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Comparative study of the post-translational processing of the mannose-binding lectins in the bulbs of garlic (*Allium sativum* L.) and ramsons (*Allium ursinum* L.)

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The biosynthesis and processing of the homodimeric and heterodimeric lectins from the bulbs of garlic (*Allium sativum*) and ramsons (wild garlic; *Allium ursinum*) were studied using pulse and pulse-chase labelling experiments on developing bulbs. By combining the results of the *in vivo* biosynthesis studies and the cDNA cloning of the respective lectins, the sequence of events leading from the primary translation products into the mature lectin polypeptides could be reconstructed. From this it is demonstrated that garlic and ramsons use different schemes of post-translational modifications in order to synthesize apparently similar lectins from totally different precursors. Both the homomeric garlic lectin (ASAII) and its homologue in ramsons (AUAII) are synthesized on the endoplasmic reticulum (ER) as nonglycosylated 13.5 kDa precursors, which, after their transport out of the ER are converted into the mature 12.0 kDa lectin polypeptides by the cleavage of a C-terminal peptide. The heterodimeric garlic lectin ASAI is synthesized on the ER as a single glycosylated precursor of 38 kDa, which after its transport out of the ER undergoes a complex processing which gives rise to two mature lectin subunits of 11.5 and 12.5 kDa. In contrast, both subunits of the heterodimeric ramsons lectin AUAI are synthesized separately on the ER as glycosylated precursors, which after their transport out of the ER are deglycosylated and further processed into the mature lectin polypeptides by the cleavage of a C-terminal peptide.

Keywords: *Allium*; biosynthesis; lectin; garlic; ramsons

Introduction

During the past few years there has been an increasing interest in a novel group of mannose-binding lectins – to date these lectins have been found in the plant families Amaryllidaceae, and Orchidaceae and *Allium* species from Alliaceae. Due to their exclusive specificity towards mannose and their potent inhibitory activity against animal and human retroviruses, several of these lectins have become useful tools in glycoconjugate and HIV research [1–3]. In addition, the group of monocotyledonous mannose-binding lectins represents an interesting biological system for two reasons. First, all Amaryllidaceae, Orchidaceae, and *Allium* lectins known thus far are encoded by large families of closely related genes [4–8]. Second, although all these lectins are very similar at the protein level, there are important differences in the way the primary translation products of their genes are converted into the mature lectin molecules. The latter peculiarity is best illustrated by the *Allium* lectins. Biochemical and molecular-biological studies have shown that the *Allium* lectins represent a rather heterogeneous group of agglutinins, which differ from each other not only in their molecular structure and biological activity but also in their biosynthesis [5–7, 9]. Basically, all *Allium* lectins known thus far are composed of lectin subunits of 11–13 kDa. However, whereas leaves, shoots and bulbs of onions, leeks and shallots contain exclusively homodimeric or homotrimeric lectins [7], bulbs of garlic and ramsons contain both a homodimeric (referred to in this paper as ASAII or *Allium sativum* agglutinin II and AUAII or *Allium ursinum* agglutinin II) and a heterodimeric lectin (referred to in this paper as ASAI or *Allium sativum* agglutinin I and AUAI or *Allium ursinum* agglutinin I) [5, 6]. Analysis of the respective cDNA clones has shown that the homomeric lectins from shallots, onions and leeks are encoded by a single set of genes and that after translation from mRNAs of approximately 800 bp the primary translation products are converted into mature lectin polypeptides by removal of a signal peptide and a C-terminal sequence [7]. In ramsons, it is more complicated. Molecular cloning of the ramsons lectin genes revealed that the two lectins, AUAI and AUAII, are encoded by three different sets of genes which not only differ in their sequences but also in the number of potential

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glycosylation sites [6]. The three cDNA clones contain 0, 1 or 2 putative glycosylation sites and hence are called LECAUAG0, LECAUAG1 and LECAUAG2. Since all *Allium ursinum* lectin polypeptides are translated from mRNAs of 800 bp, the two subunits composing the heterodimeric AUAI must be derived from two different precursors. In this respect, AUAI differs totally from ASAI. Indeed, sequence analysis of the garlic lectin genes has shown that ASAI is translated from a single mRNA of 1400 bp encoding a polypeptide of 306 amino acids [5]. A more detailed study of the cDNA encoding ASAI reveals that the lectin clone consists of a signal sequence followed by two very similar domains which share 84% homology at the deduced amino acid level. These domains are separated by a 30 amino acid sequence containing a putative glycosylation site. N-terminal sequencing of the two mature ASAI polypeptides has confirmed that both lectin subunits are derived from this precursor and coincide with the two domains within the lectin precursor sequence. In contrast to ASAI the second garlic lectin (ASAII) is translated from an mRNA of approximately 800 bp encoding a polypeptide of 155 amino acids. The cDNA clone encoding ASAII contains a sequence which is highly homologous to the second domain from the precursor encoding ASAI, flanked by a signal sequence [5].

Based on the deduced amino acid sequences of the different cDNA clones and the biochemical characterization of the mature *Allium* lectins it can be predicted that lectins of garlic and ramsons are synthesized on the endoplasmic reticulum (ER) as larger precursors which following co- and post-translational modifications are converted into the mature lectins. Since no experiments have yet been undertaken to check whether these predictions are correct, we have made a detailed study of the biosynthesis and post-translational processing of each of the two lectins in developing bulbs of both garlic and ramsons. From this it could be demonstrated that these plant species are capable of synthesizing apparently very similar lectins from totally different precursor molecules by following different schemes of post-translational modifications. In addition, these schemes could be unravelled in detail by combining the results of the *in vivo* biosynthesis experiments presented here and the previously reported data of the cDNA cloning of the ramsons and garlic lectins.

Materials and methods

Materials

Garlic (*Allium sativum* L.) bulbs were planted in early spring and grown under natural field conditions until young developing bulbs were formed. Developing bulbs of ramsons (*Allium ursinum* L.) were collected locally during late spring.

Radioactive labelling of the bulbs and tissue homogenization

Developing garlic bulbs were sliced whereas bulbs of ramsons were cut longitudinally, and incubated with a droplet (about 50 µl) of 5% sucrose containing 15 µCi ^{35}S-methionine. Bulbs were incubated for the appropriate time periods at 20 °C in a Petri dish. To prevent desiccation moistened filter paper was placed below the parafilm sheets on which the bulbs were kept. In pulse experiments bulbs were labelled with ^{35}S-methionine for the appropriate times, rinsed with distilled water and blotted dry. For the pulse-chase labelling experiments, the plant material was labelled with ^{35}S-methionine for 2 h, washed free of unincorporated radioactivity, and incubated for different periods in 5% sucrose containing 1 mM unlabelled methionine. After the chase period, the bulbs were rinsed with distilled water and blotted dry.

Unless stated otherwise, the labelled bulbs were homogenized with a mortar and pestle in five volumes (v/w) of PBS (1.5 mM KH_2PO_4, 10 mM $NaHPO_4$ (pH 7.4), 3 mM KCl, 140 mM NaCl) and the homogenates transferred into small glass tubes. After centrifugation (3000 × **g** for 5 min) the supernatants were removed with a Pasteur pipette. Samples of the extracts were withdrawn for SDS-PAGE and for the estimation of the uptake and incorporation of ^{35}S-methionine in TCA-insoluble material (using the filter paper method).

