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Purification and carbohydrate-binding specificity of *Agrocybe cylindracea* lectin

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A lectin was isolated from fruiting bodies of *Agrocybe cylindracea* by two ion-exchange chromatographies and gel filtration on Toyopearl HW55F. The lectin was homogeneous on polyacrylamide gel electrophoresis and its molecular mass was determined to be 30 000 by gel filtration, and 15 000 by sodium dodecylsulfate polyacrylamide gel electrophoresis, signifying a dimeric protein.

Its carbohydrate-binding specificity was investigated both by sugar-hapten inhibition of hemagglutination and by enzyme-linked immunosorbent assay. The inhibition tests showed the affinity of the lectin to be weakly directed toward sialic acid and lactose, and the enhanced affinity toward trisaccharides containing the NeuAc*α*2,3Gal*β*-structure. Importantly, the lectin strongly interacted with glycoconjugates containing NeuAc*α*2,3Gal*β*1,3GlcNAc-/GalNAc sequences.

***Keywords*: *Agrocybe cylindracea*, fungal lectin, carbohydrate-binding specificity, sialic acid-containing carbohydrate chain**

Introduction

Several lectins [1, 2] have been reported to interact with sialic acid-containing glycoconjugates, and some of them have been shown to recognize the sialic acid residue itself.

Knibbs *et al.* [3] reported differences in the carbohydrate binding specificity of three sialic acid-specific lectins: *Limax flavus* agglutinin (LFA), *Maackia amurensis* leuco-agglutinin (MAL), and *Sambucus nigra* agglutinin (SNA). Additionally, Konami *et al.* [4] reported that *Maackia amurensis* hemagglutinin (MAH) strongly interacted with NeuAcα2,3Galβ1,3(NeuAcα2,6)GalNAc. Considering the biological importance of sialic acid-containing conjugates [5], it would be useful to have a number of lectins with specificity toward complex sialoglycocojugates. For example, no lectin with affinity toward NeuAcα2,3Galβ1,3GlcNAc- and NeuAcα2,8Gal- sequences has been reported.

We purified a lectin from fruiting bodies of an edible fungus *Agrocybe cylindracea*, and found that this lectin had an affinity toward sialic acid. In this study, we report the purification of the lectin together with its specificity toward glycoconjugates containing sialic acid.

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Materials and methods

The fruiting bodies of *Agrocybe cylindracea* were kindly supplied by Satsuma Kako Co. (Kagoshima, Japan).

Goat antibodies against rabbit IgG, conjugated with alkaline phosphatase, were purchased from Caltag laboratories Inc. (San Francisco, CA, USA). DEAE-Toyopearl 650M and Toyopearl HW 55F were purchased from Tosoh Ltd. (Tokyo, Japan). DEAE-cellulofine A-200 was from Chisso Co. (Kumamoto, Japan).

Saccharides and glycoproteins

All monosaccharides and disaccharide were from Sigma Chemical Co. (St Louis, MO, USA). Sialotrisaccharides were purchased from Oxford Gycosystems Co. (Abingdon, UK). Murine laminin was purified according to the method of Shibata *et al.* [6]. IgA was purchased from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA, USA). κ-Casein and caseinoglycopeptide were gifts from Professor Aoki, Kagoshima University, and Dr Saitoh, Tohoku University, respectively. Asialoglycoproteins were prepared by hydrolysis with 0.1 M H_2SO_4 at 80 °C.

Intact Neu5Acα2,3Galβ1,3GalNAc and Neu5Acα2,3Galβ1,3(Neu5Acα2,6)GalNAc were obtained from carbohydrate-rich caseinoglycopeptide [7] after hydrazinolysis at 60 °C [8] followed by HPLC separation under the same condition for the reduced forms of these oligosaccharides [9].

The glycoconjugates Neu5Acα2,3Galβ-bovine serum albumin (BSA), Neu5Acα2,3Galβ1,3GlcNAcβ-BSA and Neu5Acα2,3Galβ1,4GlcNAcβ-BSA were the products of Chembiomed Co. (Edmonton, Alberta, Canada) as described in the paper by Knibbs *et al.* [3].

The gangliosides D1a, D1b, M1a, and asialo-M1 were purchased from Sigma Chemical Co. (St Louis, MO, USA).

Assay and analytical methods

Hemagglutination assay

Hemagglutinating activity was determined in wells of microtiter plates, in a final volume of 70 μl. Each well contained 50 μl of lectin solution and 20 μl of a 4% (by vol.) suspension of trypsinized erythrocytes.

