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The Major Tuber Storage Protein of Araceae Species Is a Lectin[1]

Characterization and Molecular Cloning of the Lectin from *Arum maculatum* L.

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A new lectin was purified from tubers of *Arum maculatum* L. by affinity chromatography on immobilized asialofetuin. Although this lectin is also retained on mannose-Sepharose 4B, under the appropriate conditions free mannose is a poor inhibitor of its agglutination activity. Pure preparations of the *Arum* lectin apparently yielded a single polypeptide band of approximately 12 kD upon sodium dodecyl sulfate-polyacrylamide gel electrophoresis. However, N-terminal sequencing of the purified protein combined with molecular cloning of the lectin have shown that the lectin is composed of two different 12-kD lectin subunits that are synthesized on a single large precursor translated from an mRNA of approximately 1400 nucleotides. Lectins with similar properties were also isolated from the Araceae species *Colocasia esculenta* (L.) Schott, *Xanthosoma sagittifolium* (L.) Schott, and *Dieffenbachia sequina* Schott. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and gel filtration of the different Araceae lectins have shown that they are tetrameric proteins composed of lectin subunits of 12 to 14 kD. Interestingly, these lectins are the most prominent proteins in the tuber tissue. Evidence is presented that a previously described major storage protein of *Colocasia* tubers corresponds to the lectin.

Plant lectins are a very heterogeneous group of (glyco)-proteins classified together on the basis of a single common property, namely their ability to recognize and specifically bind carbohydrate ligands (Goldstein and Poretz, 1986). Although in the past most interest in plant lectins has focused on lectins in dry seeds, especially from leguminous species, evidence has accumulated now that lectins occur also in vegetative tissues and are widespread in a large number of plant families belonging to all major taxonomic groupings.

Within the class of monocotyledonous plants lectin research has been restricted to Gramineae species (Stinissen and Peumans, 1985) for a long time. However, during the last 5 years several new lectins with interesting properties have been isolated from species belonging to the families Liliaceae (Cammue et al., 1986; Oda and Minami, 1986; Peumans et al., 1986), Orchidaceae (Van Damme et al., 1987), Amaryllidaceae (Van Damme et al., 1988), and Alliaceae (Van Damme et al., 1991a). Interestingly, these lectins strongly differ from all known dicotyledonous lectins and, in addition, exhibit unique properties especially with respect to their carbohydrate-binding specificity. It has been observed, for instance, that numerous Amaryllidaceae, Alliaceae, and Orchidaceae species contain large amounts of lectins that recognize exclusively Man (and in that respect differ basically from the Man/Glc/*N*-acetylglucosamine-binding Con A and related legume lectins) (Shibuya et al., 1988b; Kaku et al., 1990, 1992). By virtue of their unique carbohydrate-binding properties the Amaryllidaceae, Alliaceae, and Orchidaceae lectins have become very useful tools in biomedical and glycoconjugate research (Shibuya et al., 1988a; Haselbeck et al., 1990) and are also intensively used in the study of the human immunodeficiency virus infection cycle and acquired immunodeficiency syndrome chemotherapy (Balzarini et al., 1991, 1992; Van Damme et al., 1993a).

Characterization and molecular cloning of the Man-binding lectins from several Amaryllidaceae, Alliaceae, and Orchidaceae species demonstrated that they all belong to one large superfamily of Man-binding proteins that show a considerable overall sequence homology within the mature protein and, in addition, contain highly conserved domains (Van Damme et al., 1991b, 1992a, 1992b, 1993a, 1993b, 1994a, 1994b, 1995). In search of possible new members of the Man-binding monocot lectins our attention was drawn to a previously isolated but yet uncharacterized agglutinin

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Abbreviations: LECAMA, cDNA clone encoding the *Arum maculatum* agglutinin; LECCEA, cDNA clone encoding the *Colocasia esculenta* agglutinin (Hirai et al., 1993).

from tubers of *Arum maculatum* (lords and ladies), a plant species belonging to the monocot family Araceae. Based on the observation that the latter lectin behaved very much like the Amaryllidaceae lectins with respect to its binding of human and murine α-2-macroglobulin (Van Leuven et al., 1993), it seemed worthwhile to investigate its possible relationship to the other members of the family of the Man-binding monocot lectins. Therefore, the *Arum* lectin and its corresponding cDNA were isolated and subjected to a detailed characterization. In addition, the lectins from three other Araceae species, namely *Colocasia esculenta* (taro, cocoyam, dasheen, eddoe), *Xanthosoma sagittifolium* (tannia, taro cocoyam, yautia), and the ornamental plant *Dieffenbachia sequina*, were isolated for a comparative study.

The results presented here indicate that the Araceae lectins are a family of closely related proteins, the sequence of which contains the highly conserved domains of the Amaryllidaceae, Alliaceae, and Orchidaceae lectins. With respect to their specificity the Araceae lectins resemble the previously isolated Man-binding lectins from Amaryllidaceae, Alliaceae, and Orchidaceae species, except that they interact more strongly with glycoproteins. Finally, it is shown that the lectins are the most prominent proteins in the tubers of *Arum*, *Colocasia*, and *Xanthosoma* and that the recently described major storage protein of *Colocasia* corresponds to the lectin.

MATERIALS AND METHODS

Plant Material

Tubers of *Colocasia esculenta* (L.) Schott and *Xanthosoma sagittifolium* (L.) Schott and *Dieffenbachia sequina* Schott plants were purchased from a local store. Tubers and other tissues of *Arum maculatum* L. were collected locally.

Extraction and Isolation of the Lectins

The Araceae lectins were isolated from extracts of tubers or leaves by affinity chromatography on immobilized asialofetuin. The isolation procedure was essentially the same for all four Araceae lectins. Briefly, tubers of *Arum*, *Colocasia*, or *Xanthosoma* or leaves of *Dieffenbachia* were homogenized in 0.2 M NaCl containing 1 g/L ascorbic acid (10 mL/g fresh weight) at pH 7.0 using a Waring blender. The homogenates were filtered through cheesecloth and centrifuged (3000*g* for 10 min). After 20 mM $CaCl_2$ was added, the supernatant was brought to pH 9.0 (with 1 N NaOH), kept overnight in the cold, and recentrifuged at 3000*g* for 10 min. The supernatant was adjusted to pH 4.0 (with 1 N HCl) and recentrifuged at 3000*g* for 10 min. Subsequently, the cleared supernatant was adjusted to pH 7.5 (with 1 N NaOH) and solid ammonium sulfate was added to reach a final concentration of 1.0 M. After standing overnight in the cold room, the precipitate was removed by centrifugation at 9000*g* for 20 min. The final supernatant was decanted, filtered through filter paper (Whatman 3MM), and loaded on a column of asialofetuin-Sepharose 4B equilibrated with 1.0 M ammonium sulfate (in 50 mM sodium acetate, pH 6.5). After passing the extract the column was washed with 1.0 M ammonium sulfate (in 50 mM sodium acetate, pH 6.5) until the A_{280} decreased below 0.01 and the lectin desorbed with 20 mM acetic acid.

