J. Biochem. 126, 530-537 (1999)

Multi-Specificity of a *Psathyrella velutina* Mushroom Lectin: Heparin/Pectin Binding Occurs at a Site Different from the *N*-Acetylglucosamine/*N*-Acetylneuraminic Acid-Specific Site

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Received May 27, 1999; accepted June 29, 1999

An *N*-acetylglucosamine (GlcNAc)/*N*-acetylneuraminic acid-specific lectin from the fruiting body of *Psathyrella velutina* (PVL) is a useful probe for the detection and fractionation of specific carbohydrates. In this study, PVL was found to exhibit multi-specificity to acidic polysaccharides and sulfatides. Purified PVL and a counterpart lectin to PVL in the mycelium interact with heparin neoproteoglycans, as detected by both membrane analysis and solid phase assay. The pH-dependencies of the binding to heparin and $GlcNAc_{5-6}$ differ. The heparin binding of PVL is inhibited best by pectin, polygalacturonic acid, and highly sulfated polysaccharides, but not by GlcNAc, colominic acid, or other glycosaminoglycans. Sandwich affinity chromatography indicated that PVL can simultaneously interact with heparin- and GlcNAc-containing macromolecules. Extensive biotinylation was found to suppress the binding activity to heparin while the GlcNAc binding activity is retained. On the other hand, biotinyl PVL binds to sulfatide and the binding is not inhibited by GlcNAc, *N*-acetylneuraminic acid, or heparin. These results indicate that PVL is a multi-ligand adhesive lectin that can interact with various glycoconjugates. This multispecificity needs to be recognized when using PVL as a sugar-specific probe to avoid misleading information about the nature of glycoforms.

Key words: acidic polysaccharides, fungal lectin, glycoprobe, multispecificity, sulfatide.

A number of fungal lectins have been reported, some of which are considered to be responsible for possible symbiotic mechanisms in bacterium-host systems (reviewed in Refs. *1* and *2*). Only a few instances have been reported, however, of the involvement of a fungal lectin in symbiotic or parasitic recognition such as fungal-plant, fungal-fungal, fungal-algal, or fungal-nematode interactions (*2*). The nature of the involvement of lectins in saprophytic interactions of *Basidiomycetes* (mushrooms) growing in humic soil remains unclear.

On the other hand, several lectins from mushrooms have proved to be useful probes for the detection and fractionation of specific carbohydrate structures of glycoconjugates (*3-6*). A lectin from the fruiting body of *Psathyrella velutina* mushroom (PVL) is known to be specific for non-reducing terminal *N*-acetylglucosamine (GlcNAc) (*7, 8*). The remarkable specificity toward non-reducing terminal GlcNAc residues 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 several pathological states, such as IgGs from rheumatoid arthritis patients (*9*) and rat hepatoma (*10*). In fact, the use of PVL as a GlcNAc-detecting reagent in diagnostic and biochemical analyses is increasing (*11-14*).

Recently, we found that the differential binding of PVL to oligosaccharides appears to be due to both the number and linkage of non-reducing terminal NeuAc and GlcNAc residues. PVL is, therefore, capable of fractionating sialo-oligosaccharides and detecting sialoglycoconjugates when used in combination with desialylation (*15*).

In this study, the interactions of PVL with acidic polysaccharides were investigated and the relationship to GlcNAc/NeuAc binding was determined. Suitable conditions were found for suppressing the binding activity to acidic polysaccharides while retaining GlcNAc/NeuAc binding activity. It would be valuable to use the lectin as a carbohydrate-specific reagent to elucidate its correct binding specificity and find a way to regulate binding activity. Taking into account the presence of a counterpart lectin to PVL in mycelium, the biological significance of the lectin in this saprophytic fungus will be discussed.

MATERIALS AND METHODS

Materials—PVL from fruiting bodies and mycelium lectin were purified as described previously (*15*). Mycelia

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Abbreviations: EEDQ, *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline; HRP, horseradish peroxidase; NeuAc, *N*-acetylneuraminic acid; PBS, 10 mM phosphate-buffered saline (pH 7.0); PVL *Psathyrella velutina* lectin; TBS, 10 mM Tris-buffered saline (pH 7.5).

