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Screening of Wild Mushroom *Amanita* species for Occurrence of Lectins and Their Partial Purification by RP-HPLC

M. Santhiya and M. Jansi

Department of Zoology, S.T Hindu College, Nagercoil, Tamilnadu, India

Abstract: Twelve species of *Amanita* were screened for occurrence of lectins. Each of the species was investigated for the occurrence of extracellular, surface-bound and intracellular lectin activities. As many as five species namely, *Amanita phalloides, A. subjunquillea*, *A. pseudoporphyria, A. manginiana* and *A. pantherina*were found to possess both intracellular lectin activities and extracellular or surface-bound lectin activities. Each of the lectin was characterized with respect to blood group and carbohydrate specificities. All the lectins were found to agglutinate human erythrocytes, irrespective of their blood group and showed highest activity with animal rabbit erythrocytes. The lectin activity was checked by the mat formation with the four blood groups on micro titer plate. Positive results indicated the mat formation while negative results indicated the button formation. However, they did not show agglutination with mice erythrocytes. Of the various carbohydrates tested, all lectins were found to be specific for mannose, D-sucrose, Ribose and L-fucose. The minimum inhibitory concentration of each of the specific sugars was also determined. The lectins were partially purified using gel filtration purification technique and the purity was confirmed by RP-HPLC.

Key words: Heamagglutinin · Amanita sp · Mannose binding lectins · Sugar specificity · Carbohydrate inhibition

INTRODUCTION

Lectins are glycoproteins of non-immune origin and bind reversibly with specific sugars and precipitate polysaccharides, glycoproteins and glycolipids, bearing specific carbohydrate moieties present on the apposing cell,Goldstein *et al*. [1]. Lectinhave been isolated and characterized from diverse sources, including plant seeds and roots, fungi, bacteria, algae, body fluid of invertebrates, lower invertebrates and mammalian cell membranes [2] have been attributed varied roles in different organisms. Amongst the microbes, lectins widely occur in algae and higher fungi (phylum Basidiomycota and Ascomycota). Lectin from mushroom has recently been reviewed for their therapeutic potential use. Many of them are rich source of various bioactive molecules having anticancer and immunomodulatory potential. Such compounds are increasingly used in Japan as adjuvant to help supportimmune function in cancer patients during radio and chemotherapy and are reported to prolong survival timesin some types of cancer [3] *Lentinulaedodes*a type of mushrooms can lower both blood pressure and free cholesterol in plasma, as well as accelerate accumulation of lipids in the liver, by removing them from circulation [4] Medicinal mushroom Lectins display antiproliferative activity toward tumour cells. Antitumor activity and tumours mitogenic activity toward spleen cells and inhibitory activity toward HIV-1 reverse transcriptase [5, 15]. The first report on fungal lectin was appeared in *Amanita muscaria* commonly called as fly agaric [6]. Now there are numerous reports on lectin from fruit bodies of higher fungi and few from mycelium. ǂLectin activity has also been reported in culture filtrates of plant pathogenic fungus *Macrophominaphaseolina* [7]. Many species are oligotrophic and are capable of growing in environments lacking key nutrients. Some species are medically as well as commercially important, while a few of them can cause infections in humans and other animals [14]. As this vast genus has not been explored much for the occurrence of lectins, the present study was undertaken to show a comparative study on the mycelialectin of fivedifferent species of *Amanita*.

Corresponding Author: M. Santhiya, Research Department of Zoology, S.T. Hindu College, Nagercoil 629002, Kanyakumari district, Tamil Nadu, India. Tel: +91-77-08886833.

MATERIALS AND METHODS

Determination of Extracellular Lectin Activity: One loop full of culture was inoculated to the Erlenmeyer's flask containing 70ml of Dextrose broth and it was incubated at 37°C for 7 days in shaking condition. The mycelium was separated from the culture broth by filtration. Culture broth was centrifuged at 5000 rpm for 10 minutes and the mycelium free broth was used for the estimation of extracellular lectin activity [8].

