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A novel lectin with antiproliferative activity from the medicinal mushroom *Pholiota adiposa*

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Little was known about biological activities of compounds from the medicinal mushroom of the genus *Pholiota*. A lectin from the *Pholiota adiposa* has now been isolated and its properties tested. The isolation procedure included ion exchange chromatography on DEAE-cellulose and CM-cellulose, and fast protein liquid chromatography-gel filtration (FPLC) on Superdex 75. The lectin was composed of two identical subunits, each with a molecular mass of 16 kDa. Its N-terminal amino-acid sequence showed little similarity to sequences of other Agaricales lectins. The hemagglutinating activity of the lectin was stable at temperatures up to 50ºC, and in NaOH and HCl solutions with concentrations less than 25 mM. It was inhibited by inulin (12.5–200 mM), but enhanced by Cu^{2+} (6.25–25 mM), Fe^{2+} (12.5–25 mM), and Al^{3+} (6.25–25 mM) ions. The lectin showed antiproliferative activity toward hepatoma Hep G2 cells and breast cancer MCF7 cells with an IC_{50} of 2.1 μM and approximately 3.2 μM, respectively. It exhibited HIV-1 reverse transcriptase inhibitory activity with an IC_{50} of 1.9 μM. When compared with *P. aurivella* lectin, the only *Pholiota* lectin published to date, *P. adiposa* lectin differs in chromatographic behavior, molecular mass, N-terminal sequence, and effect of cations on hemagglutinating activity. In the case of the lectin from *P. aurivella*, its antifungal, antiproliferative, and HIV-1 reverse transcriptase inhibitory activities have not been determined.

Keywords: lectin, *Pholiota adiposa,* purification, mushroom, antiproliferative

INTRODUCTION

Lectins are proteins or glycoproteins of non-immune origin which have a wide distribution in animals, plants, and microorganisms. They can agglutinate cells through sugar-specific binding sites for polysaccharides and glycoconjugates (Guillot & Konska, 1997; Wang *et al.*, 1998; Ho *et al.*, 2004). In mushrooms, lectins are present in the caps, stipes, spores, and mycelia. The content of lectin varies depending on the age of the carpophore and the time and place of harvest. Mushroom lectins play an important role in dormancy, growth, morphogenesis, morphological changes consequent on parasitic infections and molecular recognition during the early stages of mycorrhization (Guillot & Konska, 1997; Ng, 2004). It has been reported that mushroom lectins manifest various exploitable actoins including antiproliferative (Liu *et al.*, 2006), antitumor (Wang *et al.*, 2000; Han *et al.*, 2005), immunomodulatory (Wang *et al.*, 1996), and mitogenic (Wang *et al.*, 2002; Ngai & Ng, 2004) activities. Lectins have been isolated from several common edible mushrooms, including *Agaricus bisporus*, *Flammulina velutipes*, *Ganoderma lucidum*, *Grifola frondosa*, *Hericium erinaceum*, *Pleurotus ostreatus*, and *Volvariella volvacea* (Pemberton, 1984; Ng, 2004).

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Abbreviations: DEAE, diethylaminoethyl; CM, carboxymethyl; HPLC, high performance liquid chromatography; ELISA, enzyme-linked immunosorbent assay; RT, reverse transcriptase; PAL, Pholiota adiposa lectin; SDS, sodium dodecyl sulfate.

Pholiota adiposa is an edible as well as a medicinal mushroom cultured in China and Japan. It belongs to Agaricales (Strophariaceae), and has antitumor, antimicrobial, antihypertensive, and antihyperlipidemic activities. An extract of *P. adiposa* inhibited the growth of sarcoma 180 and Ehrlich solid cancers in mice (Yu *et al.*, 2007). Further research suggested that the antitumor activity of *P. adiposa* polysaccharide was due to immunostimulation (Zhao *et al.*, 2007). A novel pentapeptide (Gly-Glu-Gly-Gly-Pro) with angiotensin converting enzyme inhibitory activity was isolated from the fruiting body of *P. adiposa* (Izawa & Aoyagi, 2006; Koo *et al.*, 2006). Stigmasterol purified from methanol extracts of *P. adiposa* fruiting bodies inhibited β-hydroxy-β-methyl-glutaryl coenzyme A reductase, a rate-limiting enzyme in cholesterol biosynthesis, with an IC_{50} of 6.8 μg/ml (Yu *et al.*, 2007). A *P. adiposa* extract did not produce any significant change in total triglyceride contents or epididymal fat mass, but caused a decrease in retroperitoneal fat in mice on a high-fat diet (Cho *et al.*, 2006). The present investigation aimed to isolate and characterize a lectin from the fruiting bodies of *P. adiposa* and to find out if it has some distinctive characteristics. To date only one lectin has been isolated from a representative of the *Pholiota* genus, *Pholiota aurivella* (Kawagishi *et al.*, 1991).

