Leaves of the Orchid Twayblade (*Listera ovata*) Contain a Mannose-Specific Lectin

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ELS J. M. VAN DAMME, ANTHONY K. ALLEN, AND WILLY J. PEUMANS
Laboratorium voor Plantenteelt, Katholieke Universiteit Leuven, Kardinaal Mercierlaan 92, B-3030 Leuven, Belgium (E.J.M.V.D., W.J.P.); and Department of Biochemistry, Charing Cross and Westminster Medical School, Hammersmith, London W6 8RF, United Kingdom (A.K.A.)

ABSTRACT

A new lectin was isolated from leaves of the twayblade (*Listera ovata*). It is a dimeric protein built up of two subunits of Mr 12,500. This lectin, which is the first to be isolated from a species of the family Orchidaceae, exhibits exclusive specificity towards mannose.

At present over 100 plant lectins have been isolated and characterized with respect to their biochemical, physicochemical, and carbohydrate binding properties (7, 8). Although most of these proteins have been isolated from dry seeds it is now well established that other plant parts also contain lectins as they have been found in vegetative tissues such as leaves, stems, roots, bark, tubers, and rhizomes (2, 4, 6, 12, 15). Detailed studies of a number of lectins from different vegetative tissues have shown that some of them are very similar to the seed lectins present in the same species (e.g. leaf lectins of *Griffonia simplicifolia, Dolichos biflorus* (6, 11); others, however, are completely different from all known seed lectins and occur apparently exclusively in vegetative tissue (e.g. lectins from rhizomes of *Bryonia dioica, Urtica dioica* (12, 13). In the present paper we describe the isolation and partial characterization of a mannose-specific lectin from leaves of twayblade (*Listera ovata*), a representative of the plant family Orchidaceae.

MATERIALS AND METHODS

Material. Leaves of *Listera ovata* (twayblade) were collected from flowering plants around the first half of May and used immediately or stored at −80°C.

Extraction and Purification of the *L. ovata* Agglutinin. Leaves of *L. ovata* were cut into small pieces and homogenized with a blender in 5 volumes (v/w) of a solution of 1 M (NH₄)₂SO₄ containing 10 mM thiourea. The homogenate was squeezed through a double layer of cheesecloth and centrifuged (15 min, 20,000g). The supernatant was frozen at −20°C, then thawed. The precipitate which formed as a result of the freezing and thawing was removed by centrifugation as before. Finally, the cleared supernatant was filtered through filter paper (Whatman 3 MM) to remove any particulate material. This crude filtrate was used as a source of LOA for affinity chromatography.

Affinity Chromatography on Immobilized Mannose. The crude filtrate of twayblade leaves was applied to a column of immobilized mannose (Selectin 10 from Pierce Chemical Co., Rockford, IL), equilibrated with 1 M (NH₄)₂SO₄. Unbound protein was washed off with 1 M (NH₄)₂SO₄ until the A₂₈₀ of the effluent fell below 0.01. Then the lectin was desorbed with 20 mM (unbuffered) DAP.

Ion Exchange Chromatography on Mono-Q. After the affinity chromatography step the lectin was further purified by anion exchange chromatography using a Pharmacia FPLC system (Pharmacia, Uppsala, Sweden). The lectin fractions collected after affinity chromatography were dialyzed against 20 mM DAP (pH 9.0) and applied to a column of Mono-Q type HR 5/5 (Pharmacia), equilibrated with the same buffer. After the column had been washed with 4 ml of 20 mM DAP (pH 9.0) elution was performed using a linear salt gradient (20 ml, 0–0.4 M NaCl) in DAP. Peak fractions were collected and used for further experiments.

Assays and Analysis Methods. Hemagglutination assays were carried out in small glass tubes containing, in 0.1 ml final volume, 80 μl of a 1% suspension of rabbit trypsin-treated erythrocytes and 20 μl of crude extract or lectin solution. Agglutination was determined visually (with the unaided eye) after 1 h at room temperature. The carbohydrate specificity of the lectin was determined with a series of simple sugars (glucose, galactose, glucosamine, galactosamine, N-acetylglucosamine, N-acetylgalactosamine, mannose, lactose, melibiose, fucose, arabinose, ribose, fructose, trehalose, sorbose, xylose, sucrose, maltose, and sorbitol) and glycoproteins (thryoglobulin, ovomucoid, fetuin, and asialofetuin). Lecitin preparations were analyzed by SDS-PAGE using a discontinuous system (10) on 12.5 to 25% (w/v) acrylamide gradient gels. To enhance the resolution of the polypeptide bands, the lectin was reduced and alkylated. Purified lectin (0.5 mg/ml in water) was made 0.05% (v/v) with respect to β-mercaptoethanol and heated in a boiling water bath for 1 min. After cooling, the solution was made 0.1 M in iodoacetamide (with a 1 M solution) and incubated for 30 min at 37°C. The reaction was stopped with mercaptoethanol (final concentration, 1 M).

