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Multi-specificities of the *N*-Acetylglucosamine-Binding Lectin from *Psathyrella velutina* Mushroom toward Acidic Glycoconjugates

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Abstract

A lectin from the fruiting body of *Psathyrella velutina* (PVL) has been reported to be specific for non-reducing terminal *N*-acetylglucosamine (GlcNAc) residue. PVL was found to exhibit multispecificity toward acidic glycoconjugates, i.e., polysaccharides, sialoglycoproteins, and sulfatide. PVL was purified by single-step affinity chromatography on an *N*-acetylchitooligosaccharides ($GlcNAc_{5-6}$)-Sepharose column. The binding specificities of PVL were studied using neoproteoglycans and neoglycoproteins involving heparin or $GlcNAc_{5-6}$. PVL was shown, by both membrane analysis and solid phase assay, to bind with heparin neoproteoglycans. The binding was inhibited by polygalacturonic acid, dextran sulfate, or fucoidan as well as heparin, but not by GlcNAc, other glycosaminoglycans, colominic acid, or DNA. The pH-dependencies of the binding to heparin and to $GlcNAc_{5-6}$ were different. Biotinylation of PVL destroyed the heparin binding activity while retaining the GlcNAc binding activity. In addition, circular dichroism spectroscopy of PVL indicated that a small conformational change of PVL is induced by binding with GlcNAc but not with heparin, suggesting that the binding sites for acidic polysaccharides and GlcNAc are independently located on PVL. On the other hand, PVL bound to sialoglycoproteins on solid phase assays, and the binding was inhibited with GlcNAc or desialylation treatment, indicating that PVL recognizes *N*-acetylneuraminic acid residue in glycoproteins at GlcNAc-binding site. The binding of PVL to sulfatide was not inhibited by GlcNAc, *N*-acetylneuraminic acid, or heparin. These findings indicate that the binding specificities of PVL to glycoconjugates are quite different from those reported for oligosaccharides and PVL is a novel member of multispecific lectin in higher fungi.

Introduction

A number of fungal lectins have been reported (1, 2) and several lectins from *Basidiomycetes* (mushrooms) have proved to be useful probes for detection and fractionation of the specific carbohydrate structures of glycoconjugates (3-6). A lectin from the fruiting body of *Psathyrella velutina* (PVL[1]) has been known as an *N*-acetylglucosamine (GlcNAc)-specific lectin (7). Based on analyses of its interactions with oligosaccharides, it has been reported that PVL specifically recognizes non-reducing terminal GlcNAc residue (7). In contrast to other GlcNAc-specific lectins from higher plants, which recognize internal GlcNAc in addition to the terminal GlcNAc in the oligosaccharide sequence, PVL exhibits a strong preference for the GlcNAc monomer over the chitooligosaccharides (8). Its remarkable specificity toward non-reducing terminal GlcNAc residue leads to the expectation that PVL would be a useful reagent for glycoconjugate separation and histochemical detection of specific markers, because glycosylation lacking terminal galactosylation or sialylation has been reported for

[1]Abbreviations: PVL, *Psathyrella velutina* lectin; PBS, phosphate-buffered saline; buffer A, PBS containing 10% glycerol; AGF, asialo-agalactofetuin; BSM, bovine submaxillary mucin; NeuAc, *N*-acetylneuraminic acid; AOL, *Arthrobotrys oligospora* lectin; ABL, *Agaricus bisporus* lectin

several pathological states, such as IgGs from rheumatoid arthritis patients (9) and rat hepatoma (10). In fact, use of PVL as a GlcNAc-detection reagent in diagnostic and biochemical analyses are increasing. (11-14).

A limitation occurred, however, in applying PVL to analyses of glycoconjugates, for example, by Western blotting or histochemical staining. In these assays, PVL often gave numerous positive bands on Western blotting of serum or tissue homogenates or dense staining of sectioned tissues. Even for purified glycoproteins, it was observed that PVL bound to the protein bands which were proved to be free from terminal GlcNAc residues (our unpublished data). To use PVL as a GlcNAc-specific probe avoiding these interferences, its ligand specificity toward glycoconjugates has to be elucidated. In this study, the interactions of PVL with acidic polysaccharides, sialoglycoproteins, and sulfatide are first studied and characterized. A suitable condition was found to suppress the binding activity for acidic polysaccharides while retaining the GlcNAc binding activity. The striking multispecificity of PVL is discussed in relation to its biological significance by comparison with those reported for other fungal lectins.

Materials and Methods

Materials

Fruiting bodies of *Psathyrella velutina* were collected in Gunma Prefecture, Japan and stored at -20 °C until use. Horseradish peroxidase (HRP) (Toyobo, Osaka, Japan) and biotin hydrazide (ICN Immunobiologicals, CA, USA) were used for the preparation of binding probes. Fetuin (fetal calf serum), human transferrin, *N*-hydroxysuccinimide biotin, sulfatide; Gal(3-SO_4)β1-1-ceramide (bovine brain), polygalacturonic acid (orange), and fucoidan (*Fucus vesiculosus*; sulfur content, 8.5%) were purchased from Sigma Chem. Co., St Louis, MO, USA. Colominic acid (*E. coli*) was obtained from Nacalai Tesque, Kyoto, Japan. Asialoglycoproteins were prepared by desialylation of intact glycoprotein by two methods, i.e., mild acid treatment with 0.01 M HCl at 80°C for 1 hr, or digestion with neuraminidase (from *Vibrio cholerae*, Boehringer Mannheim GmbH, Mannheim, Germany) with 0.1 unit/mg glycoprotein in acetate buffered saline (pH 5.5) containing 1 mM $CaCl_2$ and 1 mM PMSF at 37°C for 40 hr. Asialo-agalactofetuin was prepared by digestion of asialofetuin with β-galactosidase (from jack bean, Boehringer Mannheim) with 0.04 unit for 1 mg of glycoprotein in citrate buffer (pH 3.5) at 37°C for overnight. Streptavidin-biotinylated HRP complex was purchased from Amersham, Buckinghamshire, UK. Heparan sulfate (porcine kidney; sulfate content, 9.0%) (15), keratan sulfate (whale nasal cartilage, sulfate content, 15.0%) (16), bovine submaxillary mucin (BSM), and human vitronectin (17) were prepared in our laboratory as described previously. Con A-HRP, *N*-acetylchitooligosaccharides (a mixture of pentamer and hexamer; $GlcNAc_{5-6}$), chondroitin sulfate A (whale cartilage), dermatan sulfate (chondroitin sulfate B, umbilical cord), chondroitin sulfate C (shark cartilage), chondroitin (desulfated from chondroitin sulfate A), keratan sulfate (bovine cornea), and hyaluronic acid (umbilical cord) were purchased from Seikagaku Kogyo, Tokyo, Japan. Heparin (porcine intestinal mucosa), commercial PVL, and *N*-acetylneuraminic acid were from Wako Pure Chemicals (Osaka, Japan). Dextran sulfate (molecular mass, ~500,000; sulfur content, 17%) was obtained from Pharmacia Fine Chemicals AB (Uppsala, Sweden).

