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Purification and Characterization of a Mannose-Specific Lectin from Shallot (*Allium ascalonicum*) Bulbs

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A new mannose-binding lectin was isolated from shallot (*Allium ascalonicum*) bulbs by affinity chromatography on an immobilized D-mannose column. The lectin (*A. ascalonicum* agglutinin, AAA) appeared homogeneous by polyacrylamide gel electrophoresis at pH 4.3 and gave a single protein band with an apparent M_r of 11 kDa by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and a single symmetrical peak of 11 kDa by gel filtration on a Sephacryl S-200 HR column, indicating that AAA exists as a monomeric protein at neutral pH under the gel filtration condition employed. However, chemical cross-linking studies revealed that some degree of self-association of the lectin molecules occurs and that the lectin exists in solution as a mixture of monomers and oligomers. Scatchard analysis of equilibrium dialysis data showed the presence of one carbohydrate binding site for Man (α1–3) Man-α-*O*-Me per monomer, with $K_a = 1.62 \times 10^4$ M^{-1}. The carbohydrate-binding properties of the purified AAA were investigated by quantitative precipitation and hapten inhibition assays. Purified AAA precipitated asialofetuin, asialotransferrin, asialothyroglobulin, asialoorosomucoid, as well as their agalacto derivatives, but did not precipitate either sialylated glycoproteins or mucins. AAA also reacted strongly with the highly branched yeast mannan obtained from *Saccharomyces cerevisiae*. Of the monosaccharides tested only D-mannose was a hapten inhibitor of the AAA–asialofetuin precipitation system, whereas D-glucose, D-altrose, D-talose, *N*-acetyl-D-mannosamine, and derivatives of D-mannose, including 2-deoxy-, 2-deoxy-2-fluoro-, 3-deoxy-, and 6-deoxy-D-mannose were noninhibitors. These results suggest that the presence of equatorial hydroxyl groups at the C-3 and C-4 positions, an axial hydroxyl group at the C-2 position, and a free hydroxyl group at the C-6 position of the pyranose ring are the most important loci for the binding of D-mannose to AAA. Of the oligosaccharides tested, the best inhibitors were oligosaccharides containing terminal Man(α1–6)[Man(α1–3)]Man groups. Oligosaccharides containing either Man(α1–3)Man or Man(α1–6)Man units were also moderately good inhibitors of the AAA–asialofetuin precipitation system. These results indicate that AAA has an extended carbohydrate-binding site, which is most complementary to a branched mannotriosyl residue, i.e., Man(α1–6)[Man(α1–3)]Man. A comparison is presented of the detailed carbohydrate-binding properties and molecular structures of the *A. ascalonicum* lectin and several other mannose-binding lectins from different species of the plant family *Amaryllidaceae*, i.e., snowdrop lectin (*Galanthus nivalis*), daffodil lectin (*Narcissus pseudonarcissus*), and amaryllis lectin (*Hippeastrum hybr.*).

In recent years, several mannose-specific lectins have been isolated and characterized (1–4). Interestingly, these lectins were all isolated from bulbs of plants belonging to the same family (*Amaryllidaceae*) and they all exhibit the capacity of distinguishing D-mannose from D-glucose. This unique carbohydrate-binding property distinguishes these monocotyledonous lectins from the so-called D-mannose/D-glucose-binding *Leguminoseae* lectins (e.g., concanavalin A, pea lectin, lentil lectin, and *Vicia faba* lectin; 5) and makes them valuable tools in biomedical research (6).

We report here the purification and carbohydrate-binding specificities of *Allium ascalonicum* lectin (AAA)[2],

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[2] Abbreviations used: AAA, *Allium ascalonicum* agglutinin; DMA, dimethyl adipimidate; DSS, disuccinimidyl suberate; GNA, *Galanthus nivalis* agglutinin (snowdrop lectin); HHA, *Hippeastrum hybr.* agglutinin (amaryllis lectin); LAA, *Leucojum aestivum* agglutinin; LVA, *Leucojum vernum* agglutinin; Man, D-mannose; NPA, *Narcissus pseudonarcissus* agglutinin (daffodil lectin); PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline (10 mM Na phosphate containing 150 mM NaCl, pH 7.2); SDS, sodium dodecyl sulfate.

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a mannose-binding lectin present in shallot (*A. ascalonicum*) bulbs.

MATERIALS AND METHODS

Saccharides and glycopeptides. Various monosaccharides and their methyl glycosides were commercial products from Pfanstiehl Laboratories, Inc. (Waukegan, IL) and Sigma Chemical Co. (St. Louis, MO). D-Altrose and 6-deoxy-D-mannose were the gifts of Dr. N. K. Richtmyer of the National Institutes of Health. Methyl 3-*O*-α-D-mannopyranosyl-α-D-mannopyranoside [Man(α1-3)Man-α-*O*-Me], methyl 6-*O*-α-D-mannopyranosyl-α-D-mannopyranoside [Man(α1-6)Man-α-*O*-Me], and methyl 2-*O*-α-D-mannopyranosyl-α-D-mannopyranoside [Man(α1-2)Man-α-*O*-Me) were purchased from Sigma Chemical Co. Man(α1-6)Man(α1-6)Man and Man(α1-2)Man(α1-2)Man were the generous gifts of Dr. C. E. Ballou of the University of California, Berkeley. Man(α1-6)[Man(α1-3)]Man-α-*O*-Me and $Man_9GlcNAc_2Asn$ were kindly provided by Dr. J. P. Carver of the University of Toronto. $Man_6GlcNAc_2Asn$ was the gift of Dr. C. F. Brewer of Albert Einstein College of Medicine, New York.