All agglutinating activity present in the crude extracts was retained on the column using the procedure described here and could be eluted by lowering the pH (as is exemplified in Fig. 1, which shows an affinity chromatography purification of the *Arum* lectin).

Electrophoresis

Lectin preparations were analyzed by SDS-PAGE using 12.5 to 25% (w/v) acrylamide gradient gels as described by Laemmli (1970). Reduced and alkylated (carboxyamidated) samples of the lectins were electrophoresed under denaturing conditions (in 8 M urea) as described previously (Van Damme et al., 1987).

Carboxyamidation of Proteins

Protein samples were dissolved in 8 M urea containing 0.4 M Tris-HCl (pH 8.7) and 5 mM DTT and heated for 2 h at 45°C. Iodoacetamide was added to a final concentration of 20 mM and the samples were kept on ice for 30 min. The reaction was quenched by the addition of DTT to a final concentration of 40 mM, followed by heating for 30 min at 50°C.

Ion-Exchange Chromatography

Samples of purified Araceae lectins were analyzed by ion-exchange chromatography on a Mono-S column (type HR 5/5; Pharmacia, Uppsala, Sweden) using a Pharmacia fast protein liquid chromatography system and 25 mM formate, pH 3.8, as running buffer. After the samples (1–2 mg) dissolved in 25 mM formate buffer were loaded, the column was washed with 4 mL of the same buffer and the proteins were eluted using a linear gradient (36 mL) of increasing NaCl concentration (0–0.6 M) in this buffer. Peak fractions were collected manually, dialyzed against distilled water, lyophilized, and prepared for electrophoresis as described above.

Gel Filtration

Gel filtration of purified Araceae lectins was done on a Pharmacia Superose 12 column equilibrated with PBS containing 0.2 M Man (to prevent binding of the lectin to the column). Lectin samples (200 μL containing about 250 μg of pure protein) were loaded on the column and chromatographed at a flow rate of 20 mL/h. Molecular mass reference markers were BSA (68 kD), ovalbumin (45 kD), chymotrypsinogen (25 kD), and Cyt *c* (12.5 kD), as well as the previously characterized Man-binding lectins from *Galanthus nivalis* (50 kD) (Van Damme et al., 1988) and *Allium sativum* (25 kD) (Van Damme et al., 1991a).

Preparation of Antibodies against the *A. maculatum* Agglutinin

To immunize a rabbit 1 mg of purified *A. maculatum* lectin 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-d intervals. Ten days after the final injection, blood was removed from an ear marginal vein and allowed to clot overnight at room temperature. 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 sulfate (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, dialyzed against the same buffer and applied onto an anion-exchange column (10 × 2.6 cm) of Q-Fast Flow (Pharmacia), equilibrated with the same Tris buffer. Under these conditions, IgG does not bind to the column and elutes 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 in 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 Man (to prevent possible aspecific binding of the lectins to serum proteins).

Agglutination Assays

Agglutination assays were conducted using trypsin-treated rabbit erythrocytes (Van Damme et al., 1987). The carbohydrate-binding specificity of the lectins was determined using some glycoproteins (thyroglobulin, fetuin, asialofetuin, and porcine mucin) and a series of simple sugars. The sugars tested were Glc, Gal, galactosamine, GlcNAc, *N*-acetylgalactosamine, Man, lactose, melibiose, Fuc, Ara, amylose, Rib, Fru, trehalose, sorbose, Xyl, Suc, maltose, and sorbitol (all sugars of the D-configuration with the exception of L-Fuc and L-sorbose).

Amino Acid Sequence Analysis

Protein sequencing was conducted on an Applied Biosystems (Foster City, CA) model 477A protein sequencer interfaced with an Applied Biosystems model 120A on-line analyzer or a Beckman (Fullerton, CA) LF 3600 TC protein sequencer.

Protein Determination

Protein concentration was determined by the method of Bradford (1976) using BSA and purified *Arum* lectin as standards.

RNA Isolation

Total cellular RNA was prepared from plant material stored at −80°C essentially as described by Finkelstein and Crouch (1986). Alternatively small-scale isolation of total RNA was performed according to the protocol described by Wadsworth et al. (1988).

Construction and Screening of cDNA Library

A cDNA library was constructed from poly(A)-rich RNA isolated from young leaves of *A. maculatum* using the cDNA synthesis kit from Pharmacia. cDNA fragments were inserted into the *Eco*RI site of the multifunctional phagemid pT_7T_3 18U (Pharmacia). The library was propagated in *Escherichia coli* XL1 Blue (Stratagene, La Jolla, CA).

Recombinant lectin clones were screened using synthetic oligonucleotides derived from the N-terminal amino acid sequences of both *Arum* lectin polypeptides or the random primer-labeled taro cDNA clone encoding a 12-kD storage protein (Hirai et al., 1993) as probes. Hybridization using the taro cDNA clone was done overnight at 50°C as reported previously (Van Damme et al., 1992a). 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 (1987) and sequenced by the dideoxy method (Sanger et al., 1977).

Northern Blot

RNA electrophoresis was performed according to the procedure of Maniatis et al. (1982). Approximately 50 μg of total RNA were denatured in glyoxal and DMSO and separated in a 1.2% (w/v) agarose gel. Following electrophoresis the RNA was transferred to Immobilon N membranes (Millipore, Bedford, MA) and the blot hybridized using a random primer-labeled lectin cDNA insert. Hybridization was performed as reported by Van Damme et al. (1992b). An RNA ladder (0.16–1.77 kb) was used as a marker.

Genomic DNA Analysis

Total DNA from *Arum* leaves was isolated according to the procedures described by Dellaporta et al. (1983) and de Kochko and Hamon (1990). The DNA preparation was treated with RNase to remove any contaminating RNA. Approximately 50 μg of DNA were digested with restriction endonucleases and subjected to electrophoresis in a 0.8% (w/v) agarose gel. DNA was transferred to Immobilon N membranes (Millipore) and hybridized using the ^{32}P-labeled cDNA insert encoding the lectin.

Computer Analyses

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

RESULTS

Purification and Biochemical Characterization of the Lectin from Tubers of *A. maculatum*

Preliminary experiments with crude extracts from tubers of *A. maculatum* (lords and ladies) indicated the presence of a lectin that strongly agglutinated rabbit erythrocytes but was inactive with human red blood cells irrespective of their blood group. Since the agglutination of the extract

was inhibited by low concentrations of asialofetuin, affinity chromatography of the tuber extracts was performed on a column of asialofetuin-Sepharose 4B. As shown in Figure 1, virtually all initial agglutinating activity was recovered in the protein fractions desorbed from the column with 20 mM acetic acid. The overall yield of lectin was about 2 mg/g fresh weight, which corresponds to 27% of the total soluble tuber protein.

The use of 1 M ammonium sulfate during the affinity chromatography strongly enhanced the affinity of the lectin for the asialofetuin-Sepharose and by doing so allowed a prolonged washing of the column to remove unbound proteins. When an aqueous buffer (e.g. PBS) was used for the affinity chromatography on the same column, the lectin initially bound to the matrix but progressively eluted during the washing step. As a result, only a small fraction of the total lectin was retained when washing of the column was completed, so that the eventual yield was very low.