Preparation of affinity adsorbent

$GlcNAc_{5-6}$ were immobilized to amino-Sepharose by reductive amination. Briefly, Sepharose 6B was activated with epichlorohydrin and then aminated with ammonia solution (18). Amino-Sepharose (10 g) was suspended in 8 ml of 0.2 M K_2HPO_4, pH 9.0, containing 800 mg of $GlcNAc_{5-6}$ and 125 mg of $NaCNBH_3$, and incubated at 37°C for 4 days with shaking. The unreacted amino groups were *N*-acetylated as described

previously (18).

Heparin was coupled to amino-Sepharose with the aid of *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline according to the procedure reported previously (17).

Purification of PVL

All procedures were carried out at 4°C. Extraction was performed on 20 g of the fruiting bodies of *P. velutina* with 200 ml of phosphate-buffered saline, pH 7.2, containing 5 mM EDTA, 5 mM GlcNAc, and 0.5 mM phenylmethylsulfonyl fluoride and treated at pH 4.0 with acetic acid, as described previously (7). The extract was neutralized with conc. NaOH and centrifuged at 700 g for 20 min. The supernatant was mixed with 1.5 g of $GlcNAc_{5-6}$-Sepharose 6B gel in a nitrocellulose tube followed by dialysis against several changes of phosphate-buffered saline, pH 7.2, containing 5 mM EDTA and 10% glycerol (buffer A). The contents of the dialyzer were packed into the column (1 × 2.2 cm) and extensively washed with buffer A. PVL was eluted with 0.2 M GlcNAc in buffer A. Purified PVL was stored at -20°C. The protein concentration was determined by using Protein Assay Reagent (Bio-Rad Lab., Hercules, CA, USA).

Preparation of HRP- or biotin-labeled PVL

PVL was labeled with HRP in the presence of 10 mM GlcNAc by the method previously described (19). Biotinylation of PVL was performed using *N*-hydroxysuccinimide biotin (7). To 1.5 mg PVL in 1.2 ml of 10 mM sodium phosphate buffer, pH 7.0, containing 130 mM NaCl (PBS) and 10% glycerol, 150 µl of 0.1% *N*-hydroxysuccinimide biotin was added, and the mixture was allowed to stand for 15 min or 4 hr at room temperature. To protect the binding sites on PVL, GlcNAc or heparin was added at a final concentration of 10 mM or 1.6 mg/ml, respectively, in the reaction buffer. The reaction was stopped by adding 0.1 M Tris followed by dialysis against 10% glycerol.

Preparation of HRP- or biotin-labeled carbohydrates

Neoproteoglycans, heparin-HRP and heparin-BSA, were prepared by coupling with the aid of *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline. Briefly, 3 mg heparin and 4 mg of each protein were dissolved in 0.9 ml of distilled water and 3 mg *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline in 0.6 ml ethanol was added. After incubation at 4°C for 3 days with gentle shaking, the excess reagent was removed using ultrafiltration (UFP1 THK BK, exclusion MW 100,000, Nihon Millipore Ltd., Tokyo, Japan).

Biotinylation of $GlcNAc_{5-6}$ was performed as follows. Twenty mg of biotin hydrazide dissolved in 1 ml of DMSO was mixed with 50 mg of $GlcNAc_{5-6}$ in 3 ml of saline. The mixture was shaken at room temperature for 1 hr. The neoglycoprotein, $GlcNAc_{5-6}$-BSA, was prepared by coupling 2.5 mg of $GlcNAc_{5-6}$ and 40 mg of BSA by reductive amination with 7 mg of $NaCNBH_3$ in 2 ml of 0.2 M K_2HPO_4, pH 9.0. After incubation at 4°C for 7 days, the excess reagents were removed by dialysis.

SDS-PAGE and Western blotting

SDS-PAGE was carried out as described by Laemmli (20) using 9.5% polyacrylamide separation gels under reducing conditions with 5% mercaptoethanol. The electrophoresed proteins were transferred to a polyvinylidenedifluoride (PVDF) membrane (Nihon Millipore Ltd.) at 100 mA for 70 min at room temperature. The membrane was blocked with TBS containing 3% BSA, and then incubated with Con A-HRP or heparin-HRP solution (10 µg/ml in TBS) for 1 hr at room temperature. After washing membranes three times with TBS, development was carried out with 4-chloro-1-naphthol and H_2O_2 as described previously (19).

Solid phase assay

100 µl of PVL or one of the glycoprotein solutions was added to the wells of an Immulon 1 plate (Dynatech Laboratories, Chantilly, VA, USA) and immobilized for over 2 hr at room temperature. In the case of sulfatide, the wells of an Immulon 1 plate were coated with 50 µl aliquots of sulfatide solutions in ethanol, and then the lipid solutions were dried thoroughly at 37°C. Other all procedures were performed at room temperature. After washing wells with PBS and blocking with 3% BSA-PBS for 2 hr, 100 µl of HRP- or biotin-conjugated probes were added. After incubation for 1 hr, the wells were washed three times with PBS. In the case of HRP-probes, the color was developed with 0.04% *o*-phenylenediamine and 0.01% H_2O_2 in 0.1 M citrate-phosphate buffer (pH 5.0). In the case of biotin-probes, 100 µl of streptavidin-biotinylated HRP complex diluted to 1/1000 with PBS was added and incubated for 1 hr, followed by washing with PBS. Then the color was developed by the same procedure. In both cases, the absorbance of each well at 490 nm was read using a microplate reader (Model 3550, Bio Rad) and the averages of duplicate determinations were plotted. For inhibition assays, various concentrations of inhibitors were preincubated with immobilized PVL, or labeled PVL in some cases, and then incubated with ligands for 1 hr. The effects of ionic strength on the interaction of PVL with ligands were assayed by adding NaCl or Na_2SO_4 in the incubation buffer.

Affinity chromatography on heparin-Sepharose

Purified PVL (600 µg) in 500 µl of buffer A was incubated with heparin-Sepharose gel under the same conditions as $GlcNAc_{5-6}$-Sepharose gel in the purification procedure. After packing into the column (1 × 2.2 cm), the gel was washed with buffer A, and elution was tried with various eluants.