Polysaccharides and glycoproteins. All synthetic glycoproteins were available from previous studies. Yeast mannan obtained from *Saccharomyces cerevisiae* was supplied by Dr. P. A. J. Gorrin of Universidade Federal Do Panana, Brazil. Fetuin was obtained from Gibco Laboratories (Grand Island, NY). Asialofetuin, transferrin, ovalbumin, and thyroglobulin were the products of Sigma Chemical Co. Orosomucoid was the generous gift of Dr. G. W. Jourdian, University of Michigan Medical School. Asialotransferrin, asialothyroglobulin, and asialoorosomucoid were prepared by chemically desialylating the corresponding parental glycoproteins in 0.1 N sulfuric acid at 80°C for 1 h. Agalactoglycoproteins were obtained by exo-β-galactosidase digestion of the corresponding asialoglycoproteins. The digestions were carried out in 0.1 M phosphate buffer, pH 6.0, at 37°C for 48–72 h using 0.1 μunit of β-galactosidase per μmol of glycoprotein per 24 h. The release of galactose from the glycoproteins was confirmed by galactose dehydrogenase assay (7).

Miscellaneous materials. Shallot (*A. ascalonicum*) bulbs were obtained from a local supplier. Sephacryl S-200 HR, phenyl-Sepharose, and Q Fast Flow anion exchanger were products of Pharmacia LKB Biotechnology (Sweden). Immobilized D-mannose and cross-linking reagents disuccinimidyl suberate (DSS) and dimethyl adipimidate 2 HCl (DMA) were purchased from Pierce Chemical Co. (Rockford, IL). Molecular weight markers for gel filtration chromatography and SDS-polyacrylamide gel electrophoresis were obtained from Sigma Chemical Co. Bovine testicular exo-β-galactosidase was the generous gift of Dr. G. W. Jourdian, University of Michigan Medical School.

Hemagglutination assays. Hemagglutination assays were carried out in microtiter plates. A 25-μl aliquot of the agglutinin was serially diluted twofold with phosphate-buffered saline (PBS), pH 7.2 (0.01 M phosphate buffer containing 0.15 M NaCl), followed by the addition of 25 μl of 3% (v/v) rabbit erythrocyte suspension. The plates were kept at room temperature for 1 h, the hemagglutination activity was examined visually, and the hemagglutination titer was expressed as the reciprocal of the highest dilution that still gave a visible agglutination.

Purification of the A. ascalonicum lectin. *A. ascalonicum* lectin was isolated from crude extracts of shallot (*A. ascalonicum*) bulbs by affinity chromatography on mannose-Sepharose 4B and further purified by hydrophobic interaction chromatography and ion-exchange chromatography. Briefly, whole bulbs (1 kg) were homogenized and extracted overnight in 1 M $(NH_4)_2SO_4$ (approx 10 ml/g of fresh bulbs). The extract was centrifuged (20,000*g*; 15 min) and the supernatant solution applied onto a mannose-Sepharose 4B column (50 ml bed volume) equilibrated with 1 M $(NH_4)_2SO_4$. Unbound proteins were eluted with 1 M $(NH_4)_2SO_4$ and the lectin desorbed with 500 ml of 20 mM unbuffered 1,3-diaminopropane. The affinity chromatography step was repeated to remove colored impurities; the lectin fraction was adjusted to pH 7.0 and brought to 1 M ammonium sulfate by adding solid $(NH_4)_2SO_4$. After standing overnight, the precipitate was removed by centrifugation (20,000*g*; 15 min) and the supernatant fraction subjected to chromatography as above on a smaller mannose-Sepharose 4B column (10 ml bed volume). Inasmuch as the lectin fraction was still colored at this stage, it was adjusted to pH 7.0, brought to 0.5 M ammonium sulfate, and applied onto a column (1 cm in diameter; 5 ml bed volume) of phenyl-Sepharose equilibrated with 0.5 M $(NH_4)_2SO_4$. Following washing with 0.5 M $(NH_4)_2SO_4$, the lectin was desorbed with 20 mM Tris-HCl (pH 7.8), dialyzed against 20 mM Tris-HCl (pH 8.7) and applied onto a column (1 cm in diameter; 5 ml bed volume) of Q Fast Flow anion exchanger. After washing the column with the same buffer, the lectin was desorbed with 0.5 M NaCl, dialyzed against distilled water, and lyophilized.

Polyacrylamide gel electrophoresis (PAGE) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Slab gel electrophoresis was conducted at pH 4.3 in β-alanine/acetic acid buffer using a 7.5% polyacrylamide gel, according to Reisfeld *et al.* (8). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis in the presence or absence of 5% 2-mercaptoethanol was performed using a 10% slab gel according to the method of Weber and Osborn (9). Protein bands were visualized by Coomassie brilliant blue R-250 staining. The proteins used as molecular weight markers were bovine serum albumin (67 kDa), ovalbumin (45 kDa), chymotrypsinogen A (25 kDa), and cytochrome C (12.5 kDa).