To determine the molecular structure of the *Arum* lectin, the lectin preparation was analyzed by SDS-PAGE and gel filtration. Both reduced (with 2% [v/v] β-mercaptoethanol) (Fig. 2) and unreduced *Arum* lectin (results not shown) migrated as a single polypeptide band of 12 kD upon SDS-PAGE. Gel filtration of the native *Arum* lectin on a Superose 12 column in PBS containing 0.2 M Man (to prevent possible interactions between the lectins and the gel matrix) yielded a single symmetrical peak with an apparent molecular mass of 50 kD (results not shown). Based on these results, it was concluded that the *Arum* lectin is a tetramer composed of four 12-kD subunits.

To determine the amino terminal sequence of the *Arum* lectin, the lectin was run on an SDS-polyacrylamide gel and blotted onto a nitrocellulose filter. After the blot was stained, the lectin polypeptide of 12 kD was excised from the blot and used for automated amino acid sequencing. As shown in Figure 3, a double N-terminal sequence was obtained for the 12-kD purified lectin. Two amino acids were present in each sequencing cycle in equimolar amounts, suggesting that the total lectin fraction was an (equimolar) mixture of two 12-kD peptides.

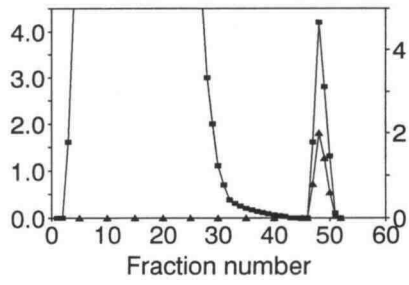

Figure 1. Affinity chromatography of the *Arum* lectin on immobilized asiolofetuin. A partially purified extract from tubers of *A. maculatum* was applied to a column of immobilized asialofetuin (2.6 × 5 cm; 25-mL bed volume). Unbound protein was eluted with 1.0 M ammonium sulfate until the A_{280} fell below 0.01 and the lectin desorbed with 20 mM acetic acid. Fractions of 5 mL were collected and the A_{280} (■) measured, and their agglutination titer ($\times 10^{-3}$) (▲) was determined with trypsin-treated rabbit erythrocytes.

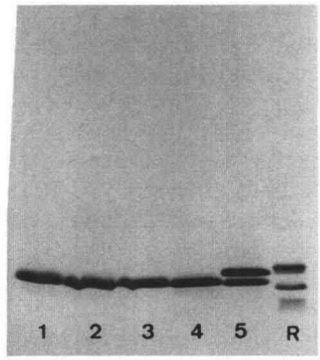

Figure 2. SDS-PAGE of purified Araceae lectins. Lectins were reduced with β-mercaptoethanol and loaded as follows: lane 1, *G. nivalis* lectin; lane 2, *A. maculatum* lectin; lane 3, *C. esculenta* lectin; lane 4, *X. sagittifolium* lectin; and lane 5, *D. sequina* lectin. About 25 μg of each lectin were loaded on the gel. Molecular mass reference proteins are shown in lane R (myoglobin, 16.9 kD; myoglobin I and II, 14.4 kD; myoglobin I, 8.2 kD).

In an attempt to separate the two different 12-kD subunits, the native lectin was subjected to ion-exchange chromatography on a Mono-S column and the resulting three isolectins (Fig. 4B) were analyzed by electrophoresis in 8 M urea (after reduction and alkylation). Thereby each isolectin showed a complex polypeptide pattern (Fig. 4A). Sequencing of the most prominent bands still yielded double N-terminal amino acid sequences except for one polypeptide (Fig. 4A, lane 2), which gave a unique N-terminal amino acid sequence (Fig. 3). When this sequence was known, the sequence of the second polypeptide could be deduced by subtraction from the double sequence.

Occurrence of Similar Lectins in Different Araceae Species

The Araceae represent a large family that, in addition to ornamental species, also comprises some economically important crop plants. To check whether similar lectins are present within different Araceae species, we looked for lectin activity in tubers of the two major edible aroids, *C. esculenta* and *X. sagittifolium*, and the leaves of the ornamental *D. sequina*. Since agglutination assays with crude extracts indicated that all three species contained an agglutinating factor with properties very similar to those of the *Arum* lectin, the same extraction and purification procedure was used to purify the different Araceae lectins. The overall yield of lectin was about 1 and 4 mg/g fresh weight for *Colocasia* and *Xanthosoma* tubers, respectively. Extracts from *Dieffenbachia* leaves contained much lower levels of lectin. The total yield of pure lectin was only 10 μg/g fresh weight.

SDS-PAGE of the purified lectins showed that the *Colocasia* and *Xanthosoma* agglutinins migrated—like the *Arum* lectin—as a single polypeptide band of about 12 kD, whereas the *Dieffenbachia* agglutinin yielded two well-resolved polypeptides of 12 and 14 kD (Fig. 2). Estimations of the molecular mass of the native *Colocasia*, *Xanthosoma*, and *Dieffenbachia* lectins by gel filtration on a Superose 12 col-

(A) Arum total lectin

V/N G/I P/T N/F Y/T H/L L/N S/M G/L E/F T/S L/G N/Q V/I D/L G/Y H/G L/D K/D

(B) Arum type 1 polypeptide

VGTNYLLSGETLNVDGHLKN

(C) Arum type 2 polypeptide

NIPFTHNMLFSGQILYGDDM

(D) Colocasia type 1 and type 2 polypeptides

Type 1 LGTNYLLSGQTLDTXXHLK

Type 2 NIPFTNNLLFSGQVXYGDG

(E) Xanthosoma type 1 and type 2 polypeptides

Type 1 LGTNYLLSGQT

Type 2 NIPFTNNLLFS

(F) Dieffenbachia 12 kD and 14 kD polypeptides

12 kD VNXNYLLSGQTINTNGXLIQGDYDL

14 kD NTPLTRN

Figure 3. N-terminal amino acid sequences of the different Araceae lectins. A, Double amino acid sequence of the total *Arum* lectin determined from a blot after SDS-PAGE. B, Unique sequence of the type 1 *Arum* lectin polypeptide determined from a blot after electrophoresis in 8 M urea of the reduced and alkylated isoform 1 (peak 1 of the chromatogram shown in Fig. 4). C, Sequence of type 2 *Arum* lectin polypeptide determined by subtracting B from A. D, Sequences of the *Colocasia* lectin (the sequence of the individual lectin polypeptides was deduced from a double sequence on the basis of their sequence homology with the type 1 and type 2 *Arum* lectin polypeptides and were confirmed by molecular cloning of the gene by Hirai et al., 1993). E, Sequences of the *Xanthosoma* lectin (the sequence of the individual lectin polypeptides was deduced from a double sequence on the basis of their sequence homology with the type 1 and type 2 *Arum* lectin polypeptides). F, Unique sequences of the *Dieffenbachia* 12- and 14-kD lectin polypeptides.

umn indicated that all three lectins eluted with an apparent molecular mass of 50 kD (results not shown). N-terminal protein sequencing of the Araceae lectins revealed high sequence similarity among the different Araceae lectins. Like the *Arum* lectin the *Colocasia* and the *Xanthosoma* lectins yielded a double amino acid sequence (Fig. 3), indicating that they are also composed of two different types of 12-kD subunits. It should be emphasized, however, that the sequences of both the *Colocasia* and *Xanthosoma* lectins are virtually identical with the N-terminal sequences of the *Arum* lectin polypeptides. Sequencing of both polypeptides of the *Dieffenbachia* lectin yielded unique N-terminal sequences (Fig. 3) with a striking homology to that of the subunits of the other Araceae lectins. It appears, therefore, that the Araceae lectins are tetramers composed of two polypeptide chains that are either identical (*Colocasia* and *Xanthosoma* agglutinins) or slightly different (*Dieffenbachia* agglutinin) in size.

