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The monomeric and dimeric mannose-binding proteins from the Orchidaceae species *Listera ovata* and *Epipactis helleborine*: sequence homologies and differences in biological activities

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The Orchidaceae species *Listera ovata* and *Epipactis helleborine* contain two types of mannose-binding proteins. Using a combination of affinity chromatography on mannose-Sepharose-4B and ion exchange chromatography on a Mono-S column eight different mannose-binding proteins were isolated from the leaves of *Listera ovata*. Whereas seven of these mannose-binding proteins have agglutination activity and occur as dimers composed of lectin subunits of 11–13 kDa, the eighth mannose-binding protein is a monomer of 14 kDa devoid of agglutination activity. Moreover, the monomeric mannose-binding protein does not react with an antiserum raised against the dimeric lectin and, in contrast to the lectins, is completely inactive when tested for antiretroviral activity against human immunodeficiency virus type 1 and type 2. Mannose-binding proteins with similar properties were also found in the leaves of *Epipactis helleborine*. However, in contrast to *Listera* only one lectin was found in *Epipactis*. Despite the obvious differences in molecular structure and biological activities molecular cloning of different mannose-binding proteins from *Listera* and *Epipactis* has shown that these proteins are related and some parts of the sequences show a high degree of sequence homology indicating that they have been conserved through evolution.

Keywords: cDNA cloning; *Epipactis*; lectin; *Listera*; mannose-binding.

Abbreviations: EHMBP, *Epipactis helleborine* mannose-binding protein; LOMBP, *Listera ovata* mannose-binding protein.

Note: The nucleotide sequences reported in this paper will appear in the Genbank™/EMBL Data library with the accession numbers L18894, L18895 and U07787.

Introduction

Since the publication in 1987 [1] of the first report of a lectin with exclusive specificity towards mannose from snowdrop (*Galanthus nivalis*) bulbs evidence has accumulated for the occurrence of a large superfamily of more or less closely related mannose-binding agglutinins within at least three different families of monocotyledonous plant species. Soon after the discovery of the snowdrop lectin, it was recognized that other members of the family Amaryllidaceae also contain lectins, which are virtually identical to that from the snowdrop [2]. Similarly, as soon as the newly discovered lectins from several *Allium* species were analysed in detail with respect to their molecular structure and specificity, and serological relationship to the Amaryllidaceae lectins, the close resemblance between these two groups of proteins became obvious [3]. Finally, a reinvestigation of the previously described mannose-binding lectin from the orchid *Listera ovata* (twayblade) [4] revealed that this agglutinin also closely resembles the Amaryllidaceae lectins not only in its specificity and molecular structure but also with respect to its serological properties [5]. Recently, evidence was obtained that mannose-specific agglutinins are widespread in different tribes of the orchid family (E. Van Damme, unpublished results). Moreover, it

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was shown that the Orchidaceae lectins from *Listera ovata*, *Epipactis helleborine* and *Cymbidium* hybrid strongly resemble each other in their biochemical and physicochemical properties [5]. From this therefore, it is evident that the Orchidaceae lectins represent a large family of mannose-binding agglutinins.

In contrast to the Amaryllidaceae and *Allium* lectins, which have been studied in great detail both at the biochemical and molecular level, our present knowledge of the group of the Orchidaceae lectins is rather limited. Until now, only three orchid lectins have been isolated and characterized in some detail [4, 5]. Molecular cloning and sequencing of the cDNA clones encoding these Orchidaceae lectins has revealed that they show a high degree of sequence homology at the amino acid level. The sequence data also show evidence of the relationship between the orchid lectins on the one hand and the Amaryllidaceae and *Allium* lectins on the other hand. Although all orchid lectins isolated thus far are considered as mannose-binding proteins, detailed carbohydrate-binding studies have only been done with the *Listera ovata* agglutinin [6]. Like the snowdrop agglutinin this orchid lectin is highly specific for α-1,3-mannose oligomers. However, it clearly differs from the snowdrop lectin in that it also strongly binds to some glycoproteins like thyroglobulin and asialofetuin. Due to its high affinity for mannans the *Listera ovata* agglutinin is of great potential use for the separation of α-mannans from α-glycans and for investigating the structure of complex carbohydrates possessing α-1,3-mannosidic linkages. In addition, because of the pronounced inhibitory effect of the twayblade lectin (relative to that of the other mannose-binding lectins) on human immunodeficiency virus (HIV)-infection [7, 8] it is a useful tool in the study of the HIV infection cycle and acquired-immuno-deficiency-syndrome (AIDS) chemotherapy.

During the screening of a cDNA library constructed using mRNA from leaves of *Listera ovata* for lectin cDNA clones some cDNA sequences showed a marked homology to the lectin clones but at the same time definitely differed from them in that the N-terminal amino acid sequences of the putative proteins they coded for strongly differed from that of the lectin. We therefore started a search for lectin-like or lectin-related proteins (and their corresponding genes) in leaves of twayblade. This report describes the characterization and molecular cloning of non-agglutinating, monomeric mannose-binding proteins in *Listera* and *Epipactis* which are clearly related to and occur along with the dimeric mannose-specific lectins of these species. Using a combination of protein purification and cDNA cloning techniques it could be demonstrated that twayblade leaves contain several closely related agglutinins as well as a non-agglutinating monomeric mannose-binding protein. Similar experiments with *Epipactis helleborine* revealed that in contrast to *Listera* this species contains only one lectin but in addition possesses a monomeric non-agglutinating mannose-binding protein. Since this is the first report of a group of monomeric mannose-binding but non-agglutinating proteins the possible significance of their discovery is discussed.

Materials and methods

Plant material

Leaves of twayblade (*Listera ovata* (L.) Robert Brown) and broad-leaved helleborine (*Epipactis helleborine* (L.) Crantz) were collected locally.

Viruses

Human immunodeficiency virus type 1 (HIV-1) (strain HTLV-III_B) was originally obtained from the culture supernatant of the persistently HIV-1-infected H9 cell line (H9/HTLV-III_B) [9] and was kindly provided by Dr R. C. Gallo (National Cancer Institute, National Institutes of Health, Bethesda, MD, USA). HIV-2 (strain LAV-2) [10] was a gift from Dr L. Montagnier (Pasteur Institute, Paris, France).

Purification of the mannose-binding proteins from Listera ovata *and* Epipactis helleborine

New purification schemes were developed for the isolation and separation of the agglutinating and non-agglutinating mannose-binding proteins from *Listera ovata* and *Epipactis helleborine*. Since the application of special chromatography conditions was essential for the isolation of these proteins, the existence of which was predicted on the basis of cDNA sequences, the purification is described in detail in the Results section. The mannose-Sepharose-4B used in the affinity chromatography was prepared by the divinylsulfone method as described previously [5].

Ion exchange chromatography

For analytical purposes protein samples were analysed by ion exchange chromatography on a Mono-S cation exchanger column (Type HR 5/5 from Pharmacia) using a Pharmacia FPLC system. Samples (1–2 mg) dissolved in formate buffer (25 mM formic acid adjusted to pH 3.8 with NaOH) were loaded on the column. After washing the column with 4 ml of buffer, proteins were eluted with a linear gradient (46 ml) of increasing NaCl concentration (0–0.4 M) (in the same buffer) at a flow rate of 2 ml min^{-1}.

For the (preparative) isolation of the individual isolectins the same FPLC system was equipped with a semipreparative Neobar CS15/4 column (Dynochrom, Lillestrom, Norway). Samples containing up to 20 mg protein (in formate buffer) were loaded on the column. After washing the column with 12 ml of buffer, proteins were eluted with a linear gradient (138 ml) of increasing NaCl concentration (0–0.4 M) (in the same buffer) at a flow rate of 6 ml min^{-1}.