Isolation of organelles

Labelled bulbs were homogenized with a cold mortar and pestle in five volumes (v/w) of precooled homogenization medium (100 mM Tris pH 7.8, 12% sucrose and 2 mM Mg-acetate) and the homogenate centrifuged at 3000 × **g** for 1 min to remove nuclei and cell wall debris. The organelles were separated from the soluble proteins and small molecules on Sepharose 4B columns (1.7 × 12 cm) (Pharmacia, Uppsala, Sweden) as described by Van der Wilden *et al.* [10]. Briefly, homogenates made in homogenization medium were applied to Sepharose 4B columns equilibrated with the same medium. The columns were eluted with the homogenization medium and fractions of 1 ml each collected. Fractions containing the light-scattering material (organelles) which eluted in the first peak and those containing the soluble proteins which eluted in the second peak were pooled and used for further analyses.

When prepared according to this procedure the organelle fraction from plant material contains (besides mitochondria) mainly vesicles of the ER and some intact Golgi-vesicles. The greater part of the Golgi apparatus and virtually all the organelles of the lysosomal-vacuolar system disintegrate during homogenization of the plant tissue [10].

Isolation of the lectin by affinity chromatography

To isolate the lectins from the extracts of labelled bulbs, fractions (500 µl) of these extracts were consecutively adjusted to the required concentrations of NaCl or

ammonium sulphate, as indicated in the legends to the Figures, and applied to small (200 µl bed volume) columns of mannose-Sepharose 4B equilibrated with the same buffer. Unbound proteins were washed off using 2 ml of the appropriate buffer. Afterwards the lectin was desorbed using 3 ml of unbuffered 20 mM diaminopropane or PBS containing 0.2 M mannose. The lectin in this fraction was precipitated with TCA (10% final concentration) and dissolved in sample buffer for subsequent analysis by SDS-PAGE and fluorography.

Deglycosylation by endoglycosidase H-treatment

Samples of crude extracts or affinity-purified lectins were dialysed against distilled water, lyophilized, and dissolved in 25 µl of 15 mM NaOAc (pH 5.5). SDS was added to a final concentration of 0.02% and the samples heated at 85 °C for 5 min. Then 3 µl of β-mercaptoethanol (1 M) and 2 mU endoglycosidase H (Boehringer, Mannheim, Germany) were added. The solution was incubated overnight at 37 °C and analysed by SDS-PAGE and fluorography.

Gel filtration

To determine the molecular structure of the native molecules containing the lectin precursors and mature lectin subunits the *de novo* synthesized lectins were analysed by gel filtration on a column of Sephacryl-100 (Pharmacia, Uppsala, Sweden). Experimental details are given in the legends to the Figures.

SDS-PAGE and fluorography

SDS-PAGE was done on 12.5 to 25% (w/v) acrylamide gradient gels using a discontinuous system as described by Laemmli [11]. After fixing and destaining gels were immersed in 1 M Na-salicylate for 30 min, dried under partial vacuum, and exposed to X-ray films (Fuji RX, Japan).

Agglutination assays

Agglutination assays with trypsin-treated rabbit red blood cells were carried out as described by Van Damme *et al.* [9].

Results

Detailed analysis of affinity-purified lectin preparations from *Allium sativum* and *Allium ursinum* has shown that bulbs of both species contain two mannose-binding lectins with slightly different molecular structures. Indeed, the heterodimeric lectins ASAI and AUAI are composed of 11.5 and 12.5 kDa subunits whereas their homodimeric counterparts ASAII and AUAII contain a single polypeptide of 12.0 kDa. Since purified garlic and ramsons lectins are (like all other mannose-specific lectins from *Allium*, Amaryllidaceae and Orchidaceae species) essentially devoid of carbohydrate [9], none of their polypeptides contain any covalently bound sugar. In spite of the obvious strong similarities between the different garlic and ramsons lectins at the protein level they are organized differently at the molecular level. Whereas the homodimeric and heterodimeric mannose-binding lectins from garlic are respectively encoded by a single one-domain and a single two-domain gene, the expression of the two different ramsons lectins is controlled by three distinct sets of lectin genes [5, 6]. To unravel the complex biosynthesis and subsequent post-translational processing of the lectin precursors, *in vivo* biosynthesis experiments were carried out. Since the garlic and ramsons lectins are synthesized differently they will be discussed separately.

Garlic

Both ASAI and ASAII are abundant proteins in mature garlic bulbs. Together, they represent up to 40% of the total soluble bulb proteins. Although the two lectins occur in roughly equal amounts in the dry bulbs, their synthesis is apparently not concerted during bulb development. In their final stage of development, for instance, garlic bulbs synthesize almost exclusively ASAI (K. Smeets, unpublished results). Since it could be predicted on the basis of the structure of the genes that the biosynthesis and processing of ASAI is much more complex than that of ASAII, most of the experiments described below were done with almost mature bulbs. When the biosynthesis of ASAII was also relevant, bulbs in an earlier developmental stage were used in the labelling experiments since they accumulate both lectins.

ASAI is synthesized in vivo *as a large precursor and processed in different steps into the mature lectin polypeptides*

Slices of developing garlic bulbs were labelled with ^{35}S-methionine for 2 h and the lectin isolated by affinity chromatography on mannose-Sepharose 4B. Subsequent analysis of the lectin fraction by SDS-PAGE and fluorography revealed a single polypeptide with an apparent molecular mass of 38 kDa (Fig. 1B, lane O). It is important to note here that no other labelled lectin polypeptide is visible on the fluorograph, which implies that the precursor of ASAII with a molecular mass of 13.5 kDa (as calculated from the sequence of its cDNA clone) is either absent or present in undetectable amounts. Evidently, the apparent absence of labelled ASAII precursors facilitates the interpretation of the results shown in Fig. 1. To follow the fate of the 38 kDa precursor, pulse-chase experiments were carried out. To do this slices of bulbs were labelled for 2 h with ^{35}S-methionine and further incubated with unlabelled methionine for different periods to follow the processing of the precursor into the mature lectin polypeptides. Isolation of the total lectin fraction after different labelling times and subsequent analysis of the protein by SDS-PAGE and fluorography showed that the labelling of the 38 kDa precursor decreased as a function of chase time (Fig. 1B). After 4 h of chase almost all radioactivity had

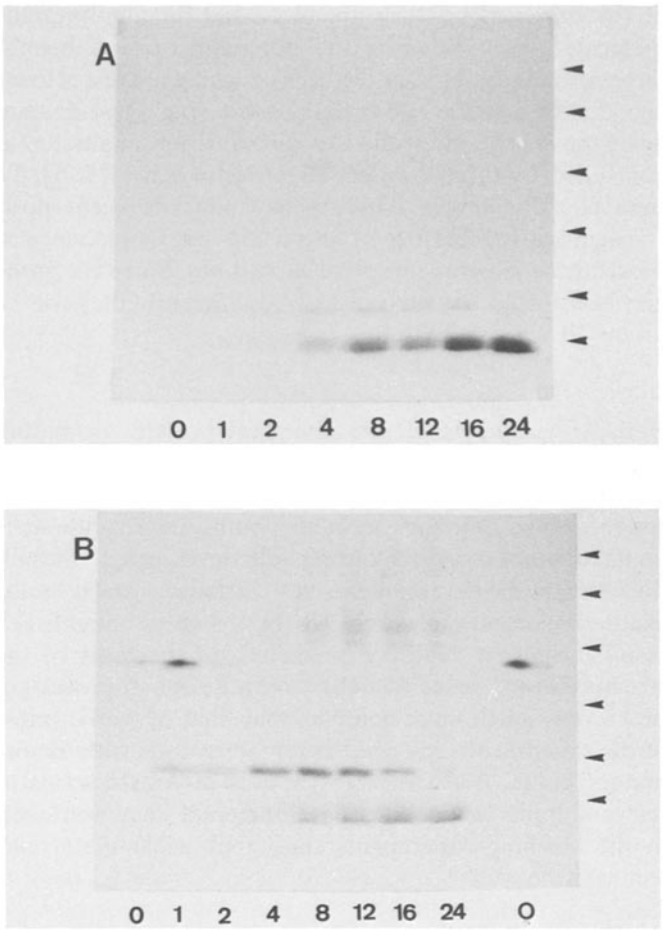


Figure 1. SDS-PAGE and fluorography of affinity-purified lectin recovered from slices of garlic bulbs labelled for 2 h with ^{35}S-methionine and further incubated with unlabelled methionine for the times indicated. (A) shows the fluorogram of the lectin fraction retained on mannose-Sepharose 4B in 0.2 M NaCl. (B) shows the fluorogram of the lectin fraction which was not retained on mannose-Sepharose 4B in 0.2 M NaCl but bound in the presence of 0.8 M ammonium sulphate. The marker proteins are indicated by the arrowheads. They are in order of increasing molecular mass: 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).

