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TWO *N*-ACETYL-D-GALACTOSAMINE-SPECIFIC LECTINS FROM *PHAEOLEPIOTA AUREA*

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Key Word Index—*Phaeolepiota aurea*; Agaricaceae; mushroom; *N*-D-acetylgalactosamine-specific lectins; PAL-I; PAL-II.

Abstract—Two lectins, PAL-I and PAL-II, were isolated from the mushroom *Phaeolepiota aurea* by affinity chromatography on acid-treated Sepharose CL-4B followed by reverse-phase FPLC on ProRPC. Both of the lectins were tetramers of 16 kDa subunits. The lectins had little agglutination activity against native erythrocytes but Pronase treatment of erythrocytes increased the sensitivity to agglutination by the lectins. Both lectins exhibited slight preferences for type A compared with type B and O erythrocytes. In haemagglutination inhibition assays, *N*-acetylgalactosamine and both anomers of methyl *N*-acetylgalactosaminide were the best inhibitors.

INTRODUCTION

Many lectins have been isolated from mushrooms and characterized [1–25]. Among them, only *Agaricus* lectins have been isolated from the Agaricaceae and compared with each other [5, 16–19, 24]. All of the those lectins are 'endolectins' that cannot recognize free simple sugars [26]. During our continuous screening aimed at finding lectins with new sugar-binding specificity, we found lectin activity with *N*-acetylgalactosamine-specificity from an extract of the fruiting bodies of *Phaeolepiota aurea*. Herein, we describe the isolation and characterization of two lectins, PAL-I and PAL-II, from this edible mushroom and compare them with other lectins from the Agaricaceae.

RESULTS AND DISCUSSION

The purification protocol is summarized in Table 1. The lectins were adsorbed to an acid-treated Sepharose CL-4B affinity column because of their binding-specificity to galactose. Lectin activity was eluted with 0.1 M galactose. As this eluted fraction showed a single band on SDS–PAGE (data not shown), it appeared to be homogeneous. However, when this fraction was applied to FPLC using a ProRP HR5/10 reverse-phase column to prepare a sample for *N*-terminal amino acid analysis, it further divided into two peaks (Fig. 1). Both peaks still exhibited lectin activities and were named PAL-I and PAL-II, respectively. Surprisingly, both lectins were stable at the last step using 0.1% TFA in aqueous acetonitrile.

Upon SDS–PAGE under reducing and nonreducing conditions, both lectins migrated almost as single bands of 16 kDa, both in the presence and the absence of 2-mercaptoethanol (Fig. 2). Gel filtration on Superose 12 in 10 mM phosphate-buffered saline containing 0.1 M galactose gave M_r values of 64000. These results indicated that PAL-I and PAL-II both occurred as tetramers of 16 kDa subunits with no disulphide bond.

Results from the amino acid analyses of the two proteins are shown in Table 2. There was a slight difference in amino acid composition of the two lectins; both lectins contained large amounts of Asx, Thr, Ser, Glx, Gly, Ala and Pro, while no Met or Tyr were detected in PAL-I. Isoelectric focusing of the two lectins indicated similar families of bands in pH zone near 6.4 (data not shown). Although 2 nmol of the proteins was applied to the sequencer, no *N*-terminal amino acids could be detected, suggesting that the *N*-termini of both proteins may be blocked.

PAL-I was inactive at 100 $\mu g\,ml^{-1}$ to all types of intact erythrocytes, whereas PAL-II weakly agglutinated all types with, a slight preference for type A over type B and O. Pronase treatments of erythrocytes increased the sensitivity of the lectins (Table 3). Haemagglutination activities of the lectins were not affected by demetalization with EDTA and addition of $CaCl_2$, $MgCl_2$, $ZnCl_2$ or $MnCl_2$ to either of the demetalized lectins did not cause

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Table 1. Purification of PAL-I and PAL-II (from 421 mg of defatted fruiting bodies)

Fraction	Total protein (mg)	Total agglutination activity (titre)	Specific agglutination activity (titre mg^{-1} protein)	Recovery of activity (%)*
Saline extract	—	2560	—	100.0
Eluate from acid-treated Sepharose CL-4B	5.2	1750	337	68.4
Eluate from ProRPC HR5/5				
PAL-I	0.9	700	778	27.3
PAL-II	2.4	820	342	32.0

*Based on saline extract.

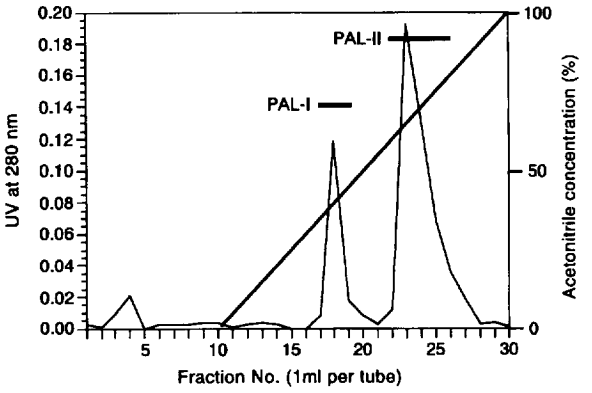

Fig. 1. FPLC of active fraction eluted from the affinity column on acid-treated Sepharose CL-4B.

