A Lectin from the Shoots of Bamboo *Phyllostachys pubescens*

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A lectin was isolated from the shoots of bamboo *Phyllostachys pubescens* by using affinity chromatography on Sephadex G-75. In hemagglutination inhibition assays, this lectin exhibited a binding specificity toward maltose and methyl α -mannoside. The N-terminal sequence was determined as Lys-Ser-Cys-Cys-Pro-Ser-Thr-Thr-Ala and identical with that of the American mistletoe toxin, ligatoxin A.

Traditionally people eat bamboo shoots in Japan and the other Asian countries. However hardly any chemical or biochemical studies on the constituents of the material have been done. In the course of our screening for new lectins from various organisms,^{1–3)} we found lectin activity in the sap of the shoots of bamboo *Phyllostachys pubescens*. Here we describe the isolation and characterization of the lectin.

MATERIALS AND METHODS

Materials. Bamboo shoots *Phyllostachys pubescens* Mazel were collected at Shizuoka City, Japan, frozen upon collection, and stored at -30°C . Sephadex G-75 and Superose 12 HR10/30 column were products of Pharmacia (Sweden). Spectra/Por 1 was a product of Spectrum Medical Industries (USA). All sugars for the hemagglutinating inhibition tests were products of Nacalai Tesque (Japan), Wako Pure Chemical (Japan) or Sigma (USA). All glycoproteins for the test were purchased from Sigma (USA). BIAcore 2000 was a product of BIAcore AB (Sweden).

Purification of PPL. All the procedures were carried out at 4°C except for defrosting. After defrosting, the bamboo shoots were homogenized in a blender and centrifuged ($10,000\times g$, 30 min). To the supernatant, 4 volumes of 5-fold concentrated PBS and ME were added, giving a crude lectin solution in PBS containing 1 mM ME. The lectin solution was applied to a Sephadex G-75 column ($1.8\times 35\text{ cm}$) equilibrated with the same buffer. The lectin activity weakly bound to the column and was eluted at the bed volume of the column. The lectin-containing fraction was concentrated by ultrafiltration (UK-500, Advantec, Japan) and applied to the Sephadex G-75 column equilibrated with the buffer again. The lectin fraction was dialyzed using Spectra/Por 1 against distilled water and lyophilized, giving a purified lectin (PPL).

Hemagglutination test. Human blood was collected in 3% sodium citrate. The erythrocytes were washed three times with PBS and suspended at a concentration of 3% in the buffer.

A 10% suspension of erythrocytes in PBS (10.0 mL) was treated with Pronase P (7.0 mg) for 30 min at 47°C , and then the erythrocytes were washed three times with the buffer and suspended at a concentration of 3% in the buffer.

A 10% suspension of erythrocytes in PBS (10.0 mL) containing 0.05% BSA was treated with *Clostridium perfringens* sialidase (Worthington Biochemical Corporation, USA, 140 mU/mL) for 4 h

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Abbreviations: PPL, *Phyllostachys pubescens* lectin; PBS, 10 mM phosphate-buffered saline (pH 7.4); HBS, 10 mM HEPES buffered saline (pH 7.4); BSM, bovine submaxillary mucin; PAGE, polyacrylamide gel electrophoresis; ME, 2-mercaptoethanol. All sugars are of D-configuration unless otherwise stated.

at 37°C, then the erythrocytes were washed three times with the buffer and suspended at a concentration of 3% in PBS.

Agglutination of 3% erythrocytes and inhibition of the agglutination by sugars and glycoproteins were done in 96-well microtiter U-plates. The titer was defined as reciprocal of the end-point dilution causing hemagglutination. Inhibition was expressed as the minimum concentration of each sugar required for inhibition of hemagglutination of titer 4 of the lectin.

SDS-PAGE. SDS-PAGE was carried out by the method of Laemmli.⁴⁾ Samples were heated in the presence or absence of ME for 10 min at 100°C. Gels were stained with Coomassie Brilliant Blue.

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 with a FPLC system. The molecular weight standards (Pharmacia, Sweden) used were ferritin (Mr 440,000), catalase (232,000), bovine serum albumin (67,000), ovalbumin (43,000), chymotrypsinogen A (25,000), and ribonuclease A (13,700).

Sugar analysis. Sugar content was measured by the phenol-sulfuric acid method with reference to glucose.

Amino acid analysis. The lectin was hydrolyzed with 6 M HCl at 110°C for 24 h in a sealed evacuated tube and analyzed on a Hitachi L-8500 A amino acid analyzer.

N-terminal amino acid analysis. The N-terminal amino acid of the protein was analyzed on a PPSQ-10 Protein Peptide Sequencer (Shimadzu, Japan).

