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Original Article

Physicochemical properties and acute toxicity studies of a lectin from the saline extract of the fruiting bodies of the shiitake mushroom, Lentinula edodes (Berk)

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Abstract: A lectin (LEL) was isolated from the fresh fruiting bodies of the shiitake mushroom Lentinula edodes by a combination of gel filtration chromatography on Sephadex G-150 and affinity chromatography on an N-acetyl-D-galactosamine-Sepharose 4B column. Its molecular mass, as determined by gel filtration, was estimated to be 71, 000 Daltons and its structure is homotetrameric with subunit molecular weight of approximately 18,000 Daltons. LEL agglutinated non-specifically red blood cells from the human ABO system as well as rabbit erythrocytes and in haemagglutination inhibition assays, exhibited sugar-binding specificity toward N-acetyl-D-galactosamine. EDTA had no inhibitory effect on its haemagglutinating activity, which was stable up to 70°C and was not affected by changes in pH. The lectin had no covalently-linked carbohydrate and amino acid composition analysis revealed that it contained 124 amino acid residues and was rich in tyrosine, proline, phenylalanine, arginine, glutamic acid and cysteine. LEL did not cause mortality neither was it observed to alter the morphology of key organs when administered intraperitoneally at concentrations up to 10,000 mg kg^{-1} body weight of mice.

Keywords: Lectin, lentinula edodes, N-acetyl-D-galactosamine, shiitake mushroom, fruiting bodies, agglutination

Introduction

Lectins are proteins (glycoproteins) of non-immune origin that specifically and reversibly bind to carbohydrates or glycoconjugates. They are widely distributed in nature and are found in plants, animals and microorganisms. Owing to their ability to bind carbohydrates, they are capable of agglutinating erythrocytes, making their detection easy. Lectins have been isolated from a number of mushrooms and are localized on the caps, stipes, and mycelia of the mushrooms. Mushroom lectins have many biological properties in common but they still represent a diversified group of proteins with respect to size, composition and structure. They have drawn the increasing attention of some investigators owing to their biological activities including anti-proliferative, anti-tumour [1] mitogenic [2] vasorelaxing [3], immunopotentiating [4], anti-insecticidal [5-6], antifungal [7], antiviral, antibacterial [8] and hypotensive [9]. The discovery of these exploitable biological activities of mushroom lectins indicates that they might be employed as drugs or therapeutic reagents for pharmaceutics. Mushroom lectins also find applications in taxonomical, embryological, and bacteriological studies, study of the modifications in membrane glycoconjugates and cancer formation, cell sorting, sorting of mutant and tumour cells, and isolation of membrane and serum glycoconjugates [10-11].

The tropical areas of Africa is a region with the most potential for mushroom lectin investigation because of the extremely high varieties of mushrooms found in the region [12-14]. Unfortunately, mushrooms remain a vast and yet largely untapped source of new pharmaceutical products especially lectins in this region. Till date, no comprehensive study has been carried out on the large variety of mushrooms found in Africa, to check for the presence of lectins and to catalogue the properties and biological activities of such lectins especially from wild mushroom species found growing freely in nature. In

view of all the potentially useful activities they exhibit, especially as drug delivery vehicles, it has become imperative to collate information on lectins present in freely growing wild mushrooms found on the African continent. Hence this study aims at purifying the lectin from Lentinula edodes to homogeneity and determining the physicochemical properties of the lectin and evaluating its toxic effect on experimental animals.

Materials and methods

Extraction and purification of lectin

Fresh fruiting bodies of the shiitake mushroom were obtained from the foot of a tree behind the Biochemistry Department, Obafemi Awolowo University, Ile-Ife, Nigeria, and were taxonomically identified by a Botanist. 250 g of the mushroom specimen were blended in 1000 ml of phosphate buffered saline (PBS), pH 7.2 and left to extract at 4°C overnight. The homogenate was then centrifuged at 4500 rpm for 15 minutes to obtain the crude saline extract. The extract was filtered through a cheese-cloth and applied on a Sephadex G-150 (2.5 × 10 cm) column and eluted with PBS, pH 7.2. The peak with lectin activity was pooled, dialysed and chromatographed on a column (1.5 × 10 cm) of N-acetyl-D-galactosamine-Sepharose 4B equilibrated with PBS. The adsorbed protein was eluted with 0.2M N-acetyl-D-galactosamine in PBS.

