

A Novel Lectin from the Fruiting Body of an Edible Mushroom *Lentinus cladopus* L é.

Huidrom Rully, Sanjenbam Kunjeshwori Devi*, Laishram Rupachandra Singh,
Senjam Sunil Singh, Sorokhaibam Jibankumar Singh, Wayenbam Sobhachandra Singh,
Helena Thongam, Hijam Kiranbala Devi

Laboratory of Protein Biochemistry, Biochemistry Department, Manipur University, Canchipur, Imphal, India

Abstract A novel lectin was purified to electrophoretic homogeneity from the fruiting body of an edible mushroom *Lentinus cladopus* L é. by successive steps of lectin extraction, ammonium sulphate (20-60%) fractionation, discontinuous gradient Q-Sepharose chromatography, DEAE-cellulose chromatography and continuous gradient Q-Sepharose chromatography. The lectin was found to be a dimeric protein of molecular weight 40kDa made up of apparently chemically identical subunits. It exhibited a specific haemagglutinating activity of 2×10^5 HAU/mg protein when tested using rabbit RBC. Its haemagglutinating activity was not inhibited by any of the simple sugars, sugar derivatives, polysaccharides or glycoproteins tested. Protein mass fingerprinting by electrospray ionisation mass spectrometry and subsequent Mascot database analysis showed the purified lectin to be a novel protein, not characterised earlier.

Keywords Mushroom, Lectin, Mushroom lectin, *Lentinus cladopus*, *Lentinus cladopus* lectin

1. Introduction

Lectins are proteins or glycoproteins of non-immune origin which bind non-covalently and reversibly to specific carbohydrates or glycoconjugates [1]. They occur in almost all life forms and have been characterised from diverse sources including plant seeds and roots, fungi, bacteria, algae, body fluid of invertebrates, lower vertebrates and mammalian cell membranes [2]. Recently, research on lectins from different mushroom species has become an interesting field as mushrooms have long been known for their nutritive and medicinal values. Many lectins have been identified and purified from mushrooms, and their applications in medicine, therapeutics and toxicity have been extensively studied. The mushroom lectins are endowed with antiproliferative, antitumor, mitogenic, hypotensive, vasorelaxing, haemolytic, anti-HIV1 reverse transcriptase and immunopotentiating activities [3]. Some of the mushroom lectins, used in clinical diagnostics, are now commercially available. They include *Agaricus bisporus* lectin marketed by Sigma Aldrich Co. and EY Laboratories Inc., USA, *Aleuria aurantia* lectin marketed by Vector Laboratories Inc., USA, and *Marasmius oreadus* and *Polyporus squamosus* lectins marketed by EY Laboratories

Inc., USA. In view of the many potential applications of mushroom lectins, investigation leading to purification and characterisation of them from diverse mushroom sources is a scientifically relevant exercise. In the present investigation, a novel lectin exhibiting no specificity for simple sugars, sugar derivatives, polysaccharides and glycoproteins tested was purified and partially characterised from the fruiting body of an edible mushroom *Lentinus cladopus* L é.

2. Materials and Methods

2.1. Lectin Source



Figure 1. The fruiting bodies of mushroom *Lentinus cladopus* L é. growing on the bark of a mango tree

* Corresponding author:

kdsanjenbam@yahoo.com (Sanjenbam Kunjeshwori Devi)

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Fresh fruiting bodies of the edible mushroom *Lentinus cladopus* L.év. (locally known as “tek-tek paan” in Manipuri), were collected during the period late August to October from Kakching in the Thoubal District, Manipur State, India as the source of lectin. The mushroom, shown in Figure 1, belongs to the family Polyporaceae of the order Agaricomycetes. The mushroom grows commonly on decaying stumps and roots of trees. 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.

2.2. Chemicals

All the saccharides and their derivatives and the glycoproteins used in the investigation were purchased from Sigma Chemical Company, St. Louis, MO, USA. DEAE-Cellulose, Q-Sepharose, Sephadex G-75, 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 also obtained from Sigma Chemical Company, St. Louis, MO, USA. Medium molecular weight (MMW) protein markers were procured from Merck, India. All other chemicals and biochemicals used were of analytical grade. Purified de-ionized water was obtained through the purification system TKA Smart2Pure UV/UF (Niederelbert, Germany).