The amino acid composition of the proteins was determined after hydrolysis under N₂ in 3 M toluene-p-sulfonic acid at 110°C for 24 and 72 h and appropriate correction factors derived for destruction or slow release of amino acids. Glucosamine was determined on the analyses after hydrolysis under N₂ in 3 M toluene-p-sulfonic acid at 100°C (1). Cystine was determined as cystic acid after performic-acid oxidation of the protein and

Abbreviations: LOA, *Listera ovata* agglutinin; DAP, 1,3 diaminopropylamine; FPLC, fast protein liquid chromatography.

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Table I. Carbohydrate Specificity of Crude Extracts and Purified LOA Isolectins

<table>
<thead>
<tr>
<th>Glycoprotein</th>
<th>Minimal Concentration of Sugar or Glycoprotein&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Crude extract&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Purified lectin&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mannose</td>
<td>6 mm</td>
<td>6 mm</td>
<td>6 mm</td>
</tr>
<tr>
<td>Fetuin</td>
<td>500 µg/ml</td>
<td>500 µg/ml</td>
<td>500 µg/ml</td>
</tr>
<tr>
<td>Asialofetuin</td>
<td>100 µg/ml</td>
<td>100 µg/ml</td>
<td>100 µg/ml</td>
</tr>
<tr>
<td>Ovalmucoid</td>
<td>500 µg/ml</td>
<td>500 µg/ml</td>
<td>500 µg/ml</td>
</tr>
<tr>
<td>Thyroglobulin</td>
<td>5 µg/ml</td>
<td></td>
<td>5 µg/ml</td>
</tr>
</tbody>
</table>

<sup>a</sup> All other sugars tested (cf. "Materials and Methods") were not inhibitory at concentrations below 100 mm.  
<sup>b</sup> Minimal concentration required for 50% inhibition of the agglutination activity in assays with trypsin-treated rabbit erythrocytes.  
<sup>c</sup> The titer of the crude extract was 100.  
<sup>d</sup> The final concentrations of LOA I and LOA II were 10 µg/ml.

FIG. 1. Affinity chromatography of LOA on immobilized mannose. A partially purified extract of twayblade leaves (250 g fresh weight) was applied to a column of immobilized mannose (10 ml bed volume). Unbound protein was eluted with 1 M (NH₄)₂SO₄ until the A₂₈₀ fell below 0.01 and the lectin was desorbed with 20 mM DAP. Fractions of 5 ml were collected and the A₂₈₀ and agglutination titer determined (with trypsin-treated rabbit erythrocytes). The overall yield was 105 mg of LOA.

FIG. 2. Ion exchange chromatography of LOA on a Mono-Q column. a, Chromatogram of total affinity-purified LOA; b, chromatogram of LOA I purified by repeated chromatography on a Mono-Q column; c, chromatogram of LOA II purified by repeated chromatography on a Mono-Q column. Experimental details are described in the "Materials and Methods" section.

FIG. 3. SDS-PAGE of LOA isolecints. Lanes a and b, LOA I and LOA II, respectively. Lane R₁, Mr reference proteins (lysozyme [Mr 14,300], soya-bean trypsin inhibitor [Mr 21,000], carbonic anhydrase [Mr 30,000], ovalbumin [Mr 45,000], BSA [Mr 67,000], and phosphorylase [Mr 93,000]). Lane R₂, Mr reference proteins (myoglobin [intact, Mr 17,201], myoglobin I + II [Mr 14,632], myoglobin I [Mr 8,235], myoglobin II [Mr 6,383]).

RESULTS

Purification of the <i>L. ovata</i> Agglutinin. Preliminary experiments with crude extracts of twayblade leaves indicated that they contain readily detectable amounts of an agglutinating factor. Indeed, when tested with trypsin-treated rabbit erythrocytes, titers of extracts prepared by homogenizing leaves in 10 volumes of PBS, went up to 100.

To work out a purification procedure the carbohydrate specificity of the agglutinin was determined using a series of simple sugars. Since these experiments indicated that mannose was the only inhibiting saccharide (Table I) mannose coupled to agarose was chosen as an affinity matrix for the purification of the LOA. Following the purification scheme described in the "Materials and Methods" section, LOA could be purified in a simple step with almost complete recovery. Virtually all the initial agglutination activity was recovered in the fraction desorbed with DAP (Fig. 1). It should be mentioned here that LOA can be desorbed equally well with mannose. However, since high concentrations (0.5 M) are required, and in addition the lectin elutes in a large
Gel filtration, in accordance with their elution position (Fig. 2a). Each isoelectin was recycled on the Mono-Q column till it yielded a single symmetrical peak (Fig. 2, b and c). LOA I and LOA II represent 33 and 67% of the total lectin content, respectively.

