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Purification and characterization of a lectin from the toxic mushroom *Amanita pantherina*

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Abstract

A lectin (APL) was isolated from the mushroom *Amanita pantherina* by means of hydrophobic chromatography on Butyl-Toyopearl, affinity chromatography on bovine submaxillary mucin (BSM)-Toyopearl and gel filtration on Superose12 HR10/30 using a FPLC system. This lectin is composed of two identical subunits of 22 kDa and the molecular mass of the intact lectin was estimated to be 43 kDa by gel filtration. In hemagglutination inhibition assays, it exhibits sugar-binding specificities towards GlcNAcβ1 → 4Manβ-pNP, Galβ1 → 4GlcNAcβ1 → 4GlcNAc, and Galβ1 → 4GlcNAcβ1 → 4GlcNAcβ1 → 4GlcNAc among mono- and oligosaccharides tested. Among glycoproteins tested, BSM and asialo-BSM were the strongest inhibitors.

Keywords: Amanitaceae; Mushroom; Fruiting body; Lectin; (*Amanita pantherina*)

1. Introduction

Many lectins from mushrooms have been isolated and characterized [1–20]. During our continuing screening for lectins in mushrooms, we found strong lectin activity in the extract of *Amanita pantherina*.

Amanita pantherina is a toxic mushroom, which can cause muscle cramp, insanity and audio-visual disorder if it is mistakenly eaten. It is well known that the main toxic principles in the mushroom are iboteric acid, muscimol, muscarine, stizolobic acid and stizolobinic acid [21–23]. However, there have been reports concerned with high molecular substances from the mushroom. This paper reports on the isolation and biochemical properties of a lectin from the mushroom.

2. Materials and methods

2.1. Materials

A. pantherina fruiting bodies were collected at Shimizu city, Shizuoka Prefecture, Japan, frozen upon collection and stored at −30°C. Butyl-Toyopearl 650M and Amino-Toyopearl 650M were purchased from Tosoh (Japan). FPLC system and Superose 12 HR10/30 column were products of Pharmacia (Sweden). ABEE reagent and Honenpak C18 column (75 mm × 4.6 mm i.d.) were obtained from Honen corporation (Tokyo, Japan). Ultrafiltration membrane PM10 was product of Amicon (USA).

2.2. Preparation of affinity adsorbent

BSM was conjugated to Amino-Toyopearl 650M by following the instructions of manufacturer.

2.3. Isolation of APL

All the procedures were carried out at 4°C. The frozen fruiting bodies of *A. pantherina* were homogenized with

Abbreviations: APL, *Amanita pantherina* lectin; PBS, 10 mM phosphate-buffered saline (pH 7.4); BSM, bovine subamxillary mucin; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; ABEE, *p*-aminobenzoic ethyl ester; TFA, trifluoroacetic acid; pNP, *p*-nitrophenyl; PA-GlcNAc, poly(GlcNAc-β-*p*-acryloylaminophenyl); All sugars are of the D-configuration unless otherwise stated.

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saline and extracted with stirring overnight. The resulting suspension was filtered and the filtrate was centrifuged (10 000 × *g*, 20 min) to remove insoluble residues. Solid ammonium sulfate was added to the supernatant to give 40% saturation and the solution was applied to a Butyl-Toyopearl 650M column equilibrated with 40% saturated ammonium sulfate. After extensive washing with 40% saturated ammonium sulfate, the lectin-containing fraction was eluted with distilled water. After the eluate was dialyzed against distilled water and PBS, and concentrated by ultrafiltration with PM10, the fraction was then applied to a BSM-Toyopearl column equilibrated with PBS. After unbound proteins were washed with PBS, the lectin fraction was desorbed with 0.2 M ammonia. The eluates were immediately neutralized with 1 M HCl, concentrated by ultrafiltration, dialyzed against distilled water, and lyophilized. The crude lectin was further purified with gel filtration on Superose 12 HR10/30 using a FPLC system, and dialyzed against distilled water and lyophilized, giving a purified lectin (APL).

2.4. Hemagglutination test

Erythrocytes and Pronase-treated erythrocytes for agglutinating test were prepared as reported previously [1,3,4]. Agglutination of erythrocytes and inhibition of the agglutination by sugars and glycoproteins were done in microtiter U-plates. The titer was defined as the reciprocal of the end-point dilution causing hemagglutination. Inhibition was expressed as the minimum concentration of each sugar or glycoprotein required for inhibition of hemagglutination of titer 4 of the lectin.

