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The α-mannosyl-binding lectin from leaves of the orchid twayblade (*Listera ovata*)

Application to separation of α-D-mannans from α-D-glucans

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The carbohydrate-binding specificity of an α-D-mannose-specific lectin isolated from leaves of the orchid twayblade (*Listera ovata*) was elucidated by quantitative precipitation of mannose-containing polysaccharides and glycoproteins, hapten inhibition, and affinity chromatography on the immobilized lectin. *L. ovata* agglutinin (LOA) interacted with various α-mannans and galactomannans of yeasts, fungi and bacteria, but not with α-glucans, e.g., dextran and glycogen, as do mannose/glucose-binding lectins. This lectin, LOA, appears to be highly specific for α1-3 mannosidic linkages. It reacted with a linear α1-3-mannan (D. P. 15) and, surprisingly, even with a linear α1-3-mannoheptasaccharide. The LOA/*C. tropicalis* mannan precipitation reaction was inhibited by α-linked mannooligosaccharides, in the order, α1-3 > α1-6 > α1-2 linkages; α1-3 $[Man]_4$ and $[Man]_5$ were the best inhibitors among various mannooligosaccharides tested, having 7-times greater potency than α1-3 $[Man]_2$, and 18-times that of methyl α-mannoside. LOA/mannan interaction was also inhibited by periodate-oxidized and reduced α1-3 $[Man]_5$ which had an inhibitory potency similar to that of α1-3 $[Man]_3$, confirming that LOA also recognizes the internal α1-3-mannosidic linkages of carbohydrate chains.

Complete resolution of mannan and glycogen from yeast cells, by affinity chromatography on an immobilized LOA column, and retention of several high-mannose-glycoproteins suggest this lectin to be a useful tool for purification and structural investigation of α-mannosyl-containing polysaccharides and glycoconjugates.

A series of α-D-mannosyl-specific lectins present in the bulbs of monocotyledonous plants has been reported by our laboratory [1–6]. The snowdrop (*Galanthus nivalis*, GNA), daffodil (*Narcissus pseudonarcissus*, NPA), amaryllis (*Hippeastrum hybr.*, HHA), garlic (*Allium sativum*, ASA), ramsons (*Allium ursinum*, AUA) and other related lectins are carbohydrate-binding proteins that are clearly different from the well-known mannose/glucose-binding lectins, such as concanavalin A and lectins from pea, lentil and *Vica faba* seeds [7], present in leguminous plants, in that they interact strongly with α-mannans and certain galactomannans of yeasts and fungi, but not with glycogen, amylopectin, dextran and other α-glucans.

The orchid twayblade (*L. ovata*) leaves accumulate a lectin (*L. ovata* agglutinin, LOA) which does not agglutinate human erythrocytes. LOA is a dimeric protein composed of two subunits of M_r 12500; it is the first lectin to be isolated from a species of the family Orchidaceae, and exhibits exclusive specificity towards D-mannose [8].

In this study, we report the carbohydrate-binding properties of the first lectin to be isolated from orchid leaves, as revealed by quantitative precipitation reactions with several microbial α-mannans and galactomannans, by hapten inhibition studies, and by affinity chromatography on the immobilized lectins.

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Abbreviations. ASA, *Allium sativum* agglutinin; AUA, *Allium ursinum* agglutinin; GNA, *Galanthus nivalis* agglutinin; HHA, *Hippeastrum* hybr. agglutinin; LBA, *Phaseolus lunatus* agglutinin; LOA, *Listera ovata* agglutinin; NPA, *Narcissus pseudonarcissus* agglutinin; PHA, *Phaseolus vulgaris* agglutinin; Me-α-D-Man*p*, methyl α-D-mannopyranoside.

MATERIALS AND METHODS

Purification of LOA

The LOA used in this study was purified from *L. ovata* leaves using a D-mannose column as reported previously [8].

Saccharides, polysaccharides and glycoproteins

Most monosaccharides and their methyl glycosides used in this study are available commercially. The α1-3-linked mannooligosaccharides and a low-molecular-mass α1-3-mannan (D. P. 15) were prepared by mild acid hydrolysis of the

glucuronoxylomannan of *Tremella fuciformis* [9]. Some α1-2-linked and α1-6-linked mannooligosaccharides were the gifts of Dr T. Nakajima of Tohoku University, Japan. Glc(α1-4) Man was available from previous studies. Man(α1-3) Man-α-*O*-Me was purchased from the Sigma Chemical Co. Man(α1-3)Man(α1-6) Man-α-*O*-Me was a gift from Dr G. Krepinsky, University of Toronto, Canada.

