Glycoconjugate Journal (1997) **14:** 331–343

Isolation and characterization of lectins and lectin-alliinase complexes from bulbs of garlic (*Allium sativum*) and ramsons (*Allium ursinum*)

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A procedure developed to separate the homodimeric and heterodimeric mannose-binding lectins from bulbs of garlic (*Allium sativum* L.) and ramsons (*Allium ursinum* L.) also enabled the isolation of stable lectin-alliinase complexes. Characterization of the individual lectins indicated that, in spite of their different molecular structure, the homomeric and heteromeric lectins resemble each other reasonably well with respect to their agglutination properties and carbohydrate-binding specificity. However, a detailed analysis of the lectin-alliinase complexes from garlic and ramsons bulbs demonstrated that only the heterodimeric lectins are capable of binding to the glycan chains of the alliinase molecules (EC 4.4.1.4). Moreover, it appears that only a subpopulation of the alliinase molecules is involved in the formation of lectin-alliinase complexes and that the complexed alliinase contains more glycan chains than the free enzyme. Finally, some arguments are given that the lectin-alliinase complexes do not occur *in vivo* but are formed *in vitro* after homogenization of the tissue.

***Keywords*: alliinase, *Allium*, garlic, lectin, ramsons**

Introduction

During the last few years evidence has accumulated for the occurrence of a large superfamily of related mannose-binding lectins in representatives of the plant families Amaryllidaceae, Alliaceae, Orchidaceae, Araceae and Liliaceae [1–10]. At present there is a large interest in these so-called monocotyledonous mannose-binding lectins because of: (i) their exclusive specificity towards mannose [11–13]; (ii) their anti-retroviral activity [14, 15]; (iii) their potent entomotoxic properties [16]; and (iv) their ability to block the adhesion receptors of mannose-fimbriated *Escherichia coli* in the small intestine of rats [17]. Moreover, the monocot mannose-binding lectins represent an interesting biological system because of the structure and organization of their genes. Unlike all other plant lectins studied thus far the Amaryllidaceae, Alliaceae, Orchidaceae and Araceae lectins are encoded by large families of closely related genes [3–8]. In addition, although all these lectins are very similar at the protein level there are important differences in the processing and post-translational modifications of the primary translation products of their genes [4–6, 18].

Of particular interest are the lectins in the bulbs of garlic (*Allium sativum*) and ramsons (*Allium ursinum*). Whereas all representatives of the Amaryllidaceae and Orchidaceae as well as most *Allium* species contain only one type of lectin, bulbs of both garlic and ramsons contain two different though related mannose-binding lectins [4, 5]. Furthermore, garlic as well as ramsons bulbs contain both a heterodimeric lectin (further referred to as ASAI or *Allium sativum* agglutinin I and AUAI or *Allium ursinum* agglutinin I) and a homodimeric lectin (further referred to as ASAII or *Allium sativum* agglutinin II and AUAII or *Allium ursinum* agglutinin II) [4, 5].

Most of information about the ramsons and garlic lectins has been obtained from the molecular cloning and sequencing of the respective cDNA clones [4, 5] and from detailed studies of their biosynthesis and post-translational processing [18]. Although the predictions made on the basis of cDNA sequences could be confirmed by N-terminal sequencing of the individual lectin polypeptides no detailed comparison could be made of the individual molecular forms [4, 5]. In this report we describe a new method for the separation of large quantities of the homodimeric and heterodimeric lectins from both garlic and ramsons bulbs and present evidence for differences between the two lectins of each species. One of the most obvious differences between the homodimeric and heterodimeric garlic and ramsons

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lectins concerns their binding to the (glycosylated) enzyme alliinase (alliin lyase or cysteine sulphoxide lyase, EC 4.4.1.4). Earlier studies have shown that this enzyme is abundantly present in bulbs of both garlic and ramsons [19–21]. Biochemical characterization of the purified garlic enzyme [19] and cloning of its cDNA [22, 23] indicated that the alliinase is a dimeric protein composed of identical subunits of about 50 kDa, which contain about 5% covalently bound carbohydrate. Recently, it has been demonstrated that ASAI binds to the glycan moiety of the alliinase from garlic bulbs whereby lectin-alliinase complexes are formed [24]. The results presented here confirm that ASAI as well as AUAI form complexes with the alliinases from bulbs of garlic and ramsons, respectively. However, a detailed characterization of the complexes, which were isolated in large quantities by affinity chromatography demonstrated that they differ from the previously described complexes.

Materials and methods

Plant material

Dry garlic (*Allium sativum* L.) bulbs were purchased from a local store. Resting ramsons (*Allium ursinum* L.) bulbs were collected locally after withering of the leaves (around July). For the *in vivo* labelling experiments developing ramsons bulbs were collected at the onset of the withering of the leaves.

Preparation of crude extracts from garlic and ramsons bulbs, and absorption of the lectins on immobilized mannose

Garlic bulbs (100 g) were homogenized in 500 ml of 1.5 M ammonium sulphate containing 1 $g\,l^{-1}$ ascorbic acid (adjusted to pH 6.5 with NaOH) using a Waring blender. The homogenate was squeezed through cheese cloth and cleared by centrifugation at $3000 \times \mathbf{g}$ for 10 min. After standing overnight in the cold room the extract was degassed under vacuum and centrifuged at $20\,000 \times \mathbf{g}$ for 15 min. The supernatant was filtered through filter paper (Whatmann 3MM) and loaded onto a column of mannose-Sepharose 4B (2.6 × 10 cm; about 50 ml bed volume) equilibrated with 1.5 M ammonium sulphate in 50 mM NaOAc (pH 6.5). After passing the extract, the column was washed with 1.5 M ammonium sulphate in the same buffer until the A_{280} fell below 0.01. Under these conditions all the agglutinating activity present in the extract was quantitatively retained on the column.

Ramsons bulbs were extracted in the same way as the garlic cloves except that the concentration of ammonium sulphate in the extraction buffer was lowered to 1 M. After passing the extract the column was washed with 1 M ammonium sulphate in the same buffer until the A_{280} fell below 0.01. All the lectin present in the extract was quantitatively retained on the column.

Sequential elution of garlic and ramsons lectins and lectin-alliinase complexes from a mannose-Sepharose 4B column

Proteins bound to the column of immobilized mannose were eluted with a linear gradient of decreasing ammonium sulfate concentration (500 ml from 1.5 to 0 M for the garlic extract and 400 ml from 1 to 0 M for the ramsons extract). Fractions of 10 ml each were collected and their A_{280} measured. Aliquots of every fifth fraction were withdrawn for the determination of their agglutination titre and ammonium sulphate concentration (which was measured by a refractometer). In addition, aliquots of the same fractions were dialysed against acetate buffer for subsequent determination of the alliinase activity. Once the elution of the lectins was completed, the column was washed with 0.2 M mannose in acetate buffer. Fractions of 10 ml each were collected and their A_{280} measured. In addition, the agglutination titre and alliinase activity of each fraction was determined.