Molecular weight determination by gel filtration. The molecular weight of the purified *A. ascalonicum* lectin was estimated by gel filtration chromatography using a Sephacryl S-200 HR column (104 × 0.9 cm, 68 ml bed volume) equilibrated with PBS, pH 7.2. The column was calibrated with bovine serum albumin (67 kDa), carbonic anhydrase (29 kDa), myoglobin (17.2 kDa), cytochrome C (12.5 kDa), and aprotinin (6.5 kDa).

Amino acid analysis. The isolated *A. ascalonicum* lectin sample was hydrolyzed for 24 h at 110°C in 6 M HCl. Amino acid composition was analyzed with an Applied Biosystems amino acid analyzer Model 420A. The tryptophan content was measured spectrophotometrically (10).

N-Terminal amino acid sequencing. Amino acid sequencing was conducted using an Applied Biosystems (Foster City, CA) Model 470 A protein sequencer interfaced with an Applied Biosystems Model 120 A on-line analyzer.

Quantitative precipitation and hapten inhibition assays. Quantitative precipitation assays were conducted by a microprecipitation technique described by So and Goldstein (11). Briefly, varying amounts of glycoproteins or polysaccharides were mixed with 18 μg of purified *A. ascalonicum* lectin in a total volume of 120 μl of PBS, pH 7.2. After incubation at 37°C for 1 h, the reaction mixtures were stored at 4°C for 48 h. The precipitates formed were centrifuged, washed three times with cold PBS, dissolved in 0.05 N NaOH, and analyzed for protein content by Lowry's method (12) using bovine serum albumin as standard. For hapten inhibition studies, increasing amounts of haptenic saccharides were added to the reaction mixture consisting of 18 μg of *A. ascalonicum* lectin and 20 μg of asialofetuin in a total volume of 80 μl of PBS, pH 7.2. The precipitated protein was determined and the percentage of inhibition was calculated.

Equilibrium dialysis. Equilibrium dialysis experiments were conducted in duplicate in multichambered dialysis cells (Technilab Instruments, Inc., Pequannock, NJ) using Man(α1-3)Man-α-*O*-Me as binding ligand. Each chamber on one side of the dialysis membrane contained 15 μM *A. ascalonicum* lectin (assuming subunit molecular weight of 11,000) in 200 μl of PBS, and chambers on the opposite side of the membrane contained varying concentrations of Man(α1-3)Man-α-*O*-Me (20–400 μM) in 200 μl of PBS. The dialysis cells were slowly rotated at 4°C for 10 days. Aliquots (25–150 μl) from each chamber were taken for the determination of carbohydrate concentration by the method of Dubois *et al.* (13). The results were analyzed according to the method of Scatchard (14).

Chemical cross-linking of A. ascalonicum lectin. Concentrated stock solutions (200 mM) of DSS and DMA were prepared in dimethyl sulfoxide and sodium carbonate-sodium bicarbonate buffer solution (pH 9.5), re-

spectively. To each sample in PBS (pH 7.2) were added aliquots of DSS or DMA solution to give a final cross-linking reagent concentration of 10 mM. After 2 h incubation at 37°C, the cross-linking reactions were quenched by the addition of 10 μl of 1 M Tris, and the proteins were precipitated with trichloroacetic acid, washed with cold acetone, and analyzed by SDS-PAGE.

RESULTS

Hemagglutination properties. The crude extracts of shallot (*A. ascalonicum*) bulbs readily agglutinated rabbit erythrocytes (both untreated and trypsin-treated cells), but did not agglutinate human red blood cells, irrespective of blood type. The minimal concentration of the purified lectin required for the agglutination of untreated rabbit red blood cells was 3.7 μg/ml. Only D-mannose inhibited the hemagglutination reaction.

Purification of A. ascalonicum lectin. Inasmuch as D-mannose inhibited the hemagglutination activity of the crude extracts of shallot (*A. ascalonicum*) bulbs, purification on an immobilized mannose column was employed. The overall yield of affinity-purified AAA was approx 9 mg/kg fresh bulb tissue. The isolated lectin appeared to be homogeneous by polyacrylamide gel electrophoresis at pH 4.3 (Fig. 1) and by gel filtration chromatography (Fig. 2). The isolated lectin was eluted as a single symmetrical peak by gel filtration on a Sephacryl S-200 HR column and gave a single protein band upon polyacrylamide gel electrophoresis.

Molecular weight and molecular structure. Both native and reduced *A. ascalonicum* lectin gave only a single protein band of M_r 11,000 upon SDS-PAGE (Fig. 1), indicating the lectin to be composed of a single type of subunit with an apparent M_r 11 kDa with no intersubunit disulfide bonds. A single symmetrical peak of M_r 11,000 was observed upon gel filtration chromatography on a Sephacryl S-200 HR column in PBS, pH 7.2, in the presence or absence of 0.2 M D-mannose (Fig. 2). Therefore, it appears that at neutral pH under the gel filtration condition described, the *A. ascalonicum* lectin exists as a monomeric protein.

Amino acid analysis and N-terminal amino acid sequence analysis. The amino acid composition of *A. ascalonicum* lectin is given in Table I. The lectin contains a high content of Asx, Glx, glycine, as well as hydrophobic and hydroxyl amino acids, but a low content of sulfur-containing amino acids. The sequence of the N-terminal 38 amino acids is shown in Table II.

