (v/v) of the freshly washed rabbit RBC, prepared as in **Preparation of rabbit RBC suspension**, was used to determine the hemagglutination activity of the lectin sample in a U-bottom 96-well microtitre plate(Tarsons Product Pvt. Ltd., Kolkata, India). 50µL of the lectin sample was two-fold serially diluted with equal volume of 50mM phosphate buffer pH 7.4 containing 0.9% NaCl (PBS) followed by addition of equal volume of 2% rabbit RBC suspension (v/v). The contents were mixed gently but thoroughly. Corresponding control wells containing a mixture 50µL of PBS and 50µL of 2% washed RBC were always included. After incubation for 1h at 37°C, hemagglutination was scored visually. The activity was taken as hemagglutination titre, the reciprocal of the highest dilution of the lectin sample at which complete hemagglutination occurred. It was expressed in terms of hemagglutination unit (HAU) that is the minimum amount of lectin required for 100% agglutination under the standard assay conditions (Sawhney *et al*., 1996).

3.2.4. Protein estimation: Protein in the lectin samples was estimated by following the method of Lowry *et al*. (1951) using crystalline bovine serum albumin as the standard:

(i) Preparation of solutions and reagent:

Solution A: Sodium carbonate (2g) was dissolved in distilled water and the volume was made up to 100mL.

Solution B: It was prepared by mixing 5.0mL of 1.1% of Rochelle salt solution with 0.5ml of 5.5% copper sulphate solution.

Solution C: 50mL of solution A was mixed with 1mL of Solution B.

1N NaOH Solution:4g of NaOH was dissolved in 100mL of distilled water.

Folin-Ciocalteau reagent: This reagent, purchased from Sisco Research Laboratories Pvt. Ltd., Bombay was diluted two-fold to obtain 1N immediately before use.

(ii) Procedure: The sample protein was suitably diluted to 0.1mL in a microcentrifuge tube, precipitated with 0.1mL of trichloroacetic acid (10%) and incubated in ice-bath for 10min and then centrifuged for 10min at 10,000 rpm. After the centrifugation, the supernatant was drained off as completely as possible. 0.1mL of 1N NaOH was added to the precipitate, vortexed thoroughly and left for 30min at room temp. 1.0mL Solution C was added and mixed thoroughly. After incubating for 10min at room temperature, 0.1mL of Folin-Ciocalteau reagent was added with constant stirring and then allowed standing for 30min. The absorbance of the resultant blue colour was measured against blank (constituted by mixing together 0.1mL of distilled water, 1.0mL Solution C and 0.1mL of Folin-Ciocalteau reagent) at 750nm (light path, 1cm). A standard curve was prepared using 1mg/mL crystalline bovine serum albumin (BSA) as standard protein, as shown in **Fig.2.**