Analytical methods

Protein concentration was determined by the method of Bradford [11] using bovine serum albumin and lectins as standards. Lectins and mannose-binding proteins were analysed by SDS-PAGE using 12.5–25% (w/v) acrylamide gradient gels as described by Laemmli [12].

Gel filtration chromatography of the purified lectins and mannose-binding proteins was performed on a Pharmacia Superose 12 column equilibrated with 20 mM phosphate buffer (pH 7.4) containing 0.2 M NaCl and 0.2 M mannose (to prevent binding of the mannose-binding lectins and proteins on the gel matrix). Samples of 200 µl (containing 1–2 mg per ml protein) were applied to the column and chromatographed at a flow rate of 20 ml h^{-1}. Molecular mass reference markers were the previously described mannose-binding lectins from *Galanthus nivalis* (50 kDa), *Narcissus pseudonarcissus* (36 kDa) [13] and *Allium sativum* (25 kDa) [14], and cytochrome c (12.5 kDa).

Agglutination assays were done using trypsin-treated rabbit erythrocytes as described previously [4].

Antiretrovirus assays

The methodology of the anti-HIV assays has been described previously [15, 16]. Briefly, CEM cells (4.5×10^5 cells ml^{-1}) were suspended in fresh culture medium and infected with HIV-1 and HIV-2 at 100 $CCID_{50}$ (cell culture infective dose-50) per ml cell suspension (1 $CCID_{50}$ being the dose infective for 50% of the cell cultures). Then, 100 µl of the infected cell suspension was transferred to microplate wells, mixed with 100 µl of the appropriate dilutions of the test compounds, and further incubated at 37 °C. After 4 days, syncytium formation was examined in the HIV-infected cell cultures. Antiviral activity was expressed as EC_{50} or effective concentration-50, being the compound concentration required to inhibit HIV-induced syncytium formation by 50%. Cytotoxic activity was expressed as CC_{50} or cytotoxic concentration-50, being the compound concentration required to reduce CEM cell viability by 50%.

Preparation of antibodies against the Listera ovata *agglutinin*

To immunize a rabbit 1 mg of highly purified isolectin 5 of *Listera ovata* dissolved in 1 ml of 0.2 M NaCl was emulsified in 1 ml of Freund's complete adjuvant and injected subcutaneously. Four booster injections were given at 10 day intervals. Ten days after the final injection, blood was removed from an ear marginal vein and allowed to clot overnight at 25 °C. Crude antiserum was obtained by centrifugation at 3000 × **g** for 5 min. Partial purification of this crude antiserum was achieved by repeated precipitation (three times) with ammonium sulphate (40% relative saturation). The final precipitate was dissolved in a volume equal to that of the original crude serum of 25 mM Tris-HCl (pH 8.7) containing 0.1 M NaCl, dialysed against the same buffer and applied on to an anion-exchange column (10 × 2.6 cm) of Q-Fast Flow (Pharmacia, Uppsala, Sweden), equilibrated with the same Tris-buffer. Under these conditions, IgG immunoglobulins do not bind to the column and elute in a large peak just behind the void volume. This IgG fraction was collected and used for further experiments.

Double immunodiffusion assay

Double immunodiffusion assays were performed in Petri dishes (9 cm diameter) filled with 12 ml of 1% (w/v) agarose in 20 mM phosphate buffer (pH 7.4) containing 0.2 M NaCl and 0.1 M mannose (to prevent possible aspecific binding of the lectins to serum proteins). The precipitin lines were visualized after staining with Coomassie blue.

Amino acid sequence analysis

Lectins and other mannose-binding proteins were separated by SDS-PAGE and electroblotted on to a nitrocellulose filter. Individual polypeptides were excised from the blot and sequenced on an Applied Biosystems model 477A protein sequencer interfaced with an Applied Biosystems model 120A on-line analyser.

RNA isolation

Total cellular RNA to be used for cDNA synthesis was prepared from plant material stored at −80 °C essentially as described by Finkelstein and Crouch [17]. Poly(A)-rich RNA was enriched by chromatography on oligodeoxythymidine cellulose (Sigma, St Louis, USA) as described by Siflow *et al.* [18] except that poly(A)-rich RNA was eluted at room temperature. Alternatively small-scale isolation of total RNA was performed according to the protocol described by Wadsworth *et al.* [19].

Construction and screening of cDNA library

cDNA libraries were constructed from poly(A)-rich mRNA isolated from young leaves of *Listera ovata*, and total RNA isolated from young shoots of *Epipactis helleborine* using a cDNA synthesis kit from Pharmacia (Uppsala, Sweden). cDNA fragments were inserted into the *Eco* RI site of the multifunctional phagemid pT_7T_3 18U (Pharmacia, Uppsala, Sweden). The library was propagated in *Escherichia coli* XL1 Blue (Stratagene, La Jolla, USA).

Recombinant lectin clones for *Listera ovata* were screened by colony hybridization using a ^{32}P-random primer labelled cDNA insert from the amaryllis lectin [13]. Hybridization was carried out overnight at 50 °C as described previously [20]. After washing filters were blotted dry, wrapped in Saran Wrap and exposed to Fuji film overnight at −80 °C. In a later stage the random-primer-labelled cDNA clone encoding the *Listera ovata* lectin was used as a probe to screen for more lectin cDNA clones in *Listera ovata* as well as to screen the cDNA libraries constructed of *Epipactis helleborine* RNA for lectin cDNA clones. Colonies that produced positive signals were

selected and rescreened at low density using the same conditions. Plasmids were isolated from purified single colonies on a miniprep scale using the alkaline lysis method as described by Mierendorf and Pfeffer [21] and sequenced by the dideoxy method [22].

Northern Blot

RNA electrophoresis was performed according to Maniatis *et al.* [23]. Approximately 3 μg of poly(A)-rich RNA or 40 μg of total RNA was denatured in glyoxal and Me_2SO and separated in a 1.2% (w/v) agarose gel. Following electrophoresis the RNA was transferred to Immobilon N membranes (Millipore, Bedford, USA) and the blot hybridized using a random-primer-labelled lectin cDNA insert as described previously [5]. An RNA ladder (0.16–1.77 kb) was used as a marker.

Computer analyses

DNA sequences were analysed using programs from PC Gene (Intelligenetics, Mountain View, CA, USA) and Genepro (Riverside Scientific, Seattle, USA).

Results

Evidence for the presence of lectin-related proteins in Listera ovata *and* Epipactis helleborine

Upon cloning and sequencing of the lectin cDNA clones from *Listera ovata* and *Epipactis helleborine*, it became evident that some clones encoded proteins which showed a considerable degree of sequence homology to the lectin cDNA clones but yielded different N-terminal sequences compared with the lectin polypeptides. Since amino acid sequencing of different lectin isoforms revealed no other sequences in our lectin preparations the question arose whether the orchids produce some other lectins or lectin-related proteins which do not copurify with the regular lectin under the conditions used in previous purification schemes. Moreover, this possibility looked worth investigating since experiments with mannose-binding lectins from *Allium* species had demonstrated that some of these lectins are not retained on a column of immobilized mannose except in the presence of relatively high concentrations of ammonium sulphate [14]. Therefore, an extract from leaves of *Listera ovata* was subjected to affinity chromatography on immobilized mannose in the presence of increasing concentrations of ammonium sulphate and the binding of proteins followed. It turned out that besides the previously described lectin several other mannose-binding proteins were retained on the column when chromatography was performed using an increased ammonium sulphate concentration. Consequently a detailed purification scheme was worked out to isolate and separate the different types of mannose-binding proteins from *Listera ovata* and *Epipactis helleborine*.

