CHAPTER 4

RESULTS

4.1. Purification of lectin: A lectin capable of hemagglutinating rabbit and human RBC was isolated and purified from the fruiting body of the mushroom*Lentinus cladopus* Lév.by a procedure involving successive steps of lectin extraction**,** ammonium sulphate fractionation, discontinuous gradient Q-Sepharose chromatography, DEAE-cellulose chromatography and continuous gradient Q-Sepharose chromatography**.** To start with, the crude lectin sample was prepared from the fruiting body of the mushroom as described under **METHODS.** The lectin activity and protein concentration of the crude lectin preparation were determined, and it was found to have a typical specific lectin activity of 1,136 HAU/mg protein**.** In the present investigation**,** various chromatographic techniques were adopted during the attempt to purify the lectin**.** To facilitate the success of the purification attempt**,** the crude lectin extract was first partially purified by subjecting to ammonium

sulphate fractionation at three alternative salt saturation ranges 0-20%, 20-60% and 60-90%. The summary of the partial purification of the lectin at the end of ammonium sulphate fractionation step are given in **Table 1**. The 20-60% ammonium sulphate fractionation achieved the best partial purification of the lectin among the three salt fractionations tested. The corresponding ammonium sulphate fraction was 2.8-fold purified with 160% recovery and found to have a typical specific lectin activity of 3,233.7 HAU/mg**.** It was then subjected for further purification to a discontinuous gradient Q-Sepharose ion exchange chromatography**.** After loading of the 20-60% ammonium sulphate fraction on to the Q-Sepharose column, it was thoroughly washed and then the bound proteins were eluted with 20mM Tris buffer pH 7.8 at increasing but fixed NaCl concentrations (0.1M, 0.2M, 0.3M, 0.4M and 0.5M). The elution profile of the ion-exchange chromatography purification step**,** obtained by analyzing protein elution by absorbance at 280 nm and lectin elution by its hemagglutination activity**,** is shown in **Fig. 3.** The protein peaks eluted at 0.1M and 0.5M did not exhibit any lectin activity. However, the protein peaks eluted at 0.2M, 0.3M and 0.4M NaCl were found to exhibit lectin activity. The active fractions of each of these protein peaks were pooled separately and dialyzed against water exhaustively and lyophilized. Each of the resulting lyophilized powders was dissolved in a minimum volume of 50mM phosphate buffer pH 7.4, and protein concentration and lectin activity were determined. The fold purification, recovery percentage and specific activity for each of the pooled fractions are given in **Table 2.** The pooled fraction of the activity peak eluted at 0.2M NaCl was found to be purified by 4.0-fold (with reference to the starting crude lectin preparation) with 69**.**0% recovery and had a specific lectin activity of 4,512.5HAU/mg protein. The pooled fractions of the other two activity peaks (eluted at 0.3M NaCl and at 0.4M NaCl) were, however, found to have specific activities (727.3 and 160.0 HAU/mg protein) that are lesser

Table 1: Summary of partial purification of lectin from fruiting body of *Lentinus cladopus* Lév. by lectin extraction followed by ammonium sulphate fractionation

Purification step		**Total protein (mg)***	**Total lectin activity (HAU)****	**Specific activity (HAU/mg)**	**Purification fold**	**Recovery %*****
Crude lectin preparation*****		33.8	38400.0	1136.0	1.0	100.0
Ammonium sulphate fractionation	0-20%	3.4	1728.0	508.2	0.5	4.5
	20-60%	19.0	61440.0	3233.7	2.8	160.0
	60-90%	10.0	2240.0	224.0	0.2	5.8

***** Protein was determined by the method of Lowry *et al.* (1951) described in **Protein estimation** under **METHODS** using crystalline BSA as the standard.

** The lectin activity was taken as the titre strength that is the reciprocal of the highest dilution showing complete agglutination in the standard **Lectin assay** under **METHODS**. It was expressed in terms of hemagglutination unit (HAU) that is the minimum amount of lectin required for 100% agglutination under the assay condition (Sawhney *et al*., 1996).

*** Recovery of activity as compared to the activity of starting crude lectin preparation taken as 100%.

**** Lectin was extracted from 10g of dried fruting body of *Lentinus cladopus* Lév. as lectin source as described in **Lectin extraction** and **Lectin purification** under **METHODS.**

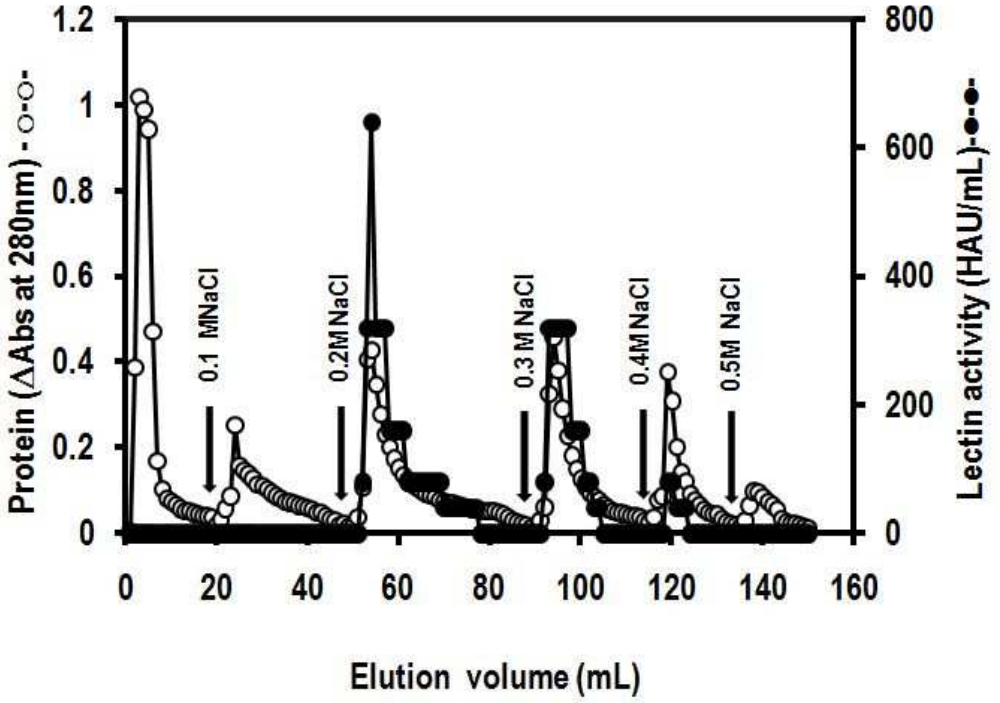


Fig.3: Elution profile of discontinuous gradient Q-Sepharose chromatography. The dialyzed 20-60% ammonium sulphate fraction (3.0mL) was loaded on to a Q-Sepharose ion exchange chromatography column (4x1cm, bed volume 4.2mL). The bound lectin was eluted at a flow rate of 30mL/h with 20mM Tris buffer pH 7.8 at increasing but fixed salt concentrations in the range 0.1- 0.5M NaCl. Fractions of 2mL each were collected. The plot with unfilled circles (○) represents elution of protein detected by absorbance at 280nm whereas that with filled circles (●) represents elution of lectin detected by its activity. For details, see **Lectin purification** under **METHODS.**