2.5. SDS-PAGE

SDS-PAGE was carried out in 13% acrylamide slab gels containing 10% SDS at pH 8.8 (Tris-HCl) by the method of Laemmli [24]. Samples were heated in the presence or absence of 2-mercaptoethanol for 10 min at 100°C. Gels were stained with Coomassie brilliant blue. The molecular weight standards (Pharmacia, Sweden) used were phosphorylase *b* (M_r 94 000), bovine serum albumin (67 000), ovalbumin (43 000), carbonic anhydrase (30 000), soybean trypsin inhibitor (20 100), and α-lactalbumin (14 400).

2.6. Gel filtration for molecular weight estimation

Gel filtration for measuring the molecular weight of native lectin was carried out on a Superose 12 HR10/30 column equilibrated with PBS using a FPLC system. The molecular weight standards (Pharmacia, Sweden) used were ferritin (440 000), catalase (232 000), bovine serum albumin (67 000), ovalbumin (43 000), chymotrypsinogen A (25 000), and ribonuclease A (13 700).

2.7. Amino acid analysis

200 μg of the lectin were hydrolyzed with 6 M HCl at 110°C for 24 h in sealed evacuated tubes, and analyzed on a Hitachi L-8500A amino acid analyzer. The content of cysteine was determined by oxidation of the lectin (200 μg) with performic acid [25] followed by hydrolysis under the same condition to that of the intact protein. The content of tryptophan was estimated by the spectrometric method of Edelhoch [26].

2.8. N-terminal amino acid analysis

The N-terminal amino acids of the proteins were analyzed on a model 477A pulsed liquid protein/peptide sequencer (Applied Biosystems) equipped with a high performance liquid chromatography system (model 120A on-line phenylthiohydantoin analyzer, Applied Biosystems)

2.9. Sugar analysis

Sugar content was measured by the phenol-sulfuric acid method with reference to glucose [27]. Composition of the sugar was determined as follows; purified lectin (200 μg) was dissolved in 20 μl distilled water in a test tube and 20 μl of 4 M TFA was added. The test tube was incubated at 100°C in a hot block bath. After 4 h, the tube was cooled to room temperature and the acid was removed by using a centrifugal concentrator at 35°C. The dried sample was derivatized with ABEE in the presence of borane-pyridine complex at 80°C. After 1 h, the reaction mixture was cooled to room temperature. The distilled water (0.2 ml) and an equal volume of chloroform were added to the reaction mixture. After vigorous vortexing, it was centrifuged (6000 × *g*, 1 min). The upper aqueous layer was analyzed by reverse-phase HPLC under the condition as follows; column, Honenpak C18 (75 mm × 4.6 mm I.D.); solvent, A 0.02%TFA/CH_3CN(92/8), B 0.02%TFA/CH_3CN(50/50); program, 0 → 45 min (B conc. 0%), 45 → 55 min (B conc. 100%), 55 → 70 min (B conc. 0%); flow rate, 1 ml/min; temp., 45°C; detection, absorbance at 305 nm. The monosaccharide and amino monosaccharide standards used were glucosamine, galactosamine, galactose, glucose, mannose, xylose, and fucose [28].

2.10. Thermostability

Samples in PBS were heated for 30 min at the temperatures indicated, cooled on ice, and titrated.

2.11. pH stability

The pH dependence of the lectin was measured by incubating the samples in the following buffers for 24 h at

Table 1
Purification of APL (from 100 g of fresh fruiting body)

Step	Total protein (mg)	Total agglutination (titer) [a]	Specific agglutination activity (titer/mg)	Recovery of activity (%)
1. Saline-extract	—	786280	—	100
2. Eluate from Butyl-Toyopearl	46.0	565248	12288	71.9
3. Eluate from BSM-Toyopearl	8.1	265420	32768	33.8
4. Eluate from Superose 12	2.2	144179	65536	18.3

[a] measured with type O Pronase-treated blood cell.

4°C, 0.02 M sodium acetate buffer (pH 3.5–5.5), 0.02 M sodium phosphate buffer (pH 6.0–7.5), 0.02 M Tris-HCl buffer (pH 8.0–9.0), and 0.02 M glycine-NaOH buffer (9.5–11.0).

2.12. *Effect of metal cations on the lectin activity*

To examine metal cation requirements of the hemagglutination by the lectin, the samples were demetalized by the method of Pandolfino and Magnuso [29].

