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The closely related homomeric and heterodimeric mannose-binding lectins from garlic are encoded by one-domain and two-domain lectin genes, respectively

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Lectin cDNA clones for two different lectins from garlic (*Allium sativum* L.) bulbs, ASAI and ASAII (ASA, *Allium sativum* agglutinin), were isolated and characterized. The first lectin, ASAI, is a heterodimer composed of two different subunits of 11.5 kDa and 12.5 kDa. It is translated from an mRNA of 1400 nucleotides encoding a polypeptide of 306 amino acids with two very similar domains. N-terminal sequencing of the two polypeptides of the mature lectin confirmed that both subunits are derived from the same precursor and that each corresponds to one of the two domains in the sequence.

In contrast to ASAI, the second garlic lectin, ASAII, is a homodimer of two identical 12-kDa subunits. It is translated from an mRNA of approximately 800 nucleotides encoding a polypeptide of 154 amino acids. Interestingly, the coding region of the ASAII cDNA clones is almost identical to that of the second domain of the ASAI cDNA clones.

Plant lectins are a very heterogeneous group of (glyco)-proteins with respect to their biochemical and physicochemical properties, as well as their carbohydrate-binding specificity. Recently, we described the isolation and characterization of a mannose-specific lectin from the bulbs of *Allium sativum* (garlic), a representative of the plant family Alliaceae (Van Damme et al., 1991a). Although this lectin was shown to be serologically related to the previously isolated Amaryllidaceae lectins from snowdrop, daffodil and amaryllis (Van Damme et al., 1987, 1988), its molecular structure was different. In contrast to the Amaryllidaceae lectins which are composed of identical 12.5-kDa subunits the garlic lectin apparently consists of two different subunits of 11.5 kDa and 12.5 kDa.

Since garlic lectin preparations clearly contain two types of subunits with slightly different molecular masses, the question arises as to whether these two lectin polypeptides are encoded by different genes or, alternatively, are products of the same gene, e.g. by different post-translational modifications. In order to answer this crucial question, the lectin genes were cloned and analyzed. As a result of this investigation, we found that the molecular structure of the garlic lectin is far more complex than it first appeared. Indeed, as described below, garlic contains two different types of lectins (further referred to as ASAI and ASAII) which are encoded by different genes. It should be emphasized here that the first indication for the occurrence of two different lectins in garlic was obtained from the cloning and sequencing of two different types of cDNA clones. Based on molecular-structure predictions deduced from the nucleotide sequences, a procedure was worked out to separate both lectins.

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Abbreviations. ASA, *Allium sativum* agglutinin; PCR, polymerase chain reaction.

Note. The nucleotide sequence data reported in this paper have been submitted to the Genbank™/EMBL data library with the accession numbers M85171, M85172, M85173, M85174, M85175, M85176 and M85177. The novel amino acid sequence data published here have been submitted to the EMBL sequence data bank(s).

EXPERIMENTAL PROCEDURES

Materials

Bulbs of garlic (*A. sativum* L.) were purchased from a local store. Oligodeoxythymidine cellulose was purchased from Sigma Chemical Co. (St Louis, MO). Radioisotopes were obtained from Amersham Corp. A cDNA synthesis kit, the multifunctional phagemid pT_7T_318U, restriction enzymes and DNA modifying enzymes were obtained from Pharmacia LKB Biotechnology Inc. *Escherichia coli* XL1 Blue competent cells were purchased from Stratagene (La Jolla, CA). *Taq* DNA polymerase was obtained from Boehringer (Mannheim, Germany).

RNA isolation

Total cellular RNA was prepared from bulbs and shoots of garlic (stored at −80 °C) essentially as described by Finkelstein and Crouch (1986). Poly(A)-rich RNA was enriched by chromatography on oligo-deoxythymidine cellu-

lose as described by Siflow et al. (1979), except that poly(A)-rich RNA was eluted at room temperature.

Protein synthesis in wheat-germ cell-free extract

Poly(A)-rich RNA was translated in a wheat-germ cell-free extract as described previously (Van Damme et al., 1991b). 1 ml incubation mixture for cell-free translation contained 0.4 ml extract, 20 mM Hepes KOH, pH 8.0, 90 mM potassium acetate, 2.5 mM magnesium acetate, 1 mM ATP, 20 μM GTP, 25 μg/ml creatine kinase, 8 mM creatine phosphate, 2 mM dithiothreitol, 0.4 mM spermidine, 19 unlabeled amino acids (100 μM each) and 50 μCi [^{35}S]methionine. Prior to adding mRNA (30 μg/ml final concentration) and labeled amino acids, the cell-free translation system was incubated for 15 min at room temperature to reduce the endogenous template activity of the system. Subsequently, the reaction mixtures were incubated for 1 h at 25 °C, and the incorporation of ^{35}S-labeled amino acid into trichloroacetic-acid-insoluble material determined.

Isolation and characterization of the *in-vitro*-synthesized lectin polypeptides

Lectin isolation from the *in-vitro*-translation mixture was accomplished either by affinity chromatography on mannose–Sepharose-4B or immunoprecipitation. Affinity chromatography on mannose–Sepharose-4B was carried out in the presence of 1 M ammonium sulphate (as described by Van Damme et al., 1988) or 1.5 mM KH_2PO_4, 10 mM Na_2HPO_4, 3 mM KCl and 140 mM NaCl, pH 7.4 (buffer A). The lectin was desorbed using unbuffered 20 mM 1,3-diaminopropane.