from *P. velutina* were cultured in medium containing 1% glucose, 1% malt extract, and 0.4% yeast extract at 25-28°C in the dark with rotary shaking for 1 month. Horseradish peroxidase (HRP) (Toyobo, Osaka) and biotin hydrazide (ICN Immunobiologicals, Costa Mesa, CA, USA) were used for the preparation of binding probes. *N*-Hydroxysuccinimide biotin, polygalacturonic acid (orange), pectin (apple), fucoidan (*Fucus vesiculosus*; sulfur content, 8.5%), and sulfatide; Gal(3-SO_4)β1-1-ceramide (bovine brain) were purchased from Sigma Chem., St Louis, MO, USA. Colominic acid (*Escherichia coli*) and alginic acid were obtained from Nacalai Tesque, Kyoto. Streptavidin-biotinylated HRP complex was purchased from Amersham, Buckinghamshire, UK. Heparan sulfate (porcine kidney; sulfate content, 9.0%) (*16*), keratan sulfate (whale nasal cartilage, sulfate content, 15.0%) (*17*), and human vitronectin (*18*) were prepared in our laboratory as described previously. *N*-Acetylchitooligosaccharides (a mixture of pentamer and hexamer, $GlcNAc_{5\text{-}6}$), 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. Heparin (porcine intestinal mucosa) was from Wako Pure Chemicals (Osaka). Dextran sulfate (molecular mass, ~500,000; sulfur content, 17%) and dextran T-500 were obtained from Pharmacia Fine Chemicals AB (Uppsala, Sweden).

Preparation of Biotinyl PVL—Biotinylation of PVL was performed as described previously (*15*) by employing reaction times of 15 min or 4 h in early experiments. For both GlcNAc- and heparin-binding, longer reaction times for biotinylation gave higher detection sensitivity, probably because more biotinyl groups could be introduced into PVL. Therefore, the reaction time of 4 h was used in this study to achieve extensive biotinylation. To protect the binding sites on PVL, GlcNAc, or heparin at a final concentration of 10 mM or 1.6 mg/ml, respectively, was added to the reaction buffer.

Preparation of Neoproteoglycans, Neoglycoprotein, or Biotinyl $GlcNAc_{5\text{-}6}$—Neoproteoglycans, heparin-HRP, and heparin-BSA, were prepared by coupling with the aid of *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ). Briefly, 3 mg heparin and 4 mg of each protein were dissolved in 0.9 ml of distilled water, and 3 mg EEDQ in 0.6 ml ethanol was added. After incubation at 4°C for 3 days with gentle shaking, the excess reagent was removed by ultrafiltration (UFP1 THK BK, exclusion MW 100,000, Nihon Millipore, Tokyo) with PBS.

The neoglycoprotein $GlcNAc_{5\text{-}6}$-BSA was prepared by coupling 2.5 mg of $GlcNAc_{5\text{-}6}$ 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. After incubation at 4°C for 7 days, the excess reagents were removed by dialysis against PBS. Biotinylation of $GlcNAc_{5\text{-}6}$ was performed by dissolving 20 mg biotin hydrazide in 1 ml DMSO mixed with 50 mg $GlcNAc_{5\text{-}6}$ in 3 ml saline and shaking at room temperature for 1 h.

SDS-PAGE and Binding Assay with Membrane-Bound Heparin-HRP—SDS-PAGE was carried out as described by Laemmli (*19*) using 11% polyacrylamide separation gels under reducing conditions with 5% mercaptoethanol. The electrophoresed proteins were transferred to a polyvinylidenedifluoride (PVDF) membrane (Nihon Millipore) at 110 mA for 70 min at room temperature. The membrane was blocked with 3% BSA-TBS, and then incubated with heparin-HRP solution diluted to 1/1,000 in TBS for 1 h at room temperature. The membranes were washed three times with TBS, and developed with 4-chloro-1-naphthol and H_2O_2 as described previously (*20*).

Solid Phase Assay—PVL, heparin-BSA, or $GlcNAc_{5\text{-}6}$-BSA in PBS (100 μl) was added to the wells of an Immulon 1 plate (Dynatech Laboratories, Chantilly, VA, USA) and immobilized overnight at 4°C. 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. All other procedures were performed at room temperature. After washing the wells with PBS and blocking with 3% BSA-PBS for 2 h, 100 μl of HRP- or biotin-conjugated probe was added. After incubation for 1 h, the wells were washed three times with PBS. In the case of HRP-probes, the color was developed and read at 490 nm using a microplate reader as described previously (*15*). In the case of biotin-probes, 100 μl of streptavidin-biotinylated HRP complex diluted 1/1,000 with PBS was added for 1 h, and then the wells were washed with PBS. The color was developed by the same procedure. In both cases, the averages of duplicate determinations were plotted. For inhibition assays, various concentrations of inhibitors were preincubated with immobilized PVL, in some cases labeled PVL, and then incubated with ligands for 1 h. The effects of ionic strength on the interaction of PVL with ligands were determined by adding NaCl or Na_2SO_4 to the incubation buffer.

Circular Dichroism Spectroscopy—CD spectra of PVL (0.1 mg/ml in PBS) were measured at 190 to 250 nm with a Model J-725 Spectropolarimeter (JASCO, Tokyo) 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 for buffer or ligand in buffer. The analysis was performed with computer software SSE-338 using the reference CD data of Yang (*21*).