Electrophoresis of the reduced and alkylated lectins yielded complex polypeptide-banding patterns (Fig. 5). However, since the results of this analysis are difficult to interpret in terms of heterogeneity at the protein level, the occurrence of multiple isoforms has also been investigated (and confirmed) at the molecular level by cDNA cloning and Southern blot analysis (see below).

Lectins Are the Predominant Proteins in Tubers of *Arum*, *Colocasia*, and *Xanthosoma*

As already mentioned above, the *Arum* lectin represents about 27% of the total soluble tuber protein and thus can be considered a major protein in this storage tissue. SDS-PAGE of a crude extract before and after passage through the affinity column shows that the most prominent protein in *Arum* tubers binds, indeed, to the asialofetuin-Sepharose 4B (Fig. 6). The same holds true for *Colocasia* and *Xanthosoma* tuber extracts.

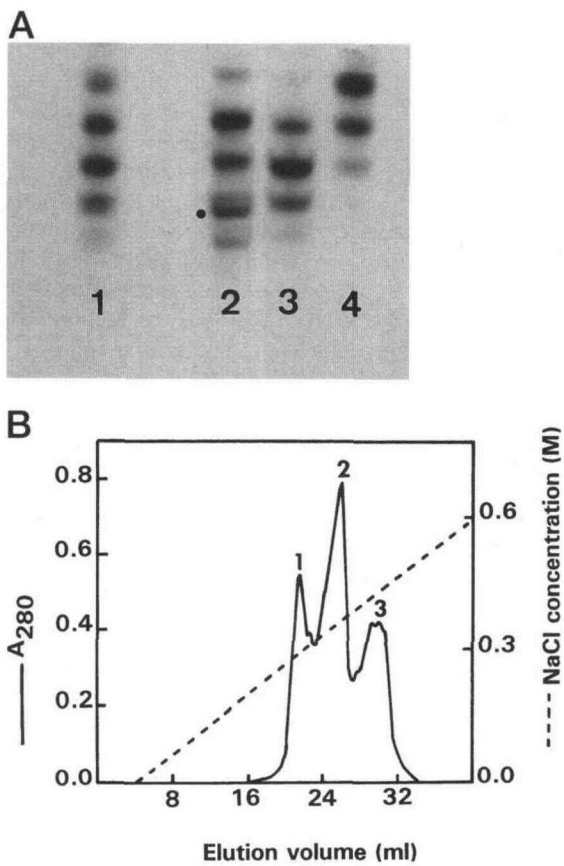

Figure 4. A, Electrophoresis of reduced and alkylated lectin from *A. maculatum* in 8 M urea. Lectins were loaded as follows: lane 1, total lectin; lanes 2, 3, and 4, peak fractions 1, 2, and 3 resolved by ion-exchange chromatography on a Mono-S column (see B). About 50 μg of lectin were loaded on each slot. The band marked by a dot in lane 2 yielded the unique amino acid sequence of type 1 lectin polypeptide shown in Figure 3B. B, Ion-exchange chromatography of the lectin from *A. maculatum*. About 1 mg of the purified lectin was chromatographed on a Mono-S column (Pharmacia) equilibrated with 25 mM formate, pH 3.8, and eluted using a linear gradient of increasing NaCl concentration (0–0.6 M) in this buffer. The flow rate was 2 mL/min.

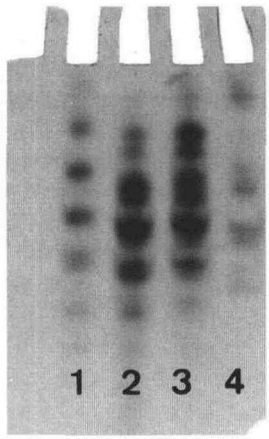

Figure 5. Electrophoresis of reduced and alkylated lectins from different Araceae species in 8 M urea. Lectins were loaded as follows: lane 1, *Arum* lectin; lane 2, *Colocasia* lectin; lane 3, *Xanthosoma* lectin; and lane 4, *Dieffenbachia* lectin. About 50 μg of each lectin were loaded on the gel.

Although the *Arum* lectin is undoubtedly the major tuber protein, it cannot be considered a tuber-specific protein, since it also occurs in all other tissues of the *Arum* plants. Agglutination assays with extracts from leaves, petioles, and different parts of the inflorescence have shown, indeed, that all of these tissues contain lectin. Their lectin content, however, is approximately 2 orders of magnitude lower than that of the tubers. A similar conclusion follows the results of northern blot experiments (see below).

Agglutination Properties and Carbohydrate-Binding Specificity of the Araceae Lectins

Like the *Arum* lectin, the *Colocasia*, *Xanthosoma*, and the *Dieffenbachia* lectins readily agglutinate rabbit erythrocytes but are completely nonreactive toward human erythrocytes irrespective of the blood group. The minimal concentration required for agglutination of trypsin-treated rabbit erythrocytes was 1.41, 0.76, 0.44, and 0.12 μg/mL for the lectins from *Arum*, *Colocasia*, *Xanthosoma*, and *Dieffenbachia*, respectively.

The carbohydrate-binding specificity of the purified Araceae lectins was assessed by hapten inhibition assays using a series of simple sugars and some glycoproteins. Of all monosaccharides tested only Man gave an inhibition of the agglutination. However, inhibition occurred only at relatively high Man concentrations (100 mM) and was transient (probably because of the much higher affinity of the lectins for the ligands on the rabbit erythrocytes than for free Man). Assays with animal glycoproteins showed that the agglutination activity of the Araceae lectins was also inhibited by low concentrations of asialofetuin (Table I). This observation is in good agreement with the fact that asiolofetuin contains several types of branched oligomannosides and indicates that, although the lectin has a weak monosaccharide specificity toward Man, the lectin-binding site is able to accommodate more complex sugar moieties.