Small aliquots were withdrawn for determination of [^{35}S]methionine incorporation. Subsequently the lectin was analyzed by SDS/PAGE on a 12.5–25% acrylamide gradient gel using a discontinuous system, as described by Laemmli (1970). After fixing and destaining, gels were immersed in 1 M sodium salicylate for 30 min, dried under partial vacuum and exposed to X-ray films (Fuji RX, Japan) at −80 °C.

Amino acid sequence analysis

Protein sequencing was conducted on an Applied Biosystems (Foster City, California, USA) model 477A protein sequencer interfaced with an Applied Biosystems model 120A on-line analyzer.

Construction and screening of cDNA library

cDNA libraries were constructed from total poly(A)-rich mRNA isolated from young and old garlic bulbs, as well as young garlic shoots, using the cDNA synthesis kit from Pharmacia. cDNA fragments were inserted into the EcoRI site of the multifunctional phagemid $pT_7T_3$18U. The library was propagated in *E. coli* XL1 Blue.

Recombinant lectin clones were screened by colony hybridization using a ^{32}P-end-labeled degenerate oligonucleotide probe (17 nucleotides; 5′-AT$^{A}/_{G}$ AA$^{A}/_{G}$ TG$^{A}/_{G}$ TAN GG$^{T}/_{C}$ TC-3′) derived from residues 19–24 of the N-terminal protein sequence determined for one isolectin (Kaku et al., 1991). Hybridization was carried out overnight at 40 °C in 0.9 M sodium chloride containing 90 mM Tris HCl, pH 7.5, 6 mM EDTA, 10 × Denhardt's solution (Denhardt et al., 1978), 0.1% SDS, 180 μg/ml hydrolyzed yeast RNA and 2 × 10^6 cpm/ml ^{32}P-labeled probe. After hybridization, filters were washed four times in 6 × NaCl/Cit (NaCl/Cit: 0.9 M sodium chloride and 0.09 M sodium citrate, pH 7.0) at room temperature for 15 min, followed by a 5-min wash at hybridization temperature in 6 × NaCl/Cit. Filters were blotted dry, wrapped in Saran Wrap and exposed to Kodak X-Omat S film at −80 °C. Colonies producing 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 Maniatis et al. (1982). Approximately 3 μg poly(A)-rich RNA was denatured in glyoxal and Me_2SO and separated in a 1.2% (mass/vol.) agarose gel. Following electrophoresis, the RNA was transferred to nitrocellulose. Hybridization was carried out as described above for screening of the cDNA library using ^{32}P-labeled synthetic oligonucleotide probes. Alternatively, blots were hybridized using random-primer-labeled cDNA insert. In the latter case, blots were prehybridized for 4 h at 42 °C in 50% formamide, 5 × NaCl/P_i/EDTA (NaCl/P_i/EDTA: 0.18 M sodium chloride, 10 mM sodium phosphate buffer, pH 7.4, 1 mM EDTA), 5 × Denhardt's solution (Denhardt et al., 1978), 10% dextran sulphate and 50 μg/ml denatured salmon sperm DNA and hybridized in the same solution containing 2 × 10^6 cpm/ml ^{32}P-labeled DNA insert from lectin cDNA clones. Hybridization was performed overnight at 42 °C. Afterwards blots were washed consecutively in 5 × NaCl/P_i/EDTA (15 min at 42 °C), NaCl/P_i/EDTA containing 0.1% SDS (30 min at 42 °C) and 0.1 × NaCl/P_i/EDTA containing 0.1% SDS (15 min at room temperature). An RNA ladder (0.16–1.77 kb) was used as a marker.

Genomic DNA analysis

Total DNA from garlic was isolated according to the procedures described by Dellaporta et al. (1983) and de Kochko (1990). The DNA preparation was treated with RNase in order to remove any contaminating RNA. Approximately 30 μg DNA was digested with restriction endonucleases and subjected to electrophoresis in a 0.8% agarose gel. DNA was transferred to Immobilon N membranes (Millipore, Bedford, USA) and hybridized using the ^{32}P-labeled cDNA insert LECASAI 2 encoding the garlic lectin ASAI. Hybridization was carried out at 68 °C for 16 h in 6 × NaCl/Cit containing 0.5% SDS, 5 × Denhardt's solution (Denhardt et al., 1978) and 100 μg/ml denatured salmon sperm DNA. After prehybridization for 4 h the ^{32}P-labeled DNA (2 × 10^6 cpm/ml) was added to this mixture. Following hybridization the membrane was washed consecutively in 2 × NaCl/Cit (30 min at 68 °C), 2 × NaCl/Cit containing 0.1% SDS (30 min at 68 °C) and 0.5 × NaCl/Cit (10 min at 68 °C).