MATERIALS AND METHODS

Isolation of lectin. Dried fruiting bodies of the mushroom *P. adiposa* (100 g) cultured in the Department of Microbiology of China Agricultural University, were homogenized in 0.15 M NaCl (10 ml/g) at 4°C and extracted overnight at 4°C. Then the homogenate was centrifuged at 8000×*g* for 15 min. To the supernatant $(NH_4)_2SO_4$ was added to 80% saturation. The mixture was left at 4°C for 4 h before centrifugation at 8000×*g* for 15 min. The precipitate was redissolved and dialyzed to remove $(NH_4)_2SO_4$ before applying to a DEAE-cellulose (Sigma) column (2.5×20 cm) in 10 mM NH_4HCO_3 buffer (pH 9.4). After removal of the unadsorbed peak (D1), three adsorbed peaks, D2, D3 and D4, were eluted stepwise with 50 mM NaCl, 150 mM NaCl and 1 M NaCl in the buffer, respectively. Fraction D3 was then passed through a CM-cellulose (Sigma) column (1.5×10 cm) which had been equilibrated and then eluted with 10 mM NH_4OAc (pH 4.6). Unbound material ((fractions C1) was eluted with the starting buffer, while bound proteins (fractions C2, C3) were desorbed sequentially with 50 mM NaCl and 1 M NaCl in the starting buffer. The active peak (C2) was subsequently purified by fast protein liquid chromatography (FPLC) on a gel filtration Superdex 75 HR 10/30 column (GE Healthcare) in 0.15 M NH_4HCO_3 buffer (pH 8.5). The second peak (SU2) obtained constituted a purified lectin.

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 (1973). Gel filtration on an FPLC-Superdex 75 column, which had been calibrated with molecular mass markers (GE Healthcare), was conducted to determine the native 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 (Wang *et al.*, 2002).

Assay for lectin (hemagglutinating) activity. In the assay, a serial twofold dilution of the lectin solution in microtiter U-plates (25 μl) was mixed with 25 μl of a 2% suspension of rabbit red blood cells in phosphate-buffered saline (pH 7.2) at 20°C. The results were recorded after about 1 h when erythrocytes in 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 (Wang *et al.*, 2000).

Hemagglutinating inhibition tests to investigate the inhibition of lectin-induced hemagglutination by various carbohydrates were performed in a manner analogous to the hemagglutination assay. The carbohydrates tested included inositol, L-sorbose, raffinose, L-rhamnose, D-fructose, D-mannose, cellobiose, L-arabinose, D-xylose, D-melibiose, lactose, inulin, maltose, D-galactose, D-glucose and *O*-nitrophenyl-β-D-galactopyranoside. Serial twofold dilutions of sugar samples were prepared in phosphate-buffered saline (pH 7.2). All of the dilutions were mixed with an equal volume (25 μl) of a solution of the lectin with 16 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 16 hemagglutination units of the lectin preparation was calculated (Han *et al.*, 2005).

The effects of temperature, NaOH, HCl, and metallic chlorides on the hemagglutinating activity of the lectin were determined as previously described (Wang *et al.*, 1996).

Assay of antiproliferative activity on tumor cell lines. The tumor cell lines, human breast cancer (MCF 7) and hepatoma (Hep G2), were purchased from American Type Culture Collection (ATCC). They were maintained in Dulbecco modified Eagle's medium (DMEM) supplemented with

Table 1. Summary of purification of *P. adiposa* lectin.

Activity refers to hemagglutinating activity.

Fraction	Yield (mg/100 g)	Specific activity (units/mg)	Total activity (units)	Recovery of activity (%)	Fold of purification
Crude extract	6301	283	1.8×10^6	100	1
D3	556	2412	1.3×10^6	72.2	8.5
C2	143	7690	1.1×10^6	61.1	27.2
SU2	70	10988	7.7×10^5	42.8	38.8

10% fetal bovine serum (FBS), 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 GE Healthcare) 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 (Li *et al.*, 2009).