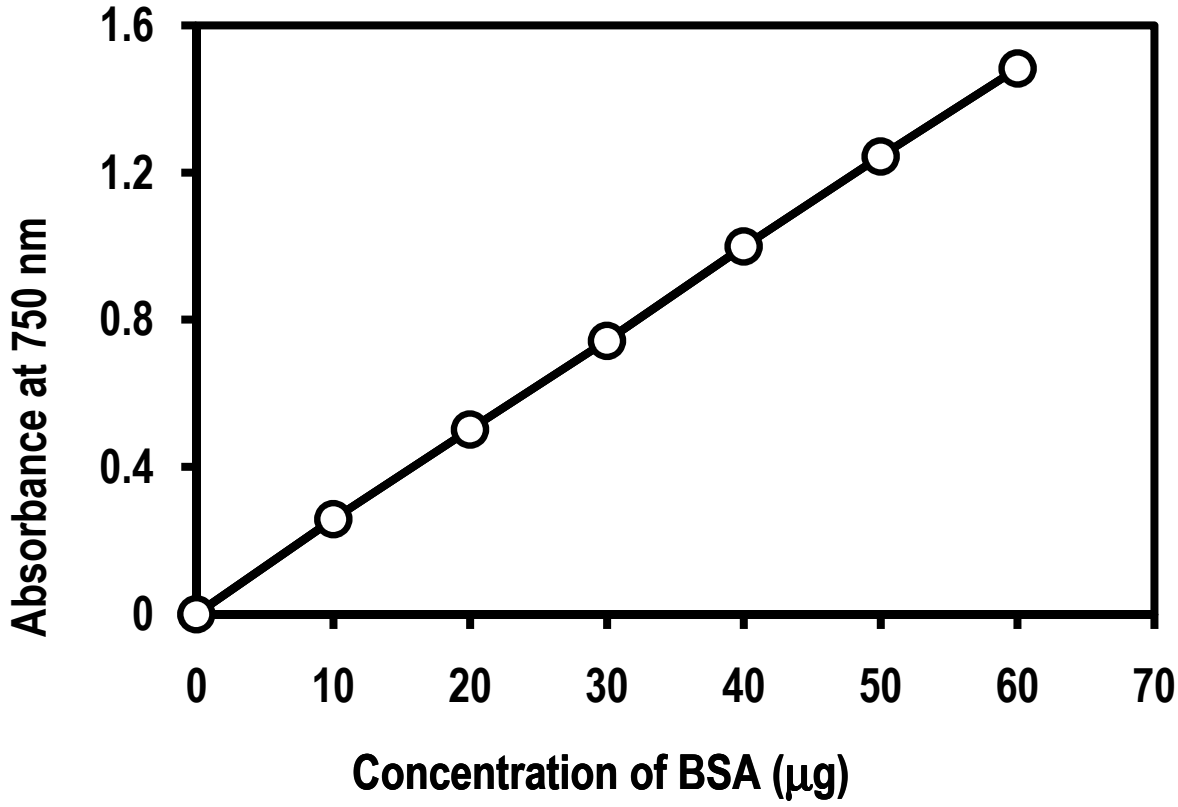

Fig. 2: Calibration of Folin-Ciocalteau's phenol reagent for protein estimation by the method Lowry *et al.* (1951) using crystalline bovine serum albumin as the standard

3.2.5. Hemagglutination inhibition assay of lectin: Hemagglutination inhibition lectin assay for determination of carbohydrate specificity of lectin was performed by a slight modification of the method of Sawhney *et al*. (1996) involving a standard serial dilution procedure. Each test carbohydrate solution (25µL) of fixed concentration [400mM in the case of sugar or 10% (w/v) in the case of polysaccharide or glycoprotein] was two-fold serially diluted with 50mM phosphate buffer pH 7.4 containing 0.9% NaCl (PBS) (25µL) in a U-bottom microtitre plate. To each well, 25µL of the lectin sample under test having 4 HAU of activity was added. After allowing 30min of incubation at 37°C to facilitate carbohydrate-lectin binding, 25µL of 2% rabbit RBC suspension (v/v) was added to each of the wells. Corresponding control wells - one for carbohydrate control (lectin plus RBC only) and another for lectin control (carbohydrate plus RBC only) were always included.The agglutination pattern was visually examined after incubation for 1h at 37°C. The inhibitory concentration of sugar, polysaccharide and glycoprotein was recorded as the lowest concentration that failed to agglutinate the rabbit RBC.

3.2.6. Lyophilization of protein sample: Thepooled lectin fractions obtained during chromatographic separations, carried out as the steps of lectin purification, were dialyzed extensively against de-ionized water. The resulting dialyzate was transferred to a lyophilization flask, frozen at -10°C and then lyophilized at -52°C by a SCANVAC Coolsafe™ lyophilizer.

3.2.7. Lectin purification: A lectin was extracted and purified from the fruiting body of *Lentinus cladopus* Lév by a procedure involving successive steps of crude lectin preparation, ammonium sulphate fractionation, discontinuous gradient Q-Sepharose ion exchange chromatography, DEAE-cellulose ion exchange chromatography and continuous gradient Q-Sepharose ion

exchange chromatography. All the operations were carried out at 0-8°C unless otherwise stated**.** Each of the purification steps is described below**:**

(i)Crude lectin preparation: The crude lectin sample was prepared from the fruiting body of mushroom *Lentinus cladopus* Lév by following the procedure described under **Lectin extraction.** The lectin activity and protein concentration of the crude lectin preparation were determined by the procedures described in **Lectin assay** and **Protein estimation** respectively. The corresponding specific activity was reported as HAU/mg protein**.**