Haemagglutinating activity

A serial two-fold dilution of the lectin solution in U-shaped microtitre plates (100 µl) was mixed with 50 µl of a 2% suspension of human as well as rabbit erythrocytes in phosphate buffered saline, pH 7.2 at room temperature (both human as well as rabbit erythrocytes were fixed with 1% glutaraldehyde). The plate was left undisturbed for 1 hr for agglutination to take place. The haemagglutination titre of the lectin expressed as the reciprocal of the highest dilution exhibiting visible agglutination of erythrocytes was reckoned as one haemagglutinating unit. Specific activity was the number of haemagglutination units per mg protein.

Blood group specificity

The blood group specificity of the crude extract was established using erythrocytes from different blood groups of the ABO system and those of the rabbit.

Hapten inhibition test

The sugar specificity of the lectin was investigated by the ability of simple sugars to inhibit the agglutination of human erythrocytes [15]. The sugars tested were: L- sorbose, Lactose, fructose, D-galactose, D-arabinose, I-o-methyl-α-o-glucopyranoside, sucrose, maltose, N-acetyl-D-glucosamine, D-glucosaminhydrochloride, D-mannitol, sorbitol, D-glucose, D- mannose and N -acetyl-D-galactosamine. 0.2 M of each sugar in PBS was prepared. A serial dilution of the sample was made until the end-point causing haemagglutination was obtained. 50 µl of the sugar solution was added to each well and allowed to stand for 30 minutes at room temperature and then mixed with 50 µl of 2% rabbit erythrocyte suspension. The haemagglutination titres obtained were compared with a non-sugar containing blank.

Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis in the presence of SDS was performed on 10% gels in Tris-glycine buffer, pH 8.9. The proteins were stained with Coomassie Brilliant Blue R, while the presence of covalently bound sugar in the lectin was detected by staining the gels with Periodic Acid Schiff reagent (PAS staining) [16].

Molecular weight determination

The purified lectin was subjected to Sodium Dodecyl Sulfate – Polyacrylamide gel electrophoresis (SDS–PAGE) for subunit molecular weight determination in accordance with the procedure of Laemmli and Favre [17] using the following protein markers: Bovine serum albumin (Mr 66,000), Creatine phosphokinase (Mr 81,000), Ovalbumin (Mr 45, 000), α-chymotrypsinogen (Mr 25, 000), Lysozyme (Mr 14, 500). Gel filtration on a Biogel P200 column (1.5 x 63 cm), which had been calibrated with molecular weight markers was carried out to determine the native molecular weight of the lectin. 5 ml of each standard was applied to the column and ran separately using a 10 mM phosphate buffer, pH 7.2 as eluant at a flow rate of 10 ml/h. Fractions of 5 ml were collected and the elution was monitored for each

protein at 280 nm. The void volume (V_0) of the column was determined using Blue dextran (elution monitored at 620 nm). The molecular weight markers used were: Creatine phosphatase (Mr 88,000; 5 mg/ml), bovine serum albumin (Mr 66,000; 5 mg/ml), Ovalbumin (Mr 45,000; 5 mg/ml), and pyruvate kinase (Mr 230,000; 5 mg/ml).

Effect of temperature on haemagglutinating activity

The effect of temperature on the haemagglutinating activity was determined as described by Sampaio et al. [18]. Aliquots of lectin were incubated at different temperatures (30-90^oC) for 30 min then rapidly cooled in ice and assayed for agglutinating activity. Agglutinating activity of the lectin sample kept at 20^oC for 30 min was used as control.

Effect of pH on haemagglutinating activity

The effect of pH on the haemagglutinating activity was measured by incubating the samples in the following buffers at different pH values; 0.2 M citrate buffer, pH 2 – 6; 0.2 M Tris-HCl buffer, pH 7 and 8; and 0.2 M glycine-NaOH buffer, pH 9 – 11. After 1 hour, the haemagglutination activity of the lectin was determined. The control values were the agglutination titre of the lectin in PBS, pH 7.2.

