Purification and Properties of an $N$-Acetylg glucosamine-specific Lectin from *Psathyrella velutina* Mushroom*

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A lectin in the fruiting bodies of *Psathyrella velutina* was purified by affinity chromatography on a chitin column and subsequent ion-exchange chromatography. *P. velutina* lectin (PVL) tends to aggregate irreversibly in buffered saline, but the addition of glycerol (10%, v/v) to lectin solutions was found to prevent aggregate formation. PVL is assumed to occur as a monomer of a polypeptide of $M_r = 40,000$ as determined by gel filtration and by gel electrophoresis in the presence of sodium dodecyl sulfate.

PVL is specific for $N$-acetylg glucosamine (GlcNAc). It was determined by equilibrium dialysis to have four binding sites/polypeptide molecule showing an average association constant of $K_a = 6.4 \times 10^3$ M$^{-1}$ toward this sugar. The binding specificity of the lectin was studied by hemagglutination inhibition assays and by avidin-biotin-mediated enzyme immunoassays using various GlcNAc-containing saccharides. The results indicate that methyl $N$-acetyl $\beta$-glucosaminide was a slightly better inhibitor than the corresponding $\alpha$-anomer. PVL binds well to oligosaccharides bearing nonreducing terminal $\beta$-GlcNAc linked 1→6 or 1→3 but poorly to those having a 1→4 linkage, such as $N$-acetylated chito-oligosaccharides. It also binds to the subterminal GlcNAc moiety when it is substituted at the C-6 position but does not interact with the moiety when substituted either at C-3 or C-4. Thus, these results show that PVL is quite different in its binding specificity from other GlcNAc-binding lectins of higher plants since they bind preferentially to $\beta$-GlcNAc in 1→4 linkage and they have a high affinity for chitin oligosaccharides.

A number of plant lectins have been extensively studied and used as probes for the saccharide structures of glycoconjugates (for reviews see Refs. 1–3). Most of these lectins are the products of higher plants. In Mycetes, including fruiting bodies of Ascomycetes and Basidiomycetes (mushrooms), the widespread occurrence of hemagglutinating substances has been reported (Ref. 4 and references cited therein), and several of these have been purified and characterized (5–8). *Aleuria aurantia* lectin, for example, is specific for fucose, and because of its unique binding property (6, 9), it has been used as a valuable tool for probing fucosylated carbohydrate chains (10–12). The presence of hemagglutinating activity in the fruiting body of *Psathyrella velutina* has been known (4), but no further investigation on the active substance was reported. This report describes the purification of *P. velutina* lectin (PVL) to homogeneity and its biochemical and immunological properties. PVL is specific for $N$-acetylg glucosamine (GlcNAc) and shows binding specificity that is quite different from that of other GlcNAc-binding lectins of higher plants. In addition, we have developed a sensitive and reproducible method for inhibition studies of lectin binding. The method proved valuable when the amounts of haptenic oligosaccharides to be used were limited.

**EXPERIMENTAL PROCEDURES**

**Materials**—Fruiting bodies of *Psathyrella velutina* (Fr.) Sing. were collected in the region of Gunma Prefecture, central Japan, and stored at $-20$ °C until use.

The structures of various saccharides used in the inhibition studies are summarized in Table IV. Among these, the following oligosaccharides were synthesized by previously reported methods: oligosaccharides V and VI (13), IX (14), X (15), XI (16), XII (17), XIII (18), XIV–XVI (19), and VII. GlcNAc and methyl $N$-acetyl $\alpha$- and $\beta$-glucosaminides were purchased from Sigma and reconstituted from methanol-diethyl ether mixtures. $N$-Acetylated chitin oligosaccharides were from Seikagaku Kogyo.

Human erythrocyte sialylglycoprotein was prepared as described previously (11), and mild alkali treatment of this material was performed by the method of Carlson (20). Asialo-agalactofetuin was prepared by Smith degradation (21) of asialofetuin (Sigma). The following materials were obtained commercially: avidin D and biotinylated alkaline phosphatase (Vector); purified chitin and 4-methylumbelliferyl phosphate (Seikagaku Kogyo); standard protein set for molecular weight determination (Boehringer Mannheim); sialyllectose and crystalline bovine serum albumin (BSA) (Sigma); and $N$-acetyl-$\beta$-[1,6-$^3$H]glucosamine, 33.1 Ci/mmol (Du Pont-New England Nuclear).

