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Biochemical and Biophysical Research Communications 336 (2005) 252–257
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A novel homodimeric lactose-binding lectin from the edible split gill medicinal mushroom *Schizophyllum commune*

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Received 2 August 2005
Available online 19 August 2005

Abstract

A homodimeric lactose-binding lectin with a molecular mass of 64 kDa was isolated from fresh fruiting bodies of the split gill mushroom *Schizophyllum commune*. The N-terminal sequence of the lectin is similar to a part of the sequence of the cell division protein from *Gleobacter violaceus*. The lectin was isolated by using a procedure which involved ion exchange chromatography on DEAE–cellulose, CM–cellulose, and Q-Sepharose, and gel filtration by fast protein liquid chromatography on Superdex 75. The hemagglutinating activity of the lectin was stable at temperatures up to 40 °C, and in concentrations of NaOH and HCl solution up to 125 and 25 mM, respectively. The lectin exhibited potent mitogenic activity toward mouse splenocytes, antiproliferative activity toward tumor cell lines, and inhibitory activity toward HIV-1 reverse transcriptase. It was devoid of antifungal activity.

Keywords: Lectin; Lactose-binding; *Schizophyllum commune*

Lectins, proteins characterized by their ability to interact with carbohydrates, can be divided into different groups according to their sugar binding specificity. Lectins have drawn the increasing attention of some investigators owing to their biological activities including antiproliferative, immunodulatory, antifungal, antiviral, and anti-insect activities [1].

Lectins have been localized on the caps, stipes, and mycelia of mushrooms, and variation in lectin content occurs depending on the carpophore age, and the time and place of harvest. In mushrooms, lectins probably play an important role in dormancy, growth, and morphogenesis, morphological changes consequent upon parasitic infections, and molecular recognition during the early stages of mycorrhization. Mushroom lectins find applications in taxonomical, embryological, and bacteriological studies, study of the modifications in membrane glycoconjugates and cancer formation, cell sorting, sorting of mutant and tumor cells, and isolation of membrane and serum glycoconjugates [2].

The split gill mushroom *Schizophyllum commune* is used as a mating type for the study of fungal genetics. It is also a medicinal mushroom. From the mushroom, a neutral glycan designated schizophyllan that can augment the production of interferon-γ and interleukin-2 from mitogen-stimulated human peripheral blood mononuclear cells [3] and directly activate peritoneal macrophages causing them to become cytotoxic [4], has been isolated. *S. commune* produces an intracellular α,α-trehalose phosphorylase during growth on D-glucose. The changes in the intracellular levels of α,α-trehalose and Pi with time are inverse of that of trehalose phosphorylase activity, suggesting a temporary utilization of the α,α-trehalose pool via phosphorolysis [5].

In view of the meager information available in the literature about the proteinaceous constituents of *S. commune*, we undertook the present study to isolate a lectin from the mushroom.

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doi:10.1016/j.bbrc.2005.08.068

Materials and methods

Purification scheme. Fresh fruiting bodies (100 g) of *S. commune* were homogenized in 0.15 M NaCl (4 ml/g) using a Waring blender and then soaked in 1 L of 0.15 M NaCl for 12 h. The slurry was then centrifuged at 8000*g* for 15 min. Ammonium sulfate was added to the resulting supernatant to 30–80% saturation. Four hours later, the precipitate was collected by centrifugation at 8000*g* for 15 min. It was then dissolved in a small amount of distilled water and dialyzed against several changes of a copious amount of distilled water, lyophilized, and then chromatographed on a 1×10 cm column of DEAE–cellulose (Sigma) in 10 mM NH_4HCO_3 buffer (pH 9.4). After removal of unadsorbed proteins in fraction D1, adsorbed proteins were desorbed sequentially in the starting buffer. The second adsorbed fraction (D3) eluted with 150 mM NaCl was lyophilized, prior to ion exchange chromatography, on a 1.5×20 cm column of CM–cellulose (Sigma) in 10 mM NH_4OAc buffer (pH 5.1). After elution of unadsorbed fraction C1, the column was eluted stepwise with 50 mM NaCl, 150 mM NaCl, and 1 M NaCl in the starting buffer. The second adsorbed fraction (C3) eluted with 150 mM NaCl was lyophilized before chromatography on a 1×10 cm column of Q-Sepharose (Amersham Biosciences) in 10 mM phosphate buffer (pH 7.5). After the unadsorbed fraction (Q1) had been eluted, the column was eluted with a linear 0–0.4 M NaCl gradient in the phosphate buffer. The first adsorbed fraction (Q2) was then subjected to gel filtration on a Superdex 75 HR 10/30 column by fast protein liquid chromatography using an AKTA Purifier (Amersham Biosciences).