Chemical composition

For carbohydrate analysis, PVL (10 µg) was completely dried in a clean hydrolysis tube and hydrolyzed with a vapor of acid (H_2O:TFA:HCl = 41:9:10) at 100°C for 4 hr in a vessel containing N_2 gas. The hydrolysate was *N*-acetylated and subjected to 2-aminopyridylation using PALSTATION (Takara Shuzo Co., Otsu, Japan) and analyzed as reported previously (21). Total sugar content was calculated from the yield of fluorescent sugars using ribose as an internal standard. For amino acid composition, PVL (12 µg) was hydrolyzed with a vapor of 6 N HCl at 110°C. Amino acids were dabsylated with Aminochrome (JASCO Corporation, Hachioji, Japan) and analyzed according to the manufacturer's instructions. *N*-terminal amino acid sequences of PVL and its trypsin fragments were analyzed by the auto Edman degradation method using an Applied Biosystems 476A sequencer (Applied Biosystems, Foster City, CA, USA), as described previously (22).

Circular dichroism spectroscopy

CD spectra of PVL (0.1 mg/ml in PBS) were measured using Model J-725 Spectropolarimeter (JASCO Corporation), from 190 to 250 nm in the presence or absence of 1 mM GlcNAc, 5 mM GlcNAc, or 0.3 mg/ml heparin in a quartz cell (cell length 0.05 cm). CD spectra of PVL were obtained by averaging 10 wavelength scans and were corrected by subtracting scans of buffer or ligand in buffer. The analysis was performed with the computer software, SSE-338 using reference CD data of Yang (23).

Results

Purification of PVL

Originally, PVL was purified by a three-step procedure: chitin-column affinity chromatography, ion-exchange chromatography, followed by rechromatography on chitin-column (7). $GlcNAc_{5-6}$-Sepharose 6B was used as an affinity absorbent instead of chitin polysaccharides in this study. As shown in Figure 1, PVL was

adsorbed onto the column and specifically eluted with 0.2 M GlcNAc but not with 50 mM GlcNAc, although that concentration was adequate to elute PVL from a chitin column (7). The yield of 20 mg of PVL from 20 g of *P. velutina* tissue was three times higher than the original method (7). The maximum adsorption capacity was 13 mg/g gel.

PVL gave a single band corresponding to about 40 kDa, as shown in Figure 2A, which agrees with the previous report (7), and the amino acid composition was almost the same as that previously reported (data not shown). PVL was thus purified by single-step affinity chromatography. The high efficiency of the affinity adsorbent used here would be due to the high ligand concentrations obtained by reductive amination of oligosaccharides and low non-specific binding to the adsorbent, which is free from charged groups (18). *N*-terminal amino acid sequencing was tried using both PVL solution and blotted membrane, but the sequence was blocked. Partial peptide sequences, VIDNFGYNQGWR, GAVRLVLKE, VIADLWXD, and ISTNNGN, were obtained from a trypsin fragment, but any protein having homology of more than 50% with each of these sequences was not found by searching the PIR, PRF, and Swiss Prot databases.

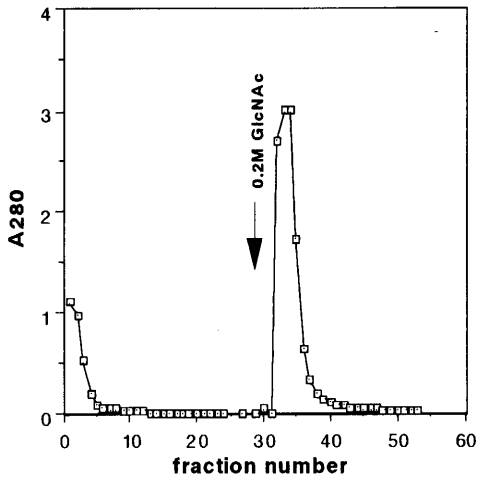

Figure 1. Purification of PVL by one-step affinity chromatography. The acid-treated extract of the fruiting body of *P. velutina* was mixed with 1.5 g of $GlcNAc_{5-6}$-Sepharose gel, followed by dialysis against buffer A, and then packed into the column (1 × 2.2 cm). After being washed with buffer A, PVL was eluted with buffer A containing 0.2 M GlcNAc at the arrow, as described in the text.

Carbohydrate chain of PVL

The carbohydrate composition of PVL was glucose, xylose, mannose, and galactose in a molar ratio of 10:7:6:2, with only trace amounts of GlcNAc, GalNAc, and fucose. The total carbohydrate content was calculated to be 10% (w/w). The presence of glucose and mannose is compatible with the positive staining of Con A-HRP shown in Figure 2B. The staining intensity was similar to that of human vitronectin, a heparin-binding glycoprotein which contains three *N*-linked carbohydrate chains of mainly biantennary complex type (24), but the carbohydrate composition of PVL suggested that the glycan structure of PVL is quite different from them.

Interaction of PVL with heparin and N-acetylchitooligosaccharide

After electrophoresis and electroblotting onto PVDF membrane, PVL was allowed to react with heparin-HRP. As shown in Figure 2C, the band of PVL was strongly stained with heparin-HRP as well as vitronectin.

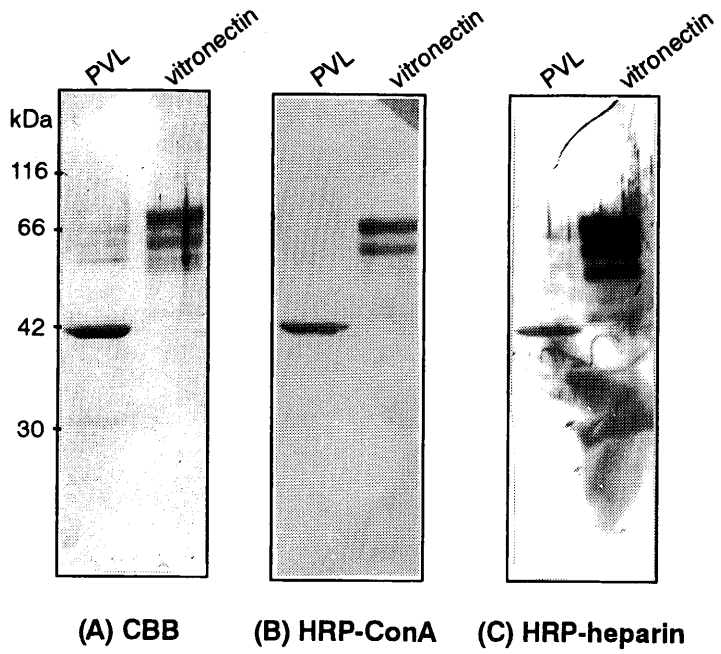

Figure 2. SDS-PAGE (A) and reactivities of PVL with Con A-HRP (B) and heparin-HRP (C) on membrane. Five μg of PVL or human vitronectin was loaded on each lane of 9.5% acrylamide gel and Western blotting was performed using PVDF membrane. Proteins were stained with Coomassie Brilliant Blue (CBB) (**A**), or allowed to react with Con A-HRP (**B**) or heparin-HRP (**C**) as described in the text. Molecular weight markers are shown in the left margin.

