Purification and Characterization of a Neu5Acα2–6Galβ1–4Glc/GlcNAc-specific Lectin from the Fruiting Body of the Polypore Mushroom *Polyporus squamosus*

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A lectin has been purified from the carpophores of the mushroom *Polyporus squamosus* by a combination of affinity chromatography on β-1-galactosyl-Synsorb and ion-exchange chromatography on DEAE-Sephacel. Gel filtration chromatography, SDS-polyacrylamide gel electrophoresis, and N-terminal amino acid sequencing indicated that the native lectin, designated *P. squamosus* agglutinin, is composed of two identical 28-kDa subunits associated by non-covalent bonds. *P. squamosus* agglutinin agglutinated human A, B, and O and rabbit red blood cells but precipitated only with human α-macroglobulin, of many glycoproteins and polysaccharides tested. The detailed carbohydrate binding properties of the purified lectin were elucidated using three different approaches, i.e. precipitation inhibition assay (in solution binding assay), fluorescence quenching studies, and glycoprotein binding by lectin staining on high-performance thin layer chromatography (solid-phase binding assay). Based on the results obtained by these assays, we conclude that although the *P. squamosus* lectin binds β-1-galactosides, it has an extended carbohydrate-combining site that exhibits highest specificity and affinity toward nonreducing terminal Neu5Acα2,6Galβ1,4Glc/GlcNAc (6′-sialylated type II chain) of *N*-glycans (200-fold stronger than toward galactose). The strict specificity of the lectin for α2,6-linked sialic acid renders this lectin a valuable tool for glycobiological studies in biomedical and cancer research.

Lectins are proteins (or glycoproteins), other than antibodies and enzymes, that bind specifically and reversibly to carbohydrates, resulting in cell agglutination or precipitation of glycoconjugates (1). They are ubiquitous in the biosphere, having been found in viruses, bacteria, fungi, plants, and animals (2).

Lectins of known specificity recognizing sialic acid serve as valuable reagents in glycobiological research. They can be employed for the detection and preliminary characterization of sialic acid-containing glycoconjugates on the surface of cells and for assaying the incorporation of sialic acid into complex carbohydrates in biosynthetic studies. In their immobilized form, these lectins can be used for the resolution and isolation of sialic acid-containing glycoconjugates. Lectins are found in greatest quantity and are most readily purified from plant sources, especially higher plants, although relatively few sialic acid-binding lectins have been identified in the plant world (including fungi), which lacks sialic acid.

During the last decade, there has been a growing interest in fungal lectins, largely due to the discovery that some of these lectins exhibit antitumor activities, e.g. *Volvariella volvacea* lectin shows antitumor activity against sarcoma S-180 cells (3), *Grifola frondosa* lectin is cytotoxic to Hela cells (4), *Agaricus bisporus* lectin possesses antiproliferation activities against human colon cancer cell lines HT29, breast cancer cell lines MCF-7 (5), and *Tricholoma mongolicum* lectin inhibits mouse mastocytoma P815 cells in vitro and sarcoma S-180 cells in vivo (6). Fungal lectins have recently been reviewed (7–9). However, apart from the lectin from *A. bisporus*, which binds to Galβ1,3GalNAc-sericin (T-disaccharide) (10), the detailed carbohydrate specificities of these fungal lectins have not been investigated in depth.

We report herein the purification and characterization of *Polyporus squamosus* lectin (designated PSA), a Neu5Acα2–6Galβ1–4Glc/GlcNAc-specific lectin present in the carpophores (fruiting bodies) of this member of the Polyporaceae family.

MATERIALS AND METHODS

Carpophores of *P. squamosus* (Huds.) Fr. were collected in late summer 1998 from a decaying Ulmus stump in Ann Arbor, Michigan. A voucher specimen (Goldstein (MICH) 27953) was deposited in the University of Michigan herbarium.

Unless stated otherwise, saccharides, their derivatives, and glycoproteins (including fetuin, asialofetuin, transferrin, thyroglobulin, α2-macroglobulin, α2-acid glycoprotein, bovine mucin, etc.) were purchased from Sigma. Ovine submaxillary mucin was a gift of Dr. R. N. Knibbs (University of Michigan). Except for asialofetuin, asialoglycoproteins were prepared by heating the corresponding native glycoproteins in 0.1 M hydrochloric acid at 80 °C for 1 h, followed by dialysis and lyophilization; the removal of sialic acid was confirmed by the thiobarbituric acid assay (11).

Neutral glycolipids and gangliosides were purchased from Matreya, Inc. (Pleasant Gap, PA), glycopentatassyl ceramide (Forssman glycolipid) was a generous gift of Dr. S.-I. Hakomori (Biomembrane Institute, Seattle, WA), aluminum-backed HPTLC sheets (HPTLC-Alufolien Kieselgel 60) were from E. Merck (Darmstadt, Germany), and EZ-Link NHS-LC-biotin (succinimidyl 6-(biotinamido) hexanoate) was a product of Pierce. Alkaline phosphatase-streptavidin was from Zymed Laboratories Inc. (San Francisco, CA).