Determination of molecular mass and N-terminal sequence. The purified lectin was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) for molecular mass determination in accordance with the procedure of Laemmli and Favre [5]. Gel filtration on an FPLC-Superdex 75 column, which had been calibrated with molecular mass markers (Amersham Biosciences), was conducted to determine the molecular mass of the lectin. The N-terminal sequence of the lectin was determined by using a Hewlett-Packard HP G1000A Edman degradation unit and an HP 1000 HPLC System [6].

Assay for lectin (hemagglutinating) activity. A serial twofold dilution of the lectin solution in microtiter U-plates (50 μl) was mixed with 50 μl of a 2% suspension of rabbit red blood cells in phosphate-buffered saline (pH 7.2) at 20 °C. The results were read after about 1 h when the blank had fully sedimented. The hemagglutination titer, defined as the reciprocal of the highest dilution exhibiting hemagglutination, was reckoned as one hemagglutination unit. Specific activity is the number of hemagglutination units/mg protein [7].

The hemagglutinating inhibition tests to investigate the inhibition of lectin-induced hemagglutination by various carbohydrates were performed in a manner analogous to the hemagglutination test. Serial twofold dilutions of sugar samples were prepared in phosphate-buffered saline. All of the dilutions were mixed with an equal volume (25 μl) of a solution of the lectin with 32 hemagglutination units. The mixture was allowed to stand for 30 min at room temperature and then mixed with 50 μl of a 2% rabbit erythrocyte suspension. The minimum concentration of the sugar in the final reaction mixture, which completely inhibited 32 hemagglutination units of the lectin preparation, was calculated [7].

The effects of temperature, NaOH solution, HCl solution, and solutions of metallic chlorides on hemagglutinating activity of the lectin were examined as previously described [7].

Assay of mitogenic activity of lectin toward mouse splenocytes. The assay of mitogenic activity was performed as described by Wang et al. [8]. Splenocytes were isolated from BALB/c mice. The cells were diluted with RPMI medium containing 10% fetal bovine serum and then seeded (2×10^6 cells/0.2 ml/well) in 96-well microplates. The lectin was then added at various concentrations. Cells cultured in the absence of lectin served as control. The cells were incubated at 37 °C in a humidified atmosphere of 5% carbon dioxide for 24 h and were viable after 24 h. During the last 6 h, the cells in one well were pulsed with 0.5 μCi and [^{3}H-methyl]thymidine (specific activity 5 μCi/mmol, Amersham Biosciences) in 10 μl and then harvested onto a glass fiber filter using a cell harvester. The radioactivity was determined using a Beckman scintillation counter. The proliferative (mitogenic) response was expressed as mean counts per min (cpm).

Assay of antiproliferative activity on tumor cell lines. The antiproliferative activity of the purified lectin was determined as follows [9]. The cell lines L1210 (leukemia), MBL2 (leukemia), and HepG2 (hepatoma) were purchased from American Tissue Culture Collection. The various cell lines were maintained in Dulbecco modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 100 mg/L streptomycin and 100 IU/ml penicillin at 37 °C in a humidified atmosphere of 5% CO_2. Cells (1×10^4) in their exponential growth phase were seeded into each well of a 96-well culture plate (Nunc, Denmark) and incubated for 3 h before addition of the lectin. Incubation was carried out for another 48 h. Radioactive precursor, 1 μCi, ([^{3}H-methyl]thymidine, from Amersham Biosciences) was then added to each well and incubated for 6 h. The cultures were then harvested by a cell harvester. The incorporated radioactivity was determined by liquid scintillation counting.

Assay of antifungal activity. The assay for antifungal activity toward various fungal species was carried out in 100×15 mm petri plates containing 10 ml of potato dextrose agar. After the mycelial colony had developed, sterile blank paper disks (0.625 cm in diameter) were placed at a distance of 0.5 cm away from the rim of the mycelial colony. An aliquot (15 μl) of the lectin was added to a disk. The plates were incubated at 23 °C for 72 h until mycelial growth had enveloped disks containing the control and had formed crescents of inhibition around disks containing samples with antifungal activity [10].