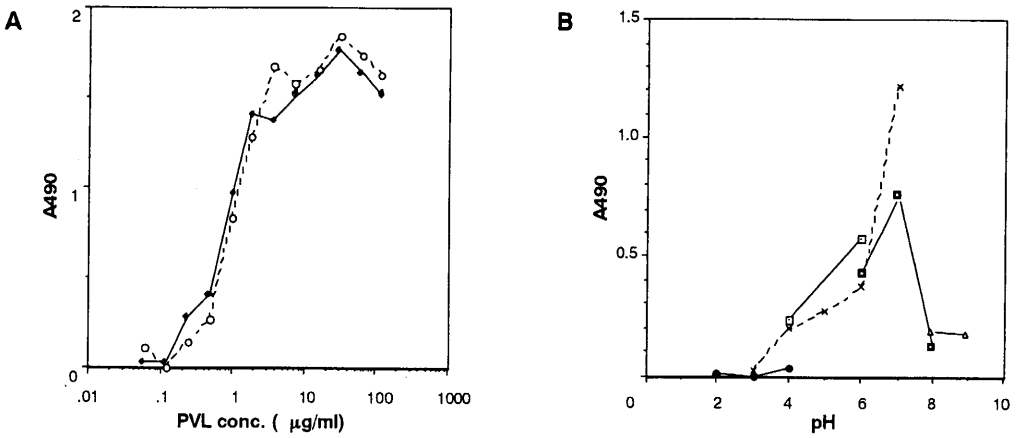

Figure 3. Reactivities of PVL with heparin-HRP by solid phase assay. The solution of PVL was serially diluted and immobilized on microtiter plates. Heparin-HRP was used diluted to 1/1000 in buffers of various pH. The following procedures were as described in the text: **A**, concentration-dependency in PBS (pH 7.0) of PVL prepared in our laboratory (—◆—), and commercial PVL (····⊙····); **B**, pH-dependency of the binding. Buffers used: pH 2-4: 10 mM glycine-HCl-0.13 M NaCl (—●—); pH 4-6: 10 mM acetate/Na-0.13 M NaCl (—□—); pH 6-8: 10 mM phosphate/Na-0.13 M NaCl (—■—); pH 8-9: 10 mM Tris/HCl-0.13 M NaCl (—△—); pH 3-7: 10 mM sodium citrate/phosphate-0.13 M NaCl (····×····).

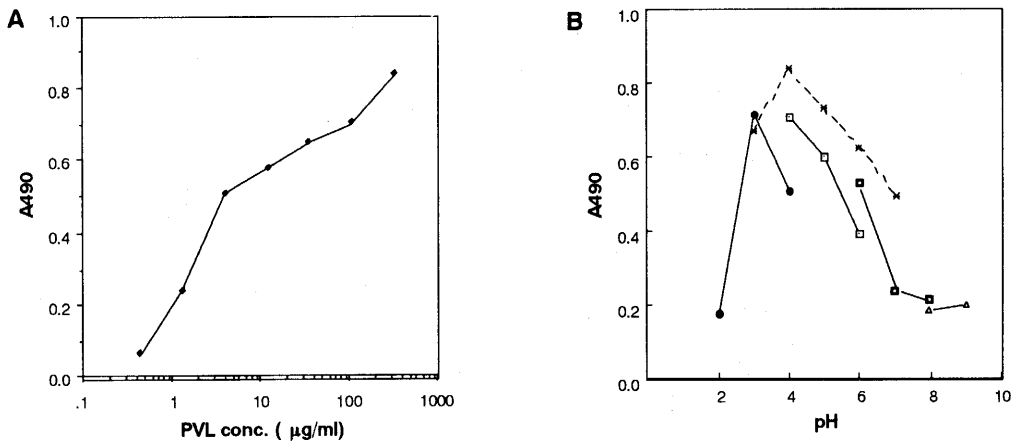

Figure 4. Reactivities of PVL with biotinyl $GlcNAc_{5-6}$ by solid phase assay. The solution of PVL was serially diluted in PBS and immobilized on microtiter plates. Biotinyl $GlcNAc_{5-6}$ (5 mg/ml) was diluted to 1/1000 in buffers of various pH. **A**, concentration-dependency in 10 mM citrate-phosphate-0.13 M NaCl at pH 4.0; **B**, pH-dependency of the binding. Buffers used: pH 2-4: 10 mM glycine-HCl-0.13 M NaCl (—●—); pH 4-6: 10 mM acetate/Na-0.13 M NaCl (—□—); pH 6-8: 10 mM phosphate/Na-0.13 M NaCl (—■—); pH 8-9: 10 mM Tris/HCl-0.13 M NaCl (—△—); pH 3-7: 10 mM sodium citrate/phosphate-0.13 M NaCl (···✖···).

Binding assays of PVL with heparin were also performed by solid phase assay. PVL immobilized in the well bound to heparin-HRP concentration-dependently at various pH from 4 to 9, as shown in Figure 3A where the result at pH 7.0 is shown as representative, in the concentration range of 60 ng/ml to 25 μg/ml of PVL. Unconjugated HRP did not bind with PVL immobilized in the well suggesting that this binding is due to the affinity of PVL to heparin rather than to HRP. Commercial PVL purified by the previous procedure (7) showed the same binding behavior to heparin (Figure 3A). As shown in Figure 3B, the maximum binding was observed at around pH 7 in two different buffer systems: combined buffers of glycine-HCl (pH 2-4), AcONa-AcOH (pH 4-6), NaH_2PO_4-Na_2HPO_4 (pH 6-8), and Tris-HCl (pH 8-9), and unified citrate-phosphate buffers (pH 2-7). Alternatively, when heparin-BSA was immobilized on the well and HRP-PVL used as a probe, a similar dose-dependency was observed and the optimal binding pH was pH 6-7 (data not shown). These results suggest that the heparin binding activity is not an artificial effect caused by the immobilization of PVL onto the solid surfaces.