Effect of EDTA and divalent cations

The effect of ethylenediaminetetraacetic acid (EDTA) and divalent cations on the lectin activity was carried out as described by Wang et al. [19]. The purified lectin sample was dialysed against 10 mM EDTA for 24 hours and the hemagglutination activity of the demetallized lectin was determined. The treated lectin was then incubated with 50 µl each of the following cations: 10 mM $ZnSO_4$, $CaCl_2$, $MgCl_2$, $MnCl_2$, $HgCl_2$, $NiCl_2$, $CoCl_2$ and $SnCl_2$ for 2 hours in order to evaluate their capacity to restore haemagglutination.

The ouchterlony double diffusion experiment

1.5% (w/v) agar solution in PBS containing 0.01% (w/v) sodium azide was prepared. The solution was slowly heated until the agar had completely dissolved and poured into clean Petri dishes. A well was made at the centre of each Petri dish and four other wells equidistant from the centre were made around it. 50 µl of the lectin sample was placed in the centre well, and 50 µl of the polysaccharide (250 mg/ml, 100 mg/ml 50 mg/ml and 10 mg/ml of each polysaccharide) was placed in the surrounding wells. The polysaccharides tested were, inulin, dextrin, glycogen, starch and the polysaccharide (galactomannan) from Afzelia africana seeds.

Amino acid analysis

The purified lectin was subjected to analysis of amino acid content. The amino acid composition of the lectin sample was determined using methods described by Spackman et al. [20]. The sample was hydrolyzed, evaporated in a rotary evaporator and loaded into the Technicon sequential Multi-sample Amino Acid Analyzer (TSM). The nitrogen content of the sample was determined by Kjeldhal method [21].

Toxicity studies

All experiments involving animals were conducted in accordance with the Obafemi Awolowo University research policy on the use of experimental animals. 15 male and female in bred mice, weighing approximately 25g, were housed under controlled laboratory conditions. They were fed a standard commercial diet and water ad libitum. The animals were fasted overnight prior to the administration of the test material. The five groups of three animals each were given single dose (i.p.) of the lectin, constituted in sterile saline solution, in concentrations of 0 (control), 10, 100, 1,000, and 10,000 mg kg^{-1} body weight of animal for a range finding test. The animals were then observed first for 48 hours to check for mortality or signs of malaise and then twice daily for 15 days while the body weights were monitored and recorded daily. Representative animals from each group were then sacrificed and key organs (the brain, the spleen the kidneys, the liver, the lungs and the heart) were excised and examined macroscopically.

Results and discussion

Isolation of the lectin

The data showed that the saline extract of the fruiting bodies of Lentinula edodes contained measurable amounts of haemagglutinating protein (lectin) which represents approximately 10 % of the protein content of the mushroom. The

