Isolation and characterization of a novel lectin with mitogenic activity from *Pleurotus ferulae*

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Abstract: Lectins are the tools for the determination of sugar chain structure. Recently, lectin arrays have become a popular new technology; therefore, lectins with specific sugar-binding properties are required. The objective of the study was to isolate a novel lectin from *Pleurotus ferulae* mushrooms and characterize its various biological activities. A novel lectin was extracted with deionized water, precipitated from the aqueous extract using 75% saturated $(NH_4)_2SO_4$, and subjected on DEAE-cellulose followed by affinity chromatography on sepharose-6B. The activity was tested using hemagglutination assays, and carbohydrate-binding specificity was determined by glycan microarray analysis. Its effects on the mitogenic activity of mouse splenocytes were determined by MTT assay. The novel lectin was adsorbed on ion-exchange chromatography DEAE-cellulose and shown as a band with the molecular mass of 17.5 kDa on a SDS-PAGE and as a single 35.0-kDa peak in gel filtration on Superdex G-75. The hemagglutinating activity of the lectin was inhibited by D-glucose, lactose, D-galactose, and galactosamine. The lectin was stable on 60°C. The hemagglutinating activity of lectin was reduced by 50% at 70°C. At 80°C, it was further reduced to 6.25% of its original activity. The hemagglutinating activity was the highest at pH 6-9. Moreover, its hemagglutinating activity was inhibited by Mg^{2+} and Ca^{2+} ions. The lectin isolated from *P. ferulae* in the current study possessed highly potent hemagglutinating and proliferative activities toward mouse splenocytes.

Keywords: Mushroom; lectin; *Pleurotus ferulae*; mitogenic activity.

INTRODUCTION

Mushrooms produce many kinds of proteins with important biological activities, including lectins (Wang and others 1998), fungal immunomodulatory proteins (Hsu and others 1997), anti-fungal proteins (Wang and Ng 2006), antibacterial peptides, (Honga 2001), polyphenol oxidase (Flurkey and Inlow 2008), and ribonucleases (Wang and Ng 1999). Among these proteins, mushroom lectins represent a class of intensely studied biomolecules, and more than 50 mushroom lectins have been isolated and purified to date. Mushroom lectins have been more widely investigated than other lectins (Wang and others 1998). Mushroom lectins have difference in molecular mass and in the number of subunits; most mushroom lectins have a molecular mass of 20 kDa or less (Wang and others 2002). Lectins are carbohydrate-binding proteins or glycoproteins that can agglutinate red blood cells or precipitate glycoconjugates (Goldstein and others 1986). Lectins have been isolated from bacteria (Zinger and others 2011), fungi (Guillot and Konska 1997), viruses (Vijayan and Chandra 1999), plants (Unitt and Hornigold 2011), and animals (Lillie and others 2005). Additionally, studies have shown that lectins possess sensitivity toward lactose (Chumkhunthod and others 2006; Han and others 2005), arabinose (Wang and Ng 2005), N-acetylneuraminic acid (Ueda and others 2003), N-glycolyneuraminic acid (Kobayashi and others 2004), N-acetylgalactosamine (Chumkhunthod and others 2006; Sumisa and others 2004), D(+)-galactosamine (Ngai and Ng 2004), D(+)-galactose (Ngai and Ng 2004), and N-acetylglucosamine (Wang and Ng 2003) and have been isolated from various mushroom species, such as *Xylaria hypoxylon* (Liu and others 2006). *Schizophyllum commune* (Chumkhunthod and others 2006), *Boletus edulis* (Zheng and others 2007). and *Pleurotus citrinopileatus* (Li and others 2008). In mushrooms, lectins possess anti-fungal (Charungchitrak and others 2011), mitogenic, antiproliferative (Faheina-Martins and others 2012), immunomodulatory (Clement and others 2010), antitumor (Fu and others 2012), and HIV-1 reverse transcriptase-inhibiting activities (Wang and others 2007). The antiviral activity of lectins appears to depend on their ability to bind mannose-containing oligosaccharides present on the surface of viral envelope glycoproteins.

P. ferulae is a cultivated or wild mushroom species found in northwest China. *P. ferulae* is also highly valued as functional food for its physiological benefits (Liu and others 2008). Research has been performed to study the antioxidant activities and tyrosinase inhibition of the fruiting bodies of *P. ferulae* extracted with acetone, methanol, and hot water (Alam and others 2012). The results suggested that consumption of *P. ferulae* may be beneficial against oxidative damage and others complications.