Galβ1,3GalNAc-Synsorb (β-galactosyl-Synsorb) was the product of Chemibiomed Ltd. (Edmonton, Alberta, Canada). Bio-Gel P-150 (50–100 mesh) was from Bio-Rad, and DEAE-Sephacel was obtained from Amersham Pharmacia Biotech.

Methyl 3-O-β-p-galactopyranosyl-2-acetamido-2-deoxy-β-p-glucopyranoside (Galβ1, 3GlcNAcβ1-O-Me) and methyl 4-O-β-p-galactopyranosyl-...
syl-2-acetamido-2-deoxy-β-D-glucopyranoside (αGal[1,4GlcNAcβ1-OMe]) were synthesized in this laboratory.

Molecular mass standards used in SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (i.e. BenchMark protein ladders) and alkaline phosphatase substrate package (i.e. 5-bromo-4-chloro-3-indolylphosphate p-toluidine hydrochloride and nitroblue tetrazolium chloride) were from Life Technologies, Inc.

Purification of the Lectin—All procedures were conducted at 4 °C. The fruiting bodies from P. squamosus (fresh weight, 80 g) were homogenized and extracted overnight with 400 ml of extraction buffer (10 mM sodium phosphate, 0.15 mM NaCl, 0.04% sodium azide, pH 7.2) containing 10 mM thio-urea, 0.25 mM phenylmethylsulfonyl fluoride, and 1 g/liter ascorbic acid. The homogenate was squeezed through two layers of cheesecloth and centrifuged at 10,000 × g for 30 min. To the supernatant solution was added solid ammonium sulfate to 25% saturation. After standing overnight, the precipitate was removed by centrifugation, and the supernatant solution was applied directly onto a β-D-galactosyl-Synsorb 100 column (18 × 1.2 cm; bed volume, 80 ml), which had been.equilibrated with 10 mM PBS (pH 7.2) containing 1 mM ammonium sulfate. The column was washed with the same solution until the absorbance at 280 nm of the effluent had fallen below 0.01. The affinity-adsorbed lectin was desorbed with 0.2 M lactose in 10 mM PBS, collected, dialyzed extensively against distilled water, and lyophilized (designated crude PSA). Approximately 18 mg of crude lectin was obtained from fresh fruiting body.

Crude lectin was reconstituted in 50 mM phosphate buffer, pH 7.8, and percolated slowly through a DEAE-Sephalac column (17 × 1.2 cm, bed volume 75 ml) preequilibrated and eluted with the same buffer (50 mM phosphate buffer, pH 7.8). The elution was monitored by absorbance at 280 nm until it become negligible, whereupon the adsorbed protein was eluted with 1 M sodium chloride. Both the DEAE-unbound (pass through) and DEAE-bound peak fractions were collected, dialyzed against distilled water, lyophilized, reconstituted in 10 mM PBS, pH 7.2, and tested for electrophoretic homogeneity and agglutination activity.

Protein Estimation—Protein concentration was determined by the method of Lowry et al. (12), using bovine serum albumin as a standard.

PAGE and SDS-PAGE—Native gel electrophoresis using a 12.5% slab gel was conducted in Tris/tricine buffer system as previously described by Schagger and von Jagow (14).

Hemagglutination Assay—The hemagglutinating activity of the lectin was determined by a 2-fold serial dilution procedure using formaldehyde-treated (15) human and rabbit erythrocytes as described previously (16). Briefly, varying amounts of glycoconjugate were mixed with 50 μl of PBS, pH 7.2, containing 0.1 M sodium chloride and incubated at 37 °C for 1 h, the reaction mixtures were stored at 4 °C for 24 h. The precipitates formed were centrifuged, washed three times with 150 μl of ice-cold PBS, dissolved in 0.05 M NaOH, and determined for protein content by Lowry's method using bovine serum albumin as standard.

For hemagglutination assays, increasing amounts of various haptenic saccharides were added to the reaction mixture consisting of 10 μg of the purified lectin and 5 μg of α2-macroglobulin in a final volume of 160 μl of PBS, pH 7.2. After incubation at 37 °C for 1 h and storage at 4 °C for 48 h, the precipitated proteins were centrifuged, washed, and determined. The percentage of inhibition was calculated, and inhibition phatase-streptavidin diluted with 0.1M Tris/HCl, pH 9.5, containing 100 mM NaCl and 5 mM MgCl2, incubated at 37 °C for 1 h, washed five times with the same buffer, and finally visualized with 5-bromo-4-chloro-3-indolylphosphate-nitroblue tetrazolium chloride. α2,6-Sialylation of Asialofetuin—Asialofetuin, 2.5 mg (40 nmol) in 1.0 ml of 50 mM MES buffer, pH 6.0, containing 10 mM MnCl2, 0.15M NaCl, 10% glycerol, 2 μmol of CMP-sialic acid, and 10 milliliters of rat liver α2,6-sialyltransferase (Roche Molecular Biochemicals, 8 units/mg of protein) was incubated 24 h at 37 °C, followed by addition of another 1 μmol of CMP-sialic acid and 5 milliliters of alysintransferase. After an additional 24 h of incubation, the reaction mixture was passed through a column (1 × 15 cm) of BioGel P-10. Fractions containing protein were pooled, dialyzed against distilled water, and lyophilized. The resialylated fetuin contained 3.1 mol of sialic acid/mol of protein, compared with a negligible amount (<0.1 mol of sialic acid/mol of protein) in the asialofetuin and approximately 10 mol of sialic acid/mol of protein in native fetuin.