α-Mannans of *Saccharomyces cerevisiae* (Oriental Baker's yeast), *Candida tropicalis* [10], *Candida albicans*, *Aspergillus fumigatus* [11], *Alternaria kikuchiana* and *Mycobacterium tuberculosis* [12], and galactomannans of *Audiobasidium pullulans* [13] were prepared as described previously. *Candida lyptica* mannan (a gift of Dr Gorin), *Elucione leucospila* [14], and the arabinomannan of *M. tuberculosis* [12] were also available. The galactomannans of *A. fumigatus* and *A. kikuchiana* which contain galactofuranosyl side chains were treated with 0.1 M H_2SO_4 at 90°C for 1 h, and the resulting mannan preparations were used in precipitation studies. Dextran B-1355-S was a gift of Dr. A. Jeane, Peoria, IL. Ovalbumin was purchased from Sigma Chemical Co. Lima bean lectin (LBA) and *Phaseolus vulgaris* lectin (PHA) were available in our laboratories.

α1-3-linked mannan and mannooligosaccharides

Glucuronoxylomannan of *T. fuciformis* which contains an α1-3-linked mannan backbone was oxidized with 50 mM $NaIO_4$ for 7−10 days at 4°C, followed by reduction with sodium borohydride. The resulting polysaccharide polyalcohol was hydrolyzed with 0.4 M trifluoroacetic acid at 90°C for 5 h and a series of α1-3-linked mannooligosaccharides, including a linear α1-3 mannan (D. P. 15), was purified by gel filtration on a column of BIO-gel P-2; the content of 1-3-mannosidic linkages was confirmed by methylation analysis. An α1-3-mannopentaose was also subjected to periodate oxidation/reduction employing the same conditions, as described above.

Precipitation and hapten inhibition assay

Quantitative precipitation reactions were caried out by a microprecipitation technique [1]. LOA (20 μg or 50 μg) was added to varying amounts of polysaccharides in a total volume of 100, 150, 200 or 250 μl. After incubation at 30°C for 1 h the mixtures were kept at 4°C for 48 h and centrifuged, and protein in the precipitates was determined by the method of Lowry [15] using bovine serum albumin as a standard.

Sugar inhibition of the precipitation reactions was carried out by adding increasing amounts of sugar or derivative to precipitation systems containing LOA and *C. tropicalis* mannan.

Immobilization of lectin and affinity chromatography

An aliquot of purified LOA (10 mg) was immobilized by coupling with AF-Tresyl Toyopearl 650 (Toyosoda Co.). The LOA conjugated Toyopearl contained approximately 1.5 mg protein/ml gel.

Polysaccharides (250−1000 μg) or glycoproteins (100 μg carbohydrate) were applied to the LOA-Toyopearl column (1 cm×10 cm). The column was washed first with NaCl/P_i (10 mM sodium phosphate, 0.15 M NaCl, pH 7.2) followed by elution with NaCl/P_i containing 0.5 mM methyl α-D-mannoside (Me α-D-Man*p*). The amount of carbohydrate present in each tube was determined by the phenol/sulfuric acid method [16].

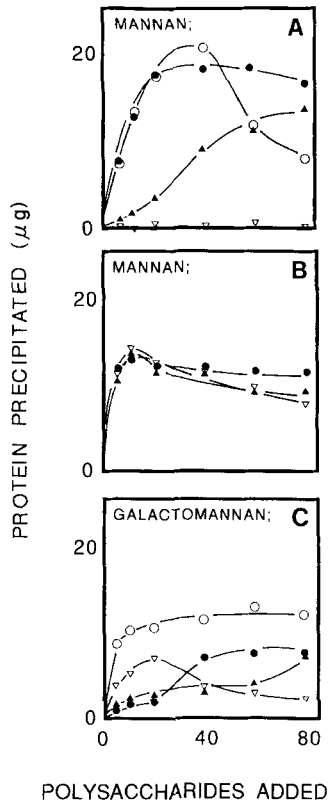

Fig. 1. Quantitative precipitation curves of yeast mannans (A, B), galactomannans and arabinomannan (C) bv LOA. The amount of protein was 50 μg (in 200 μl in each tube). (A) Mannans of *S. cerevisiae*,(●); *C. tropicalis*, (○); *A. fumigatus*, (▲); dextran 1355-S, (▽). (B) Mannans of *C. albicans*, (▽); *A. kikuchiana*, (●); *M. tuberkulosis Aoyama*, (▲); (C) Galactomannans of *A. pullulans*, (○); *C. lyptica*, (▽); *E. leucospila*, (●); and an arabinomannan, (▲) were also examined.