(ii) Ammonium sulphate fractionation: The crude lectin preparation obtained above was subjected to ammonium sulphate fractionation to remove bulk of the unwanted material and to achieve concentration of the desired lectin. It was first brought to initial ammonium sulphate (0/20/60%) saturation by gradual addition of the calculated amount of the salt in small portions with constant stirring at 0°C**.** The stirring was continued further for 2h to precipitate as much proteins as possible**.** The resulting suspension was centrifuged at 17,000g for 30min at 4°C to discard the pellet**.** The supernatant obtained was then brought to respective final ammonium sulphate (20/60/90%) saturation with constant stirring at 0°C**.** The stirring was continued further for 2h to precipitate as much protein as possible**.** The resulting suspension was centrifuged as before and the pellet obtained was re-dissolved in minimum volume of 20mM Tris-HCl buffer, pH 7.8 containing 25mM NaCl and then dialyzed extensively against 1L of the same buffer at 0°C with four buffer changes. The lectin activity and protein concentration of the dialyzed ammonium sulphate (0-20%, 20-60% or 60-90%) fraction were determined as usual to ascertain the corresponding specific activity.

(iii) Discontinuous gradient Q-Sepharose anion exchange chromatography: The dialyzed ammonium sulphate (20-60%) fraction

(3mL), having the highest lectin activity among the above three ammonium sulphate fractions, was loaded on to a Q-Sepharose column (1x4cm**,** bed volume 4.2mL) pre-equilibrated with 20mM Tris-buffer pH 7**.**8 containing 25mM NaCl. The unabsorbed materials were washed out from the column by eluting with same buffer at a flow rate of 30mL/h**.** The washing was continued until the absorbance was <0**.**010 at 280nm on a Multiskan GO (Thermo Scientific) recording spectrophotometer**.** After the completion of the washing**,** the elution of the bound lectin from the charged column was effected by discontinuous gradient elution with the 20mM Tris buffer pH 7**.**8 containing varying salt concentrations (100mM, 200mM, 300mM, 400mM, and 500mM NaCl). The flow rate was kept constant at 30mL/h using a peristaltic pump (Ravel Hiteks Pvt. Ltd., Chennai, India). Individual fractions of 2mL each were collected. Protein elution was monitored by absorbance at 280nm and lectin elution by hemagglutination assay as described in the standard **Lectin assay.** The whole chromatography procedure was carried out at room temperature**.** The individual fractions of each lectin activity peaks eluted at different salt concentrations (0.2M, 0.3M and 0.4M NaCl) were pooled separately, dialyzed extensively against de-ionized water and concentrated by lyophilization carried out as described in **Lyophilization of protein sample**. The lectin activity and protein concentration of each of the three lyophilized pooled lectin fractions were determined as usual to ascertain the corresponding specific activities.

(iv) DEAE-Cellulose anion exchange chromatography**:** The lyophilized powder prepared from the 200mM NaCl-eluted lectin activity peak obtained in the above discontinuous gradient Q-Sepharoseanion exchange chromatography was dissolved in a minimum volume of 50 mM phosphate buffer pH 7.4 containing 20mM NaCl. The resulting dissolved fraction (1.1mL)

was loaded on to a DEAE-cellulose column (1x4cm, bed volume 4.2mL) pre-equilibrated with the same buffer. The column was washed with 3 bed volumes of the equilibration buffer. The bound proteins were then eluted by a continuous linear NaCl gradient ranging from 20mM to 250mM in 50mM phosphate buffer pH 7.4 at a flow rate of 6mL/h.Individual fractions of 1mL each were collected. Protein elution was monitored by absorbance at 280nm and lectin elution by hemagglutination assay as usual. Whole of the chromatography process was carried out at room temperature**.** Individual fractions exhibiting hemagglutinating activity were pooled together and dialyzed extensively against 20mM Tris-buffer, pH 7.8 containing 25mM NaCl. The lectin activity and protein of the dialyzed pooled lectin fraction were determined as usual to ascertain the corresponding specific activity.

(v) Continuous gradientQ-Sepharose anion exchange chromatography: The DEAE-cellulose lectin fraction (11mL) obtained above was subjected to anion exchange chromatography using a Q-Sepharose column (1x4cm, bed volume 4.2mL) pre-equilibrated with 20mM Tris-buffer, pH 7.8 containing 25mM NaCl. The column was washed with 3 bed volumes of the equilibration buffer. The bound proteins were then eluted from the column by a continuous linear NaCl gradient ranging from 25 to 200mM in 20mM Tris-buffer, pH 7.8 at a flow rate of 30mL/h. Individual fractions of 1mL each were collected. Protein elution was monitored by absorbance at 280nm and lectin elution by hemagglutination assay as usual. Whole of the chromatography process was carried out at room temperature**.** Individual fractions exhibiting high hemagglutinating activity were pooled together and dialyzed extensively against water and then lyophilized as usual. The lectin activity and protein of the dialyzed pooled lectin fraction were determined as usual to ascertain the corresponding specific activity.