Purification of free alliinase from garlic and ramsons bulbs

After removal of the lectins by affinity chromatography on mannose-Sepharose 4B the remaining proteins were precipitated with ammonium sulphate (50% saturation). The precipitate was collected by centrifugation at $9000 \times \mathbf{g}$ for 20 min and redissolved in 100 ml of PBS (1.5 mM KH_2PO_4, 10 mM $NaHPO_4$ (pH 7.4), 3 mM KCl, 140 mM NaCl). Insoluble material was removed by centrifugation at $20\,000 \times \mathbf{g}$ for 20 min and the cleared solution applied onto a column (2.6 × 10 cm; about 50 ml bed volume) of Con A-Sepharose 4B. The column was washed with PBS until the A_{280} fell below 0.01 and the bound proteins eluted with 0.1 M methyl α-D-mannoside in PBS. Fractions with the highest A_{280} were pooled, concentrated by lyophilization and chromatographed on a column (30 × 2.6 cm; 150 ml bed volume) of Sephacryl-100 (Pharmacia, Uppsala, Sweden) using PBS as a running buffer. The alliinase, which eluted in the major peak yielded a single polypeptide band of approximately 50 kDa upon SDS-PAGE (results not shown), was used for further experiments.

Radioactive labelling of the ramsons bulbs and tissue homogenization

Developing bulbs of ramsons were cut longitudinally and each half put on top of a droplet (about 50 µl) of 5% sucrose containing 15 µCi ^{35}S-methionine. Bulbs were incubated for 4 h at 20 °C in a Petri dish. To prevent dessication moistened filter paper was placed below the parafilm sheets on which the bulbs were kept. After labelling, the bulbs were rinsed with distilled water, blotted dry and homogenized in a mortar with pestle in 5 volumes (v/w) of PBS. The homogenate was centrifuged in a microcentrifuge ($12\,000 \times \mathbf{g}$, 5 min) and the supernatant taken off with a Pasteur pipette.

Gel filtration

For analytical purposes gel filtration was performed using an FPLC system (type GP-250 from Pharmacia, Uppsala, Sweden) equipped with a Superose 12 column (type HR 10/30). Preparative gel filtration was carried out using a column (30 × 2.6 cm; 150 ml bed volume) of Sephacryl-100 (Pharmacia, Uppsala, Sweden). Details about the running buffers and elution conditions are given in the legends to the figures.

Ion exchange chromatography

Purified lectins were analysed by ion exchange chromatography on a Mono-Q column (Type HR 5/5 from Pharmacia) using a Pharmacia FPLC system. Samples (1–2 mg) dissolved in Tris buffer (20 mM Tris-HCl, pH 8.7) were loaded on the column. After washing the column with 4 ml of Tris buffer, proteins were eluted using a linear gradient (28 ml) of increasing NaCl concentration (0–0.5 M) (in the same buffer) at a flow rate of 2 ml min^{-1}.

SDS-PAGE and fluorography

SDS-PAGE was done on 12.5–25% (w/v) acrylamide gradient gels using a discontinuous system as described by Laemmli [25]. 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).

Analytical methods

Protein concentration was determined by the method of Bradford using bovine serum albumin and lectins as standards [26]. Total neutral sugar content was determined by the phenol-sulfuric acid method [27] using D-glucose as a reference. Agglutination assays with trypsin-treated rabbit red blood cells were carried out as described by Van Damme *et al.* [2]. The agglutination titre is determined as the highest dilution which still gives a visible agglutination after 1 h.

Cyanogen bromide cleavage

Lyophylized alliinase (1 mg) was dissolved in 0.1 ml of 70% formic acid. Ten mg of solid cyanogen bromide was added and the mixture incubated overnight at 37 °C (in the dark). Peptides were recovered by evaporation under vacuum.

Alliinase assay

Alliinase was assayed by determining the production of pyruvate from exogenous ethyl cysteine sulphoxide according to the method described by Schwimmer and Guadagni [28]. The standard reaction mixture contained 100 mM sodium phosphate buffer (pH 7) and 10 mM ethyl cysteine sulphoxide. The mixture was incubated at room temperature and 100 µl aliquots were taken to determine the production of pyruvate. One unit of alliinase activity is defined as the amount of enzyme which produces 1 µmol pyruvate per min.

Amino acid sequence analysis

Lectins and alliinase were separated by SDS-PAGE as described above and electroblotted on a PVDF membrane. Individual polypeptides were excised from the blots and sequenced on an Applied Biosystems model 477A protein sequencer interfaced with an Applied Biosystems model 120A on-line analyser.

Preparation and staining of tissue prints of garlic and ramsons bulbs

Tissue prints of garlic were made by pressing freshly cross-sectioned half bulbs on dry Immobilon P membranes (Millipore) for a few seconds. Filters were air-dried, immersed in methanol (3 s) and water (5 min), and the proteins immunocytochemically visualized by the peroxidase-anti-peroxidase method. Polyclonal antibodies against total bulb lectin and alliinase were raised in rabbits. Animals were injected subcutaneously with 1 mg protein dissolved in 1 ml of PBS and emulsified with 1 ml of Freund's complete adjuvant. Five injections were given with intervals of 10 days. Ten days after the final injection, blood was collected from an ear marginal vein, allowed to clot, and the serum collected by centrifugation. An IgG enriched fraction was prepared by repeated precipitation of the serum proteins with ammonium sulphate (40% saturation) followed by ion exhange chromatography on a Q Fast Flow column [8].

Results

Lectins and alliinase are predominant proteins in ramsons and garlic bulbs

Previous work has shown that the most prominent band (12 kDa) in the polypeptide pattern of crude extracts from bulbs of garlic and ramsons (Figure 1A, lane 1; Figure 1B, lane 1) corresponds to a mixture of the respective homodimeric and heterodimeric lectins [4, 5]. The second most prominent band (50 kDa), which stains less intensively than the lectin polypeptides corresponds to the subunits of the respective alliinases (EC 4.4.1.4) [19, 21].

Separation of the garlic lectins ASAI and ASAII by differential elution from mannose-Sepharose 4B

Elution of the garlic lectins from the mannose-Sepharose 4B column with a linear gradient of decreasing ammonium sulphate concentration yielded two major protein peaks. The A_{280} values of both peaks coincided well with the agglutinating activity except that the specific agglutinating activity 'of the firstly eluting lectin was considerably lower than that of the second (Figure 2A). SDS-PAGE of the proteins indicated that the fractions of the first peak contained a single 12 kDa polypeptide whereas these of the second peak yielded two polypeptide bands of 11.5 and 12.5 kDa, respectively (Figure 1A). N-terminal sequencing confirmed that the 12 kDa polypeptide corresponded to the