RESULTS AND DISCUSSION

Precipitation assay

The precipitation curves of LOA with various polysaccharides are shown in Fig. 1. LOA interacted strongly with the highly branched α-mannans isolated from *S. cerevisiae* and *C. tropicalis* [10] which contain side chains of α1-2-linked and α1-3-linked D-mannosyl residues attached to a backbone of α1-6-linked mannose residues. The lectin did not give a precipitation reaction with glycogen or dextran B-1355-S. The arabinomannan from *M. tuberculosis* Aoyama B [12], and cell surface galactomannans from *C. lyptica* and *E. leucospila* [14] in which some residues in the α1-6-mannose backbone, are substituted by α-D-galactofuranosyl and mannobiosyl or triosyl units gave approximately 25−50% the amount of protein precipitation as that with the *S. cerevisae* mannan. The differences in these precipitation reactions may be attributed to the structures of the mannans, particularly the position of short side chains of α1-3 or α1-2-mannose units, which may affect accessibility to the lectin. The essentially linear α1-3 mannan (D. P. 15) prepared from *T. fuciformis* glucuronoxylomannan [9] reacted strongly with LOA. This lectin also reacted with the linear α1-3-manno-

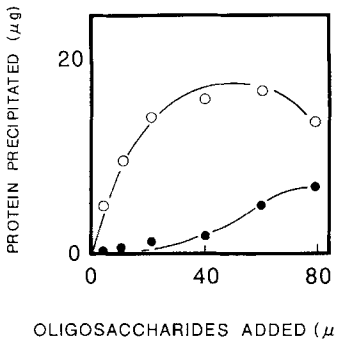

Fig. 2. Quantitative precipitation curve of a linear α1-3-mannan (D. P. 15) (○) and α1-3-Man$_7$ (●) by LOA. The protein concentration was 50 μg/tube.

Table 1. Inhibition by various sugars of LOA/*C. tropicalis* mannan precipitation.

Sugars	Concentration for 50% inhibition
	mM
D-Mannose	440
D-Glucose	No inhibition at 2000 mM
D-Galactose	No inhibition at 2000 mM
PNP α-D-Mannoside	32% inhibition at 67 mM
PNP β-D-Mannoside	12% inhibition at 80 mM
Me α-D-Mannoside	220
Me β-D-Mannoside	1000
Man (α1-2) Man	125
Man (α1-2) Man (α1-2) Man	23% inhibition at 50 mM
Man (α1-3) Man	85
Man (α1-3) Man (α1-3) Man	21
Man (α1-3) Man (α1-3) Man (α1-3) Man	14
Man (α1-3) Man (α1-3) Man (α1-3) Man (α1-3) Man	12
Periodate-oxidized, $NaBH_4$-reduced α1-3 $[Man]_5$	28
Man (α1-3) Man-α-*O*-Me	17
Man (α1-6) Man	100
Man (α1-6) Man (α1-6) Man	29% inhibition at 50 mM
Glc (α1-4) Man	14% inhibition at 50 mM
Man α1 \ 6 Man-α-*O*-Me 3 / Manα1	30

heptasaccharide to give a precipitation reaction as shown in Fig. 2. These results suggest that LOA recognizes sequences of internal α-D-mannosyl residues, in addition to terminal α-mannosyl units.

Inhibition of precipitation reaction by haptenic sugars

The carbohydrate-binding specificity of LOA was studied by sugar hapten inhibition of the interaction of LOA with *C. tropicalis* mannan. It was confirmed that LOA is a mannose-specific lectin and shown that neither D-glucose nor D-galactose inhibited the lectin/mannan reaction at a concentration of 2 M. Methyl α-D-mannopyranoside was twofold better an inhibitor than that of D-mannose (Table 1) whereas the

Table 2. Inhibition by manno-oligosaccharides of four α-mannosyl-binding lectins. Data for LOA are based on inhibition of the LOA/*C. tropicalis* mannan precipitation system. Data for *Galanthus nivalis* (GNA) bulb lectin are taken from Shibuya et al. [1]. Data for *Narcissus pseudonarcissus* (NPA) and *Hippeastrum hybr.* (HHA) bulb lectins are taken from Kaku et al. [3].