Polymerase chain reaction (PCR)

The reaction mixture for amplification of genomic DNA sequences contained 10 mM Tris HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 100 μg/ml gelatin, 0.4 mM of each dNTP, 2.5 U *Taq* DNA polymerase, 100–1000 ng genomic (RNA-free) DNA and 720 ng of each of two appropriate primer mixtures in a 100-μl reaction volume. The reaction was over-

laid with 75 µl mineral oil. After denaturation of the DNA for 5 min at 95 °C amplification was performed for 30 cycles through a regime of 1 min template denaturation at 92 °C, followed by primer annealing for 1 min at 55 °C and primer extension for 3 min at 72 °C using an automatic thermal cycler. The PCR primers were derived from both ends of the coding region of cDNA clones encoding ASAI and ASAII. Restriction sites were introduced at the 5′ end of each primer to facilitate cloning of the PCR fragments. The high sequence similarity between both lectin genes at the C-terminal indicated that the primer was identical for ASAI and ASAII, 5′-GCC CTA GGC AAG CAG CAC CGG CCA GAG T-3′. The primers for the N-termini of the coding regions of LECASAI 2 and LECASAII 1 were 5′-CGG GAT CCG AAT GGG CCG TAA TAC TCC A-3′ and 5′-CGG GAT CCT AAT GAG CAT AGC CAC TGT A-3′, respectively. PCR products were analyzed by agarose gel electrophoresis.

Computer analyses

DNA sequences were analyzed using programs from PC Gene and Genepro.

RESULTS

Construction of cDNA libraries

Since preliminary experiments indicated that the lectin concentration in garlic was developmentally regulated, poly(A)-rich RNA was isolated from three different tissues, young shoots and both old and young developing bulbs, and used to construct a cDNA library. Initially the cDNA libraries were screened using a degenerate oligonucleotide probe (17 nucleotides, 5′-AT$^{A}/_{G}$ AA$^{A}/_{G}$ TG$^{A}/_{G}$ TAN GG$^{T}/_{C}$ TC-3′) derived from amino acids 19–24 of the N-terminal protein sequence determined for one isolectin of garlic (Kaku et al. 1991). At a later stage, a lectin cDNA insert was used to screen the cDNA libraries.

In contrast to the cDNA library constructed with mRNA from shoots and old bulbs, which contained no or very few lectin sequences, respectively, about 4% of the bacterial colonies containing the cDNA library constructed with mRNA from young developing bulbs reacted positively with the oligonucleotide probe, indicating that they contained lectin sequences. Thus, young bulbs seem to accumulate high concentrations of mRNA encoding the garlic lectin.

Characterization of lectin cDNA clones with a 940 bp open reading frame

The nucleotide sequence of LECASAI 1 and the deduced amino acid sequences of several cDNA clones selected by colony hybridization are depicted in Fig. 1. Although several lectin clones were isolated which definitely differ from each other at certain positions within their nucleotide sequences and deduced amino acid sequences all clones show a high degree of overall similarity within both their coding sequence and their 3′ untranslated region.

The lectin cDNA clone LECASAI 1 contains an open reading frame of 940 bp with three possible initiation codons at positions 8, 18 and 35 of the deduced amino acid sequence (Fig. 2). According to the criteria of Kozak (1981), the first initiation codon is most likely to be used since there is a purine at position −3 and a G at position +4 relative to the A from the ATG codon. Translation starting with this ATG codon