Table 2: Summary of partial purification of lectin from fruiting body of *Lentinus cladopus* Lév.by a procedure involving successive steps of lectin extraction, ammonium sulphate fractionation and discontinuous gradient Q-Sepharose ion exchange chromatography.

Purification step		**Total Protein (mg)***	**Total Activity (HAU)****	**Specific Activity (HAU/mg)**	**Fold Purification**	**Recovery %*****
Crude lectin preparation****		33.8	38400.0	1136.0	1.0	100.0
20-60% ammonium sulphate fractionation		19.0	61440.0	3233.7	2.8	160.0
Discontinuous gradient Q-Sepharose chromatography	Pooled fraction eluted at 0.2M NaCl	5.9	26624.0	4512.5	4.0	69.3
	Pooled fraction eluted at 0.3M NaCl	1.1	800.0	727.3	0.6	2.1
	Pooled fraction eluted at 0.4M NaCl	0.5	80.0	160.0	0.1	0.2

***** Protein was determined by the method of Lowry *et al.* (1951) described in **Protein estimation** under **METHODS**.

****** The lectin activity was taken as the titre strength that is the reciprocal of the highest dilution showing complete agglutination in standard **Lectin assay** under **METHODS**. It was expressed in terms of hemagglutination unit (HAU) that is the minimum amount of lectin required for 100% agglutination under the assay condition (Sawhney *et al*., 1996).

******* Recovery of activity as compared to the activity of starting crude lectin preparation taken as 100%.

******** Lectin was extracted from 10g of dried fruting body of *Lentinus cladopus* Lév. as lectin source as described in **Lectin extraction** and **Lectin purification** under **METHODS**.

than that of the starting crude lectin preparation (1,136 HAU/mg protein), and therefore, these lectin fractions were not considered for further purification. The pooled fraction of the activity peak eluted at 0.2M NaCl during the Q-Sepharose ion exchange chromatography was dissolved in 50mM phosphate buffer pH 7.4 containing 20mM NaCl and then loaded on to a DEAE-Cellulose column pre-equilibrated with the same buffer. The column was thoroughly washed with the same buffer and the bound proteins were then eluted by a continuous linear NaCl gradient ranging from 20 to 250mM in 50mM phosphate buffer pH 7.4 at a flow rate of 6mL/h. The elution profile**,** obtained by analyzing protein elution by absorbance at 280nm and lectin elution by its hemagglutination activity**,** is shown in **Fig.4.** A single lectin activity peak was observed. Fractions with hemagglutinating activity, covered by the horizontal bar shown within the figure, were pooled and dialyzed extensively against 20mM Tris-HCl buffer pH 7.8 containing 25mM NaCl. The resulting DEAE-cellulose chromatography fraction was found to have a typical specific activity of 70,400 units/mg protein with a purification fold of 62 and a recovery of 36.6% with reference to the starting crude lectin preparation. It was loaded on to a column of Q-Sepharose pre-equilibrated with 20mM Tris buffer pH 7.8 containing25mM NaCl and washed with the same buffer.The bound proteins were eluted with a continuous salt gradient ranging from 0.025M to 0.2 M NaCl in the same buffer. The elution profile, obtained by analyzing protein absorbance at 280nm and lectin activity by hemagglutination assay of the eluted fractions (1mL each)**,** is shown in **Fig. 5.** A single lectin activity peak was observed. Those active fractions covered by the horizontal bar shown within the figure were pooled together and dialyzed exhaustively against water and then lyophilized. The lyophilized powder, thus obtained, was dissolved in a minimum volume of 50mM phosphate buffer pH 7.4 containing 0.9% NaCl (PBS). The resulting purified protease preparation was found to have a specific activity of 2,04,800 HAU/mg protein. It was purified 180-fold with a recovery of 16% as compared to the crude preparation. The summary of purification of the lectin from the fruiting body of *Lentinus cladopus* Lév. is given in **Table 3.**