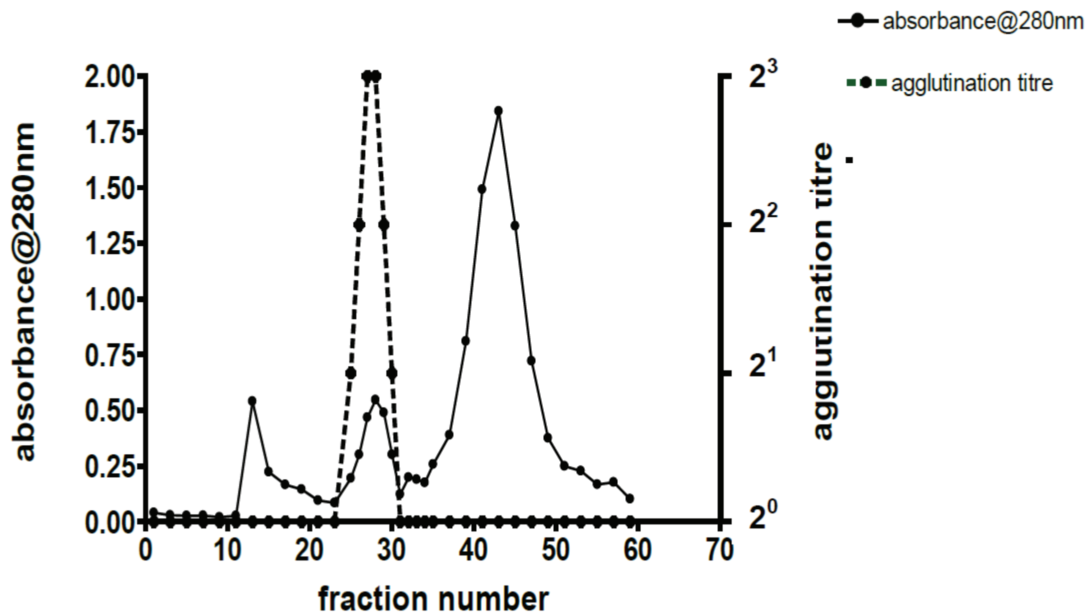

Figure 1. Gel filtration of the crude extract of the mushroom Lentinula edodes on Sephadex G-150. Eluant was PBS, pH 7.2. The flow rate was 18 ml/h and the fraction size was 4ml.

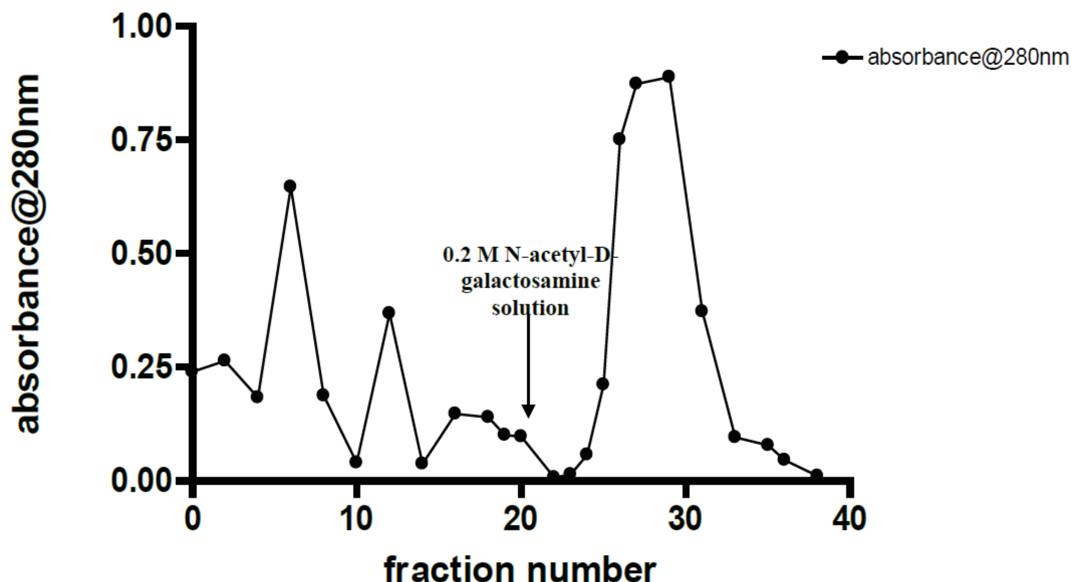

Figure 2. Affinity chromatography of the active gel filtration peak on N-acetyl-D-galactosamine-Sepharose 4B. The column was first washed with PBS to remove the unadsorbed proteins then the adsorbed lectin was eluted with 0.2 M N-acetyl-D-galactosamine in PBS. The flow rate was 18 ml/h and fraction size was 1 ml.

results of chromatography of L. edodes crude extract on Sephadex G-150 are illustrated in **Figure 1**. Three protein peaks were obtained only one of which exhibited haemagglutination activity. This active peak was used for affinity chromatography on N-acetylgalactosamine-Sepharose column (**Figure 2**). Lectin activity resided in the fraction adsorbed on the immobilized sugar, which was eluted with 0.2 M N-acetylgalactosamine in PBS and constituted the

Table 1. Summary of purification procedures

Fraction	Total protein (mg)	Total activity (HU)	Specific activity (HU/mg)	Yield (%)	Purification fold
Crude Extract	1007.50	64	0.06	100	1.00
Gel filtration of crude extract on sephadex G-150	39.90	32	0.80	3.96	13.33
Affinity chromatography of active gel filtration peak on sepharose 4B-N-acetyl-D-galactosamine	5.54	16	2.89	0.55	48.17

