Optimization of culture conditions and characterization of a new lectin from *Aspergillus niger*

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Received: 8 December 2007 / Accepted: 21 April 2008
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Abstract Lectin activity was determined on solidified medium containing agar and in broth cultures of *Aspergillus niger*. The fungus was found to express 16 times higher activity in broth cultures, when grown in a medium adjusted to pH 5.5 at 30°C under stationary condition. Lectin activity was found to be expressed by 6-day-old mycelial cultures with maximum activity being expressed on 9th day of incubation. The crude lectin (total titer 1280) was found to be precipitated at 50% saturation of ammonium sulphate with 2.4-fold purification and 83% yield in the precipitate. The partially purified lectin was found to agglutinate all human, rat, mice and pig erythrocytes. It was found to have a strong binding affinity to mucin, asialofetuin and inulin.

Keywords *Aspergillus niger* · Hemagglutination · Sugar specificity · Minimum inhibitory concentration (MIC)

Introduction

Lectins are ubiquitous 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 cells [1]. They exhibit the ability to agglutinate erythrocytes, lymphocytes, fibroblasts, spermatozoa, yeast, fungal, bacterial and plant cells, owing to the non-covalent interaction of surface sugars and sugar-binding sites on lectin molecules [2]. They play a significant role in host-parasite interactions and are known to contribute to the specificity in mycoparasitism [3]. Their specificity has found immense applications in developmental processes, phagocytosis of cells, tumor cell adhesion, cell differentiation and organ formation and they are also used as ligands in affinity chromatography of many biologically active compounds containing carbohydrates [4]. The ability of membrane lectins to internalize their ligands has manifested their use as drug carriers and metabolite inhibitors and has selective advantage for targeted drug delivery [2, 5].

The lectins in biological sources can be detected by their hemagglutinating ability, based on their interaction with carbohydrates present on surface of erythrocytes. Owing to their carbohydrate-binding ability, the interaction of lectins with cells can be inhibited by specific sugars [6]. Lectins have been reported from varied natural sources including plants, microbes, protozoans and vertebrates [7]. Although many fungal lectins have been reported, most of them are from fruiting bodies of mushrooms [8]. Mycelial lectins have been reported from *Rhizopus stolonifer* [9] and *Arthrobotrys oligospora* [10]. There are also reports on extracellular lectins from *Lentinus edodes* [11] and *Sclerotium*

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rolfsii [3]. Fungal lectins have been found to be specific for mucin and fetuin [12, 13]. Among the genus *Aspergillus*, lectins have been reported from *A. fumigatus* [14, 15] and *A. oryzae* [16]. In the present study, we investigated a new mucin-specific lectin from the mycelium of *A. niger*. The present study was aimed to investigate the effect of culture conditions on lectin activity of *A. niger* and characterizing the lectin with respect to erythrocyte and sugar-binding specificities.

Materials and methods

Organism and culture conditions

The culture of *A. niger* was procured from Microbial Type Culture Collection, Institute of Microbial Technology, Chandigarh, India and maintained on potato dextrose agar slants. Subculturing was done at fortnight intervals and culture slants were stored at $4\pm 1^{\circ}\text{C}$, until further use. The culture was grown in potato dextrose agar and potato dextrose broth at 30°C for 7 days. The mycelium recovered from each of the medium was assayed for lectin activity.

Optimization of culture conditions to enhance the lectin activity

Various culture conditions (pH, temperature, agitation and culture age) were varied to determine their effect on amount of lectin expressed by *A. niger*. One culture disc of 5 mm diameter was inoculated per 50 ml of the potato dextrose medium. The pH of the medium in batch cultures was adjusted in the range of 5.0–7.5, to determine the lectin activity as a function of pH. To investigate the effect of temperature on the amount of lectin expressed, cultures were incubated at $25\text{--}35^{\circ}\text{C}$. The fungus was grown under stationary as well as under agitation (150 rpm) conditions to determine the mode of cultivation in liquid medium. The lectin activity was also determined as a function of culture age (3–10 days).