2.3. Preparation of Rabbit RBC

Blood (1 mL) was withdrawn from the posterior marginal vein of rabbit (New Zealand White) external ear by a procedure performed in accordance with the *Guide for the Care and Use of Laboratory Animals* [4], causing minimal pain to the experimental animal. RBC was separated from the blood by a low-speed centrifugation, re-suspended in 50mM phosphate buffer pH 7.4 containing 0.9% NaCl (PBS) (5mL) with gentle mixing and then washed by centrifugation. This procedure of re-suspension and subsequent centrifugation was repeated four times to obtain washed RBC. The washed RBC (0.2mL) was diluted with PBS (10mL) to obtain 2% (v/v) RBC suspension.

2.4. Lectin Assay

Lectin assay was carried out according to the method of Devi *et al.* [5] by two-fold serial dilution of 50 μ L of the lectin sample in a U-bottom 96-well microtitre plate with equal volume of PBS followed by addition of equal volume of the washed 2%(v/v) rabbit RBC suspension. After incubation for 1hr at 37 °C, haemagglutination was scored visually. The lectin activity was taken as the reciprocal of the highest dilution of the lectin sample at which complete haemagglutination occurred. It was expressed in terms of haemagglutination unit (HAU) that is the minimum amount of lectin required for complete haemagglutination under the assay condition. Protein concentration was estimated by the method of Lowry *et al.* [6] using crystalline bovine serum

albumin as the standard. The specific haemagglutination activity was expressed as HAU/mg protein.

2.5. Lectin Purification

The lectin was purified from the fruiting body of the edible mushroom *Lentinus cladopus* L.év. by a procedure carried out at 0-8°C unless otherwise stated. 10g of the coarse mushroom powder prepared from the air-dried fruiting bodies of the mushroom was soaked in 70mL of 20mM Tris-HCl buffer pH 7.8 containing 25mM NaCl, 0.1mM EDTA and 1mM β -mercaptoethanol. After soaking for 1hr, it was homogenised in a pestle and mortar under ice-cold condition. The homogenate was filtered through a double layered cotton cloth and then centrifuged at 17,000g for 30min at 4 °C. The resulting light brown supernatant was treated with activated charcoal (90 mg/mL) for 15 min in ice-cold condition. After the charcoal treatment, the suspension was centrifuged and the resulting supernatant was filtered through glass wool to obtain a clear solution as the crude lectin preparation. The lectin preparation was then brought to initial 20% ammonium sulphate saturation at 0°C. The resulting suspension was centrifuged at 17,000g for 30min at 4°C and the supernatant obtained was then brought to 60% ammonium sulphate saturation at 0°C. The suspension obtained was centrifuged, the pellet obtained as the ammonium sulphate (20-60%) fraction was re-dissolved in a minimum volume of 20mM Tris-HCl buffer pH 7.8 containing 25mM NaCl (TBS), and then dialysed extensively against the same buffer at 0°C. The dialysed fraction was then subjected to discontinuous gradient Q-Sepharose chromatography using a column (1x4 cm, bed volume 4.2 mL), pre-equilibrated with TBS. The charged column was extensively washed and the bound protein was eluted by the same buffer at a flow rate of 30 mL/hr at increasing but different salt concentrations (0.1 to 0.5M). The peak fractions with haemagglutinating activity eluted at 0.2M NaCl were pooled, dialysed against de-ionized water and then lyophilised. The lyophilised powder was re-dissolved, dialysed extensively against 50mM phosphate buffer pH 6.8, and then subjected to anion exchange chromatography using a DEAE-cellulose column (1x4cm, bed volume 4.2mL) pre-equilibrated with the same buffer. The charged column was thoroughly washed, and then the bound proteins were eluted with the same buffer at a continuous gradient of 0.02 to 0.25M NaCl at a flow rate of 6mL/hr. Fractions having activity were pooled, dialysed extensively against TBS, and then subjected to a continuous gradient Q-Sepharose chromatography using a column (1x4cm, bed volume 4.2mL) pre-equilibrated with TBS. The charged column was extensively washed and then the bound protein was eluted with the same buffer at a continuous gradient ranging from 0.025 to 0.20M NaCl at a flow rate of 30mL/hr. Fractions with activity were pooled, extensively dialysed, and then lyophilised to obtain the purified lectin preparation. The activity and protein of the purified lectin preparation were determined to ascertain its

specific activity.

2.6. SDS-PAGE

The purified lectin preparation was subjected to 14% reducing SDS-PAGE by the method of Laemmli [7]. The electrophoresed protein bands were visualised by the silver staining method of Merrill [8]. The MWM protein markers (Merck, India) were used as reference proteins. The relative subunit molecular weight of the purified lectin was determined using the standard plot of $\log M_r$ vs. R_f .