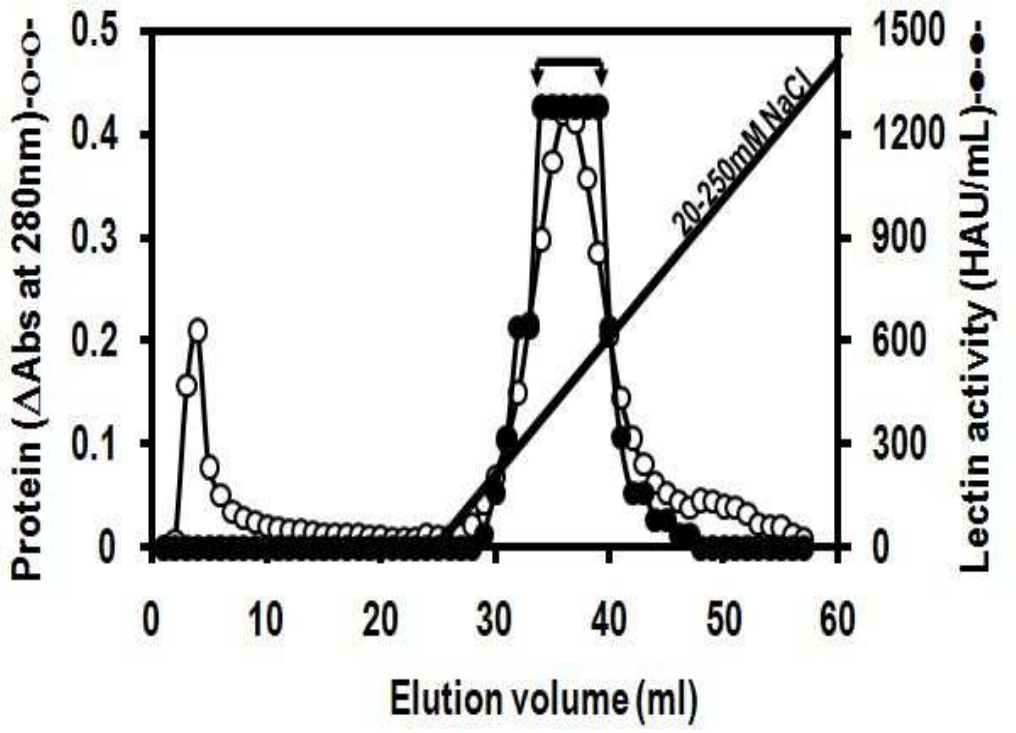


Fig.4: Elution profile of continuous gradient DEAE-cellulose chromatography. The lyophilized 0.2M NaCl fraction obtained from the discontinuous gradient Q-Sepharose chromatography was dissolved in a minimum volume of 50mM phosphate buffer pH 7.4, and was then loaded on to a DEAE-cellulose column (4×1cm, bed volume 4.2mL)**.** The bound lectin was eluted at a flow rate of 6mL/h with the same buffer at continuous gradient from 0.02 to 0.25M NaCl. Fractions of 1mL each were collected. The plot with unfilled circles (○) represents elution of protein detected by absorbance at 280nm whereas that with filled circles (●) represents elution of lectin detected by its activity. For details**,** see **Lectin purification** under **METHODS.**

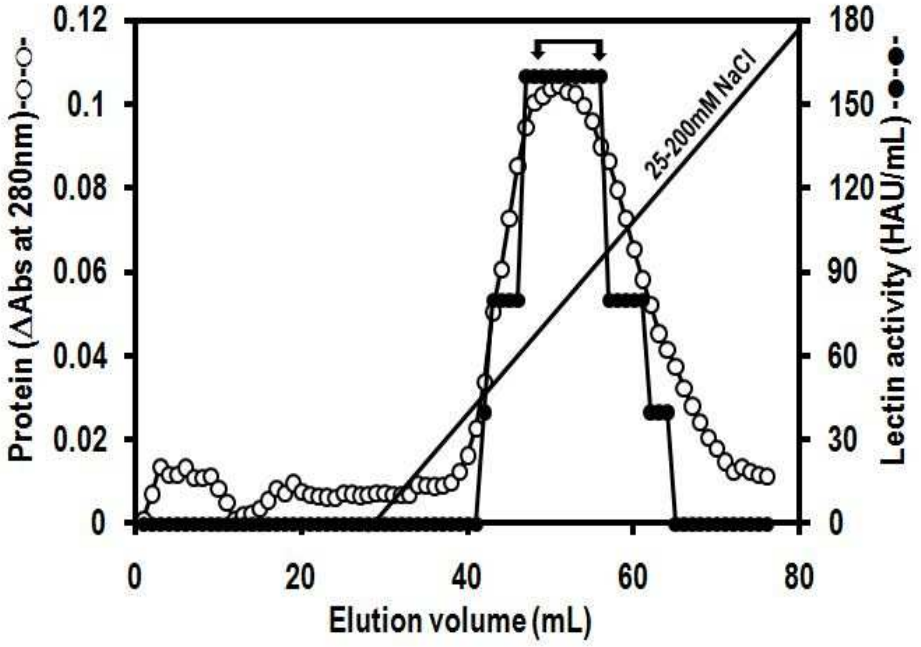


Fig.5: Elution profile of continuous gradient Q-Sepharose chromatography. The dialyzed DEAE-cellulose chromatography fraction was loaded on to a Q-Sepharose column (4×1cm, bed volume 4.2mL) and washed. The bound lectin was eluted at a flow rate of 30 mL//h with 20 mM Tris-buffer, pH 7.8 at continuous gradient from 0.02 to 0.2M NaCl. Fractions of 1mL each were collected. The plot with unfilled circles (○) represents elution of protein detected by absorbance at 280nm whereas that with filled circles (●) represents elution of lectin detected by its activity. For details, see **Lectin purification** under **METHODS.**

Table 3. Summary of the purification of lectin from the fruting body of *Lentinus cladopus* Lév.

Purification step	**Total protein (mg)***	**Total activity (HAU/mL)****	**Specific activity (HAU/mg)**	**Fold purification**	**Recovery %*****
Crude lectin preparation****	33.80	38400.0	1136.0	1.0	100.0
20-60% Ammonium sulfate fractionation	19.00	61440.0	3233.7	2.8	160.0
Discontinuous gradient Q-Sepharose Chromatography	5.90	26624.0	4512.5	4.0	69.3
DEAE-cellulose Chromatography	0.20	14080.0	70400.0	62.0	36.7
Continuous gradient Q-Sepharose Chromatography	0.03	6144.0	204800.0	180.0	16.0

***** Protein was determined by the method of Lowry *et al.* (1951) described in **Protein estimation** under **METHODS**.

****** The lectin activity was taken as the titre strength that is the reciprocal of the highest dilution showing complete agglutination in the standard **Lectin assay** under **METHODS**. It was expressed in terms of hemagglutination unit (HAU) that is the minimum amount of lectin required for 100% agglutination under the assay condition (Sawhney *et al*., 1996).

******* Recovery of activity as compared to the activity of starting crude lectin preparation taken as 100%.

******** Lectin was extracted from 10g of dried fruting body of *Lentinus cladopus* Lév. as lectin source as described in **Lectin extraction** and **Lectin purification** under **METHODS**.

4.2. Homogeneity of the purified lectin preparation: The lectin preparation purified from the fruiting body of *Lentinus cladopus* Lév.was found to migrate as a single protein band on polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE) with reduction performed according to the method of Laemmli (1970) coupled with silver staining by a minor modification of the method of Merril (1990). The lectin sample was heated in boiling water bath in a sample buffer containing 2-mercaptoethanol before subjecting to the electrophoresis using a 12% resolving slab gel**.** The result is shown in **Fig.6**. The migration as single protein band showed that the purified lectin preparation was electrophoretically homogeneous**.**

Fig.6: Behaviour of the the lectin purified from the fruting body of *Lentinus cladopus* Lév. on SDS-PAGE (12% gel). The lectin (0.6µg) was electrophoresed according to the method of Laemmli (1970) with reduction described in **Polyacrylamide gel electrophoresis** and **Silver staining of protein**under **METHODS.** The protein band was silver stained by a minor modification of the method of Merril (1990).