Absorbance and Fluorescence Spectra—Ultraviolet absorbance spectra were recorded on a Shimadzu model UV160U spectrophotometer; fluorescence spectra were recorded on an ISA JodinYvon-Spek Fluoromax-2 spectrofluorometer.

RESULTS

Hemagglutinating Activity—In a survey of mushrooms for hemagglutination activity by Pemberton (20), it was found that the extract of P. squamosus exhibited strong hemolytic activity. In the present study, by using hemolysis-resistant, formaldehyde-treated erythrocytes, we observed that the crude extract of P. squamosus contained a lectin(s) that agglutinated rabbit and human erythrocytes, irrespective of blood group type (type A and O). The hemagglutinating activity was inhibited by D-galactose and D-galactose-related carbohydrates, such as D-fucose, L-arabinose, melibiose, and lactose.

Purification of the Lectin—Because the hemagglutination activity of the crude extract of P. squamosus was inhibited by D-galactose and lactose, β-D-galactosyl-Synsorb was used as an affinity absorbent for isolation of the lectin. As shown in Fig. 1, using affinity chromatography on β-D-galactosyl-Synsorb showed two protein bands, which we later found could be separated from each other by ion exchange chromatography on DEAE-Sephalac in 0.05 M phosphate buffer, pH 7.8 (Fig. 1). Under these conditions, the major portion of the lectin activity was not retained on the DEAE column. This DEAE-unbound

P. squamosus Lectin

butter—P. squamosus Lectin Conjugate—The biotinylation of the purified lectin was achieved using EZ-Link NHS-LC-Biotin according to the manufacturer's instructions, except that 0.2 μl lactose was added to the reaction mixture to protect the carbohydrate-binding sites. After coupling, the lectin activity was ascertained by hemagglutination assay.

HPTLC of Glycolipids and Lectin Staining—Two identical sets of glycolipids were separated chromatographically in parallel on the same aluminum-backed silica gel 60 HPTLC plate (Merck, Darmstadt, Germany) using chloroform/methanol/water (65:25:4, by volume) for neutral glycolipids or chloroform/methanol/aqueous 0.25% KCl (50:48:10, by volume) for gangliosides as developing solvent. The reference chromatogram was chemically visualized by spraying the plate with orcinol reagent.

The lectin staining was performed as follows: after drying, the plates were blocked by overlaying with 1% gelatin in PBS containing 0.1% bovine serum albumin and 0.05% Tween 20, and incubated at 4 °C for 2 h. After rinsing the plates five times with 10 mM Tris/HCl buffer, pH 9.5, containing 0.05% Tween 20, they were overlaid with alkaline phosphatase-streptavidin diluted with 0.1 M Tris/HCl, pH 9.5, containing 100 mM NaCl and 5 mM MgCl2, incubated at 37 °C for 1 h, washed five times with the same buffer, and finally visualized with 5-bromo-4-chloro-3-indolylphosphate-nitroblue tetrazolium chloride. α2,6-Sialylation of Asialofetuin—Asialofetuin, 2.5 mg (40 nmol) in 1.0 ml of 50 mM MES buffer, pH 6.0, containing 10 mM MnCl2, 0.15M NaCl, 10% glycerol, 2 μmol of CMP-sialic acid, and 10 milliliters of rat liver α2,6-sialyltransferase (Roche Molecular Biochemicals, 8 units/mg of protein) was incubated 24 h at 37 °C, followed by addition of another 1 μmol of CMP-sialic acid and 5 milliliters of alysintransferase. After an additional 24 h of incubation, the reaction mixture was passed through a column (1 × 15 cm) of BioGel P-10. Fractions containing protein were pooled, dialyzed against distilled water, and lyophilized. The resialylated fetuin contained 3.1 mol of sialic acid/mol of protein, compared with a negligible amount (<0.1 mol of sialic acid/mol of protein) in the asialofetuin and approximately 10 mol of sialic acid/mol of protein in native fetuin.

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fraction was electrophoretically pure (designated purified PSA). The minimal concentration of the purified lectin required for the agglutination of formaldehyde-treated human type B erythrocytes was 0.6 μg/ml. Removal of divalent metal ions by extensive dialysis of this fraction against buffer containing 1.25 mM EDTA had little effect on its agglutination activity. The DEAE-bound fraction exhibited about 30 times lower agglutinating activity, 19 μg/ml being required for agglutination; therefore, in the present study, characterization of only the DEAE-unbound fraction was pursued.