Molecular Structure of LOA I and LOA II. The molecular structure of both LOA isolecitins was analyzed by SDS-PAGE, gel filtration, and sucrose-gradient centrifugation. As shown in figure 3, both isolecitins yielded a polypeptide band of Mr 12,500 upon SDS-PAGE.

The Mr of native LOA was determined by gel filtration of LOA I and LOA II on Sephadex G-100 and Seprose 12. Both isolecitins eluted with an apparent Mr of 3,400 on Sephadex G-100 and 5,600 on Seprose 12 (Figs. 4a and 5a). Since these Mr values are lower than the apparent Mr of the lectin polypeptides determined by SDS-PAGE, interactions between LOA and both types of matrices became obvious and gel filtration experiments were repeated in the presence of 0.1 M mannose. Under these conditions LOA isolecitins eluted with an apparent Mr of 12,000 and 14,000 from a Sephadex G-100 and Seprose 12 column, respectively, which indicated that in the presence of this specific sugar the interactions between lectin and the gel matrices were reduced (Figs. 4b and 5b). Because of the uncertainty inherent to Mr estimations based on gel filtration LOA was centrifuged in a sucrose gradient. From their sedimentation position (relative to that of Mr marker proteins) a Mr between 20,000 and 25,000 was calculated for both native LOA isolecitins (Fig. 6).

These results, taken together with the SDS-PAGE pattern, indicate that both LOA I and LOA II are dimers composed of two identical subunits of Mr 12,500.

Amino acid analyses revealed that LOA I and LOA II have a similar amino acid composition typified by a high content of asparagine/aspartic acid, glutamine/glutamic acid, glycine and serine. LOA lacks methionine and histidine (Table II). Sugar determination of purified LOA indicated no covalently bound carbohydrate, suggesting that the lectin is not a glycoprotein.
Carbohydrate-binding Specificity and Agglutination Properties of LOA I and LOA II. Purified LOA isolectins readily agglutinate rabbit erythrocytes, the minimal concentration required for agglutination of trypsin-treated and untreated cells being 1.6 and 12.5 μg/ml respectively, for both isolectins. Human red blood cells, irrespective of their blood group, were not agglutinated even at lectin concentrations as high as 2 mg/ml. 

Hapten inhibition assays carried out with both LOA isolectins confirmed that from all sugars which were tested only mannose inhibits the agglutination activity, the concentration required for 50% inhibition being 6 mM (Table I). From the glycoproteins we tested, thyroglobulin proved to be the most potent inhibitor, being effective at a concentration as low as 5 μg/ml (Table I). Ovomucoid and fetuin were 100 times less potent inhibitors than thyroglobulin; asialofetuin inhibited the agglutination at a 5-fold lower concentration than native fetuin. As far as can be concluded from hapten inhibition assays both LOA isolectins behave identically with respect to their carbohydrate-binding specificity.

**DISCUSSION**

Leaves of twayblade (Listera ovata) contain a lectin which can easily be purified by affinity chromatography on immobilized mannose. This lectin is a mixture of two different molecular forms which can be separated by ion exchange chromatography on a Mono-Q column. Both isolectins are dimers of two identical sized subunits of Mr 12,500 and each isolectin exhibits the same agglutination properties and carbohydrate-binding specificity. The sugar-binding specificity of LOA is unique in that it binds exclusively mannose. This is in contrast to other mannose-specific lectins from dicots such as concanavalin A, pea, and lentil lectin, which bind glucose almost equally well as mannose. Furthermore, the greater inhibitory power of thyroglobulin over the other glycoproteins indicates that the twayblade lectin preferentially binds to a high-mannose structure. Recently another monocot lectin with exclusive specificity towards mannose has been isolated from snowdrop (Galanthus nivalis) bulbs (14). However, unlike LOA, the latter lectin has no higher affinity for mannose oligosaccharides than for the monomer of mannose. Another difference from these mannose-specific monocot lectins is that the snowdrop lectin does not bind to fetuin-agarose whereas LOA can readily be isolated on this affinity matrix.

LOA is the first lectin to be isolated from a species belonging to the family Orchidaceae and it differs in specificity and structure from all other lectins so far described. The absolute specificity towards mannose and especially towards mannose oligosaccharides is unique and might be quite useful in glycoconjugate research.

**LITERATURE CITED**

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