4.3. Molecular weight on SDS-PAGE: The subunit molecular weight of the purified lectin from the fruiting body of *Lentinus cladopus* Lév was determined by SDS-PAGE (14% gel) according to the method of Laemmli (1970) with reduction using protein molecular weight markers (Medium Molecular Weight Markers, Merck, India), *viz.* phosphorylase b (M_r 97.4kDa), bovine serum albumin (M_r 66kDa), ovalbumin (M_r 43kDa), carbonic anhydrase (M_r 29kDa), lactoglobulin (M_r 18.4kDa) and aprotinin (M_r 6.5kDa). The electrophoretic migrations of the protein bands are shown in **Fig.7 (A).** The ratio of the distance migrated by marker protein to the distance migrated by dye front (R_f) is plotted against logarithm of relative molecular weight (M_r) in **Fig.7 (B).** The molecular weight of the lectin under denaturing condition with reduction**,** deduced from the standard R_f *vs.* log M_r plot**,** was found to be 20kDa**.**

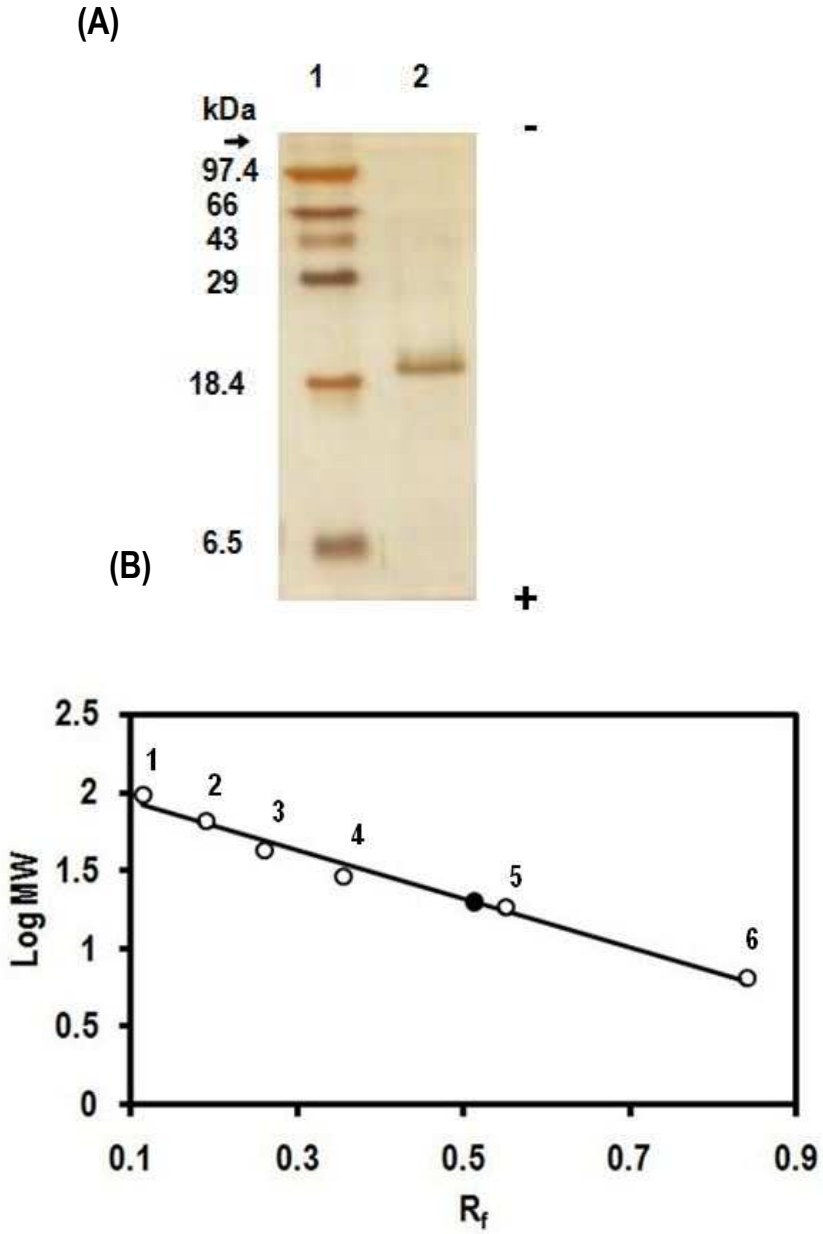


Fig.7 (A): Electrophoretic mobility of the purified lectin from the fruting body of *Lentinus cladopus* Lév. on SDS-PAGE (14% gel)**.** The arrow indicates the origin of the electrophoretic migration**.** The protein bands were silver stained by a minor modification of the method of Merril (1990)**.** The detailed procedure performed according to Laemmli (1970) is described in **Determination of subunit molecular weight** under **METHODS**. Lane 1: molecular weight marker proteins (Merck, medium range), Lane 2: the purified lectin (0.6µg). **Fig.7 (B):** Determination of subunit molecular weight of the purified lectin based on the data of SDS-PAGE in **Fig. (A).** Relative mobility (R_f)**,** calculated with reference to dye front**,** is plotted against logarithm of subunit molecular weight (log MW). The unfilled circles (○)**,** labelled 1 to 6**,** represent the experimental points corresponding to phosphorylase b (M_r 97.4kDa), bovine serum albumin (M_r 66kDa), ovalbumin (M_r 43kDa), carbonic anhydrase (M_r 29kDa), lactoglobulin (M_r 18.4kDa) and aprotinin (M_r 6.6kDa)**,** respectively**.** The unlabelled filled circle (●) represents the experimental point corresponding to the purified lectin**.**

4.4. Native molecular weight: The native molecular weight of the lectin purified from the fruiting body of *Lentinus cladopus* Lév was determined by gel filtration through a column of Sephadex G-75 using bovine serum albumin (M_r 66kDa)**,**chicken egg ovalbumin (M_r 44.3kDa), α-chymotrypsin (M_r 29kDa), lysozyme (M_r 18.4kDa) as the standard proteins**.** The results of the gel filtration are shown in **Fig.8** in which the ratio of the elution volume to void volume (V_e/V_o) is plotted against logarithm of relative molecular weight (M_r)**.** The native molecular weight of the purified lectin, deduced from the standard V_e/V_o *vs***.** log M_r plot, was found to be 40kDa**.** By considering this result of native molecular weight determination together with that of the subunit molecular weight determination by SDS-PAGE**,** the purified lectin from the fruiting body of *Lentinus cladopus* Lév was inferred to be a dimeric protein made up of apparently chemically identical subunits.