Agglutination was assessed after incubation for 1 h at room temperature, and hemagglutinating activity was expressed as titer, namely, the reciprocal of the highest dilution that gave a positive result. The specific hemagglutinating activity was defined as titer (mg lectin)$^{-1}$.

Quantitation of protein and carbohydrate

Protein was quantified by the method of Lowry *et al.* [10] with bovine serum albumin as the standard, and carbohydrate was quantified by the phenol-sulfuric acid method of Dubois *et al.* [11] with D-mannose as the standard.

Electrophoresis

Polyacrylamide gel electrophoresis (PAGE) was carried out by the method of Davis [12].

Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using a discontinuous system, as described by Laemmli [13].

Proteins on all gels were stained with Coomassie brilliant blue R-250.

Estimations of molecular mass

The molecular mass of the purified lectin was estimated by gel filtration on a column of Toyopearl HW 55F (2.5 cm i.d. × 140 cm), by SDS-PAGE, and by HPLC. HPLC was performed with a model 880 PU pump, a model 875 UV detector and a Fine Pak SIL AF-102 column (JASCO, Tokyo, Japan). The column was eluted with 10 mM sodium phosphate buffer, pH 6.8, that contained 0.2 M sodium sulfate at a flow rate of 0.5 ml min^{-1}, and column effluents were monitored by absorption at 280 nm.

Amino acid analysis and determination of N-terminal amino acid sequence

Each purified lectin (50 μg) was hydrolysed with constant-boiling HCl in a sealed evacuated glass tube for 24 and 72 h at 110 °C. The hydrolysates were analysed with an amino acid analyser (model 835; Hitachi, Tokyo, Japan). Half-cystine was determined as cysteic acid by the method of Hirs [14]. Tryptophan was determined by the spectrophotometric method of Edelhoch [15].

Amino-terminal sequencing was performed with a model 492 protein sequencer fitted with a model 140C PTH analyser (Applied Biosystems-Perkin Elmer Japan, Tokyo).

Immunological analyses

Rabbit antisera against the lectin from *A. cylindracea* were prepared with Freund's complete adjuvant (Nacalai Tesque Inc. Kyoto, Japan).

The antibodies were used for examining carbohydrate binding specificity by the enzyme-linked immunosorbent assay (ELISA).

ELISA

The following four procedures were adopted as the ELISA assay.

(1) The wells of flat-bottomed titer plates were incubated with a solution of BSA-glycoconjugates (5 μg per 50 μl) or murine laminin (3 μg per 50 μl) in phosphate-buffered saline (PBS), pH 7.2, overnight at 4 °C followed by washing three times with PBS containing 0.05% Tween 20 (PBS-T). After washing, 50 μl of 3% BSA in PBS was added to each well and incubated for 3 h. The plates were emptied again and washed with PBS-T three times. The lectin solution (1 ng to 500 ng per 50 μl PBS) was added to the wells and incubated for 2 h. The wells were washed three times with PBS-T. The antiserum against *Agrocybe* lectin was diluted 1000-fold with PBS, and wells were incubated with the diluted solution of antiserum for 2 h at room temperature. The plates were emptied again and washed twice with PBS-T. The wells were further incubated with the second antibody (antibodies raised in goat against rabbit IgG, conjugated with alkaline phosphatase and diluted 1000-fold with PBS) for 1.5 h at room temperature. The wells were washed twice with PBS-T in order to remove unbound conjugates. The phosphatase reaction was initiated by the addition, at 30 °C, of 50 μl of 0.1 M glycine-NaOH buffer, pH 10.3, containing 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$, 0.2 mg ml^{-1} BSA and 0.55 mM *p*-nitrophenyl phosphate to each well, and the reaction was quenched by the addition of 50 μl of 1 M NaOH after 30 min. The developed colour of liberated *p*-nitrophenol was monitored at 405 nm with a model 450 microplate reader (BioRad, Richmond, CA, USA).

(2) Lectin solution (2 μg per 50 μl PBS) was used for coating the wells, and then the BSA-conjugates (5 μg) were added to the wells to react with the coated lectin. The reaction was carried out for 2 h. PBS-T was used for washing the wells in a similar manner as (1).

As a blocking solution, 50 μl of 3% ovalbumin in PBS was added to each well and incubated. After 3 h, the plate was emptied and the wells were washed with PBS-T three times. Rabbit IgG against BSA was diluted 1000-fold and added to the wells. The following procedures were the same as described in (1).