Assay for HIV reverse transcriptase inhibitory activity. The lectin was tested for this activity since some lectins possess an inhibitory activity toward human immunodeficiency virus type 1 (HIV-1) reverse transcriptase. The assay for ability to inhibit HIV-1 reverse transcriptase was assessed by using an enzyme-linked immunosorbent assay kit from Boehringer–Mannheim (Germany) as described by Wang and Ng [11]. The assay takes advantage of the ability of reverse transcriptase to synthesize DNA, starting from the template/primer hybrid poly(A)–oligo$(dT)_{15}$. In place of radio-labeled nucleotides, digoxigenin- and biotin-labeled nucleotides in an optimized ratio are incorporated into one and the same DNA molecule, which is freshly synthesized by the reverse transcriptase (RT). The detection and quantification of synthesized DNA as a parameter for RT activity follows a sandwich enzyme-linked immunosorbent assay protocol. Biotin-labeled DNA binds to the surface of microtiter plate modules that have been precoated with streptavidin. In the next step, an antibody to digoxigenin, conjugated to peroxidase (anti-DIG-POD), binds to the digoxigenin-labeled DNA. In the final step, the peroxidase substrate is added. The peroxidase enzyme catalyzes the cleavage of the substrate, producing a colored reaction product. The absorbance of the samples at 405 nm can be determined using a microtiter plate (ELISA) reader and is directly correlated to the level of RT activity. A fixed amount (4–6 ng) of recombinant HIV-1 reverse transcriptase was used. The inhibitory activity of the lectin was calculated as percent inhibition as compared to a control without the protein.

Results

Hemagglutinating (lectin) activity was detected mainly in the absorbed fraction eluted with 150 mM NaCl from the DEAE–cellulose column, i.e., fraction D3. No activity was found in the much larger unadsorbed fraction D1 (Fig. 1). Fraction D3 was resolved into an unadsorbed fraction C1 and three adsorbed fractions C2, C3, and C4 upon ion exchange chromatography on CM–cellulose. Fraction C3 was a much higher peak than all other three fractions. Lectin activity resided in fraction C3 (Fig. 2). Ion exchange chromatography of fraction C3 on Q-Sepharose resulted in a small unadsorbed fraction Q1 and

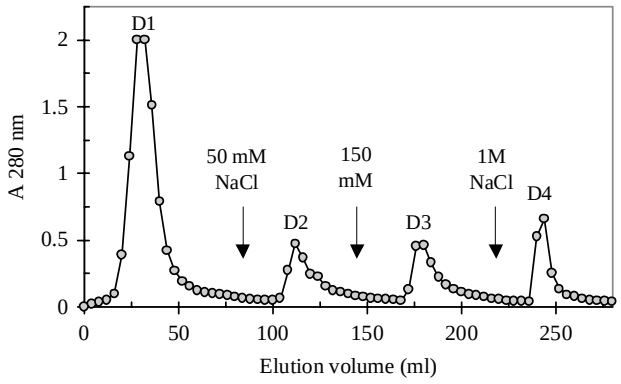

Fig. 1. Anion exchange chromatography of protein derived from the $(NH_4)_2SO_4$ precipitate of *S. commune* fruiting body extract on a DEAE–cellulose column (1 × 10 cm), which was equilibrated and eluted with 10 mM NH_4HCO_3 buffer (pH 9.4) and then stepwise with 50, 150 mM, and 1 M NaCl in the same buffer. Arrows indicate the point at which buffer was changed.

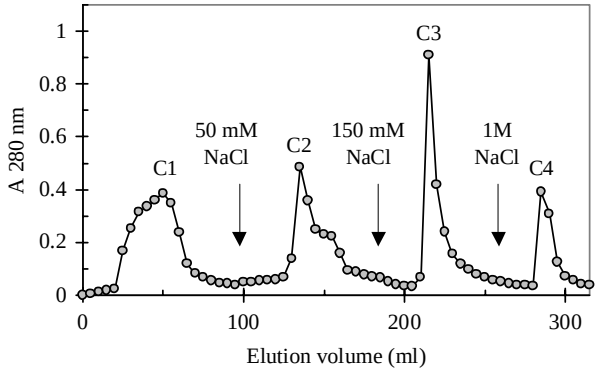

Fig. 2. Cation exchange chromatography of fraction D3 (adsorbed on DEAE–cellulose) on CM–cellulose. The column (1.5 × 20 cm) was first eluted with 10 mM NH_4OAc buffer (pH 5.1) and then with 50, 150 mM, and 1 M NaCl, respectively, in the same buffer. Arrows indicate the point at which buffer was changed.

