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Isolation of a Novel Agglutinin with Complex Carbohydrate Binding Specificity from Fresh Fruiting Bodies of the Edible Mushroom *Lyophyllum shimeiji*

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A hemagglutinin, with a molecular weight of 30,000 and expressing hemagglutinating activity which could not be inhibited by simple sugars and glycoproteins, was isolated from fresh fruiting bodies of the edible mushroom *Lyophyllum shimeiji*. The protein was adsorbed on CM-Sepharose even in 20 mM ammonium acetate (pH 5.5) containing 1 M NaCl and was desorbed by 20 mM ammonium bicarbonate (pH 9). The hemagglutinating activity was subsequently adsorbed on Mono S in 20 mM ammonium acetate (pH 5.5) and was desorbed by a linear gradient of 0.2–0.5 M NaCl in ammonium acetate buffer. The hemagglutinin exhibited a novel N-terminal sequence not found in any lectin and hemagglutinin reported so far. It was devoid of antifungal activity. © 2002 Elsevier Science

Lectins have arrested the attention of numerous researchers. The enormous number of books and articles on lectins speaks to the importance of lectins. Lectins express exploitable activities such as immunomodulatory (1–3), antiproliferative/antitumor (1, 4–6), antifungal (7, 8), antiviral (8–10), and anti-insect (11, 12) activities. These carbohydrate-binding proteins have been purified from innumerable animals, plants, and microorganisms.

Mushrooms are renowned for their nutritive and medicinal values. Recent research on mushroom biochemistry has focused on lectins (13–17), proteases (18–26), ribonucleases (27–30), ribosome inactivating proteins (31–35), antifungal proteins (31), polysaccharide–protein complexes (36), polysaccharopeptides (37–40), and polysaccharides (41). Some of these mushroom constituents such as lectins (15, 16), polysaccharides (41–43), polysaccharopeptides (37–40) and ribosome inactivating proteins (31) elicit antiproliferative/antitumor and/or immunomodulatory effects. Ribosome inactivating proteins exert an immunomodulatory action (31). Antifungal proteins (32) and ribosome inactivating proteins (31) retard fungal growth.

Mushroom lectins have been reviewed (17). Although a huge number of mushroom species exists, the number of studies on the isolation and characterization of mushroom lectins is relatively small. Lectins have been purified from a number of edible mushrooms including straw mushroom (44), button mushroom (45), monkey head mushroom (46), oyster mushroom (15), *Ganoderma lucidium* (47) and winter mushroom (48). However, it is unknown whether the edible mushroom *Lyophyllum shimeiji* produces a lectin. It has been reported that the biological activity of some lectins may persist after passage through the gastrointestinal tract (49). This adds to the exploitable value of lectins. An antifungal protein and a ribosome-inactivating protein have been isolated from this mushroom (31). The intent of the present study was to isolate a lectin from the fruiting bodies of *L. shimeiji*. The study disclosed the presence of an agglutinin with unique features.

MATERIALS AND METHODS

Purification. Fresh fruiting bodies of *Lyophyllum shimeji* (500 g) purchased from a local market were extracted in 20 mM NH_4OAc buffer (pH 5.5). After centrifugation of the homogenate, the supernatant was saved and loaded on a CM-Sepharose (Amersham–Pharmacia Biotech) column which had previously been equilibrated with and was then eluted with 20 mM NH_4OAc buffer (pH 4.5), and finally with 20 mM NH_4HCO_3 (pH 9). All fractions were assayed for hemagglutinating activity. The most strongly adsorbed fraction, in which hemagglutinating activity resided, was subsequently dialyzed, lyophilized and chromatographed on an FPLC–Mono S column (Amersham–Pharmacia Biotech) which had previously been equilibrated with and was then eluted with 20 mM NH_4OAc (pH 5.5). Following removal of the unadsorbed fraction the column was eluted successively with a linear gradient of 0–0.2 M NaCl, 0.2 M NaCl, a linear gradient of 0.2–0.5 M NaCl, and 1 M NaCl. All fractions were then assayed for hemagglutinating activity as described below.