Precipitation of AAA by glycoproteins and polysaccharides. Figure 3 shows the precipitation curves of several glycoproteins and polysaccharides with the *A. ascalonicum* lectin. AAA readily interacted with many desialylated N-glycosylated glycoproteins such as asialofetuin, asialoagalactofetuin, asialotransferrin, asialoagalactotransferrin, asialothyroglobulin, asialoagalactothyroglobulin, and asialoorosomucoid, forming distinct precipitates; however, it did not interact with fetuin, transferrin, orosomucoid,

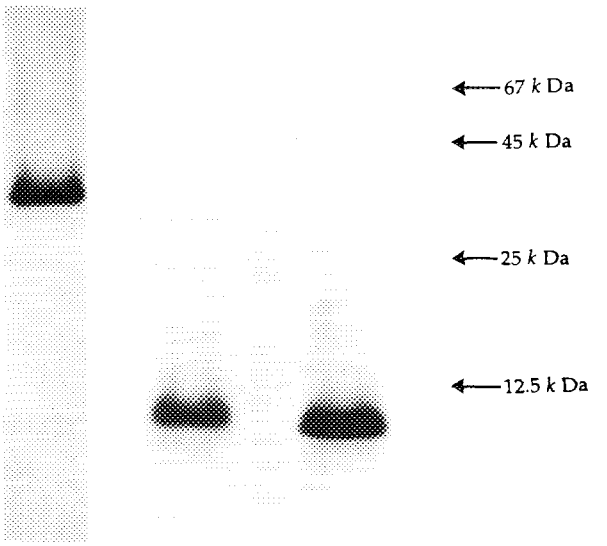

FIG. 1. Polyacrylamide gel electrophoresis of the purified *A. ascalonicum* agglutinin. From left to right: lane 1, native gel at pH 4.3 in β-alanine/acetic acid buffer system; lane 2, SDS-PAGE in the presence of 5% mercaptoethanol; lane 3, SDS-PAGE in the absence of mercaptoethanol; lane 4, molecular weight standards.

thyroglobulin, ovine submaxillary mucin, asialo ovine submaxillary mucin, bovine submaxillary mucin, and asialo bovine submaxillary mucin, suggesting that AAA recognizes some common structural feature in N-linked glycans but not in O-linked glycans and that the presence of terminal sialic acids in N-linked glycans hinders their interaction with the lectin.

The highly branched yeast mannan obtained from *S. cerevisiae,* containing numerous terminal α-1,3-linked mannobiosyl residues (16), reacted with AAA so strongly that the precipitation reaction could not be readily inhibited by any simple saccharides. Therefore, asialofetuin was chosen as the precipitant in the inhibition experiments. On the contrary, the galactomannan of *Candida lipolytica* in which most of the terminal mannose residues are masked by α-D-galactosyl groups did not precipitate the *A. ascalonicum* lectin.

Inhibition of precipitation by haptenic saccharides. To investigate the detailed carbohydrate-binding specificities of AAA, haptenic sugar inhibition experiments were conducted using asialofetuin as the precipitating glycoprotein. Concentrations of carbohydrates required for 50% inhibition were obtained from complete inhibition curves (Fig. 4) and are tabulated in Table III.

Of the monosaccharides tested, only D-mannose was inhibitory; epimers of D-mannose, i.e., glucose (C-2), altrose (C-3), and talose (C-4), all were noninhibitory up to 200 mM. The 2-deoxy, 2-deoxy-2-fluoro, 3-deoxy, 6-deoxy derivatives of D-mannose, and *N*-acetylmannosamine were also noninhibitory up to 100–200 mM. These results suggest that the presence of equatorial hydroxyl