been chased from the 38 kDa precursor into a 18.5 kDa polypeptide and to a lesser extent into a 12.5 kDa polypeptide. After 8 h of chase radioactivity gradually appeared in the lectin polypeptides which migrate with an apparent molecular mass of approximately 12.5 kDa (Figs 1a, b). Since the lectin precursors were isolated by affinity chromatography on mannose-Sepharose 4B it is evident that they exhibit the same carbohydrate binding activity as the lectin itself. However, as shown in Fig. 1 the different precursor forms showed a markedly different affinity for the mannose-Sepharose 4B column. Whereas part of the 18.5 kDa polypeptide and the mature lectin (11.5 and 12.5 kDa) polypeptides are retained on the affinity column in 0.2 M NaCl (Fig. 1A) the 38 kDa precursor binds only in the presence of 0.8 M ammonium sulphate (Fig. 1B), indicating that the large precursor has a weaker affinity for mannose under conditions of weak hydrophobic interactions. Taking into consideration that mature garlic lectin ASAI consists of two subunits with molecular masses of 11.5 and 12.5 kDa, respectively, these results suggest that both ASAI lectin polypeptides are synthesized on one large precursor which is post-translationally converted into mature lectin subunits.

ASAI is synthesized as a glycosylated precursor

Since the cDNA clone encoding ASAI contains one putative *N*-glycosylation site in its sequence [5] the possible glycosylation of the 38 kDa precursor and its processing products was investigated. Slices of bulbs were pulse-labelled with ^{35}S-methionine and subsequently incubated with unlabelled methionine for 0, 2 and 4 h respectively. The total lectin fraction was isolated by affinity chromatography on immobilized mannose, treated with endoglycosidase H and analysed by SDS-PAGE and fluorography. As shown in Fig. 2, this treatment resulted in a shift in molecular mass of approximately 3.5 kDa of the 18.5 and 38 kDa precursors, indicating that an *N*-glycan was removed from the polypeptides. Treatment of the mature lectin polypeptides (which appear in Figs 1A and 1B as a single band of 12.5 kDa) had no effect (results not shown) as

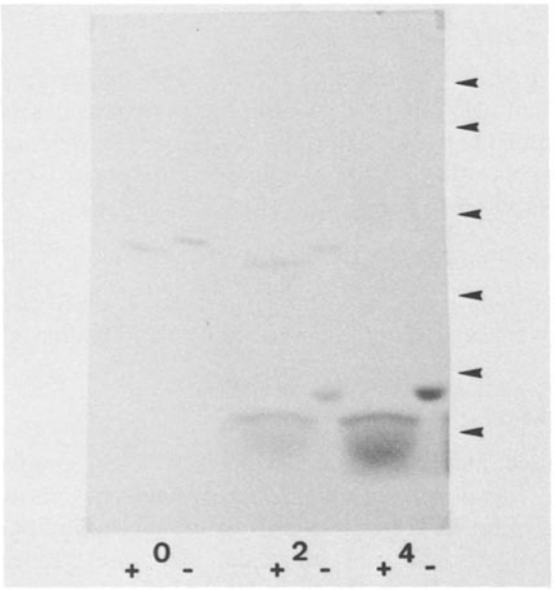


Figure 2. Deglycosylation of the *in vivo* synthesized garlic lectin precursors with endoglycosidase H. Affinity-purified lectin from slices of garlic bulbs, labelled with ^{35}S-methionine for 2 h and further incubated with unlabelled methionine for 0, 2 and 4 h was treated with endoglycosidase H overnight and analysed by SDS-PAGE and fluorography. Control and endoglycosidase H-treated samples are indicated by (−) and (+), respectively. The position of the marker proteins is indicated by the arrowheads. They are the same as in Fig. 1.

could be expected since ASAI does not contain any covalently bound sugar [9]. The glycosylation of the 38 and 18.5 kDa lectin precursors could be confirmed by their binding to Con A-Sepharose (results not shown).

ASAI and ASAII are initially synthesized on the ER

The amino acid sequences deduced from the lectin cDNA clones indicate that the primary translation products of both ASAI and ASAII are proproteins, which suggests that they are synthesized on the ER [5]. To check whether the garlic lectins are synthesized on the ER, slices of young developing bulbs (which synthesize both ASAI and ASAII) were labelled with ^{35}S-methionine for 4 h, homogenized in an isotonic medium (100 mM Tris pH 7.8, 12% sucrose, 2 mM Mg-acetate) and fractionated on a Sepharose 4B column to separate the soluble proteins from the organelles. Analysis of the different fractions by SDS-PAGE and fluorography revealed that the 38 kDa precursor is predominantly associated with the organelle fraction (Fig. 3, lane O) indicating that ASAI is synthesized on the rough ER. Since both the 18.5 and 12.5 kDa polypeptides are exclusively found in the soluble fraction (Fig. 3, lane S), it is evident that the processing of the precursor into mature lectin polypeptides occurs after transport of the precursor out of the ER. In addition to the 38 kDa polypeptide, the organelle fraction also contained a 13.5 kDa lectin polypeptide which corresponds to the precursor form of ASAII. Thus ASAII is also synthesized as a precursor on the ER. Moreover, since all mature lectin polypeptides are exclusively found in the soluble fraction, the post-translational processing of ASAII must take place after the precursor is transported out of the ER.

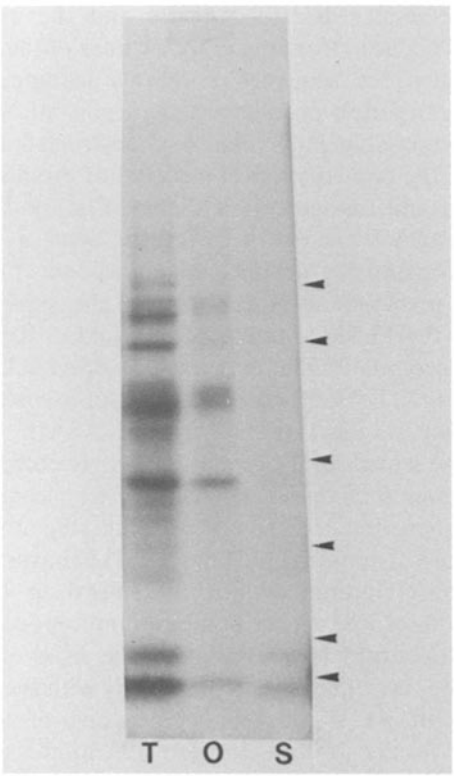
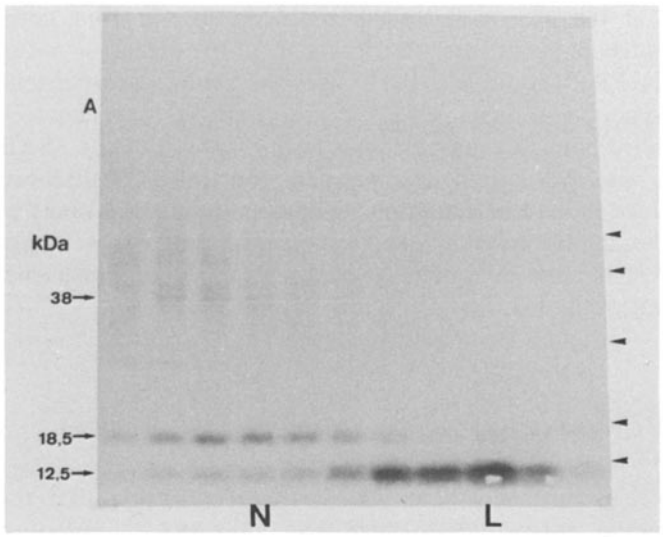