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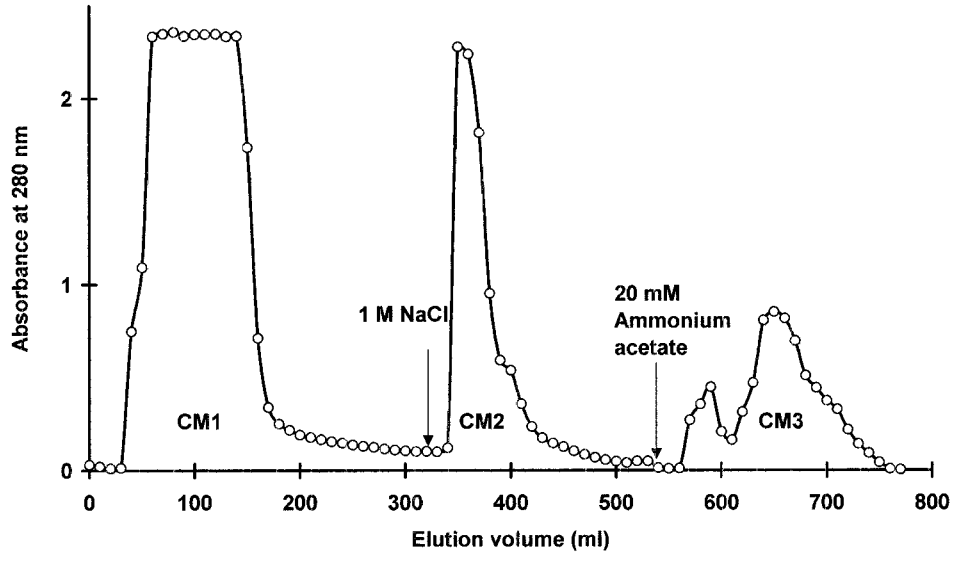

FIG. 1. Ion-exchange chromatography of an extract of *Lyophyllum shimeiji* fruiting bodies (0.5 kg) on a CM-Sepharose column (1.5 × 8 cm). Flow rate, 3 ml/min.

Sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) (SDS–PAGE). It was conducted according to the method of Laemmli and Favre (50). After electrophoresis the gel was stained with Coomassie brilliant blue. The molecular weight of the purified aggluatinin was determined by comparison of its electrophoretic mobility with those of molecular weight marker proteins from Amersham–Pharmacia Biotech.

Amino acid sequence analysis. The N-terminal amino acid sequence of the purified agglutinin was analyzed by means of automated Edman degradation using a Hewlett–Packard 1000A protein sequence equipped with an HPLC system.

Assay of antifungal activity. The assay for antifungal activity toward *Botrytis cinerea, Mycosphaerella arachidicola, Fusarium oxysporum, Rhizoctonia solani,* and *Coprinus comatus* was carried out in 100 × 15-mm petri plates containing 10 ml of potato dextrose agar. After the mycelial colony had developed, at a distance of 0.5 cm away from the rim of the mycelial colony were placed sterile blank paper disks (0.625 cm in diameter). An aliquot of a solution of the agglutinin 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 (31, 32).

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 per mg protein (15).

The hemagglutinating inhibition tests to investigate inhibition of agglutinin-induced hemagglutinating 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 agglutinin with 8 hemagglutination units. The mixture was allowed to stand for 30 min at room temperature and then mixed with 50 μl of 2% rabbit erythrocyte suspension. The minimum concentration of the sugar in the final reaction mixture which completely inhibited 8 hemagglutination units of the agglutinin was calculated (15).