**Purification of PVL**—All of the procedures in this section were carried out at 4 °C. Fruiting bodies of *P. velutina* (100 g) were homogenized with 1,000 ml of 145 mM NaCl in 5 mM sodium phosphate buffer, pH 7.2 (PBS), containing 5 mM EDTA and 0.5 mM phenylmethylsulfonyl fluoride. The homogenate was centrifuged at 20,000 $\times g$ for 20 min, and the pellet was re-extracted with 500 ml of the same solution. To the combined extracts was added drops of 20% (v/v) acetic acid with stirring, and the pH of the solution was brought to 4.0. After standing for several hours, the insoluble material was removed by centrifugation at 20,000 $\times g$ for 20 min, and the supernatant was neutralized with 1 M NaOH.

The acid-treated extract was passed through a chitin column (3.5 $\times$ 30 cm) at a flow rate of 60 ml/h, and the column was washed with extraction buffer until the $A_{280}$ of the effluent was less than 0.1. Then the bound material was eluted with 50 mM GlcNAc in PBS containing 10% (v/v) glycerol (Fig. 1A). The eluate was concentrated by ultrafiltration.

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† All the sugars in this report refer to the D form except for fucose which has the L form.

‡ The abbreviations used are: PVL, *Psathyrella velutina* lectin; BSA, bovine serum albumin; PBS, sodium phosphate-buffered saline; TBS, Tris-HCl-buffered saline; ELISA, enzyme-linked immunosorbent assay; HPLC, high pressure liquid chromatography.

1. K.-L. Matta, manuscript in preparation.
filtration through an Advantec Q100 membrane (Toyo Roshi). The concentration was then applied to columns of DEAE-cellulofine (1.7 × 25 cm, Chisso) and CM-Sepharose CL-6B (1.7 × 25 cm, Pharmacia LKB Biotechnology Inc.), which had been connected serially and equilibrated with 10 mM sodium phosphate buffer, pH 7.0, containing 10% (v/v) glycerol. After washing through the same buffer, the columns were disconnected and the material bound to the CM-Sepharose CL-6B column was eluted with 0.4 M NaCl (Fig. 1B).

Finally, the lectin was chromatographed on a chitin column (1.5 × 20 cm) which had been equilibrated with PBS-glycerol. The bound lectin was eluted with 50 mM GlcNAc (Fig. 1C).

**Molecular Sieve Chromatography—** A column of Toyopearl HW-55 (1 × 50 cm, Toyoda Soda) was calibrated by using the standard proteins for M, determination. The solution used in these experiments was 140 mM NaCl in 10 mM Tris-HCl buffer, pH 7.5 (TBS), containing 10% (v/v) glycerol, and the flow rate was kept constant (6 ml/h) with a peristaltic pump. Purified PVL (about 200 µg) in the same solution containing 10 mM GlcNAc was applied to the column, and the A280 of the elute was monitored. Apparent M, was estimated graphically as described in Ref. 22.

**Gel Electrophoresis—** Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was performed according to the method of Laemmli (23) using a 4-20% acrylamide gradient slab gel.

**Chemical Analysis—** Purified PVL was desalted by extensive dialysis against water, lyophilized, and weighed. This was dissolved in warm 6 M HCl, and an aliquot containing about 500 µg of protein was hydrolyzed in a sealed evacuated tube at 110 °C for 24 h and 72 h. The hydrolysates were analyzed with a Shimadzu HPLC model LC6A assembled for p-phenylalanine dehydrogenase amino acid analysis. The values for serine and threonine were corrected by extrapolating to 0-h hydrolysis, and those of isoleucine and valine were of 72-h hydrolysis. Tryptophan was estimated spectrophotometrically (24). Protein content was determined by a dye-binding protein assay (Bio-Rad) with BSA as the standard.

**Equilibrium Dialysis—** This was conducted by using a dialysis chamber made of polycrlic plastic resin (Sanplatec). A solution of 750 µg of GlcNAc, from 25 to 750 µM, in TBS containing 10% (v/v) glycerol, 0.02% NaN₃ and 0.1% BSA, plus 50 µl of tritiated GlcNAc (0.07 µM) was added to a half-compartment, and 500 µl of the purified lectin (916 pg) was added to the counterpart. After the chamber was allowed to stand for 48 h at room temperature, an aliquot was withdrawn from each compartment, and the radioactivity was measured in an Aloka model 673 liquid scintillation spectrometer.