Since the inhibition of the Araceae lectins by Man was much less pronounced than that of the Amaryllidaceae, Alliaceae, and Orchidaceae lectins, their specific interaction with this sugar was further investigated by affinity chromatography of the purified lectins on immobilized Man. When PBS was used as a running buffer the Araceae lectins were only retarded on the Man-Sepharose 4B column. However, in the presence of 1.5 M ammonium sulfate, the lectins were quantitatively retained on the same column. When a gradient of decreasing ammonium sulfate concentration was applied on the column the lectins eluted in a symmetrical peak (as is illustrated for the *Arum* lectin in Fig. 7) at a concentration of about 0.7 M ammonium sulfate. It is worth mentioning here that the homodimeric Man-binding lectins from garlic (*A. sativum*) and ramsons (*Allium ursinum*) (Van Damme et al., 1991a), as well as the monomeric Man-binding proteins from orchids (Van Damme et al., 1994a), require similar concentrations of ammonium sulfate for binding to Man-Sepharose 4B. Finally, the specificity of the binding of the *Arum* lectin to the immobilized Man is further illustrated by the observation that the lectin did not bind to Sepharose 4B or Gal-Sepharose 4B (in the presence of 1.5 M ammonium sulfate) and that the addition of 0.1 M Man to the lectin solution (in 1.5 M ammonium sulfate) prevented its binding to the Man-Sepharose 4B column.

Serological Relationship among the Araceae Lectins

Since all Araceae lectins strongly resemble each other with respect to their molecular structure and carbohydrate-binding specificity, it seemed worthwhile to examine the possible serological relationship among these lectins. A

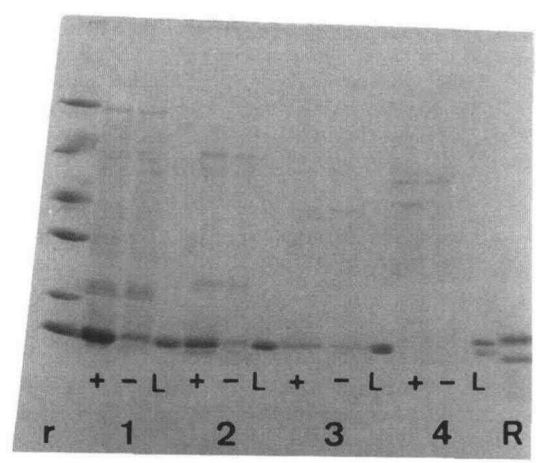

Figure 6. SDS-PAGE of crude extracts from tubers of *Arum* (lane 1), *Colocasia* (lane 2), and *Xanthosoma* (lane 3) and leaves of *Dieffenbachia* (lane 4) before (+) and after (−) affinity chromatography on immobilized asialofetuin. In the lanes marked L, the respective purified lectins were loaded. Molecular mass markers shown in lane r are, in order of increasing molecular mass, lysozyme (14 kD), soybean trypsin inhibitor (20 kD), carbonic anhydrase (30 kD), ovalbumin (43 kD), BSA (67 kD), and phosphorylase b (94 kD). Reference proteins shown in lane R are myoglobin (16.9 kD), myoglobin I and II, (14.4 kD) and myoglobin I (8.2 kD).

Table I. *Carbohydrate-binding specificity of the Araceae lectins*

The carbohydrate-binding specificity of the Araceae lectins was determined by hapten inhibition assays using a series of simple sugars and some glycoproteins. The following sugars were not inhibitory at a final concentration of 200 mM: Glc, Gal, galactosamine, GlcNAc, *N*-acetylgalactosamine, lactose, melibiose, Fuc, Ara, amylose, Rib, Fru, trehalose, sorbose, Xyl, Suc, maltose, and sorbitol. The concentration required for 50% inhibition refers to the inhibition of agglutination of trypsin-treated rabbit erythrocytes.

Sugar/Glycoprotein	Concentration Required for 50% Inhibition in the Presence of the Lectins from			
	Arum	*Colocasia*	*Xanthosoma*	*Dieffenbachia*
Man	100 mM	100 mM	100 mM	100 mM
Fetuin	200 μg/mL	200 μg/mL	200 μg/mL	50 μg/mL
Asialofetuin	12.5 μg/mL	25 μg/mL	25 μg/mL	12.5 μg/mL
Thyroglobulin	100 μg/mL	100 μg/mL	150 μg/mL	12.5 μg/mL
Porcine mucin	200 μg/mL	200 μg/mL	200 μg/mL	50 μg/mL

polyclonal antiserum prepared against the *Arum* lectin was challenged with the different Araceae lectins. As shown in Figure 8, all Araceae lectins reacted or cross-reacted with the antibodies raised against the *Arum* agglutinin. It is evident that only the *Colocasia* and the *Xanthosoma* lectins form precipitin lines of identity with each other and thus can be considered serologically identical. The precipitin lines of the *Dieffenbachia/Arum* lectins, *Dieffenbachia/Colocasia-Xanthosoma* lectins, and *Arum/Colocasia-Xanthosoma* lectins clearly do not fuse, which indicates that the *Dieffenbachia* and *Colocasia-Xanthosoma* lectins differ serologically from the *Arum* lectin.

Molecular Cloning of the Lectin from *A. maculatum*

Although tubers and young shoots of *A. maculatum* contain high concentrations of lectin, the isolation of RNA from these plant materials was very cumbersome. In addition, the quality of the RNA was poor, which led to an early arrest of the synthesis of the first cDNA strand. Since the lectin is also present in the leaves of *Arum*, a cDNA library was constructed from poly(A)-rich RNA isolated from young leaves of *Arum*. Initial screenings of the cDNA library constructed from *Arum* were performed using degenerate oligonucleotide probes derived from the N-terminal amino acid sequences of the lectin polypeptides. In a later stage we also used a cDNA insert encoding a major storage protein from *Colocasia* tubers (Hirai et al., 1993), since the deduced amino acid sequence of this cDNA clone showed a striking homology to the N-terminal sequence of the two *Arum* lectin polypeptides.

Multiple lectin cDNA clones were isolated from cDNA libraries constructed from leaves and tubers of *Arum* and their sequences analyzed. The complete nucleotide sequences of two cDNA clones, LECAMA1 and LECAMA2, encoding the *Arum* leaf lectin were determined and compared. As shown in Figures 9 and 10, the lectin cDNA clones LECAMA1 and LECAMA2 clearly differ from each other at some positions in their nucleotide sequences (Fig. 9) as well as in their deduced amino acid sequences (Fig. 10). The cDNA clone LECAMA1 contains a 820-bp open reading frame encoding a 273-amino acid precursor with one possible initiation codon at position 15 of the deduced amino acid sequence. Translation starting with this Met residue results in a protein of 259 amino acids with a calculated molecular mass of 27,964 D, which after cotranslational cleavage of the signal peptide of 24 amino acids yields a lectin precursor polypeptide of 25,533 D.

A detailed analysis of the deduced amino acid sequence of the cDNA clone reveals that the N-terminal amino acid sequences of the type 1 and type 2 *Arum* subunits are present in the beginning and the middle, respectively, of the presumed 25-kD lectin precursor. It is evident, therefore, that both types of lectin polypeptides are synthesized

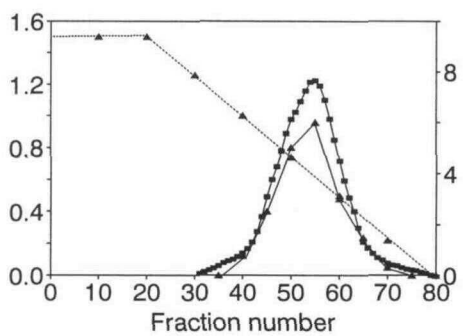

Figure 7. Affinity chromatography of purified *Arum* lectin on a column of immobilized Man. About 50 mg of purified *Arum* lectin dissolved in 1.5 M ammonium sulfate were applied onto the column (2.6 × 10 cm; 50-mL bed volume) and eluted using a linear gradient of 1.5 M ammonium sulfate to water. Fractions of 5 mL were collected and the A_{280} (■), the agglutination titer ($\times 10^{-2}$) (▲—▲), and the ammonium sulfate concentration (M) (▲- - - -▲) were determined.