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The aim of the present study was to isolate, purify, and characterize the chemical and biological properties (including hemagglutinating activity, structure, and antibacterial effects) of *P. ferulae* lectin.

MATERIALS AND METHODS

Materials

Fruit bodies of *P. ferulae* mushrooms were collected from their natural environment in Qinghe County, Xingjiang, China for extraction of lectin. All the other chemicals and reagents were of analytical grade.

Isolation and purification of lectin

Fresh fruiting bodies (100g) were homogenized with distilled water (1000mL) in a blender and extracted overnight. The resulting suspension was filtered with filter paper under reduced pressure, and the supernatant was then centrifuged at 10,000 × *g* for 30 min. Proteins were precipitated with 75% $(NH_4)\ _2SO_4$. The precipitate was dialyzed against distilled water and lyophilized. The crude extract material (50 mg) was redissolved in 10 mM Tris-glycine buffer (pH 7.5) and applied to a DEAE-cellulose column (Bio-Rad, BioLogic LP system, low-pressure chromatography system 1.5cm × 30cm, USA), which had previously been equilibrated with 10 mM Tris-glycine buffer (pH 7.5). Materials were eluted in the same buffer, and adsorbed materials on the column were eluted successively with 0.1 M NaCl and then with 0.5 M NaCl to show fractions D1 and D2. D1 contained hemagglutinating activity.

Fraction D1 was subjected to affinity chromatography on a sepharose-6B column (Bio-Rad, BioLogic LP system, low-pressure chromatography system 1.0 × 20 cm, USA) in 10 mM phosphate buffer (pH 7.0). After removal of the unadsorbed fraction SP1, adsorbed proteins were eluted with 10 mM phosphate buffer (pH 7.0) and a gradient of 0–0.3 M galactose in10 mM phosphate buffer (pH 7.0). Fraction SP2 with hemagglutinating activity was tested to gel filtration by fast protein liquid chromatography on a Superdex 75 HR 10/30 column of AKTA Purifier with frac-900 (GE Healthcare, USA) in 50 mM Tris-HCl buffer (pH 7.5), containing 0.3 M NaCl, with 0.2 M lactose to prevent interactions between the lectin and column matrix (Pohleven, 2009). The peak fraction (S1) was the purified lectin (*P. ferulae* lectin, PFL).

Determination of molecular mass

The purified lectin was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for molecular mass determination (Laemmli and, Favre, 1970). Gel filtration on an FPLC-Superdex 75 column was conducted to determine the molecular mass of the lectin based on molecular markers.

Measurement of lectin hemagglutinating activity

A serial 2-fold dilution of the lectin solution in microtiter U-plates (50mL) was mixed with 50mL of rabbit red blood cells, obtained from the Department of Experimental Animal, College of Animal Science, Shihezi University (Xinjiang, China), prepared as a 2% suspension in phosphate-buffer (pH 7.2) at 20°C. The results were tested after about 1 h, when the blank had fully sedimented (Wang and others 2002).

Effects of carbohydrates on hemagglutination

Hemagglutinating inhibition tests were performed to investigate the inhibition of lectin-induced hemagglutination by various carbohydrates using methods analogous to those of the hemagglutination test (Li and others 2008).

Effects of temperature, metal ions, and pH on hemagglutinating activity

The effects of temperature on the hemagglutinating activity of 64 hemagglutination units of lectin was determined by incubating the lectin for 30 min at 10, 20, 30, 40, 50, 60, 70, 80, 90, or 100°C, followed by measurement of hemagglutinating activity (table 1). The effects of metallic chlorides on the hemagglutinating activity of 64 hemagglutinating units of the purified lectin were determined by addition of 40 mM Ca^{2+}, Mn^{2+}, Mg^{2+}, or Zn^{2+} ions for 30 min. The effects of pH were evaluated by examining the hemagglutinating activity of 8 hemagglutination units of the purified lectin in the pH range of 4 to 10 using the following buffers: 0.02 M acetate buffer (pH 4-5), 0.02 M phosphate buffer (pH 6-7), 0.02 M Tris-HCl buffer (pH 8-9), and 0.02 M glycine/NaOH buffer (pH 9-10). Lectin (0.3mL, 0.5 mg/mL) was incubated with each buffer solution for 2 h, and the hemagglutinating activity was evaluated (Molchanova and others 2007).