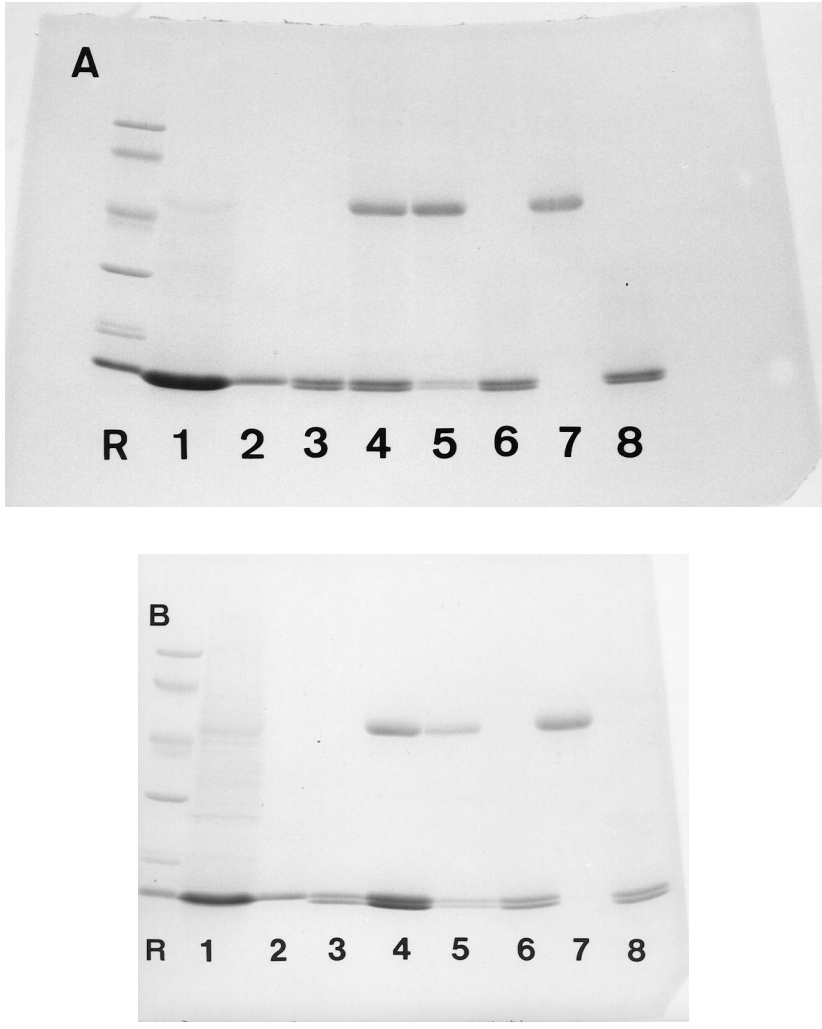

Figure 1. SDS-PAGE of crude extracts, lectins and lectin-alliinase complexes from garlic and ramsons bulbs.

(A) Lane 1 was loaded with the crude extract from garlic bulbs. Samples of fractions 28, 45 and 69 of Figure 2A were loaded in lanes 2, 3 and 4, respectively. Top fractions of peak 1 and peak 2 of Figure 3A were run in lanes 5 and 6, respectively. Top fractions of peak 1 and 2 of Figure 4A were loaded in lanes 7 and 8, respectively. Molecular mass markers shown in lane R are, in order of increasing molecular mass: lysozyme (14 kDa), soybean trypsin inhibitor (20 kDa), carbonic anhydrase (30 kDa), ovalbumin (43 kDa), BSA (67 kDa) and phosphorylase b (94 kDa).

(B) Lane 1 was loaded with the crude extract from ramsons bulbs. Samples of fractions 25, 41 and 60 of Figure 2B were loaded in lanes 2, 3 and 4, respectively. Top fractions of peak 1 and peak 2 of Figure 3B were run in lanes 5 and 6, respectively. Top fractions of peak 1 and 2 of Figure 4B were loaded in lanes 7 and 8, respectively. Molecular mass markers shown in lane R are the same as in A.

ASAII subunit whereas the 11.5 and 12.5 kDa polypeptides of fraction 50 (from Figure 2A) corresponded to the subunits of ASAI (Table 1).

Separation of the ramsons lectins AUAI and AUAII by differential elution from mannose-Sepharose 4B

Elution of the ramsons lectins from the mannose-Sepharose 4B column with a linear gradient of decreasing ammonium sulphate concentration yielded two well resolved peaks (Figure 2B). The agglutination activity of the different fractions coincided with the measured A_{280} values and the specific agglutination activity of the lectins in both peaks was very similar. SDS-PAGE of the proteins indicated that the fractions of the first peak contained a single 12 kDa polypeptide whereas these of the second peak yielded two polypeptide bands of 11.5 and 12.5 kDa respectively (Figure 1B). N-terminal amino acid sequencing confirmed that the 12 kDa polypeptide corresponded to the AUAII subunit whereas the 11.5 and 12.5 kDa polypeptides corresponded to the subunits of AUAI (Table 1).

Characterization of the garlic and ramsons lectins

The availability of reasonable quantities of the two different lectins from garlic and ramsons enabled a detailed

characterization of the proteins. SDS-PAGE showed that ASAI and AUAI are composed of 11.5 and 12.5 kDa subunits whereas ASAII and AUAII are built up of identical subunits of 12 kDa (Figure 1). Gel filtration on a Superose 12 column further showed that all four lectins eluted with an apparent M_r of 25 kDa (results not shown), indicating that ASAI and AUAI are heterodimers whereas ASAII and AUAII are homodimers.

The specific agglutination activity (i.e. the minimal concentration required to agglutinate trypsin-treated rabbit erythrocytes) of ASAI and ASAII was 10 and 25 $\mu g\,ml^{-1}$, respectively, suggesting that ASAI is slightly more active than its homodimeric homologue ASAII. AUAI and AUAII were equally active, their specific agglutination activity being 1 $\mu g\,ml^{-1}$. Evidently, the ramsons lectins are about one order of magnitude more active than the garlic agglutinins.

Total preparations of the garlic and ramsons lectins exhibit an exclusive specificity towards mannose [13]. To check possible (major) differences in specificity between the two types of lectins from garlic and ramsons, hapten inhibition experiments were carried out with mannose and a series of glycoproteins (fetuin, asialofetuin, thyroglobulin, mucin and asialomucin). Using the inhibition of the agglutination of trypsin-treated rabbit erythrocytes as a test system no differences in sugar specificity could be observed between ASAI and ASAII or between AUAI and AUAII (Results not shown).

Isolation of lectin-alliinase complexes from garlic bulbs

When extracts from garlic or ramsons bulbs were passed through a mannose-Sepharose 4B column, the top of the affinity matrix progressively turned deep yellow-orange. The coloured material was still present after elution of the lectins with a gradient of decreasing ammonium sulphate concentration but eluted in a sharp peak with 0.2 M mannose (Figure 2A). Agglutination assays of the fractions containing the yellow-orange material definitely showed an agglutination activity comparable to that of the lectins (Figure 2A). SDS-PAGE revealed two lectin polypeptide bands of 11.5 and 12.5 kDa (Figure 1A) but also indicated that these lectin polypeptides were only minor bands in comparison with the major protein band of 50 kDa. Since the M_r of the latter polypeptide corresponded to that of the alliinase, and pure preparations of alliinases from different *Allium* species have a yellow-orange colour because of the presence of the cofactor pyridoxal 5-phosphate [19], the possible presence of the alliinase was checked with a specific enzyme assay. The high alliinase activity found in the fractions desorbed from the affinity column with mannose confirmed the presence of the enzyme (Figure 2A). Moreover, N-terminal sequencing of the 50 kDa polypeptide (lane 4 of Figure 1A) yielded a sequence identical to previously reported sequences of the garlic alliinase (Table 1).