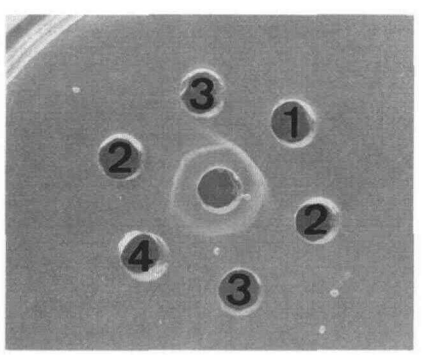

Figure 8. Double immunodiffusion of purified Araceae lectins. Antiserum against *A. maculatum* agglutinin (central well) was challenged with 5 μg of purified lectin from *A. maculatum* (1), *C. esculenta* (2), *X. sagittifolium* (3), and *D. sequina* (4).

on a single large precursor that is posttranslationally processed into two different polypeptides of similar size. If we assume that no further processing occurs at the C terminus of the lectin polypeptides, cleavage of the precursor between amino acids 140 and 141 (Fig. 10) will yield the two lectin polypeptides (116 and 119 amino acids) with calcu-

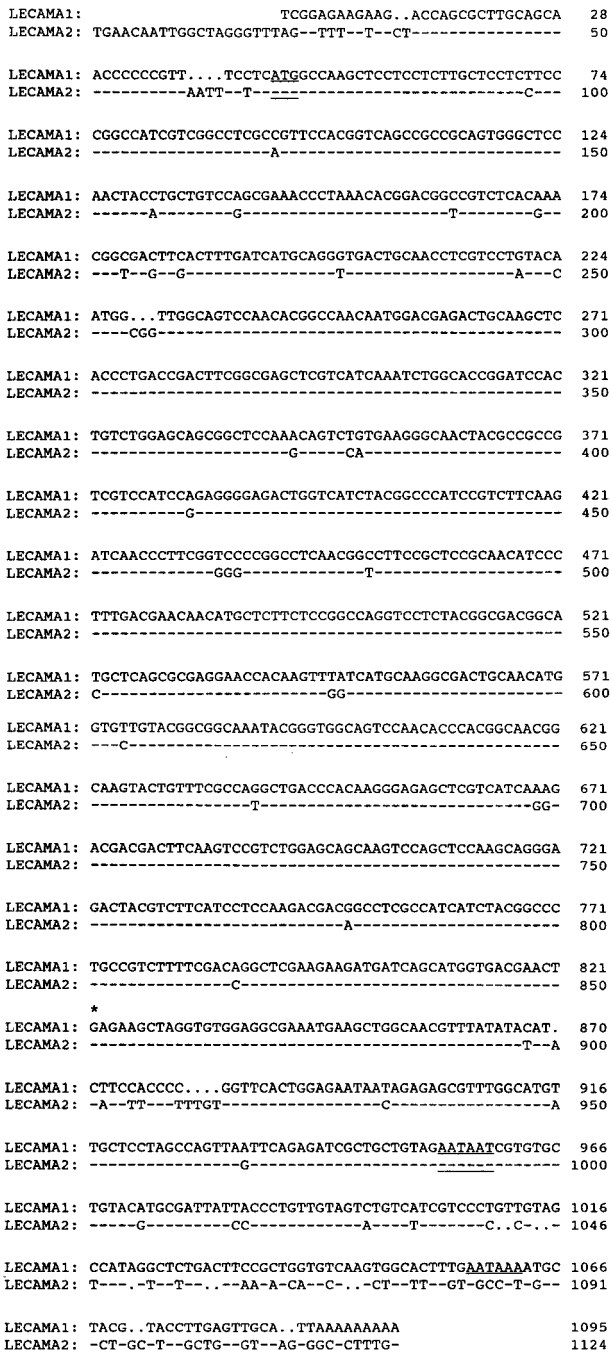

Figure 9. Comparison of nucleotide sequences of two cDNA clones encoding the leaf lectin from *A. maculatum*. The sequences were aligned to maximize homology. Dashes denote sequence similarity to LECAMA1. Dots represent gaps introduced for maximal alignment. Putative start codons and polyadenylation signals are underlined. The stop codon is indicated by an asterisk.

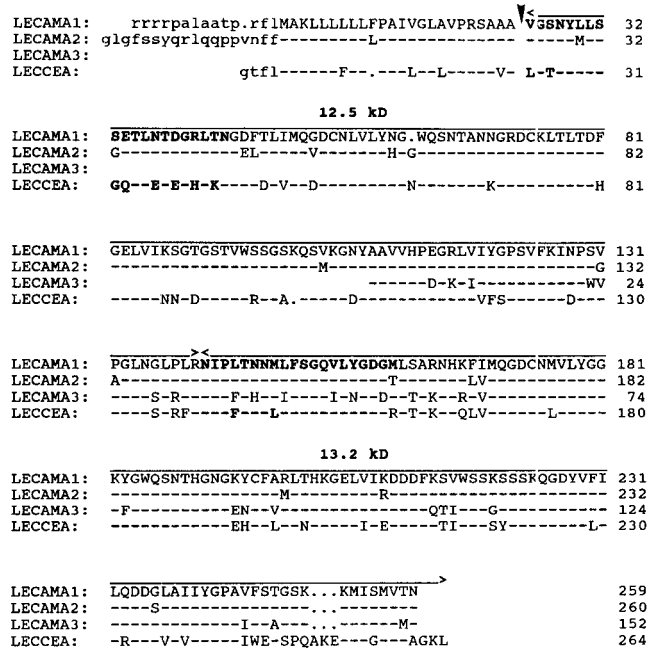

Figure 10. Sequence alignment of deduced amino acid sequences of lectin cDNA clones from *A. maculatum* and comparison with the amino acid sequence of the 12-kD storage protein from *C. esculenta* (LECCEA) (Hirai et al., 1993). Since the first Met is most likely to be used as the translation-initiation site, the deduced amino acids preceding this Met are shown in lowercase letters. The arrowhead indicates the cleavage site for the signal peptide. The N-terminal amino acid sequences of the lectin polypeptides (see Fig. 3) are shown in bold in LECAMA1 and LECCEA. Dashes denote sequence identity with LECAMA1. LECAMA1 and LECAMA2 represent lectin cDNA clones from *Arum* isolated from a cDNA library from leaves, whereas LECAMA3 represents a partial cDNA clone isolated from a cDNA library constructed from total RNA isolated from *Arum* tubers. The deduced amino acid sequence of the 12-kD taro storage protein shown in this figure is the sequence after correction of the sequencing error in the signal peptide. Dots represent gaps introduced for maximal alignment.

lated molecular masses of 12,364 and 13,187 D, respectively. These molecular masses of the lectin polypeptides predicted from the deduced amino acid sequence of the lectin precursor are in good agreement with the molecular masses of the mature lectin polypeptides determined by SDS-PAGE.