Isolation and characterization of lectins and lectin-related proteins from Listera ovata

Basically the different mannose-binding proteins from twayblade leaves were isolated and separated by a combination of affinity chromatography (under different conditions) on immobilized mannose and ion exchange chromatography on a Mono-S column. Leaves (200 g) were homogenized in 2 l of 0.2 M NaCl containing 1 g l^{-1} ascorbic acid adjusted to pH 4.0 using a Waring blender. The homogenate was squeezed through cheese cloth and cleared by centrifugation at 3000 × **g** for 10 min. Afterwards the supernatant was decanted, the pH adjusted to 9.0 using 1 M NaOH and recentrifuged (3000 × **g** for 10 min). The resulting supernatant was adjusted to pH 7.0 with 1 M acetic acid and solid ammonium sulphate was added to a final concentration of 1 M. After standing overnight in the cold room at 4 °C the extract was degassed under vacuum, centrifuged at 20000 × **g** for 15 min and the supernatant filtered through filter paper (Whatmann 3MM). The cleared extract was loaded on to a column of mannose-Sepharose-4B (2.6 × 10 cm; 50 ml bed volume), equilibrated with 1 M ammonium sulphate. After passing the extract the column was washed with 1 M ammonium sulphate until the A_{280} was less than 0.01. Under these conditions all the agglutinating activity present in the extract was quantitatively retained and firmly bound to the column even upon prolonged washing of the column. It is worth mentioning here that when the affinity chromatography is performed in a buffer without 1 M ammonium sulphate (as has been described in earlier reports) the lectin progressively elutes upon prolonged washing of the column. A first crude separation of the different isolectins was achieved by eluting the column using a linear gradient of decreasing ammonium sulphate concentration (from 1 M to water). As shown in Fig. 1A, a gradient elution of the lectins yields a typical pattern in which two broad peaks can be distinguished. Since the agglutination activity of the different fractions coincides with the measured A_{280} values, it is evident that all fractions contain lectin(s). Analysis of the proteins in the different fractions by SDS-PAGE further confirmed that they exclusively contain lectin polypeptides (results not shown). To find out about the isolectin composition of the fractions desorbed from the affinity column small portions of a number of them were dialysed against an Na-formate buffer (25 mM formic acid adjusted to pH 3.8 with NaOH) and analysed by ion exchange chromatography on a Mono-S column. As shown in Fig. 2, the isolectin pattern of the fractions clearly changes as the elution from the mannose column proceeds, illustrating the apparent differences of the individual isolectins in their affinity for the immobilized sugar.

To isolate the different isolectins, the fractions eluted from the mannose column were pooled as indicated in the legend to Fig. 1, dialysed against water (to remove the

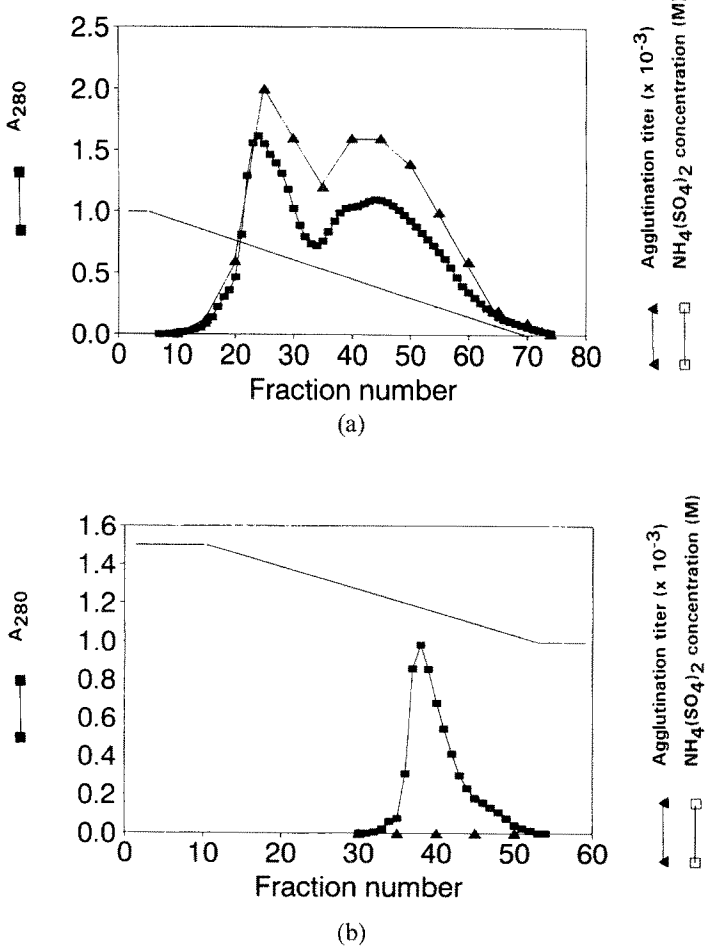

Figure 1. Affinity chromatography of the mannose-binding protein from *Listera ovata* on immobilized mannose. (A) Elution of the mannose-binding proteins retained in 1 M ammonium sulphate with a gradient of decreasing ammonium sulphate concentration. The mannose-binding proteins retained on the column (2.6 cm × 10 cm; 50 ml bed volume) in 1 M ammonium sulphate were eluted with a linear gradient (700 ml) of decreasing ammonium sulphate concentration (from 1 to 0 M). Fractions of 10 ml each were collected and their A_{280} measured. Aliquots of every fifth fraction were withdrawn for the determination of their agglutination titre and ammonium sulphate concentration (which was measured by a refractometer). Small portions (1 ml) of fractions 25, 35, 45 and 55 were removed and prepared for analytical ion exchange chromatography (cf. Fig. 2). Fractions 11–32, 33–41, 42–50 and 51–72 were pooled and prepared for semi-preparative ion exchange chromatography (cf. Fig. 3). (B) Elution of the mannose-binding proteins retained in 1.5 M ammonium sulphate with a gradient of decreasing ammonium sulphate concentration. The mannose-binding proteins which were not retained in 1 M ammonium sulphate but bound to the column (1.6 cm × 5 cm; 10 ml bed volume) in 1.5 M ammonium sulphate were eluted with a linear gradient (140 ml) of decreasing ammonium sulphate concentration (from 1.5 to 1 M). Fractions of 2.5 ml each were collected and their A_{280} measured. Aliquots of every fifth fraction were withdrawn for the determination of their agglutination titre and ammonium sulphate concentration.