Sandwich Affinity Chromatography of Heparin Neoproteoglycan on PVL Preadsorbed by diN-Acetylchitobiamyl Sepharose—All procedures were carried out using PBS at 4°C. Two milliliters of di*N*-acetylchitobiamyl Sepharose was incubated with purified PVL (500 μg) overnight and then packed into the column (0.75×4.5 cm) and washed with 15 ml of PBS. One milliliter of heparin-HRP diluted 1/1,000 was applied to the column and eluted with 15 ml of PBS and then by 0.2 M GlcNAc. A 100 μl aliquot of each eluted fraction (1 ml) was added to a well of a microtiter plate and protein immobilization was performed overnight. The elution of heparin-HRP was monitored by measuring the peroxidase activity, and color was developed by the same procedure as described for the solid phase assay. A control experiment was performed by the same procedures without the preincubation of the di*N*-acetylchitobiamyl Sepharose gel with PVL.

RESULTS

Interaction of PVL and Mycelium Lectin with Heparin on the Membrane After electrophoresis and electroblot-

ting onto a PVDF membrane, PVL and the mycelium lectin of *P. velutina* were allowed to react with heparin-HRP. As shown in Fig. 1, membrane analysis revealed that both *P. velutina* lectins bind to heparin-HRP. The staining intensity is similar to that of human vitronectin, a heparin-binding adhesive glycoprotein in the plasma.

The mycelium lectin, a novel lectin purified in this study by the same procedures used for PVL purification (*15*), shows hemagglutinating activity toward rabbit erythrocytes. The minimum inhibiting concentration of D-GlcNAc was found to be 1.56 μM for mycelium lectin and 6.25 μM for PVL, but neither lectin was inhibited by 0.1 M D-Gal, D-Glc, or D-Man. On SDS-PAGE, the purified mycelium lectin gave a single band at the same migration position as PVL, corresponding to about 40 kDa, as shown in Fig. 1. Based on these combined results, the lectin was considered to be a mycelial counterpart of PVL. The carbohydrate specificity of *P. velutina* lectin for acidic polysaccharides was further analyzed by solid phase assay using PVL because of the limited amount of mycelium lectin (1.5 mg mycelium lectin from 25 g of diploid mycelium culture).

Interaction of PVL with Heparin by Solid Phase Assay—PVL immobilized in the well was found to bind to heparin-HRP in a concentration-dependent manner at various pH values from 4 to 9, as shown in Fig. 2A where the result at pH 7 is shown as representative, in the concentration range of 60 ng/ml to 25 μg/ml of PVL. Unconjugated HRP did not bind to PVL immobilized in the well, suggesting that this binding depends on the affinity of PVL for heparin rather than HRP (data not shown). Alternatively, when heparin-BSA was immobilized on the well and biotinyl PVL was used as a probe, a similar dose-dependency was observed, as shown in Fig. 2B. The binding activity for heparin-BSA was retained in PVL biotinylated in the presence of heparin, while it was completely lost by biotinylation in the absence of heparin; the presence of GlcNAc during biotinylation provided no protection of the heparin-binding activity (Fig. 2B). PVL biotinylated in all cases retained GlcNAc-binding activity as detected by solid phase assay using immobilized asialoagalactofetuin, and the binding was not inhibited by heparin at a concentration of 6 mg/ml (data not shown). This result suggests that the heparin binding site is susceptible to biotinylation. Since the N-terminal sequence of PVL is blocked (our unpublished results), certain Lys residue(s) in PVL are thought to be essential for binding to heparin but not to GlcNAc.

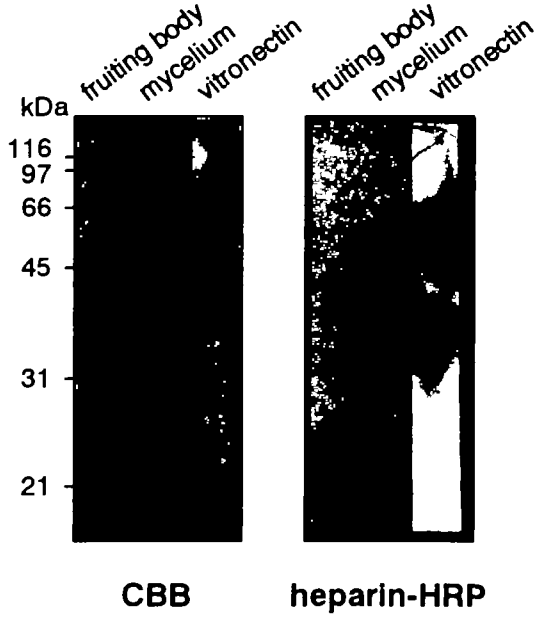

Fig. 1. **Reactivities of PVL and mycelium lectin with heparin-HRP on membranes.** Five micrograms of PVL, mycelium lectin purified from *P. velutina*, or human vitronectin was subjected to SDS-PAGE on 11% polyacrylamide gels and Western blotting was performed on the PVDF membrane. Proteins were stained with Coomassie Brilliant Blue (CBB) or allowed to react with heparin-HRP as described in the text. Molecular mass markers are shown on the left.