3.2.8. Polyacrylamide gel electrophoresis in presence of sodium dodecyl sulphate: The lectin sample was subjected to polyacrylamide gel eletrophoresis in presence of sodium dodecyl sulphate (SDS-PAGE) according to the procedure of Laemmli (1970). The gel eletrophoresis system consisted of two contiguous but distinct gels - a resolving (lower) gel and a stacking (upper) gel. The gel casting apparatus was assembled after thoroughly cleaning the glass plates, spacers, and comb and buffer reservoirs. The polymerizing mixture for resolving gel (12%) was prepared by mixing together 4mL of 30% acrylamide solution (29.2g acrylamide and 0.8g N,N'-methylene-bis-acrylamide dissolved in 100mL of distilled water), 2.5mL of resolving gel buffer (1.5M Tris- HCl pH 8.8), 0.1mL of 10% SDS solution and 3.35mL of distilled water. After degassing with a vacuum pump, the polymerizing mixture was thoroughly mixed with 50µL of 10% ammonium persulphate solution and 6µL of N,N,N',N'-tetramethylethylenediamine (TEMED) and then quickly put between the glass plates up to the mark delimiting the resolving gel. After putting a layer of water on the top, the gel was left to set for 30-60 min. Meanwhile monomer solution of the stacking gel (6%) was prepared by mixing together 0.39mL of 30% acrylamide solution, 0.5mL of stacking gel buffer (0.5M Tris-HCl pH 6.8), 0.02mL of 10% SDS solution and 1.09mL distilled water. After de-aeration, the monomer solution was mixed with appropriate amount of ammonium persulphate (10.0µL of 10% solution) and TEMED (6.0µL). After pouring off the water over the resolving gel, top of the gel was rinsed with a little volume of stacking gel solution. Then, the stacking gel solution was put into the space above the resolving gel, well-cleaned comb was placed in the stacking gel, and the gel was left to set for 30-45min. After the polymerization of the stacking gel, the buffer reservoirs were filled with electrode buffer (prepared by dissolving 0.6g of Tris, 2.88g glycine and 0.2g SDS in a total volume of 200mL of distilled

water) and the comb was removed carefully without distorting the shape of the wells. The stock sample buffer was prepared by mixing 0.12mL of 0.5M Tris-HCl buffer pH 6.8, 0.2mL of 10% SDS, 0.1mL of 100% glycerol, 50µL of 0.5% bromophenol blue solution and 50µL of 2-mercaptoethanol. Onevolume of the above stock sample buffer was mixed with equal volume of the protein sample in a microfuge tube and heated in a boiling water bath for 5min, cooled to room temperature, centrifuged for a min at 3000g and then loaded on to the well of stacking gel using a microlitre Hamilton syringe. Finally the electrophoresis unit was connected to a power supply (Electophoresis Power Supply, Amersham Pharmacia Biotech) in such a way that the lower electrode was made anode and the upper one, the cathode. The current was adjusted to 15mA until the dye front traveled through the stacking gel. Then, the current was increased to 25mA and the electrophoresis was continued until the dye front reached the bottom of the resolving gel. After the run was complete, the power supply was disconnected. The gel was carefully removed from the glass plates and then stained by the procedure described under **Silver staining of protein on SDS-PAGE** below.

3.2.9. Silver staining of protein on SDS-PAGE: The proteins separated on polycryamide gel electrophoresis in presence of sodium dodecyl sulphate were visualized by silver staining carried out by a slight modification of the method of Merril (1990). The successive steps involved in the staining procedure are given below:

(i) The SDS-PAGE gel was fixed by soaking overnight in a solution containing 50% methanol, 12% acetic acid (glacial) and 0.075% formaldehyde.

(ii) The gel was washed by soaking in 50% ethanol with occasional shaking with the help of a gel rocker (Bangalore Genei Pvt. Ltd., Bangalore,

India), and then subsequent decantation of the ethanol solution after 10min. This washing was repeated three times.

(iii) Then the gel was treated with 0.02% $Na_2S_2O_3$ (sodium thiosulphate) for 1min.

(iv) The gel was washed three times in water (each wash lasting about 20s).

(v) The gel was treated with 0.2% $AgNO_3$ solution containing 0.075% formaldehyde for 20min with occasional shaking with the help of the shaker.