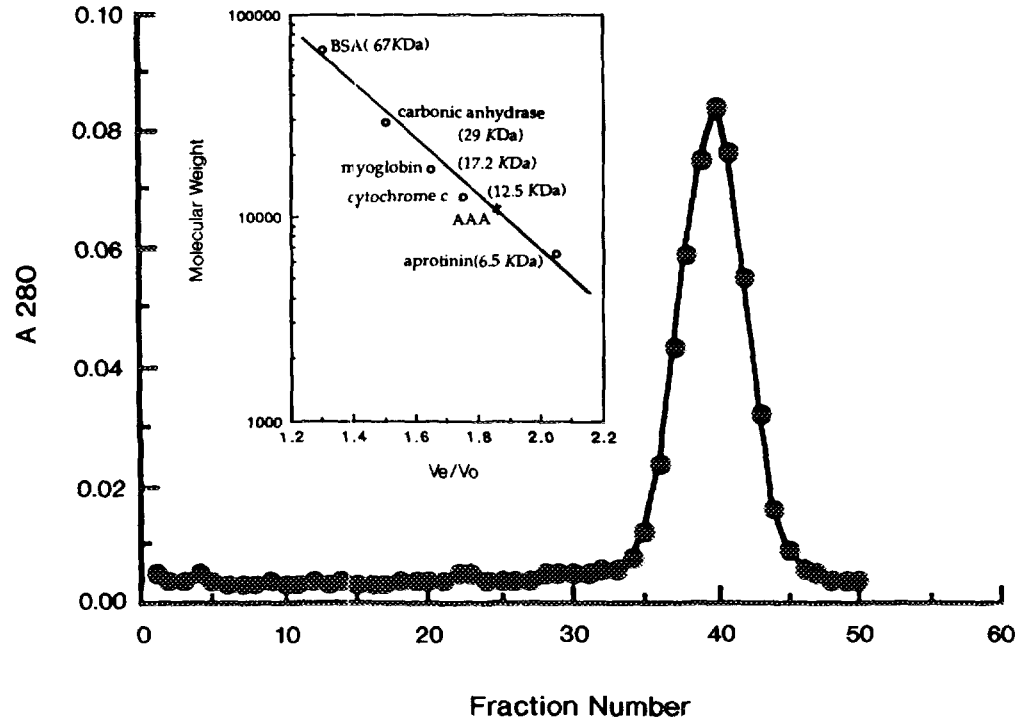

FIG. 2. Gel filtration chromatography of *A. ascalonicum* lectin on a Sephacryl S-200 column. The column (0.9 × 104 cm, bed volume 68 ml) was loaded with 0.5 mg of the purified AAA and eluted with PBS in the absence or presence of 0.2 M mannose. Fractions of 40 drops/tube were collected and monitored at A_{280}. (Inset) Determination of molecular weight of AAA by gel filtration. V_e, elution volume; V_o, void volume.

groups at the C-3 and C-4 positions, an axial hydroxyl group at the C-2 position, and a free hydroxyl group at the C-6 position of the D-pyranose ring are important loci for the binding of D-mannose to AAA. Both methyl α- and β-D-mannopyranoside were better inhibitors than D-mannose, with the α-anomer (3-fold) being somewhat preferred over the β-anomer (1.7-fold better than D-manose).

Of the disaccharides tested, Man(α1–3)Man-α-*O*-Me was a good inhibitor exhibiting an inhibitory potency 20 times greater than that of D-mannose. Man(α1–6)Man-α-*O*-Me was also found to be a good inhibitor, being 10–12 times better than D-mannose. On the other hand, Man(α1–2)Man-α-*O*-Me was only threefold better than D-mannose, being equivalent to methyl α-D-mannopyranoside.

Among the oligosaccharides tested, the mannotriose Man(α1–6)Man(α1–6)Man was a better inhibitor than the corresponding mannobiose Man(α1–6)Man-α-*O*-Me, showing 1.6 times higher inhibitory potency than Man(α1–6)Man-α-*O*-Me and 18-fold greater potency than D-mannose.

A branched mannotriose, Man(α1–6)[Man(α1–3)]Man-α-*O*-Me, was found to be the most potent inhibitor, exhibiting 50–60 times greater inhibitory potency than D-mannose.

It was interesting to note that $Man_6GlcNAc_2Asn$ had an inhibitory potency equivalent to the branched mannotriose Man(α1–6)[Man(α1–3)]Man-α-*O*-Me (5 mM of $Man_6GlcNAc_2Asn$ gave 47% inhibition of precipitation); further substitution of this oligosaccharide with additional α 1–2-linked mannosyl residues, resulting in $Man_9GlcNAc_2Asn$, appeared to abolish its inhibitory potency (2 mM of $Man_9GlcNAc_2Asn$ gave no inhibition).

Equilibrium dialysis. The Scatchard analysis of the equilibrium dialysis data revealed a linear relationship (Fig. 5), indicating that *A. ascalonicum* lectin possesses one carbohydrate-binding site per subunit (M_r 11000) with an apparent association constant of 1.62×10^4 M^{-1}. The absence of curvature in the Scatchard plot suggests that the carbohydrate-binding sites of AAA are homogeneous and noninteracting.

Chemical cross-linking of AAA. As shown in Fig. 6, higher molecular weight species appeared in cross-linking reagent-treated AAA samples but were not detected in native AAA. The apparent molecular weights of these species were 22–25, 33, and 44 kDa, corresponding respectively to the predicted dimeric, trimeric, and tetrameric structures of AAA.

DISCUSSION

In the process of purification of *A. ascalonicum* lectin, a 1 M ammonium sulfate solution was used both in extraction and affinity chromatography in order to reduce the activity of polyphenol oxidase and to enhance the

TABLE I

A Comparison of the Amino Acid Compositions of NPA, GNA, and AAA

Amino acid	Mol % NPA[a]	GNA[a]	AAA
Asx	15.4	15.4	13.7
Thr	5.9	7.7	5.9
Ser	8.7	10.5	6.9
Glx	7.1	7.2	8.8
Pro	6.2	4.1	2.9
Gly	10.5	12.0	12.7
Ala	5.2	3.5	5.9
Cys	2.8	1.7	1.0
Val	7.2	4.8	8.8
Met	0.0	0.0	1.0
Ile	5.8	5.4	4.9
Leu	8.6	8.9	6.9
Tyr	3.6	5.0	4.9
Phe	2.1	2.1	2.9
His	2.7	1.0	1.0
Lys	3.1	4.2	3.9
Trp	2.6	3.0	1.9
Arg	2.5	3.5	5.9

[a] These data are from Van Damme *et al.* (3).