As shown in Fig. 2C, the maximum binding to heparin-HRP was observed at pH 7 in two different buffer systems: combined 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) buffers, and unified citrate-phosphate buffers (pH 3-7). PVL immobilized in the well bound to biotinyl $GlcNAc_{5\text{-}6}$ in a concentration-dependent manner at pH 2 to 9 (data not shown) and, as shown in Fig. 2D, the maximum binding to biotinyl $GlcNAc_{5\text{-}6}$ was observed at pH 3-4, which is different from heparin binding. Alternatively, when $GlcNAc_{5\text{-}6}$-BSA neoglycoprotein was immobilized on the well, HRP-PVL showed the same pH dependency of binding with an optimal pH of 4 (data not shown).

Effects of Ionic Strength on Heparin Binding of PVL—As shown in Fig. 2E, the heparin binding of PVL at pH 7 decreased with increasing concentrations of NaCl or Na_2SO_4, as well as several heparin binding proteins of animal origin (*18, 22*), with some binding still existing at salt concentrations above 0.5 M. This suggests that the binding of PVL to heparin is due mainly to electrostatic interactions and that these interactions occur under physiological conditions.

Inhibition Studies on Heparin Binding of PVL—Inhibition studies of solid phase assays were performed using a 10 μg/ml PVL solution and incubation at pH 7, based on the binding characteristics to heparin (Fig. 2, A and C). As shown in Fig. 3, pectin, polygalacturonic acid, fucoidan, dextran sulfate, alginic acid, and heparin inhibit the binding with heparin, whereas chondroitin, chondroitin sulfate A, B, and C, heparan sulfate, hyaluronic acid, colominic acid, keratan sulfate, dextran, DNA, and GlcNAc do not inhibit at concentrations of 6 mg/ml, as summarized in Table I. PVL binding did not occur in parallel with the amount of anionic charges. Pectin and polygalacturonic acid showed high inhibitory activities among the polysaccharides tested even though they are unsulfated and, furthermore, esterified. These results suggest that sulfate or carboxyl groups on acidic polysaccharides may contribute to the binding, but the binding does not depend simply on electrostatic interaction.

None of the inhibitory polysaccharides except heparin contains any GlcNAc or NeuAc residues; and the finding that heparan sulfate and hyaluronic acid do not inhibit the binding indicates that PVL does not recognize the internal GlcNAc residues of heparin because heparan sulfate and hyaluronic acid contain more GlcNAc than heparin, which contains *N*-sulfated GlcN as a major hexosamine. The binding specificities found for PVL are, therefore, quite