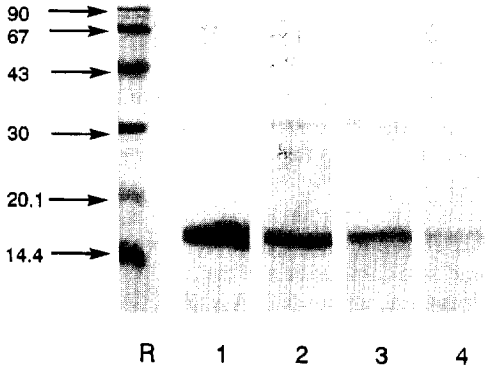

Fig. 2. SDS–PAGE of PAL-I and PAL-II. Lane R, marker proteins; 1, PAL-I in the absence of 2-mercaptoethanol (2ME); 2, PAL-I in the presence of 2ME; 3, PAL-II in the absence of 2ME; 4, PAL-II in the presence of 2ME.

Table 2. Amino acid compositions of PAL-I and PAL-II

Amino acid	(Mol %) PAL-I	PAL-II
Asx	11.9	13.6
Thr	9.0	10.0
Ser	10.1	9.0
Glx	11.8	8.7
Gly	12.5	9.7
Ala	10.5	10.3
Val	5.2	6.3
Met	0.0	1.8
Ile	4.4	4.9
Leu	5.2	6.0
Tyr	0.0	2.0
Phe	2.4	3.2
Lys	4.3	3.8
His	0.9	1.1
Arg	2.3	1.6
Pro	9.5	8.0

Table 3. Agglutination profiles of PAL-I and PAL-II ($100\ \mu g\ ml^{-1}$)

Group of human erythrocytes	Agglutination activity (titre) PAL-I	PAL-II
Native A	0	4
B	0	2
O	0	2
Pronase-treated A	512	2048
B	256	1024
O	256	1024

any change of the activities, suggesting the lectin activities of PAL-I and PAL-II were independent of metal cations.

In haemagglutination inhibition assays, the binding specificities to mono- and oligosaccharides of the two lectins were similar (Table 4). *N*-Acetylgalactosamine and both anomers of methyl *N*-acetylgalactosaminide were the best inhibitors. Because *N*-acetylgalactosamine and its glycosides were much stronger inhibitors than Gal $[\beta(1 \rightarrow 4)\text{GalNAc}]_2$ and $\text{Gal}[\beta(1 \rightarrow 4)\text{GalNAc}]_3$, it was concluded that these lectins recognized mainly terminal *N*-acetylgalactosaminide residue. This conclusion was supported by the preferences of the two lectins for type

Table 4. Inhibition of haemagglutination of PAL-I and PAL-II by mono- and oligosaccharides

Inhibitor*	Minimum inhibitory concentration (mM)	
	PAL-I	PAL-II
N-Acetylgalactosamine	0.0977	0.0488
Methyl α-*N*-acetylgalactosaminide	0.0977	0.0977
Methyl β-*N*-acetylgalactosaminide	0.0977	0.0977
Fucose	12.5	12.5
Galactose	12.5	12.5
Methyl α-galactoside	50.0	50.0
Methyl β-galactoside	50.0	50.0
Lactose	6.25	6.25
Gal[β(1 → 4)GalNAc]$_2$	50.0	50.0
Gal[β(1 → 4)GalNAc]$_3$	50.0	50.0

*Glucose, glucosamine, *N*-acetylglucosamine, 2-deoxyglucose, galactosamine, 2-deoxygalactose, galacturonic acid, D,L-arabinose, xylose, mannose, L-sorbose, L-rhamnose, L-fucose, *N*-acetyllactosamine, lacturose, lactobionic acid and Galβ(1 → 6)GlcNAc did not inhibit at all at concentrations up to 200 mM. *N*-Acetylneuramic acid did not inhibit at all at concentrations up to 25 mM.

A erythrocytes over others (Table 3); the structures of the antigenic determinant of type A erythrocytes are GalNAcα(1 → 3)[Fucα(1 → 2)]Galβ(1 → 3)GlcNAc-R and GalNAcα(1 → 3)[Fucα(1 → 2)]Galβ(1 → 4)GlcNAc-R [27].

All our data allow us to conclude that PAL-I and PAL-II, although similar to each other, are definitely different proteins. As the source of the lectins was a single species, it was deduced that the lectins are isoforms. However, they might simply arise from genetic variants of a single gene in a genetically variable population. This question is still to be addressed.