PVL immobilized on the well bound concentration-dependently to biotinyl $GlcNAc_{5-6}$ at pH 2 to 9, as shown in Figure 4A, where the result at pH 4.0 is shown as representative. The maximum binding was observed at pH 3-4 as shown in Figure 4B, which differed from that of heparin binding. When $GlcNAc_{5-6}$-BSA neoglycoprotein was immobilized on the well, HRP-PVL showed the same pH dependency in binding with an optimal pH of around 4 (data not shown).

Effects of polysaccharides or ionic strength on heparin binding of PVL

Based on its binding characteristics to heparin (Figures 3A and B), a PVL solution of 10 μg/ml and incubation at pH 7.0 were used for the inhibition studies with various acidic polysaccharides. Fucoidan, polygalacturonic acid, dextran sulfate, heparin (Figure 5A), pectin, and alginic acid (data not shown) inhibited the binding with heparin, whereas chondroitin, chondroitin sulfate A, B, and C, heparan sulfate, hyaluronic acid, colominic acid, keratan sulfates from both bovine cornea and whale nasal cartilage, DNA, and GlcNAc did not inhibit it even at the concentration of 6 mg/ml. These results indicate that PVL does not recognize internal GlcNAc residue of heparin although it contains α1-4 linked GlcNAc as a component.

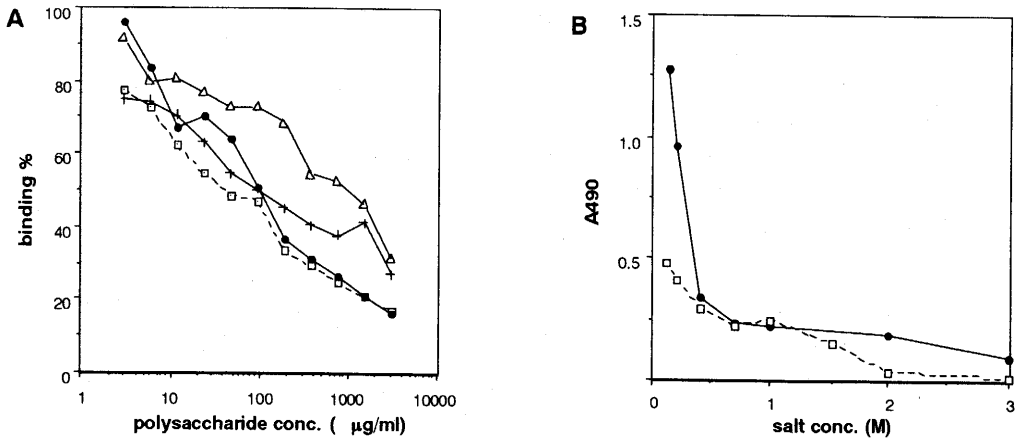

Figure 5. Effects of various polysaccharides (A) and ionic strength (B) on the interaction between heparin-HRP and PVL. The solution of PVL (10 μg/ml) was immobilized on microtiter plates for inhibition assay in PBS (pH 7.0). **A**, various concentrations of polysaccharides were preincubated with immobilized PVL for 1 hr before addition of heparin-HRP. Polysaccharides: heparin (—△—), fucoidan (···□··), dextran sulfate (—+—), and polygalacturonic acid (—●—). The following substances showed no inhibition: GlcNAc, chondroitin sulfates A, B, C, heparan sulfate, hyaluronic acid, keratan sulfate, colominic acid, and DNA. The same results were obtained in triplicate experiments. The binding % represents the proportion of the absorbance at 490 nm to that in the absence of inhibitors. **B**, Heparin-HRP was prepared in buffers of various salt concentrations and added to the well. Symbols used are: NaCl (—●—), Na_2SO_4 (·····□·····).

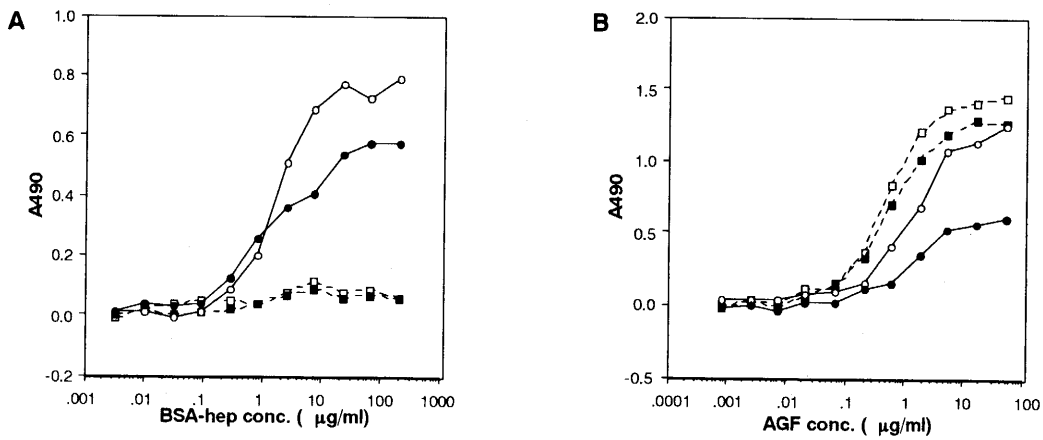

Figure 6. Effects of protecting carbohydrates during biotinylation of PVL on the binding of heparin-BSA (A) or AGF (B). Solutions of heparin-BSA (**A**) or AGF (**B**) in PBS were serially diluted and immobilized on microtiter plates. PVL was biotinylated in the presence of heparin for 15 min (—●—) or 4 hr (—○—), or GlcNAc for 15 min (····■····) or 4 hr (····□····). Biotinyl PVL was used at a concentration of 10 μg/ml in PBS (pH 7.0) and added to the wells, following procedures as described in the text.

The heparin binding of PVL decreased with the increasing concentration of NaCl or Na_2SO_4, as shown in Figure 5B. These findings suggested that the binding of PVL to heparin is due mainly to the electrostatic interaction and that sulfate or carboxyl groups of acidic polysaccharides may contribute to the binding. The comparison between polygalacturonic acid and colominic acid, as well as that between fucoidan and heparan sulfate, suggested that not only the anionic charges but their spatial location on polysaccharides is significant for binding with PVL.