The identification of alliinase as the major protein in the fractions, which were eluted with mannose raised the question whether the enzyme exhibits mannose-binding and/or hemagglutinating activity. Whereas mannose-binding activity seemed evident on the basis of the retention of the alliinase on the immobilized mannose, its possible agglutination activity was less obvious because of the presence of detectable quantities of lectin. To remove the contaminating (free) lectin the alliinase-containing fraction was passed through a column of Sephacryl-100. As shown in Figure 3A, the first peak contained all the alliinase activity and definitely exhibited an agglutinating activity whereas the second peak, the elution position of which coincided with that of pure lectin, was completely devoid of alliinase activity but

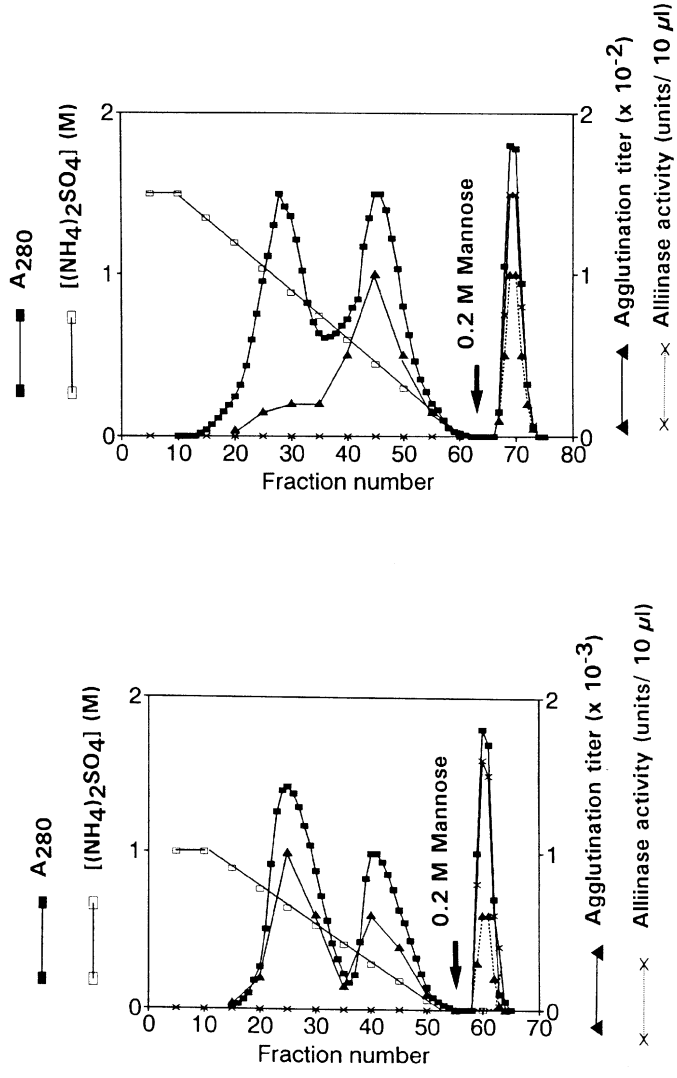

Figure 2. Sequential elution of garlic (A) (top) and ramsons (B) (bottom) lectins and lectin-alliinase complexes from a mannose-Sepharose 4B column. Extracts prepared from 100 g of garlic and ramsons bulbs, respectively, were loaded in (A) and (B). The total yield of the chromatography shown in (A) was 160, 140 and 30 mg protein in the first, second and third peak, respectively. The chromatography shown in (B) yielded 95, 80 and 22 mg protein in the first, second and third peak, respectively.

Table 1. Comparison of the N-terminal amino acid sequences of lectin and alliinase polypeptides from garlic and ramsons bulbs and the deduced amino acid sequences of the respective cDNA clones.

Deduced sequence of ASAII subunit	RNILM	NDEGL	YAGQS	
12 kDa polypeptide lane 2 Figure 1A	– – – – –	– – – –	– – – – –	
Deduced sequence of ASAI 12.5 kDa subunit	RNLLT	NGEGL	YAGQS	
12.5 kDa polypeptide lane 3 Figure 1A	– – – – –	– – – –	– – – – –	
12.5 kDa polypeptide lane 5 Figure 1A	– – – – –	– – – –	– – – – –	
Deduced sequence of ASAI 11.5 kDa subunit	RNILR	NDEGL	YAGQS	
11.5 kDa polypeptide lane 3 Figure 1A	– – – – –	– – – –	– – – – –	
11.5 kDa polypeptide lane 5 Figure 1A	– – – – –	– – – –	– – – – –	
Deduced sequence of garlic alliinase	KMTWT	MKAAE	EAEAV	ANINC
50 kDa polypeptide lane 5 Figure 1A	– – – – –	– – – –	– – – – –	– – – – –
50 kDa polypeptide lane 5 Figure 1B	– V – – –	Q – – – –	– – – –	– N – D –
Deduced sequence of AUAII subunit	RNILG	NEQAL	YADQS	
12 kDa polypeptide lane 2 Figure 1B	– – – – –	– GEG –	– – – – –	
Deduced sequence of AUAI 12.5 kDa subunit	RNILG	NEEGL	YADQS	
12.5 kDa polypeptide lane 3 Figure 1B	– – L – –	– – – – –	– – – –	
12.5 kDa polypeptide lane 5 Figure 1B	– – L – –	– – – – –	– – – –	
Deduced sequence of AUAI 11.5 kDa subunit	RNILG	NEEGL	YADQS	
11.5 kDa polypeptide lane 3 Figure 1B	– – – – –	– – – – –	– – G – –	
11.5 kDa polypeptide lane 5 Figure 1B	– – – – –	– – – – –	– – – – –	

showed a high agglutination activity. SDS-PAGE further revealed that the second peak contained exclusively 11.5 and 12.5 kDa lectin polypeptides suggesting that ASAI is the only protein present in this peak (Figure 1A). The first peak contained predominantly the 50 kDa alliinase subunits (Figure 1A) but also small amounts of 11.5 and 12.5 kDa polypeptides (Figure 1A), which based on their N-terminal amino acid sequences (Table 1) correspond to the subunits of ASAI. Rechromatography of the top fractions of the first peak (of Figure 3A) on a Superose 12 column yielded a single symmetrical peak (eluting with an apparent M_r of 170 kDa) (results not shown).

The apparent M_r of the mannose-binding alliinase (170 kDa) was considerably higher as that of previously described garlic alliinases (100 kDa) [20]. To explain these discrepancies in M_r and the coelution of ASAI and alliinase upon gel filtration, the hypothesis was put forward that the glycosylated enzyme and the mannose-binding lectin form stable complexes. To test this hypothesis, the presumed complexes were dialysed against 20 mM unbuffered 1,3-diaminopropane (under which condition the lectin has no carbohydrate-binding activity) and chromatographed on a Sephacryl-100 column with 20 mM unbuffered 1,3-diaminopropane as running buffer. Two peaks with an apparent M_r of about 50 and 12 kDa, respectively, appeared in the elution pattern (Figure 4A). SDS-PAGE revealed that the first peak contained exclusively alliinase polypeptides whereas the second peak contained only the 11.5 and 12.5 kDa subunits of ASAI (Figure 1A). Due to the high pH, the alliinase was irreversibly inactivated and could not be traced by its enzymatic activity. Conversely the lectin regains its activity upon lowering the pH and hence could be traced by its agglutinating activity. Calculations made on the basis of the results shown in Figure 4A indicated that the ratio alliinase:ASAI in the complexes is about 2:1 on a weight base. Taking into consideration that native alliinase is about four-fold larger than native ASAI, the molar ratio alliinase:ASAI roughly equals 0.5 which implies that each alliinase subunit carries one ASAI heterodimer.