(3) Gangliosides were dissolved in ethanol and an aliquot (7 nmol) was dried on the wells. Then 50 µl of 3% BSA was added to each well and incubated for 18 h. The plates were washed with PBS-T three times, and after the addition of lectin solution (1–500 ng) in 50 µl PBS were incubated for 2 h. The following procedures after the use of antibodies against lectin were the same as described in (1).

(4) Each well of the ELISA plate was incubated with κ-Casein (20 µg) in 50 µl of PBS at 4 °C overnight. The plates were emptied and were washed with PBS-T three times. Then 50 µl of 3% BSA was added to each well and incubated for 18 h. The wells were washed with PBS-T three times. Lectin (0.5 µg) or lectin-gangliosides solution was preincubated in 10 µl PBS at room temperature for 20 min, and incubated for 2 h in the wells after five-fold dilution. The procedures after this step were the same as described in (1).

For estimating the interaction with lectin and Neu5Acα2, 3Galβ1, 3GalNAc or NeuAcα2, 3Galβ1, 3(NeuAcα2,6) GalNAc, the preincubation described above was not carried out.

Purification procedure

Hemagglutinating activity was measured throughout all purification procedures with trypsinized human type A and trypsinized rabbit erythrocytes. A total of 300 g of fruiting bodies of *A. cylindracea* was homogenized with 1.5 l of PBS at 4 °C, and the homogenate was filtered through two layers of gauze and centrifuged at 8000 × **g**. Ammonium sulfate was added to the supernatant solution to give 90% saturation. The precipitate was collected by centrifugation and dialysed against 2 l of 10 mM sodium phosphate buffer, pH 7.5, with five changes of the buffer over the course of 48 h. The dialysate (450 ml) was loaded on to a column of DEAE-cellulofine A-200 (2.7 cm i.d. × 45 cm) that had previously been equilibrated with 10 mM sodium phosphate buffer, pH 7.5. After washing of the column with 1500 ml of the same buffer, the hemagglutinating activity was eluted with a linear gradient of 0–1.0 M NaCl (total 2 l) in the same buffer. The active fractions were combined and protein was precipitated with ammonium sulfate (100% saturation).

The precipitated protein was dissolved in 80 ml of 10 mM sodium phosphate buffer, pH 6.6, and dialysed against the same buffer with five changes of the buffer over the course of 48 h. The dialysate was loaded on to a column of DEAE-Toyopearl 650 M (2.5 cm i.d. × 35 cm) that had been equilibrated with the same buffer. The column was washed with 500 ml of 10 mM sodium phosphate buffer, pH 6.6, and then protein was eluted with a linear gradient of NaCl (0–0.5 M, total 600 ml) in the same buffer. The active fractions, eluted by a NaCl gradient, were combined and the protein was concentrated by precipitation with ammonium sulfate. The precipitated protein was dissolved in 5 ml of 0.1 M phosphate buffer, pH 7.0, and loaded on a column of Toyopearl HW 55F (2.4 cm i.d. × 141 cm). The single resultant peak of activity coincided with a peak of protein.

Results

Purification of a lectin from fruiting bodies of A. cylindracea

From 300 g of fruiting bodies of *A. cylindracea*, 25.2 mg of the purified lectin was obtained. Figure 1 shows the second ion-exchange chromatography on DEAE-Toyopearl 650 M. Table 1 summarizes the purification. The yield of the lectin after gel filtration was 40% from the crude extract on the basis of hemagglutination with trypsinized rabbit erythrocytes.

The lectin hemagglutinated trypsinized human A, B, O erythrocytes and trypsinized rabbit erythrocytes. The relative hemagglutinating activity toward these blood cells was 1 : 1 : 1 : 16 (A : B : O : R) and the minimum concentration of protein for hemagglutinating trypsinized rabbit erythrocytes was 30 ng ml^{-1}.

Homogeneity and molecular mass of the lectin

Figure 2 shows the purity of the lectin. By SDS-PAGE, a single protein band of 15 kDa was found irrespective of the treatment with 2-mercaptoethanol. From the results by two gel filtrations (Toyopearl HW 55F and FinePak SIL AF102), the molecular mass of the native lectin was estimated to be 30 kDa.

Amino acid composition and N-terminal amino acid residue

The amino acid composition of the lectin is shown in Table 2, in comparison with those of two other Agaricales lectins. The composition of this lectin was apparently different from those of other lectins in the content of serine and basic amino acids.

N-Terminal amino acid residue of the lectin was not detected by the sequencer, suggesting that the lectin was blocked at its N-terminus.