Assay for HIV-1 reverse transcriptase inhibitory activity. The inhibitory activity towards human immunodeficiency virus type 1 (HIV)-1 reverse transcriptase (RT) was assessed by using an enzyme-linked immunosorbent assay (ELISA) kit from Boehringer Mannheim (Germany). The assay takes advantage of the ability of reverse transcriptase to synthesize DNA, starting from the template/primer hybrid poly(A)-oligo(dT)$_{15}$. The digoxigenin- and biotin-labeled nucleotides in an optimized ratio are incorporated into the DNA molecule synthesized by the RT. The detection and quantification of the synthesized DNA as a measure of RT activity follows sandwich ELISA protocol. A fixed amount (4–6 ng) of recombinant HIV-1 RT was used. The inhibitory activity of the lectin was calculated as percent inhibition as compared to a control without the protein (Wang & Ng, 2004).

Assays for antifungal and ribonuclease activities. The assays were conducted as described in (Han *et al.*, 2005; Wang & Ng, 2006), respectively.

RESULTS

Isolation of lectin

One of the fractions adsorbed on DEAE-cellulose, fraction D3, showed hemagglutinating activity (Table 1). Ion exchange chromatography on CM-cellulose resulted in an unadsorbed fraction C1 and two adsorbed fractions C2 and C3 (Fig. 1). The lectin activity resided in fraction C2 (Table 1). Subsequently, fraction C2 was resolved into a smaller peak SU1 and a larger peak SU2 upon gel filtration on a Superdex 75 HR 10/30 column by FPLC on an AKTA Purifier (GE Healthcare; Fig. 2). The hemagglutinating activity was

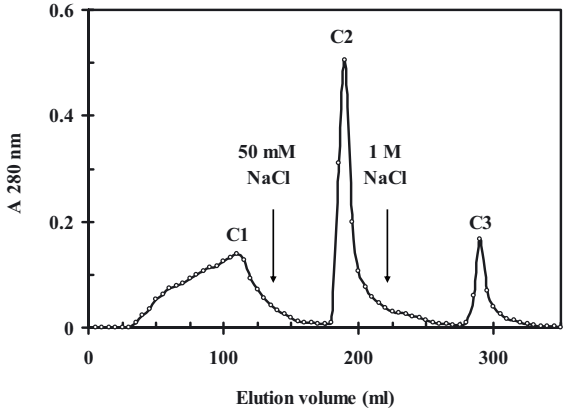

Figure 1. Ion exchange chromatography of fraction D3 (adsorbed on DEAE-cellulose) on CM-cellulose.
A CM-cellulose column (1.5×10 cm) was equilibrated and eluted with 10 mM NH_4OAC buffer (pH 4.6) and then stepwise with 50 mM and 1 M NaCl in the same buffer. Arrows indicate the points at which buffer was changed.

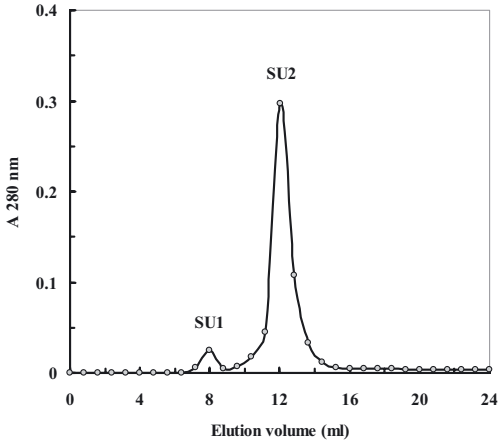

Figure 2. Gel filtration of fraction C2 from CM-cellulose column on Superdex 75.
A Superdex 75 (HR 10/30) column was eluted with 0.15 M NH_4HCO_3 buffer (pH 8.5). The flow rate was 0.4 ml/min. Fraction SU2 represents purified lectin.

Table 2. Comparison of N-terminal sequence of *P. adiposa* lectin with sequences of known Agaricales lectins.

Species	N-terminal sequence	Reference
Pholiota adiposa	DILMGTYGML	This study
Pholiota aurivella	YSVTTPNSVKGGTNQPGA	Kawagishi *et al.* (1991)
Agaricus bisporus	MGGSGTSGSL	Crenshaw *et al.* (1995)
Agrocybe aegerita	NISAGTSVDL	Zhao *et al.* (2003)
Coprinopsis cinerea	IPLEGTFGDR	Walti *et al.* (2008)
Flammulina velutipes	TSLTFQLAYL	Ko *et al.* (1995)
Laccaria bicolor	SHLYGDGVAL	Martin *et al.* (2008)
Marasmius oreades	YILDGEYLVL	Kruger *et al.* (2002)
Pleurotus cornucopiae	SDSTWTFAML	Oguri *et al.* (1996)

Table 3. Effect of temperature on hemagglutinating activity of *P. adiposa* lectin.