Assay for mitogenic activity in mouse splenocytes

All animal procedures were approved by the Animal Care and Use Committee of Shihezi University (Xinjiang, China). Four BALB/C mice (female, 6 weeks old, 20-25 g) were sacrificed by cervical dislocation, and their spleens were aseptically removed. Splenocytes were isolated by pressing the tissue through a sterilized 100-mesh stainless steel sieve. After the red blood cells were removed by hemolytic Gey's solution, splenocytes were resuspended to a density of 4×10^6 cells/mL in RPMI 1640 culture medium supplemented with 10% fetal bovine serum, 100units/mL penicillin, and 100μg/mL streptomycin. The splenocytes were seeded into a 96-well culture plate, and various concentrations (2.5- 40μg/mL) of PFL in 20μL medium were added. Controls included wells with no cells and well where water was added instead of PFL. Splenocytes were then incubated at 37°C in a humidified atmosphere of 5% CO_2 for 24 h, and 10μL (5 mg/mL) 3-(4,5-dimethylthiazole-2-yi)-2,5-dipheny-

ltetrazolium bromide (MTT) in RPMI 1640 culture was then added to each well. The plates were incubated for another 4 h under the same conditions. After incubation, the plates were centrifuged at 1500*g* for 10 min. The culture medium was discarded. The plates were carefully washed with RPMI 1640 culture r 3 times. Positive controls were incubated with 20µL ConA (5µg/mL) and oscillated for 30 min (Lin and Tang 2007). The absorbance was measured at 570 nm on a plate reader (ELISA reader, ASYS Hitech GmbH, Austria). The stimulation index (SI) was calculated based on the following formula: SI= the absorbance value for mitogen-stimulated cultures divided by the absorbance value for nonstimulated cultures.

STATISTICAL ANALYSIS

All experiments were repeated at least 2 times. The standard deviation was calculated and the statistical significance of differences was defined using Student's t-test and Mann-Whitney rank sum test (p<0.05).

RESULTS

Purification of PFL

PFL was purified to homogeneity by a combination of ion-exchange chromatography on a DEAE-cellulose column (fig. 1) and affinity chromatography on a sepharose 6B column (fig. 2). The purified lectin showed a single peak on a Superdex 75 HR 10/30 column using an FPLC system.

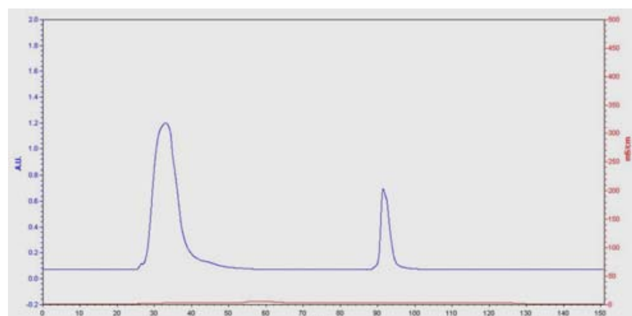

Fig. 1: Anionic chromatography of extracts of *P. ferulae* fruiting bodies on a DEAE-cellulose column (1.5 cm × 30 cm) in 10 mM Tris-glycine buffer (pH 7.5) at a flow rate of 1mL/min. Unbound materials were eluted in Tris-glycine buffer, and adsorbed materials on the column were eluted successively with 0.1 M NaCl and then with 0.5 M NaCl to yield fractions D1 and D2, respectively.

PFL molecular mass

Upon gel filtration on a Superdex 75 column, SP2 was resolved into a large peak (S1) with a molecular mass of 35.0 kDa (fig. 3). SDS-PAGE under reducing conditions (β-mercaptoethanol) revealed a single band of about 17.5 kDa (fig. 4). This indicated the presence of a disulfide bond and 2 subunits of identical molecular mass.

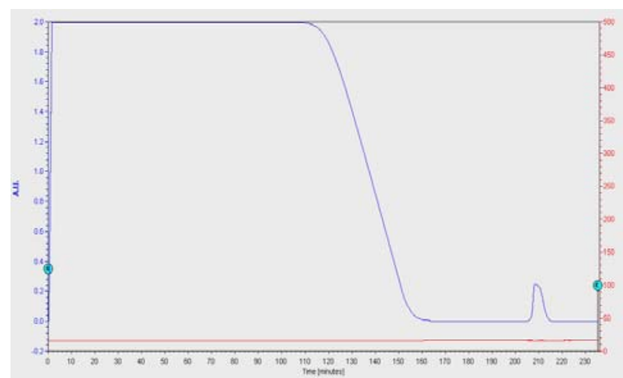

Fig. 2: Fraction D1 was subjected to affinity chromatography on a sepharose 6B column (1.0 × 20 cm) in 10 mM phosphate buffer (pH 7.0). After removal of the unadsorbed fraction SP1, adsorbed proteins (SP2) were eluted with 10 mM phosphate buffer (pH 7.0) and a gradient of 0-0.3M galactose in10mM phosphate buffer (pH 7.0).