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

ice, and titrated.

pH Stability. The pH dependence of the lectin was measured by incubating the samples in the following buffers for 24 h at room temperature: 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 (pH 9.5–10.5).

Surface plasmon resonance analysis. Real time detection of PPL binding to glycoproteins was recorded by using a BIAcore 2000. PPL was immobilized covalently via its primary amines to carboxyl groups within a dextran layer on the sensor chip CM-5 according to the manufacturer's specifications. During the immobilization, maltose (1 mM) was added to the coupling buffer in order to protect the sugar-binding site in the lectin. Glycoproteins at various concentrations in HBS were injected over immobilized PPL at 20 mL/min. After injection of each protein, HBS was introduced onto the sensor surface to start the dissociation. The data were analyzed by BIAevaluation 3.0 software (Biacore AB, Sweden).¹²⁾

RESULTS AND DISCUSSION

The purification procedure is summarized in Table 1. Since the lectin activity was unstable, the purification procedure was done successively without lyophilization. Therefore the protein amounts in the first two steps are not given in Table 1. Fresh shoots of bamboo *P. pubescens* were homogenized in a blender and centrifuged. To the obtained supernatant, 5-fold concentrated PBS and ME were added. Without ME, the lectin was deactivated gradually. Since this crude lectin solution showed a binding specificity toward Glc and Man,

Table 1. Purification of PPL (from 117 g of fresh bamboo shoots).

Step	Total protein (mg)	Total agglutination activity (titer) ^a	Specific agglutination activity (titer/mg)	Recovery of activity (%)
1. Supernatant after homogenization	—	3840	—	100
2. Eluate from first affinity chromatography	—	3328	—	88.7
3. Eluate from second affinity chromatography	1.1	3072	2800	80.0

^a Measured with type A Pronase-treated blood cells.

Sephadex G-75 was chosen as the affinity support. The lectin activity weakly bound to the affinity column and eluted at the bed volume of the column. The obtained lectin-containing fraction was applied to the affinity column again. Pure lectin, PPL, was obtained by the two affinity chromatography processes.

The molecular weight of PPL estimated by gel filtration was 48,000 (data not shown). Figure 1 shows SDS-PAGE of the protein. Unreduced lectin appeared as a broad single band of molecular weight of about 13,000. The reduced one showed a diffused band of molecular weight of less than 13,000. These results show that this lectin is composed of several subunits. Although size homogeneity was suggested by gel filtration and SDS-PAGE in the absence of ME, isoelectric focusing over the pH 3.50–9.50 indicated a family of bands focusing in the pH zone between 4.5 and 5.3 (data not shown). Sugar content of the lectin was 8.8%. Amino acid analysis revealed a high content of Asx, Gly, and Ala, and a low content of Tyr, Met, Pro, and His (Table 2). Amino acid sequence analysis gave no evidence of heterogeneity in the primary structure of the first 9 *N*-terminal residues

Table 2. Amino acid composition of PPL.

Amino acid	mol%
Asx	15.9
Thr	7.4
Ser	4.8
Glx	9.6
Gly	15.4
Ala	13.7
Val	4.2
Met	0.9
Trp	n.d. ^a
Ile	4.4
Leu	5.3
Tyr	0.8
Phe	2.7
Lys	8.1
His	1.4
Arg	4.1
Pro	1.3
Cys	n.d.

^a n.d., not determined.

Fig. 1. SDS-PAGE of PPL.

Lane 1, PPL (reduced with 2-mercaptoethanol); lane 2, PPL (non-reduced); lane M, marker. The molecular weight standards (Pharmacia, Sweden) used were phosphorylase b (Mr 94,000), albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), trypsin inhibitor (20,100), and α -lactalbumin (14,400).

(Lys-Ser-Cys-Cys-Pro-Ser-Thr-Thr-Ala). The reason for the diffusion of the band in SDS-PAGE in the presence of ME is not clear but the subunits might have some intramolecular S-S linkage(s)² or might be due to heterogeneity of the sugar chains among the subunits of the protein. Interestingly, the *N*-terminal sequence of the lectin was identical with that of ligatoxin A which is a toxin isolated from the American mistletoe *Phoradendron liga*.^{5,6} Taxonomically the bamboo and the mistletoe belong to different subclasses. The LD₅₀ of the toxin, determined by intraperitoneal injection in mice, is 1.00 ± 0.08 mg/kg bodyweight.⁶ In Japan and China, people never eat raw bamboo shoots and usually eat them after heat treatment such as boiling because the raw shoots are toxic. The lectin activity was stable under 40°C; however temperatures higher than 45°C caused rapid deactivation of the lectin activity after incubation for 30 min. The molecular weight of ligatoxin A is about 5000 and composed of a single chain of 46 amino acids. It would be interesting to know whether the whole primary structure of the lectin still has homology to ligatoxin A and the raw shoots' toxicity is due to the lectin or not. The lectin was stable for a pH range from 4.0 to 10.5 after incubation at room temperature for 24 h.