Initial hemagglutinating activity was 16 U.

Temperature (°C)	20	30	40	50	60	70	80	90	100
Hemagglutinating activity remained [%]	100	100	100	100	25	0	0	0	0

enriched in SU2. About 40-fold purification was achieved (Table 1).

Molecular mass determination and N-terminal sequence analysis

The purified lectin appeared as a single band with a molecular mass of 16 kDa in SDS/PAGE (Fig. 3) and a single peak with a molecular mass of 32 kDa in FPLC gel filtration (Fig. 2). Thus it appears that the purified lectin is composed of two identical subunits, each with a molecular mass of 16 kDa. The N-terminal sequence of the lectin was DILMGTYGML, different from the other mushroom lectins shown in Table 2.

Sugar specificity, thermostability, pH stability, and effect of cations on hemagglutinating activity

Among the variety of sugars tested, inulin at a concentration of 25 mM was able to inhibit 16 hemagglutinating units of the lectin. The hemagglutinating activity of the lectin was stable between 20 and 50°C. The activity decreased by 75% at 60°C. At and above 70°C the hemagglutinating activity was undetectable (Table 3). The lectin was stable in 6 mM (pH 2.2), 12.5 mM (pH 1.9), and 25 mM (pH 1.6) HCl. The activity was reduced by 75% in 50 mM HCl (pH 1.3), and disappeared altogether at 100 mM HCl (pH 1.0) (Table 4). Full hemagglutinating activity was observed in 6 mM (pH 11.8), 12.5 mM (pH 12.1), 25 mM (pH 12.4), and 50 mM NaOH (pH 12.7). Only 25% of the activity remained in 100 mM NaOH (pH 13.0). The activity vanished in 200 mM NaOH (pH 13.3) (Table 4). The hemagglutinating activity was unaffected in the presence of Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Hg^{2+}, and Fe^{3+} (6.25–50 mM), but was increased by Cu^{2+}, Al^{3+} (12.5–50 mM), and Fe^{2+} (25–50 mM) (Table 5).

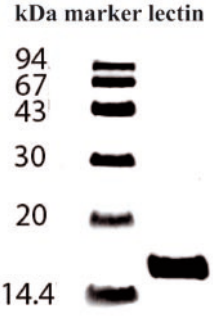

Figure 3. SDS/PAGE of purified *P. adiposa* lectin.
Fraction SU2 from gel filtration was run on SDS/polyacrylamide gel. Molecular mass markers (left lane) are: phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa) and α-lactalbumin (14.4 kDa), all from GE Healthcare.

Other biological activities

The lectin inhibited the proliferation of Hep G2 and MCF tumor cells with an IC_{50} value of 2.1 μM and about 3.2 μM, respectively (Fig. 4). HIV-1 reverse transcriptase was inhibited with an IC_{50} of 1.9 μM (Fig. 5). The lectin lacked antifungal and ribonuclease activities (not shown).

DISCUSSION

Pholiota adiposa lectin (PAL) isolated in the present investigation differs from lectins purified from other Agaricales mushrooms. PAL was purified using three kinds of columns: DEAE-cellulose, CM-cellulose and Superdex 75. PAL is adsorbed on DEAE-cellulose and CM-cellulose columns and eluted with 150 mM NaCl and 50 mM NaCl, respectively.

Table 4. Effects of NaOH and HCl on hemagglutinating activity of *P. adiposa* lectin.

Initial hemagglutinating activity was 16 U.

pH Value	Hemagglutinating activity remaining (%)	pH Value	Hemagglutinating activity remained (%)
0.7	0	9.0–11.5	100
1.0	0	11.8	100
1.3	25	12.1	100
1.6	100	12.4	100
1.9	100	12.7	100
2.1	100	13.0	25
2.1–4.5	100	13.3	0

Table 5. Effects of cations on hemagglutinating activity of *P. adiposa* lectin.

Initial hemagglutinating activity was 16 U.