Sugar	Relative inhibitory potency on			
	LOA	GNA	NPA	HHA
D-Mannose	1.0	1.0	1.0	1.0
Me α-D-Mannoside	2.0	1.6	1.2	1.5
Man (α1-2) Man	3.5	2.1	3.3	3.2
Man (α1-3) Man	5.2	12.1	2.8	5.9
Man (α1-3) Man-α-*O*-Me	25.9	14.2	3.1	10.5
Man (α1-2) Man-α1-2 Man	> 8.8	3.4	1.7	3.6
Man (α1-3) Man (α1-3) Man	21.0			
Man (α1-6) Man (α1-6) Man	> 8.8	5.7	12.4	20.0
Man α1 \ 6 Man-α-*O*-Me 3 / Manα1	14.5	28.3	3.8	13.8

β-anomer (methyl β-D-mannopyranoside) was a poor inhibitor exhibiting only 25% the activity of the α-anomer.

Among a series of α-linked manno-oligosaccharides tested, it is apparent that the α1-3-mannopentasaccharide is the best inhibitor of the LOA/mannan interaction. As shown in Table 1 α1-3-mannobiose had a significantly higher inhibitory activity than α1-2 and α1-6 mannobiose indicating that the α1-3 linkage is most complementary to the sugar-binding sites of LOA. The α1-3 mannobiose had an inhibitory activity 2.5-times higher than that of the methyl α-D-mannoside. The mannotriose was 10-times, mannotetraose 15-times and mannopentaose 18-times more inhibitory than methyl α-D-mannoside. Man(α1-3)Man-α-*O*-Me was also an excellent inhibitor, better than Man(α1-3)Man, and nearly equivalent to Man(α1-3)Man(α1-3)Man, suggesting that the α-configuration of the reducing unit makes an important contribution to its binding activity. It is interesting to note that the inhibitory potency of the branched trisaccharide Manα1-3(Man-α-1-6)-Man-α-*O*-Me was approximately 50% that of Man(α1-3)-Man-α-*O*-Me suggesting it is the Man(α1-3)Man residue which is recognized by the lectin. The fact that periodate-oxidized and $NaBH_4$-reduced α1-3-mannopentasaccharide, in which both terminal mannosyl ends were modified, had the same activity as α1-3-linked mannotriose (Table 1), strongly suggests that the combining site(s) of LOA appears to be most complementary to three consecutive mannosyl units linked α1-3, even if both terminal mannosyl units are not involved. In similar fashion, LOA gave a strong precipitation with a linear α1-3-mannan (D. P. 15) and the periodate-oxidized and reduced glucuronoxylomannan of *T. fuciformis* hich contains an (α1-3)-mannan backbone. In Table 2, the mannosyl-binding specificity of LOA is compared with several other mannose-specific lectins.

Binding characteristics of immobilized LOA

The capability of a LOA affinity column to bind various polysaccharides and glycoproteins was also investigated. Yeast mannan bound strongly to the LOA column and was

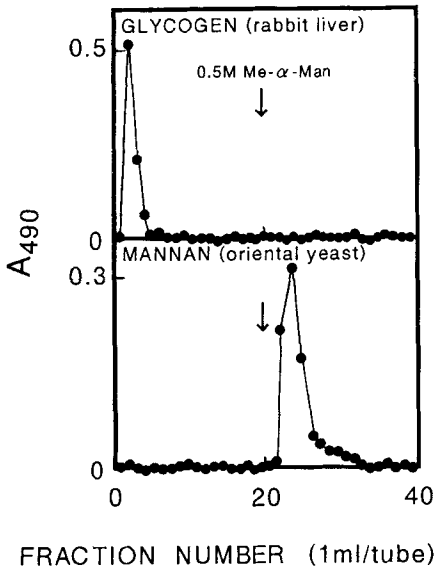

Fig. 3. Elution profile of mannan and glycogen on a LOA-Toyopearl column (1 cm×1 cm). Rabbit liver glycogen (1 mg) was applied to the LOA-Toyopearl column; also shown, *C. tropicalis* mannan (250 μg) was applied to the same column. The arrows indicate the addition of 0.5 mM methyl α-D-mannoside. The methyl glycoside in each fraction was removed by dialysis before assay for total carbohydrate.