**Molecular Mass and Molecular Structure**—Upon gel filtration chromatography on Bio-Gel P-150, the purified lectin eluted as a single, symmetric peak, irrespective of the presence of 0.2 mM lactose, at an elution volume corresponding to an apparent molecular mass of 52 kDa (not shown). On the other hand, upon SDS-PAGE, with or without β-mercaptoethanol, purified PSA gave a single band with an apparent mass of 28 kDa (Fig. 2). Taken together, these data suggest that at neutral pH, the lectin exists as a homodimer of 28-kDa subunits associated by noncovalent bonds. No neutral carbohydrate was detected using the phenol-sulfuric acid assay.

**Amino Acid Composition and N-terminal Amino Acid Sequence**—As shown in Table I, purified PSA contains an extremely high proportion of hydrophobic amino acids (Ala, Ile, Leu, Val, and Phe) that account for one-third of the total amino acids, high contents of acidic and hydroxyl amino acids (Asx and Glx account for 22%; Ser and Thr, 14%), and relatively high amounts (11.5%) of aromatic amino acids, accounting for the high absorbency at 280 nm of the lectin (A280 = 29.0). The lectin also contains two residues each of methionine and cysteine. No free sulphydryl groups were detected by reaction with 5,5'-dithiobis(2-nitrobenzoic acid); together with the observation that β-mercaptoethanol has no effect on SDS-PAGE migration, the presence of an intrachain disulfide linkage is indicated. A single N-terminal amino acid sequence, H2N-IPFEGHGIY-HIPSVNTANVRI, was determined. A search of the protein data base revealed no significant homology of this N-terminal sequence to any sequence in the data base.

**Quantitative Precipitation and Precipitation Inhibition**—Inasmuch as the hemagglutinating activity of the crude extract of *P. squamosus* was specifically inhibited by galactose and the lectin was initially isolated by affinity chromatography on β-D-galactosyl-Synsorb, various glycoproteins were chemically desialylated to expose their penultimate D-galactosyl residues and tested for their ability to precipitate the lectin. However, none of these asialoglycoproteins formed a detectable precipitate with the lectin. The galactomannan from *Cassia alata*, which contains multiple terminal α-D-galactosyl residues, also failed to precipitate with the lectin. To our great surprise, of the many native glycoproteins tested, including fetuin, transferrin, thyroglobulin, α1-acid glycoprotein, bovine mucin, and ovine submaxillary mucin, only human α2-macroglobulin, but not its desialylated form, gave a pronounced precipitation reaction with the lectin. Therefore, human α2-macroglobulin was employed as a precipitant in the inhibition assays.

![Fig. 1. Native PAGE of PSA on a 12.5% gel in Tris/glycine running buffer at pH 8.3. Lane 1, crude lectin preparation from affinity chromatography on β-D-galactosyl-Synsorb containing two protein bands; lane 2, purified PSA from pass-through fraction from DEAE-Sepharcl in 0.05 M phosphate buffer, pH 7.8; lane 3, the DEAE-bound fraction having low hemagglutinating activity.](http://www.jbc.org/content/106/25/A10625/F1)

![Fig. 2. SDS-PAGE of purified PSA on a 12.5% slab gel in Tris/Tricine running buffer, pH 8.3, showing a single band with an apparent subunit mass of 28 kDa. The same result was obtained in the presence of β-mercaptoethanol. Left lane, purified PSA; right lane, molecular mass standards (BenchMark Protein Ladders) from Life Technologies, Inc.](http://www.jbc.org/content/106/25/A10625/F2)

**TABLE I**

| Amino acid | Mol % | Residues/subunit |
|------------|-------|------------------|
| Asx        | 14.5  | 38               |
| Glx        | 7.53  | 20               |
| Ser        | 6.51  | 17               |
| Gly        | 10.7  | 28               |
| His        | 1.33  | 3                |
| Arg        | 2.71  | 7                |
| Thr        | 7.72  | 20               |
| Ala        | 9.60  | 25               |
| Pro        | 3.50  | 9                |
| Tyr        | 2.94  | 8                |
| Val        | 5.71  | 15               |
| Met        | 0.89  | 2                |
| Cys        | 0.65  | 2                |
| Ile        | 4.25  | 11               |
| Leu        | 7.65  | 20               |
| Phe        | 5.52  | 14               |
| Lys        | 4.94  | 13               |
| Trp        | 3.18  | 8                |
| Total residues | 260 |

*Estimated by 5,5'-dithiobis(2-nitrobenzoic acid) after denaturation (6 M guanidine HCl) and reduction.
*Estimated spectrophotometrically by the method of Edelhoch (19).
The results of sugar hapten inhibition are shown in Table II. Among the monosaccharides tested, only D-galactose, its derivatives, and D-galactose-related carbohydrates (i.e. D-fucose and L-arabinose) were inhibitory, whereas epimers of D-galactose (i.e. D-talose (C-2 epimer), D-gulose (C-3 epimer), and D-glucose (C-4 epimer)) were all non-inhibitory up to 100 mM (D-glucose to saturation).