	Hemagglutinating activity (%)			
Cation	50 mM	25 mM	12.5 mM	6.25 mM
Cu^{2+}	200	200	200	100
Fe^{2+}	200	200	100	100
Al^{3+}	200	200	200	100

It is strongly adsorbed on SP-Sepharose column and no activity can be eluted with 3 M NaCl. Compared with PAL, a lectin named PAA isolated from *Pholiota aurivella*, which belongs to the same genus as *P. adiposa*, is adsorbed during affinity chromatography on a fetuin, asialofetuin, BSM, or asialo-BSM column, and during ion exchange chromatography on DEAE-Toyopearl and CM-Toyopearl. No lectin activity is found when a variety of eluents is used. Only 0.1% SDS can elute the lectin from the adsorbents or matrices (Kawagishi *et al.*, 1991).

PAL possesses an N-terminal sequence that bears little resemblance to some of the published Agaricales mushroom lectins, such as those from *Agaricus bisporus*, *Agrocybe aegerita*, *Coprinopsis cinerea*, *Flammulina velutipes*, *Laccaria bicolor*, *Marasmius oreades*, *Pholiota aurivella*, and *Pleurotus cornucopiae* (shown in Table 2) (Kawagishi *et al.*, 1991; Crenshaw *et al.*, 1995; Ko *et al.*, 1995; Oguri *et al.*, 1996; Kruger *et al.*, 2002; Zhao *et al.*, 2003; Nagata *et al.*, 2005; Martin *et al.*, 2008; Walti *et al.*, 2008). Similarly to the *Agaricus edulis* II, *Ischnoderma resinosum*, *Pleurotus citrinopileatus*, and *P. comucopiae* PCL-a lectins (Guillot *et al.*, 1997; Li *et al.*, 2008), PAL is composed of two identical subunits with a molecular mass of 16 kDa.

Carbohydrate specificity is an important characteristic of lectins. It is interesting in that only inulin, a plant polysaccharide, is able to inhibit the hemagglutinating activity of PAL. To date only a few inulin-specific lectins have been reported (Liu *et al.*, 2004; Feng *et al.*, 2006). Hence PAL may be used in the production of immobilized lectin for affinity chromatography.

In the present study, Cu^{2+}, Fe^{2+}, and Al^{3+} ions increased the hemagglutinating activity of PAL when present at 12.5 mM, 25 mM, and 12.5 mM, respectively, while Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Hg^{2+}, and Fe^{3+} ions were devoid of any effect. Different mushroom lectins may be affected differently by the ions. Al^{3+} ions strongly increase the hemagglutinating ac-

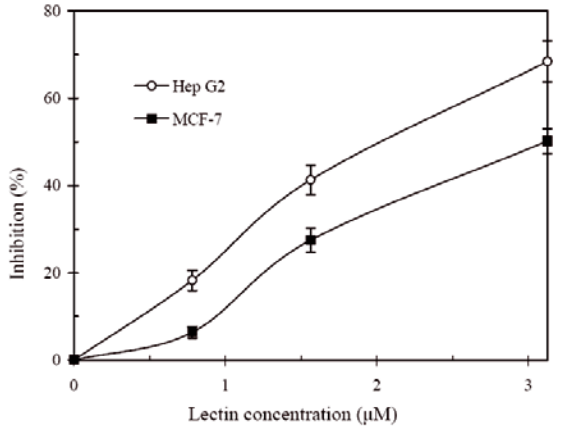

Figure 4. *In vitro* inhibitory effect of *P. adiposa* lectin on proliferation of Hep G2 and MCF-7 cancer cell lines.
Results represent mean ±S.D. (n = 3). The IC_{50} toward Hep G2 cells and MCF7 cells is 2.1 μM and close to 3.2 μM, respectively.

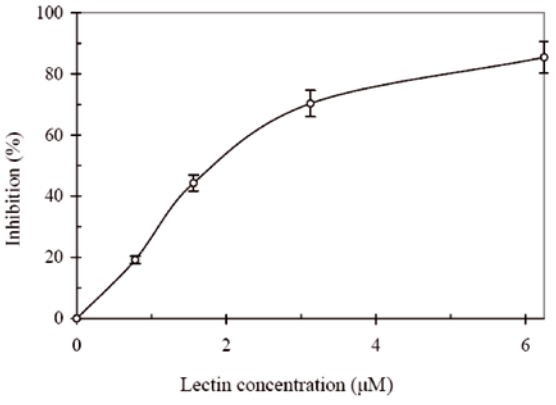

Figure 5. Inhibitory effect of *P. adiposa* lectin on activity of HIV-1 reverse transcriptase.
Results represent mean ±S.D. (n = 3). The IC_{50} is 1.9 μM.