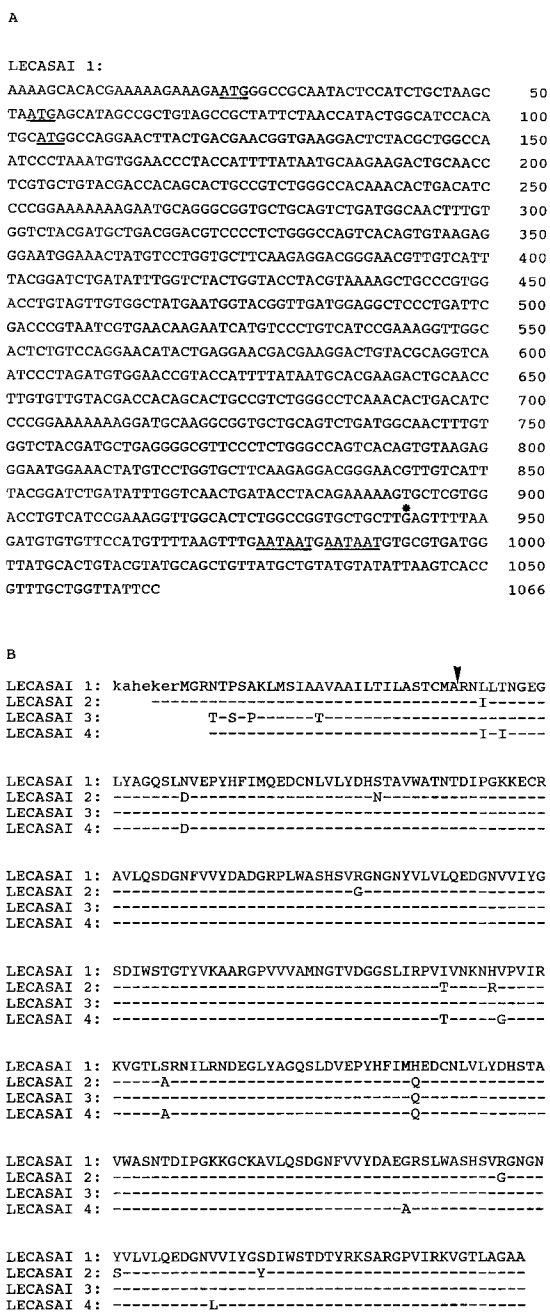

Fig. 1. Nucleotide sequence of LECASAI 1 and deduced amino acid sequences of LECASAI 1–4. (A) Nucleotide sequence of cDNA clone LECASAI 1 encoding the garlic lectin ASAI. Putative start codons and polyadenylation signals are underlined. The stop codon is indicated by an asterisk. (B) Comparison of deduced amino acid sequences of different cDNA clones for the garlic lectin ASAI. The arrowhead indicates the possible processing site for the cleavage of the signal peptide. (–) Sequence similarity to LECASAI 1. Since the first ATG codon is most probably used as the translation-initiation site, the deduced amino acids preceding this methionine are shown in lower case letters.

generates a 306-amino-acid polypeptide with a calculated molecular mass of 33020 Da. The 3′ untranslated region of the clone contains three in-frame stop codons and two putative polyadenylation signals (Dean et al., 1986; Fig. 1).

A more detailed study of the lectin cDNA sequence reveals that the lectin clone consists of two domains which share 91%

kahekerMGRNTPSAKLMSIAAVAAILTILASTCMARNLLTNGEGLYAGQSLNVEPYHF
RNLLTNGEGLYAGQSLDVE

IMQEDCNLVLYDHSTAIWSTNTDIPGKKECRAVLQSDGNFVVYDADGRPLWASHSVRGN

GNYVLVLQADGNVVIYGSDIWSTGTYVKAARGPVVVAMNGTVDGGSLIRPVIVNKNHVP

VIRKVGTLSRNILRNDEGLYAGQSLDVEPYHFIMHEDCNLVLIDHNTAVWASNTDIPGK
TLARNILRNDEGLYAGQVLDVE

KGCKAVLQSDGNFVVYDAEGRSLWASHSVRGNGNYVLVLQEDGNVVIYGSDIWSTDTYR

KSARGPVIRKVGTLAGAA

Fig. 2. Deduced amino acid sequence of lectin cDNA clone LECASAI 1. Each of the two domains in the lectin sequence is indicated by the bar above the sequence. The N-terminal sequence of the two mature lectin polypeptides is shown below the corresponding deduced sequence. A putative glycosylation site is denoted by an asterisk. The arrowhead indicates the putative processing site for the signal peptide. Since the first ATG codon is most probably used as the translation initiation site, the deduced amino acids preceding this methionine are shown in lower case letters.

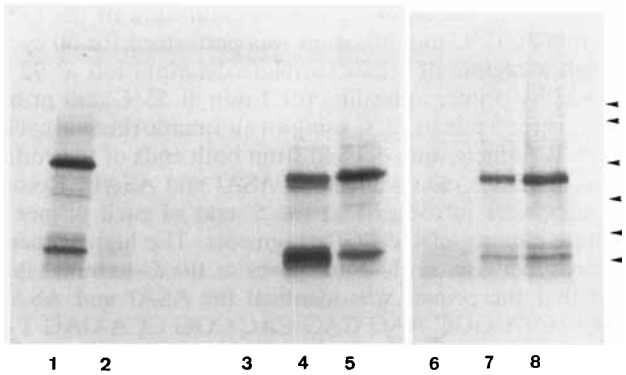

Fig. 3. Fluorograph of translation products of poly(A)-rich RNA from young garlic bulbs in a wheat-germ cell-free system. Lane 1, total translation products synthesized in response to poly(A)-rich RNA from garlic; lane 2, control experiment (no mRNA added); lane 3, immunoprecipitate of translation products from a control experiment using the antiserum against the garlic lectin; lanes 4 and 5, immunoprecipitates of translation products from poly(A)-rich RNA from garlic in buffer A (lane 4) or ammonium sulphate (lane 5); lane 6, affinity-purified translation products from control experiment; lanes 7 and 8, affinity-purified lectin polypeptides from translation products of a wheat-germ cell-free system supplemented with RNA from garlic using buffer A (lane 7) or ammonium sulphate (lane 8) as running buffer. The marker proteins are lysozyme (14 kDa), soybean trypsin inhibitor (20 kDa), carbonic anhydrase (30 kDa), ovalbumin (43 kDa), bovine serum albumin (67 kDa) and phosphorylase *b* (94 kDa).

and 84% similarity at the nucleotide deduced amino acid levels, respectively. Both domains are separated by an amino acid sequence containing a putative glycosylation site (Fig. 2).