3. Results and discussion

Isolation procedure is summarized in Table 1. No precipitate was obtained when ammonium sulfate was added into the saline extract of *A. pantherina* until 80% saturation, suggesting that the lectin was a highly hydrophilic protein. Therefore, the saline extract was adjusted to 40% saturation with ammonium sulfate, and directly applied for hydrophobic chromatography on Butyl-Toyopearl. The activity was completely adsorbed to the column and the lectin-containing fraction was eluted with distilled water.

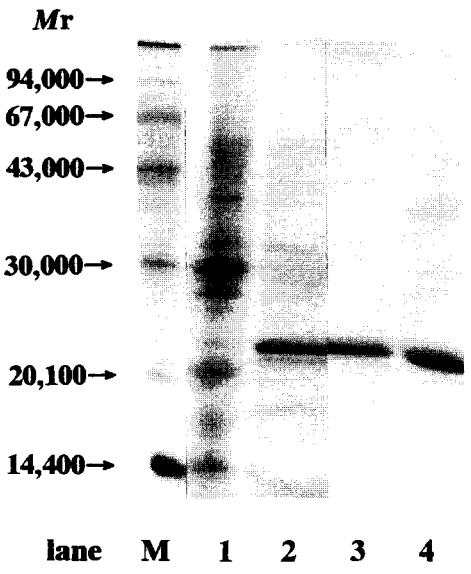

Fig. 1. SDS-PAGE of APL. lane M, marker; lane 1, step 2; lane 2, step 3; lane 3, step 4 (reduced with 2-mercaptoethanol); lane 4, step 4 (non-reduced).

Since the fraction exhibited no strong binding specificity to any monosaccharides and showed specificity for BSM in preliminary hemagglutination inhibition assays, BSM-Toyopearl was chosen as the affinity support. The lectin activity of the fraction was adsorbed to the affinity column and eluted with 0.2 M ammonia. The eluate from the column was further purified by gel filtration on Superose 12 and a pure lectin which was named APL was obtained.

SDS-PAGE of each lectin fraction in the process of purification (steps 2–4 in Table 1) is shown in Fig. 1. The purified lectin, APL, (step 4 in Table 1) appeared as a single band corresponding to the molecular mass of 22 kDa regardless of the presence (lane 3) or absence (lane 4) of 2-mercaptoethanol. Since the molecular weight of the intact lectin was estimated to be 43 000 by gel filtration on Superose 12, it was considered to be a dimer containing two identical subunits. The isoelectric focusing gave a family of bands in pH zone 4.8–5.3 (not shown), indicating that this lectin was an acidic protein.

The result of amino acid analyses revealed a high content of Gly, Glx, Ser, Asx and a low content of Met, Lys, Tyr, His (Table 2). Amino acid sequence analysis gave no evidence of heterogeneity in the primary structure of the first 10 N-terminal residues (Table 3). The carbohydrate contents of APL was 4.3%, and the components of the sugar were determined as Glc, Man, Xyl and GlcNAc (5.65:4.61:1.00:4.16 in molar ratio) by the analysis of their ABEE-derivatives. Among mushroom lectins, there is only one paper which reported the sugar compositions of the lectins [15]; four isolectins, ABA-I to IV from *Agaricus bisporus* contained Fuc, Man, Gal, and $GlcNH_2$. To our knowledge, this type of sugar chain in APL has not been

Table 2
Amino acid composition of APL

Amino acid	mol%	Amino acid	mol%
Asx	8.2	Ile	4.5
Thr	6.8	Leu	6.3
Ser	9.1	Tyr	2.2
Glx	9.9	Phe	3.0
Gly	11.1	Lys	5.0
Ala	7.5	His	2.4
Val	6.3	Arg	3.6
Cys	2.1	Pro	5.7
Met	0.4	Trp	5.9

Table 3
N-terminal amino acid sequence of APL

1	2	3	4	5	6	7	8	9	10
Ile	Phe	Ala	Val	Gly	Glu	Thr	Gln	Gly	Glu

reported yet. The determination of the structures of the sugar chains in APL is now in progress.

This lectin was quite stable between pH 4 and 9.5, and below 40°C. Its hemagglutinating activity was not affected by demetalization with EDTA, and in the presence of $CaCl_2$ and $MgCl_2$, the lectin did not cause any change of the activity. The results demonstrated that the activity of the lectin was not concerned with metal cations.