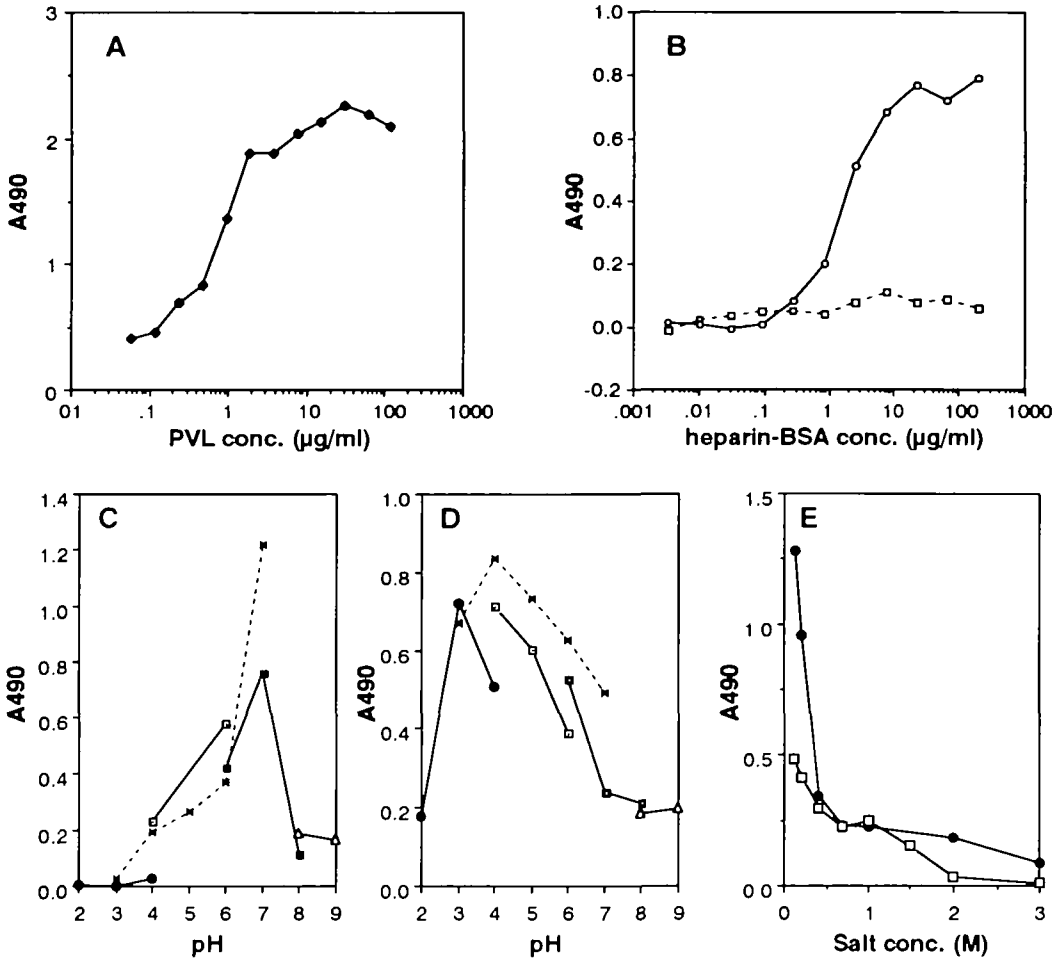

Fig. 2. **Reactivities of PVL with heparin-HRP (A), and biotinyl PVL with heparin-BSA (B) by solid phase assay. pH Dependencies of heparin binding (C) and GlcNAc binding (D), and the effects of ionic strength on the interaction between heparin-HRP and PVL (E).** A: PVL was immobilized on microtiter plates, and heparin-HRP diluted 1/1,000 in PBS was added. The following procedures were performed as described in the text. B: Heparin-BSA was immobilized on microtiter plates and 10 μg/ml of biotinyl PVL in PBS was added. Symbols used in B are: PVL biotinylated in the presence of heparin ( ) or GlcNAc ( ). C and D: PVL was immobilized on microtiter plates and heparin-HRP diluted 1/1,000 (C) or 5 μg/ml of biotinyl $GlcNAc_{6\text{-}6}$ (D) in buffers of various pH was added. 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 (▪). E, PVL solution (10 μg/ml) was added to the microtiter plates and PVL immobilization was performed. Heparin-HRP prepared in phosphate buffer (pH 7) at various salt concentrations was added to the well. Symbols used are: NaCl (●), Na_2SO_4 ( ).

distinct from those reported previously for oligosaccharides (*7, 8, 15*).

Circular Dichroism Spectra—In order to examine the conformational changes of PVL caused by ligand binding, the circular dichroism of PVL was measured in the presence or absence of specific sugars, GlcNAc, and heparin. As shown in Fig. 4A, the intensities around 230 nm increased and those around 210 nm decreased in the presence of GlcNAc. The calculated α-helix content of PVL decreased slightly 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 $67 \pm 1.2\%$. The spectrum in the presence of heparin (0.3 mg/ml), which corresponds to a glucosamine concentration of about 0.4 mM, was unchanged from that of native PVL (Fig. 4B). These results suggest that the binding of GlcNAc induces a conformational change in PVL, but the binding of heparin does not.

Sandwich Affinity Chromatography on diN-Acetylchitobiamyl Sepharose—Purified PVL binds completely to a di*N*-acetylchitobiamyl Sepharose gel after incubation, and, when heparin-HRP was subsequently applied to the column, the heparin neoproteoglycan bound to the column and was eluted with 0.2 M GlcNAc in association with PVL, as shown in Fig. 5A. Since heparin-HRP did not bind to the column in the absence of PVL, as shown in Fig. 5B, these results indicate that the heparin neoproteoglycan binds to the column *via* PVL that is preadsorbed to the di*N*-acetylchitobiamyl Sepharose gel. The sandwich affinity chromatography suggests that PVL can bind to heparin- and GlcNAc-containing macromolecules simultaneously at different binding sites. This finding suggests that PVL may serve as an adhesion molecule by forming a multicomponent complex including acidic polysaccharides and other glycoconjugates containing GlcNAc/NeuAc.

Interaction of PVL with Sulfatide—As shown in Fig. 6A,

biotinyl PVL prepared in the presence of GlcNAc binds to sulfatide in a concentration dependent manner, but the binding is not inhibited by GlcNAc, heparin, or NeuAc (data not shown). When PVL biotinylated in the presence of heparin was used, the same inhibition potency results were obtained (data not shown). In contrast to heparin binding, PVL binding to sulfatide is not dependent on either chloride or sulfate ions (Fig. 6B), suggesting that the binding is not due to an electrostatic interaction. The binding mode with