Processing of the ASAI preproprotein, deduced from the nucleotide sequence of the lectin cDNA clone and the N-terminal amino acid sequence of the mature lectin polypeptides

Since the size of the polypeptide encoded by the cDNA clones exceeds that of the mature lectin polypeptides by a factor of two the primary translation product of these lectin genes must undergo a series of post- translational modifications. Using the rules for protein processing of von Heijne (1986), one possible cleavage site for the signal peptide was identified between residues 36 and 37 of the deduced amino acid sequence of the lectin clone LECASAI 1. Cleavage at this site is consistent with the N-terminal amino acid sequence of the mature protein (Kaku et al., 1991) and will result in a precursor lectin with an apparent molecular mass of 30112 Da. However, since the mature lectin was found to be composed of two different subunits of approximately 11.5 kDa and 12.5 kDa (Van Damme et al. 1991b), determined by SDS/PAGE of the purified lectin, further processing of the lectin precursor is likely to occur. Since at that time it was already evident that the garlic lectin is a mixture of two different molecular forms encoded by mRNA of different size, both ASAI and ASAII were purified and the N-termini of their polypeptides determined. As shown in Fig. 2, the N-terminal amino acid sequences of the two mature ASAI polypeptides demonstrate very high sequence identity to the deduced amino acid sequence of the lectin cDNA clone LECASAI 1. Whereas the N-terminal sequence of the 11.5-kDa polypeptide coincides with the amino acid sequence deduced from residues 37–55, the N-terminal sequence of the 12.5-kDa polypeptide is almost identical to residues 184–205 from the lectin clone LECASAI 1, indicating that both subunits are derived from a single large precursor. Apparently, each lectin subunit corresponds to one domain of the lectin cDNA sequence.

In vitro translation of poly(A)-rich RNA from garlic in a wheat-germ cell-free system

Poly(A)-rich RNA isolated from old and young developing bulbs of *A. sativum* was translated in a wheat-germ cell-free system and the lectin isolated either by immunoprecipitation or affinity chromatography on mannose–Sepharose-4B. As shown in Fig. 3, two polypeptide bands, with estimated molecular masses of 36 kDa and 17 kDa, were purified from the translation mixture supplemented with poly(A)-rich RNA from young developing bulbs by either immunoprecipitation or affinity chromatography, whereas in the translation mixture containing RNA from old bulbs, no mannose-binding proteins could be detected. These results indicate that two proteins were purified from the translation mixture both by affinity chromatography (indicating that both proteins are mannose binding) and by immunoprecipitation (which suggests a serological relationship between the proteins). It should be noted that these two polypeptides represent the most prominent proteins among the translation products synthesized in response to poly(A)-rich RNA from young garlic bulbs indicating that the RNA encoding these proteins are abundant at this developmental stage.

Whereas the occurrence of the 36-kDa mannose-binding protein in the *in vitro* translation mixture can easily be explained, since it corresponds in size to the preprolectin expected on the basis of the nucleotide sequence of the lectin cDNA LECASAI, there is, at this point, no plausible explanation for the 17-kDa polypeptide which is so abundant among the *in-vitro*-synthesized polypeptides. As discussed below, however,

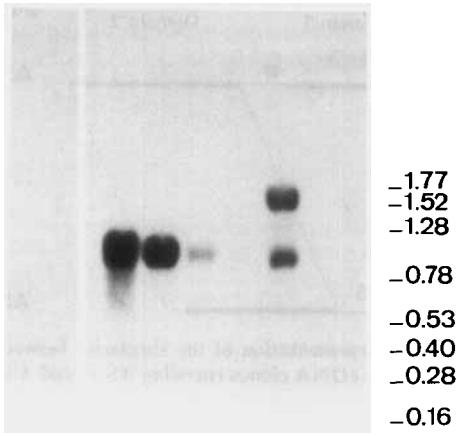

Fig. 4. Northern blot of poly(A)-rich RNA isolated from Amaryllidaceae and Alliaceae species. Poly(A)-rich RNA was isolated from ovaries of snowdrop (lane 1), daffodil (lane 2), amaryllis (lane 3) and young garlic bulbs (lane 4). The blot was hybridized using the ^{32}P-labeled cDNA insert from LECASAI 2. The numbers on the right show RNA size (kb).

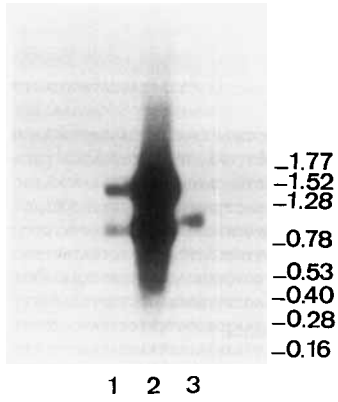

Fig. 5. Northern blot of poly(A)-rich RNA isolated from different tissues of garlic. RNA was isolated from old bulbs (lane 1), young bulbs (lane 2) and shoots (lane 3). The blot was hybridized using the ^{32}P-labeled cDNA insert from LECASAI 2. The numbers on the right show RNA size (kb).

this finding, in combination with the results of Northern-blot analysis of garlic mRNA, led to the conclusion that there are two types of garlic lectin.

Northern blot analysis

Nothern-blot analysis of garlic mRNA, using the degenerate oligonucleotide probe derived from the N-terminal sequence of the protein, revealed a single band of approximately 1400 nucleotides. However, when the same blot was screened using the cDNA insert from garlic lectin clone LECASAI 2, an additional signal of approximately 800 nucleotides was detected (Fig. 4). Interestingly the second signal corresponds in size to the length of the mRNA encoding the Amaryllidaceae lectins (Van Damme et al., 1991 b). This result was identical when a lectin cDNA insert from snowdrop was used.