The inhibition of hemagglutination of Agrocybe lectin by various sugars and glycoconjugates

Table 3 shows the inhibition of hemagglutinating activity of the lectin by sugars and glycoproteins. *N*-Acetylneuraminic acid (NeuAc) and *N*-glycolylneuraminic acid (NeuGc) were inhibitory at 5 mM, but no other monosaccharides were inhibitory. Lactose, *N*-acetyllactosamine and lacto-*N*-biose were poor inhibitors. Neu5Acα2,3lactose and Neu5Acα2, 3*N*-acetyllactosamine were potent inhibitors, Neu5Acα2, 3*N*-acetyllactosamine being slightly more potent than Neu5Acα2,3lactose. However, the reduced form of Neu5Acα2,3lactose was much weaker towards the lectin, indicating the necessity of an intact trisaccharide unit for recognition by the lectin. Neither Neu5Acα2,6lactose nor Neu5Acα2,6*N*-acetyllactosamine were inhibitory. Of the glycoproteins tested, bovine submaxillary mucin (Type I, Sigma), glycophorin A, and IgA were strong inhibitors.

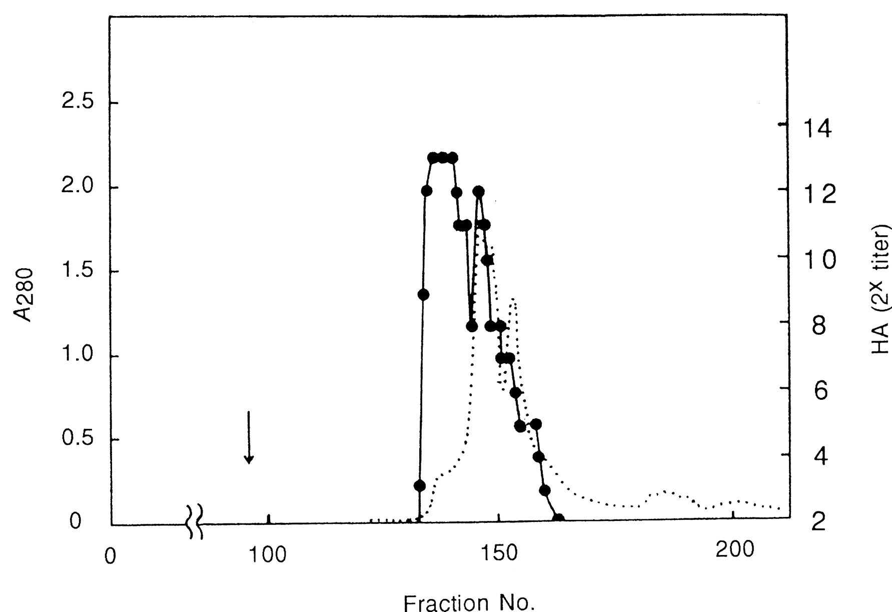

Figure 1. DEAE-Toyopearl 650 M column chromatography of lectin preparation after DEAE-Cellulofine A-200 chromatography. The dialysed supernatant was applied to a column (2.5 cm i.d. × 35 cm) equilibrated with 10 mM soldium phosphate buffer, pH 6.6. The column was washed with 500 ml of the same buffer, and then protein was eluted with a linear gradient of NaCl (0–0.5 M, total 600 ml) in the same buffer. The arrow indicates the starting position of the gradient. Broken line, A_{280}; Closed circles, hemagglutination activity (HA) with trypsinized rabbit erythrocytes. Volume of each fraction was 5 ml.

Table 1. Purification of *A. cylindracea* lectin.

Purification procedure	*Protein (mg)*	*Hemagglutinating activity (titer)*	*Specific activity (titer per mg)*
Crude extract	1760	2080000	1180
DEAE-cellulofine A-200	180	1500000	8300
DEAE-Toyopearl 650M	30.3	850000	28000
Toyopearl HW55F	25.2	820000	33000

These are glycoproteins with mucin-type carbohydrate chains. However, ovine submaxillary mucin (OSM) and porcine submaxillary mucin (PSM) were nonihibitors. On the other hand, α_1-acid glycoprotein, ovalbumin, ovomucoid and transferrin were not inhibitory. Bovine fetuin, thyroglobulin, prothrombin (Factor II) and κ-casein were inhibitory to the same extent. Most asialoglycoproteins (asialofetuin, asialoBSM, asialoglycophorin A, asialo-κ-casein and asialoprothrombin) were not inhibitory, except for asialothyroglobulin. Asialothyroglobulin inhibited the hemagglutination twice as strongly as thyroglobulin.