two adsorbed fractions Q2 and Q3. Q2, in which lectin activity resided, was smaller than Q3 (Fig. 3). Fraction Q2 was fractionated into a larger fraction SU1 and a smaller fraction SU2 upon gel filtration on a Superdex 75 HR 10/30 column by fast protein liquid chromatography on an AKTA Purifier (Amersham Biosciences). Lectin activity resided in fraction SU1 (Fig. 4). The purified lectin appeared as a single band with a molecular mass of 32 kDa in SDS–PAGE (Fig. 5). The yields and specific hemagglutinating activities of the various chromatographic fraction are given in Table 1. The N-terminal sequence of the lectin was similar to that of the cell division protein from *Gleobacter violaceus* and α subunit of inhibin (Table 2). The lectin was stable between 20 and 40 °C. The activity declined to 25% of that at 50 °C and to negligible levels at 60 °C (Table 3). The lectin activity was stable in 6, 12.5, and 25 mM HCl. The activity was reduced to 50% in 50 and 100 mM HCl, and disappeared altogether at 0.2 M HCl (Table 4). Full activity was observed in 6 and 12.5 mM NaOH. Only 25% of the activity was left in 25 mM NaOH and 12.5% left in 50 mM NaOH (Table 4). The hemagglutinating activity was unaffected by the presence of $FeCl_3$ and $AlCl_3$, but was reduced by Zn^{2+}, Ca^{2+}, Mg^{2+}, and Mn^{2+} ions (Table 5). Lactose potently and inulin less potently inhibited the activity (Table 6).

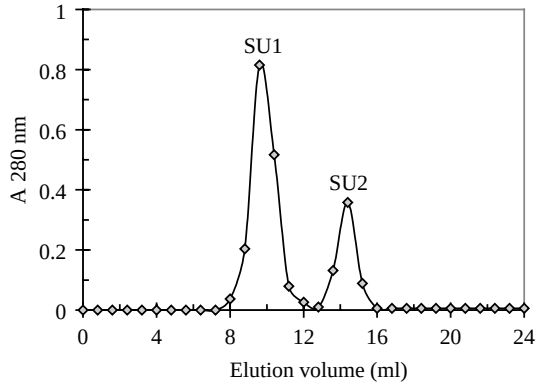

Fig. 4. Gel filtration of fraction Q2 from Q-Sepharose column on a Superdex 75 column, which was eluted with 10 mM phosphate buffer (pH 7.5) containing 150 mM NaCl. The flow rate was 0.4 ml/min.

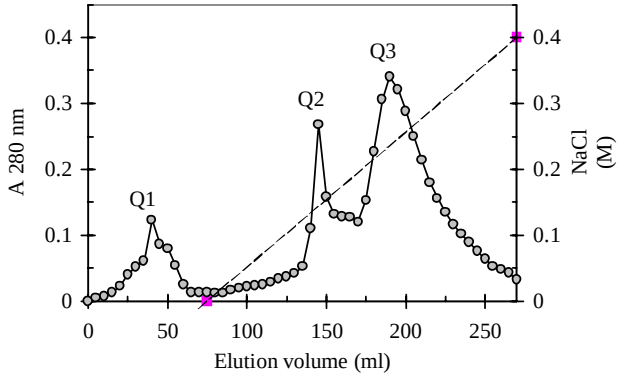

Fig. 3. Anion exchange chromatography of fraction C3 from CM–cellulose column on a Q-Sepharose column (1 × 10 cm). The column was initially eluted with 10 mM phosphate buffer (pH 7.5) and subsequently with a linear gradient of 0–400 mM NaCl in the same buffer.

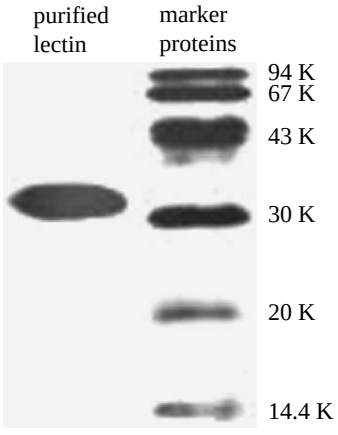

Fig. 5. SDS–PAGE of fraction SU1 from Superdex 75 column.