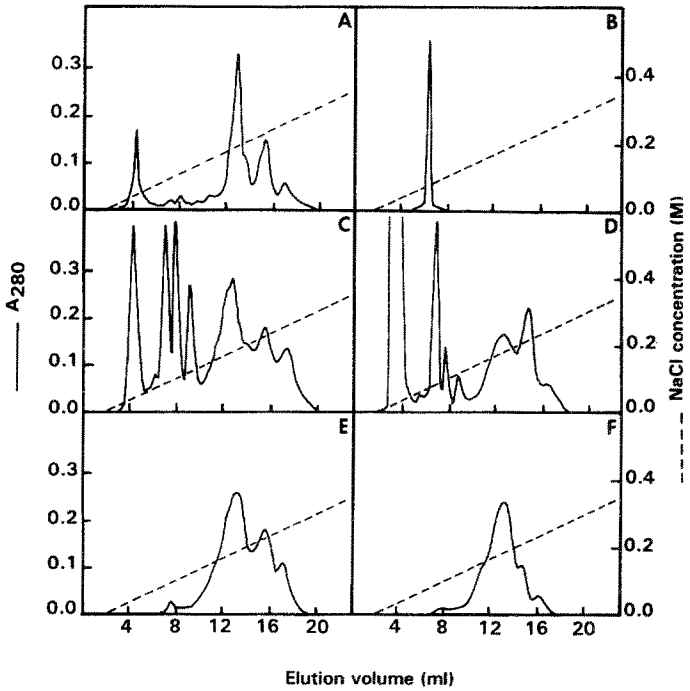

Figure 2. Ion exchange chromatography of the mannose-binding proteins from *Listera ovata* on a Mono-S column. Elution patterns of total lectin (mixture of equal volumes of fractions 11–75 of Fig. 1A) and LOMBP (mixture of equal volumes of fractions 31–50 of Fig. 1B) are shown in A and B, respectively. Elution patterns of samples prepared from fractions 25, 35, 45 and 55 of Fig. 1A are given in C, D, E and F, respectively.

ammonium sulphate) and lyophilized. The freeze dried lectins were dissolved in 50 ml of Na-formate buffer and aliquots of 10–20 mg loaded on to a semi preparative FPLC ion exchange column. The column was eluted using a linear gradient of increasing NaCl concentration in an Na-formate buffer and peak fractions collected (cf. example shown in Fig. 3). Peak fractions of different runs were pooled and dialysed against water (for subsequent lyophilization) or an appropriate buffer for further analysis. Using the procedure described here, seven individual isolectins could be isolated from *Listera ovata* leaves.

Besides the agglutinins which are retained on the mannose column in 1 M ammonium sulphate, extracts of *Listera* leaves also contain a non-agglutinating mannose-binding protein (further referred to as *Listera ovata* mannose-binding protein or LOMBP). To isolate this protein, the fraction which ran through the mannose column during the affinity chromatography in 1 M ammonium sulphate was brought to 1.5 M ammonium sulphate by adding the solid salt, centrifuged, degassed and filtered as described above and applied to the same mannose column equilibrated with 1.5 M ammonium sulphate. After passing the extract, the column was washed with 1.5 M ammonium sulphate until the A_{280} was less than 0.01 and the bound protein eluted using a linear gradient of decreasing ammonium sulphate concentration (from 1.5 M to 1 M). As

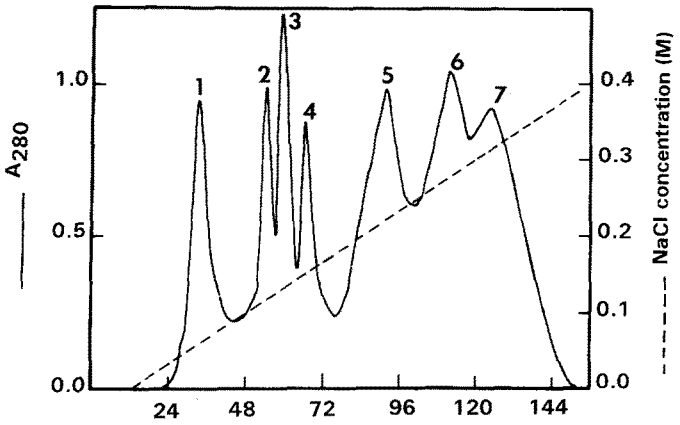

Figure 3. Ion exchange chromatography of the agglutinating isolectins of *Listera ovata* on a Neobar CS15/4 column. Twenty mg of protein prepared from the pooled fractions 11–32 of Fig. 1A was loaded on the column. The individual peaks, which are numbered in the order of their appearance, correspond to the seven different isolectins referred to throughout the paper.

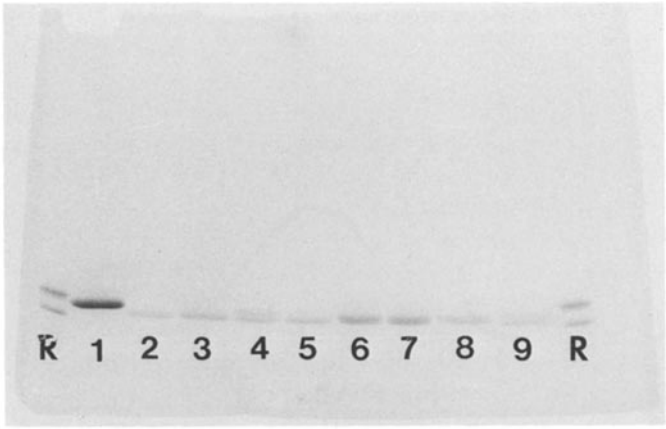

Figure 4. SDS-PAGE of purified mannose-binding proteins from *Listera ovata*. Approximately 25 μg of each protein was loaded on to the gel. The mannose-binding proteins were loaded as follows: lane 1, LOMBP; lane 2, isolectin 1; lane 3, isolectin 2; lane 4, isolectin 3; lane 5, isolectin 4; lane 6, isolectin 5; lane 7, isolectin 6; lane 8, isolectin 7; lane 9, isolectin 3. Molecular mass reference proteins are shown in lane R (myoglobin, 16.9 kDa; myoglobin I and II, 14.4 kDa; myoglobin I, 8.2 kDa).

shown in Fig. 1B, the mannose-binding protein eluted in a symmetrical peak at a concentration of about 1.2 M ammonium sulphate. None of the fractions exhibited any agglutination activity. Fractions containing the protein were pooled, dialysed and lyophilized as described above for the lectins. In contrast to the true lectins, the mannose-binding protein elutes as a single symmetrical peak upon ion exchange chromatography on the Mono-S column (Fig. 2).

The total yield of the lectins and LOMBP was 75 and 3 mg, respectively, per 100 g of leaves. Evidently, LOMBP is present in much lower concentrations than the lectins as it represents only 4% of the total amount of mannose-binding proteins in *Listera ovata* leaves.

To determine the molecular structure of the different *Listera* isolectins and LOMBP, purified protein fractions were analysed by SDS-PAGE and gel filtration. The apparent molecular mass of all seven isolectins determined by gel filtration is 25 kDa whereas that of LOMBP is only 14 kDa. Since SDS-PAGE of the LOMBP yielded a single polypeptide of 14 kDa, the native protein must be a monomer (Fig. 4). Gel electrophoresis of the different isolectins yielded a rather complex overall pattern of lectin polypeptides. Indeed, as shown in Fig. 4 isolectins 1, 2 and 4 yielded a single polypeptide band of 12 kDa upon SDS-PAGE and hence must be considered as homodimeric proteins composed of two identical subunits. Isolectin 3, however, clearly showed two different polypeptides of 12 and 13 kDa, respectively, which demonstrates that it is a heterodimer composed of two different subunits. Finally, isolectins 5, 6 and 7 also yielded two protein bands upon SDS-PAGE (of 11.5 and 12 kDa) suggesting that they are

Listera ovata	
isolectin 1	DSQLG AGERL NSGE
isolectin 2	DNQLG AGERL NSGE
isolectin 3	SNTLG GGERL NSGEG LTEG (upper band)
	DNQLG AGERL NSGEG LTQG (lower band)
isolectin 4	DNQLG AGERL NGGES L
isolectin 5	LNHLL GGERL NTGQS LTDGG
isolectin 6	LNHLL GGERL NTGQS LTDGG
isolectin 7	LNHLL GGERL NTGQS LTDGG
LOMBP	SDRLN AGQSL GTGG
Epipactis helleborine	
lectin	DNHLL TGQRL TTGXF LLEGG
EHMBP	ADRLN AGQSL GTGGS

Figure 5. N-terminal sequences of the lectins and mannose-binding proteins from *Listera ovata* and *Epipactis helleborine*. No amino acid could be identified at position 14 of the *Epipactis* lectin.

heterodimers built up of slightly different subunits. Further identification of the subunits composing the different isolectins was done by N-terminal amino acid sequencing of the different polypeptides resolved by SDS-PAGE. As shown in Fig. 5, the homodimeric isolectins 1, 2 and 4 all contain polypeptides with the same N-terminal sequence. The smaller polypeptide of isolectin 3 also yielded the same sequence, which definitely differs from that of the larger subunit of the same isolectin. In contrast to isolectin 3, all

polypeptides composing isolectins 5, 6 and 7 yielded an identical N-terminal sequence which is well distinguishable from the N-terminal sequence of the other isolectins. Finally, the sequence of LOMBP showed little homology to that of the isolectins indicating that the dimeric isolectins are more closely related than the lectins and the monomer.