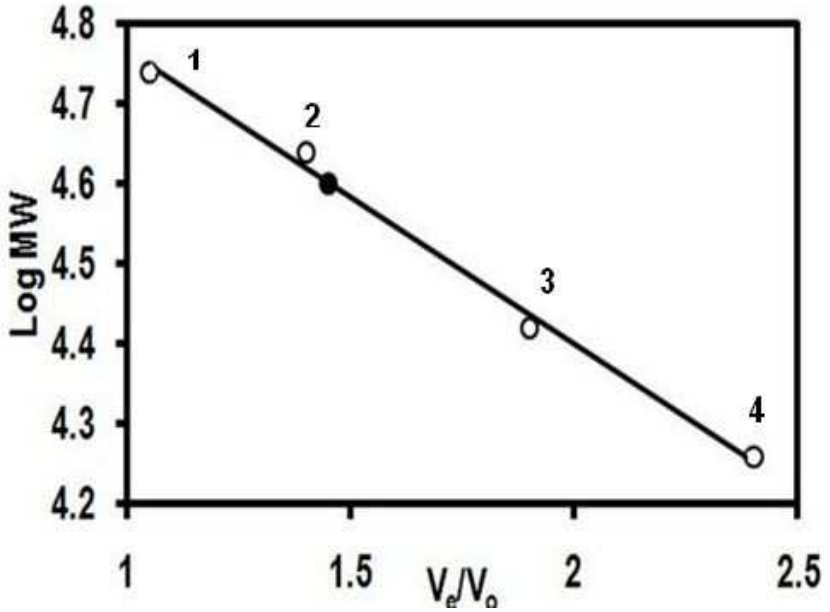


Fig.8: Determination of native molecular weight of the purified lectin from the fruiting body of *Lentinus cladopus* Lév based on the data of gel filtration through a Sephadex G-75 column (Sigma) (82x0.9cm**,** bed volume 62mL), the detailed procedure of which is described in **Determination of the native molecular weight** under **METHODS.** The ratio of elution volume to void volume**,** V_e/V_o**,** was plotted against logarithm of molecular weight of standard protein markers**.** The void volume (V_o) was determined using blue dextran (M_r 2,000kDa) whose elution was monitored by absorbance at 625nm. The unfilled circles (**O**)**,** labelled 1 to 4**,** represent the experimental points corresponding to the standard protein markers, *viz***.** bovine serum albumin (M_r 66kDa), chicken egg ovalbumin (M_r 44.3kDa), α –chymotrypsin (M_r 29kDa), lysozyme (M_r 18.4kDa). The unlabelled filled circle (●) represents the experimental point corresponding to the purified lectin**.**

4.5. Sugar specificity: The carbohydrate specificity of the lectin purified from the fruiting body of *Lentinus cladopus* Lév was determined by hemagglutination inhibition lectin assay**.** It was performed by a slight modification of the method of Devi *et al***.** (2009) involving a standard two-fold serial dilution procedure using 2% suspension (v/v) of freshly washed rabbit RBC**.** The inhibitory concentration of the catbohydrate was taken as its minimum concentration required for complete inhibition of the hemagglutination activity of 4 HAU of the lectin**.** The sugars and sugar derivatives taken for the inhibition test were 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 and N-Acetyl-D-lactosamine**.**Some polysaccharides *viz*.heparin*,* mannan, dextran, gum tragacanth, gum ghatti were also examined for their inhibitory effect on the hemagglutination activity of the lectin. None of these polysaccharides was, however, found to be inhibitory to the hemagglutinating activity of the lectin. Certain glycoproteins *viz*. thyroglobulin, ovalbumin and peroxidase at a concentration as high as 10% (w/v) were also investigated for their inhibitory effect on the hemagglutination activity of the lectin. Again, none of them was found to inhibit the hemagglutinating activity of the lectin. The summary of the results of the hemagglutination inhibition assay of the lectin is given in **Table 4.**

Table 4. Hemagglutination inhibition test for the purified lectin preparation from the fruiting body of *Lentinus cladopus* Lév.

Simple sugar and its derivatives	**Minimum inhibitory concentration***
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-Acetamide-D-deoxy-Galactonolactone, 4-Methyl-D-Galactoside, D-Raffinose, N-acetyl-D-Lactosamine,	Each of the sugar or sugar derivatives was non-inhibitory even up to 66.7mM in test
Polysaccharides	
Mannan, Dextran, Heparin, Gum ghatti, Gum tragacanth	Each of the polysaccharides was non-inhibitory even up to 1.67% in test
Glycoprotein	
Thyroglobulin, Ovalbumin, Peroxidase	Each of the glycoproteins was non-inhibitory even up to 1.67% in test

*The minimum concentration of the sugar or its derivatives, polysaccharide or glycoprotein required to completely inhibit the hemagglutination by the purified lectin sample having 4 HAU activity in the standard **Hemagglutination Inhibition Assay** as described under **METHODS.**

4.6. Effect of metal ion: To determine the effect of divalent metal ion on the activity of lectin purified from the fruiting body of *Lentinus cladopus* Lév., the activity was determined after (i) subjecting the purified lectin to treatment with EDTA followed by subsequent extensive dialysis at 0°C to remove any bound metal ion, and (ii) subsequent treatment of the EDTA-dialyzed lectin with externally added metal ion. The experimental results are given in **Table 5.** The expression of an optimum activity by the EDTA-dialyzed lectin preparation remained same in the absence or presence of externally added divalent metal ion Ca^{2+} (calcium chloride) / Mg^{2+} (magnesium chloride)/ Mn^{2+} (manganese chloride), in the concentration range of 0.5 to 16 mM. In a parallel control experiment, the same sample of the purified lectin was subjected to extensive dialysis and then the lectin activity was determined in the absence or presence of externally added divalent metal ion as before. The activity of the resulting buffer-dialyzed lectin was also found to be same as that of the EDTA-dialyzed purified lectin either in the absence or presence of externally added divalent metal ion. These experimental results showed that the activity of the lectin is independent of divalent metal ions such as Ca^{2+}, Mg^{2+} and Mn^{2+}.