Figure 3. SDS-PAGE and fluorography of total protein in the organelle and soluble fraction from slices of garlic bulbs pulse-labelled for 4 h with ^{35}S-methionine. Lanes O and S were loaded with samples of the organelle and soluble fraction, respectively. Lane T was loaded with a sample of the homogenate from which two organelle and soluble fractions were prepared. The marker proteins indicated by the arrowheads were the same as in Fig. 1.

Molecular structure of the native molecules containing ASAI and ASAII precursors

The experiments described above demonstrate that the mature polypeptides of both ASAI and ASAII are derived from larger precursor molecules. However, from the results discussed thus far no conclusions can be drawn concerning the molecular structure of the native molecules which contain the respective lectin precursor polypeptide(s). To answer the question whether the different precursors occur as monomers or as oligomers, the molecular weight of the native proteins had to be determined. Therefore both the soluble and the organelle fraction of pulse-labelled garlic bulbs were subjected to gel filtration on Sephacryl 100. Fractions were collected and analysed by SDS-PAGE and fluorography. Such an analysis of the organelle fraction (which contains exclusively the 38 kDa precursor of ASAI and the 13.5 kDa precursor or ASAII) demonstrated that the 38 kDa ASAI precursor polypeptide eluted with an apparent molecular mass of approximately 40 kDa, whereas the 13.5 kDa precursor of ASAII eluted with a molecular mass of approximately 27 kDa (Fig. 4B). Thus the 38 kDa precursor of ASAI behaves as a monomer in the ER whereas the 13.5 kDa precursors of ASAII associate into dimers. A similar analysis was done with the soluble fraction, which contains the partially processed precursors of ASAI as well as both mature lectins. As shown in Fig. 4A, the 18.5 kDa precursor polypeptide of ASAI coeluted with a 12.5 kDa polypeptide with an apparent molecular mass of approximately 30 kDa indicating that these two polypeptides form heterodimers which are definitely larger than the mature lectins (23 kDa) and the dimers of two ASAII precursors (27 kDa). It follows, therefore, that the two polypeptides of these heterodimers must be derived from the original 38 kDa ASAI precursor. Most likely, the latter undergoes a proteolytic cleavage thereby giving rise to an intermediate precursor form in which the two final lectin polypeptides are already separated from each other.

The elution position of the mature lectins could be traced by determining the agglutination activity of the fractions. It is evident, however, that the mature forms of ASAI and ASAII coelute since their native molecular mass is virtually identical.



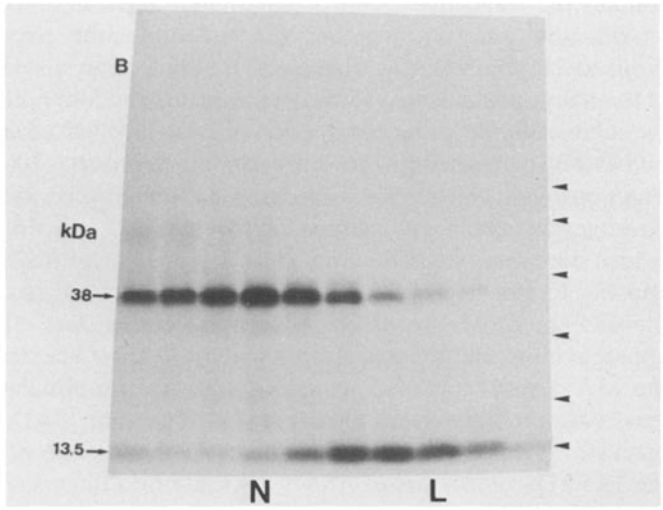


Figure 4. SDS-PAGE and fluorography of organelle-associated and soluble garlic lectin after gel filtration on Sephacryl 100. Slices of garlic bulbs were labelled with ^{35}S-methionine for 4 h and the homogenate fractionated on Sepharose 4B into organellar and soluble fraction. Both fractions were brought to 0.1% Triton X-100 and 1.5 ml of each fraction chromatographed on a column of Sephacryl 100 (28 cm × 2.6 cm; about 150 ml bed volume). One mg of pure ASAI was added to the organellar fraction in order to trace the elution position of the mature lectin. The column was eluted with PBS containing 0.2 M mannose at a flow rate of 1 ml min^{-1}. Fractions of 1.75 ml were collected and analysed by SDS-PAGE and fluorography. The results of the chromatography of the soluble and organellar fraction are shown in A and B, respectively. In both chromatograms the elution position of the mature lectin from Sephacryl 100 (which was determined by agglutinating assays) is indicated by 'L'. In addition, the elution position of the related mannose-binding lectin from *Narcissus pseudonarcissus* (36 kDa), which was determined in a separate run is indicated by 'N'. The position of the marker proteins of the SDS-PAGE is indicated by the arrowheads. They are the same as in Fig. 1.

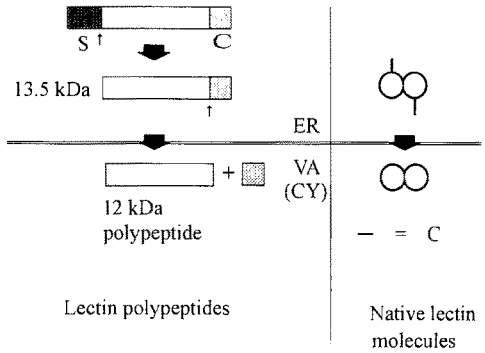


Figure 5. Model of the biosynthesis and processing of ASAII and AUAII. The left side of the figure gives a schematic representation of the processing of the original lectin precursor into the mature lectin subunits whereas the right side depicts the structure of the native molecules containing the different precursors. The compartment above the double line (indicated by ER) refers to the endoplasmic reticulum. The lower compartment corresponds to the vacuoles (VA) or cytoplasm (CY). S and C refer to signal peptide and C-terminal peptide, respectively.

Model of the biosynthesis, processing and topogenesis of ASAI and ASAII

From the results described above and the amino acid sequences deduced from the cDNA clones of the respective garlic lectins the sequence of events leading from the primary translation products of the lectin mRNAs to the mature lectin polypeptides can be reconstructed. In the case of ASAII, the construction of a model of the biosynthesis, processing and topogenesis is simple (Fig. 5). The cDNA clone of ASAII encodes a precursor with a calculated molecular mass of 16.7 kDa which is converted into a 13.9 kDa precursor after cleavage of the signal peptide. Evidently the 13.5 kDa polypeptide found in the organelle fraction accounts for the precursor of ASAII. After transport out of the ER the 13.5 kDa precursor is converted into the 12 kDa subunits of the mature ASAII. Since the N-terminal sequence of the mature ASAII corresponds to the N-terminus of the cDNA clone after cleavage of the signal sequence, the second processing step involves the cleavage of a C-terminal peptide. ASAII behaves exactly as the mannose-binding lectin from snowdrop (*Galanthus nivalis*) with regard to its biosynthesis and topogenesis [12]. Taking into consideration the molecular mass of the lectin polypeptides and the sequence homology with the *Galanthus nivalis* lectin we assume that processing of the ASAII precursor occurs around position 105 of the proprotein which implies that a C-terminal peptide of 22 amino acids is removed.