**Hemagglutination Assay and Hemagglutination Inhibition Assay—** These were performed in a microtiter plate. The lectin sample (25 µl) was serially diluted with PBS and mixed with 25 µl of 2% human serum albumin (BSA) as the standard. The minimum amount of protein (pg/ml) giving positive reaction after mixing 25 pl containing 8 units of the purified PVL. After standing for 2 h at room temperature followed by dialysis against TBS containing 10% (v/v) glycerol, the lectin was eluted purified by affinity chromatography on a chitin column (1.5 × 20 cm).

The method of avidin-biotin-mediated ELISA was essentially based on that described in Ref. 25. The erythrocyte sialoglycoprotein, was incubated at 45 °C for 2 h. The plate could be stored at 4 °C for several days. After washing the plate with TBS containing 0.05% Tween 20, 0.02% NaN₃ (buffer B), and 0.1% BSA, 100 µl of the biotinylated PVL (14 µg) in the preceding solution was added to the wells and incubated at 37 °C for 1 h.

For inhibition studies, 60 µl of the biotinylated PVL solution (28 µg in 100 µl) was mixed with an equal volume of serially diluted inhibitor in buffer B, and preincubated for 2 h at 4 °C, then 100 µl of the solution was used for the assay. After the plate was washed with buffer B, 100 µl of a solution containing 0.5 µg of avidin D, 0.01 unit of biotinylated alkaline phosphatase, and 40 mM MgCl₂ in buffer B was added to the wells. The plate was incubated at 37 °C for 2 h, washed with buffer B plus 40 mM MgCl₂ and buffer A plus 40 mM MgCl₂ successively. Then, 100 µl of 2 µM 4-methylumbelliferyl phosphate in buffer A was added. The plate was incubated at 37 °C for 12 h, and the fluorescence was measured in a Dynatec fluorescer after adding 25 µl of 0.2 M NaOH.

In preliminary experiments, the amount of the antigen used for coating the well, concentration of the biotinylated lectin, and the composition of avidin-biotinylated enzyme were examined for proper conditions to give optimum results. The conditions described here were chosen to be suitable when 10 µg of the sialoglycoprotein was used for coating the well. The amounts of synthetic oligosaccharides used as inhibitors were less than 2 mg, which gave 5 to 10 µM in 200 µl of the starting solution, and were enough for assays in triplicate experiments.

**RESULTS AND DISCUSSION**

**Purification of PVL—** It was found in our preliminary experiments that PVL could not be recovered completely by salting out with either ammonium sulfate or sodium sulfate even at 100% saturation. Therefore, the acid-treated extract of the fruiting bodies was subjected directly to affinity chromatography using a chitin column, and the active fractions after each chromatographic step were concentrated by ultrafiltration. Moreover, PVL tends to aggregate irreversibly to give an insoluble sediment in a dilute buffer (up to 50 mM) or a buffered saline. We found that the presence of glycerol (10–20%, v/v) prevents aggregate formation and the solution was suitable for handling and storage without apparent loss of material. Therefore, the solutions containing 10% glycerol were used throughout the purification procedures except for the extraction buffer.

The contaminants in the elute from the first affinity column (Fig. 1A) were nearly completely removed by subsequent ion-exchange chromatography. The lectin passed through the DEAE-cellulofine column and was retained on the CM-Sepharose CL-6B column at pH 7.0, then eluted with 0.4 M NaCl as shown in Fig. 1B. The second affinity chromatography using the chitin column gave a purified lectin

**FIG. 1. Purification of P. velutina lectin.** A, first affinity chromatography on chitin column (3.5 × 30 cm). The column was eluted at the arrow with 50 mM GlcNAc in PBS containing 10% (v/v) glycerol, and the fractions of 8 ml were collected. B, elution profile from the CM-Sepharose CL-6B column. The column was eluted at the arrow with 0.4 M NaCl in sodium phosphate buffer containing 10% (v/v) glycerol. Fractions of 1.6 ml were collected and analyzed for A₂₈₀ and hemagglutinating activity. C, second affinity chromatography on chitin column (1.5 × 20 cm). The column was eluted at the arrow with the same solution as in A. Fractions of 1.2 ml were collected and analyzed for A₂₈₀ and hemagglutinating activity.
TABLE I
Purification of P. velutina lectin