Isolation of lectin-alliinase complexes from ramsons bulbs

Lectin-alliinase complexes were also isolated from ramsons bulbs using the same isolation procedure as described above for the garlic lectin-alliinase complexes (Figures 2B and 3B). Analysis of the complexes by gel filtration in 1,3-diaminopropane (Figure 4B) and subsequent SDS-PAGE of the resulting peaks (Figure 1B) revealed that they consist of alliinase and the heterodimeric ramsons lectin AUAI. It should be noted, however, that the lectin-alliinase complexes from ramsons eluted with an apparent M_r of 220 of kDa upon gel filtration on a Superose 12 column (results not shown). Gel filtration of free alliinase from ramsons on the same column yielded an M_r of about 110 kDa, which is lower than the M_r reported in an earlier paper (150 kDa) [21]. Calculations made on the basis of the results shown in Figure 4B indicated that the ratio alliinase:AUAI in the complexes is about 1.3:1 on a weight base. Taking into

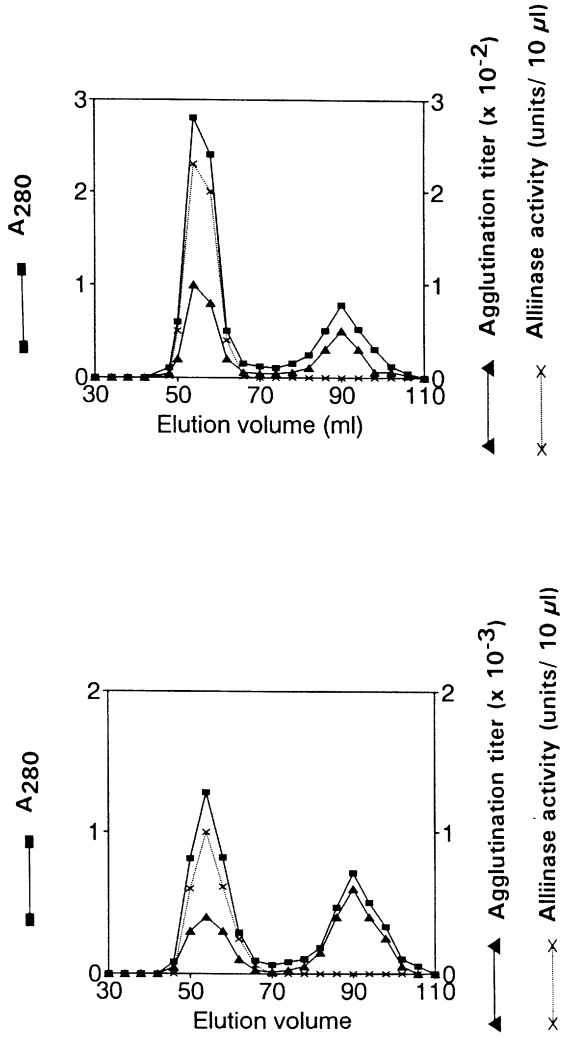

Figure 3. Gel filtration on a Sephacryl-100 column of the garlic and ramsons proteins desorbed from the mannose-Sepharose 4B column with mannose. (A) (top) Elution pattern of the garlic proteins from fractions 69 and 70 of the chromatogram shown in Figure 2A. (B) (bottom) Elution pattern of the ramsons proteins from fractions 59 and 60 of the chromatogram shown in Figure 2B.

Fractions 69–70 of the chromatogram shown in Figure 2A and fractions 59–60 of the chromatogram shown in Figure 2B were lyophilized and reconstituted with 4 ml of distilled water. The concentrated solution was loaded onto a column (30 cm × 2.6 cm; 150 ml bed volume) of Sephacryl-100 preequilibrated with 50 mM NaOAc (pH 6.5) containing 0.2 M NaCl and 0.2 M mannose (to prevent binding of the lectins to the gel matrix). Proteins were eluted using the same buffer at a flow rate of 1 ml min^{-1}. Fractions of 4 ml each were collected and their A_{280} measured. In addition, small aliquots were withdrawn from each fraction for determination of their agglutination titre and alliinase activity. From the total protein (30 mg) loaded in (A), 20 mg and 10 mg were recovered in the first and second peak, respectively. Starting from 18 mg total protein in (B), 12 mg and 6 mg were recovered in the first and second peak, respectively.

consideration that native alliinase is about four-fold larger than native AUAI, the molar ratio alliinase:AUAI roughly equals 0.3, which implies that on average each alliinase subunit carries two molecules of AUAI.

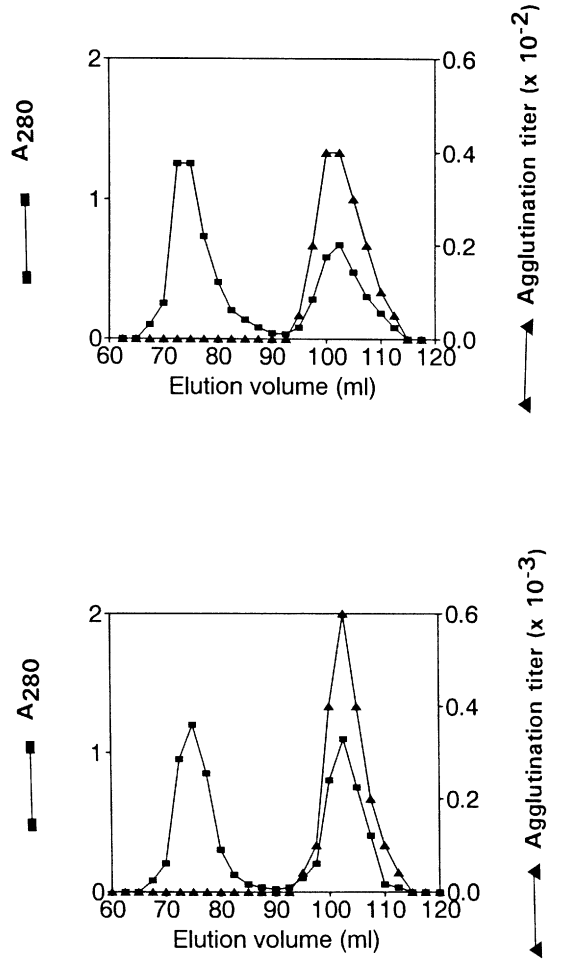

Figure 4. Gel filtration of lectin-alliinase complexes from garlic (A) (top) and ramsons (B) (bottom) bulbs on Sephacryl-100 at high pH. The peak fractions of the chromatograms shown in Figure 3A and 3B were combined, dialysed against 20 mM unbuffered 1,3-diaminopropane, lyophilized and reconstituted with 4 ml of distilled water. The concentrated solution was loaded onto a column (30 cm × 2.6 cm; 150 ml bed volume) of Sephacryl-100 preequilibrated with 20 mM unbuffered diaminopropane and the proteins eluted using the same buffer at a flow rate of 1 ml min^{-1}. Fractions of 2.5 ml each were collected and their A_{280} measured. In addition, small aliquots were withdrawn from each fraction for determination of their agglutination titre. Calculations based on the measured A_{280} values of the fractions and the specific absorbance of the garlic alliinase and ASAI (determined from the respective cDNA clones) indicated that the chromatography shown in (A) yielded 6.1 mg alliinase and 3.1 mg ASAI. Similar calculations using the specific absorbance of AUAI and the garlic alliinase (since the ramsons alliinase has not been cloned yet) revealed that the chromatography shown in (B) yielded 5.7 mg alliinase and 4.2 mg AUAI.