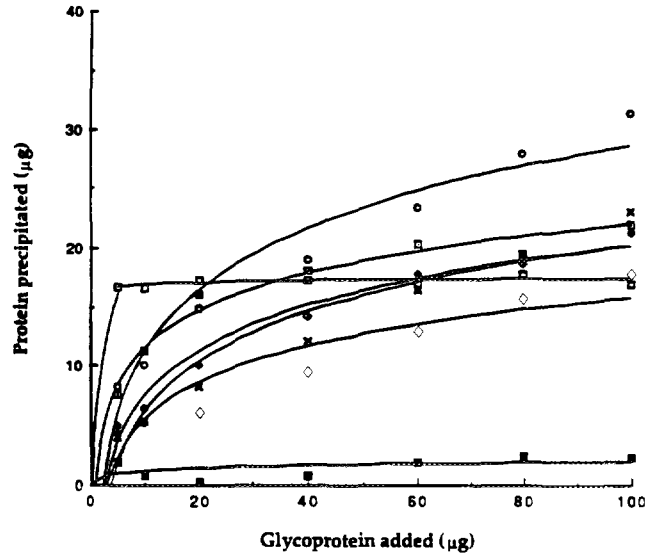

FIG. 3. Quantitative precipitation of *A. ascalonicum* lectin by several glycoproteins and polysaccharides. Varying amounts of glycoproteins were allowed to react with 20 μl (18 μg) of AAA. ⊡, asialoagalactofetuin; ♦, asialoorosomucoid; ○, asialoagalactotransferrin; ◇, asialofetuin; ■, ovalbumin; □, yeast mannan; ×, asialotransferrin.

binding of the lectin to the affinity column. 1,3-Diaminopropane was chosen in lieu of D-mannose to elute the bound lectin from the affinity column, because it required a large volume (approx 20 bed volume) of a high concentration of mannose (>0.2 M). The diaminopropane-eluted lectin appeared, in all respects, identical to that eluted with D-mannose (data not shown).

As indicated in Table III, the *A. ascalonicum* lectin has a strict specificity for D-mannose; any modification of the C-2, 3, 4, or 6 hydroxyl groups of this sugar abolished or strongly diminished its capacity to occupy the lectin's carbohydrate-binding site. Of the oligosaccharides tested, the linear mannotriose Man(α1–6)Man(α1–6)Man was a better inhibitor than the corresponding disaccharide Man(α1–6)Man-α-*O*-Me. The branched mannotriose Man(α1–6)[Man(α1–3)]Man-α-*O*-Me was the most potent inhibitor tested, being three times more potent than the linear mannotriose, Man(α1–6)Man(α1–6)Man, five times more potent than Man(α1–6)Man-α-*O*-Me and 50- to 60-fold more inhibitory than D-mannose; on the other hand, $Man_6GlcNAc_2Asn$ was only equivalent in its inhibitory potency to the aforementioned branched mannotriose. Interestingly, further addition of α-(1,2)-linked mannosyl residues to $Man_6GlcNAc_2Asn$ to give $Man_9GlcNAc_2Asn$ abolished its ability to inhibit the precipitation reaction.

TABLE II

A Comparison of the N-Terminal 38 Amino Acid Sequence between the *A. ascalonicum* Lectin and the Snowdrop (*Galanthus nivalis*) Lectin

N-Terminal

	5 10 15 20 25 30 35	
1	RNVLVNNEGLYAGQSLVEEQYTFIMQDDDNLVLYEYST	38 (AAA)
1	DNILYSGETLSTGEFLNYGSFVFIMQEDCNLVLYDVDK	38 (GNA)

Taken together, these observations imply that *A. ascalonicum* lectin has an extended carbohydrate-binding site (up to, but probably not exceeding, three monosaccharide residues), which is most complementary to the branched trisaccharide Man(α1–6)[Man(α1–3)]Man, the core mannotriose present in all N-linked glycans. This conclusion was further supported by the observations that *A. ascalonicum* lectin readily formed precipitates with many N-linked asialoglycoproteins, such as asialofetuin, asialothyroglobulin, asialoorosomucoid (Table IV). Ovalbumin which did not precipitate *A. ascalonicum* lectin was the only exception, perhaps because of its monovalent nature (It contains only one N-linked glycan chain per ovalbumin molecule). However, the *A. ascalonicum* lectin was retarded on an ovalbumin-Sepharose column (data not shown).

Native, sialylated N-linked glycoproteins did not precipitate the *A. ascalonicum* lectin, due perhaps to steric hindrance or electronic repulsion by terminal sialic acid groups (Table IV).

The strong precipitation reaction observed between AAA and the yeast mannan of *S. cerevisiae* could be ascribed to its clustered terminal α-1,3-linked mannobiosyl residues and the marked preference of *A. ascalonicum* lec-