Determination of Loosely Surface-bound and Intracellular Lectin Activity: Mycelium was harvested by filtration, washed thoroughly with t~~T~~ris base saline buffer briefly pressed dry and analyzed for lectin activity. For the determination of loosely surface bound lectin activity, mycelium in tris base saline buffer extract was recovered by centrifugation at 5000 rpm for 10 minutes and the supernatant was used for the estimation of surface bound lectin activity. For the determination of intracellular lectin activity, fungal extracts were prepared by homogenizing the recovered mycelium in TBS buffer and was centrifuged at 5000 rpm for 15 minutes and the supernatant was assayed for lectin activity. Singh *et al*. [8].

Preparation of Erythrocyte Suspension: Human blood was collected in Alseviers medium (anticoagulant) (30 mMtrisodium citrate, 77 mMNaCl and 114 mM glucose) and stored at 4°C up to 2 weeks. The erythrocyte suspension was freshly prepared by washing the erythrocytes three times with 10 volt of 100 mMTris-HCl, pH 7.6 containing 50 mMNaCl and 10 mM $CaCI_2$ and suspended in the same buffer as 1.5% suspension (v/v).

Hemagglutination Assay: The hemagglutination assays were performed in V-bottomed micro titre plates by serial two-fold dilution of a 25µl serum sample with an equal volume of TBS. After dilution, 25µl RBC suspension was added to each well and incubated for 1 h at room temperature. The hemagglutination titre was recorded as the reciprocal of the highest dilution of the sample causing complete agglutination of normal RBC. Each experiment was performed in duplicate and the hemagglutinating activities were expressed as the median hemagglutination titre. Finally the purified lectin was assayed for the hemagglutination activity.

The hemagglutinating activity ($HA=HUmL^{-1}$) was defined as the hemagglutination units per volume used. Specific hemagglutinating activity was expressed as the activity per mg of protein.

Hemagglutination Inhibition Assay: Purified lectin (64 HU) was allowed to react with equal volume of several carbohydrates and glycoprotein (Sigma) solutions and incubated at room temperature for 1 h. After incubation, 25µl of pre-prepared 1.5% human erythrocyte suspension was then added to the mixture and after 1 h of incubation the hemagglutination was examined. The results were expressed as the minimum concentration of the inhibitor required to completely terminate the agglutination.

Partial Purification of Lectin from Fungal Sample Using Sephadex G-75 [16]: The swelled gel was packed in the glass column. (0.6 X 100 cm).Sephadex gel was uniformly packed without air bubbles. Then the gel was equilibrated with elution buffer. After equilibration, the dialyzed active sample was applied to the column. Elution was done with the help of elution buffer and the fractions were collected in 2ml propylene tubes. The OD value of each fraction was observed at 280 nm. Active fractions were identified through the hemagglutination assay and protein content was estimated using Lowry's method.

Lectin Purification by RP-HPLC: The gel filtration purified lectin derived from *Amanita*spp. was applied to the HPLC C18 column ((250 X 4.6mm) Varian (Lake forest, CA, USA,) Cyberlab, USA). The column was previously equilibrated with required percentage of acetonitrile and water. Fractions were carried out at the flow rate of 1ml/min. The active peak was collected and concentrated using Speed Vac

RESULTS

Screening of *Amanita* Sp. For Lectin Activity: In the present study, Lectins have a crucial role to play in drug discovery programs and some species of mushrooms are reported to contain lectins. All the five wild species of *Amanita* were evaluated for the presence of lectins.