The reactivity of lectin towards BSA-glycoconjugates and murine laminin with NeuAcα2,3Galβ1,4GlcNAc-sequences

Figure 3 shows the reactivity of lectin towards BSA-oligosaccharides. In this study, BSA-conjugates were first coated on the ELISA plate and then lectin-binding to the conjugates were estimated [method 1 in Materials and methods]. In an another method, the lectin was first coated on the ELISA plates [method 2 in Materials and methods]. Method 2 (data not shown) gave the same result as method 1. The lectin interacted with NeuAcα2,3Galβ1,3GlcNAc-somewhat more strongly than NeuAcα2,3Galβ1,4GlcNAc-BSA, but the interaction with NeuAcα2,3Galβ-BSA and murine laminin was very weak.

The inhibition of the lectin – κ-casein interaction by gangliosides or oligosaccharides

Figure 4A shows the reactivity of the lectin with gangliosides by ELISA. When gangliosides were first coated on the ELISA plate followed by estimating the interaction [method 3 in Materials and methods], relatively strong non-specific interaction between the lectin and gangliosides

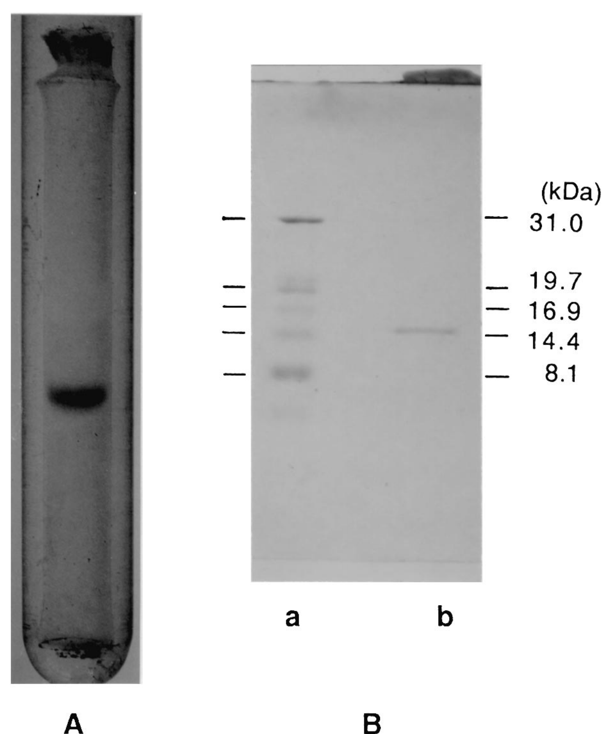

Figure 2. Results of PAGE and SDS-PAGE of the final preparation. (A) PAGE (7.5% gel) at pH 8.9; (B) SDS-PAGE (15% gel), a and b, molecular-mass marker (Wako Pure Chemical, Osaka, Japan) and *Agrocybe* lectin in the absence of 2-mercaptoethanol, respectively.

Table 2. Amino acid compositions of lectins from *Agrocybe cylindracea* and other fungi.

Amino acid	A. cylindracea	Agaricus[a] campestris	*PCL*[b]
Asx	15.9 (16)	19.4	17.2
Thr	14.0 (14)	12.9	10.2
Ser	14.8 (15)	6.3	9.4
Glx	11.4 (11)	12.2	14.5
Pro	4.6 (5)	2.0	3.2
Gly	12.9 (13)	11.4	14.4
Ala	12.2 (12)	11.8	12.9
1/2Cys	0	1.0	–
Val	12.9 (13)	10.6	10.9
Met	0.9 (1)	1.0	3.7
Ile	6.7 (7)	5.6	6.9
Leu	9.5 (10)	6.9	9.4
Tyr	4.8 (5)	7.6	4.8
Phe	5.8 (6)	6.4	5.7
Lys	3.9 (4)	6.3	5.9
His	1.3 (1)	1.1	2.2
Arg	2.6 (3)	8.5	9.6
Trp	2.2* (2)	2.6	1.0
Subunit MW	15 K	15.5 K	16 K

*Determined spectrophotometrically.
[a]calculated from data by Sage and Connett [17].
[b]lectin of *Pleurotus cornucopiae* [18].

was found to occur (data not shown). When the reactivity of gangliosides toward the lectin was estimated by the inhibition of the lectin – κ-casein interaction (method 4 in Materials and methods), gangliosides containing sialic acid apparently inhibited the interaction. Compared with the control without the lectin, 7 nmoles of gangliosides D1b and M1a gave almost the same values, suggesting the complete inhibition of the interaction of *Agrocybe* lectin – κ-casein (Figure 4A). The inhibition by 1.4 nmol of ganglioside D1a was about 50%. However, the inhibition by 7 nmol of D1a was almost the same as by 7 nmol of D1b (data not shown).