Inhibition of precipitation of P. squamosus agglutinin with α2-macroglobulin by oligosaccharides

| Sugara       | IC50 b | Relative potency |
|--------------|--------|------------------|
| Galactose    | 30     |                  |
| GalMe        | 45     | 0.67             |
| GalEMe       | 15     | 2.0              |
| 2-Deoxy galactose | 14     | 2.1              |
| Melibiose (Gal-1,6Glc) | 5.3     | 5.7              |
| D-Fucose     | 40     | 0.75             |
| D-Glucose    | 0.04   | 0.75             |
| 2-Deoxy galactose | 6.4     |                  |
| 2-Mannosamine | 100      | <0.3             |
| 2,8-Linked polymer (Colominic acid) | 100 (15%) | <0.3             |
| 2,3 Lactose  | 1.6    |                  |
| 2,3 LacNAc   | 0.17   |                  |
| 2,6 Lactose  | 0.16   |                  |
| 2,6 LacNAc   | 0.014  | 2143             |
| 2,3 LacNAc   | 0.15   |                  |
| 2,6 LacNAc   | 0.015  |                  |
| 2,3 LacNAc   | 0.005  |                  |
| 2,6 LacNAc   | 0.001  |                  |
| 2,6 LacNAc   | 0.05   |                  |
| p-Nitrophenyl-α-mannoside | 100 (16%) | <0.3             |
| p-Nitrophenyl-α-glucoside | 100 (15%) | <0.3             |
| p-Nitrophenyl-α-fucoside | 100 (15%) | <0.3             |
| p-Nitrophenyl-α-arabinoside | 100 (15%) | <0.3             |
| p-Nitrophenyl-α-L-rhamnoside | 100 (15%) | <0.3             |
| P. squamosus Lectin

* Maximum concentration of inhibited by inhibition observed.

a N-Acetyl-glucosamine, D-arabinose, 2-deoxy-ribose, D-fucose, D-mannose, L- and D-rhamnose, L- and D-ribose, L- and D-xylene, cellobiose, gentiobiose, maltose, isomaltose, sucrose and trehalose were all non-inhibitory up to 200 mM (D-glucose to saturation).

b Minimum concentration required for 50% inhibition of the PSA/α2-macroglobulin precipitation reaction, unless otherwise noted.

The results of sugar hapten inhibition are shown in Table II. Among the monosaccharides tested, only D-galactose, its derivatives, and D-galactose-related carbohydrates (i.e. D-fucose and L-arabinose) were inhibitory, whereas epimers of D-galactose (i.e. D-talose (C-2 epimer), D-gulose (C-3 epimer), and D-glucose (C-4 epimer)) were all non-inhibitory up to 100 mM. However, the most striking observation was that both Neu5Ac and 2,3-linked Neu5Ac at the β-linked galactosyl residue, also failed to react with the lectin, further confirming that this lectin is exclusively specific for α2,6-linked Neu5Ac.

Spectral and Fluorescence Studies—Changes in absorbance or fluorescence spectra were examined as probes for binding studies. It had been observed that p-nitrophenyl α-d-mannoside, upon binding to the mannose/glucose-specific lectin concanavalin A, undergoes a small but definite spectral change with a maximum decrease in absorbance at 317 nm, apparently due to interaction of an aromatic residue with the chromophore (21). Accordingly, we looked for such a spectral change with both pNPβGal and pNPβLac upon reaction with PSA, but none was observed (data not shown). Another indication of the interaction of an aromatic aglycon with residues in the lectin is the complete quenching of fluorescence of methylumbelliferyl α-d-mannoside (excitation at 320 nm, emission peak at 375 nm) upon binding to concanavalin A (22). Again, however, fluorescence of methylumbelliferyl β-d-galactoside was unaffected by titration with PSA, suggesting that an aromatic residue of the protein is not in a position near the binding site to interact with the chromophore or fluorophore of these β-galactosides.

We also examined the effects of ligands on the intrinsic fluorescence of tryptophanyl residues of the protein, as measured by excitation at 280 nm and emission at 300–400 nm. Free monosaccharides, free oligosaccharides, or methyl glycosides had no effect on the intrinsic fluorescence peak at 340 nm. As expected because of its strong UV absorbance, all p-nitrophenyl glycosides quenched intrinsic fluorescence and caused a slight shift in the emission peak toward longer wavelengths. However, we observed that quenching by pNPβGal or pNPβLac was significantly greater than that caused by a nonreactive p-nitrophenyl glycoside, such as p-nitrophenyl α-d-mannoside, at the same concentration. Furthermore, addition of competing oligosaccharides, such as lactose or LacNAc, reversed this additional quenching in a saturable manner, indicating that it is caused by the p-nitrophenyl β-galactosides binding in the vicinity of a fluorophoric group on the lectin. We thus refer to this phenomenon as “specific quenching.”