The model describing the events leading from the ASAI preproprotein to the mature lectin subunits is more complicated (Fig. 6). Basically, the cDNA clone of ASAI encodes a 306 amino acid polypeptide with a calculated molecular mass of approximately 33 kDa. Cleavage of a

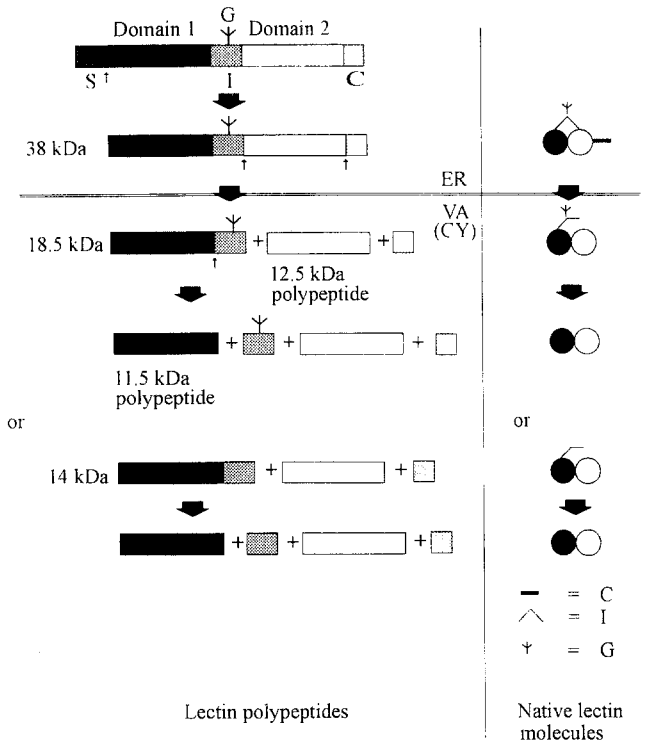


Figure 6. Model of the biosynthesis and processing of ASAI. The left side of the figure gives a schematic representation of the processing of the original lectin precursor into the mature lectin subunits whereas the right side depicts the structure of the native molecules containing the precursors. The compartment above the double line (indicated by ER) refers to the endoplasmic reticulum. The lower compartment corresponds to the vacuoles (VA) or cytoplasm (CY). S, I, C and G refer to signal peptide, intervening sequence, C-terminal peptide and glycan chain, respectively.

signal peptide of 36 amino acid residues yields a polypeptide of 270 amino acid residues (30.1 kDa) which contains two very homologous domains separated from each other by an intervening sequence of 30 amino acids possessing a putative glycosylation site (Fig. 6). Our experiments confirm that ASAI is synthesized on the ER as a glycosylated precursor with an apparent molecular mass of approximately 38 kDa. Since the signal peptide is removed co-translationally, this 38 kDa precursor can only correspond to the glycosylated proprotein of 270 amino acids. Taking into consideration the calculated molecular mass of the proprotein (30.1 kDa) and the extra increase of 2–4 kDa due to the presence of the glycan chain, the size of the 38 kDa is probably overestimated. The same holds true for the apparent molecular mass of the deglycosylated precursor (35 kDa) which is also higher than the calculated 30.1 kDa. It should be mentioned, however, that the polypeptides of the snowdrop lectin (11.7 kDa as determined by complete sequencing of both the protein and the cDNA) also migrate with an apparent molecular mass of 13 kDa upon SDS-PAGE [12].

After its transport out of the ER, the 38 kDa precursor is cleaved into a glycosylated 18.5 kDa and a non-glycosylated 12.5 kDa polypeptide which form a native 30 kDa molecule. Based on the molecular mass of the resulting products and the fact that the N-terminal sequence of the 12.5 kDa subunit of ASAI corresponds to the second domain of the lectin gene [5], the most likely cleavage site of the 38 kDa precursor is located at the beginning of the second domain. However, a cleavage at this position would result in a (glycosylated) 18.5 kDa and a (non-glycosylated) 14 kDa polypeptide. Since the latter 14 kDa polypeptide cannot be detected in the soluble fraction and the 18.5 and 12.5 kDa polypeptides appear simultaneously in the pulse-chase experiment, we assume that the 14 kDa polypeptide is rapidly converted into the mature 12.5 kDa polypeptide by cleavage of a C-terminal peptide.

The final step in the processing of the ASAI precursor consists of a proteolytic cleavage of the glycosylated 18.5 kDa polypeptide at the end of the first domain of the original 38 kDa precursor. By removal of a carboxy-terminal peptide (which contains the glycan) the 18.5 kDa polypeptide is converted into the mature 11.5 kDa subunit. At this point there is some uncertainty since it is not clear whether the 18.5 kDa precursor is processed in a single step by the removal of a glycosylated C-terminal peptide or whether it is first deglycosylated (yielding a 15 kDa non-glycosylated intermediate) and subsequently cleaved at its C-terminus. As already mentioned above, no 15 kDa intermediate could be detected. However, the apparent absence of the hypothetical intermediate does not preclude its existence since its half-life may be too short to build up a detectable pool.

A final point concerns the intracellular location of the processing steps and the topogenesis of the lectins. As indicated in Figs 5 and 6, it is evident that the cotranslational removal of the signal peptide of the primary translation products of ASAI and ASAII and the glycosylation of the ASAI precursor take place in the ER. Since only the 38 kDa precursor of ASAI and and 13.5 kDa fraction are found in the organelle fraction all further processing steps must take place after the transport of these precursors out of the ER. The exact intracellular location of the mature lectins is unknown. However, the fact that they are found in the soluble fraction does not imply that they are cytoplasmic proteins. On the contrary, by analogy with most plant lectins which occur in large quantities in typical vegetative storage tissues it is possible that they are located in specialized vacuole-like organelles.

Ramsons

AUAI and AUAII are synthesized in vivo *as precursors*

To study the *in vivo* biosynthesis of the two ramsons lectins developing bulbs were cut longitudinally and labelled with ^{35}S-methionine for 3 h. Afterwards the total lectin fraction was isolated by affinity chromatography on mannose-Sepharose 4B and analysed by SDS-PAGE and fluorography.

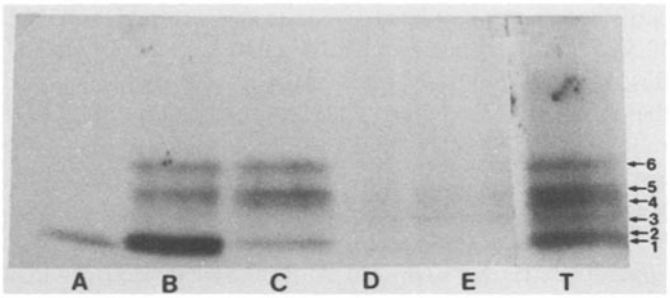


Figure 7. SDS-PAGE and fluorography of affinity-purified lectin from ramsons bulbs labelled for 3 h with ^{35}S-methionine. Lane A contains the lectin bound to the column of mannose-Sepharose 4B in 0.5 M ammonium sulphate. Lectin fractions which did not bind in 0.5 M ammonium sulphate but were retained by successively increasing the ammonium sulphate concentration to 1 M, 1.5 M and 2 M are shown in lanes B, C and D/E, respectively. The lane marked T contains the total lectin fraction (i.e. the lectin isolated from the original extract by affinity chromatography mannose-Sepharose 4B directly in 2 M ammonium sulphate). The different lectin polypeptides are indicated by the numbers on the right. They are in order of increasing molecular mass: 12 kDa (1), 13.5 kDa (2), 16.2 kDa (3), 19.0 kDa (4), 19.9 kDa (5) and 23.4 kDa (6).

