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Purification and characterization of an *N*-acetyl-D-galactosamine-specific lectin from the edible mushroom *Schizophyllum commune*

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Abstract

An *N*-acetyl-D-galactosamine (GalNAc)-specific lectin was purified from the edible mushroom, *Schizophyllum commune*, using affinity chromatography on a porcine stomach mucin (PSM)-Sepharose 4B column. Under reducing and non-reducing conditions, SDS-polyacrylamide gel electrophoresis gave a major band of 31.5 kDa. The *Schizophyllum commune* lectin (SCL) showed high affinity toward rat erythrocytes and the sugar inhibition assay exhibited its sugar specificity highly toward lactose and *N*-acetyl-D-galactosamine. It was stable at 55 °C for 30 min and at pH 3–10 for 18-h test. The lectin was shown to be a glycoprotein with cytotoxic activity against human epidermoid carcinoma cells. The N-terminus of SCL was blocked but amino acid sequences of internal tryptic peptides showed moderately sequence similarities with some other fungal and plant lectins. Crystals of SCL were obtained by the sitting drop vapour-diffusion method using polyethylene glycol 8000 as the precipitant, and gave an X-ray diffraction pattern to approximately 3.8 Å resolution.

Keywords: Lectin; Edible mushroom; *N*-acetyl-D-galactosamine-specific lectin; Lectin crystal; *Schizophyllum commune*; X-ray diffraction

1. Introduction

Lectins are a heterogeneous group of proteins or glycoproteins of non-immune origin that specifically and reversibly bind to carbohydrates of glycoconjugates [1,2]. These proteins are ubiquitous in nature, and occur in animals, plants, bacteria, viruses, and fungi [3,4]. Most lectins play a crucial role in diverse biological processes, particularly in host defense mechanisms, inflammation, and metastasis [5]. Owing to their binding specificities, lectins are employed in numerous biochemical and clinical research areas [6–8]. *N*-acetyl-D-galactosamine (GalNAc)-specific lectins are of a great interest since they have been reported as a detecting agent for tumor-associate antigens (Thomson–Friedenreich or T-antigens) of malignant cells [9,10].

Lectins with high affinity to GalNAc have been isolated and characterized from both vertebrates and invertebrates. For the purpose of the anticancer drug production, work on GalNAc lectins has been rapidly increasing. A novel mouse macrophage (M∅) C-type lectin (MGL) which has Gal/GalNAc-specificity has been well characterized [11,12]. The cDNA cloning of GalNAc-specific lectin from starfish, *Asterina pectinifera* was also attempted. Most recently, X-ray crystallographic analysis of a sea cucumber, *Cucumaria echinata* lectin (CEL), which recognizes GalNAc, was revealed [13].

In addition, several fungal lectins, which possess immunomodulatory, mitogenic, and antitumor activities, have been isolated, for example from *Agaricus bisporus*, *Volvariella volvacea*, *Grifola frondasa*, and *Tricholoma mongolicum* [14–17]. The discovery of lectin in a mushroom from tropical areas with cytotoxic activity could possibly be highly interesting.

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Although some of GalNAc-specific lectins have been intensively studied, only a few reports on crystallization of the GalNAc-specific lectin from edible mushrooms have been published.

In the present study, a GalNAc-specific lectin from the edible mushroom, *Schizophyllum commune* collected from a local market in Thailand was purified and characterized. The cytotoxic property of its lectin was elucidated. Additionally, single crystals of the *Schizophyllum commune* lectin (SCL) were prepared for the first time, and proved suitable for X-ray diffraction studies.

2. Materials and methods

2.1. Purification of the SCL

Fresh fruiting bodies of the edible mushroom *Schizophyllum commune* were obtained from a local market in Nakhon Pathom Province, Thailand. The mushroom specimens were dried at 40 °C for 2 days. The dried specimens were ground into powder using a blender and stored in sealed plastic bag at temperature below 25 °C until use. The lectin was extracted by homogenizing the mushroom powder with 10 times (w/v) of 10 mM phosphate buffer saline (PBS), pH 7.2, containing 1 mM benzamidine, 0.1% (v/v) 2-mercaptoethanol, and 1.5% (w/v) polyvinylpyrrolidone (PVPP) and stirring overnight at 4 °C. The homogenate was filtered through cheesecloth, and the filtrate was centrifuged at 14000×*g* for 30 min at 4 °C. Then solid ammonium sulfate was added to the supernatant to obtain 30% saturation. After stirring overnight at 4 °C, the supernatant was collected by centrifugation at 100000×*g* for 30 min at 4 °C. The supernatant was loaded on to a porcine stomach mucin (PSM)-Sepharose 4B column which was equilibrated with 10 mM Tris–HCl, pH 8 containing 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 0.02% (w/v) NaN_3. Unbound proteins were eluted and the lectin was desorbed with 20 mM 1, 3-diaminopropane (DAP). All fractions were then neutralized with 1 M Tris–HCl, pH 7. The adsorbed fractions were assayed for hemagglutinating activity before repeating the loading on to the column in order to increase their purity. Then the final fractions were represented as purified lectin.