Thus, intrinsic fluorescence titration of oligosaccharide ligands in the presence of a fixed amount of pNPβGal yielded apparent inhibition constants (IC50 values) for the titrant from a plot of 1/ΔF versus 1/[L], where ΔF is the increase in peak fluorescence caused by the titrant at a concentration of [L], in a manner analogous to Lineweaver-Burk plots. The ordinate intercept gives the reciprocal of the maximum fluorescence change ΔFmax (in arbitrary fluorescence units), which is a function of concentration and binding affinity of the quenching probe. Table IV summarizes data for several oligosaccharides and chromophoric probes. At a lectin concentration of 0.09 mg/ml (3.2 μM in monomers) and pNPβGal of 100 μM, the maximum fluorescence change was approximately 120,000 units, as compared with a total quenching of about 800,000, or about 15% of the total quenching. The use of pNPβGal at the same concentration also caused a specific quenching signal, but the ΔFmax was only about 40,000 units, consistent with the weaker interaction of the lectin with α-galactosides.

DISCUSSION

By definition, a lectin is a sugar-binding protein or glycoprotein of nonimmune origin that agglutinates cells and/or precipitates glycoconjugates (1). In order to form detectable precipitate, however, both the lectin and the glycoconjugate must be multivalent as well as in an appropriate stoichiometric ratio.
respectively) are unreactive. The reactivity of specifically at the 6-position of nonreducing terminal lin, failed to precipitate with PSA. In fact, we observed that contain a few nonreducing terminal Neu5Ac precipitation reaction (data not shown), indicating that they do of the tri- and tetraantennary structures, thus precluding lec-
mimic acids. In addition to the prevalence of 2,6-linked Neu5Ac residues at the nonreducing termini of these glycoproteins, many
methyl-D-galactose are nearly equal to D-galactose in in-
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inhibitory potentcy suggests that the 6-hydroxyl group is not
1,4Glc/NAc, indicate that the 2-hydroxyl group is also important in

Of all the sialoglycoproteins we assayed for their ability to form a precipitate with the P. squamosus agglutinin, only hu-
man α2-macroglobulin precipitated the lectin. Human α2-mac-
roglobulin is composed of four identical subunits, each of which contains 1451 amino acid residues and eight N-linked sugar chains (23) that are 70% sialylated, exclusively in Neu5Aco2,6 linkage (24). On the other hand, most native glycoproteins, including α1-acid glycoprotein, fetuin, transferrin, and thyro-
globulin, contain both α2,3- and α2,6-linked N-acetyleneur-
aminic acids. In addition to the prevalence of α2,3-linked sialic acids at the nonreducing termini of these glycoproteins, many α2,6-linked Neu5Ac residues occur not at the nonreducing term-
lin binding. It is noteworthy that these α2,6-linked sialic acid groups are also resistant to cleavage by neuraminidase from Clostridium perfringens (25). To further confirm the specificity of PSA for terminal α2,6-linked Neu5Ac, we resialylated asialo-

Taking these facts into consideration, it is understandable why the native glycoproteins tested, except for α2-macroglo-

To elucidate the detailed carbohydrate binding specificity of purified PSA, precipitation inhibition assays were carried out using α2-macroglobulin as the precipitant. The data in Table II permit some conclusions to be drawn regarding the specificity of the lectin. The 2-, 3-, and 4-hydroxyl groups in the galacto-

Both methyl β-d-galactopyranoside and p-nitrophenyl β-d-galactopyranoside are three times more potent inhibitors than the corresponding α-glycosides, as indicated both by precipitin inhibition and extent of specific fluorescence quenching, sug-

The carbohydrate structures of neutral and acidic glycosphingolipids

| Name (trivial name) | Carbohydrate structure |
|--------------------|------------------------|
| Neutral glycosphingolipids | Galβ1,4Glβ1,1Cer |
| Lactosyl ceramide | Galα1,4Galβ1,4Glβ1,1Cer |
| (Lac-cer, CDH) | GalNAcβ1,3Galα1,4Galβ1,4Glβ1,1Cer |
| Globotrihexosyl ceramide | GalNAcβ1,3Galα1,4Galβ1,4Glβ1,1Cer |
| Globotetrahexosyl ceramide | GalNAcβ1,3Galα1,4Galβ1,4Glβ1,1Cer |
| Globopentaosyl ceramide | GalNAcβ1,3Galα1,4Galβ1,4Glβ1,1Cer |
| (Forsman glycolipid, Glb5) | GalNAcβ1,3Galα1,4Galβ1,4Glβ1,1Cer |
| Gangliosides (acidic glycosphingolipids) | GalNAcβ1,3Galα1,4Galβ1,4Glβ1,1Cer |
| Monosialogangliosides | GalNAcβ1,3Galα1,4Galβ1,4Glβ1,1Cer |
| GM3 | GalNAcβ1,3Galα1,4Galβ1,4Glβ1,1Cer |
| GM2 | GalNAcβ1,3Galα1,4Galβ1,4Glβ1,1Cer |
| GM1 | GalNAcβ1,3Galα1,4Galβ1,4Glβ1,1Cer |
| Disialogangliosides | GalNAcβ1,3Galα1,4Galβ1,4Glβ1,1Cer |
| GD3 | GalNAcβ1,3Galα1,4Galβ1,4Glβ1,1Cer |
| GD1a | GalNAcβ1,3Galα1,4Galβ1,4Glβ1,1Cer |
| GD1b | GalNAcβ1,3Galα1,4Galβ1,4Glβ1,1Cer |