Most of the alliinase in garlic and ramsons bulbs is not associated with lectin

A comparison of the alliinase activity of the crude extract and the unbound (on mannose-Sepharose 4B) lectin-depleted fraction indicated that less than 5% of the total enzyme activity was retained on the affinity column.

Evidently, only part of the alliinase molecules is associated with the lectin.

Lectin-alliinase complexes are soluble only in the presence of mannose

The results described above indicate that the binding of heterodimeric garlic and ramsons lectins to the respective glycosylated alliinases is not disrupted by free mannose at a concentration of 0.2 M. Attempts to dissociate the complexes by increasing the mannose concentration were unsuccessful. Even when the complexes from garlic and ramsons were kept in 1 M mannose for several days and chromatographed on a Superose 12 column in the presence of 1 M mannose, only a single peak with an apparent M_r of 170 and 220 kDa, respectively, was detected (results not shown).

Upon removal of the mannose from a solution of purified lectin-alliinase complexes by dialysis or gel filtration on Sephadex G-25 the solution rapidly turned turbid and eventually most of the complexes precipitated. The precipitate obtained after centrifugation of the turbid solution readily redissolved in 0.2 M mannose but not in 0.2 M glucose, sucrose, galactose or mannitol.

Since the extracts used for the isolation of the lectin-alliinase complexes have been centrifuged at $20\,000 \times \mathbf{g}$ before loading on the mannose-Sepharose column, the complexes (or at least part of them) must be present in a soluble form in the crude extracts. Most likely, the crude extracts contain carbohydrate compounds which prevent the formation of large aggregates of lectin-alliinase complexes. To test this assumption, crude extracts prepared as described in the Materials and methods section, were extensively dialysed against 0.2 M NaCl (in 50 mM NaOAc, pH 6.5) and centrifuged at $20\,000 \times \mathbf{g}$ for 15 min. The resulting supernatants of the garlic and ramsons extracts were brought at 1.5 and 1 M ammonium sulphate, respectively, and chromatographed on a mannose Sepharose 4B column exactly as described for the undialysed extracts. Both dialysed extracts yielded a normal elution pattern for the lectins but in neither case lectin-alliinase complexes were eluted with mannose (results not shown).

Stability of lectin-alliinase complexes from garlic and ramsons

Experiments were done to check whether the purified lectin-alliinase complexes are – like the pure enzyme preparations – labile upon storage [20, 29]. Taking into consideration the stabilizing effect of mannose on the structure of the complexes all experiments were done with preparations of purified complexes in 50 mM NaOAc (pH 6.5) containing 0.2 M NaCl, 0.2 M mannose and 0.01% Na-azide. Repeated freezing and thawing of the solutions did not affect the lectin or enzyme activity of the complexes. Similarly, repeated lyophilization and reconstitution (with water) of the solutions did not result in a decrease of any of the activities of the complexes. The effect of prolonged storage at room temperature as well as in the cold (0–2 °C) was followed by measuring the lectin and alliinase activity of the complexes as a function of storage time. After 2 years of storage in the cold no measurable loss of either lectin or enzyme activity occurred. Even at room temperature, the complexes kept their activity for months (at least in the dark).

Lectin-alliinase complexes are possibly artefacts

To see whether the lectin-alliinase complexes from garlic and ramsons bulbs occur *in vivo* or are artefacts formed during their preparation the possible association between the lectin and the alliinase during or immediately following their synthesis was checked by gel filtration analysis of the *de novo* synthesized proteins (after pulse-labelling with ^{35}S-methionine). Since the difference in M_r between the lectin-alliinase complexes and free alliinases is larger in ramsons (220 kDa versus 100 kDa) than in garlic (170 kDa versus 100 kDa) developing ramsons bulbs were used for these experiments. A control experiment indicated that overnight incubation of purified complexes with 0.1% Triton X-100 did not affect their elution behaviour. Figure 5 shows that *de novo* synthesized (ER-associated) ramsons alliinase eluted with an apparent M_r of about 100 kDa whereas the labelled lectin precursors eluted with an apparent M_r around 40 kDa (which corresponds to the M_r of the AUAI precursor [18]). Thus, newly synthesized AUAI and alliinase do not form complexes within the ER, which, however, does not preclude that both proteins associate with each other in a later stage of their processing and/or topogenesis.

If the complexes do not exist *in vivo* but are formed after homogenization of the tissue one can expect that their concentration increases upon storage of the extract. Due to their higher M_r the complexes can easily be traced in the elution pattern of the proteins upon gel filtration of crude extracts from garlic or ramsons bulbs. As shown in Figure 6, lectin-alliinase complexes clearly occurred in freshly prepared extracts. Moreover, the amount of complexes did not increase as a function of storage time, which suggests that the lectin-alliinase complexes are either present in the cells or are formed immediately upon homogenization of the tissue.

In a third approach the location of the lectins and the alliinase in the bulb tissue was studied in some detail using tissue prints. As shown in Figure 7, the lectins are distributed all over the bulb whereas the alliinase is confined to the tissue in the direct vicinity of the vascular bundles (which is in good agreement with the immunocytochemical localization of the garlic alliinase [30]). The different location of both proteins at the tissue level implies that most of the alliinase and the lectins cannot associate with each other *in vivo*.

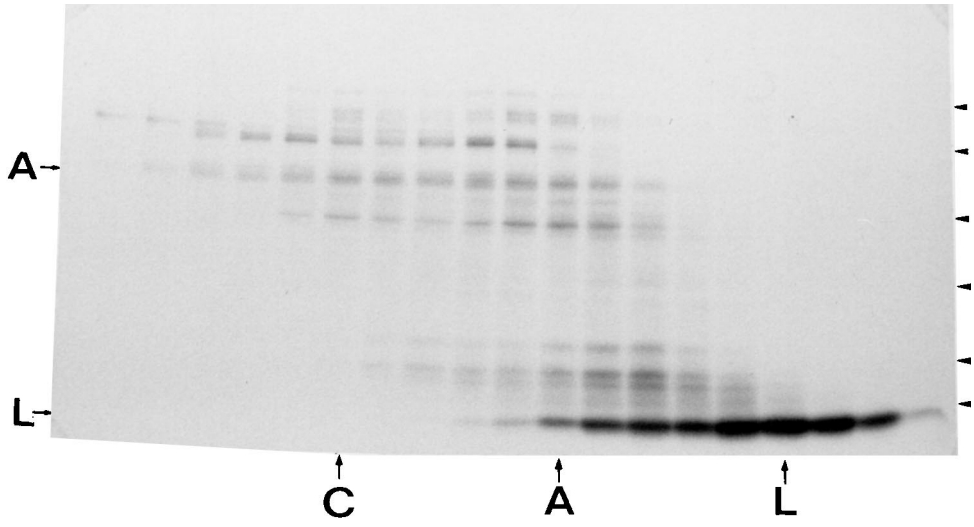

Figure 5. SDS-PAGE and fluorography of organelle-associated proteins after gel filtration on a Superose 12 column. Ramsons bulbs were labelled with ^{35}S-methionine for 3 h and the homogenate fractionated on Sepharose 4B into organellar and soluble fraction as described previously [18]. The organellar fraction was brought at 0.1% Triton X-100 and chromatographed on a Superose 12 column. The elution position (from the Superose 12 column) of the lectin-alliinase complexes (C), free alliinase (A) and AUAI (L), which were determined in a separate run, is indicated by the arrowheads at the bottom of the figure. The position of the molecular mass markers of the SDS-PAGE (which are the same as in Figure 1) is indicated by the arrowheads at the right hand side of the figure. Similarly, the arrowheads at the left hand side indicate the position of the mature alliinase (A) and lectin (L) polypeptides, respectively. The labelled polypeptides migrating with an apparent M_r between 30 kDa and 15 kDa correspond to the different precursor molecules of AUAI and AUAII [18].