As shown in Fig. 7 such an analysis revealed a complex pattern of six lectin polypeptides with molecular masses of 12, 13.5, 16.2, 19, 19.9 and 23.4 kDa, respectively. Taking into consideration that mature lectin polypeptides in ramsons bulbs have apparent molecular masses of approximately 12 kDa this implies that there are at least five lectin precursors. Since the total lectin fraction was isolated by affinity chromatography on mannose-Sepharose 4B it is evident that all these lectin precursors have a mannose-binding activity comparable to that of the lectins themselves. It should be mentioned, however, that the different lectin precursors behave differently in their affinity for immobilized mannose. Indeed, whereas the mature lectin polypeptides bind to the column in 0.5 M ammonium sulphate as running buffer, the precursor molecules require a higher concentration of this salt in order to be retained on the same affinity column (Fig. 7).

Attempts to follow the fate of the different precursors by pulse-chase labelling experiments were unsuccessful. When ramsons bulbs were labelled for 3 h and further incubated in the presence of an excess of unlabelled methionine for up to 24 h, the banding pattern on the fluorographs of the purified lectin fraction was virtually unchanged. It seems, therefore, that in contrast to slices of garlic bulbs which remain metabolically active for at least 24 h, longitudinally cut ramsons bulbs lose most of their activity within a few hours.

AUAI and AUAII are synthesized on the ER

The amino acid sequences deduced from the lectin cDNA clones of ramsons indicate that the primary translation

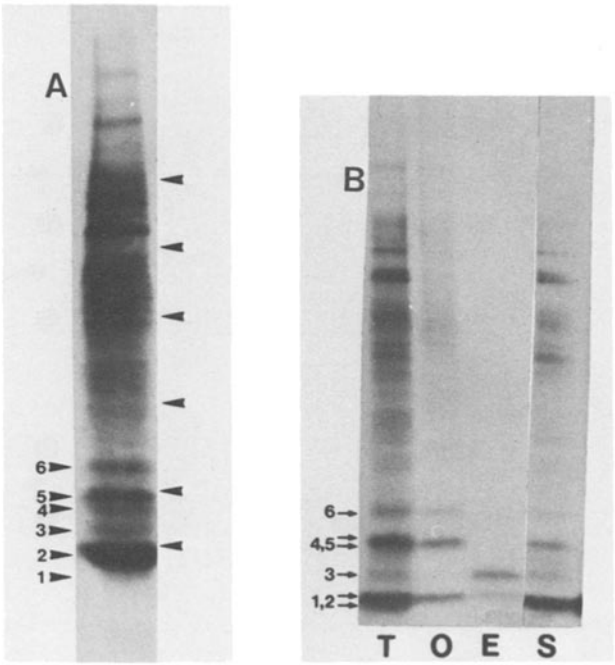


Figure 8. (A) SDS-PAGE and fluorography of a total extract from ramsons bulbs labelled for 3 h with ^{35}S-methionine. The extract loaded on the gel was made in 20 mM unbuffered diaminopropane in order to ensure complete extraction of the proteins. The position of the lectin polypeptides (which appeared in the fluorogram of the purified lectin fraction (Fig. 7, lane T) are indicated. The positions of the marker proteins are indicated by the arrowheads. They are the same as in Fig. 1. (B) SDS-PAGE and fluorography of total protein in the organelle and soluble fraction from ramsons bulbs pulse labelled for 3 h with ^{35}S-methionine. Lanes O and S were loaded with samples of the organelle and soluble fraction, respectively. Lane T was loaded with a sample of the homogenate from which the organelle and soluble fractions were prepared. In lane E, an endoglycosidase H-treated sample of the organellar fraction was loaded. The different lectin polypeptides are indicated by numbers (cf. Fig. 7).

products of both AUAI and AUAII are preproproteins. To check whether the ramsons lectins are synthesized on the ER, bulbs were labelled with ^{35}S-methionine for 3 h, homogenized in an isotonic medium (Fig. 8A) and fractionated on a Sepharose 4B column to separate the soluble proteins from the organelles (Fig. 8B). Analysis of the organelle fraction (which consists mainly of ER vesicles) by SDS-PAGE and fluorography revealed the presence of lectin polypeptides with molecular masses of 23.4, 19.9, 19.0 and 13.5 kDa (Fig. 8B). Since the ER is partly ruptured during homogenization of the tissue and isolation of the organelle fraction, it is evident that the soluble fraction is contaminated with the 23.4, 19.9, 19.0 and 13.5 kDa precursors (Fig. 8B). However, as shown in Fig. 8B, most of the radioactivity, which is incorporated in the lectin isolated from the soluble fraction, is clearly associated with a 12 kDa polypeptide (corresponding to the mature lectin

subunits). It is also worth mentioning that the soluble fraction contains an additional labelled polypeptide of 16.2 kDa which is not detectable in the organellar fraction. Although these results do not allow any conclusion about the processing of the different lectin polypeptides in ramsons bulbs, they demonstrate that AUAI and AUAII are initially synthesized on the ER and are processed after transport out of the ER.

Some of the lectin precursors of ramsons are glycosylated

Since the amino acid sequences deduced from the ramsons lectin cDNA clones LECAUAG1 and LECAUAG2 indicated that the precursors of the AUA I subunits possess one or two putative glycosylation sites, it seemed worthwhile to find out whether some of the lectin precursors are glycosylated. Therefore the organelle fraction was isolated from a homogenate of ^{35}S-methionine labelled bulbs and treated with endoglycosidase H overnight. Analysis of the resulting digest by SDS-PAGE and fluorography revealed that the polypeptides with apparent molecular masses of approximately 23.4, 19.9 and 19.0 kDa were converted into a 16.2 kDa polypeptide, whereas the molecular mass of the 13.5 kDa polypeptide was not affected by endoglycosidase H-treatment (Fig. 8B). It is evident therefore that the lectin precursors with apparent molecular masses of 23.4, 19.9 and 19.0 kDa, respectively, are glycosylated. The non-glycosylated 16.2 kDa precursor is not detectable in the organelle fraction but is clearly visible in the fluorogram of the lectin isolated from the soluble fraction as well as in the total extract (Fig. 8A, B).

Molecular structure of the native molecules containing AUAI and AUAII precursors

To determine the molecular structure of the native molecules which contain the precursor polypeptide(s) of the ramsons lectins both the soluble and the organelle fraction of pulse-labelled ramsons bulbs were subjected to gel filtration on Sephacryl 100. Fractions were collected and analysed by SDS-PAGE and fluorography. Such an analysis of the organelle fraction demonstrates that the glycosylated precursors of AUAI elute from the column with an apparent molecular mass of 40 kDa (Fig. 9B). Therefore, there is no doubt that these precursors occur as (hetero)dimers. Similarly, the 13.5 kDa precursor of AUAII elutes before the mature lectins and thus forms dimers in the ER.

Analysis of the soluble fraction also reveals that the native molecules containing the 16.2 kDa precursors elute behind the dimers composed of the glycosylated 23.4 kDa and 19.9–19.0 kDa precursors but before the mature lectins (Fig. 9A). However, the differences in elution volume do not allow us to conclude whether the 16.2 kDa precursors form exclusively homodimers or also associate with mature AUAI polypeptides into heterodimers.