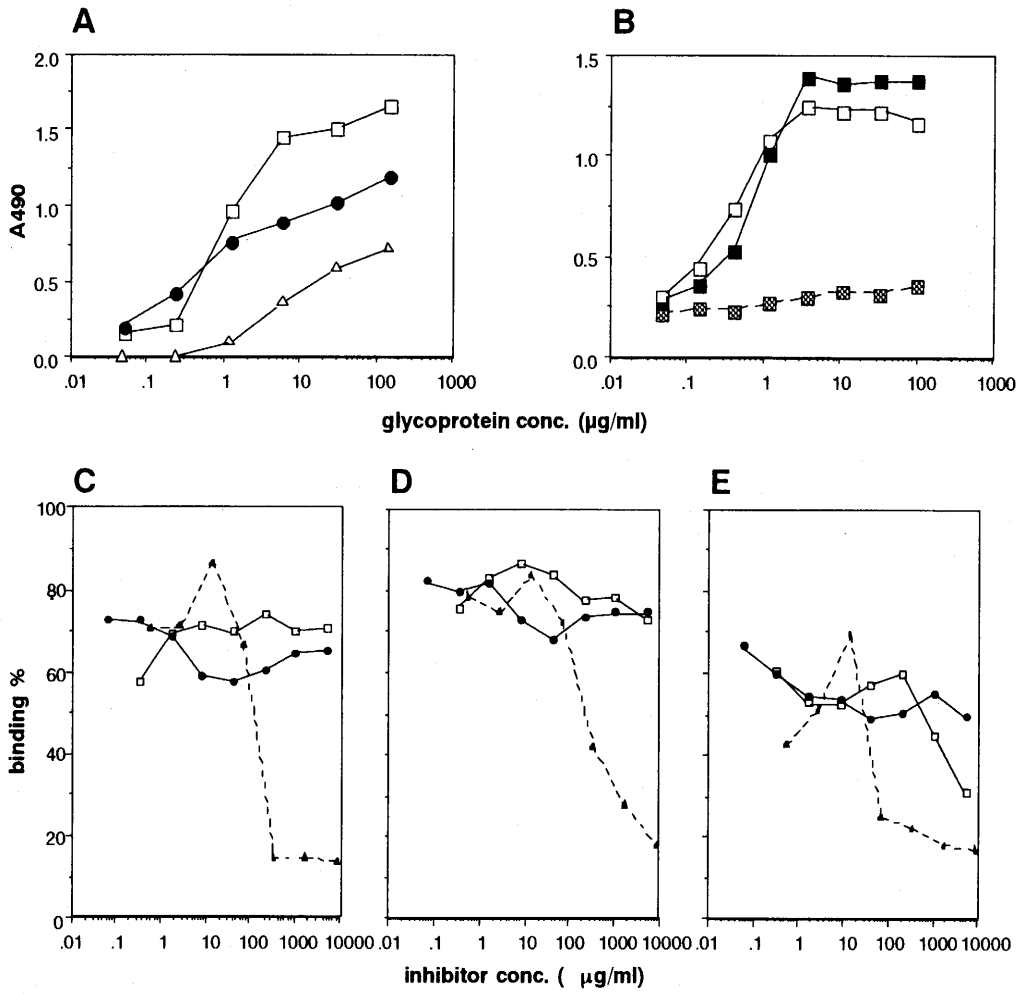

Figure 7. Reactivities of biotinyl PVL with sialoglycoproteins by solid phase assay. Solutions (100 μl) of glycoproteins were serially diluted in PBS and immobilized on microtiter plates. Biotinyl PVL (10 μg/ml) was added to the wells and a solid phase assay was performed as described in the text. **A**, reactivity with sialoglycoproteins: fetuin (—□—), BSM (—●—), and transferrin (—△—). **B**, effects of desialylation and degalactosylation of fetuin on binding of biotinyl PVL. Symbols used are: fetuin (—□—), asialofetuin (– – ▣ – –), and asialo-agalactofetuin (—■—). **C-E**, effects of GlcNAc, NeuAc, or heparin on the interaction between sialoglycoprotein and biotinyl PVL. After immobilization of 10 μg/ml of fetuin (**C**), BSM (**D**), or transferrin (**E**), biotinyl PVL (10 μg/ml) preincubated with various concentrations of GlcNAc (·····▲····), NeuAc (—□—), or heparin (—●—) for 1 hr was added to each well. The binding percentage is represented by the proportion of the absorbance at 490 nm to that in the absence of inhibitors.

Effect of protecting carbohydrates during biotinylation of PVL on the binding activities

In the course of the study, labeling of PVL with NHS-biotin instead of HRP was found to attenuate the heparin-binding activity. The sensitivities of each binding site for heparin and GlcNAc to biotinylation were therefore compared. As shown in Figure 6A, the binding activities for heparin-BSA were completely retained in PVL biotinylated in the presence of heparin, at both reaction times of 15 min and 4 hr, while it was completely lost by biotinylation in the absence of heparin. The presence of GlcNAc during biotinylation did not protect the heparin-binding activity at all. On the contrary, the binding activity for AGF was retained in all cases, as shown in Figure 6B, suggesting that some Lys residues of PVL are essential for binding to heparin but not to GlcNAc. These findings, plus the observation that heparin binding was not inhibited with GlcNAc in the inhibition assay, indicate that the binding sites for acidic polysaccharides and GlcNAc are independently located on PVL. For both GlcNAc- and heparin-binding, longer reaction times for biotinylation give higher sensitivity

for detection, probably because more biotinyl groups could be introduced into PVL.

Affinity chromatography on heparin-Sepharose

Purified PVL was completely adsorbed onto a heparin-Sepharose column, but it was not eluted with any eluants tested: heparin (8 mg/ml in buffer A), 4 M NaCl, 1 M Na_2SO_4, 0.1 M $Na_2B_4O_7$, 0.01 M glycine-HCl (pH 2.0 and 3.0), 0.1 M EDTA, 0.1 M AcOH, and 50% glycerol. The binding behavior on heparin-Sepharose was apparently irreversible and unusual among heparin-binding proteins hitherto examined on this adsorbent (17, 25). As the irreversible binding was not observed for $GlcNAc_{5-6}$-Sepharose column (Figure 1), it is considered to be caused by the strongly binding to multi-valent heparin ligands on a Sepharose gel bead.

Interaction of PVL with sialoglycoproteins

Fetuin, transferrin, or BSM were used as representatives of sialoglycoproteins and immobilized on a microtiter plate for the binding assays using biotinyl PVL. As shown in Figure 7A, the binding of biotinyl PVL was observed for the sialoglycoproteins examined. The fetuin binding of biotinyl PVL was found to be completely prevented by treatment with neuraminidase, as shown in Figures 7B, or by acid treatment of fetuin (data not shown). Biotinyl PVL bound to asialo-agalactofetuin (Figure 7B) because GlcNAc had been exposed at the non-reducing terminals. In addition, HRP-PVL was shown to bind to fetuin and asialo-agalactofetuin, but not to asialo-fetuin, by Western blot analysis (data not shown). As shown in Figures 7C-E, GlcNAc commonly inhibited the interaction of PVL with the sialoglycoproteins. These results clearly suggest that PVL bound to intact sialoglycoproteins by recognizing non-reducing terminal NeuAc residues at GlcNAc-binding site but not by interacting with other parts of the glycoproteins.