Northern-blot analysis of mRNA isolated from different tissues revealed very strong hybridization signals in the young bulb for both the large (1400 nucleotides) and the small (800 nucleotides) mRNA. Both signals were also detected in the mRNA isolated from the old bulb, although at much lower concentration. Although no lectin cDNA clones could be isolated from the cDNA library constructed from RNA isolated from garlic shoots, a weak hybridization signal of approximately 850 nucleotides could be detected in the lane containing poly(A)-rich RNA isolated from shoots (Fig. 5).

The results of the Northern-blot analysis leave no doubt that garlic contains an mRNA which is about half the size of that of the mRNA encoding the lectin ASAI, but nevertheless must have a considerable similarity to the ASAI mRNA. Moreover, the presence of this mRNA of 800 nucleotides can account for the 17-kDa mannose-binding polypeptide that was found among the *in-vitro*-translation products synthesized under the direction of garlic bulb mRNA in a wheat-germ cell-free system. To determine whether both mRNA code for related proteins, we searched for cDNA clones corresponding to this small mRNA.

Isolation and characterization of cDNA clones for ASAII with an open reading frame of 467 bp

A differential screening, using the oligonucleotide probe which was used to screen for ASAI lectin cDNA clones and a garlic lectin cDNA insert encoding ASAI, was set up to isolate cDNA clones translated from the RNA of 800 nucleotides reactive on the Northern blot. Since the cDNA clones derived from this mRNA were unreactive with the oligonucleotide probe, but reacted strongly with the lectin insert under very stringent conditions, it was possible to discriminate between the clones derived from the small 800 nucleotides mRNA and the lectin clones encoded by the mRNA of 1400 nucleotides.

Sequence analysis of the cDNA clones resulting from the differential screening revealed a protein sequence in which a signal sequence is flanked by a sequence almost identical to that of the second domain, including the C-terminal sequence of ASAI lectin clones (Figs 6–8). The cDNA clone LECASAII 1 encodes an open reading frame of 155 amino acids with three putative ATG start codons. Translation starting with the first initiation codon, which conforms to the rules of Kozak (1981), results in a 154-amino-acid precursor polypeptide (16 743 Da). Following the rules of von Heijne (1986), the signal peptide is likely to be processed between amino acid residues 28 and 29 of the deduced amino acid sequence of the cDNA clone LECASAII 1 and will result in a 13 947-Da polypeptide.

Southern blot analysis

Digestion of genomic DNA isolated from *A. sativum* bulbs, using various restriction enzymes (BamHI, BglII, EcoRI, HincII and HindIII), revealed a complex pattern of bands after hybridization of the blot with the lectin cDNA insert from LECASAI 2 (Fig. 9). Similar results were obtained when hybridization of the blot was performed using an oligonucleotide probe (which will only react with ASAI). Since none of the restriction enzymes used cleaves within the lectin insert, these results suggest that the garlic lectin is encoded by multiple genes.

PCR analysis of genomic DNA

Starting from genomic DNA, the coding region of both garlic lectins was amplified using PCR. The amplification of DNA, using specific primers derived from both ends of the transcribed region of the genes encoding ASAI and ASAII, yielded single bands of approximately 930 bp and 450 bp,

A

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LECASAII 1:
AAAGAATGACTACTCCATCTCCTAAAGTAATGAGCATAGCCACTGTAGCC    50
GCTATTCTAACCATATGGGCATCCACATGCATGGCCAGGAACATACTGAT    100
GAACGACGAAGGTCTGTACGCAGGCCAATCCCTAGATGTGGAACCGTACC    150
ATCTTATAATGCAGGAAGACTGCAACCTCGTGCTGTACGACCACAGCACT    200
GCCGTCTGGACCTCAAACACTGACATCCCCGGAAAAAAAGGATGCAAGGC    250
GGTGCTGCAGTCTGATGGCAACTTTGTGGTCTACGATGCCGAAGGACGTT    300
CCCTCTGGGCCAGTCACAGTGTAAGAGGGAATGGAAACTATGTCCTGGTG    350
CTTCAAGAGGACGGTAACGTTGTCATTTACGGATCTGATATTTGGTCTAC    400
TAATACCTACAAAGGAAGTGCTCGTGGACCTGTCATCCGAAAGGTTGGCA    450
CTCTGGCCGGTGCTGCTTGAGTTTTAGGATGTGTGTTCCATGTTTTAAGT    500
TTGAATAATGAATAATGAATAATGTGCGTGATGGTGATGCACTGTACTTA    550
TGTCAACTGTTATGCTGCATTATATATATTAAGCACAGTTTGCTGGTTA    599
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B

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LECASAII 1: rMTTPSPKVMSIATVAAILTIWASTCMARNILMNDEGLYAGQSLD
LECASAII 2:             ----------L----------------------
LECASAII 3:     ----------------L----------------------

LECASAII 1: VEPYHLIMQEDCNLVLYDHSTAVWTSNTDIPGKKGCKAVLQSDGN
LECASAII 2: ----------------------A--------------------
LECASAII 3: ----------------------------------------------

LECASAII 1: FVVYDAEGRSLWASHSVRGNGNYVLVLQEDGNVVIYGSDIWSTNT
LECASAII 2: ----------------------------------------------
LECASAII 3: ----------------------------------------------

LECASAII 1: YKGSARGPVIRKVGTLAGAA
LECASAII 2: --------------------
LECASAII 3: --------------------
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Fig. 6. Nucleotide sequence of LECASAII 1 and deduced nucleotide sequences of LECASAII 1 – 3. (A) Nucleotide sequence of cDNA clone LECASAII 1 encoding the second garlic lectin ASAII. Putative start codons and polyadenylation signals are underlined. The stop codon is indicated by an asterisk.(B) Comparison of deduced amino acid sequences of different cDNA clones encoding the second garlic lectin ASAII. The arrowhead indicates the possible cleavage site of the signal peptide. (–) Sequence similarity to LECASAII 1. Since the first ATG codon is most probably used as the translation-initiation site, the deduced amino acid preceding this methionine is shown in lower case letter.