The reaction between the lectin and κ-casein was inhibited by two purified oligosaccharide preparations from caseinoglycopeptide (Figure 4B). Both of the oligosacharide chains, NeuAcα2,3Galβ1,3GalNAc and NeuAcα2,3Galβ1,3(NeuAc2,6)GalNAc were inhibitory. However, inhibition by the trisaccharide was slightly stronger than by the tetrasaccharide.

Discussion

Kawagishi *et al.* [16] reported that the hemagglutinating activity of a lectin from the fruiting bodies of *Hericium* was inhibited by NeuAc and NeuGc. However, asialo-BSM was the best inhibitor of this lectin and glycoconjugates containing sialic acid were poor inhibitors. In this respect, the *Hericium* lectin appeared to differ from other sialic acid-recognizing lectins [1, 2]. The lectin in the present study showed low affinity toward sialic acid itself, but higher specificity toward glycoconjugates containing sialic acid (Table 3) as reported for many other sialic acid-recognizing lectin.

Tichá *et al.* [19] reported carbohydrate-binding specificity of *A. aegerita*, the species closely related to *A. cylindracea*. The *A. aegerita* lectin is galactose-specific, and its molecular mass is 22 K, differing from the *A. cylindracea* lectin in this study.

Knibbs *et al.* [3] characterized the carbohydrate-binding properties of three sialic-acid specific lectins, LFA, MAL and SNA. LFA was specific for sialic acid itself, at any position and in any sequence. The specificity of LFA toward many sialic acid monosaccharides was later clarified [20]. Wang and Cummings [21] first described that MAL was specific for NeuAcα2,3Gal-sequence but Knibbs *et al.* [3] showed that MAL recognized the NeuAcaα2,3Galβ1,4GlcNAc-sequence more strongly and had the affinity toward many sialic acid containing N-linked sugar chains. This lectin did not precipitate NeuAcα2,3Galβ1,3GlcNAc-BSA or NeuAcα2,3Galβ1,3GalNAc-BSA. MAL also did not show affinity toward mucin-containing NeuAcα2,3Galβ1,3GalNAc-sequences. On the contrary, *Agrocybe* lectin reacted with NeuAcα2,3Galβ1,4GlcNAc-BSA and NeuAcα2,

Table 3. Inhibition of hemagglutination of Agrocybe lectin by various sugars and glycoconjugates. Trypsinized rabbit erythrocytes were used.

Sugars and glycoconjugates	*Concentration (mM or μg ml^{-1}) for complete inhibition of titer 4 hemagglutination*
Lactose	10 mM
N-acetyllactosamine	10
Lacto-*N*-biose	10
Gal*β*1,3GalNAc*α*-OCH_2Ph	No inhibition at 5 mM
N-Acetyl neuraminic acid	5
N-Glycolyl neuraminic acid	5
NeuAc*α*2,3lactose	0.07
NeuAc*α*2,3lactose (reduced)	1.5
NeuAc*α*2,3*N*-acetyllactosamine	0.035
NeuAc*α*2,6lactose	No inhibition at 1.5 mM
NeuAc*α*2,6*N*-acetyllactosamine	No inhibition at 1.5 mM
Colominic acid	No inhibition at 1000 μg ml^{-1}
Bovine submaxillary mucin (BSM)	11
Asialo-BSM	No inhibition at 1000 μg ml^{-1}
Ovine submaxillary mucin (OSM)	No inhibition at 350 μg ml^{-1}
Porcine submaxillary mucin (PSM)	No inhibition at 350 μg ml^{-1}
Human IgA	11
Glycophorin A	11
Asialo-glycophorin A	No inhibition at 1000 μg ml^{-1}
κ-Casein	90
Asialo *κ*-casein	No inhibition at 1000 μg ml^{-1}
Fetuin (bovine)	90 μg ml^{-1}
Fetuin (mucin-free)	350
Asialo-fetuin	350
Prothrombin (factor II)	90
Asialoprothrombin	No inhibition at 1000 μg ml^{-1}
α_1-acid glycoprotein	No inhibition at 1000 μg ml^{-1}
Murine laminin	No inhibition at 1000 μg ml^{-1}
Ovalbumin	No inhibition at 1000 μg ml^{-1}
Ovomucoid	No inhibition at 1000 μg ml^{-1}
Thyroglobulin	180
Asialo-thyroglobulin	90
Transferrin	No inhibition at 350 μg ml^{-1}

N-Acetyl D-galactosamine, *N*-acetyl D-glucosamine, L-fucose, D-galactose, D-glucose, D-mannose were not inhibitory at 100 mM. *p*-Nitrophenyl *α*-D-galactopyranoside, *p*-nitrophenyl *β*-D-galactopyranoside and *p*-nitrophenyl *β*-D-glucopyranoside were not inhibitory at 50 mM.