Table 5: Effect of metal ions on activity of the lectin purified from the fruting body of *Lentinus cladopus* Lév.

Concentration of metal ion (Mg^{2+}/Mn^{2+} /Ca^{2+}) in the lectin assay mixture (mM)	**Lectin activity (HAU/mL)**	
	EDTA-dialyzed purified lectin*	**Buffer-dialyzed purified lectin****
0.00	160	160
0.50	160	160
1.00	160	160
2.00	160	160
4.00	160	160
8.00	160	160
16.0	160	160

***The activity of each lectin sample (25μL containing 0.02μg of protein) was determined in the presence of metal ion at different but fixed concentrations. Detailed procedure is given in **Determination of metal ion requirement** under **METHODS**.

****EDTA –dialyzed lectin was prepared by incubating purified lectin (5.6μg/mL) with 10mM EDTA in PBS, followed by extensive dialysis against TBS. A similar procedure without EDTA treatment was carried out to obtain the buffer-dialyzed lectin preparation

4.7. Effect of pH on lectin activity: The effect of pH on the activity of the lectin purified from the fruiting body of *Lentinus cladopus* Lév. was determined as a function of the assay pH. The pH was varied from 4.0 to 12.0 in the otherwise standard hemagglutination assay mixture by using different buffers at 50 mM: citrate-phosphate buffer (pH 4.0 to 6.0), phosphate buffer (pH 6.5 to 7.5 and pH 11.0 to 12.0), Tris buffer (pH 8.0 to 9.0) and Glycine buffer (pH 9.5 to 10.5). The results are shown in **Fig. 9.** The pH optimum of the lectin activity was found to be relatively broad ranging from 7.5 to 9.0. The activity declined on either side of the pH range at which optimum (100%) lectin activity is expressed. On the lower pH side, the activity decreased to 25% and 0% of the optimum value at pH 6.0 and 5.0 respectively. On the higher pH side, the lectin activity was also reduced to 50%, 25% and 0% of the optimum value at pH 10.0, 11.0 and 11.5 respectively.

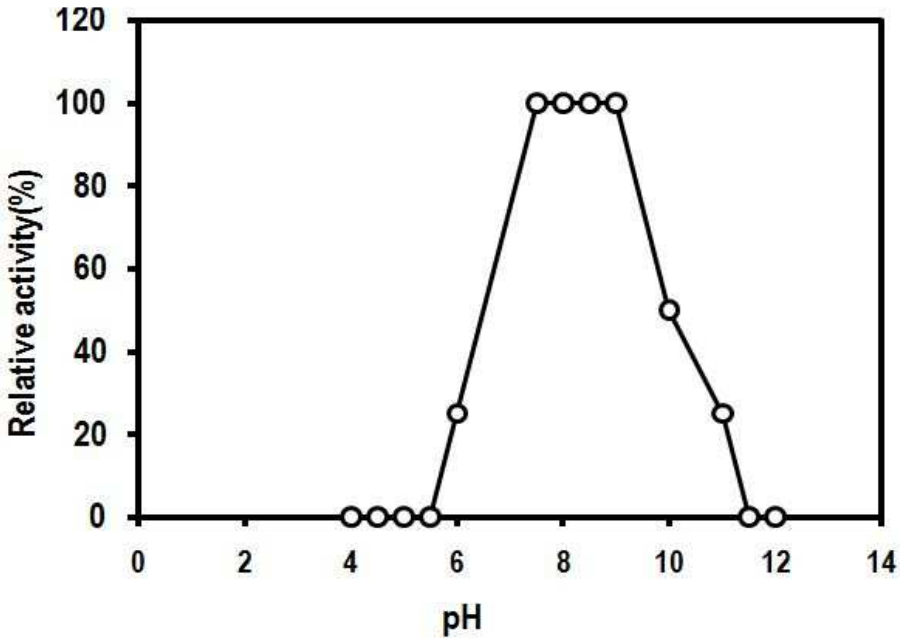


Fig.9: Effect of pH on activity of the lectin purified from the fruiting body of *Lentinus cladopus* Lév. The hemagglutinating activity of the purified lectin (0.075μg) was studied as a function of assay pH in the otherwise standard **Lectin assay** system. The assay pH was plotted against the lectin activity relative to the optimum (100%) activity. For details, see **Determination of effect of pH** under **METHODS.**

4.8. Effect of temperature: The effect of temperature on the activity of lectin purified from the fruiting body of *Lentinus cladopus* Lév. was studied by incubating the hemagglutination assay mixture at different designated temperatures ranging from 10-90°C in the otherwise standard lectin assay procedure described in **Lectin assay** under **METHODS.** The results are shown in **Fig. 10.** A maximum lectin activity was observed in the temperature range from 10 to 50°C under the prevailing experimental conditions**.** The activity was reduced rapidly at assay temperatures greater than 50°C**.** At assay temperature of 80°C, only 25% of the activity remained**.** No activity was observed at all at 90°C**.**

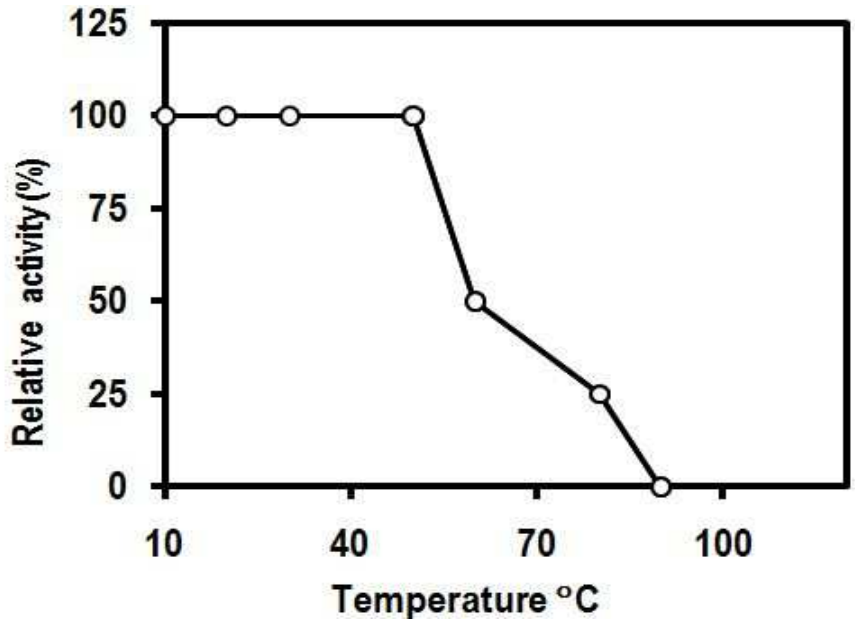


Fig.10: Effect of temperature on activity of the lectin purified from the fruiting body of *Lentinus cladopus* Lév. The hemagglutinating activity of the purified lectin (0.075µg) was studied as a function of the assay temperature (varied from 10 to 90°C) in the otherwise standard **Lectin assay** system. The assay temperature was plotted against the lectin activity relative to the maximum (100%) activity. For details, see **Determination of effect of temperature** under **METHODS.**