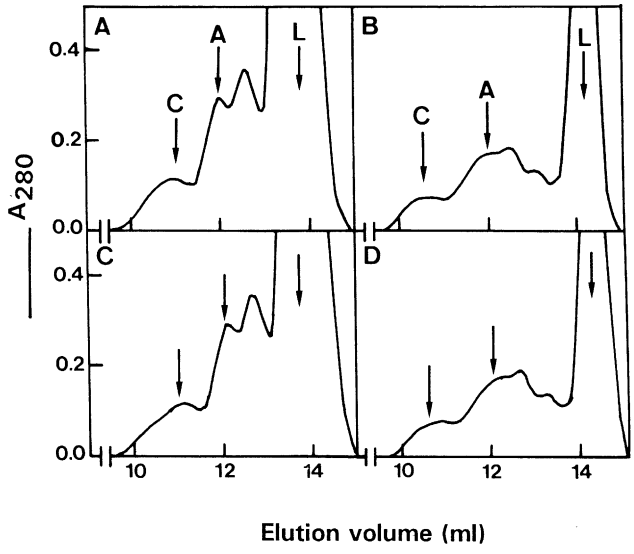

Figure 6. Gel filtration of crude extracts from garlic and ramsons bulbs on a Superose 12 column. Crude extracts prepared in 50 mM NaOAc (pH 6.5) containing 0.2 M NaCl and 0.2 M mannose were passed through a column of Sephadex G-25 in the same buffer and the excluded fraction chromatographed on a Superose 12 column. Elution profiles of freshly prepared garlic and ramsons extracts are shown in panels A and B, respectively. Panels C and D show the elution patterns of the same extracts after storage for 24 h. The elution position of lectin-alliinase complexes (C), free alliinase (A) and free lectin (L) is indicated by the arrowheads.

Differences in glycosylation between free and complexed alliinases from garlic and ramsons

Since only a fraction of the total alliinase from garlic and ramsons bulbs associates with ASAI and AUAI, respectively, there may be important differences in glycosylation between the free and complexed enzyme molecules. Estimations of the total sugar content indicated that free and complexed garlic alliinase contained 5.4 and 8.5% covalently bound carbohydrate, respectively. Assuming that the oligosaccharide side chains contain 10 monosaccharide units, these values indicate that free and complexed alliinase contain one and two glycan chains, respectively. The total carbohydrate content of free and complexed ramsons alliinase amounted to 9.2 and 13.4%, respectively, suggesting that the free and complexed alliinase contain two and three glycan chains, respectively, per subunit. Carbohydrate staining of the cyanogen bromide cleavage fragments of the respective alliinases confirmed the results of the carbohydrate determinations. As shown in Figure 8, free alliinase from garlic yielded a major glycopeptide (of about 17 kDa) and an additional larger fragment of 24 kDa. Since the latter fragment stains less intensively than the 17 kDa glycopeptide, it probably represents a uncompletely cleaved fragment. The cleavage products of the complexed enzyme contained two (equally) intense bands of 20 and 15 kDa, respectively, which confirms that the complexed alliinase carries two oligosaccharide side chains per polypeptide.

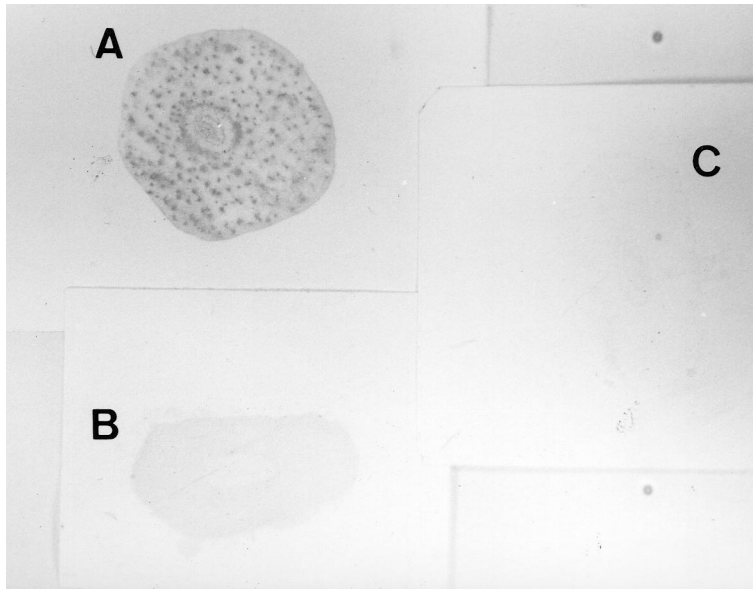

Figure 7. Localization of garlic alliinase and lectin on tissue prints of the bulbs. Panels A and B show the distribution of the alliinase and the lectin, respectively. Controls (developed with preimmune serum) are shown in panel C.

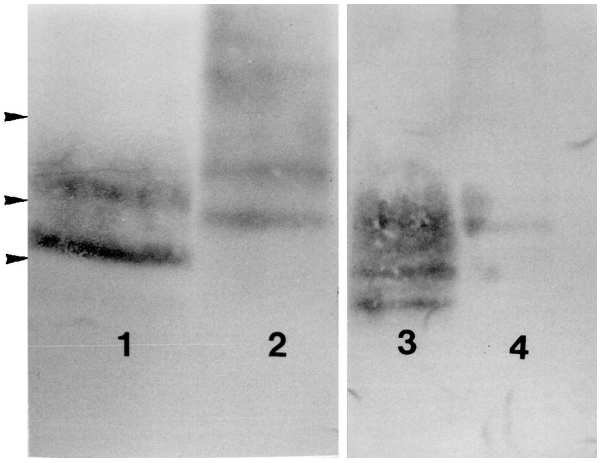

Figure 8. Carbohydrate staining of cyanogen bromide cleavage products of free and complexed alliinases from garlic and ramsons bulbs. Polypeptides obtained by cyanogen bromide cleavage were separated by SDS-PAGE, blotted on an Immobilon P membrane and stained for carbohydrate with the DIG glycan detection kit from Boehringer. Samples (containing 50 μg of protein) were loaded as follows: lane 1, complexed garlic alliinase; lane 2, free garlic alliinase; lane 3, complexed ramsons alliinase; lane 4, free ramsons alliinase. The molecular mass markers are indicated by the arrows; they are in order of increasing M_r: lysozyme (14 kDa), soybean trypsin inhibitor (20 kDa) and carbonic anhydrase (30 kDa).