Hemagglutinating activity and carbohydrate-binding activity

The carbohydrate binding specificity of PFL was studied by sugar-hapten inhibition of hemagglutination (Li and others 2008). The minimum concentrations of sugars inhibited hemagglutination are shown in table 1. The results showed among the monosaccharides tested D-Galactose was the most effective inhibitor.

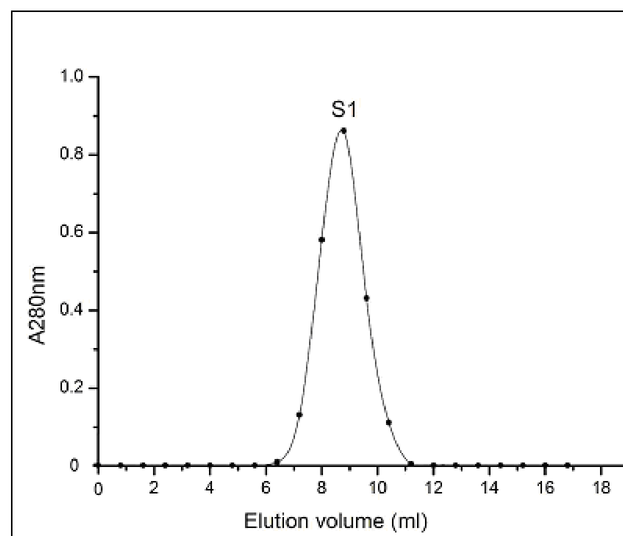

Fig. 3: Affinity chromatography of fraction SP2 on a Superdex 75 HR 10/30 column using FPLC. Bound material was eluted with 50 mM Tris/HCl buffer, pH 7.5, containing 0.3 M NaCl, with 0.2 M lactose. The flow rate was 0.8 mL/min. Fraction S1 represented purified lectin. The peak fraction (S1) was the purified lectin (*P. ferulae* lectin; PFL).

pH, metal, and temperature dependencies

Studies of the effects of pH on PFL revealed that the hemagglutination activity of the lectin was stable at pH 6–

9 (table 2). Moreover, PFL was temperature sensitive, and it lost 50% of its original activity after incubation at 70°C for 30 min. Full irreversible loss of activity was observed following incubation for 2 h at 60°C (table 3). The hemagglutinating activity of PFL was found to be independent of Ca^{2+} and Mg^{2+} (table 4).

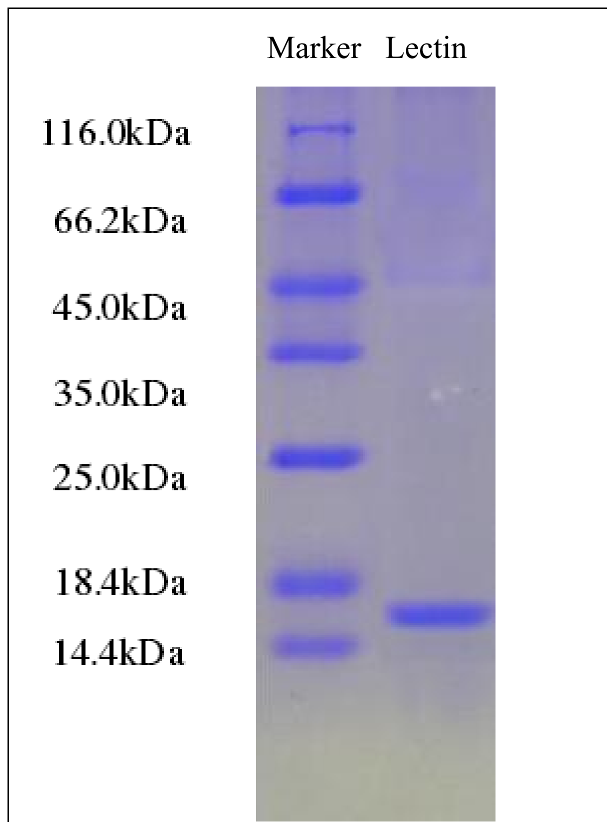

Fig. 4: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the purified lectin (12% separating gel; 5% stacking gel). 1: PFL with 2-mercaptoethanol (running time 3 h at a constant 80 V).