Some properties of lectins from mushrooms of the Agaricaceae are summarized in Table 5. All the lectins other than PALs are from *Agaricus* mushrooms and have a striking similarity to each other. Gallagher [26] classified lectins into two groups according to their carbohydrate specificity: First, 'exolectins', which will bind to appropriate external, nonreducing sugars in complex saccharides, the agglutination by which can be inhibited by low concentrations of free sugars or their methyl glycosides and second, 'endolectins', which will recognize complex oligosaccharides, the agglutination by which can be inhibited only by specific sugar sequences. All the *Agaricus* lectins can be defined as endolectins. Conversely, PAL-I and PAL-II, which are from the genus *Phaeolepiota* in the same family as the genus *Agaricus*, are apparently endolectins. To our knowledge, PAL-I and PAL-II are the first lectins from the genus *Phaeolepiota* and the first exolectins reported from the Agaricaceae.

EXPERIMENTAL

Materials. Phaedepiota aurea fruiting bodies were collected at Kushiro-city, Hokkaido, Japan, frozen upon collection, and stored at −20°. Sepharose CL-4B, Superose 12 HR10/30 and ProRPC HR5/10 columns were products of Pharmacia. Spectra/Por 1 was a product of Spectrum Medical Industries (U.S.A). All the sugars for the haemagglutinating tests were of the D-configuration unless otherwise stated and obtained from Nacalai Tesque. All other chemicals were reagent grade.

Preparation of affinity adsorbent. Acid-treated Sepharose CL-4B was prepared by the method of ref [28].

Isolation of PAL. All procedures were done at 4° except for defrosting of fruiting bodies. Frozen fruiting bodies were defrosted at room temp., immediately homogenized in a blender with Me_2CO and extracted overnight. The resulting suspension was filtered and defatted fruiting bodies extracted in saline with stirring. The resulting suspension was filtered through gauze and the filtrate centrifuged (10000 *g*) to remove insoluble residues. The supernatant was neutralized with 0.1 N NaOH and applied to a column of acid-treated Sepharose CL-4B equilibrated with 10 mM Pi-buffered saline (PBS). After extensive washing with PBS, lectin activity was desorbed with 0.1 M galactose. Eluates were dialysed against dist. H_2O with Spectra/Por 1 and lyophilized. Lyophilized material was redissolved in 0.1% TFA and applied to a ProRPC HR5/10 column. After washing the column with solvent, a linear gradient elution from 0 to

Table 5. Some properties of the lectins from mushrooms of the Agaricaceae

Source (lectin name)	Human blood-type specificity	Monosaccharides specificity	M_r	Subunits per molecule	Reference
Phaeolepiota aureae (PAL-I)	A	GalNAc	64000	4	
(PAL-II)	A	GalNAc	64000	4	
Agaricus bisporus (ABA I-IV)	no	no	64000	4	[16,19]
A. blazei (ABL)	no	no	64000	4	[5]
A. campestris	no	no	64000	4	[17,18]
A. edulis (I)	no	no	60000	4	[24]
(II)	no	no	32000	2	

100% MeCN gave the two purified lectins, PAL-I and PAL-II.

Erythrocytes. Human blood was collected in 3% Na citrate. Erythrocytes were washed ×3 with PBS and suspended at a concn of 3% in buffer.

Enzyme treatment of erythrocytes. A 10% suspension of erythrocytes in PBS (10 ml) was treated with Pronase P (7 mg) for 30 min at 47°, then the erythrocytes were washed ×3 with buffer and suspended at a concn of 3% in buffer.

Haemagglutination test. Agglutination of 3% erythrocytes and inhibition of the agglutination by sugars were done in microtiter U-plates. The titre was defined as the reciprocal of the end-point dilution causing haemagglutination. Inhibition was expressed as the min. concn of each sugar required for inhibition of haemagglutination of titre 4 of the lectin.

SDS–PAGE. SDS–PAGE was done by the method of ref. [29]. Samples were heated in the presence or absence of 2-mercaptoethanol for 10 min at 100°. Gels were stained with Coomassie brilliant blue. The M_r standards (Pharmacia) used were phosphorylase B (M_r 94 000), albumin (67 000), ovalbumin (43 000), carbonic anhydrase (30 000), trypsin inhibitor (20 100) and α-lactalbumin (14 400).

Gel filtration for M_r *estimation.* Gel filtration for measuring the M_r of native lectin was done on a Superose 12 HR10/30 column with a FPLC system using PBS containing 0.1 M galactose as running buffer.

Isoelectric focusing. This was carried out on LKB Ampholine Pagplate, pH 3.5–9.5. The developed gel was stained with Coomassie brilliant blue.

Amino acid analysis. Amino acids were analysed with an automatic amino acid analyser after hydrolysis of samples in 6 M HCl at 110° for 20 hr in sealed evacuated tubes.

N-*terminal amino acid analysis.* *N*-terminal amino acids of proteins were analysed on a model 477A Pulsed Liquid Protein/Peptide Sequencer (Applied Biosystems) equipped with a HPLC system (Model 120A on-line PTH Analyzer, Applied Biosystems).

Effect of metal cations on lectin activity. To examine metal cation requirements of the haemagglutination by the lectins, samples were demetalized by the method of ref [30].

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