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Protein</th>
<th>Total H.U.*</th>
<th>Recovery</th>
<th>Specific activity</th>
<th>Purification</th>
</tr>
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<tbody>
<tr>
<td>Acid-treated crude extract</td>
<td>251</td>
<td>17,120</td>
<td>100</td>
<td>68.2</td>
<td>1</td>
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<tr>
<td>Eluate from the first affinity column</td>
<td>33.0</td>
<td>10,048</td>
<td>59</td>
<td>305</td>
<td>4.5</td>
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<tr>
<td>Eluate from CM-Sepharose CL-6B</td>
<td>30.6</td>
<td>10,240</td>
<td>59</td>
<td>335</td>
<td>4.9</td>
</tr>
<tr>
<td>Eluate from the second affinity column</td>
<td>28.3</td>
<td>19,304</td>
<td>60</td>
<td>364</td>
<td>5.3</td>
</tr>
</tbody>
</table>

* Hemagglutinating unit (H.U.) as defined under "Experimental Procedures."

FIG. 2. Electrophoresis of purified P. velutina lectin. Samples containing about 10 μg of protein were submitted and stained with Coomassie Blue. Lane 1, human IgG H chain (Mr = 50,000); Lane 2, BSA (Mr = 68,000), ovalbumin (Mr = 45,000), chymotrypsinogen (Mr = 25,000), and cytochrome c (Mr = 12,500); lane 3, P. velutina lectin reduced with 2-mercaptoethanol; lane 4, P. velutina lectin, nonreduced.

FIG. 3. Binding of P. velutina lectin with GlcNAc. Binding assays were performed in duplicate. See the text for details.
Inhibition of hemagglutination

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration*</th>
</tr>
</thead>
<tbody>
<tr>
<td>GlcNAc</td>
<td>1.56</td>
</tr>
<tr>
<td>Methyl α-GlcNAc</td>
<td>3.13</td>
</tr>
<tr>
<td>Methyl β-GlcNAc</td>
<td>0.78</td>
</tr>
<tr>
<td>GlcNAcβ1→4GlcNAc</td>
<td>2.5</td>
</tr>
<tr>
<td>GlcNAcβ1→4GlcNAcβ1→4GlcNAc</td>
<td>2.5</td>
</tr>
<tr>
<td>GlcNAcβ1→4GlcNAcβ1→4GlcNAcβ1→4GlcNAc</td>
<td>2.5</td>
</tr>
</tbody>
</table>

*Minimum concentration required for complete inhibition of hemagglutination with 8 units of P. velutina lectin.

Figure 4. Effects of various saccharides on P. velutina lectin binding in avidin-biotin-mediated ELISA. The lectin binding is expressed as the fluorescence intensity relative to that without inhibitor substance. See Table IV for the structures of substances employed. A, monosaccharides; B, disaccharides having nonreducing terminal GlcNAc; C, trisaccharides having nonreducing terminal GlcNAc; D, oligosaccharides having subterminal GlcNAc.

Table IV

<table>
<thead>
<tr>
<th>Saccharide Structure</th>
<th>Concentration*</th>
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<tbody>
<tr>
<td>GlcNAc</td>
<td>0.27</td>
</tr>
<tr>
<td>GlcNAc α-Me</td>
<td>0.47</td>
</tr>
<tr>
<td>GlcNAc β-Me</td>
<td>0.38</td>
</tr>
<tr>
<td>GlcNAcβ1→4GlcNAc</td>
<td>2.3</td>
</tr>
<tr>
<td>GlcNAcβ1→3GalNAcβ1→Bzl</td>
<td>1.1</td>
</tr>
<tr>
<td>GlcNAcβ1→3GalNAcβ1→Bzl</td>
<td>0.68</td>
</tr>
<tr>
<td>GlcNAcβ1→6Galβ-Np</td>
<td>0.18</td>
</tr>
<tr>
<td>GlcNAcβ1→4GlcNAcβ1→4GlcNAc</td>
<td>2.4</td>
</tr>
<tr>
<td>GlcNAcβ1→3GalNAcβ1→Bzl</td>
<td>0.6</td>
</tr>
</tbody>
</table>

Table V

<table>
<thead>
<tr>
<th>Glycoprotein</th>
<th>Concentration*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asialofetuin</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>Asialo-agalactofetuin</td>
<td>10</td>
</tr>
<tr>
<td>Alkali-treated sialoglycoprotein</td>
<td>380</td>
</tr>
<tr>
<td>Sialoglycoprotein</td>
<td>0.62</td>
</tr>
</tbody>
</table>

*Concentration of substance giving 50% inhibition of the lectin binding.