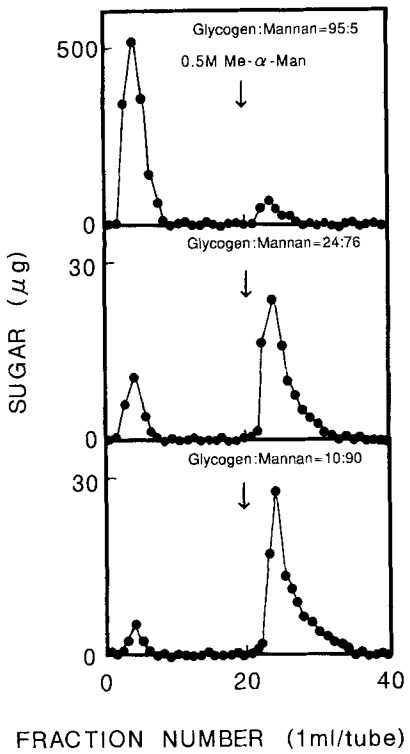

Fig. 4. Elution profiles of yeast polysaccharides containing mannan and glycogen on LOA-Toyopearl. Conditions were similar to those of Fig. 3.

completely eluted with methyl α-D-mannoside, whereas glycogen readily passed through the column unretarded (Fig. 3). When the yeast mannan fraction prepared from *S. cerevisiae* cells, which is usually contaminated with glycogen, was applied to the LOA column, glycogen was resolved completely from the mannan, which was eluted subsequently with 0.5 mM methyl α-D-mannoside, as shown in Fig. 4. Similar results were obtained in three experiments using yeast mannan fractions containing varying proportions of glycogen or cell wall glucans (Fig. 4). This represents a dramatic example of the utility of immobilized LOA for the separation of α-mannans from α-glucans.

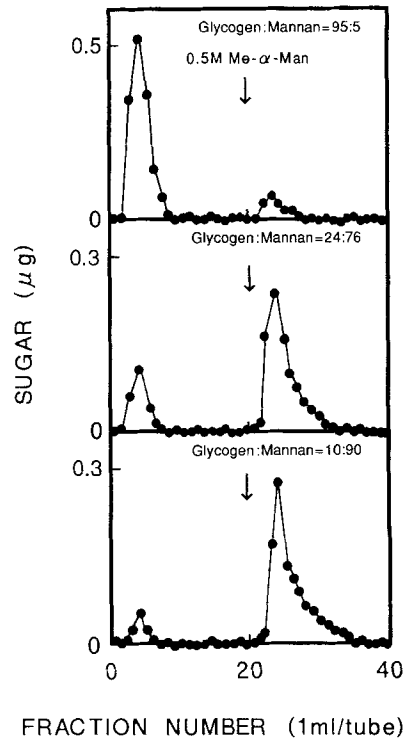

Fig. 5. Elution profiles of some glycoproteins on a LOA-Toyopearl column. Various glycoproteins were applied to a Toyopearl column (1 cm × 1 cm), followed by elution with NaCl/P_i and NaCl/P_i containing 0.5 M Me-α-D-Man.

In another experiment, some glycoproteins containing various high levels of α-mannosyl units in their carbohydrate chains were applied to the LOA-Toyopearl column and eluted with NaCl/P_i and NaCl/P_i containing 0.5 mM Me-α-D-Man*p*. The results are shown in Fig. 5. Lima bean lectin which carries a terminal Man(α1-3)Man unit on both carbohydrate chains [17] bound strongly to this column and was eluted with Me-α-D-Man*p* (NaCl/P_i) whereas the *Phaseolus vulgarus* lectin was resolved into two components signifying heterogeneity of the glycosyl moiety [18]. However, ovalbumin, which contains a large number and variety of high-mannose and hybrid-type glycosyl moieties, was not retained on the column as was the case with the immobilized-snowdrop-lectin column [1]. This may be due either to the presences of a single carbohydrate chain, or the location of the (α1-3)-mannosyl sequences on the molecule.

We believe the orchid twayblade lectin should be of great utility for the separation of α-mannans from α-glucans, and for investigating the structure of complex carbohydrates, especially those carrying α1-3-mannosidic linkages.

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