Table 1
Yields and hemagglutinating activities of various chromatographic fractions (from 100 g fresh fruiting bodies)

Fraction	Yield (mg)	Specific activity (U/mg)	Total activity (U/10^5)	Recovery of activity (%)
Ammonium sulfate precipitate	1739.06	520	9.04	100
D1	746.25	—	—	—
D2	99.27	330	0.33	3.65
D3	**94.40**	**4650**	**4.39**	**48.56**
D4	79.08	260	0.21	2.32
C1	26.31	—	—	—
C2	13.58	1290	0.18	1.99
C3	**18.83**	**13000**	**2.45**	**27.10**
C4	6.02	570	0.03	0.33
Q1	2.5	—	—	—
Q2	**6.10**	**19390**	**1.18**	**13.05**
Q3	6.4	620	0.04	0.44
SU1	**1.72**	**53100**	**0.91**	**10.07**
SU2	0.89	—	—	—

Bold font indicates lectin-enriched fraction.

Table 2
N-terminal composition of *S. commune* lectin

	Sequence	Protein length
Schizophyllum commune lectin	APPNFPLLV-PGNDKLLAVISAMTP	—
Gleobacter violaceus Cell division protein (205–222)	------LIVIP-NDKLLSVISEQTP	419
Schizophyllum commune lectin	APPNFPLLVPG	—
Feline inhibin α subunit precursor (87–97)	APPDLPLLVPG	154
Lectin LZ-8 from *Ganoderma lucidum* (27–58)	GNPNNFIDTVTFPKVLTDKAYTYRVAV-SGRNL	—
Schizophyllum commune lectin	APPN-FPLLV-PG-NDKLL----AVISAMTP	110

Identical amino acid residues are underlined.

Table 3
Effect of temperature on hemagglutinating activity of *S. commune* lectin

Temperature (°C)	20	30	40	50	60	70	80	90	100
Hemagglutinating activity (U)	64	64	64	16	4	1	0	0	0

Initial hemagglutinating activity was 64 hemagglutinating units as indicated by 7 wells in the plate exhibiting hemagglutination.

Table 4
Effects of NaOH and HCl solutions on hemagglutinating activity of *S. commune* lectin

HCl (M)	0.006	0.0125	0.025	0.05	0.1	0.2
Hemagglutinating activity (U)	16	16	16	8	8	0
NaOH (M)	0.006	0.0125	0.025	0.05	0.1	0.2
Hemagglutinating activity (U)	16	16	4	2	0	0

Initial hemagglutinating activity was 16 hemagglutinating units.

Table 5
Effects of cations on hemagglutinating activity of *S. commune* lectin

	1.25 mM	2.5 mM	5 mM	10 mM
Fe^{3+}	32	32	32	32
Al^{3+}	32	32	32	32
Zn^{2+}	16	16	16	16
Ca^{2+}	32	16	8	8
Mg^{2+}	16	16	16	16
Mn^{2+}	16	16	16	16

Initial hemagglutinating activity was 32 hemagglutinating units.

The mitogenic activity of the lectin toward mouse splenocytes is shown in Fig. 6. Maximal stimulation (about 15-fold over control value) was achieved at a lectin concentration of 4 μM. The lectin inhibited [^{3}H-methyl]thymidine uptake by L1210, MBL2, and HepG2 cells with an IC_{50} value of 0.5, 1.1, and 1.4 μM, respectively. HIV-1 reverse transcriptase was inhibited with an IC_{50} of 1.2 μM. The lectin lacked antifungal activity (data not shown).

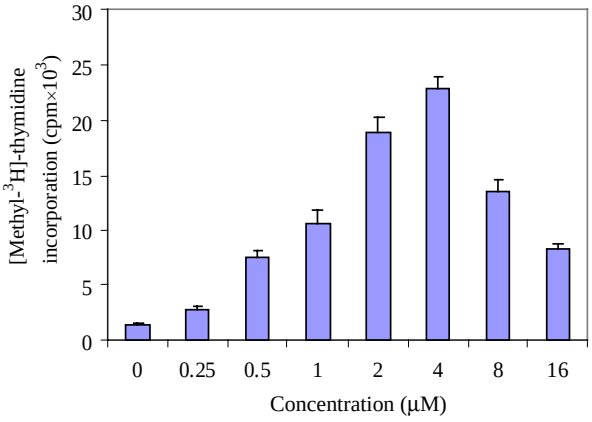

Fig. 6. Effect of *S. commune* lectin on mitogenic response of mouse splenocytes. Results represent means ± SD ($N = 3$).