Summarizing one can conclude that *Listera* leaves contain four types of mannose-binding proteins which can be distinguished on the basis of their molecular structure and N-terminal amino acid sequences. Besides the monomeric LOMBP, three types of dimers are found. In contrast to the first type, which are homodimers built up of identical 12 kDa subunits with identical N-terminal sequences (namely isolectins 1, 2 and 4), the second and third types are heterodimers. However, whereas some of these heterodimers are composed of two subunits which have the same N-terminal sequence and differ only slightly in size (such as isolectins 5, 6 and 7), at least one isolectin (namely isolectin 3) is built up of two subunits which have a different N-terminal sequence and size.

Isolation and characterization of lectins and lectin-related proteins from Epipactis helleborine

The lectin and the non-agglutinating mannose-binding protein from *Epipactis helleborine* leaves were isolated using the same purification procedure as described above for *Listera ovata*. As shown in Fig. 6A the *Epipactis* lectin eluted as a single symmetrical peak from the mannose column upon applying a gradient of decreasing ammonium sulphate concentration. Compared with the *Listera* lectin, this elution pattern is much less complex. Moreover, it is evident that the desorption of the *Epipactis* lectin starts only when the concentration of ammonium sulphate is almost zero indicating that its binding to the affinity column is stronger than that of the *Listera* lectin. Ion exchange chromatography of the different fractions of the *Epipactis* lectin obtained from the affinity column yielded the same pattern (results not shown), indicating that its isolectin composition is far less complex than that of the *Listera* lectin. Moreover, analysis of the lectin fractions by SDS-PAGE revealed only a single polypeptide.

Similar to the monomeric mannose-binding protein from *Listera* the non-agglutinating mannose-binding protein from *Epipactis* (further referred to as *Epipactis helleborine* mannose-binding protein or EHMBP) also eluted as a single symmetrical peak upon elution from the mannose column with a gradient of decreasing ammonium sulphate concentration. Moreover, EHMBP eluted from the mannose column at the same ammonium sulphate concentration as observed for the elution of LOMBP, namely 1.2 M (Fig. 6B).

The total yield of *Epipactis* lectin and EHMBP was 43 and 34 mg, respectively per 100 g of fresh leaves. Although the total yield of mannose-binding proteins was almost the same as that in leaves of twayblade, the relative content of

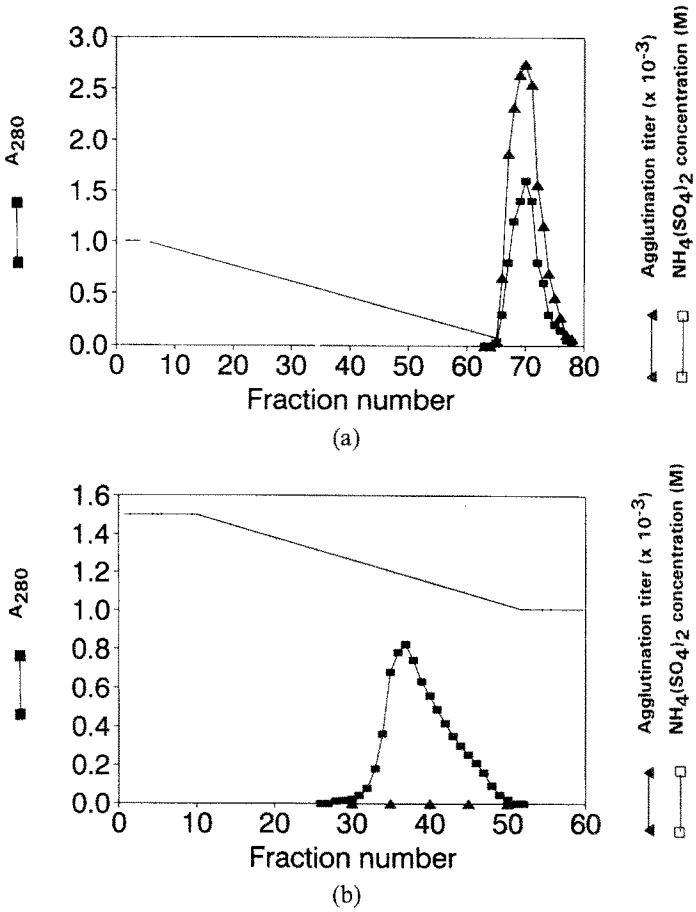

Figure 6. Affinity chromatography of the mannose-binding proteins from *Epipactis helleborine* on immobilized mannose. (A) Elution of the mannose-binding proteins retained in 1 M ammonium sulphate with a gradient of decreasing ammonium sulphate concentration. The mannose-binding proteins retained on the column (2.6 cm × 10 cm; 50 ml bed volume) in 1 M ammonium sulphate were eluted with a linear gradient (700 ml) of decreasing ammonium sulphate concentration (from 1 to 0 M). Fractions of 10 ml each were collected and their A_{280} and agglutination activity determined. Aliquots of every fifth fraction were withdrawn or the determination of ammonium sulphate concentration (which was measured by a refractometer). (B) Elution of the mannose-binding proteins retained in 1.5 M ammonium sulphate with a gradient of decreasing ammonium sulphate concentration. The mannose-binding proteins which were not retained in 1 M ammonium sulphate but bound to the column (2.6 cm × 10 cm; 50 ml bed volume) in 1.5 M ammonium sulphate were eluted with a linear gradient (500 ml) of decreasing ammonium sulphate concentration (from 1.5 to 1 M). Fractions of 10 ml each were collected and their A_{280} measured. Aliquots of every fifth fraction were withdrawn for the determination of their agglutination titre and ammonium sulphate concentration.

the non-agglutinating protein (44%) was much higher than in *Listera* where LOMBP represents only 4% of the total content of mannose-binding proteins.