PPL exhibited a slight preference for type B erythrocytes. Sialidase and Pronase treatments increased the sensitivity of erythrocytes to agglutination by the lectin (Table 3).

In the hemagglutination inhibition assay,^{1,2)} maltose was the strongest inhibitor among the mono- and oligo-saccharides used (Table 4). Methyl α -mannoside, mannose and methyl α -glucoside were also inhibitory. However, methyl β -glucoside did not inhibit PPL-mediated hemagglutination even at concentrations up to 400 mM. These results indicate that this lectin is an α -glucosyl specific lectin. All the glycoproteins used were completely inert even at 1 mg/mL in the assay. However, surface plasmon resonance analysis using BIAcore

2000 gave kinetic data for the binding between immobilized PPL and 4 glycoproteins (Fig. 2 and

Table 4. Inhibition of the hemagglutination activity of PPL by mono-, oligo-, polysaccharides and glycoproteins.

Inhibitors ^a	Minimum inhibitor concentration (mM) ^b	Specific factor ^c
Maltose	12.5	1.0
Methyl α -mannoside	25	2.0
Mannose	50	4.0
Methyl α -glucoside	50	4.0
Methyl β -mannoside	100	8.0
Glucose	100	8.0
Fructose	100	8.0
Lactose	200	16.0
Sorbose	200	16.0

Table 3. Agglutination profiles of PPL (100 μ g/mL).

Group of erythrocytes	Titer ^a		
	Untreated	Pronase treated	Sialidase treated
Human A	16	256	128
Human B	32	512	256
Human O	16	256	128

^a Defined as reciprocal of the end dilution causing hemagglutination.

^a Galactose, methyl β -glucoside, GalNAc, GlcNAc, ManNAc, LacNAc, L-arabinose, mannitol, L-rhamnose, xylose, D- and L-fucose, sorbitol, glucuronic acid, *N*-acetylneuraminic acid, and GlcNAc β 1 $\rightarrow$ 4Man did not inhibit at all at concentrations up to 400 mM. BSM, asialo-BSM, fetuin, asialo-fetuin, transferrin, and α_1 -acid glycoprotein did not inhibit at all at concentrations up to 1 mg/mL. ^b Minimum concentration required for inhibition of 4 hemagglutination dose of the lectin. ^c Calculated in relation to maltose.

Fig. 2. Sensorgrams showing the interaction between immobilized PPL and glycoproteins.

(A), asialo-BSM; (B), BSM; (C), asialo-fetuin; (D), fetuin.

Table 5. Kinetic analysis of the interaction of immobilized PBL and glycoproteins by using BIAcore.

Analyte	k_{on} ($M^{-1}s^{-1}$)	k_{off} (s^{-1})	K_A (M^{-1})
Asialo-BSM	3.40×10^3	1.92×10^{-3}	1.78×10^6
BSM	1.93×10^3	4.97×10^{-3}	3.89×10^5
Asialo-fetuin	3.40×10^3	1.79×10^{-3}	1.90×10^6
Fetuin	4.37×10^3	1.22×10^{-3}	3.59×10^6

Table 5). Usually in the case of binding analysis of lectins to sugars or glycoproteins by using BIAcore, sugars or glycoproteins are immobilized as ligands and lectins are used as analytes, because lectins are deactivated in the immobilizing conditions.⁶⁻¹²⁾ However, after various attempts, we succeeded in immobilization of PPL without deactivation in the presence of a specific binding sugar, maltose. The bindings of the lectin to all the glycoproteins were very weak; the association rates (k_{on}) were very slow and the dissociation rates (k_{off}) were very rapid compared with previously reported kinetic data on the binding between other lectins and glycoproteins or sugars.⁷⁻¹²⁾ There is no significant difference among those kinetic data for the glycoproteins.

PPL is produced by the shoots of bamboo *P. pubescens*. However, lectin activity was not detected in the extract of the mature bamboo (data not shown). So far as we are aware, this is the first lectin isolated from the genus *Phyllostachys*.

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タケノコ由来のレクチンについて

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未熟モウソウチク (タケノコ) からレクチンを Sephadex G-75 を用いたアフィニティークロマトグラフィーによって単離, 精製した。このレクチンはゲル濾過によって分子量 48,000 と推定され, 数個の同一サブユニットから成っていた。赤血球凝集阻害反応によって, マルトースや α -メチルマンノシドに結合特異性を示すことが判明した。N 末端配列は Lys-Ser-Cys-Cys-Pro-Ser-Thr-Thr-Ala であり, これはヤドリギから得られている ligatoxin A と同一であった。