Model of the biosynthesis, processing and topogenesis of AUAI and AUAII

The results of the experiments described above in combination with the data obtained from the cDNA cloning of the respective lectins [6] allowed reconstruction of the sequence of events which lead from the primary translation products of the ramsons lectin mRNAs to the mature lectin polypeptides. Since the two subunits of AUAI as well as that of AUAII are each translated from a separate set of mRNAs of 800 nucleotides [6] it is evident that ramsons bulbs synthesize three different lectin precursors with a comparable size. Fortunately, the precursors of each of the three lectin polypeptides can be distinguished from each other on the basis of the differences in their glycosylation.

As for ASAII, the model of the biosynthesis, processing and topogenesis of the homomeric AUAII is simple (Fig. 5). The cDNA for AUAII (LECAUAG0) encodes a precursor of 148 amino acid residues (calculated molecular mass of 15.0 kDa) without a possible glycosylation site, which is converted into a 13.5 kDa proprotein by the co-translational removal of the signal peptide of 15 amino acid residues. Evidently, the (non-glycosylated) 13.5 kDa polypeptide found in the organelle fraction accounts for the precursor of AUAII. After its transport out of the ER the 13.5 kDa precursor is converted into the 12 kDa subunits of the mature AUAII by the cleavage of a C-terminal peptide (as can be inferred from the N-terminal sequence of the mature AUAII and the N-terminus of the cDNA clone after cleavage of the signal sequence). It appears, therefore, that the biosynthesis and processing of ASAII and AUAII are virtually identical.

Although all three lectin polypeptides of ramsons are translated from mRNAs of 800 nucleotides, the primary translation products of the genes encoding the two subunits of AUAI are definitely larger than that of the gene encoding AUAII (Fig. 10). From the nucleotide sequence of the cDNA encoding the 12.5 kDa subunit of AUAI (LECAUAG1), it is evident that the primary translation product is a polypeptide of 165 amino acid residues (17.2 kDa), which after removal of the signal peptide of 15 amino acid residues yields a 15.7 kDa peptide with one possible glycosylation site. Similarly, the cDNA for the 11.5 kDa subunit of AUAI (LECAUAG2) encodes a 18.1 kDa preproprotein of 174 amino acids, which is co-translationally converted into a 15.6 kDa proprotein with two putative glycosylation sites. Since the calculated molecular mass of the proproteins encoded by LECAUAG1 and LECAUAG2 yields values of 15.7 and 15.6 kDa (exclusive of the glycan chains), respectively, they certainly cannot correspond to the 13.5 kDa lectin polypeptide found in the organelle fraction of labelled ramsons bulbs. In other words, the latter polypeptide represents exclusively the

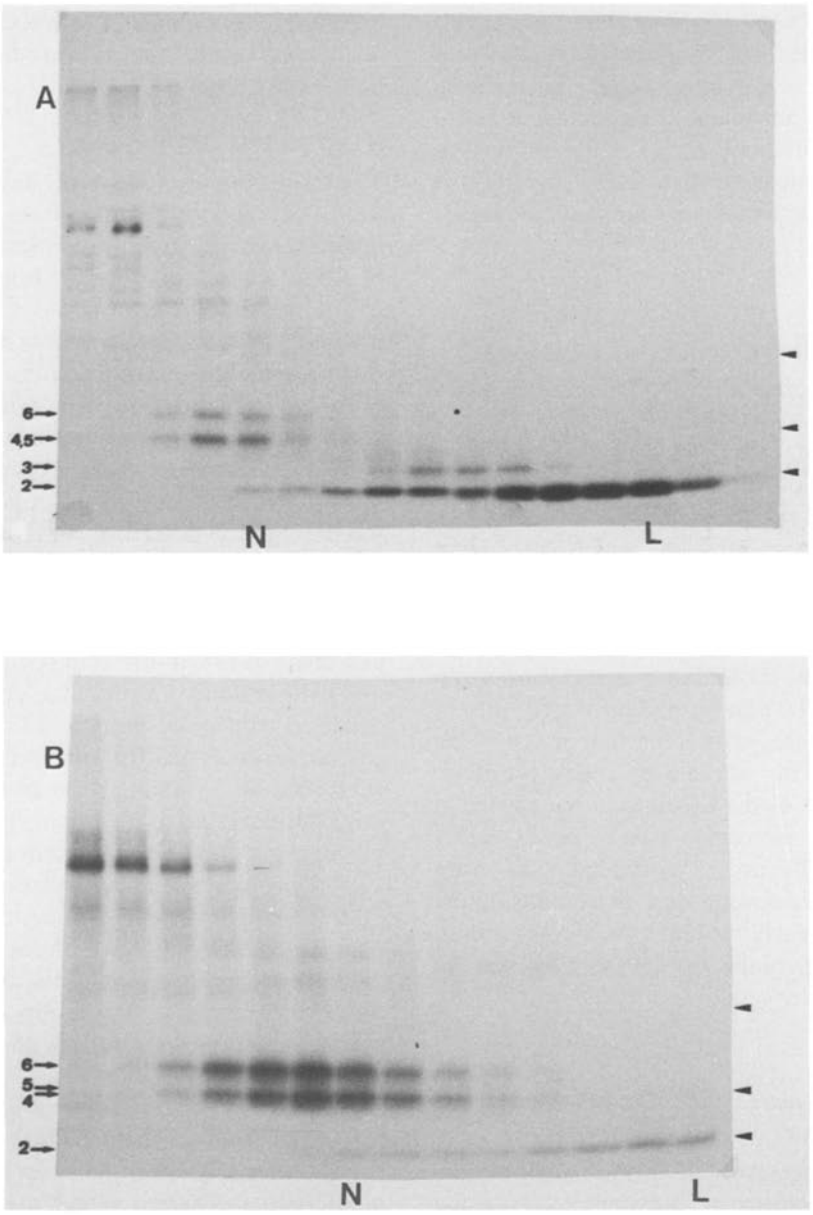


Figure 9. SDS-PAGE and fluorography of organelle-associated and soluble ramsons lectin after gel filtration on Sephacryl 100. Ramsons bulbs were labelled with ^{35}S-methionine for 3 h and the homogenate fractionated on Sepharose 4B into organellar and soluble fraction. Both fractions were brought at 0.1% Triton X-100 and 1.5 ml of each chromatographed on a column of Sephacryl 100 as described in the legend to Fig. 4. One mg of pure AUAI was added to the organellar fraction in order to trace the elution position of the mature lectin. The results of the chromatography of the soluble and organellar fraction are shown in A and B, respectively. In both chromatograms the elution position of the mature lectin (which was determined by agglutinating assays) is indicated by 'L'. The elution position of the related mannose-binding lectin from *Narcissus pseudonarcissus* (36 kDa), which was determined in a separate run is indicated by 'N'. The position of the marker proteins of the SDS-PAGE is indicated by the arrowheads – they are the same as in Fig. 1.

proprotein of the AUAII subunits. Moreover, since in the organelle fraction no lectin polypeptides of 15.7 or 15.6 kDa can be detected whereas three bands of 23.4, 19.9 and 19.0 kDa, respectively, are clearly visible on the fluorogram, most, if not all, of the proproteins of both AUAI subunits must be glycosylated. This presumed glycosylation could be confirmed by the shift in molecular mass of the 23.4, 19.9 and 19.0 kDa polypeptides towards 16.2 kDa upon treatment with endoglycosidase H.