(vi) The gel was again washed three times with water (each wash lasting about 20s).

(vii) The gel was developed in 6% Na_2CO_3 containing 0.05% formaldehyde and 1 drop of $Na_2S_2O_3$ solution (1mg/mL) until bands appeared.

(viii) The gel was again washed three times with water (each wash lasting about 60s).

(ix) The gel was placed in a solution containing 50% methanol and 12% acetic acid to arrest further colour development.

3.2.10. Determination of subunit molecular weight of purified lectin by SDS-PAGE: The purified lectin preparation (0.6μg protein) was subjected to SDS-PAGE (14%) with reduction adopting the procedure of Laemmli (1970) described in **Polycryamide gel electrophoresis in presence of sodium dodecyl sulphate.** Protein molecular weight markers (Merck, medium range) consisting of phosphorylase b (M_r 97.4kDa), bovine serum albumin (M_r 66kDa), egg white ovalbumin (M_r 43kDa), carbonic anhydrase (M_r 29kDa), lactoglobin (M_r 18.4kDa), and aprotinin (M_r 6.5kDa) were used as reference proteins. The protein bands in the gel was fixed in a solution containing 50% methanoland 12% glacial acetic acid in distilled water overnight at room

temperature. The protein bands were stained with silver staining procedure described in **Silver staining of protein on SDS-PAGE.**The relative molecular weight of the purified lectin under the denaturing condition was calculated from the standard plot of R_f *vs.* log M_r.

3.2.11. Determination of the native molecular weight: The native molecular weight of the purified lectin was determined by gel filtration through a Sephadex G-75 column (Sigma) (82 x 0.9cm, bed volume 62mL) pre-equilibrated with 50mM phosphate buffer, pH 7.4 containing 0.9% NaCl (PBS). The void volume (V_o) of the gel was determined using blue dextran (M_r 2,000kDa) whose elution was monitored by absorbance at 625nm. The column was calibrated by using protein standards *viz*., bovine serum albumin (M_r 66.43kDa), chicken egg ovalbumin (M_r 44.3kDa), α-chymotrypsin (M_r 29kDa), lysozyme (M_r 18.4kDa) respectively. Each of the protein standards (1mL containing 1mg protein) and the purified lectin sample (1mL at 24.8μg/mL, containing 5120 HAU) was loaded separately on to the column, and was eluted by PBS at a flow rate of 6mL/h. Individual fractions (1mL each) were collected. The elution of protein standards was monitored by absorbance at 280nm. The elution of the lectin under investigation was however monitored through its hemagglutination activity. The native molecular weight of the purified lectin was calculated from the standard plot of V_e/V_o *vs*. log M_r.

3.2.12. Determination of metal ion requirement: The purified lectin sample was incubated at 10mM EDTA for 24 h at 8°C by mixing 0.9mL of the purified lectin sample containing 1,280 HAU with 0.1mL 0.1M EDTA in 50mM phosphate buffer pH 7.4 containing 0.9% NaCl (PBS). At the end of incubation, the resulting mixture was dialyzed against 500mL of 50mM Tris buffer pH 7.4, containing 0.9% (TBS) at 0°C with four buffer changes. The

activity of the EDTA-dialyzed lectin sample (25µL containing 0.02µg protein) was checked in the presence of each of the divalent metal ions *viz*. Ca^{2+} (calcium chloride), Mg^{2+} (magnesium chloride), or Mn^{2+} (manganese chloride) at different but fixed concentrations (0.5 to 16mM in test). 25µL of the EDTA-treated lectin sample was two-fold serially diluted with 25µL of TBS followed by successive addition of 25µL of the divalent metal ion solution to each well and incubated for 15min at room temp to facilitate lectin-metal binding. After incubation, 50µL of 2% rabbit RBC suspension (v/v) was added and the contents were mixed thoroughly. Corresponding control wells containing a mixture of TBS and RBC suspension in presence of metal ion were always included. After incubation for 1h at 37°C, the hemagglutination titre was scored visually. In a parallel experiment, 0.9mL of purified lectin sample containing 1,280 HAU was dialyzed extensively against 50mM TBS in the same manner as that applied to the EDTA-treated purified lectin and activity of the resulting buffer-dialyzed purified lectin was checked in absence or presence of the metal ions as described for the EDTA-dialyzed purified lectin.