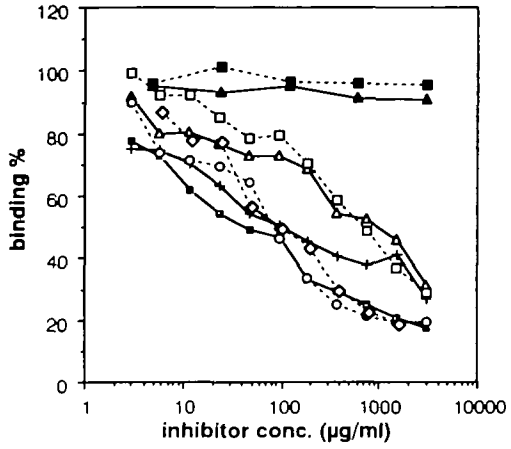

Fig. 3. **Effects of various polysaccharides on the interaction between heparin-HRP and PVL.** PVL solution (10 μg/ml) was added to microtiter plates and PVL immobilization was performed. Various concentrations of polysaccharides were preincubated in PBS with the immobilized PVL for 1 h before the addition of heparin-HRP. Binding % represents the proportion of the absorbance at 490 nm compared to that in the absence of inhibitors. The averages of triplicate experiments were plotted. Polysaccharides: heparin (△), fucoidan (▽), dextran sulfate (+), polygalacturonic acid (◇), pectin (○), and alginic acid (□). The following substances showed no inhibition: GlcNAc, chondroitin sulfates A, B, and C, heparan sulfate, hyaluronic acid, keratan sulfate, colominic acid, and DNA. GlcNAc (■) and chondroitin sulfate B (▲) are shown in the figure as representatives.

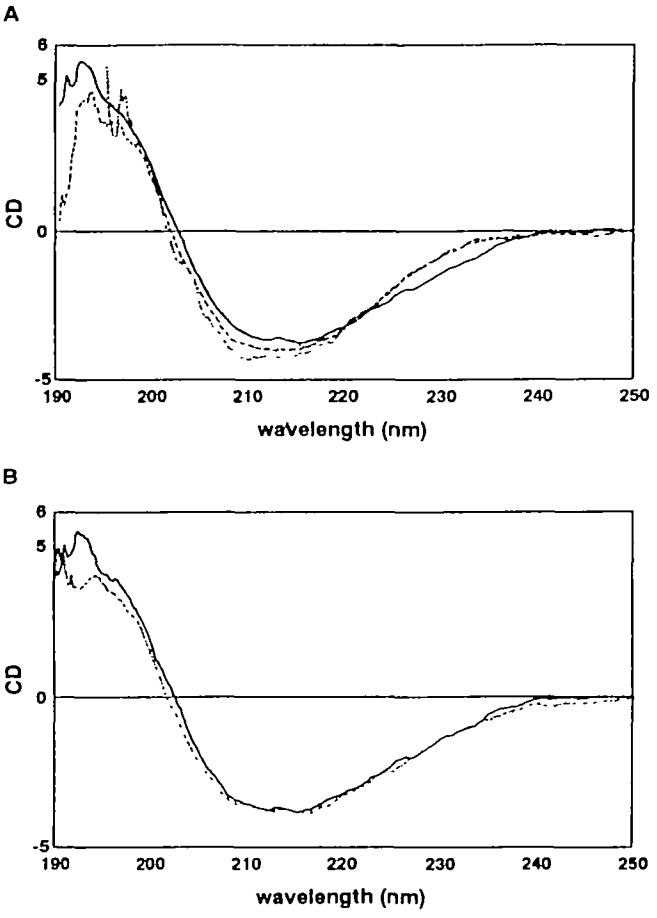

Fig. 4. **CD spectra of PVL.** CD spectra of PVL (0.1 mg/ml) in PBS were measured in the absence of carbohydrate (——), or in the presence of 1 mM GlcNAc (- - -) or 5 mM GlcNAc ( ); (A), or in the presence of 0.3 mg/ml heparin (·····); (B), as described in the text.

TABLE I. **Inhibition of the interaction between PVL and heparin-HRP.**

Inhibitor	IC_{50}[a] (μg/ml)	Constituting carbohydrates[b]	Sulfate content (%)
Sulfated polysaccharides			
Fucoidan	40	Fuc-S	25
Dextran sulfate	100	Glc di-S	51
Heparin	1,000	IdU2-S, GlcN-S, GlcN2, 6-diS, GlcU	30
Heparan sulfate	>6,000	GlcN-S, GlcU, IdU, GlcNAc	9
Chondroitin sulfate A	>6,000	GalNAc4-S, GlcU	18[c]
Chondroitin sulfate B	>6,000	GalNAc4-S, IdU-S	21[c]
Chondroitin sulfate C	>6,000	GalNAc6-S, GlcU	18[c]
Keratan sulfate	>6,000	GlcNAc6-S, Gal	15
Unsulfated polysaccharides & other substances			
Pectin	80	MeGalU, GalU	–
Polygalacturonic acid	90	GalU	–
Alginic acid	700	GulU, ManU	–
Chondroitin	>6,000	GlcU, GalNAc	–
Colominic acid	>6,000	NeuAc	–
Hyaluronic acid	>6,000	GlcU, GlcNAc	–
Dextran	>6,000	Glc	–
DNA	>6,000	Deoxyribose	–
GlcNAc	>6,000	–	

[a]Concentration inhibiting 50% of the lectin binding. [b]Abbreviations used in the table: S, sulfate; IdU, iduronic acid; GlcU, glucuronic acid; GalU, galacturonic acid; GulU, guluronic acid; ManU, mannuronic acid; MeGalU, galacturonic acid methyl ester. [c]Sulfate contents were from Ref. *26*.