Table 2. Amino acid composition of Lentinula edodes lectin

AMINO ACID	Concentration of amino acid in g/100g protein	Calculated residues to the nearest integer	Residue weight
Lysine	3.03	4	584
Histidine	1.26	4	620
Arginine	3.02	12	2088
Aspartic acid	4.28	4	532
Threonine	2.37	4	476
Serine	2.55	4	420
Glutamic acid	5.07	11	1617
Proline	2.65	14	1610
Glycine	2.26	3	225
Alanine	3.30	5	445
Cysteine	0.46	9	1089
Valine	2.05	4	468
Methionine	0.47	4	596
Isoleucine	2.28	4	524
Leucine	3.03	4	524
Norleucine	-	-	-
Tyrosine	1.43	22	3982
Phenylalanine	2.01	12	1980
Total		124	≈ 18,000

Total number of amino acids = 124, Minimum molecular weight calculated from the amino acid composition = 18,000 Daltons

homogenous preparation of the L. edodes lectin which gave a distinct band in SDS- PAGE. The yields and specific haemagglutinating activities of the different chromatographic fractions are presented in **Table 1**.

Properties of lentinula edodes lectin

The phosphate buffered saline extract from the fruiting bodies of L. edodes mushroom agglutinated non-specifically red blood cells of the human A, B and O system as well as rabbit erythrocytes. The hapten inhibition studies to define the sugar specificities of the saline extract of the fruiting bodies of L. edodes showed that N-acetyl-glucosamine and D-arabinose slightly enhanced the haemagglutinating activity of the lectin while D-Mannitol, D-Glucose, D-Sorbitol and D-Maltose slightly inhibited the activity. The activity of the lectin was completely inhibited by N-acetyl-D-galactosamine. The minimum concentration of N-acetyl-D-galactosamine which brought about a 100 % inhibition of haemagglutinating activity was 0.4 M.

Exhaustive dialysis of the lectin with EDTA did not bring about any change in the haemagglutinating activity, even at EDTA concentrations up to 100 mM. This could suggest that either the lectin activity was not dependent on the metal ions or that the metal ions present were too strongly held in the lectin structure and could not be removed by dialysis with chelating agent. This conclusion is in concurrence with recent observations by Khan and Khan [22] that mushroom lectins generally do not require metal ions for their activity.

Amino acid composition

Amino acid composition analysis revealed that LEL is made up of 124 amino acid residues (**Table 2**). The amino acid composition was char-

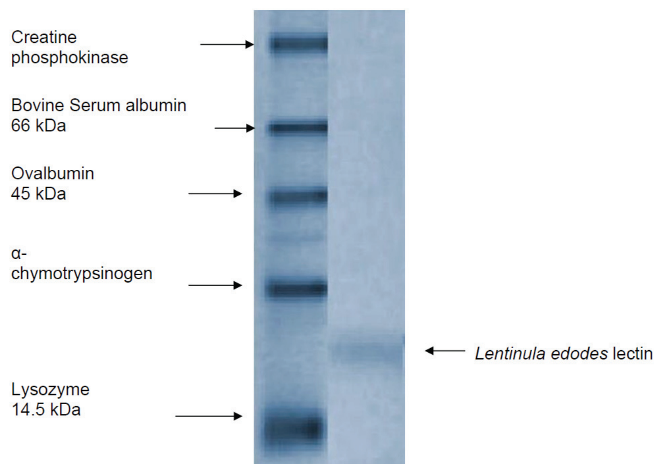

Figure 3. Subunit molecular weight determination by SDS-PAGE. The protein markers used were Creatine phosphokinase (Mr 81,000), Bovine serum albumin (Mr 66,000), Ovalbumin (Mr 45, 000), α-chymotrypsinogen (Mr 25, 000) and Lysozyme (Mr 14, 500).

acterized by a high content of tyrosine (17.74 %), proline (11.29 %), phenylalanine (9.68 %), arginine (9.68 %), glutamic acid (8.87 %) and cysteine (7.26 %). Basic amino acids constituted about 16.1%, acidic amino acids – 12.1%, polar (neutral) amino acids - 34%, and hydrophobic amino acids - 37% of all the amino acid residues. The high content of hydrophobic amino acids suggests that the protein is tightly folded.