3Gal*β*1,3GlcNAc-BSA. But the affinity of lectin toward NeuAcα2,3Gal*β*1,3GlcNAc-BSA was somewhat stronger than toward NeuAcα2,3Gal*β*1,4GlcNAc-BSA. *Agrocybe* lectin did not recognize murine laminin with NeuAcα2,3Gal*β*1, 4GlcNAc-sequences [22]. Glycoproteins with NeucAα2, 3Gal*β*1,4GlcNAc-sequence in their N-linked sugar chains appeared not to be active toward this lectin (Table 3). The discrepancy between the results by glycoproteins and by BSA-conjugates may be due to the C_9 spacer arm of BSA-conjugates. The extended structure and/or hydrophobicity of the spacer arm may be involved in the interaction between the *Agrocybe* lectin and the BSA-conjugates. On the contrary, steric hindrance may inhibit the interaction between *Agrocybe* lectin and glycoproteins.

On the other hand, bovine prothrombin (Factor II) which is known to contain both NeuAcα2,3Gal*β*1,3GlcNAc- and NeuAcα2,3Gal*β*1,3(NeuAcα2,6)GlcNAc- sequences [23] was inhibitory to the hemagglutination of the lectin. Therefore, it is presumed that the *Agrocybe* lectin recognized the N-linked chain with the NeuAcα2,3Gal*β*1,3GlcNAc-sequence. However, it was not clear whether or not both of the sequences NeuAcα2,3Gal*β*1,3GlcNAc- and NeuAcα2,3Gal*β*1, 3(NeuAcα2,6)GlcNAc- were active to the lectin.

To date, no lectin recognizing NeuAcα2,3Gal*β*1,3GlcNAc-sequences has been reported.

MAH, another lectin obtained from *Maackia amurensis* was reported by Kawaguchi and Osawa [24], and later by Konami *et al.* [4] to recognize the tetrasaccharides

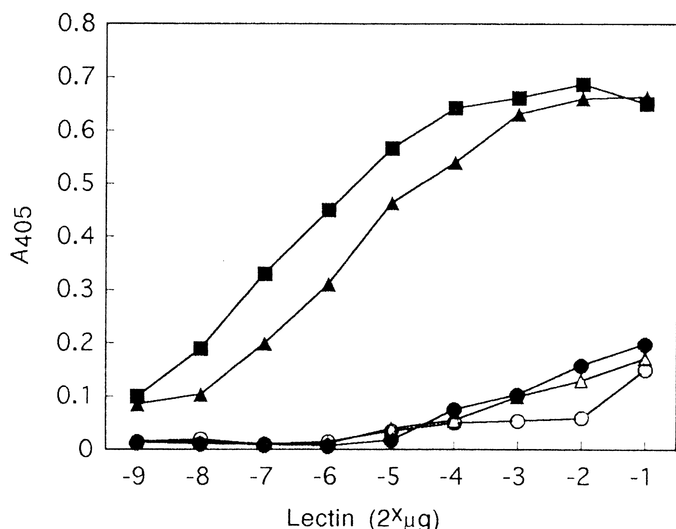

Figure 3. Binding of *Agrocybe* lectin to BSA-conjugates on ELISA plate. All the values were average of three determinations. Open circle, control (only BSA); closed circle, NeuAcα2,3Gal-BSA; NeuAcα2,3Galβ1, 3GlcNAc-BSA, ■; and NeuAcα2,3Galβ1,4-GlcNAc-BSA, ▲; murine laminin, △.