Discussion

Schizophyllum commune is a unique mushroom characterized by splitting of the fruiting body. It is noteworthy that the N-terminal sequence of its lectin closely resembles a partial sequence of cell division protein from *G. violaceus*. It is likely that the splitting of the fruiting body is related to accelerated cell division. The mitogenic activity of *S. commune* lectin toward spleen cells is in line with its sequence homology to cell division protein. It is interesting that the N-terminal sequence of *S. commune* lectin is similar to a partial sequence of the α subunit of feline inhibin, which inhibits pituitary secretion of follicle-stimulating hormone. Activin formed from the β subunit of inhibin has an action opposite to that of inhibin [12]. Follicle-stimulating hormone stimulates cell division in the seminiferous tubule during the process of spermatogenesis in the testis. Thus, the N-terminal sequence of *S. commune* lectin may be related to its role in cell division. It is noteworthy that *S. commune* produces the auxin indoleacetic acid, which is a plant growth hormone [13], and that *S. commune* has a rapid growth. It remains to be elucidated whether its rapid growth and splitting of fruiting body are due to the auxin only or also attributed to its lectin.

The antiproliferative activity of *S. commune* lectin in agreement with previous reports on lectins from the mushroom *Tricholona mongolicum* [9], *Pleurotus ostreatus* [7], and *Agaricus bisporus* [14]. Potent activity with an IC50 around 1 μM is observed. The mitogenic activity of *S. commune* lectin toward murine splenocytes is in accordance with reports on lectins from the mushrooms *Volvariella volvacea* [15,16] and *Agrocybe cylindracea* [8]. Considerable (15-fold) stimulation was attained at a dose of 4 μM. The lectin also displays potent anti-HIV reverse transcriptase activity ($IC_{50} = 1.2$ μM). Some lectins are devoid of such activity [17].

The instability of *S. commune* lectin to high temperatures, low pH, and high pH indicates that it is less robust than *T. monogolicum* lectin [9]. The hemagglutinating activity of *S. commune* lectin is unaffected in the presence of the trivalent ferric and aluminum ions, but is curtailed by divalent cations including Ca^{2+}, Zn^{2+}, Mg^{2+}, and Mn^{2+}.

Schizophyllum commune lectin is dimeric, like lectins from mushrooms including *A. cylindracea* [8], *Coprinus cinereus* [18], *Hericium erinaceum* [19], *Oudemansiella platyphylla* [20], *Polyporus squamosus* [21], *T. mongolicum* [9], and *V. volvacea* [15,16].

A nearly 100-fold purification was achieved in the present study on the purification of *S. commune* lectin. It is also outstanding in that it has a very high specific hemagglutinating activity. Four chromatographic steps including three ion exchange steps and one gel filtration step are involved in the purification protocol. Like many other lectins, *S. commune* lectin is adsorbed on DEAE–cellulose and Q-Sepharose.

The co-existence of a lectin and a polysaccharide, both with antiproliferative/antitumor and immunostimulating activities in *S. commune*, provides a scientific basis for the medicinal value of this mushroom.

Table 6
Effects of different concentrations of various carbohydrates on hemagglutination induced by *S. commune* lectin

	200 mM	100 mM	50 mM	25 mM	12.5 mM	6.25 mM	3.125 mM	1.5625 mM	0.781 mM	0.39 mM	0.195 mM
Inositol	+	+	+	+	+	+	+	+	+	+	+
O-Nitrophenyl-β-D-galacto-pyranoside	+	+	+	+	+	+	+	+	+	+	+
Sorbose	+	+	+	+	+	+	+	+	+	+	+
Raffinose	+	+	+	+	+	+	+	+	+	+	+
L(+)-Rhamnose	+	+	+	+	+	+	+	+	+	+	+
D(−)Fructose	+	+	+	+	+	+	+	+	+	+	+
D(+)-Mannose	+	+	+	+	+	+	+	+	+	+	+
Cellobiose	+	+	+	+	+	+	+	+	+	+	+
L(+)-Arabinose	+	+	+	+	+	+	+	+	+	+	+
D(+)-Xylose	+	+	+	+	+	+	+	+	+	+	+
D-Melibiose	+	+	+	+	+	+	+	+	+	+	+
Lactose	−	−	−	−	−	−	−	+	+	+	+
Inulin	−	−	−	+	+	+	+	+	+	+	+

Initial hemagglutinating activity was 32 hemagglutinating units.
+, hemagglutinating; −, no hemagglutinating.

Acknowledgment

We thank Miss Fion Yung for her expert secretarial assistance.

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