Biological Action Spectrum: In the present study, twelve different *Amanita* spp. were screened for lectin activity among this five showed lectin activity in culture filtrate, buffer and mycelium extract. All the samples were tested for lectinhemagglutinating activity using a bettery of

Table 1: Heamagglutination activity titre of *Amanita sp*lectin against various blood samples

Species		A^+	A_1B^+	O^+	B^+
Amanita phalloides	Culture filtrate	512	64	1024	8
	buffer	64	16	512	4
	mycelium	256	16	1024	4
Amanita subjunquillea	Culture filtrate	256	512	512	8
	buffer	64	256	64	8
	mycelium	64	64	256	2
Amanita pantherina	Culture filtrate	1024	64	512	4
	buffer	512	128	128	2
	mycelium	512	64	512	2
Amanita pseudoporphyria	Culture filtrate	4	4	256	4
	buffer	2	2	128	2
	mycelium	2	Nil	256	2
Amanita manginiana	Culture filtrate	16	1	64	8
	buffer	4	4	16	8
	mycelium	4	4	64	4

Table 2: Hemagglutination inhibition (HAI) assay of agglutinin from *A. phalloides*by using various carbohydrates

Inhibitor tested (n=10)	Maximum concentration tested (mM)	Hemagglutination inhibition (HAI)	Minimum inhibitory concentration (mM)[a]	Relative inhibitory potency (%)
N-acetyl neuraminic acid	200	-[b]	-[b]	-[b]
Galactose	200	-[b]	-[b]	-[b]
Arabinose	200	-[b]	-[b]	-[b]
Glucosamine	200	-[b]	-[b]	-[b]
Glucose	200	-[b]	-[b]	-[b]
Lactose	200	-[b]	-[b]	-[b]
Mannose	200	1	200	3.125
D-Sucrose	200	64	3.125	100
Fructose	200	-[b]	-[b]	-[b]
Xylose	200	-[b]	-[b]	-[b]
Ribose	200	64	3.125	100
Maltose	200	-[b]	-[b]	-[b]
Chitin	200	-[b]	-[b]	-[b]
L-fucose	200	1	200	3.125
Melibiose	200	-[b]	-[b]	-[b]
Trehelose	200	-[b]	-[b]	-[b]
Raffinose	200	-[b]	-[b]	-[b]
Cellobiose	200	-[b]	-[b]	-[b]
Dextrose	200	-[b]	-[b]	-[b]
Sorbitol	200	-[b]	-[b]	-[b]
Galactosamine	200	-[b]	-[b]	-[b]
N-acetyl glucosamine	200	-[b]	-[b]	-[b]
N-acetyl galactosamine	200	-[b]	-[b]	-[b]
Glucuronic acid	200	-[b]	-[b]	-[b]
Methyl β –Dgalactopyranoside	200	-[b]	-[b]	-[b]
Nitro α-D-galactopyranoside	200	-[b]	-[b]	-[b]

human erythrocytes. The result was represented in Table 1 the present investigation reports that*Amanita phalloides*posseshighlectin activity and this species was choosed for further analysis.

Heamagglutination Inhibition Assay: Carbohydrate specificity profile of fungal lectin was elaborated in Table: 2 lectin was tested with a panel of carbohydrates: N-acetyl neuraminic acid, Galactose, Arabinose, Glucosamine, Glucose, Lactose, Mannose, D-Sucrose, Fructose, L-Fucose, Ribose, Maltose, Chitin, Xylose, Melibiose, Trehelose, Raffinose, Cellobiose, Dextrose, Sorbitol, Galactosamine, N-acetyl glucosamine, N-acetyl galactosamine, Glucuronic acid, Methyl β-D galactopyranoside, Nitro α-D- galactopyranoside. In this *A. phalloides*showed specificity to Ribose and D-sucrose

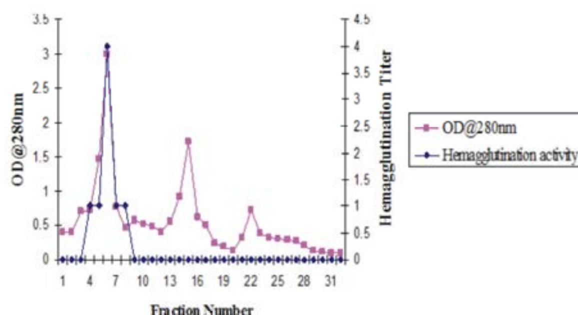

Fig. 1:

Fig. 2:

and showed the lower inhibitory titre value for mannose (HAI. titre = 1) and L-fucose. The other carbohydrates do not show inhibitory potency.