As indicated in Table 4, APL did not agglutinate any type of native human erythrocytes, but it could cause agglutination of Pronase-treated blood cells, and exhibited a remarkable preference for type A Pronase-treated erythrocytes rather than type O and B. The results of hemagglutination inhibition assays of APL are shown in Table 5. Seventy-two monosaccharides and oligosaccharides were tested. However, only 3 oligosaccharides, GlcNAcβ1 → 4Manβ-pNP, Galβ1 → 4GlcNAcβ1 → 4GlcNAc, and Galβ1 → 4GlcNAcβ1 → 4GlcNAcβ1 → 4GlcNAc, could inhibit the lectin-mediated hemagglutination. Among glycoproteins tested, BSM and asialo-BSM, which have glycosidically linked sugar chains, had much stronger inhibitory activity than fetuin and asialo-fetuin which have both glycosidically and *N*-glycosidically linked sugar chains. Since the inhibitory activities of BSM and asialo-BSM were very strong, these glycoproteins were treated with Pronase in order to examine the structure–activity relationship. The inhibitory activities of Pronase-treated BSM and asialo-BSM were much weaker than those of untreated ones, suggesting that their activities were not due to only simple binding between their sugar chains and the sugar binding sites of APL.

APL was the first lectin isolated from the family *Amanitaceae*. The binding sugar chain(s) in the type A Pronase-treated blood cells and the common structure of the binding sugar remain undetermined.

Table 4
Agglutination profiles of APL (1 pg/ml)

Group of erythrocytes		APL (titer)
Untreated	Human O	—
	Human A	—
	Human B	—
Pronase-treated	Human O	4
	Human A	256
	Human B	4

Table 5
Inhibition of the hemagglutinating activity of APL by mono- andoligosaccharides and glycoproteins

Inhibitor [a]	Minimum inhibitory concentration [b]	Specificity factor [c]
	(mM)	
GlcNAcβ1 → 4Manβ-pNP	4.00	1.00
Galβ1 → 4GlcNAcβ1 → 4GlcNAc	12.5	3.13
Galβ1 → 4(GlcNAc)2β1 → 4GlcNAc	25.0	6.25
	(μg/ml)	
BSM	0.1	
asialo-BSM	0.1	
Fetuin	1000	
asialo-Fetuin	500	

[a] Glucose, galactose, mannose, D,L-fucose, xylose, fructose, L-rhamnose, ribose, L-arabinose, galactosamine, glucosamine, mannosamine, *N*-acetyl galactosamine, *N*-acetyl glucosamine, *N*-acetyl mannosamine, α-methyl glucoside, β-methyl glucoside, α-methyl galactoside, β-methyl galactoside, α-methyl mannoside, β-methyl mannoside, α-methyl-*N*-acetyl galactosaminide, β-methyl-*N*-acetyl galactosaminide, α-methyl-*N*-acetyl glucosaminide, β-methyl-*N*-acetyl glucosaminide, sorbose, lactose, cellobiose, sorbitol, lactitol, mannitol, dulcitol, inositol, glucuronic acid, galacturonic acid, Galβ1 → 4GlcNAc, and Manβ1 → 4Man, exhibited no inhibition at concentrations up to 400 mM; melibiose, lactulose, lactobionic acid, Galβ1 → 4GalNAc, and Galβ1 → 6GlcNAc at concentrations up to 200 mM; GlcNAc(β1 → 4GlcNAc)$_n$ (n = 1–4) and Galβ1 → 6GlcNAcβ-pNP at concentrations up to 100 mM; *N*-acetyl neuraminic acid at concentrations up to 50 mM; α-phenyl-*N*-acetyl glucosaminide, β-phenyl-*N*-acetyl glucosaminide, *N*-glycolyl neuraminic acid, GlcNAcβ1 → 6GlcNAc, Manβ-pNP, Galβ1 → 4GlcNAcβ-pNP, Galα1 → 6Galb-pNP, and 3-fucosyllactose at concentrations up to 25 mM; GlcNAcβ1 → 4Man at concentrations up to 8 mM; PA-GlcNAc at concentrations up to 3 mg/ml; methyl-cellulose, pectic acid, and heparin at concentrations up to 2 mg/ml; maltose, colominic acid, and transferrin at concentrations up to 1 mg/ml.

[b] Minimum concentration required for inhibition of 4 hemagglutinating dose of the lectin.

[c] Calculated in relation to GlcNAcβ1 → 4Manβ-pNP.

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