4.9. Thermal stability of the lectin: In a thermal stability experiment carried out in the present investigation, the lectin purified from fruiting body of *Lentinus cladopus* Lév. was dissolved in 50 mM phosphate buffer pH 7.4 containing 0.9% NaCl (PBS) at 3.1µg/mL. It was then incubated for 10 min at the designated temperatures ranging from 10 to 90°C in a sealed microfuge tube using a water thermostat (sensitivity ±0.01°C). At the end of the incubation time, the lectin sample was cooled in ice, brought to room temperature, and then the residual activity was assayed as in standard **Lectin assay** described under **METHODS.** The results are shown in **Fig. 11** in which the percent residual activity was plotted against the incubation temperature. The lectin remained fully active when incubated at temperatures ranging from 10 to 50°C under the experimental conditions. However, the lectin was rapidly inactivated at incubation temperatures higher than 50°C. The $T_{1/2}$ for the thermal inactivation, the temperature at which 50% of the full lectin activity is lost, was found to be 55°C under the prevailing experimental conditions. Only 25% activity remained by incubation at 70°C and complete inactivation was observed by incubation at 90°C.

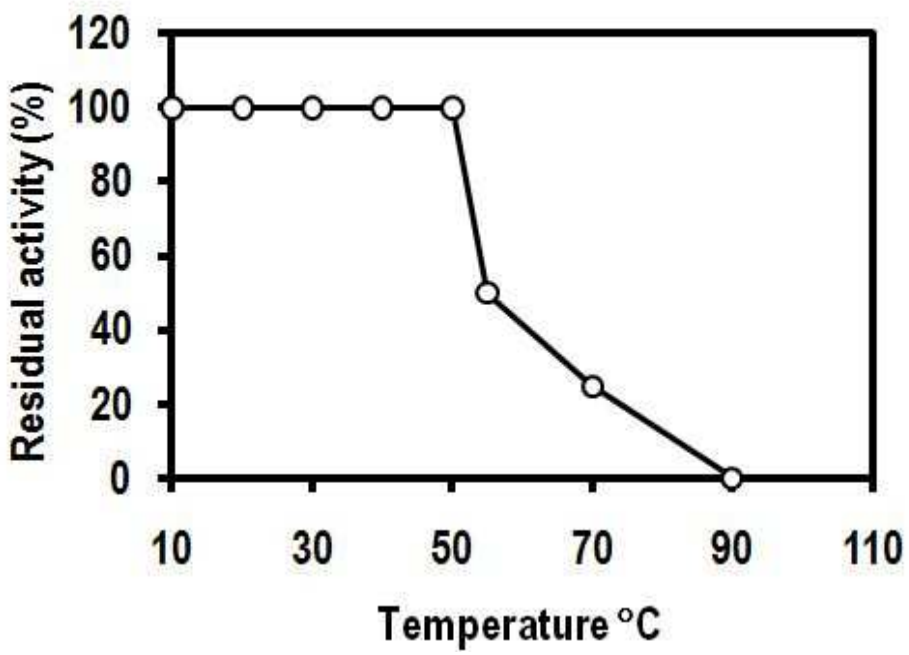


Fig.11: Thermostability behaviour of the lectin purified from the fruiting body of *Lentinus cladopus* Lév. The lectin dissolved in PBS at 3.1μg/mL was incubated for 10 min at designated temperatures (10 to 90°C) and then the residual activity was measured by the standard **Lectin assay** as described in **Determination of thermal stability** under **METHODS.**

4.10. Blood group specificity: To study the human blood group specificity of the lectin purified from fruiting body of *Lentinus cladopus* Lév**.,** the lectin activity was determined as usual adopting the standard assay method described in **Lectin assay** under **METHODS** replacing the rabbit RBC by those prepared from human blood samples belonging to the different blood groups A**,** B and O. This lectin did not show remarkable specificity towards any of the human ABO blood groups. It could agglutinate RBC belonging to all A, B and O groups, with O group being only 2 times more specific than either A or B group. However**,** the observed hemagglutination activity of the lectin towards RBC belonging to the human blood groups was 2-4 times lesser than that observed towards rabbit RBC**.** The results are given in **Table 6.**

Table 6: Blood group specificity of the lectin purified from the fruting body of *Lentinus cladopus* Lév.

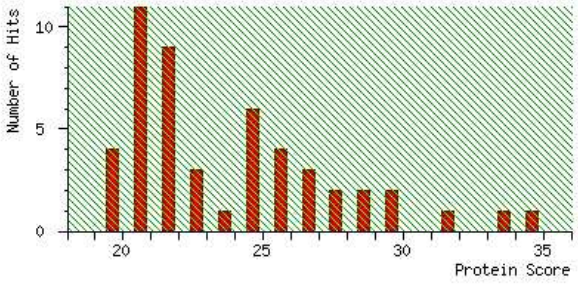
RBC type	**Lectin activity (HAU/mL)***
Human blood group A	160
Human blood group B	160
Human blood group O	320
Rabbit	640

***The activity of the purified lectin (0.14μg) was determined by taking different RBC types in the otherwise standard **Lectin assay** as described in **Determination of blood group specificity** under **METHODS.**

4.11. Protein identification by mass spectrometry: The peptide mass fingerprinting of the lectin purified from the fruiting body of *Lentinus cladopus* Lév**.**was carried out at the international proteomics facility at Proteomics International Pty Ltd, located at Bayliss Building, UWA Campus, Crawley, Australia & MRF Building, 50 Murray Street, Perth, Australia. The purifiedlectinwas subjected to protein separation by SDS-PAGE coupled with Coomassie staining. 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 with Mascot Sequence matching software (Matrix Science). The resulting peptide mass fingerprint was found to have no significant match with any of those of known fungal proteins in the database Ludwig NR, taxonomy: Fungi (1483294 sequences as of October 2013) as shown in **Fig.12.** The detailed results of the peptide mass fingerprinting analysis of the purified lectin are provided in **Appendix I**.