2.7. Native Molecular Weight Determination

The native molecular weight of the purified lectin was determined by molecular sieving gel chromatography under native condition. The lectin was eluted through a Sephadex G-75 column (82x0.9cm, bed volume 62 mL), pre-equilibrated with PBS. The void volume (V_o) of the gel column was determined using blue dextran (M_r 2,000kDa). 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) and lysozyme (M_r 18.4kDa). The elution of protein standards were monitored by the absorbance at 280nm and that of lectin by haemagglutination activity. The native molecular weight of the purified lectin was calculated from the standard plot of $\log M_r$ vs. V_e/V_o .

2.8. Haemagglutination Inhibition Assay

Haemagglutination inhibition lectin assay was performed by a standard serial dilution procedure adopted by Devi *et al.* [5]. Each test carbohydrate solution (25 μ L) of fixed concentration [400mM in the case of sugar/sugar derivative, or 10% (w/v) in the case of polysaccharide/glycoprotein] was two-fold serially diluted with PBS (25 μ L) in a U-bottom microtitre plate. To each well, 25 μ L of the lectin sample under test having 4HAU of activity was added. After allowing 30min of incubation at 37°C to facilitate carbohydrate-lectin binding, 25 μ L 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 haemagglutination pattern was visually examined after incubation for 1hr at 37°C. The inhibitory concentration of the carbohydrate was recorded as the lowest concentration that can inhibit the complete haemagglutination.

2.9. Protein Identification by Mass Spectrometry

The purified protein was first run on SDS-PAGE, stained with Coomassie blue and then the single protein band obtained was subjected to analysis for identification in the Proteomics International Facility, Bayliss Building, UWA Campus, Perth, Australia. The protein sample was trypsin digested and resulting peptides extracted according to standard technique of Bringans *et al.* [9]. The tryptic peptides were loaded onto an Agilent Zorbax 300SB-C18, 3.15 μ m [Agilent Technologies] and separated with a linear gradient of water/acetonitrile/0.1% formic acid (v/v). The peptides were analyzed by electrospray ionization mass spectrometry using the Shimadzu Prominence nano HPLC system coupled to a 5600 triple TOF mass Spectrometer [AB Sciex]. Spectra were analysed to identify the protein using Mascot Sequence matching software [Matrix Science] applied to Ludwig NR database for Taxonomy: Fungi.

3. Results

A lectin was purified to electrophoretic homogeneity from the fruiting body of the edible mushroom *Lentinus cladopus* L.év. by successive steps of lectin extraction, ammonium sulphate (20-60%) fractionation, discontinuous gradient Q-Sepharose chromatography, DEAE-Cellulose chromatography and continuous gradient Q-Sepharose chromatography. The summary of the lectin purification is given in Table 1.

Table 1. Summary of the purification of lectin from the fruiting body of *Lentinus cladopus* L.év.

Purification step	Total protein (mg) ^a	Total activity (HAU/mL) ^b	Specific activity (HAU/mg protein)	Fold purification	Recovery % ^c
Crude lectin preparation ^d	33.8	38,400.0	1,136.0	1.0	100.0
Ammonium sulphate (20-60%) fractionation	19.0	61,440.0	3,233.7	2.8	160.0
Discontinuous gradient Q-Sepharose Chromatography	5.9	26,624.0	4,512.5	4.0	69.3
DEAE-cellulose Chromatography	0.2	14,080.0	70,400.0	62.0	36.7
Continuous gradient Q-Sepharose Chromatography	0.03	6,144.0	2,04,800.0	180.0	16.0

^a Protein was determined by the method of Lowry *et al.* [6] using crystalline bovine serum albumin as the standard.

^b The lectin activity was taken as the titre strength that is the reciprocal of the highest dilution showing complete haemagglutination in the standard **Lectin assay** under **Materials and Methods**. It was expressed in terms of haemagglutination unit (HAU) that is the minimum amount of lectin required for complete haemagglutination under the lectin assay condition [5].

^c Percent recovered activity was obtained by comparing with the total activity of the starting crude lectin preparation.

^d Lectin was extracted from 10g dried powder of the fruiting body of *Lentinus cladopus* L.év.