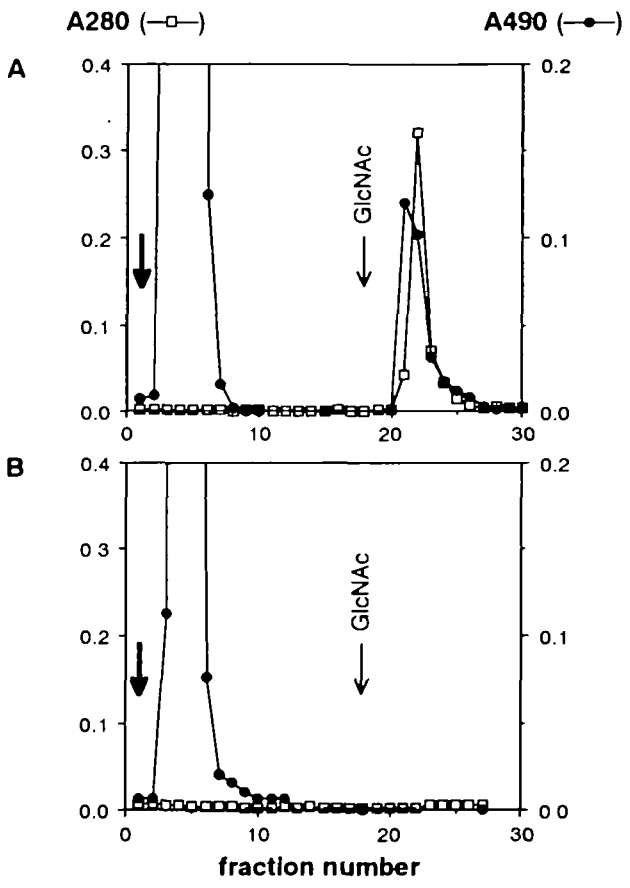

Fig. 5. **Sandwich affinity chromatography of heparin-HRP on di*N*-acetylchitobiamyl Sepharose *via* PVL.** A: Purified PVL (500 μg) was incubated with 2 ml of di*N*-acetylchitobiamyl Sepharose in PBS overnight at 4°C with shaking and packed into a column (0.75 × 4.5 cm). The column was washed and heparin-HRP diluted 1/1,000 in PBS was applied at the bold arrow (↓); the column was then washed with PBS again. The bound material was eluted with 0.2 M GlcNAc in PBS at the arrow (↓ GlcNAc). Elution was monitored at 280 nm for protein (□) and 490 nm for heparin-HRP (●) as described in the text. B: For the control experiment, heparin-HRP was applied to a di*N*-acetylchitobiamyl-Sepharose column without preincubating the gel with PVL, and elution and monitoring were performed in the same way as A.

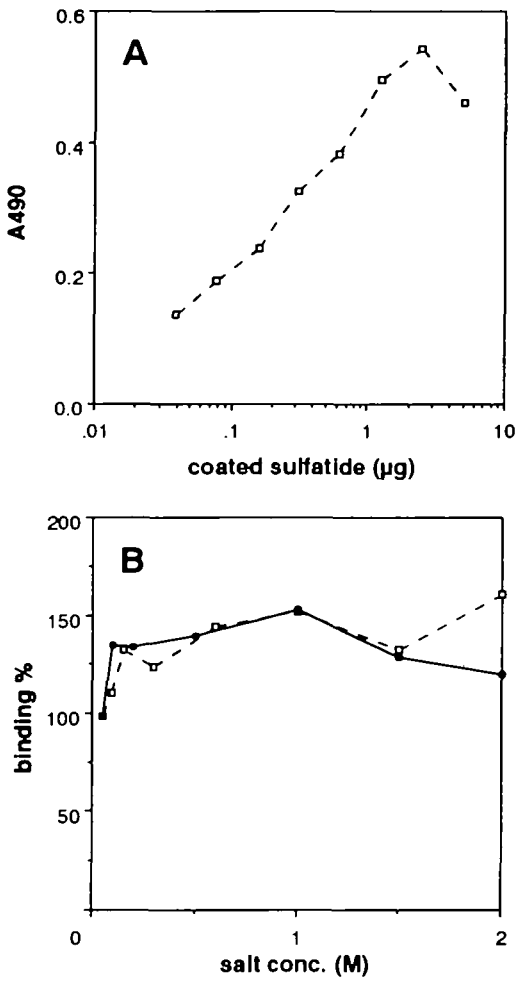

Fig. 6. **Reactivities of biotinyl PVL with sulfatide by solid phase assay.** Sulfatide in EtOH was immobilized on microtiter plates by evaporization of EtOH. PVL (10 μg/ml) biotinylated in the presence of GlcNAc was added to the wells and a solid phase assay was performed in PBS (pH 7). A, concentration-dependency 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 (□).

sulfatide seems distinct from those with other ligands, but the nature of sulfatide binding remains unclear.