Mitogenic activity of PFL in mouse splenocytes

To assay the general effects of PFL on immune cells, we examined splenocyte proliferation in the presence of different concentrations of PFL. As shown in fig. 5, treatment with 5, 10, 20, or 40μg/mL PFL caused a significant increase in proliferation compared with the control.

DISCUSSION

Recently, another group (Gauto and others 2011) described the isolation and characterization of lectin from another mushroom; this peaked our interest in identifying the lectin protein from *P. ferulae*. In this study, we identified and characterized a D-galactose-dependent lectin isolated from *P. ferulae* extracts. Two chromatographic steps, including an ion exchange step and an affinity chromatography step, were applied in the purification protocol. Our results were similar to those of some lectins isolated from *Amanita virosa*, adsorbed on CM-cellulose and eluted on DEAE-Toyopearl (Antonyuk and others 2010). In our study, PFL isolated by affinity chromatography exhibited an apparent molecular mass of 35 kDa as determined by FPLC on a Superdex 75 system and a subunit molecular mass of 17.5 kDa on reducing and nonreducing SDS-PAGE.

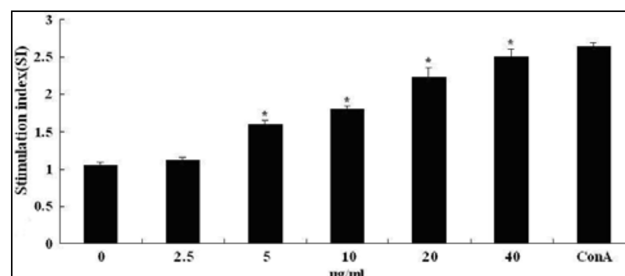

Fig. 5: Splenocyte proliferation in response to PFL treatment. Murine splenocytes (7×10^5 cells/mL) were stimulated with PFL at the indicated concentrations or with 2.5μg/mL Con A (positive control) for 72 h at 37°C in a humidified atmosphere containing 5% CO_2. The values are presented as means ± SDs (n=6). *P<0.01, significantly different compared to the 0 μg/mL control.

Lectins are unique in that a variety of sugars, including D-galactose, D-glucose and lactitol are able to inhibit the hemagglutinating activity of lectins (Wang and others 1998). Thus, sugars can be used in affinity chromatography media to evaluate interactions with specific lectins. In this study, we used D-galactose (sepharose-6B, composed of mannose and galactose) in the affinity chromatography media to interact with PFL. In comparison, some mushroom lectins, such as *Tricholoma mongolicum* lectin (Chang and others 1995), *Psilocybe barrerae* lectin (Hernandez and others 1993), and *Schizophyllum commune* lectin (Han and others 2005) are inhibited by only one sugar. PFL was not distinct from previously described lectins in *P. ferulae* mushrooms, adding to the growing list of recognized lectins from this mushroom. The D-galactose requirement suggested that this lectin may be similar to the D-galactose-dependent lectin described by Mizukami (2008).

The isolated lectin was moderately thermostable up to a temperature of 60°C. At 70°C, 50% of the hemagglutinating activity remained. The activity was further reduced to 6.25% of the original activity at 80°C and was completely destroyed at 90°C. Based on these properties, our PFL was more thermostable than *S. commune* lectin (Chumkhunthod and others 2006), *Mycoleptodonoides aitchisonii* lectin (Kawagishi and others 2001), *Xylaria hypoxylon* lectin (Liu and others 2006), which were reported to be stable up to 55, 45 and 35°C, respectively. PFL was stable in solutions with pH values ranging from 6.0 to 9.0 and hemagglutinating activity was detectable in a solution at pH 3.0. In this regard, PFL was less stable than some other mushroom lectins.