Upon ion exchange chromatography on a Mono-S column both the *Epipactis helleborine* lectin and EHMBP

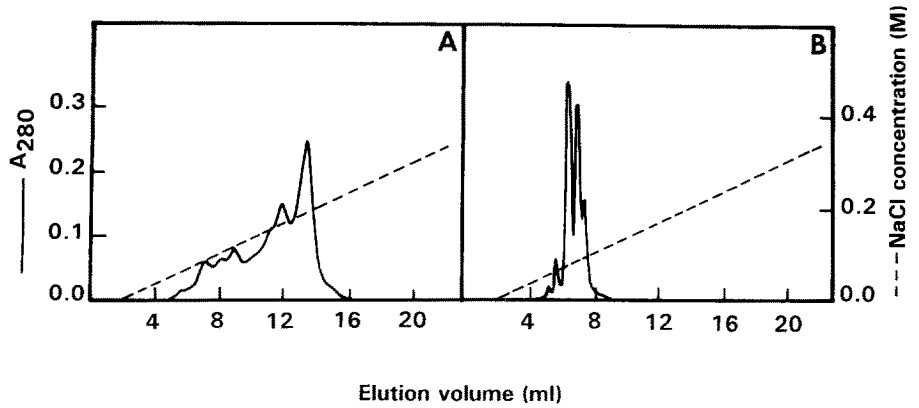

Figure 7. Ion exchange chromatography of the mannose-binding proteins from *Epipactis helleborine* on a Mono-S column. Elution patterns of the lectin (fraction 70 of Fig. 6A) and EHMBP (fraction 38 of Fig. 6B) are shown in A and B, respectively.

yielded a more or less complex elution pattern (Fig. 7). Several of the most prominent peaks were further purified on a semi-preparative Neobar CS column and analysed by SDS-PAGE. From this it became evident that all isolectins from *Epipactis helleborine* yielded a single 12 kDa and all EHMBP-isoforms a single 14 kDa polypeptide (Fig. 8). Moreover, N-terminal sequencing of the polypeptides from the different fractions yielded identical sequences for all isolectins and for all EHMBP isoforms (Fig. 5). It should be emphasized, however, that the N-terminal sequences of the lectins and the non-agglutinating mannose-binding protein show little homology.

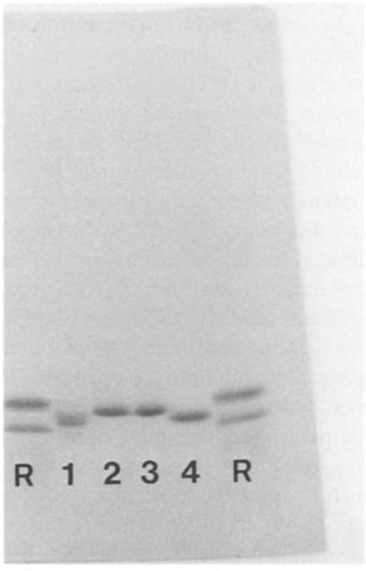

Figure 8. SDS-PAGE of purified mannose-binding proteins from *Listera ovata* and *Epipactis helleborine*. Approximately 25 μg of each protein was loaded on the gel. The mannose-binding proteins were loaded as follows: lane 1, *Listera ovata* isolectin 5; lane 2, LOMBP; lane 3, EHMBP; lane 4, *Epipactis helleborine* lectin. Molecular mass reference proteins are shown in lane R (myoglobin, 16.9 kDa; myoglobin I and II, 14.4 kDa; myoglobin I, 8.2 kDA).

To determine the molecular structure of the *Epipactis* lectin and EHMBP, purified fractions were further analysed by gel filtration. Similar to *Listera* the apparent molecular mass of the lectin is 25 kDa whereas that of EHMBP is only 14 kDa. Since, as already mentioned, both proteins yield a single polypeptide band upon SDS-PAGE, it can be concluded that the native EHMBP is a monomer whereas the lectin is a homodimer of two identical subunits.

Biological activities of the mannose-binding proteins from Listera ovata *and* Epipactis helleborine

Since the agglutinating and non-agglutinating mannose-binding proteins from *Listera ovata* and *Epipactis helleborine* clearly differ with respect to their binding properties to immobilized mannose and molecular structure, some of their biological activities were compared in some detail. As shown in Table 1, all the dimeric mannose-binding proteins clearly exhibit agglutination activity when tested with

Table 1. Agglutinating activity of the mannose-binding proteins from *Listera ovata* and *Epipactis helleborine* in assays with trypsin treated rabbit erythrocytes.

Mannose-binding protein	*Minimal concentration required for agglutination* ($\mu g/ml^{-1}$)
Listera ovata	
Isolectin 1	0.375
Isolectin 2	0.750
Isolectin 3	1.500
Isolectin 4	0.750
Isolectin 5	0.500
Isolectin 6	0.500
Isolectin 7	0.500
LOMBP	>5000[a]
Epipactis helleborine	2.000
EHMBP	>5000[a]

[a] No agglutination was observed.

Table 2. Antiretroviral activity and cytotoxic properties of the mannose-binding proteins from *Listera ovata* and *Epipactis helleborine.*

Compound	EC_{50}^{a} ($\mu g\ ml^{-1}$)		CC_{50}^{b} ($\mu g\ ml^{-1}$)
	HIV-1	*HIV-2*	
Listera ovata			
Isolectin 1	0.87 ± 0.30	0.87 ± 0.30	$\geqslant 40.5$
Isolectin 2	0.51 ± 0.35	0.25 ± 0.13	> 50
Isolectin 3	0.75 ± 0.12	0.20 ± 0.05	> 50
Isolectin 4	0.71 ± 0.18	0.59 ± 0.16	> 50
Isolectin 5	0.098 ± 0.032	0.098 ± 0.032	> 50
Isolectin 6	0.21 ± 0.032	0.20 ± 0.06	> 50
Isolectin 7	0.94 ± 0.64	1.13 ± 0.89	> 50
LOMBP	$\geqslant 22$	$\geqslant 22$	> 50
Epipactis helleborine			
Lectin	0.046 ± 0.0004	0.046 ± 0.0004	$\geqslant 41$
EHMBP	> 50	> 50	> 50

[a] 50% effective concentration of compound concentration required to protect CEM cells against the cytopathicity of HIV by 50%.
[b] 50% cytotoxic concentration or compound concentration required to reduce CEM cell viability by 50%.

rabbit erythrocytes. Although there are some differences in specific agglutination activity between the individual isolectins of *Listera* in the sense that, for instance, isolectin 3 is four-fold less active than isolectin 1, the observed values are quite similar. No measurable differences were observed between the specific agglutination activities of the isolectins of *Epipactis.* However, compared with the *Listera* lectins their activity is about four-fold lower. In contrast to the dimeric lectins, neither LOMBP nor EHMBP exhibited any agglutination activity even when their concentration surpassed 5 mg ml^{-1}. It appears, therefore, that because of their monomeric nature both mannose-binding proteins are completely devoid of agglutination activity.

Since it was already known that some orchid lectins are potent inhibitors of human and animal retroviruses *in vitro* [7, 8] the anti-HIV activity of the different mannose-binding proteins from *Listera ovata* and *Epipactis helleborine* was evaluated and compared. As shown in Table 2, all dimeric lectins from both species show strong antiretroviral activity. All isolectins of *Listera ovata* exhibited a comparable antiretroviral activity except for isolectins 5 and 6 which were about five- to eight- and two- to four-fold more active against HIV-1, and two- to eight- and one- to four-fold more active against HIV-2, respectively. The lectin from *Epipactis* apparently is a more potent agent against HIV than any of the *Listera* isolectins since its EC50 value is two- to 20-fold more pronounced. In contrast to the true lectins, neither LOMBP nor EHMBP showed any antiretroviral effect in this test system, indicating that both monomeric mannose-binding proteins behave quite differently with respect to their biological activities.