Similarly, LECAMA2 encodes a 260-amino acid precursor of 27,939 D, which upon cleavage of the signal peptide is converted into a polypeptide of 25,542 D (236 amino acids). The molecular mass of the lectin polypeptides encoded by this precursor is comparable to the lectin polypeptides encoded by LECAMA1, being 12,418 and 13,141 D, respectively, except that the first lectin polypeptide contains 1 additional amino acid. The lectin precursors encoded by LECAMA1 and LECAMA2 are devoid of putative glycosylation sites. Because of the poor quality of the tuber mRNA, the resulting cDNA library contained only incomplete lectin cDNAs. However, as shown in Figure 10, the sequence of a partial cDNA clone from tubers is highly

homologous to the corresponding part of the complete clones from the leaf cDNA library.

Sequence Homology between Lectin cDNA Clones from *A. maculatum* and Previously Isolated cDNA Clones Encoding Man-Binding Lectins

The results described above leave no doubt that the two subunits of the *Arum* lectin are encoded by two separate domains of a single gene. To corroborate the possible homology between both domains, the deduced amino acid sequences of the mature type 1 and type 2 polypeptides were compared. Alignment of these sequences revealed an overall homology of only 40% at the amino acid level but at the same time demonstrated that some parts of the sequences are identical (Fig. 11). Interestingly, the conserved parts of both domains of the *Arum* lectin cDNA clones show a remarkable homology to the deduced amino acid sequence of the previously isolated lectin cDNA clones encoding the Man-binding lectins from Amaryllidaceae, Alliaceae, and Orchidaceae species. Figure 11 shows only the sequence of the snowdrop (*G. nivalis*) lectin. However, as was demonstrated earlier, all known Man-binding lectins from representatives of Amaryllidaceae, Alliaceae, and Orchidaceae contain highly conserved parts in their respective sequences (Van Damme et al., 1995). Obviously, the high degree of conservation within these particular parts of the sequences indicates that the amino acids that compose them play an important role in the folding and/or carbohydrate-binding activity of the lectins.

Sequence Homology between Lectin cDNA Clones from *A. maculatum* and a Previously Isolated cDNA Clone Encoding the Putative Major Storage Protein of *Colocasia* Tubers

A comparison of the deduced amino acid sequences of the lectin cDNA clones from *Arum* and the cDNA clone of the putative major storage protein of *Colocasia* tubers reveals a 65 to 68% sequence homology for the total coding region (depending on the *Arum* lectin cDNA clone under consideration) (Fig. 10). In spite of the high homology between both proteins there was almost no sequence homology between the signal peptides of the *Arum* lectin and the *Colocasia* storage protein (Hirai et al., 1993). However, resequencing of the *Colocasia* storage protein cDNA clone (provided by Dr. M. Hirai, Mie, Japan) revealed an error within the region of the signal peptide. The addition of a single nucleotide resulted in a frame shift and yielded a signal peptide that showed approximately 80% sequence homology with the signal peptide determined for the *Arum* lectin cDNA clones.

```
AMA1pp1:     VGSNYLLSSETLNTDGRLTNGDFTLIMQGDCNLVLYN....GWQSNTA
             .  : : :   :  :: :.      :::::::.:::     ::::::
AMA1pp2: NIPLTNNMLFSGQVLYGDGMLSARNHKFIMQGDCNMVLYG.GKYGWQSNTH
           ::.:.::  :     :   :  :::: :::.:::   :  : .::
LECGNA1:       NNILYSGETLSAGEFLNQGNYVFIMQEDCNLVLYDVDKPLWETNTG

AMA1pp1: NNGRDCKLTLTDFGELVIKSGTGSTVWSSGSKQSVKGNYAAVVHPEGRLVI
          ::. :   ::  ::::::     .:::: :  :  : :  ..  .:  .:
AMA1pp2: GNGKYCFARLTHKGELVIKDDDFKSVWSSKSS.SKQGDYVFILQDDGLAII
         :  . :.  .  : ::.     : .:.: .    .: :: ::: ::   :
LECGNA1: GLSRRCYLNMQTDGNLVVYNPSNKPIWASNTG.GENGNYVCILQKDGNIAI

AMA1pp1: YGPSVFKINPSVPGLNGLPLR
         :::.::          .  .
AMA1pp2: YGPAVFSTGSK..KMISMVTN
         ::::...::
LECGNA1: YGPAIWATG
```

Figure 11. Sequence comparison of the cDNA-derived amino acid sequences of the two *Arum* lectin polypeptides encoded by LECAMA1 and the mature *G. nivalis* lectin encoded by LECGNA1 (Van Damme et al., 1992b). The amino acids have been aligned by introducing gaps (.) to maximize identity. Colons between lines indicate identical amino acid residues, whereas single dots indicate similar amino acids.

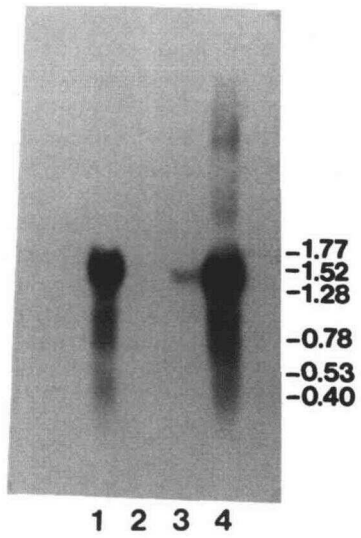

Figure 12. Northern blot of total RNA isolated from different tissues of *A. maculatum*. Total RNA was isolated from shoots (lane 1), flowers (lane 2), leaves (lane 3), and tubers (lane 4). The blot was hybridized using the ^{32}P-labeled cDNA insert LECAMA1, washed, and exposed overnight. Numbers on the right show RNA size (kb).

Northern Blot Analysis

To determine the total length of the RNA encoding the *Arum* lectin and the expression of the lectin in different plant tissues, a blot containing total RNA isolated from different tissues was hybridized with the labeled lectin clone encoding the *Arum* lectin. Hybridization of the blot revealed a single band of lectin mRNA with an estimated length of approximately 1400 nucleotides (Fig. 12). Very strong hybridization signals were detected in the tuber and the young shoots. Lectin mRNA was also detected in the leaves, although at a much lower concentration. Upon longer exposure of the blot, the RNA from the flower tissue also yielded a weak signal. It can be concluded, therefore, that the *Arum* lectin is highly expressed in the tubers. However, since the lectin as well as its mRNA is found all over the plant, the *Arum* lectin cannot be considered as a tuber-specific protein in the strict sense.