From the shifts in the molecular mass of the glycosylated precursors after treatment with endoglycosidase H it can be concluded that the 23.4 kDa polypeptide contains two glycan chains since its apparent molecular mass decreases by 7 kDa whereas both the 19.9 and the 19.0 kDa

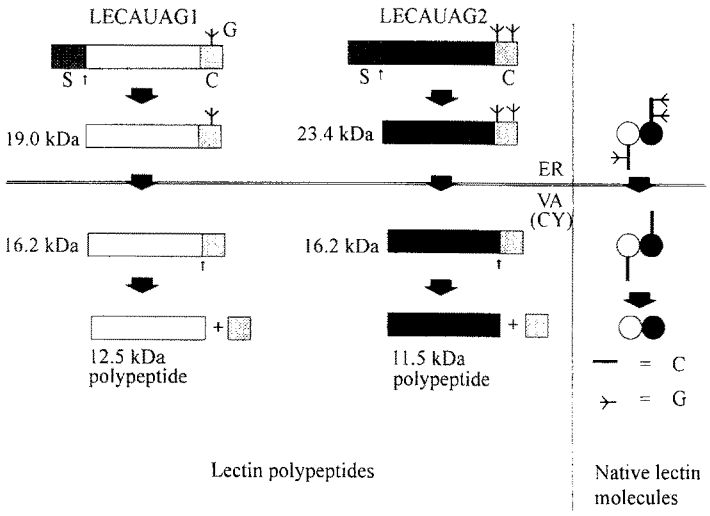


Figure 10. Model of the biosynthesis and processing of AUAI. The left side of the Figure gives a schematic representation of the processing of the original lectin precursors into the mature lectin subunits whereas the right side depicts the structure of the native molecules containing the different precursors. Symbols are the same as in Fig. 6.

polypeptides possess a single carbohydrate side chain as their apparent molecular mass decreases by only 3–4 kDa. Since only the precursor of the 11.5 kDa AUAI subunit contains two putative glycosylation sites the 23.4 kDa lectin polypeptide can be identified unambiguously as the proprotein of the 11.5 kDa subunit in which the two glycosylation sites are occupied. Following the same reasoning, either the 19.0 or the 19.9 kDa lectin polypeptide correspond to the proprotein of the 12.5 kDa subunit in which the single glycosylation site is effectively used. The other one most likely represents the proprotein of the 11.5 kDa subunit in which only one of the two glycosylation sites is actually used. An argument for the occurrence of partially glycosylated proproteins of the 11.5 kDa subunit is the observation that the relative intensity of the 23.4 kDa band on the fluorogram is considerably lower than that of the sum of the 19.9 and 19.0 kDa bands. Indeed, a concerted synthesis of fully glycosylated proproteins (with exactly the same methionine content) would result in 23.4 kDa and 19.9 or 19.0 kDa bands of the same intensity on the fluorograms.

Gel filtration of the lectin isolated from the organelle fraction demonstrated that the glycosylated precursors of the AUAI subunits occur as dimers composed of 23.4 and 19.9 or 19.0 kDa subunits. It appears, therefore, that immediately upon their synthesis, the respective precursors of both AUAI subunits associate into (hetero)dimers, which implies that further processing of each of the two precursors must take place on these heterodimers. Since mature AUAI subunits cannot be detected in the organelle fraction, it is evident that the processing of the 23.4 and 19.9 or 19.0 kDa precursors takes place after their transport out of the ER. Since the N-terminal sequences of the mature AUAI subunits correspond to the N-termini of the respective cDNA clones after cleavage of the signal sequences, the processing of the glycosylated precursors into the mature 11.5 and 12.5 kDa subunits must involve the cleavage of a C-terminal peptide containing the carbohydrate chain(s). Such a proteolytic removal is in full agreement with the fact that the mature lectin polypeptides are not glycosylated [9]. The occurrence of a clearly detectable pool of a 16.2 kDa lectin precursor in the soluble fraction may indicate that the glycosylated precursors of AUAI are deglycosylated before their C-terminal peptide is cleaved.

Finally, the intracellular location of the processing steps and the topogenesis of the ramsons lectins needs some clarification. As indicated in Figs 5 and 10, the removal of the signal peptide of the primary translation products of AUAI and AUAII and the glycosylation of the AUAI precursors take place in the ER whereas all further processing steps occur after the transport of these precursors out of the ER. Evidently the fact that the ramsons lectins and some of their precursors are found in the soluble fraction does not imply that they are cytoplasmic proteins. There is a possibility, indeed, that they are located in specialized vacuole-like organelles. However, until the exact location of the mature lectins is known, one can only speculate about the intracellular route the lectin precursors follow to their final destination.

Conclusions

Summarizing we can conclude that the homodimeric garlic and ramsons lectins are synthesized and processed following the same scheme. Moreover, both the organization of the genes and the biosynthesis of ASAII and AUAII strongly resemble that of all other homomeric mannose-binding lectins like the Amaryllidaceae and Orchidaceae agglutinins and the lectins from several *Allium* species such as onions, shallots and leeks. The heterodimeric lectins ASAI and AUAI, however, differ from all other known mannose-binding lectins with respect to the organization of their genes and the biosynthesis and processing of their gene products. Although the complex mechanisms by which different sets of genes eventually direct the synthesis of apparently closely related proteins have been unravelled, several questions remain to be answered. A first question is why garlic and ramsons contain two different lectins which differ only slightly from each other at the protein level. The second question deals with the totally different organization of the genes encoding the heterodimeric lectins ASAI and AUAI. It is very surprising that sequences which have been conserved so well during evolution are used in a completely different manner for the eventual synthesis of virtually identical proteins. Finally, a third question concerns the glycosylation of the ASAI and AUAI precursors. Why are these precursors glycosylated whereas the precursors of the

homomeric lectins, which occur in the very same tissues are not glycosylated at any stage of their biosynthesis?

Acknowledgements

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References

1. Shibuya N, Berry JE, Goldstein IJ (1988) *Arch Biochem Biophy.* **267**:676–80.
2. Haselbeck A, Schickaneder E, von der Eltz H, Hösel W (1990) *Anal Biochem* **191**:25–30.
3. Balzarini J, Schols D, Neyts J, Van Damme E, Peumans W, De Clercq E (1991) *Antimicrob Agents* **35**:410–16.
4. Van Damme EJM, Goldstein IJ, Vercammen G, Vuylsteke J, Peumans WJ (1992) *Physiol Plant* **86**:245–52.
5. Van Damme EJM, Smeets K, Torrekens S, Van Leuven F, Goldstein IJ, Peumans WJ (1992) *Eur J Biochem* **206**:413–20.
6. Van Damme EJM, Smeets K, Torrekens S, Van Leuven F, Peumans WJ (1993) *Eur J Biochem* **217**:123–9.
7. Van Damme EJM, Smeets K, Engelborghs I, Aelbers H, Balzarini J, Pusztai A, Van Leuven F, Goldstein IJ, Peumans WJ (1993) *Plant Mol Biol* **23**:365–76.
8. Van Damme EJM, Smeets K, Torrekens S, Van Leuven F, Peumans WJ (1994) *Eur J Biochem* **221**:769–77.
9. Van Damme EJM, Goldstein IJ, Peumans WJ (1991) *Phytochemistry* **30**:509–14.
10. Van der Wilden W, Gilkes NR, Chrispeels MJ (1980) *Plant Physiol* **66**:390–4.
11. Laemmli UK (1970) *Nature* **227**:680–5.
12. Van Damme EJM, Kaku H, Perini F, Goldstein IJ, Peeters B, Yagi F, Decock B, Peumans WJ (1991) *Eur J Biochem* **202**:23–30.