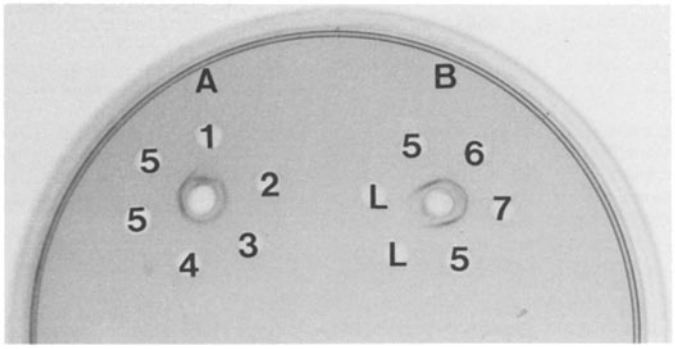

Figure 9. Double immunodiffusion analysis of purified mannose-binding proteins from *Listera ovata.* Antisera against the *Listera ovata* isolectin 5 were challenged with 5 µg of purified isolectin 1 (1), isolectin 2 (2), isolectin 3 (3), isolectin 4 (4), isolectin 5 (5), isolectin 6 (6), isolectin 7 (7) and LOMBP (L).

Serological relationships between the mannose-binding proteins from Listera ovata *and* Epipactis helleborine

To test the possible serological relationship between the different mannose-binding proteins from *Listera ovata*, all isolectins and LOMBP were challenged with a partially purified antiserum raised against the major (agglutinating) isolectin (No. 5) in an immunodiffusion assay. As shown in Fig. 9 all the lectins reacted very well with the antiserum in contrast to LOMBP which did not form a precipitin line. A closer examination of the precipitin lines indicates that isolectins 5, 6 and 7 react identically. Isolectins 1, 2, 3 and 4 also yielded precipitin lines which completely fuse suggesting that they are serologically identical. There are, however, some serological differences between the two groups of isolectins since there is clear spur formation at the boundary between the precipitin lines of, for instance, isolectins 1 and 5. When the *Epipactis helleborine* mannose-binding proteins were challenged with the same antiserum, a weak cross-reaction was observed between the *Epipactis* lectin and isolectin 5 of *Listera ovata.* Like LOMBP, the EHMBP did not react in the immunodiffusion assay (results not shown).

Isolation and characterization of cDNA clones encoding different mannose-binding proteins from Listera ovata *and* Epipactis helleborine

As already mentioned above, a search for cDNA clones encoding the lectins from *Listera ovata* and *Epipactis helleborine* resulted in the isolation of some cDNA clones which did not match the N-terminal sequences of the lectin polypeptides. However, once it became evident that both orchids contain additional mannose-binding proteins which are undoubtedly related to the originally described lectins, efforts were undertaken to assign a cDNA to each type

```
LECLOA2:         MASSIMCILLCAASLALLLAIPSSG▼SNTLGGGERLNSGESLT  42
LECLOA3:         -----K-------------------- ------------QY--  42
LECLOA1:         ---------------T--------- L-H-L------T-Q---  42
LECEPA :         ------TL-------T--------- D-H-LT-Q--TT-SF--  42

LOMBP1 :     MTVSFLSMSTILLVSTMALMSLLATPVSA SDRLNAGQSLGTGGSLA  46
LOMBP2 :     -A---------------------------- -----------------  46
EHMBP1 :      MA----LR----L--A------------ A-----------------  45
EHMBP2 :      MA-L--LR----L-AA--I----M---- A------K---------  45
EHMBP3 :      MA-L--LR----L-AA-----------G A-----------------  45

LECLOA2: EGACVFIMQEDCNLVLYESSRPTWASGSYHQGSGCYVTLQNDGNLVVYNN  92
LECLOA3: ----------------F--N------RTRWRAT-------------I-D-  92
LECLOA1: D-GNA----------------------T--R-----LAM---------D-  92
LECEPA : EGGFT----S--K----DLN--I----T-GK-T--FLSM-S-------DV  92

LOMBP1 : LGGYIFIIQGDCNLVLYDNNRAVWASGTNGRGSNCILSMQRDGNLVIYSS  96
LOMBP2 : --S-------------------------------------------------  96
EHMBP1 : ------K--A----------------------ATD---------------G  95
EHMBP2 : ----L-T--R----------------------A-----------------G  95
EHMBP3 : ----L-T--R----------------------A-----------------G  95

LECLOA2: RNRAVWASDTVRENIGNVILILQKDHNVVLY...SLPIWATGTNRYGS.G 138
LECLOA3: ------------------------------...--------------.- 138
LECLOA1: ----I---Q-D-Q-V-T--------------...TN---------F--P- 139
LECEPA : --I-I---N-A-N-.--YL------R---I-...-Q-----T--IR--A- 138

LOMBP1 : .GRAIWASNTNRQN.GNYYLILQRDRNVVIYDNSNNAIWATGTN.VGNAA 143
LOMBP2 : .--V----------.-----------------------------.----- 143
EHMBP1 : .S-V---------S.-----------------------------.----- 142
EHMBP2 : .S------------.------------C----------------.I---- 142
EHMBP3 : .S------------.------------C----------------.I---- 142

LECLOA2: VVVSPARNGTVGISGAEQNKVSEIVRIVDVTGSV  172
LECLOA3: -------------------------G--------  172
LECLOA1: --ITA----------------------G--------  173
LECEPA : --IA--L-----V----------MTN-LE--AN-  172

LOMBP1 : IAVIPHNNGTAAASGAKQNKVRELNP  169
LOMBP2 : --------------------------  169
EHMBP1 : ----------------Q-------Y-  168
EHMBP2 : ------K---------Q-------Y-  168
EHMBP3 : ------K---------Q----H--Y-  168
```

Figure 10. Comparison of the deduced amino acid sequences of cDNA clones encoding the lectins and mannose-binding proteins from *Listera ovata* and *Epipactis helleborine*. (–) denotes sequence similarity of LECLOA2 and LOMBP1. Dots indicate gaps introduced to obtain maximal homology. The processing site for the cleavage of the signal peptide is indicated by the arrow. The putative glycosylation site is underlined.

of mannose-binding protein. The deduced amino acid sequences of different cDNA clones encoding mannose-binding proteins from *Listera* and *Epipactis* including the previously reported lectin cDNA clones (LECLOA1 and LECEPA) [5] are shown in Fig. 10.

Similar to the previously reported lectin cDNA clone LECLOA1, cDNA clones LECLOA2 and LECLOA3 encode lectin polypeptides with a calculated molecular mass of approximately 18.7 Da. Cleavage of a signal peptide between residues 25 and 26 of LECLOA2 and LECLOA3 will result in a 147 amino acid lectin polypeptide of about 16 kDa with an N-terminal sequence corresponding to the sequence of the upper band of isolectin 3 whereas processing of the signal peptide in LECLOA1 yields a lectin polypeptide with an N-terminal sequence identical to that of isolectins 5, 6 and 7 of *Listera* (Fig. 5). Since the molecular mass of the lectin polypeptides after cleavage of the signal peptide is approximately 3 kDa larger than the molecular mass determined for the mature lectin polypeptides on SDS-PAGE, a second post-translational modification is most likely to occur at the C-terminus of the lectin precursor, as previously reported for the mannose-binding lectins from Amaryllidaceae and *Allium* species [14, 20]. Until now no cDNA clone encoding the isolectins 1, 2 and 4 could be isolated.

The cDNA clones LOMBP1 and LOMBP2 encode the non-agglutinating mannose-binding protein from *Listera ovata* (Fig. 10). Both cDNA clones encode a polypeptide of 169 amino acids with a calculated molecular mass of approximately 18 kDa which after cleavage of the signal peptide between residues 29 and 30 is converted into a polypeptide of 14.9 kDa which is in good agreement with the molecular mass of the mannose-binding protein determined by SDS-PAGE. Similar to the lectin cDNA clones LECLOA1 and LECLOA2 the clones LOMBP1 and LOMBP2 also contain one putative glycosylation site in their sequence.