Southern Blot Analysis

Sequence analysis of different lectin cDNA clones of *Arum* revealed minor differences in their sequences. These

results taken together with the complex isolectin patterns revealed by ion-exchange chromatography and gel electrophoresis under denaturing conditions suggested that the *Arum* lectin is encoded by multiple genes. To demonstrate the occurrence of a lectin gene family in *Arum*, genomic DNA was subjected to Southern blot analysis. As shown in Figure 13, hybridization of a blot containing restriction fragments of *Arum* with a labeled lectin cDNA clone reveals a complex pattern of bands. Since none of the restriction enzymes used except *Bam*HI cleaves in the coding region of the genes, the results of this Southern blot analysis indicate that the lectin is encoded, indeed, by a small family of genes.

DISCUSSION

During the last few years several novel lectins with interesting properties have been isolated and characterized from species belonging to the class Monocotyledoneae. The Man-binding lectins (and lectin genes) from the plant families Amaryllidaceae, Alliaceae, and Orchidaceae have in particular been intensively studied, since they represent a superfamily of evolutionary well-conserved proteins with unique biological, physiological, and molecular biological properties (Van Damme et al., 1991b, 1992a, 1992b, 1993a, 1993b, 1994a, 1994b, 1995). Moreover, the finding that several members of this lectin family exhibited insecticidal activity in feeding experiments with both artificial diets and transgenic plants (Powell et al., 1993; Gatehouse et al., 1995) not only suggested that they are involved in the plant's defense but also demonstrated their potential use in crop protection against phytophagous invertebrates.

In 1990 Sandhu et al. reported the occurrence of lectin activity in extracts from several representatives of the Araceae. More recently, Singh et al. (1993) described the isolation and partial characterization of an asiolofetuin-binding lectin from tubers of *Alocasia indica*. Until now, however, few efforts have been made to purify and characterize other araceous lectins or to clone Araceae lectin genes. Since preliminary experiments with the *Arum* lectin indicated a resemblance to the previously isolated Man-binding lectins, it seemed worthwhile to characterize both the protein and its corresponding gene(s) to check its possible sequence homology with the different members of the superfamily of monocotyledonous Man-binding proteins.

Isolation and characterization of the Araceae lectins from *A. maculatum*, *C. esculenta*, *X. sagittifolium*, and *D. sequina* demonstrated that they are—like the previously reported Man-binding lectins from Amaryllidaceae, Alliaceae, and Orchidaceae species—composed of subunits of approximately 12 kD. Similar to the previously described Man-binding lectins the Araceae lectins exhibit agglutination activity with rabbit erythrocytes but are completely nonreactive with human red blood cells. Although the Araceae lectins strongly bind to a column of Man-Sepharose 4B in the presence of ammonium sulfate, their agglutination activity is only poorly inhibited by low concentrations of Man, which suggests that they bind preferentially more complex Man-rich oligosaccharides. As already mentioned above, some other members of the superfamily of Man-binding monocotyledonous lectins, such as the garlic (*A. sativum*) and ramsons (*A. ursinum*) lectins, also behave very similarly with respect to their specificity toward Man (Van Damme et al., 1991a). Furthermore, since the *Arum* lectin shows a striking sequence homology to the previously reported Man-binding lectins from the Amaryllidaceae, Alliaceae, and Orchidaceae species, we believe that it belongs to the same superfamily of monocot lectins.

N-terminal sequencing of the 12-kD lectin polypeptides from *Arum* revealed a double amino acid sequence, suggesting that two different lectin polypeptides of the same size are present in the *Arum* lectin. Molecular cloning of the *Arum* lectin has shown that both lectin subunits are synthesized on one large precursor that is posttranslationally cleaved into the mature lectin polypeptides. Although sequence alignment of the two lectin subunits revealed only 40% sequence homology, it is clear that some parts of the sequence are identical. In this regard the *Arum* lectin resembles the heterodimeric garlic (*A. sativum* L.) lectin. The two different polypeptides of the latter lectin are also derived from a single precursor, which is translated from an mRNA of 1400 nucleotides and contains two very homologous (80%) domains (which coincide with the mature lectin polypeptides) separated from each other by an intervening sequence of 30 amino acids (Van Damme et al., 1992a).

A search for sequence homology to the N-terminal amino acid sequences of the *Arum* lectin revealed a striking homology to a 12-kD storage protein from taro (*C. esculenta*), which was reported to be corm specific and highly regulated during tuber development (de Castro et al., 1992; Hirai et al., 1993). Since the N-terminal sequence of the lectin we isolated from *C. esculenta* tubers revealed a sequence identical with that reported for the taro storage protein and, in addition, the molecular cloning of the *Arum* lectin revealed a high degree of sequence homology to the

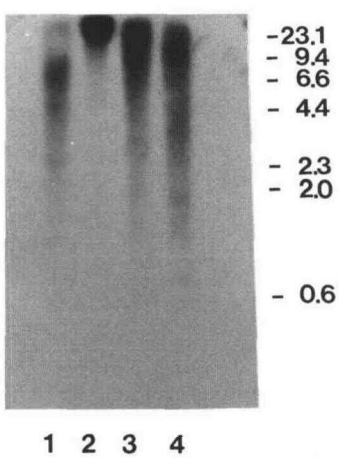

Figure 13. Southern blot of genomic DNA isolated from leaves of *A. maculatum*. DNA was digested with *Eco*RI (lane 1), *Pst*I (lane 2), *Bam*HI (lane 3), or *Hind*III (lane 4) and hybridized with the ^{32}P-labeled cDNA insert LECAMA1. Numbers on the right show DNA size (kb).

Colocasia storage protein cDNA, it can only be concluded that the presumed taro storage protein is a Man-binding lectin. The same holds true for the other major aroid food crop *Xanthosoma*. Although the lectin gene of this species has not been cloned, the N-terminal sequences of the lectin that we isolated from the *Xanthosoma* tubers as well as its serological identity to the *Colocasia* lectin leave no doubt that in this species the major tuber (storage) protein is also a Man-binding lectin. Finally, it should be mentioned that the agglutination properties as well as the molecular structure of the lectin isolated from *Alocasia* tubers indicate that this agglutinin closely resembles the above-described Araceae lectins. Moreover, since approximately one-tenth of the total *Alocasia* tuber protein was retained on the affinity matrix, the lectin is apparently a major (storage) protein in this tissue (Singh et al., 1993).

The obvious similarities in carbohydrate-binding specificity and sequence homologies between the Araceae lectins and the previously described Man-binding monocotyledonous lectins indicate that the Araceae lectins also belong to the same superfamily of proteins as the Amaryllidaceae, Alliaceae, and Orchidaceae lectins. It appears, therefore, that most of the monocotyledonous lectins that hitherto have been identified belong to a single group of evolutionarily well-conserved proteins.

A final point to discuss concerns the abundance of the Araceae lectins in the tuber (storage) tissue. Since in *Arum*, as well as in *Colocasia* and *Xanthosoma* (and perhaps in *Alocasia* as well), the lectin is a major storage protein, the question of its physiological role has to be addressed. By analogy to the function of other plant lectins that are found in vegetative storage organs, it is possible that the Araceae tuber lectins are major storage proteins that in addition to their storage role can be recruited for a defense-related function when necessary (Peumans and Van Damme, 1993).

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