DISCUSSION

Solid phase assays utilizing neoproteoglycans, neoglycoproteins, and biotinyl oligosaccharides, in combination with HRP- or biotin-labeled lectin, showed that PVL binds to acidic polysaccharides and sulfatide. We previously reported that PVL interacts not only with non-reducing terminal GlcNAc but also with NeuAc and GalNAc, and further, with internal GlcNAc of the *N*-acetyllactosamine structure at common binding sites, and that the binding is inhibitable by GlcNAc. In those interactions, the *N*-acetyl group plays an essential role in PVL binding (*15*). The sites that interact with acidic polysaccharides and GlcNAc are considered to be independent for the following reasons: (i) the most potent inhibitory polysaccharides do not contain GlcNAc or *N*-acetyl groups, and the binding activity to heparin is not inhibited by GlcNAc and *vice versa* (Table I and Fig. 3); (ii) the binding of heparin and GlcNAc show different pH dependencies (Fig. 2, C and D); (iii) the heparin-binding activity is destroyed by biotinylation of PVL while the GlcNAc-binding activity is retained (Fig. 2B); (iv) the CD spectrum of PVL was affected by GlcNAc but not by heparin (Fig. 4). PVL has four GlcNAc-binding sites per monomer, and may form a multimer in the absence of 10% glycerol (*7*). The fact that heparin-binding activity is not inhibited by GlcNAc and *vice versa*, however, suggests that different sites are involved in GlcNAc and heparin binding. In accordance with this, PVL could bind to di*N*-acetylchitobiamyl Sepharose and heparin neoproteoglycan simultaneously and be eluted specifically by GlcNAc in association with heparin (Fig. 5).

PVL exhibited heparin binding even after SDS-PAGE (Fig. 1), similar to vitronectin. Heparin-binding consensus motifs such as XBBXBX and XBBBXXBX, where B is a

basic amino acid (*23*), are contained in many heparin binding proteins including vitronectin but not in the primary sequence of PVL. PVL contains basic motifs in its peptide sequence comprising seven homologous repeats, *i.e.* five repeats of RX(D or E)BHXR and four repeats of BB, where B = K or R and X = V, I, L, or P, and these motifs may serve as heparin-binding motifs.

Among the acidic polysaccharides tested, PVL binding was observed for only a limited number of polysaccharides. The fact that the heparin binding of PVL is effectively inhibited by pectin and polygalacturonic acid but not by chondroitin sulfates, colominic acid, or other acidic polysaccharides indicates that the affinity is not merely an electrostatic interaction. A certain spatial location of anionic charges and hydroxyl groups may be required for PVL binding and that of pectin polysaccharide may fit these requirements.

On the other hand, the use of PVL as a sugar-specific probe in histochemical and biochemical analyses of glycoconjugates could produce misleading information concerning the nature of glycoforms, disregarding the multispecificity. When using PVL as a detection reagent, the extensive biotinylation of amino groups on PVL, or incubation at pH 4 instead of pH 7 for the binding assay would be useful to circumvent the interference by acidic polysaccharides without harming the GlcNAc-binding activity.

The binding of PVL and the mycelium lectin to acidic polysaccharides observed in this study may be biologically relevant to a saprophytic interaction in a humic soil originating from plant or wood. As shown here, PVL can simultaneously interact with different receptors and cross-link them, *e.g.*, pectin or polygalacturonic acid, components of the plant cell wall, *N*-acetyl containing sugar residues, alginic acid, or sulfatide on bacterial cell surfaces.

Other *Eumycota* lectins from *Arthrobotrys oligospora*, a nematode-trapping fungus, and *Agaricus bisporus* have been reported to interact with sialoglycoproteins, dextran sulfate, and fucoidan (*24*). The affinity for pectin, polygalacturonic acid, alginic acid, and heparin is unique to PVL, although some similarities in binding specificity are observed despite the lack of sequence similarity between lectins and PVL. As reported recently, *A. bisporus* lectin might be a defense molecule that has an antiproliferative effect on epithelial cell growth without cytotoxity by being internalized and blocking the uptake of nuclear proteins into the nucleus (*25*). Some possibilities are that multiligand adhesive lectins function in chemotactic responses to mobilize defense systems, to link fungi with sources of nutrition, or to form fruiting bodies. Further investigation of the biological receptors of PVL as well as its spatial and developmental regulation needs to be clarified in order to elucidate the function of this lectin.

We thank Mr. Takashi Takakuwa (JASCO Corporation) for his kind support in the CD spectrometric analysis.

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