The results of monosaccharides shown in Fig. 4A indicate that methyl N-acetyl α-glucosaminides have an inhibiting activity for PVL, the α anomer being somewhat more potent than the β anomer. Chitin oligosaccharides proved to be less active than the monosaccharide (GlcNAc); the inhibitory activity did not increase with an increase in chain length up to pentasaccharide. These results imply that PVL is distinctive in its binding property from other GlcNAc-specific lectins, since they generally have high affinity for chitin oligosaccharides rather than the monosaccharide (see next section also).

Inhibition Studies of Avidin-Biotin-mediated ELISA—In another approach to elucidate the binding specificity of PVL, the inhibiting activity of various saccharides in the avidin-biotin-mediated ELISA was examined. The biotinylated PVL was preincubated with a serial dilution of the inhibitor substance, and then the mixture was employed for the ELISA system. Fig. 4 shows the fluorescence intensity relative to that of a control experiment in which the same volume of buffer B was used instead of the inhibitor solution. Table IV summarizes the structures of the inhibitors in Fig. 4 and their inhibiting potencies. The latter values were estimated graphically as the concentration (mM) in the preincubation mixture at which 50% inhibition of the lectin binding occurred.

The results of monosaccharides shown in Fig. 4A indicate that methyl N-acetyl β-glucosaminide is slightly better than the corresponding α anomer. Apparently, PVL has no strong preferences for either the α or the β anomer. Similar results
were observed in the hemagglutination inhibition assays in the preceding section. Other monosaccharides tested had no activity at all. Results for disaccharides having non-reducing terminal GlcNAc appear in Fig. 4B. Interestingly, the linkage position of GlcNAc influenced inhibitory activity. Thus, an order of 1→6 > 1→3 > 1→4 in the inhibiting potency is presumed from the results with oligosaccharides IV, V, and VI. However, the nature of the glycone may influence these results since p-nitrophenyl glycoside (VII) was more potent than benzyl glycoside (VI), although they have identical terminal structures. Fig. 4C illustrates the inhibition curves given by trisaccharides having terminal GlcNAc. The most striking observation is that N-acetylated chitotriose (VIII), and also chitotriose (IV) in Fig. 4B, showed lower activity than the monosaccharide. These results are in good agreement with those obtained in the hemagglutination inhibition assays. Therefore, it is apparent that PVL binding specificity is in contrast to other GlcNAc-specific lectins, which are effectively inhibited by the chitin oligosaccharides (Ref. 30 and references cited therein). A lectin of Grifonia simplicifolia seed (GS II) interacts with terminal nonreducing α- and β-N-acetylglucosaminides and has no strong preference for the chitin oligosaccharides (31, 32). Fig. 4C shows that oligosaccharide IX, with a terminal 1→6-linked GlcNAc, was less active than X which has a 1→3 linkage. This is in conflict with the order of affinity described above but is probably due to the steric interference of branched galactose in IX. The terminal disaccharide structures are identical in trisaccharides X and XI, but the latter was more inhibitory. The difference may be attributed to the benzyl residue in X, although this observation needs further investigation.

Among the series of fucosyl oligosaccharides (XIV–XVI) assayed, only XVI showed the ability to inhibit PVL (Fig. 4D, Table IV). Moreover, oligosaccharides XII and XIII (N-acetyllactosamine) failed to inhibit. These results point out that PVL can recognize subterminal GlcNAc substituted at C-6 more than benzyl glycoside (VI), although they have identical terminal disaccharide structures are identical in trisaccharides (IV) in Fig. 4B, showed lower activity than the monosaccharide. These results are in good agreement with those obtained in the hemagglutination inhibition assays. Therefore, it is apparent that PVL binding specificity is in contrast to other GlcNAc-specific lectins, which are effectively inhibited by the chitin oligosaccharides (Ref. 30 and references cited therein). A lectin of Grifonia simplicifolia seed (GS II) interacts with terminal nonreducing α- and β-N-acetylglucosaminides and has no strong preference for the chitin oligosaccharides (31, 32). Fig. 4C shows that oligosaccharide IX, with a terminal 1→6-linked GlcNAc, was less active than X which has a 1→3 linkage. This is in conflict with the order of affinity described above but is probably due to the steric interference of branched galactose in IX. The terminal disaccharide structures are identical in trisaccharides X and XI, but the latter was more inhibitory. The difference may be attributed to the benzyl residue in X, although this observation needs further investigation.

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