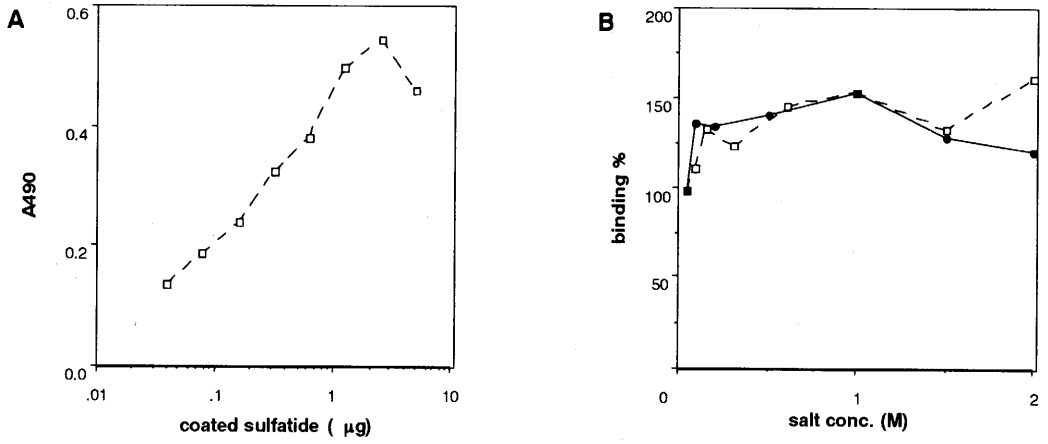

Figure 8. Reactivities of biotinyl PVL with sulfatide by solid phase assay. Solutions of sulfatide in ethanol was immobilized on microtiter plates by evaporization of ethanol. PVL (10 μg/ml) biotinylated in the presence of GlcNAc was added to the wells and a solid phase assay was performed in PBS (pH 7.0). **A**, concentration-dependency of sulfatide; **B**, biotinyl PVL (10 μg/ml) was incubated with immobilized sulfatide (2 μg/well) in the presence of various concentrations of salts. The binding % represents the proportion of the absorbance at 490 nm to that in the absence of salts. Symbols used are NaCl (—●—), Na_2SO_4 (·····□·····).

Interaction of PVL with sulfatide

As shown in Figure 8A, biotinyl PVL bound to sulfatide concentration-dependently, but the binding was not inhibited by GlcNAc, heparin, or NeuAc (data not shown). When PVL biotinylated in the presence of

heparin was used, the same results for inhibition potency were obtained (data not shown). In contrast to the heparin binding, sulfatide binding of PVL was not inhibited by either chloride or sulfate ions (Figure 8B), suggesting that the binding is not due to an electrostatic interaction. The binding mode with sulfatide seems distinct from those with other ligands, but the nature of the sulfatide binding is yet unclear.

Circular dichroism spectra

In order to examine the conformational changes of PVL, circular dichroism of PVL was measured in the presence or absence of specific sugars, GlcNAc, and heparin. As shown in Figure 9A, the intensities around 230 nm increased and those around 210 nm decreased in the presence of GlcNAc. Calculated α-helix contents of PVL decreased to about 13% in the presence of 5 mM GlcNAc from 15% in native PVL. The β-sheet content of PVL was unaffected by GlcNAc and calculated to be 67% ± 1.2%. The spectrum in the presence of heparin was the same as that of native PVL (Figure 9B). These results suggested that the binding of GlcNAc induced a certain conformational change in PVL.

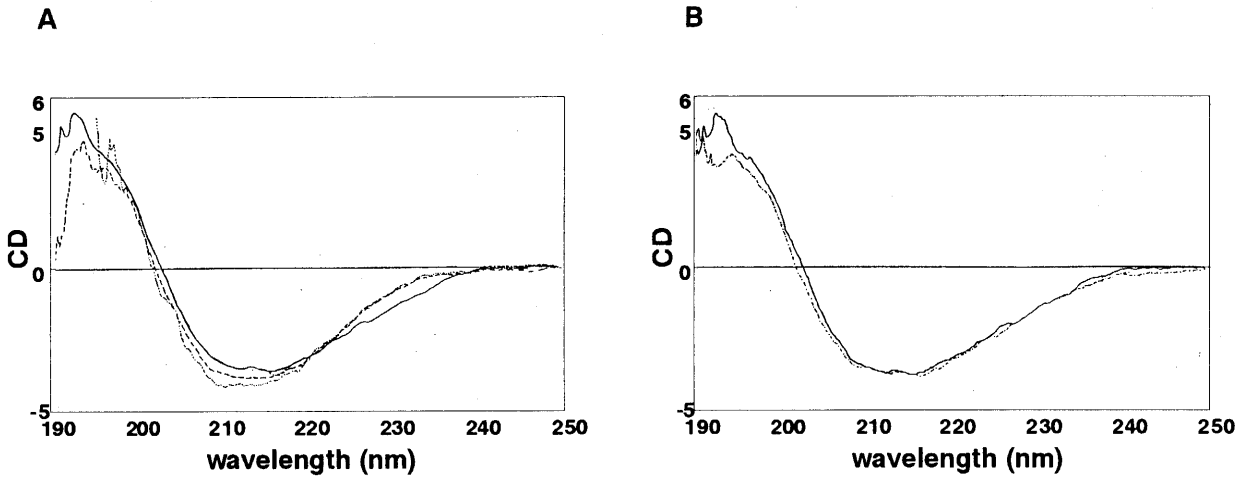

Figure 9. CD spectra of PVL. CD spectra of PVL (0.1 mg/ml) in PBS were measured in the absence of carbohydrate (———), or in the presence of 1 mM GlcNAc (— — —) or 5 mM GlcNAc (·····) (**A**), or in the presence of 0.3 mg/ml heparin (· — · — ·) (**B**), as described in the text.

Discussion

Solid phase assays utilizing neoproteoglycans, neoglycoproteins, and biotinyl oligosaccharides, in combination with HRP- or biotin-labeled lectin, showed that PVL binds to the glycoconjugates: acidic polysaccharides, sialoglycoproteins, and sulfatide, as summarized in Table 1. Most of these glycoconjugates do not contain non-reducing terminal GlcNAc residues; therefore, the binding specificities of PVL to glycoconjugates are quite distinct from those toward oligosaccharides reported previously (7, 26). Disregarding the multispecificity of PVL, use of PVL as a sugar-specific probe in histochemistry or analyses glycoconjugates could produce misleading information about the nature of glycoforms.