2.2. SDS-PAGE

SDS-PAGE was performed on 17.5% (w/v) polyacrylamide gel containing 0.1% (w/v) of SDS according to the method of Laemmli [18]. After electrophoresis, the gel was stained with Coomassie brilliant blue R-250. The molecular weight of the purified lectin was determined by comparing its electrophoresis mobility with the standard molecular weight marker proteins, LMW-SDS marker (Amersham-Pharmacia Biotech, Uppsala, Sweden). In order to determine carbohydrate of the lectin, periodic acid Schiff's (PAS) staining was investigated according to the method described by Segrest and Jackson [19].

2.3. Protein content estimation

Protein concentration was determined by the method of Bradford [20].

2.4. Enzymatic deglycosylation

In order to determine the *N*-glycosidic linkage of the SCL, the deglycosylation was performed using the Glycoprofile II, enzymatic in-solution *N*-deglycosylation kit (Sigma, St. Louis, USA). Then molecular mass of the lectin subunits after deglycosylation was detected by SDS-PAGE.

2.5. Amino acid sequence analysis

After SDS-PAGE, the lectin band was immobilized on a polyvinylidene difluoride (PVDF) membrane by electroblotting, which was performed at 14 V for 30 min with a semi-dry transfer cell for N-terminal sequencing by Edman degradation using an Applied Biosystems model 471A Protein Sequenator. In-gel digestion was carried out using an adaptation of the method of Rosenfeld et al. [21]. Gel slices from SDS-PAGE were washed (2×30 min) with 50% acetonitrile, 0.2 M ammonium bicarbonate pH 8.9 and then freeze-dried. The slices were re-swollen in 50 μl of RHB (0.2 M ammonium tricarbonate pH 7.8, 0.02% tween 20)/2 M urea containing 0.5 μg trypsin and incubated at 37 °C overnight. Excess RHB/2 M urea was then removed to an Eppendorf tube and peptides were extracted from the gel slices with 2 lots of 60% acetonitrile and 0.1% TFA. The latter and the excess RHB/2M urea were pooled, concentrated by centrifugal evaporation, and applied to a reverse phase HPLC column to separate the peptides. Suitable peptides were then subjected to sequencing by Edman degradation using a model 471A Protein Sequenator (Applied Biosystems, Warrington, UK).

2.6. Heat stability and pH stability tests

The heat stability and pH stability of the selected lectin was examined according to the modified method of Freire et al. [22] and Kobayashi et al. [23]. Heat stability was determined by heating aliquots of the lectin (1 mg/ml lectin in PBS) for 30 min at various temperatures of 40, 50, 55, 65, 80, and 100 °C. Then the heated aliquots were cooled rapidly on ice, centrifuged to remove any precipitate, and assayed for hemagglutination in comparison with a control sample of lectin. Results were expressed by calculating the percentage of hemagglutination shown by the heated aliquots (titration value) compared with the control sample representing 100%.

For the pH stability test, the lectin was measured by incubating the lectin samples (1 mg/ml) in the following buffers varying from pH 3–10 for 18 h at 4 °C. Different buffers were used according to pH range as follows; 50 mM glycine–HCl buffer (pH 2.0–3.0), 50 mM sodium acetate buffer (pH 4.0–5.5), 50 mM Tris–HCl buffer (pH 8.0–8.5), and 50 mM glycine–NaOH buffer (pH 9.0–11.0). The residual hemagglutinating activity was assayed after dialysis the lectin samples against PBS for 18 h at 4 °C. Three replicates were done for each test. Results were calculated by expressing the titration values of the lectin as percentages of the titration value of the control.

2.7. Hemagglutinating activity and sugar inhibition assays

A serial two-fold dilution of the mushroom lectin solution (50 μl) in microtiter U-plates was mixed with the same volume of a 2% suspension of rabbit erythrocytes in PBS, pH 7.2 and incubated at 4 °C. The results were read after about 1 h when the negative control had fully sedimented. The hemagglutination titer, defined as the reciprocal of the highest dilution exhibiting hemagglutination, was reckoned as one hemagglutination unit.

Sugar inhibition assay was investigated for studying sugar specificity of the mushroom lectin. The sugar-inhibition of lectin-induced hemagglutination using various sugars was performed in a manner analogous to the hemagglutination test. Serial two-fold dilutions of test sugar samples were prepared in PBS, pH

Table 1
Hemagglutinating activities of SCL chromatographic fractions from 15 g of dried fruiting body powder against rabbit erythrocytes

Step	Total protein (mg)	Specific hemagglutinating activity (unit/mg)	Total hemagglutinating activity (unit)	Recovery of hemagglutinating activity (%)	Fold of purification
Crude extraction	7548	86.5	652,800	100	–
30% NH_2SO_4 precipitation	4371	87.9	384,000	58.8	1.0
PSM-Sepharose 4B affinity column	32.4	2496.1	80,640	12.4	28.9