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rMTTPSPKVMSIATVAAILTIWASTCMARNILMNDEGLYAGQSLDVEPYHLIMQEDCNL
                          RNILMNGEGLYAGQSLDVEP

VLYDHSTAVWTSNTDIPGKKGCKAVLQSDGNFVVYDAEGRSLWASHSVRGNGNYVLVLQ

EDGNVVIYGSDIWSTNTYKGSARGPVIRKVGTLAGAA
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***Fig.* 7. Deduced amino acid sequence of lectin cDNA clone LECASAII 1.** The arrowhead indicates the possible processing site for the cleavage of the signal peptide. The domain in the sequence similar to the two domains found in LECASAI clones is indicated by the thick line. The N-terminal sequence of the mature lectin polypeptide is shown in the second line. Since the first ATG codon is most probably used as the translation-initiation site, the deduced amino acid preceding this methionine is shown in lower case letter.

respectively. Subsequent sequencing of the amplified DNA showed that no introns are present in the coding region of these proteins, indicating that the coding region of the lectin is encoded entirely by a single exon. Hence, the two mannose-binding lectins from garlic are the results of the expression of different genes.

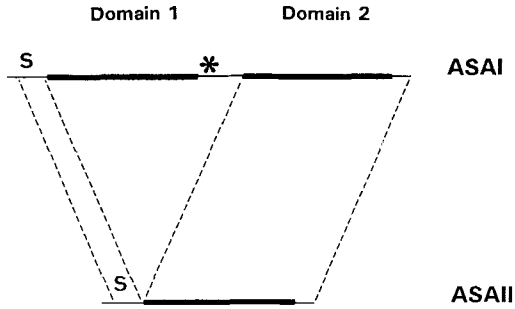

Fig. 8. Schematic representation of the similarity between the coding regions of the lectin cDNA clones encoding ASAI and ASAII.

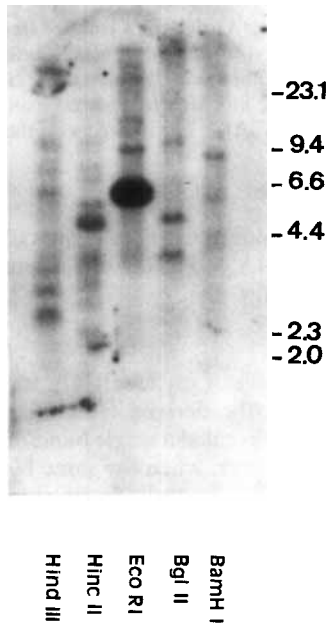

Fig. 9. Southern blot of genomic DNA isolated from garlic bulbs. DNA was digested with HindIII, HincII, EcoRI, BglII and BamHI and hybridized with the ^{32}P-labeled cDNA insert from LECASAI 2. Numbers on the right show DNA size (kb).

Isolation and partial characterization of ASAI and ASAII

Due to the apparent need for pure preparations of both garlic lectins, a method was developed to separate ASAI and ASAII. Since ASAI and ASAII are complex mixtures of isolectins, all efforts to accomplish a separation by either ion-exchange chromatography or hydrophobic-interaction chromatography failed. Therefore, attempts were made to resolve the two molecular forms of the garlic lectin by affinity chromatography under different conditions. Eventually, after numerous trials a procedure based on repeated affinity chromatography steps was developed, which allowed complete separation of ASAI and ASAII in reasonable quantities. Briefly, an extract of garlic bulbs made in 0.2 M NaCl was adjusted to pH 6.5 and centrifuged (20 000 *g* for 20 min). The supernatant solution was filtered (Whatman 3 MM) and applied to a column of mannose – Sepharose-4B equilibrated with 0.2 M NaCl. Under these conditions, only ASAI binds to the column. After thorough washing of the column with 0.2 M NaCl, ASAI was desorbed with 50 mM sodium tetraborate (pH 9.0). To remove any contaminating ASAII, the fraction desorbed with sodium tetraborate was adjusted to pH 6.5,

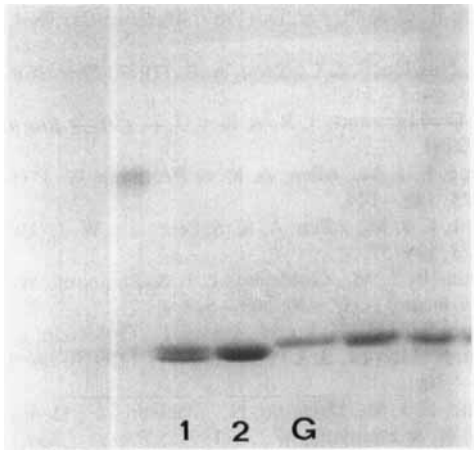

Fig. 10. SDS/PAGE of purified garlic lectins. About 30 μg of the purified lectins ASAI (lane 1) and ASAII (lane 2) was loaded into each slot. The lectin from snowdrop was used as a marker protein (lane G).

brought to 0.2 M NaCl and rechromatographed on the mannose–Sepharose-4B column. After the second affinity-chromatography step, the ASAI preparation is essentially pure, as could be judged by SDS/PAGE (Fig. 10).