3.2.13. Determination of effect of pH: The effect of pH on the hemagglutination activity of the purified lectin (0.075µg) was determined by varying the pH from 4.0 to 12.0 in the otherwise standard **Lectin assay**. Variation in pH of the assay mixture was achieved by taking different assay buffers at 50mM having different pH values, *viz*. citrate-phosphate buffer (for pH 4.0, 4.5, 5.0, 5.5 and 6.0); phosphate buffer (for pH 6.5, 7.0 and 7.5); Tris buffer (for pH 8.0, 8.5 and 9.0); Glycine buffer (pH 9.5, 10.0 and 10.5) and phosphate buffer (for pH 11.0, 11.5 and 12.0).

3.2.14. Determination of effect of temperature: The effect of temperature on the activity of the purified lectin (0.075μg) was studied by incubating the hemagglutination assay mixture at different designated temperatures, *viz*. 10, 30, 40, 50, 60, 70, 80, and 90°C, in the otherwise standard lectin assay procedure described under **Lectin assay.**

3.2.15. Determination of thermal stability: To determine the thermal stability, 50μL of purified lectin, dissolved in 50mM phosphate buffer pH 7.4 containing 0.9%NaCl (PBS) at 3.1μg/mL, was taken in microfuge tube and incubated at designated temperatures ranging from 10 to 90°C for 10min using a water thermostat (accuracy ±0.01°C). At the end of the incubation, the lectin sample was cooled in ice for 1min, then kept for 5min at room temperature and the residual lectin activity was assayed by the standard **Lectin assay.**

3.2.16. Determination of blood group specificity: Human blood samples belonging to different blood groups (group A, B and O) were collected from healthy donors with informed consent through a blood bank in the Regional Institute of Medical Sciences, Imphal, India in presence of heparin as anticoagulant. 2% (v/v) of human RBC suspension was prepared as described in **Preparation of rabbit RBC suspension.** The blood group specificity of the purified lectin (0.14μg) was determined by the standard hemagglutination assay described in **Lectin assay** replacing the rabbit RBC by the human RBC belonging to different blood groups. Parallel experiments were run using rabbit RBC for comparison of hemagglutination activities.

3.2.17. Protein identification by Mass Spectrometry: The purifiedlectinwas subjected to SDS-PAGE coupled with Coomassie staining and the gel plug obtained was then subjected to analysis through the help of international proteomics facility at Proteomics International Pty Ltd, located at Bayliss Building, UWA Campus, Crawley, Australia & MRF Building, 50 Murray Street, Perth, Australia. The protein sample was trypsin digested and peptides extracted according to standard techniques of Bringans *et al*. (2008). Tryptic peptides were loaded on to a HPLC column Agilent Zorbax 300SB-C18, 3.15 μm (Agilent Technologies) and separated with a linear gradient of water / acetonitrile / 0.1% formic acid (v/v). Peptides were analyzed by electrospray ionization mass spectrometry using the Shimadzu Prominence nano HPLC system (Shimadzu) coupled to a Triple TOF-5600 Mass Spectrometer (AB Sciex). Spectra were analyzed to identify protein of interest using Mascot Sequence matching software (Matrix Science) with the database: Ludwig NR, taxonomy: Fungi (October 2013, 1483294 sequences).

3.2.18. *De novo* sequencing and MS BLAST analysis: The purified lectin was subjected to SDS-PAGE coupled with Coomassie staining and the gel plug obtained was then subjected to analysis through the help of international proteomics facility at Proteomics International Pty Ltd, located at Bayliss Building, UWA Campus, Crawley, Australia & MRF Building, 50 Murray Street, Perth, Australia. The protein sample was trypsin digested and peptides extracted according to standard techniques (Bringans *et al*., Proteomics 2008). Tryptic peptides were loaded onto a HPLC column Agilent Zorbax 300SB-C18, 3.15μm [Agilent Technologies] and separated with a linear gradient of water/ acetonitrile/0.1% formic acid (v/v). Peptides were analyzed by electrospray ionization mass spectrometry using the Shimadzu

Prominence nano HPLC system (Shimadzu) coupled to a Triple TOF-5600 Mass Spectrometer [AB Sciex]. MS/MS spectra were analyzed using PEAKS Studio Version 4.5 SP2 [Bioinformatics solutions] and manual interpretation to deduce the amino acid sequence of a major peptide present in the lectin. The resulting fragment sequence was used to interrogate protein databases using the specialized algorithm for mass spectrometric analysis, MS BLAST available at http://dove.embl-heidelberg.de/blast2/msblast.html as suggested by Shevchennko *et al*. (2001).