Preparation of mycelial extract

The mycelium was recovered by filtration from broth cultures and were scrapped free of agar from solidified medium. Recovered mycelium was washed extensively in phosphate buffered saline (PBS, 0.1M, pH 7.2) and pressed dry. The fungal biomass and PBS in the ratio of 1:2 (w/v) was homogenized at high speed (22,000 rpm) for 3–5 min in an ice bath, using an ultra high speed homogenizer (Ultra-Turrax® T25 basic IKA®-werke), followed by grinding with acidified river sand (40–200 mesh EP, s d fine-chem Ltd., India) for 25 min in pestle and mortar in an ice bath. The

extract was centrifuged at $3,000 \times g$ for 20 min at 4°C and supernatant obtained was assayed for lectin activity.

Partial purification of lectin

Lectin was partially purified using ammonium sulphate precipitation. To 10 ml of mycelial extract, ammonium sulfate (10–100%) was added in small fractions with constant stirring on an ice bath for 20–30 min and then kept overnight undisturbed at 4°C . The resultant sample was centrifuged ($3000 \times g$, 20 min, 4°C) and the pellet obtained was dissolved in 2 ml of PBS (0.1M, pH 7.2). The lectin titer and protein content [17] of the precipitate was quantified.

Erythrocyte suspension

Each type of blood from three different human volunteers and animals was withdrawn in Alsever's solution in the ratio 1:3 and centrifuged at $400 \times g$ for 5 min at 4°C . The erythrocyte pellet was washed thrice in PBS (0.1 M, pH 7.2) and resuspended in PBS to a final concentration of 2% (v/v).

Hemagglutination assay

Hemagglutination assay was carried out in U-bottom 96-well microtiter plates (Tarsons Products Pvt. Ltd., India). To 20 μl of serially diluted lectin sample, an equal volume of erythrocyte suspension (10^6 cells/ml) was added. Incubation of microtiter plate was carried out at room temperature for 30 min, and then at 4°C for 1–2 h. Formation of mat indicated lectin activity in the sample, while button formation indicated a negative reaction. Lectin titer was defined as highest dilution capable of visible agglutination. Hemagglutination assay was carried out using human type A, B, AB, O, rat, mice, pig, sheep and goat erythrocytes.

Hemagglutination inhibition assay

Hemagglutination inhibition assay was performed by incubating 20 μl of appropriately diluted lectin (twice the lowest concentration capable of visible agglutination) with an equal volume of sugar stock solution in microtiter plates at room temperature for 1 h and then 40 μl of 2% erythrocyte suspension was added to each well. Plates were further incubated for 30 min at room temperature. A control was also run containing 20 μl of PBS, instead of sugar solution. The plates were stabilized at 4°C for 2–3 hours. Formation of button indicated specific sugar, while mat formation in the presence of sugar indicated non-specific sugar.

Various sugars tested as inhibitors were D-ribose, L-rhamnose, D-raffinose, D-xylose, L-fucose, D-glucose,

D-fructose, D-mannitol, D-mannose, D-arabinose, L-arabinose, inositol, meso-inositol, D-trehalose, D-glucuronic acid, D-galacturonic acid, D-glucosamine hydrochloride, D-galactosamine hydrochloride, N-acetyl-D-glucosamine, N-acetyl-D-galactosamine, 2-deoxy-D-glucose, 2-deoxy-D-ribose, D-galactose, D-sucrose, D-maltose, D-lactose, melibiose, thiodigalactoside, inulin, mucin, asialofetuin, chondroitin-6-sulfate and pullulan (Sigma, USA). Simple sugars were tested at a concentration of 100 mM while complex sugars and glycoproteins were tested for inhibition studies at a concentration of 1 mg/ml. Minimum inhibitory concentration (MIC) of each of the specific sugar was determined by serial double dilution of the sugar solution. MIC was defined as the lowest concentration of sugar capable of complete inhibition of lectin-mediated hemagglutination.