The substitution of the C-6 hydroxyl group of the nonreduc-
ting terminal β-galactosyl residue with α 2,6-linked N-acetyl-
neuraminic acid increased the inhibitory potency by 3 orders of magnitude (2000-fold) as compared with galactose and 250–
P. squamosus Lectin

![Image](http://www.jbc.org/jbc/article-lookup/10.1074/jbc.201807.117/A1)

**FIG. 3.** HPTLC of glycolipids and lectin staining. Two identical sets of glycolipids were separated in parallel on the same aluminum-backed silica gel 60 HPTLC plate. Solvents were chloroform/methanol/water (65:25:4, by volume) (A) and chloroform/methanol/0.25% KCl (50:40:10, by volume) (B). The reference chromatograms were visualized chemically by orcinol reagent; lectin staining was conducted as described under "Materials and Methods." A, neutral glycosphingolipids (for structures, see Table III). Lane 1, lactosyl ceramide; lane 2, globotriosyl ceramide (note: the two bands are due to heterogeneity of the lipid moiety; the upper band contains nonhydroxy fatty acid side chain, and the lower band contains mostly hydroxylated fatty acid side chains); lane 3, globotetraosyl ceramide; lane 4, globopentaosyl ceramide; lanes 5 and 6, mixture of the four glycolipids. Lanes 1–5 were detected by orcinol reagent, and lane 6 was stained with biotinylated lectin. Among neutral glycolipids tested, only lactosyl ceramide reacted with the lectin. B, acidic glycosphingolipids (gangliosides; see Table III for structures). Lanes 1 and 3, monosialoganglioside mixture including GM3, GM2, and GM1; lanes 2 and 4, disialoganglioside mixture containing GD3, GD1a, and GD1b. Lanes 1 and 2 were visualized by orcinol spray, and lanes 3 and 4 were stained with biotinylated PSA. None of the gangliosides tested (all have α2,3-linked Neu5Ac, but lack α2,6-linked Neu5Ac) were bound by the lectin, further confirming the results obtained by precipitation inhibition assays that PSA is exclusively specific for α2,6-linked Neu5Ac.

300 times compared with the parent sugars (i.e. lactose and LacNAc). However, free N-acetylneuraminic acid, ρ-nitrophe- nyl α-sialoside, and the Neu5AcO2.8 polymer colominic acid did not react, whereas galactose derivatives having an acidic function near the C-6 position (α-galacturonic acid, ρ-galactose 6-sulfate, and α-galactose 6-phosphate) are moderate to very weak inhibitors. Furthermore, the addition of sialic acid to the C-3 position of the nonreducing terminal galactose abolished the lectin binding almost totally. This is in contrast to S. nigra agglutinin, a plant lectin isolated from elderberry (Sambucus nigra) bark, which recognizes not only the terminal Neu5Ac2,6Gal/GalNAc sequence but also the α2,3-linked iso- mer, to a 100-fold smaller extent (28). The exclusive specificity of PSA is consistent with the C-3 equatorial hydroxyl group of the terminal galactosyl group being a critical locus, so that its substitution abrogates the lectin binding, probably due to steric effects. However, the C-6 hydroxy group of the α-pyranose ring is not involved in lectin binding, so that substitution at that position of the galactose is tolerated. Furthermore, sialylation at the C-6 position evidently creates additional interactions via hydrogen bonds and/or charge interactions between the carboxylate group and positively charged amino acids in the vicinity of the carbohydrate-binding site of the lectin. However, because free sialic acid, or sialosides, its polymer, or its 2,3-substituted form does not react, the lectin does not appear to have an independent binding site for sialic acid and cannot be consid- ered as an N-acetylneuraminic acid-binding lectin.

A lectin with similar affinity was isolated from tubers of Trichosanthes japonica (Cucurbitaceae) (29). That lectin also recognized β-galactosyl residues and was greatly enhanced by α2,6-sialylation but blocked by α2,3 sialylation. However, that lectin did not react with the Galβ1,3GlcNAc group, showed tolerance for C-2 epimerization of the galactose (i.e. β-talose), and had little or no preference for β-galactosides versus α-galactosides. Those workers also observed that 6-sulfated lactose and 6′-sialylacto-N-neotetraose strongly interacted with the lectin. We have tested neither of these latter compounds, but would expect them to react at least as well as lactose and 6′-sialylactose, respectively. We did, however, observe that galactose 6-sulfate is equivalent in its inhibitory potency to galactose. On the other hand, a lectin isolated from a related polypore mushroom, Laetiporus sulphureus, appears to be completely different in its molecular structure, amino acid composition, and carbohydrate binding specificity (30).