Mascot Score Histogram

Ions score is -10*Log(P), where P is the probability that the observed match is a random event.
Individual ions scores > 47 indicate identity or extensive homology (p<0.05).
Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.



Peptide Summary Report

Format As: Peptide Summary			Help
	Significance threshold p< 0.05	Max. number of hits AUTO	
	Standard scoring ◉ MudPIT scoring ○	Ions score or expect cut-off 0	Show sub-sets 0
	Show pop-ups ◉ Suppress pop-ups ○	Sort unassigned Decreasing Score	Require bold red ☐

Select All | Select None | Search Selected | ☐ Error tolerant | Archive Report

No significant hits to report

Peptide matches not assigned to protein hits: (no details means no match)

Fig.12: Mascot score histogram of peptide fingerprinting analysis of the lectin purified from fruiting body of *Lentinus cladopus* Lév.

4.12. *De novo* sequencing and MS BLAST analysis: The *De novo* sequence analysis of the lectin purified from the fruiting body of *Lentinus cladopus* Lév**.** was carried out at the international proteomics facility at Proteomics International Pty Ltd, located at Bayliss Building, UWA Campus, Crawley, Australia & MRF Building, 50 Murray Street, Perth, Australia. The purifiedlectinwas subjected to protein separation by SDS-PAGE coupled with Coomassie staining. 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). MS/MS spectra were analyzed using PEAKS Studio Version 4.5 SP2 (Bioinformatics solution) and manual interpretations to deduce the amino acid sequences of peptides. The *de novo* sequencing results for a major peptide having m/z value of 512.25 obtained from the purified lectin are shown/given in **Fig.13** and **Table 7** and **8**.

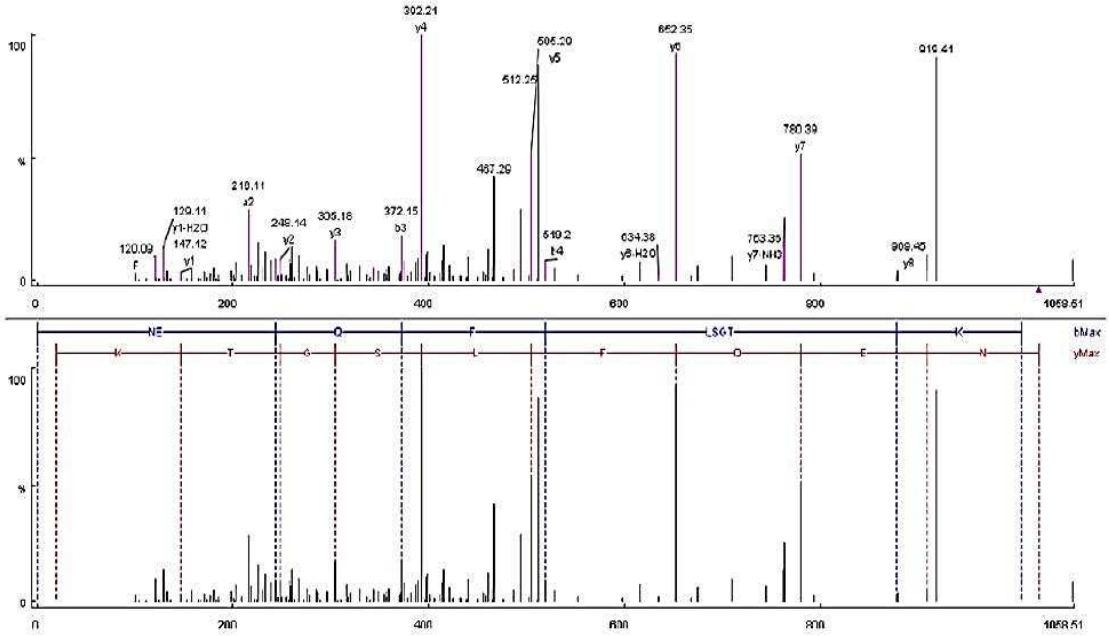


Fig.13: *De novo* spectra for a major peptide 512.25 $(MH_2)^{2+}$ obtained from the lectin purified from the fruiting body of *Lentinus cladopus* Lév**.**

Table 7: Sequence of the major peptide 512.25$(MH_2)^{2+}$ deduced from the *de novo* spectra in **Fig.13**: Sequence: NEQFLSGTK

#	b	sequence	y	#
1		N		9
2	244.097	E	909.454	8
3	372.152	Q	780.393	7
4	519.204	F	652.349	6
5		L	505.287	5
6		S	392.212	4
7		G	305.185	3
8	877.37	T	248.143	2
9		K	147.119	1

= detected y and b ions

Table 8: *De novo* sequencing result of the lectin purified from the fruiting body of *Lentinus cladopus* Lév**.**

No.	m/z	Peptide	Score (%)	Rank
1	512.25	NEQFLSGTK	91	0
		GGEQFLSGTK	9	1

Footnote: The first and second columns show the sequential numbers and the m/z of the parent ions while the third column contains the deduced sequences. A maximum of 5 sequences *i.e.* interpretations are presented for any given ion. These are ranked based on their likelihood (0 to 4) with ranking shown in the fifth column. The fourth column (titled Score) is also related to this ranking and shows the likelihood of any sequence amongst all possible interpretations for an ion. Colors used to display the actual amino acids are indicative of the confidence in each amino acid assignment. The color-coded confidences are: Red, >90% confident; Purple, 90-80% confident; Blue, 80-60% confident; Black, <60% confident.

The *de novo* sequence NEQFLSGTK determined for the major nanopeptide present in the lectin purified from the fruiting body of *Lentinus cladopus* Lév**.** was used to interrogate protein database using MS BLAST, a specialized algorithm for mass spectrometric analysis. The algorithm was found at: **http://dove.embl-heidelberg.de/Blast2/msblast.html.** The resulting sequence was found to have no significant match with any of the known sequences of the database NCBI nr95 (MS-BLAST) (21,671,167 sequences; 7,484,321,361 total letters as of January 2014). The detailed results of the MS BLAST analysis are given in **Appendix II**.