Results and discussion

Optimization of culture conditions

The extract of mycelium recovered from broth cultures of *A. niger* exhibited 16 times higher hemagglutinating activity (titer 512) as compared to the mycelium growing in solidified PDA medium. Mycelial extract of *Rhizopus stolonifer* obtained from solidified medium exhibited agglutinating activity, with no such activity in mycelial extract from liquid medium [3]. Maximum lectin activity was observed in cultures growing at pH 5.5 (Fig. 1) and a temperature of 30°C. Temperatures above or below 30°C were accompanied by a decrease in lectin activity. Elevated temperature was more detrimental to the lectin activity. For the cultures incubated at 35°C (titer 64), eight times lower activity was achieved while the activity dropped only four times for cultures grown at 25°C (titer 128). Similar results

Fig. 1 Effect of pH on lectin activity

have been reported for *Lentinus edodes*, where a drastic reduction in lectin activity was observed at 32°C (optimum 26°C) and the activity was not much affected at temperature below optimum upto 16°C [11]. Broth cultures when grown under agitation formed a pellet of hyphal mass and exhibited only marginal lectin activity (titer 2 as compared to titer 512 observed in cultures incubated under stationary condition). When investigating the effect of culture age on lectin activity, it was found that lectin began to be expressed by 6-day-old mycelium, with no lectin activity upto 5 days of incubation (Fig. 2). Lectin activity reached its stationary value on 9 days of incubation (titer 1024). These observations further confirm that lectin activity is not a function of growth rate alone [18].

Partial purification of lectin

Crude lectin was found to be precipitated at 50% saturation of ammonium sulphate and was purified 2.4-fold with a specific activity of 37.17 titer/mg of protein and 83% yield. Lectin produced by *A. oryzae* has been reported to be precipitated at 30–75% saturation using ammonium sulfate [16].

Characterization of partially purified lectin

The partially purified lectin was found to agglutinate all human, pig, mice and rat erythrocytes while no agglutination was observed with goat and sheep erythrocytes. The non-specificity of lectin to different blood groups suggest that the lectin falls under the category of panagglutinins or non-specific lectins and that the lectin binds to sugar moieties on the erythrocytes other than blood group determinants [6]. The lectin was found to be specific for D-galacturonic acid, N-acetyl-D-galactosamine, chondroitin-6-sulphate, melibiose,

Fig. 2 Effect of incubation period on lectin activity

Table 1 Minimum inhibitory concentration (MIC) of specific sugars

Sugar	MIC
D-Ribose	>12.5 mM
L-Rhamnose	>50 mM
L-Fucose	>25 mM
D-Arabinose	>25 mM
D-Fructose	>12.5 mM
D-Mannitol	>50 mM
D-Galacturonic acid	>50 mM
N-Acetyl-D-galactosamine	>6.25 mM
D-Sucrose	>12.5 mM
Melibiose	>50 mM
Inulin	>62 µg/ml
Mucin	>15.5 µg/ml
Asialofetuin	>62 µg/ml
Chondroitin-6-sulphate	>1 mg/ml

D-ribose, D-rhamnose, L-fucose, D-arabinose, D-fructose, D-mannitol and D-sucrose with strong binding affinity to inulin, mucin and asialofetuin. MIC of the specific sugars is depicted in Table 1. Other sugars tested did not inhibit lectin-mediated hemagglutination at the concentration tested. The sugar specificity of *A. niger* was found to be similar to the sugar-binding profile of other fungal lectins which are reported to be specific for glycoproteins fetuin and mucin [12, 13].

Conclusions

The present study reports a new lectin from *A. niger* specific for D-ribose, L-fucose, D-arabinose, D-fructose, D-sucrose and N-acetyl-D-galactosamine, with a high binding affinity to inulin, mucin, asialofetuin and chondroitin-6-sulphate. There are two reports on lectin produced by *A. fumigatus*, one specific for L-fucose [14] and the other specific for mucin [15]. Lectin reported from *A. oryzae* is also specific for L-fucose [16]. This might suggest some degree of similarity among lectins produced by *Aspergillus* species.

Acknowledgments Authors are thankful to the Department of Biotechnology, Punjabi University, Patiala for providing the necessary facilities to execute this work. The financial assistance received from Department of Science and Technology, Govt. of India, New Delhi, under FIST programme, is duly acknowledged.

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