Molecular weight

Native molecular masses of fungal lectins have been observed to range from 15-90 kDa [22]. The apparent molecular mass of LEL, as determined by gel filtration chromatography, was 70,796 Daltons. The subunit molecular mass as determined from SDS-PAGE was 19,907 Daltons while the molecular mass estimated from the amino acid composition was 17,780 Daltons (**Figure 3**). It would appear, therefore, that the native protein is homotetrameric in a manner that is similar to that observed for the lectins from Pleurocybella porrigens [25] and Lactarius lignyotus [26] lectins.

Molecular stability

The effect of temperature on the haemagglutinating activity of LEL is shown in (**Figure 4**). The haemagglutinating activity of LEL was relatively stable up to 60°C, declined markedly at 80°C, and was totally inactivated at 90°C. The examination of LEL activity at different pH values (pH 2.0-11.0) showed that the haemagglutinating activity of the lectin was unaffected by pH (**Figure 5**). Some of the known mushroom lectins also display tolerance over a wide range of temperature and pH. Examples include the lectin from Ganoderma capense, which showed remarkable thermostability at 100°C for 1 h and tolerance over pH range of 4-11 [23]. The lectin isolated from the edible mushroom Schizophyllum commune, was also found to be stable at temperatures up to 55°C and at extremes of pH [24].

Carbohydrate conten

PAS staining revealed that LEL is not a glycoprotein. An application of the modified anthrone reaction method [27] further confirmed this observation.

Interaction with polysaccharides

The lectin when examined for possible interactions with some polysaccharides by Ouchterlony double diffusion experiment formed no precipitin band with any of the polysaccharides tested. This could be interpreted to mean that there was no interaction between the lectin and the carbohydrate moieties of these polysaccharides

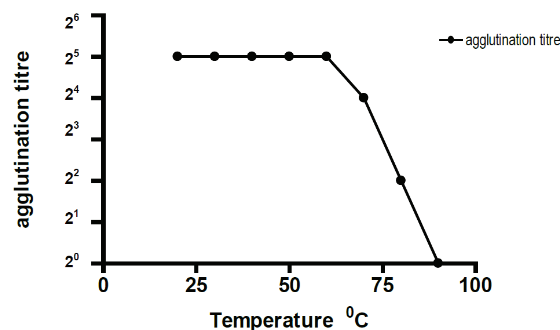

Figure 4. Effect of temperature on the haemagglutinating activity of Lentinula edodes lectin. Aliquots of lectin were incubated at different temperatures (30-90°C) for 30 min then rapidly cooled in ice and assayed for agglutinating activity. The control was agglutinating activity of lectin sample kept at 20°C for 30 min.

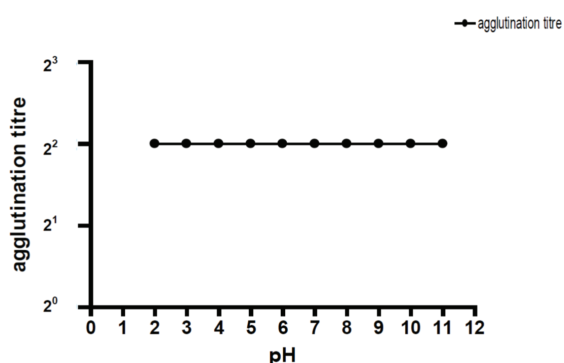

Figure 5. Effect of pH on the haemagglutinating activity of Lentinula edodes lectin. Lectin samples were icubated in the following buffers at different pH values; 0.2 M citrate buffer, pH 2 – 6; 0.2 M Tris-HCl buffer, pH 7 and 8; and 0.2 M glycine-NaOH buffer, pH 9 – 11. After 1 hour, the haemagglutination activity of the lectin was determined. The control values were the agglutination titre of the lectin in PBS, pH 7.2.

and glycoproteins. This could however be due to many factors such as the concentration of the protein as well as the molecular size of the polysaccharides.