Table 6. Comparison of characteristics of *P. adiposa* and *P. aurivella* lectins.

Characteristics	*P. adiposa* lectin	*P. aurivella* lectin
Chromatographic behavior		
DEAE ion exchanger	adsorbed, eluted with 150 mM NaCl	tenaciously adsorbed, eluted only with 0.1% SDS
Affi-gel blue gel	unadsorbed	not used
CM ion exchanger	adsorbed, eluted with 50 mM NaCl	tenaciously adsorbed, eluted only with 0.1% SDS
Molecular mass (kDa)	32	more than several hundred
Subunit molecular mass (kDa)	16	18
N-terminal sequence	DILMGTYGML	YSVTTPNSVKGGTNQPGA
Thermostability	up to 50°C	not determined
pH stability	pH 1.6–12.7	pH 6–10.5
Sugar specificity	hemagglutinating activity inhibited by the polysaccharide inulin and not by simple sugars	hemagglutinating activity inhibited by glycoproteins and not by simple sugars
Effect of cations on hemagglutinating activity	activity enhanced by Cu^{2+}, Fe^{2}, and Al^{3+}	no effect
Antifungal activity	no effect	not determined
Antiproliferative activity	IC_{50} = 2–3 μM	not determined
HIV-1 RT inhibitory activity	IC_{50} = 1.9 μM	not determined

tivity of *Pleurotus ostreatus* lectin (Wang *et al*., 2000), but do not affect the lectin from *Schizophyllum commune* (Han *et al*., 2005). Ca^{2+}, Mg^{2+}, Mn^{2+}, and Zn^{2+} ions do not affect the hemagglutinating activity of lectins isolated from *Amanita pantherina* (Zhuang *et al*., 1996), *G. frondosa* (Kawagishi *et al*., 1990), and *Hericium erinaceum* (Kawagishi *et al*., 1994), but inhibit a *Schizophyllum commune* lectin (Han *et al*., 2005).

PAL is stable in the presence of 25 mM HCl and 50 mM NaOH. It exhibits some similarity to the lectin from *Armillaria luteo-virens* (Feng *et al*., 2006). Mushroom lectins differ from one another in thermostability. The hemagglutinating activity of PAL decreases when the lectin is exposed to temperatures above 50°C. At and above 70°C the lectin activity is completely abolished. The lectin from *Ganoderma capense* is not affected after exposure to 100°C for 60 min (Ngai & Ng, 2004), while the hemagglutinating activity of lectins from *P. ostreatus* is reduced at or above 40°C (Wang *et al*., 2000).

PAL is characterized by an ability to inhibit proliferation of two tumor cell lines, Hep G2 and MCF-7. The IC_{50} toward Hep G2 cells and MCF7 cells is 2.1 μM and near 3.2 μM, respectively. Lectins from *A. bisporus*, *P. ostreatus*, *Tricholoma mongolicum*, and *Volvariella volvacea* have antitumor activity *in vivo* or antiproliferative activity *in vitro* (Wang *et al*., 1996; 1998; 2000). The potent antiproliferative activity of PAL is remarkable and hopefully it can be developed into an agent for cancer therapy. HIV RT is a key enzyme of the HIV life cycle. Screening of HIV RT inhibitors is currently a strategy to search for anti-HIV drugs. It is worth mentioning that PAL manifests a potent inhibitory activity toward HIV-1 RT with an IC_{50} of 1.9 μM. Compared with many other lectins, *P. adiposa* lectin has significant inhibitory activity toward HIV-1 reverse transcriptase. It is possible that the mechanism of inhibition is analogous to the protein–protein interaction involved in the inhibition of HIV-1 reverse transcriptase by the homologous protease (Bottcher & Grosse, 1997).

When compared with *P. aurivella* lectin, the *P. adiposa* lectin displays many differences in chromatographic behavior, molecular mass, N-terminal sequence, and effect of cations on hemagglutinating activity. For *P. aurivella* lectin, many biological activities, including antifungal, antiproliferative, and HIV-1 RT inhibitory activities have not been determined (Table 6) (Kawagishi *et al*., 1991).

In summary, a lectin with a distinctive N-terminal sequence, carbohydrate specificity, and potent antiproliferative activity was isolated from *P. adiposa* fruiting bodies. It represents an addition to the existing list of mushroom lectins since it is the second Strophariaceae lectin and the second lectin isolated from a mushroom belonging to the *Pholiota* genus.

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