In the case of *Epipactis helleborine* three different cDNA clones encoding the non-agglutinating mannose-binding protein were isolated. All clones contain an open reading frame of 168 amino acids encoding a polypeptide of about 17.8 kDa with one potential glycosylation site. Using the rules for protein processing of von Heijne [24] a possible cleavage site for the processing of the signal peptide was identified between residues 28 and 29 which is in good agreement with the N-terminal amino acid sequence of the protein EHMBP. Cleavage of the signal peptide at this position will result in a polypeptide of approximately 15 kDa. The occurrence of several cDNA clones encoding EHMBP which differ in their deduced amino acid sequences confirmed the complex elution pattern of EHMBP upon ion exchange chromatography on a Mono-S column (Fig. 7).

Northern blot analysis

Northern blot analysis of total RNA isolated from young leaves of *Listera ovata* and young shoots of *Epipactis helleborine* revealed a single band of approximately 800 nucleotides after hybridization of the blot with a random primer labelled cDNA insert from LOMBP. These results were identical after probing the blot with different lectin clones from *Listera ovata* or *Epipactis helleborine*, or the cDNA clone encoding the monomeric mannose-binding protein from broad-leaved helleborine.

To study the expression of the different mannose-binding proteins in *Listera* and *Epipactis* total RNA was isolated

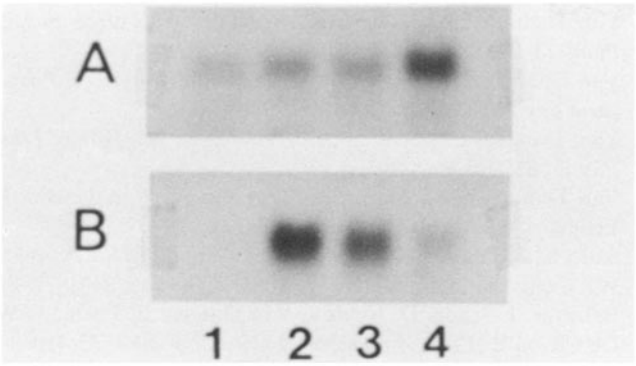

Figure 11. Northern blot analysis of total RNA isolated from different tissues of *Listera ovata*. RNA isolated from rhizomes (1), leaves (2), stems (3) and flowers (4) was hybridized to the lectin (A) and the monomeric mannose-binding protein (B).

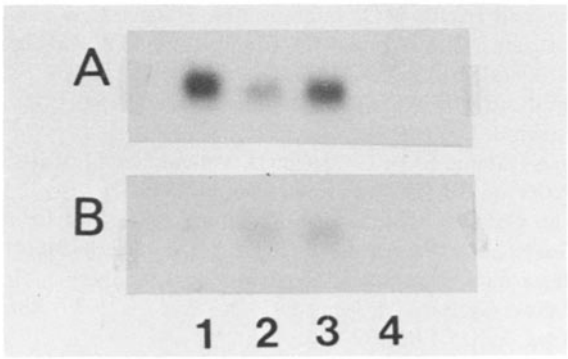

Figure 12. Northern blot analysis of total RNA isolated from different tissues of *Epipactis helleborine*. RNA isolated from flowers (1), stems (2), leaves (3) and rhizomes (4) was hybridized to the lectin (A) and the monomeric mannose-binding protein (B).

from various tissues of these plants and subjected to Northern blot analysis. As shown in Fig. 11 the highest concentrations of mRNA encoding the *Listera* lectins are found in the flower. However, the lectin mRNAs are also present in leaves, stems and rhizomes though in lower concentrations. Identical results were obtained when the blot was hybridized with the labelled lectin clones LECLOA1, LECLOA2 and LECLOA3. The non-agglutinating mannose-binding protein from *Listera* is highly expressed in the leaf and is also detected in the stem and the flower tissue. In the case of *Epipactis helleborine* high lectin concentrations were found in the flower and the leaf whereas low levels of lectin mRNA were found in the stem. The non-agglutinating mannose-binding protein EHMBP is expressed in the leaves and the stems of *Epipactis* (Fig. 12).

Sequence similarity between cDNA clones encoding different mannose-binding proteins in Listera *and* Epipactis

A detailed comparison of the deduced amino acid sequences of the cDNA clones encoding different mannose-binding proteins from *Listera* and *Epipactis* reveals a high degree of sequence similarity between the lectins on the one hand and the non-agglutinating proteins on the other hand. Indeed, whereas the sequence similarity for the total coding region ranged from 64 to 88% for the lectins and 82 to 99% for the non-agglutinating mannose-binding proteins from *Listera* and *Epipactis*, the overall sequence similarity between the lectins and the non-agglutinating mannose-binding proteins is approximately 50%. However, despite the obvious differences between the sequences of both classes of mannose-binding proteins alignment of the sequences reveals that some parts of the sequences are highly conserved. For instance, the amino acid sequences 53–60, 98–123, 127–133 and 152–159 of LECLOA2 are highly conserved regions which occur in all the sequences of the lectins and the monomeric mannose-binding proteins from *Listera* and *Epipactis*. Furthermore, the position of the cysteine residues is identical in all orchid lectins and monomeric mannose-binding proteins except for EHMBP2 and EHMBP3 which contain an additional cysteine at position 121.

Discussion

This paper deals with the isolation and characterization of different mannose-binding proteins from the orchid species *Listera ovata* and *Epipactis helleborine*. Judging from the biochemical properties and biological activities of the different proteins they can be divided into two distinct classes. Whereas the first class comprises the dimeric lectins which clearly show agglutination activity with rabbit erythrocytes and pronounced inhibitory activity against HIV, the second class represents the monomeric, non-agglutinating mannose-binding proteins that are devoid of marked anti-HIV activity. In spite of the obvious differences between both classes of mannose-binding proteins in their molecular structure and biological activities, the striking overall homologies between their amino acid sequences and especially the occurrence of highly conserved domains indicate that their genes are closely related. Moreover, a detailed comparison of the nucleotide and deduced amino acid sequences of the different mannose-binding proteins from the Orchidaceae species described here to the previously reported mannose-binding lectins from Amaryllidaceae and *Allium* species reveals that these proteins all belong to the same superfamily of mannose-binding proteins. However, the Orchidaceae species (or at least part of them) are unique in the sense that they use closely related genes to direct the synthesis of monomeric and dimeric mannose-binding proteins. It should be mentioned that, in spite of intensive efforts, neither in snowdrop (*Galanthus nivalis*) nor garlic (*Allium sativum*) could mannose-binding monomeric proteins or their genes be detected (E. Van Damme, unpublished results).

The discovery of the monomeric, non-agglutinating mannose-binding proteins from *Listera ovata* and *Epipactis helleborine* once again questions the definition of the term

lectin. Although it is evident that they are unable to agglutinate erythrocytes (or other cells) because of their monomeric nature, the carbohydrate binding activity of LOMBP and EHMBP is specific and reversible, which implies that according to the definition of Barondes [25] they should be considered as lectins. However, since their biological activities clearly differ from these of the true (agglutinating) lectins from the same species, both types of mannose-binding proteins should be distinguished from each other in one way or another. In addition, the discovery of LOMBP and EHMBP highlights another important problem in lectinology. Since these proteins are totally devoid of agglutinating activity they were overlooked in all searches based on hemagglutination assays and, as already mentioned, their identification was the fortunate result of the isolation of cDNA clones which were related to but not identical with these of the true lectins. It is evident, therefore, that a negative result of an agglutination assay with an extract from a given plant species does not preclude the presence of a carbohydrate-binding protein. On the contrary, the monomeric mannose-binding proteins from orchids are possibly just one example of a large group of yet undiscovered non-agglutinating carbohydrate-binding plant proteins.

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