To isolate ASAII, the fraction which ran through the mannose–Sepharose-4B column in 0.2 M NaCl was rechromatographed under the same conditions (to remove any ASAI) and subsequently adjusted to 1 M ammonium sulphate by adding the solid salt. After standing overnight in the cold, the solution was centrifuged and filtered as described above and applied to the mannose–Sepharose-4B column equilibrated with 1 M ammonium sulphate. Unbound proteins were eluted by washing the column with 1M ammonium sulphate until the A_{280} of the eluate fell below 0.01. Finally, ASAII was desorbed from the column with 0.2 M NaCl. The ASAII preparation obtained in this manner was essentially pure, as judged by SDS/PAGE (Fig. 10).

The purified ASAI and ASAII preparations were analyzed by SDS/PAGE and gel filtration. As shown in Fig. 10, SDS/PAGE of ASAI yields two polypeptide bands of 11.5 kDa and 12.5 kDa, whereas ASAII migrates as a single band of 12 kDa. Since the molecular mass of the native lectins determined by gel filtration on a Superose 12 column (using the snowdrop and the daffodil lectins as molecular mass marker proteins) was approximately 26 kDa for both ASAI and ASAII, it is evident that the former is a dimer built up of two different subunits, whereas the latter is a dimer composed of identical subunits.

To determine the N-terminal sequence of the lectin polypeptides, ASAI and ASAII were run on a SDS/polyacrylamide gel and blotted onto a nitrocellulose filter. After staining of blots, individual polypeptides were cut from the blot and used for automated amino acid sequencing. As shown in Figs 2 and 7, both ASAI polypeptides and the ASAII polypeptide yielded unique sequences which, in addition, correspond to the deduced amino acid sequences of the ASAI and ASAII cDNA clones.

DISCUSSION

This paper describes the isolation and characterization of cDNA clones for two mannose-binding lectins (ASAI and ASAII) from *A. sativum*, one of which (ASAI) is the lectin described recently by Van Damme et al. (1991a). Interestingly, the sequences of both mannose-binding proteins show a high degree of similarity both at the nucleotide level and at the deduced amino acid level. Whereas the lectin ASAI is composed of two domains interrupted by an amino acid sequence containing a putative glycosylation site, the sequence of the lectin ASAII contains only one domain and a C-terminal sequence. Compared to the previously cloned mannose-specific lectins from Amaryllidaceae species (Van Damme et al., 1991c), the garlic lectins ASAI and ASAII only show about 50% and 48% similarity, respectively, at the nucleotide level. Computer searches in the EMBL DNA sequence databases revealed no significant similarity of the garlic lectin cDNA sequences to any other known sequence.

Several lectin cDNA clones have been isolated from the cDNA library constructed from poly(A)-rich RNA isolated from young and old garlic bulbs which definitely differ from each other at certain positions within their sequence. However, in contrast to the cDNA clones encoding the Amaryllidaceae lectins, the garlic lectin clones show a higher degree of similarity both in their coding region and 3′ untranslated region. Furthermore, the overall similarity between the different lectin clones is much higher for the garlic lectin clones, being 95% or more. Similar to the Amaryllidaceae lectin clones, some of the differences in the sequence result in different charges along the polypeptide which can account for the occurrence of multiple lectin isoforms in garlic (Van Damme et al., 1991c). Taken together with the results from Southern blot analysis, this indicates that the garlic lectin is also encoded by a family of closely related genes.

Since the garlic lectin ASAI is translated from an mRNA of 1400 nucleotides encoding a 306-amino-acid polypeptide, the question arises as to how this precursor is processed into the mature lectin polypeptides of 11.5 kDa and 12.5 kDa. N-terminal sequencing of the two lectin subunits revealed that both subunits are encoded by this large precursor. Although *in-vivo*-biosynthesis studies are required to unravel the biosynthesis and processing of ASAI, it can already be concluded that the processing of its precursor represents a unique example of protein processing in plants, in the sense that two highly similar (though different) lectin polypeptides which eventually constitute the (heterodimeric) lectin molecule are generated from a single precursor.

Similar to the Amaryllidaceae lectins, the second garlic lectin ASAII is translated from a mRNA of approximately 800 nucleotides, encoding a 154 amino acid polypeptide which is highly similar to the 12.5-kDa ASAI polypeptide. Moreover, preliminary studies concerning the biosynthesis of the garlic lectin ASAII have indicated that this protein is most probably processed in a manner similar to the Amaryllidaceae lectins.

In summary, it can be concluded that the two mannose-binding garlic lectins, which are undoubtedly very closely related proteins, are encoded by different genes. It is very striking, therefore, that two sets of structurally different genes eventually give rise to virtually identical proteins.

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