Partial Gel Filteration Purification Profile of Mushroom Lectin by Using Sephadex G-75: Separation performed on the Sephadex G-75 column had one major peak with additional minor two peaks at 280 nm. From this, the major peak showed the hemagglutination activity. Fraction number 5,6,7,8,9 showed higher hemagglutination activity.Subsequently the 6th fraction with high hemagglutinin activity was used in further characterization studies. The result was fraphically represented in Figure 1.

The peak at the 6th fraction shows the higher heamagglutination titre.

RP-HPLC Purification: Separation of RP-HPLC with the C_{18} column showed a major single peak at 280 nm in 1.75 min (retention time of 1.75) and obtained 68% of total protein concentration during a isocratic elution run of 0.1% TFA in 65% acetonitrile and 35% dH_2O. This peak eluted from the column before the start of the acetonitrile gradient and this was true for numerous other attempts under a variety of conditions (varying the concentration of acetonitrile and water). No further resolution was obtained with this column and fraction collected from the column C_{18} did not have any substantial bands with any gel preparation or stain (not shown). This showed the purity of the single hemagglutininlectin.

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DISCUSSION

All the five wild species of *Amanita* were evaluated for the presence of lectins. The tests were performed by taking human erythrocytes of different blood groups viz. A, B, A_1B and O. The highest titer value of hemagglutination was observed in *A. phalloides*and the other species showed significantly low when compared to *A. Phalloides*. The result indicated that lectin present in *Amanita*spp agglutinate all the human erythrocytes. Mushroom lectins are endowed with antiproliferative, antitumor, mitogenic, hypotensive, vasorelaxing, haemolytic, anti-HIV1 reverse transcriptaseand immune potentiating activities [9, 10].

Due to their specificity to the different sugars, twenty six different sugars namely, N-acetyl neuraminic acid, Galactose, Arabinose, Glucosamine, Glucose, Lactose, Mannose, Sucrose, Fructose, L- Fucose, Ribose, Maltose, Chitin, Xylose, Melibiose, Trehelose, Raffinose, Cellobiose, Dextrose, Sorbitol, Galactosamine, N-acetyl glucosamine, N-acetyl galactosamine, Glucuronicacid, Methyl β-D galactopyranoside, Nitro α-D-galactopyranoside. In this *A. phalloides* showed specificity to Ribose, D-sucrose and showed the lower inhibitory titre value for mannose (HAI. titre = 1) and L-fucose. The other carbohydrates do not show inhibitory potency.Parslewwhile working on *Agaricusbisporus* observed that besides the effect of lectins on tumour, these also inhibit cell proliferation, a potentially useful property in the treatment of psoriasis [11].

The study of sugar specificity of lectins has application as it leads us to a better understanding of the function of sub cellular mechanisms in diverse fields ofbiological sciences such as pathological markers of diseases, tissue metastasis and for controlling a variety of infections [12, 13]. This is the first report of isolation of lectin from *Amanita sp* and the RP-HPLC profile shows the purity of isolated lectin. This opens up new array for exploring the occurrence of lectins within this vast genus. Their unique carbohydrate specificity advocates their commercial purification for isolation of oligosaccharides

from complex mixtures using lectin affinity chromatography. Further investigations are however required to ascertain their potential for biomedical applications.

CONCLUSION

The present study reports a new lectin from Amanita sp specific for carbohydrate such as mannose, Ribose, D-Sucrose and L-Fucose with a high binding affinity. Thus there are reports on lectin produced by*Amanita muscaria*which also specific for most of the same sugars. [6, 11]. This might suggest some degree of similarity among lectins produced by mushroom species.The purification of such lectins have a crucial role to play in drug discovery programes.

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