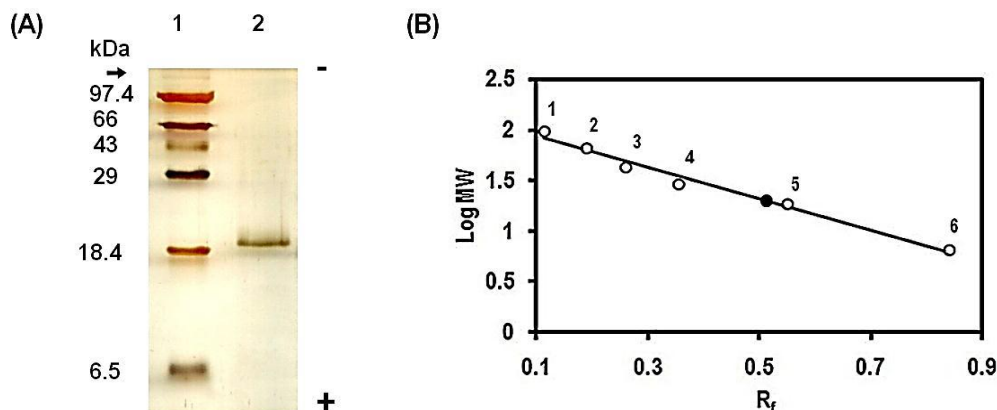


Figure 2. (A) Electrophoretic mobility of the purified lectin from the fruiting body of *Lentinus cladopus* L.év. on 14% reducing SDS-PAGE carried out according to the method of Laemmli [7]. The arrow indicates the origin of the electrophoretic migration. The protein bands were silver stained by the method of Merrill [8]. Lane 1: molecular weight marker proteins (Merck, medium range), Lane 2: the purified lectin (0.6 µg). (B) Determination of subunit molecular weight of the purified lectin based on the data of SDS-PAGE in Figure 2(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

The purified lectin was found to migrate as a single protein band on 14% reducing SDS-PAGE that was carried out according to the method of Laemmli [7] coupled with silver staining by the method of Merrill [8]. The electrophoretic migration is shown in Figure 2(A). Lane 1 in the figure corresponds to the migration of molecular marker proteins, and lane 2, that of the purified lectin. The molecular weight of the lectin under denaturing condition, deduced from the standard R_f vs. log M_r plot shown in Figure 2(B) based on the data for electrophoretic migration in Figure 2(A), was found to be 20kDa.

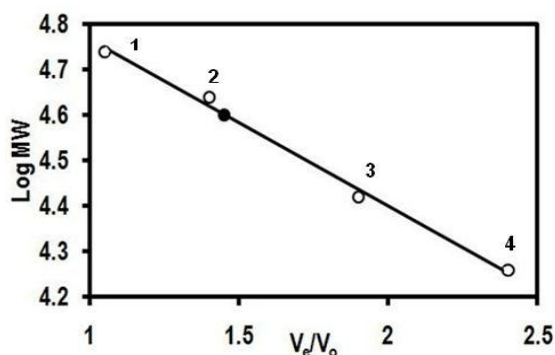


Figure 3. Determination of native molecular weight of the purified lectin from the fruiting body of *Lentinus cladopus* L.év. based on the data of gel filtration through a Sephadex G-75 column (82x0.9cm, bed volume 62mL), the detailed procedure of which is described in *Native molecular weight determination* under **Materials and 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 (○), 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) and lysozyme (M_r 18.4kDa) respectively. The unlabelled filled circle (●) represents the experimental point corresponding to the purified lectin

On the other hand, the native molecular weight of the purified lectin was determined by gel filtration through a

column of Sephadex G-75 using bovine serum albumin (M_r 66kDa), chicken egg ovalbumin (44.3kDa), α -chymotrypsin (29kDa) and lysozyme (18.4kDa) as the protein molecular weight markers. The native molecular weight, calculated from the corresponding standard plot of log M_r vs. V_e/V_o , shown in Figure 3, was found to be 40kDa. By considering the result of native molecular weight determination together with that of the molecular weight determination by SDS-PAGE, the purified lectin was found to be a dimeric protein made up of apparently chemically identical subunits.

The sugar specificity of the purified lectin was determined by haemagglutination inhibition lectin assay according to the method of Devi *et al.* [5] involving a standard two-fold serial dilution procedure using freshly washed rabbit RBC as described in **Haemagglutination inhibition assay**. The sugars and sugar derivatives taken for the inhibition test included 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-melibiose, 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. Polysaccharides tested in the inhibition assay were heparin, mannan, dextran, gum tragacanth and gum ghatti. None of these carbohydrates at concentrations up to 66.7mM in test (for sugars and their derivatives) or at concentrations up to 1.7% (w/v) in test (for polysaccharides) showed any inhibition to the haemagglutinating activity of the lectin. The glycoproteins - thyroglobulin, ovalbumin and peroxidase taken at concentrations up to 1.7% (w/v) in test were also investigated. None of them was also found to inhibit the haemagglutinating activity of the lectin.