RESULTS

Ion-exchange chromatography of the fruiting body extract of *Lyophyllum shimeiji* on CM-Sepharose yielded a large unadsorbed peak (CM1), a smaller, sharp peak (CM2) eluted with 1 M NaCl, and then a small peak (CM3) eluted with 20 mM NH_4HCO_3 (pH 9) (Fig. 1). Hemagglutinating activity was concentrated in CM3. CM3 was resolved by FPLC on Mono S into three fractions: a large unadsorbed fraction (MS1), followed by a sharp adsorbed peak (MS2), and finally by a small adsorbed peak (MS3) (Fig. 2). Hemagglutinating activity was concentrated in MS3, with a 9-fold increase over the activity of the crude extract. The yield of MS3 was 4.5 mg from 0.5 kg fruiting bodies (Table 1). MS3 yielded a single band with a molecular weight of 30,000 in SDS–PAGE (Fig. 3) and also a single peak with a molecular weight of 30,000 in FPLC-gel filtration on Superdex 75 (data not shown). Thus MS3 represents the purified agglutinin. The N-terminal sequence of the agglutinin is presented in Table 2. The first 20 N-terminal residues demonstrate homology to mitogen-activated protein kinase from flowering plants. From the 16th to the 29th residue of the mushroom agglutinin, similarity to mitotic centromere-associated kinesin is detected. However, all of these proteins are much larger than the agglutinin and sequence homology commences, in most cases, half way down the amino acid sequence of the protein.

The hemagglutinating activity of the purified agglutinin was not affected by glucose, D-mannose, D(+)raffinose, D(+)galactose, α-lactose, D-mannosamine, D(+)galactosamine, α-D(+)melibiose, *N*-acetylglucosamine, D(+)galactosamine, α-D(+)melibiose, *N*-acetylglucosamine, L-rhamnose, L-arabinose, *N*-acetylneuraminic acid, and

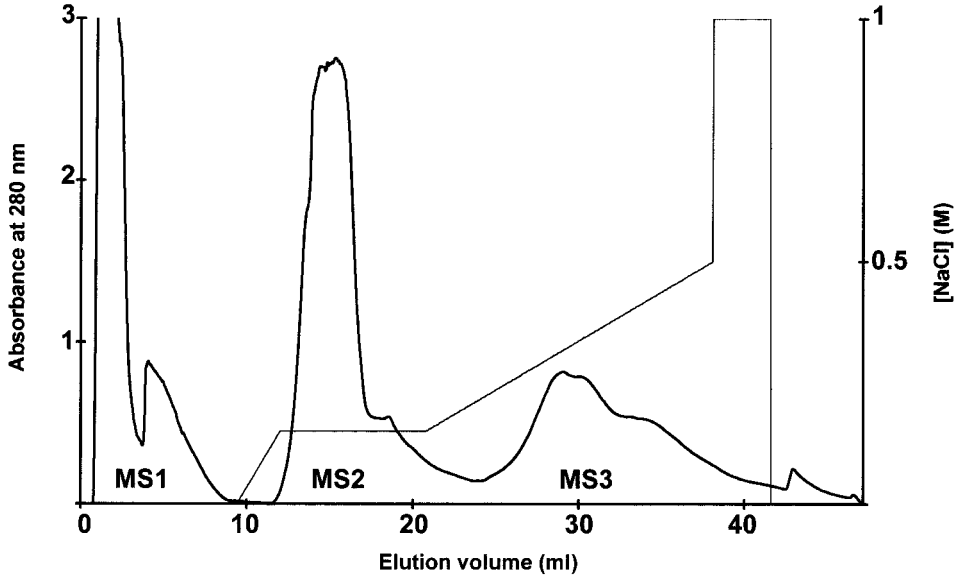

FIG. 2. Ion-exchange chromatography on a Mono S (1 × 10 cm) by fast protein liquid chromatography. Fraction CM3 derived from CM Sepharose chromatography was dialyzed, lyophilized, and applied on the Mono S column in 20 mM NH_4OAc buffer (pH 5.5). The line across the chromatogram indicates the application of different concentrations of NaCl in the elution buffer to desorb bound proteins. Flow rate, 1 ml/min.

glycoproteins including human chorionic gonadotropin or lactoferrin at 200 mM concentration.