The inhibitory potency of LacNAcβOMe and the isomeric Galβ1,3GlcNAcβOMe are similar, suggesting that PSA would recognize Neu5Ac2,6Galβ1,3GlcNAc as well as it does Neu5Ac2,6Galβ1,4GlcNAc. However, until an authentic sample of this sialylated β1,3 oligosaccharide is assayed, we can only speculate on its recognition by the mushroom lectin.

| Quencher   | Ligand          | $K_i$ | Δ$F_{\text{max}}$ (specific quenching) |
|------------|-----------------|------|--------------------------------------|
| pNPβGal   | Sialo2,6Lac     | 1.88 | 135                                  |
|           | Sial-Lac        | 2.08 | 123                                  |
|           | Sialo2,3Lac     | NR   |                                       |
|           | Sialo2,6LacNAc  | 1.38 | 138                                  |
|           | Sialo2,5LacNAc  | 1.05 | 119                                  |
| Lactose    | 105             | 119  |                                       |
|           | LacNAc          | 128  | 116                                  |
|           | LacNAc-βOMe     | 96   | 114                                  |
|           | Lac-N-biose-βOMe| 227  | 126                                  |
| pNPβLac   | Lactose         | 111  | 87                                   |
| pNPβLacNAc| Lactose         | 259  | 96                                   |
| pNPβLacNAc| NR              | 0    |                                       |

* Mixed α2,3 and α2,6-linked from human milk (~80% α2,6).

† 10% of Δ$F$ given by Sialo2,6LacNAc at 5 μM; double reciprocal plot extrapolated below origin.

NR, no reaction.
P. squamosus Lectin

10629

Of equal importance is the finding that ovine submaxillary mucin, which contains a prodigious number of Neu5Acα2,6GalNAc1-Ser/Thr moieties per molecule, is neither a precipitant nor an inhibitor of PSA, in contrast to S. nigra agglutinin, which requires only a siulio2,6Gal/GalNAc moiety (28). This observation is consistent with the preference of PSA for three structural features lacking in the mucin O-linked glycans: a β-galactosidic linkage (cf. Galβ1OMe versus GaloOMe), the necessity of a free equatorial 2-OH group on the galactosyl residue (cf. galactose versus GalNAc, 2-deoxy galactose, or D-talose), and the preference for an additional sugar at the β-galactosidic linkage (cf. lactose or LacNAc versus GalβOMe).

The relative inhibitory constants of oligosaccharides tested in the specific fluorescence quenching assay (Table IV) are consistent with those observed by precipitin inhibition (Table II), although absolute values are lower in the former case. This difference is understandable, because the reporter ligand in the fluorescence assay is monovalent, whereas in the precipitin assay, it is a polyvalent ligand leading to precipitation of an extensive network of lectin and glycoprotein molecules, requiring higher concentrations of a given competitive monovalent ligand to cause inhibition. Preliminary measurements of ligand binding using isothermal titration calorimetry showed PSA to bind methyl-β-galactoside, lactose, and Neu5Acα2,6Lac with $K_d$ values of 393, 92, and 0.59 μM, respectively (data not shown), values that are also 30–40-fold lower than the IC₅₀ values for precipitation inhibition (Table II) but comparable to $K_i$ values for specific fluorescence quenching (Table IV). Calorimetric studies with PSA, as well as further studies of this specific quenching phenomenon, which we have observed with several lectins, are in progress and will be published subsequently.

In conclusion, the P. squamosus agglutinin possesses an extended carbohydrate-combining site with strict specificity and high affinity for nonreducing terminal Neu5Acα2,6Galβ14GlcNAc residues. Thus, it appears that PSA has a binding site that accommodates three carbohydrate moieties. This specificity could make this lectin an invaluable tool for glycobiochemical studies, especially for cancer research and diagnosis. For example, it has been well documented in NIH3T3 (or FR3T3) cells transformed with ras oncogene that there is an increased β-galactoside α-2,6-sialyltransferase activity (31, 32) and a concomitant decreased CMP-Neu5Ac: Galβ13GalNAc α-2,3-sialyltransferase activity (33). Furthermore, some tumors, e.g. hepatocellular carcinoma (34) and human colorectal tumors (35, 36), express a high level of α2,6-sialylation of N-acetylgalactosaminic sequences on their cell surface, which is correlated with high metastatic potential (37, 38).

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Purification and Characterization of a Neu5Acα2−6Galβ1−4Glc/GlcNAc-specific Lectin from the Fruiting Body of the Polypore Mushroom *Polyporus squamosus*

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