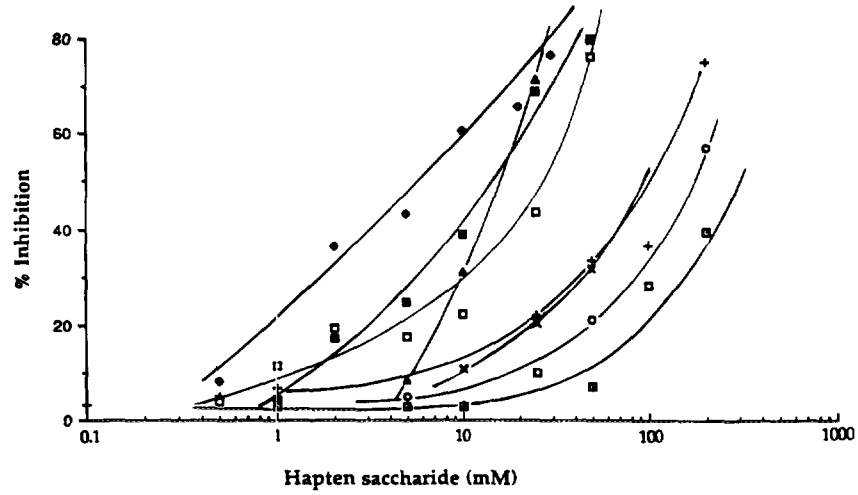

FIG. 4. Inhibition of AAA/asialofetuin precipitation by various hapten saccharides. ⊡, mannose; +, mannose-α-*O*-methyl; ○, mannose-β-*O*-methyl; ◇, Man(α1-6)[Man(α1-3)]Man-α-*O*-Me; ■, Man(α1-3)Man-α-*O*-Me; □, Man(α1-6)Man-α-*O*-Me; ▲, Man(α1-6)Man(α1-6)Man; ×, Man(α1-2)Man-α-*O*-Me.

tin for α-1,3-linked mannobiose over α-1,6-linked and α-1,2-linked mannobioses.

In recent years, we reported on the isolation and characterization of a number of mannose-binding lectins, i.e., snowdrop (GNA, *Galanthus nivalis* agglutinin) (1, 2), daffodil (NPA, *Narcissus pseudonarcissus* agglutinin), and amaryllis lectins (HHA, *Hippeastrum hybr.* agglutinin; 4; LAA, *Leucojum aestivum* agglutinin; LVA, *Leucojum vernum* agglutinin) (3).

It is of great interest to note that all these mannose-specific lectins bear a striking resemblance to each other but differ in their detailed, fine carbohydrate-binding specificity and molecular structure.

Upon SDS–PAGE, under both reducing and nonreducing conditions, all these mannose-binding lectins yield only a single low molecular weight polypeptide band (approx 11–13 kDa), indicating that they are all composed of a single type of low molecular weight subunit and do

TABLE III

Inhibition of AAA/Asialofetuin Precipitation by Saccharides, and a Comparison of Carbohydrate-Binding Properties of GNA,[a] NPA,[b] HHA,[b] and AAA

Saccharide	Concentration required for 50% inhibition (mM)	Relative inhibitory potency			
		AAA	GNA	NPA	HHA
D-Mannose	300	1.0	1.0	1.0	1.0
Man-β-*O*-Me	173	1.7	0.3	0.2	0.5
Man-α-*O*-Me	105	2.9	1.6	1.2	1.5
Man (α1–6) Man-α-*O*-Me	28	10.7	4.3	5.1	8.3
Man (α1–3) Man-α-*O*-Me	15	20	14.2	3.1	10.5
Man (α1–6) Man (α1–6) Man	17	17.6	5.7	12.4	20
Man (α1–6) \ Man-α-*O*-Me / Man (α1–3)	5.5–6	50–60	28.3	3.8	13.8
Man (α1–2) Man-α-*O*-Me	95	3.2	2.1	3.3	3.2
Man (α1–2) Man (α1–2) Man	17.3% inhibition at 50 mM				
$Man_6GlcNAc_2Asn$	46.6% inhibition at 5 mM				
$Man_9GlcNAc_2Asn$	No inhibition up to 2 mM				

Note. There was no inhibition by D-glucose, D-altrose, D-talose, D-galactose, D-fucose, L-fucose, L-rhamnose, L-arabinose, D-xylose, 2-deoxy-D-mannose, 2-deoxy-2-fluoro-D-mannose, 3-deoxy-D-mannose, 6-deoxy-D-mannose, and *N*-acetyl-D-mannosamine up to 200 mM.

[a] These data are from Shibuya *et al.* (2) using the GNA–*H. capsulata* mannan precipitation system.

[b] These data are from Kaku *et al.* (4) using the NPA–, HHA–*Pichia pastoris* yeast mannan precipitation system.

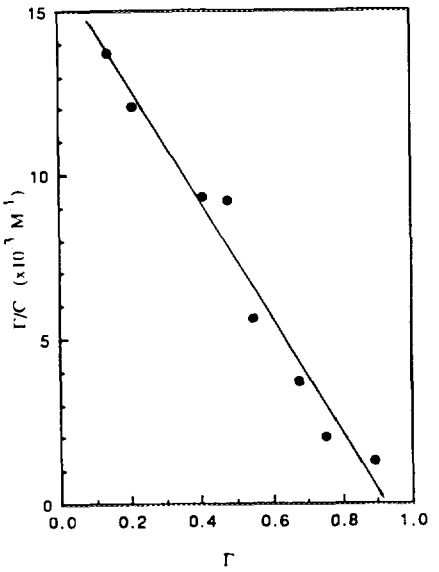

FIG. 5. Scatchard analysis of Man(α1-3)Man-α-*O*-Me binding to AAA. Each point represents the average of duplicate experiments. Γ, the ratio of the molar concentration of bound Man(α1-3)Man-α-*O*-Me to that of the lectin; *C*, the molar concentration of free Man(α1-3)Man-α-*O*-Me.