The lectin did not exhibit antifungal activity (data not shown).

DISCUSSION

Lyophyllum shimeiji agglutinin is distinctive in that its hemagglutinating activity cannot be inhibited by simple sugars or glycoproteins. Mushroom lectins can be divided into groups with different carbohydrate-binding specificities (17). However, lectin from the straw mushroom *Volvariella volvacea* exhibits hemagglutinating activity which is inhibited by glycoproteins but not by simple sugars (44). Neither simple sugars nor glycoproteins are able to curtail the hemagglutinating activity of *Smilax glabra* agglutinin (51). *Lyophyllum shimeiji* agglutinin represents one of the few mushroom proteins known to date with hemagglutinating activity unaffected by simple sugars and glycoproteins. The others include *Agaricus edulis* lectin (52), *Flammulina velutipes* lectin (48) and straw mushroom lectin (44). The specific hemagglutinating activity of

TABLE 1

Yields and Specific Hemagglutinating Activities of Various Chromatographic Fractions Derived from Extract of *Lyophyllum shimeiji* Fruiting Bodies

Chromatographic fraction	Yield (mg)	Specific hemagglutinating activity (U/mg)	Fold of purification
Crude extract	361	974	1
CM1	272	88	—
CM2	26	0	—
CM3	31.6	1240	1.4
CM3MS1	11.5	1260	—
CM3MS2	10.2	1430	—
CM3MS3 (agglutinin)	4.5	8823	9.2

Note. Five-hundred-gram fruiting bodies were used.

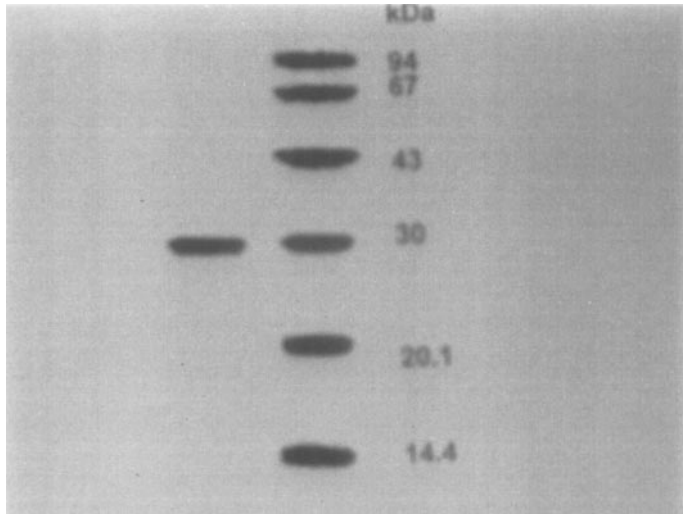

FIG. 3. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis. (Left lane) *Lyophyllum Shimeiji* agglutinin. (Right lane) Molecular weight markers from top downward phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), and lactalbumin (14.4 kDa).

TABLE 2

Comparison of N-Terminal Sequence of *Lyophyllum shimeiji* Agglutinin with Other Proteins (Results of BLAST Search)

	Residue No.		Protein length
Lyophyllum shimeiji agglutinin	1	PVVFELKFPNNNPESLLALAACAR····NKAH	
Mitogen-activated protein kinase from *Cicer arietinum*	163	PVPFEQKFPNADP····LAL	492
Mitogen-activated protein (MAP) kinase from *Medicago sativa*	275	PVPFERKFPNADP····LAL	608
MAP kinase from *Arabidopsis thaliana*	263	PIPFAQKFPNADPLSKLLERLLA	594
Homo sapiens kinesin-like 6	519	SLLALKECIRALGQNKAH	568
Mitotic centromere-associated kinesin from *Cricetulus griseus*	497	SLLALKECIRALGQNKAH	732
Homo sapiens testis-specific mitotic centromere-associated kinesin	465	SLLALKECIRALGQNKAH	671

Note. "·," space left to maximize similarity. Residue No. 263 in MAP kinase from *Arabidopsis thaliana* refers to P being the No. 263 amino acid in the enzyme.

the *L. shimeiji* agglutinin is comparable to previously isolated lectins such as *Smilax glabra* agglutinin (51) but lower than *Pleurotus ostreatus* lecitn (15). The yield of *L. shimeiji* is also lower than that of *Pleurotus ostreatus* lectin (15).