Acidic polysaccharide-binding activity has been found for the first time in mushroom lectins. The sites that interact with GlcNAc and acidic polysaccharides are considered to be independently located on the PVL molecule for the following reasons: 1. the binding activity for GlcNAc was not inhibited with heparin, and *vice versa*; 2. biotinylation of PVL destroyed the heparin-binding activity while retaining GlcNAc-binding activity

(Figures 6); 3. heparin and GlcNAc binding showed different pH dependencies (Figures 3B and 4B); and 4. a distinct conformational change of CD spectra of PVL was induced by GlcNAc but not by heparin (Figure 9). The binding of PVL was observed in only limited acidic polysaccharides suggesting that a particular spatial location of anionic charges on the polysaccharide is required for binding. When using PVL as a detection reagent, extensive biotinylation of amino groups of PVL, in addition to employing the incubation at pH 4.0 instead of pH 7.0 for the binding assay, proved useful to circumvent the interference with acidic polysaccharides without harming the GlcNAc-binding activity.

Table I Multispecificity of PVL compared with AOL

Lectin	Ligands		Nature of interaction	Reference
PVL	oligosaccharide	non-reducing terminal GlcNAc	carbohydrate specific (GlcNAc)	7, 26
	acidic polysaccharide	heparin dextran sulfate fucoidan polygalacturonic acid pectin alginic acid	electrostatic but requires certain location of negative charges not inhibited with GlcNAc	this work
	sialoglycoprotein	fetuin transferrin bovine submaxillary mucin	carbohydrate specific (NeuAc) inhibited with GlcNAc	this work
	sulfated glycolipid	sulfatide	not electrostatic not inhibited with GlcNAc & heparin	this work
AOL	oligosaccharide	Galβ3GalNAcαSer/Thr	carbohydrate specific	35
	acidic polysaccharide	fucoidan dextran sulfate	electrostatic but not depend on charge density	33
	glycoprotein	fetuin asialofetuin porcine stomach mucin	carbohydrate specific but requires intact peptide backbone	33, 35
	sulfated glycolipid	sulfatide	electrostatic not inhibited with gangliosides	33

On the other hand, PVL bound to sialoglycoproteins and this binding was prevented completely by desialylation (Figure 7A and B). In parallel experiments, biotinyl B-SJA-I, a galactose-specific lectin from bark of *Sophora japonica*, bound well to asialofetuin but not to fetuin (data not shown). Degalactosylation of asialofetuin considerably lowered the binding to B-SJA-I. The reversed reactivity of PVL and B-SJA-I to fetuin and its derivatives indicate that these glycoproteins were immobilized enough for lectin detection, regardless of their oligosaccharide structures, and these lectins respectively recognized the specific carbohydrates in oligosaccharides of glycoproteins. In addition, sialoglycoprotein-binding of PVL was inhibited with GlcNAc (FIgure 7C-E), suggesting that PVL recognized NeuAc residues of sialoglycoproteins at GlcNAc-binding site although previous works reported that immobilized PVL interacted with the non-reducing terminal GlcNAc residue but not with the non-reducing terminal NeuAc residue (26). Since all the tested sialoglycoproteins contain GlcNAc as a constituent sugar, the fine oligosaccharide structures have to be taken into account in interpreting the results. Bovine fetuin contains both *N*-linked glycans and *O*-linked glycans on a molecule.

The major oligosaccharides have been elucidated to be *N*-glycans of a sialylated triantennary complex type (27) and *O*-glycans having a sialylated core-type 1 (28, 29), but the presence of GlcNAc residues at non-reducing end has not been found yet. Human transferrin contains only *N*-linked complex-type glycans. Though glycans of a sialylated biantennary complex type are a major component, transferrin contains, as a minor component (5%), a tetraantennary glycan having an incomplete structure with one antenna terminated by GlcNAc (30). Such a tetraantennary glycan would not have high affinity to PVL since the three other sialylated branches on the same glycan would interfere the PVL binding. On the other hand, BSM has been reported to possess *O*-linked oligosaccharides containing a core-type 4 structure having non-reducing terminal β1-3- and β1-6-linked GlcNAc residues (31), and thus BSM and asialo-BSM may interact with PVL at the GlcNAc residues.

The multispecificity of PVL is compared with a GalNAc-specific lectin from the nematophagous fungus, *Arthrobotrys oligospora* lectin (AOL) in Table 1 (32). AOL showed affinity to fucoidan and dextran sulfate, but not to heparin, as detected by inhibition of sulfatide binding (33). *Agaricus bisporus* lectin (ABL), a mushroom lectin of the *Agaricoles* order like PVL, exhibited binding specificities similar to AOL and bound with sulfatide, some sialoglycoproteins, and phospholipids, in addition to a GalβGalNAcα-Ser/Thr structure (34). AOL and ABL are suggested to belong to a novel family of saline soluble fungal lectins sharing similar molecular structures, molecular weights (16-17 kDa) and binding specificities (34). In contrast, PVL differed from AOL and ABL in its molecular weight and oligosaccharide specificity, and has a more diverse binding spectrum than AOL and ABL, especially in having affinities to heparin and polygalacturonic acid. The secondary structure of PVL calculated from CD spectroscopy differed considerably from that of AOL: 34% β-sheet and 21% α-helix. In addition, we did not find a sequence similarity between PVL and these lectins. PVL may be classified, therefore, as a novel multispecific lectin of the higher fungi that is structurally different from AOL and ABL.

Despite their diversities, however, the multispecific lectins may be distributed widely in higher fungi. The biological significance of the multispecific saline-soluble lectins is very interesting. We have shown that PVL can interact simultaneously with different receptors, e.g., polygalacturonic acid, a component of the plant cell wall, and GlcNAc on bacterial cell surfaces. The irreversible binding of PVL to heparin-Sepharose gel observed in this study might be biologically relevant to its function because strong binding to a multivalent ligand such as a cell surface would be physiologically advantageous for a saprophyte. PVL and other lectins may function as multi-ligand adhesion molecules which are necessary for survival of both symbiotic and parasitic fungi, e.g., they can initiate chemotactic responses to link fungi with plant or animal host surfaces, to form fruiting bodies, or to mobilize defense systems. Further investigation of the biological receptors of PVL as well as its developmental regulation is under way to elucidate the function of this lectin.

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