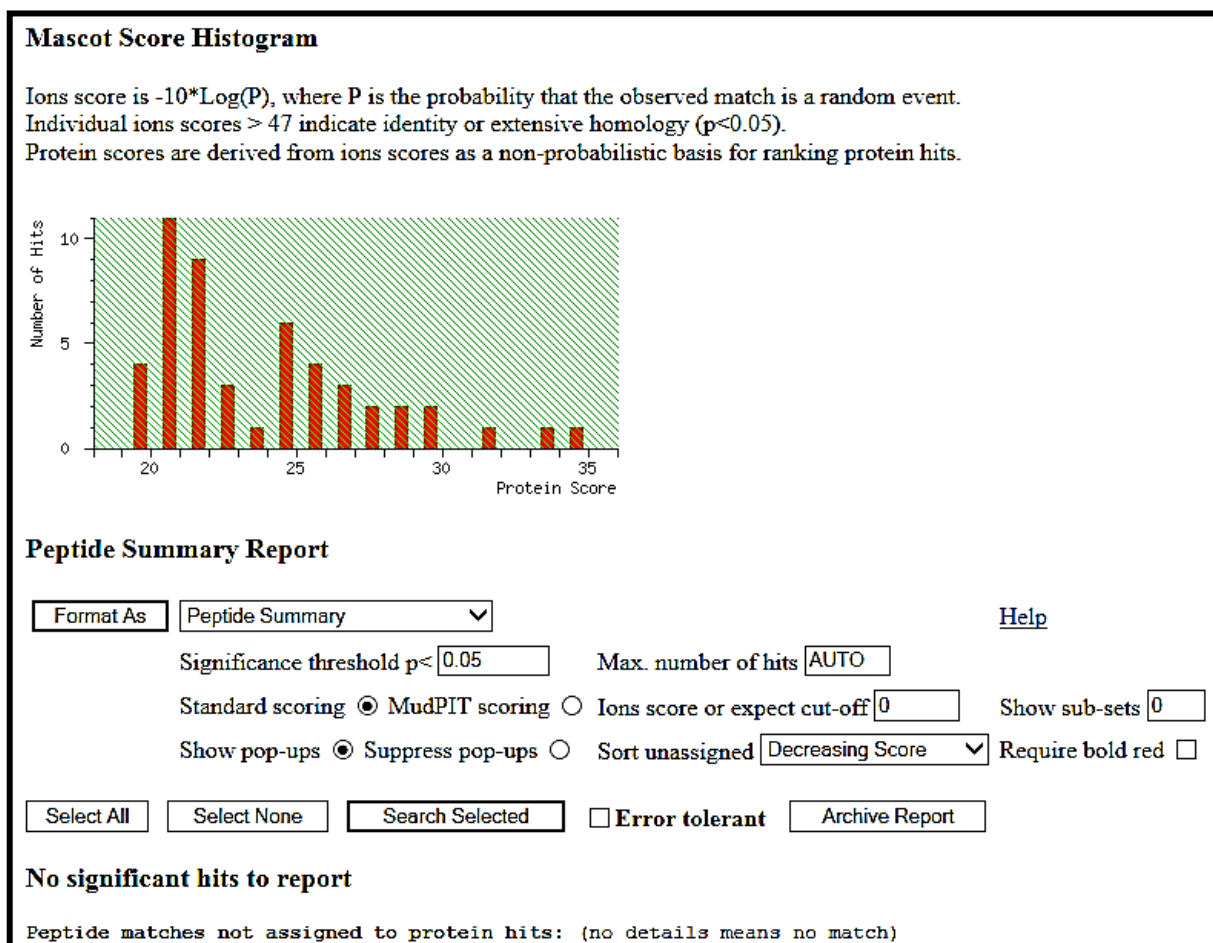


Figure 4. Mascot score histogram of peptide fingerprinting analysis of the lectin purified from the fruiting body of *Lentinus cladopus* L.év.

The peptide mass fingerprinting of the lectin purified from the fruiting body of *Lentinus cladopus* L.év. was carried out at the Proteomics International Pty Ltd, Australia. The resulting peptide mass fingerprint, analyzed to identify protein of interest using the Mascot sequence matching software (Matrix Science), was found to have no significant match with any of the known fungal proteins in the database, Ludwig NR, taxonomy: Fungi, as shown Mascot score histogram in Figure 4.