Toxicity studies

In general, toxicity of lectins is characterized by growth inhibition in experimental animals, and by diarrhoea, nausea, bloating and vomiting when ingested orally [28-29]. Ironically, the discovery of lectins in mushrooms was as a result of investigations into the toxicity of higher fungi. The first fungal lectin was reported in the mushroom Amanita muscaria by Ford [30] and lectin activity was found to be associated with the toxicity of the mushroom. Later accounts on lectins in edible mushrooms such as Lactarius deliciosus [31], Schizophyllum commune [11] and Pleurotus Ostreatus [32] demonstrated the independence of toxicity and lectin activity. Toxicity studies on LEL showed that there was no significant loss in body weight of the animals after intraperitoneal administration of the lectin (**Table 3**). The animals also showed no sign of malaise. No mortality was observed in the experimental animals at concentrations up to 10,000 mg kg^{-1} body weight. This is an indication that either the mode of administration, or treatment regimen or the lectin extract itself does not have any significant effect on whole body homeostasis. In the histopathological investigation there were no gross changes in the morphology of any of the organs investigated and no visible pathological lesions were observed. Thus, it can be suggested that the lectin is not acutely toxic when administered via i.p at concentrations up to 10,000 mg kg^{-1} body weight.

Jeune et al. [33] reported a procedure for purifying a lectin from the fruiting bodies of Lentinula edodes mushrooms cultivated in Asia. The procedure involved extraction with 0.15 M NaCl, precipitation with 80 % saturated $(NH_4)_2SO_4$, dialysis, adsorption of lectin on DEAE Sephadex A-50 and elution with a salt gradient and finally adsorption on hydroxyapatite followed by elution with a phosphate gradient. The procedure however proved inadequate as the final lectin preparation exhibited four sharp bands in polyacrylamide gel electrophoresis. The amino acid compositions of 3 fractions, namely, the sample ready for ion exchange chromatography on DEAE-Sephadex, the fraction eluted with 0.05 M

Table 3. Changes in body weight following intraperitoneal administration of LEL

Dose mg kg^{-1} b.wt.	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
0	18.8± 5.8	19.6± 5.7	20.1± 6.2	20.7± 6.2	20.6± 6.1	21.0± 5.2	21.6± 4.8
10	19.0 ± 3.7	19.3± 4.0	20.3± 3.8	20.9± 3.6	20.9± 3.7	21.6± 3.2	22.4± 2.8
100	20.5± 1.1	20.8± 0.9	21.4± 1.0	21.7± 0.9	21.3± 1.3	22.2± 0.9	22.3±1.1
1000	17.2± 1.2	17.6± 1.1	18.1± 0.9	18.1± 0.7	18.2± 0.8	17.6± 0.9	18.3± 0.8
10, 000	22.3± 3.2	22.0± 3.6	22.3± 3.5	22.3± 3.6	22.0± 3.8	22.2± 4.4	22.7± 3.9

Values are expressed as Mean ± SEM (n = 3)

NaCl from the DEAE-Sephadex column and the fraction eluted with 0.05 M phosphate from the hydroxyapatite column, were found to be disparate. Wang et al. [34] sought to purify the Lentinula edodes lectin in view of the apparent heterogeneity of the lectin prepared by the aforementioned group of investigators. Their purification procedure involved extraction with 0.15 M NaCl, precipitation with 45 % saturated $(NH_4)_2SO_4$, dialysis, gel filtration on Sephadex G 100 and finally affinity chromatography on N-acetylgalactosamine. The results of their experiments were not in accord with that reported by Jeune and co workers [33].

The Lentinula edode lectin reported in this study showed some similarities to the lectin isolated by Wang and co workers. In both cases, the use of gel filtration followed by affinity chromatography proved effective in purifying the lectin to homogeneity. The lectin had a native molecular weight of 43,000 Daltons and showed sugar specificities for N-acetyl-D-galactosamine, mannose and N-acetyl-D- glucosamine and the minimum concentration of N-acetyl-D-galactosamine needed to inhibit haemagglutinating activity by 100 % was 0.5 M. In contrast, the lectin in this study has a native molecular weight of 71,000 Daltons and showed sugar specificity for only N-acetyl-D-galactosamine. The minimum concentration of N-acetyl-D-galactosamine needed to inhibit haemagglutinating activity by 100 % was 0.4 M.

Conclusion

The lectin from Lentinula edodes found growing wild in the African region has shown similar physicochemical properties with the lectin from the cultivated mushroom of the Asian origin. The functional understanding of these lectins from different varieties of mushrooms with their various exploitable biological properties will provide new applications in the food and pharmaceutical industries in the future.

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