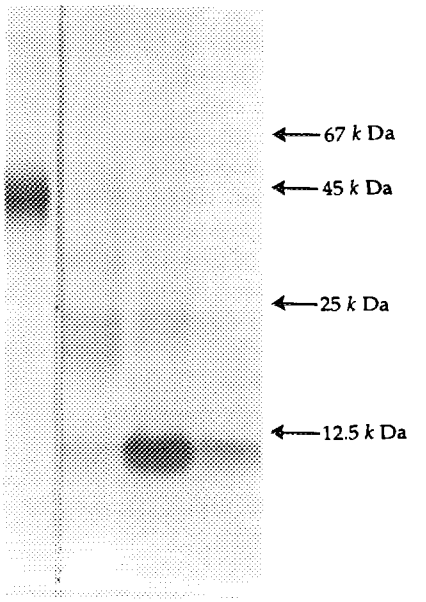

FIG. 6. Oligomeric structures of AAA revealed by SDS–PAGE analysis of the chemical cross-linking products. From left to right: lane 1, DSS-treated ovalbumin; lane 2, DSS-treated AAA; lane 3, DMA-treated AAA; lane 4, untreated AAA; lane 5, molecular weight standards.

not contain intersubunit disulfide bonds. Furthermore, these lectins have very similar amino acid compositions (Table I), featuring high contents of Asx, glycine, hydroxy amino acids, Glx, and hydrophobic amino acids, but a low content of sulfur-containing amino acids.

Not only the amino acid compositions, but also the amino acid sequences strongly resemble each other. A comparison was made of the sequence of the N-terminal 38 amino acids of the *A. ascalonicum* lectin and the snowdrop lectin (GNA). As shown in Table II, there are 60% similarities (43% identities) between the two sequences.

However, the molecular weight determined by gel filtration revealed that the *A. ascalonicum* lectin possesses a molecular structure different from the other lectins. At neutral pH, AAA eluted with an apparent molecular weight of 11 kDa, no change in molecular weight was observed when the gel filtration was repeated in the presence of 0.2 M D-mannose, indicating that at neutral pH, under the chromatographic condition described, AAA exists as a monomeric lectin; under the same conditions, NPA, LAA, LVA are homodimers, and GNA is a tetramer (3).

While these D-mannose-binding lectins are very similar in terms of their hemagglutinating activities toward rabbit erythrocytes and their exclusive specificity for D-mannose, a comparison of their detailed carbohydrate-binding properties sheds light on the subtle differences in their carbohydrate-binding properties. As shown in Table III, the *A. ascalonicum* lectin has an extended carbohydrate-binding site which is most complementary to the branched mannotriose, Man(α1-6)[Man(α1-3)]Man. It also exhibits a preference for the α-1,3-linkage over α-1,6- and α-1,2-linkages. The carbohydrate-binding site of GNA also appears to be most complementary to Man(α1-6)[Man(α1-3)]Man, but this branched mannotriose is only 28 times more active than D-mannose as an inhibitor of GNA. On the contrary, the linear α-1,6-linked mannotriose is by far the best inhibitor for both HHA (being 20 times more active than D-mannose) and NPA (12 times more potent than D-mannose), indicating that both NPA

TABLE IV

A Comparison of Reactivities toward Various Glycoproteins and Polysaccharide of NPA, HHA, GNA, and AAA

Glycoproteins and polysaccharide	AAA	NPA	HHA	GNA
Fetuin	N.D.	N.D.	N.D.	N.D.
Asialofetuin	+	+	+	N.D.
Asialoagalactofetuin	++	++	++	+
Orosomucoid	N.D.	N.D.	N.D.	N.D.
Asialoorosomucoid	++	+	+	N.D.
Ovalbumin	N.D.	N.D.	N.D.	N.D.
Transferrin	N.D.	N.D.	N.D.	N.D.
Asialotransferrin	++	+	+	N.D.
Asialoagalactotransferrin	+++	++	++	+
Asialothyroglobulin	+++	+++	+++	++
Yeast mannan (*Saccharomyces cerevisiae*)	++++	++++	++++	++++

Note. N.D., no precipitate detected.

and HHA also have extended binding sites which are most complementary to the linear α-1,6-linked mannotriose, i.e., Man(α1–6)Man(α1–6)Man.

In order to agglutinate cells, a lectin must be at least bivalent. However, the results obtained from gel filtration indicate that the *A. ascalonicum* lectin appears to be monovalent. This apparent discrepancy could be rationalized as follows. In aqueous solution, *A. ascalonicum* lectin exists as a mixture of monomers, dimers, and oligomers, due to self-association of the lectin molecules. Between the formation and dissociation of the oligomers, there is an equilibrium, which is both concentration and pH dependent. The gradual dilution due to diffusion of the lectin sample, which occurs during gel filtration, may have shifted the equilibrium to dissociation of the oligomers, so that under the chromatographic condition used for molecular weight determination, neither dimers nor oligomers could be detected.

To verify the existence of oligomers resulting from self-association, chemical cross-linking experiments were carried out. As shown in Fig. 6, higher molecular weight species (oligomers) did appear in cross-linking reagent-treated AAA samples, but were not detected in an untreated AAA sample. DSS-treated ovalbumin did not form aggregates under the identical experimental conditions, indicating that the observed AAA dimers and oligomers resulted from specific self-association of the lectin rather than from random collision of the molecules. The oligomerization of AAA through self-association confers the property of being multivalent.

Last but not least, the striking similarities between *A. ascalonicum* lectin and the other mannose-binding lectins obtained from the plant family *Amaryllidaceae* provide evidence on a molecular level to support the placing of the genus *Allium* in the *Amaryllidaceae* family rather than in the *Liliaceae* family, a long-disputed taxonomic controversy (15).

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