From the fruiting bodies of *Lyophyllum shimeiji* a ribosome inactivating protein and an antifungal protein have been isolated (31). It is known that some lectins may manifest antifungal activity, e.g., lectins from red kidney bean (8) and potato (7). Other lectins, e.g., that from sugar snap legumes, are devoid of antifungal activity (53). The ribosome inactivating protein and antifungal protein from *L. shimeiji* exhibit antifungal activity (31). However, *L. shimeiji* agglutinin is devoid of antifungal activity. The antifungal lectin from red kidney beans possesses an N-terminal sequence with some homology to chitinases (8). *L. shimeiji* agglutinin demonstrates no sequence similarity whatsoever to antifungal proteins.

The N-terminal sequence of *L. shimeji* agglutinin does not manifest any resemblance to any mushroom lectin nor to any lectin. Contrarily, its N-terminal sequence demonstrates some likeness to proteins unrelated to lectins. It can thus be inferred that *L. shimeiji* agglutinin is a novel agglutinin. The resemblance of its N-terminal sequence to mitogen-activated protein kinase, which plays an important role in the signal transduction pathway of hormones inducing cell proliferation such as insulin and growth factors (54), may be related to the fact that many lectins such as Con A act as growth factors (49) and manifest mitogenic activity toward splenocytes (55). The similarity of *L. shimeiji* agglutinin to mitotic centromere-associated kinesin is likely also a reflection of the mitogenic activity of lectins.

L. shimeij agglutinin is a single-chained protein with a molecular weight of 30 kDa. About three-quarters of mushroom lectins are composed of subunits (17). Only a few including lectins of *Auricularia polytricha, Boletus satanas, Coprinus cinereus, Ganoderma lucidum, Laccaria amethystine, Psathyrella laurymabunda, Psathyrella velutina,* and *Xerocomus chrysenteron* consist of only a single chain (17). A wide range of molecular weights has been reported for mushroom lectins. Some are as low as 15 kDa (56) while some have a value exceeding 190 kDa (57). *L. shimeiji* agglutinin exhibits an intermediate molecular weight. About 60% of known mushroom lectins are larger and 25% are smaller compared with *L. shimeiji* agglutinin. About 80% of the known mushroom lectins have a subunit molecular weight (circa 16,000) smaller than the molecular weight of *L. shimeiji* agglutinin (17). Only lectins of *Aleuria aurantia, Boletus satanas, Laetiporus sulfureus, Pleurotus ostreatus, Psathyrella lacrymabunda, P. velutina,* and *Rigidoporus lignosus* display a subunit molecular weight (36,000–63,000) larger than the molecular weight of *L. shimeiji* (17).

Lyophyllum shimeiji is a popular edible mushroom in the Orient, like the straw mushroom *Volvariella volvacea,* the shiitake mushroom *Lentinus edodes,* the oyster mushroom *Pleurotus ostreatus,* and the button mushroom *Agaricus bisporus.* A great deal of literature on these mushrooms except *L. shimeiji* is available. Regarding *L. shimeiji,* an acidic polysaccharide with mitogenic activity, a ribosome inactivating protein (31) and an antifungal protein (31) represent the only known constituents of its fruiting body. The present report of the isolation of a lectin from *L. shimeiji* fruiting bodies adds to the meager literature.

In sum, a simple and convenient method has been used to isolate a novel agglutinin from *L. shimeiji* fruiting bodies. Affinity chromatography on immobilized sugar is not useful because a variety of sugars tested are not capable of inhibiting the hemagglutinating activity of the agglutinin.

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