Table 1: Hemagglutination inhibition assay of PFL by various sugars (initial hemagglutinating activity, 4 hemagglutinating units)

Test sugars	Concentration of sugar (mM)								
	100	50	25	12.5	6.25	3.12	1.56	0.78	0.39
D-Glucose	+	+	-	-	-	-	-	-	-
Lactose	+	+	+	-	-	-	-	-	-
Rhamnose	-	-	-	-	-	-	-	-	-
Arabinose	-	-	-	-	-	-	-	-	-
Raffinose	-	-	-	-	-	-	-	-	-
Fucose	-	-	-	-	-	-	-	-	-
Xylose	-	-	-	-	-	-	-	-	-
Mannose	-	-	-	-	-	-	-	-	-
D-Galactose	+	+	+	+	+	+	-	-	-
Me-α-Ga	-	-	-	-	-	-	-	-	-
p-Nitrophenyl-N-acetyl-α-D-galactosaminide	-	-	-	-	-	-	-	-	-
p-Nitrophenyl-β-D-galactopyranoside	-	-	-	-	-	-	-	-	-
Galactosamine	+	-	-	-	-	-	-	-	-
Galβ1→4Glcitol (Lactitol)	-	-	-	-	-	-	-	-	-
Fucα1→2Galβ1→4Glc (2′-Fucosyllactose)	-	-	-	-	-	-	-	-	-

*Initial hemagglutinating activity was 4 hemagglutinating units. +: hemagglutination activity; –: no hemagglutination activity. The experiment was repeated twice, and the results were reproducible.

Table 2: Hemagglutinating activity of a solution of *P. ferulae* lectin in distilled water after exposure to solutions with various pH values for 30 min (initial hemagglutinating activity: 8 hemagglutinating units).

pH	1	2	3	4	5	6	7	8	9	10	11
Hemagglutinating activity (U)	0	0	4	4	32	64	64	64	64	0	0

Table 3: Hemagglutinating activity of a solution of *P. ferulae* lectin in phosphate-buffered saline (pH 7.2) after exposure to various temperatures for 30 min (initial hemagglutinating activity: 64 hemagglutinating units).

Temperature (°C)	10	20	30	40	50	60	70	80	90	100
Hemagglutinating activity (U)	64	64	64	64	64	64	32	4	0	0

*Initial hemagglutinating activity was 64 hemagglutinating units, as indicated by 6 wells in the plate exhibiting hemagglutination. The experiment was repeated 3 times, and the results were reproducible.

The hemagglutinating activity of PFL was affected by Mg^{2+} and Ca^{2+} ions. The hemagglutinating activity of other lectins, such as *A. pantherina* lectin, is not altered by the presence of divalent metal ions (Wang and others 1998). The activity of the lectin ConA is affected by Mn^{2+}, Mg^{2+} and Ca^{2+} ions. Moreover, the hemagglutinating activities of *Russula lepid*a lectin (Zhang and others 2010) and *Inocybe umbrinella* lectin (Zhao and others 2009) are also depressed by Mn^{2+} ions. In contrast, the hemagglutinating activities of some lectins are not inhibited by Fe^{3+} ions, including *R. lepida* lectin (Zhang and others 2010). Some lectins, such as *Penicillium chrysogenum* lectin (Francis and others 2011), *Rhizoctonia bataticola* lectin (Pujari and others 2010), and *Grifola frondosa* lectin (Lee and others 2011), show immunomodulatory effects.

PFL was capable of mitogenic response to mouse splenocytes. Although the magnitude of the response to PFL was not as high as that of Con A, the lectin concentration of the maximal response was 40μg/mL. However, 16 times as much Con A was required to obtain the maximal response. The effects of PFL were similar to those of a ricin B-like lectin from the mushroom *Clitocybe nebularis* (Pohleven and others 2009). Similar effects have also been described for other mushroom lectins (Yu and others 1993; Zhao and others 2003).

Table 4: Effects of cations on hemagglutinating activity of a solution of lectin in deionized water.

Cations	50 mM	25 mM	12.5 mM	6.25 mM
Ca^{2+}	32	32	64	64
Zn^{2+}	64	64	64	64
Mg^{2+}	32	32	32	64
Mn^{2+}	64	64	64	64
Cu^{2+}	64	64	64	64
Co^{2+}	64	64	64	64
Fe^{2+}	64	64	64	64
Al^{3+}	64	64	64	64
Fe^{3+}	64	64	64	64

*The lectin solution had been dialyzed extensively against deionized water containing 1mM EDTA (initial hemagglutinating activity: 64 hemagglutinating units). The experiment was repeated twice, and the results were reproducible.

In summary, *P. ferulae* lectin (PFL) is a novel lectin with potent and potentially exploitable activities. Our results have provided a theoretical foundation for the further exploration of lectins isolated from mushrooms, which may have immunomodulatory properties, such as that described in the mushroom *Clitocybe nebularis* (Pohleven and others 2009).

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