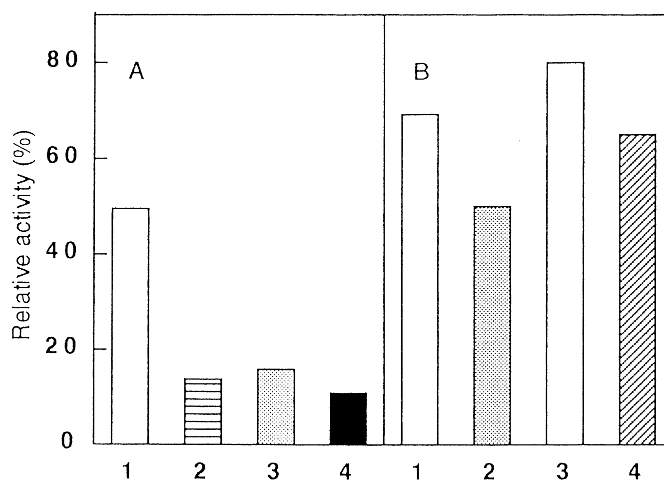

Figure 4. Inhibition by gangliosides of lectin – κ-casein interaction. (A) Wells were coated with κ-casein (20 μg), and lectin (0.5 μg) or lectin – ganglioside mixtures previously incubated in 10 μl PBS for 20 min were diluted five-fold with PBS and added to the wells. All the values were average of four determinations on the same plate. (1) D1a (1.4 nmol); (2) D1b (7 nmol); (3) M1a (7 nmol); (4) M1a (7 nmol) without lectin. The values were expressed as % relative to the absorbance (A_{405} = 0.686) obtained with only lectin. (B) Differing from A, preincubation was not performed. All the values were average of three determinations on the same plate. 1, 20 μM NeuAcα2,3Galβ1, 3GalNAc; 2, 40 μM NeuAcα2,3Galβ1,3GalNAc; 3, 20 μM NeuAcα2,3Galβ1,3(NeuAcα2,6) GalNAc; 4, 40 μM NeuAcα2,3Galβ1,3(NeuAcα2,6)GalNAc. The values were expressed as % relative to the absorbance (A_{405} = 0.808) obtained with only lectin.

NeuAcα2,3Galβ1,3(NeuAcα2,6)GalNAc. This tetrasaccharide is present in glycophorin A [25], IgA [26] and κ-casein [27, 28]. The *Agrocybe* lectin in this study showed affinity toward all these glycoproteins (Table 3) as well as the tetrasaccharide (Figure 4B). Many glycoproteins with mucin-type carbohydrate chain contain not only the tetrasaccharide but also the trisaccharide NeuAcα2,3Galβ1, 3GalNAc [26–28]. Therefore, inhibition of hemagglutination by glycoproteins cannot answer the question of whether the lectin recognizes both of the carbohydrate chains. Figure 4B shows that the trisaccharide is slightly more potent than the tetrasaccharide.

With respect to mucin-type and N-linked oligosaccharides, α2,6-linked sialyl conjugates are generally present. SNA [29] is known to be a lectin recognizing these NeuAcα2,6Gal/GalNAc- sequences. However, OSM, PSM and the two oligosaccharides with NeuAcα2,6Gal- structure were not inhibitory to the hemagglutination of the *Agrocybe* lectin. Exceptionally, BSM was inhibitory to the hemagglutination of *Agrocybe* lectin. The reason why only BSM was inhibitory remains unclear at this time.

Table 3 shows that the *Agrocybe* lectin has weak affinity toward N-linked carbohydrate chains. Asialothyroglobulin was inhibitory to the lectin and its potency was slightly stronger than the intact thyroglobulin. It is apparent that the NeuAcα2,6Galβ1,4GlcNAc structure in thyroglobulin [30] is not effective as an inhibitor. Mucin-free fetuin and asialofetuin were weakly inhibitory to the lectin and the inhibition by the two modified fetuin preparations was not as effective as the intact fetuin. Bovine fetuin [31–33] has NeuAcα2,3Galβ1,3GlcNAc- and NeuAcα2,3Galβ1,3Gal NAc-sequences in its N-linked and O-linked saccharides, respectively. The inhibitory effect of mucin-free fetuin was elucidated by the former N-linked trisaccharide. However, asialofetuin was weakly inhibitory also. This might be due to the same effect of N-linked sugar chain as asialothyroglobulin.

Several sialogangliosides were reactive with the lectin. All the gangliosides tested showed non-specific affinity toward the lectin (data not shown). However, taking the nonspecific interaction into consideration, the reactivity of the lectin toward the carbohydrate chain of the sialoganglioside was apparent (Figure 4A). In the inhibition of the hemagglutination study (Table 3), NeuAcα2,3Galβ1,4Glc was inhibitory. All the sialogangliosides tested have this sequence and ganglioside D1a additionally, has the NeuAcα2,3Galβ1,3GalNAc-sequence. We did not study the fine difference of the reactivity of the ganglioside, but the results in Figure 4A suggested that the presence of an additional NeuAcα2,8-residue (ganglioside D1b) or sugar branch (gangliosides D1b and M1a) did not conclusively affect the supposed recognition of NeuAcα2,3Galβ1,4Glc- sequence in gangliosides by this lectin.

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