4. Discussion

A lectin was purified from the fruiting body of edible mushroom, *Lentinus cladopus* L.év (Figure 1) by successive steps of (i) lectin extraction, (ii) ammonium sulphate (20-60%) fractionation, (iii) discontinuous gradient Q-Sepharose chromatography, (iv) DEAE-cellulose chromatography, and (v) continuous gradient Q-Sepharose chromatography. The final electrophoretically homogeneous lectin preparation obtained was purified by 180-folds with 16% recovery and had a specific lectin activity of 204,800 HAU/mg protein. No lectin has been purified and characterised till date from *Lentinus cladopus* L.év., though

lectins from some other *Lentinus* species such as *Lentinus squarrosulus* [10] and *Lentinus edodes* (Berk.) Singer [11] were isolated and characterised earlier. The native molecular weight of the purified lectin, determined by gel filtration through Sephadex G-75, was found to be 40kDa (Figure 3). Though the molecular weights of lectins from mushrooms may be as low as 12kDa as reported for the lectin from the mushroom *Flammulina velutipes* [12] or may be as high as 190kDa as reported for the lectin from *Laetiporus sulphureus* [13], the native molecular weight of the lectin purified in the present investigation compares well with those of many mushroom lectins including those from *Agrocybe aegerita* (44kDa, [14]), *Amanita pantherina* (43kDa, [15]), *Lentinus edodes* (43kDa, [16]), and *Psathyrella asperospora* (41.8kDa, [17]). The subunit molecular weight of the purified lectin, determined by SDS-PAGE with reduction coupled with silver staining, was found to be 20kDa (Figure 2). Combining the result of native molecular weight determination by gel filtration with that of the subunit molecular weight determination by SDS-PAGE, it could be deduced that the lectin is a dimeric protein made up of apparently chemically identical subunits. This homodimeric subunit structural nature is in agreement with the earlier reports on other lectins from mushrooms

including *Agaricus edulis* [18], *Paxillus atrotomentosus* [19], *Pleurotus ostreatus* [20], *Boletus edulis* [21], *Lactarius flavidulus* [22] and *Clavaria purpurea* [23]. The present *Lentinus cladopus* lectin is notable in that its haemagglutinating activity cannot be inhibited by the simple sugars and sugar derivatives tested. Some polysaccharides and glycoproteins tested also did not inhibit the haemagglutinating activity. As such, the lectin belongs to a group of a few mushroom lectins known to date showing haemagglutinating activity that is not inhibited by simple sugars. Some other lectins belonging to the group are *Volvarelliella volvacea* lectin [24], *Mycoleptodonoides aitchisonii* lectin [25], *Lyophyllum shimeiji* lectin [26], *Agrocybe aegerita* lectin [27], *Armillaria luteo-virens* lectin [28], *Boletus venenatus* isolectins [29] and *Oudemansiella radicata* lectin [30]. At this juncture, it is relevant to mention that the non-inhibition of the haemagglutinating activity of the *Lentinus cladopus* lectin by the externally added test carbohydrates might be due to absence of, or inaccessibility to, the required specific inhibitory covalent sugar sequence in them. The peptides obtained from tryptic digestion of the purified lectin were subjected to electrospray ionization mass spectrometry and the resulting spectra obtained were analyzed to identify the protein using Mascot sequence matching software [Matrix Science] applied to Ludwig NR database for Taxonomy: Fungi. The mass spectrometric analysis did not yield any significant hit (Figure 4). The result, therefore, showed that the lectin, purified from *Lentinus cladopus* L.év. in the present investigation, is a novel protein (lectin), not characterised earlier.

5. Conclusions

A novel lectin was purified from an edible mushroom *Lentinus cladopus* L.év. by successive steps of lectin extraction, ammonium sulphate fractionation, discontinuous gradient Q-Sepharose chromatography, DEAE-cellulose chromatography and continuous gradient Q-Sepharose chromatography. The lectin was found to be a protein of molecular weight 40kDa made up of two apparently chemically identical subunits. Its lectin (haemagglutinating) activity was not inhibited by any of the simple sugars, sugar derivatives, polysaccharides or glycoproteins tested. Protein mass fingerprinting by electrospray ionisation mass spectrometry and subsequent Mascot database analysis showed the purified lectin to be a novel protein.

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