A similar analysis of the ramsons alliinases indicated that the complexed enzyme yielded four glycopeptides whereas the free enzyme showed only a single band after staining with digoxigenin (Figure 8). Again, these figures are in good agreement with the total carbohydrate content of the respective alliinase preparations.

Discussion

This paper describes a novel method for the separation of the homodimeric and heterodimeric lectins from ramsons and garlic by sequential elution of the bound lectins from a column of immobilized mannose with a gradient of decreasing ammonium sulphate concentration. Apart from the obvious differences in their molecular structure, ASAI and ASAII differ slightly with respect to their specific agglutination activity. In the case of AUAI and AUAII no differences could be found in agglutination activity. Although no detectable differences in carbohydrate specificity were observed between ASAI and ASAII or between AUAI and AUAII, their differential elution from the affinity column indicates that the binding sites of the homodimeric and heterodimeric lectins are not identical. Moreover, the exclusive involvement of the heterodimeric lectins in the formation of lectin-alliinase complexes implies that there must be differences in fine specificity between the heterodimeric and homodimeric lectins. Such differences can be explained by the fact that – on the analogy of the snowdrop lectin – the garlic and ramsons lectins contain three mannose-binding sites per subunit [31].

The elaboration of a method to separate the garlic and ramsons lectins also resulted in the isolation of complexes between the heterodimeric lectins and the alliinases. A comparison of the garlic lectin-alliinase complexes described here and those reported by Rabinkov *et al.* [24] revealed

several important differences. First, the complexes we isolated are soluble only in the presence of free mannose, whereas these described by Rabinkov *et al.* [24] were apparently soluble in a buffer without any free sugar. Second, the M_r of the garlic lectin-alliinase complexes reported by Rabinkov *et al.* [24] (240 kDa) is considerably higher than the value we obtained (170 kDa). Third, the alliinase present in the complexes we isolated contains two glycan chains per polypeptide whereas Rabinkov *et al.* [24] found only one glycan chain per alliinase monomer. To explain these obvious differences the structure and possible origin of the lectin-alliinase is discussed in some detail.

Any model of the structure of the lectin-alliinase complexes should comply with the M_r data and give an explanation for the behaviour of the complexes in the presence and absence of mannose. Moreover, it should also explain why only part of the alliinase is complexed with the lectin and why only the heterodimeric lectins ASAI and AUAI are involved in the complexes.

The observed M_r value of the lectin-alliinase complexes of garlic (170 kDa) as well as the molar ratio alliinase:ASAI (0.5) corresponds best to the structure Aas_2Las_2 whereby Aas stands for the garlic alliinase subunit (50 kDa) and Las for an ASAI heterodimer (25 kDa). Similarly, the lectin-alliinase complexes of ramsons, which have an apparent M_r of 220 kDa and a molar ratio alliinase:AUAI of 0.3, most likely have a Aau_2Lau_4 structure whereby Aau stands for the ramsons alliinase subunit (50 kDa) and Lau for an AUAI heterodimer (25 kDa). The proposed structure Aas_2Las_2 implies that only one of the two glycan chains of the garlic alliinase subunits is occupied by a lectin molecule. Similarly, the proposed structure Aau_2Lau_4 implies that two of the four glycans of the ramsons alliinase subunits are complexed with a lectin molecule. Accordingly, ASAI and AUAI bind – at least in the presence of 0.2 M mannose – only to part of the carbohydrate chains available on the respective alliinases, which suggests the occurrence of two different types of glycan chains on the alliinases. The obvious homogeneity and stability of the lectin-alliinase complexes also implies that ASAI and AUAI do not cross-link the alliinase molecules, which in turn suggests that the lectins do not simultaneously bind two glycan chains on two different alliinase molecules. Hence, only one of the two binding sites of ASAI and AUAI is involved in the binding of glycan chains of the alliinase in the presence of 0.2 M mannose. Evidently, the two binding sites of the heterodimeric ASAI and AUAI differ in their fine specificity and/or affinity for mannose and mannose-containing oligosaccharides. The lectin-alliinase complexes of garlic and ramsons form large aggregates and eventually precipitate in the absence of mannose. Since the formation of such aggregates implies a lectin-mediated cross-linking between alliinase molecules, ASAI and AUAI must be capable of simultaneously binding two glycan chains on two different alliinase molecules when no excess free mannose is present in the medium. This means: (i) that both binding sites of the heterodimeric lectins are occupied by a glycan chain of the alliinase molecules; and (ii) that all glycan chains (i.e. the presumed two different types) of the alliinase polypeptides are bound by the lectins. A schematic representation of the complexes and aggregates is depicted in Figure 9. According to this model: (i) the alliinase polypeptides contain two different types of glycan chains; and (ii) the heterodimeric lectins ASAI and AUAI possess two different carbohydrate-binding sites. In the presence of excess free mannose only one of the binding sites of the lectins is capable of binding one of the two types of

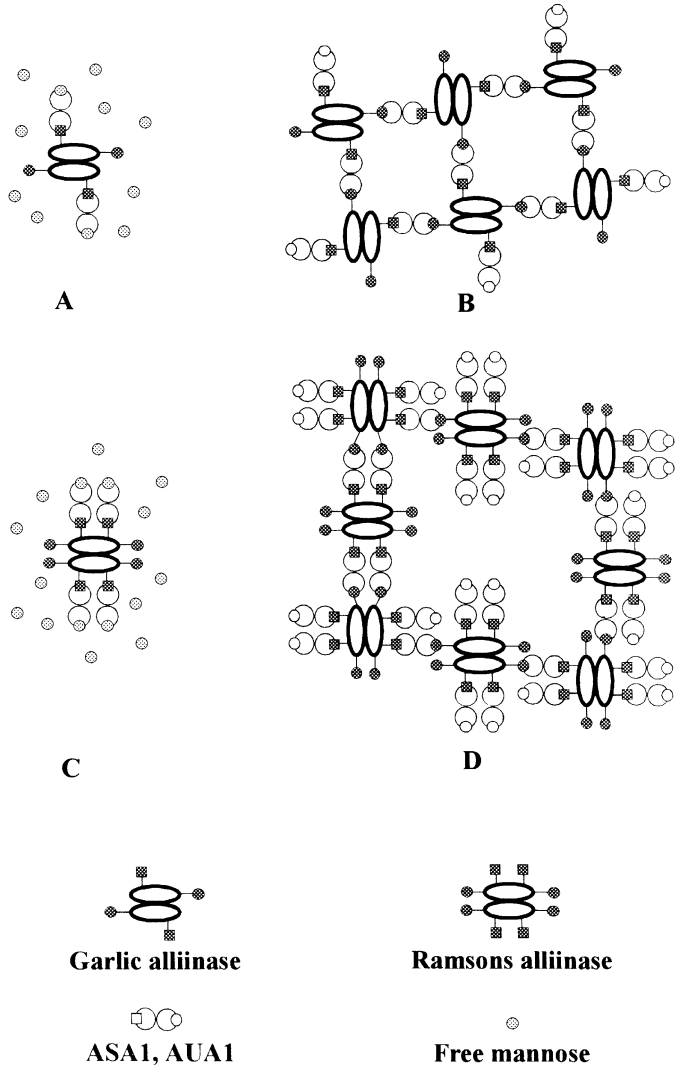

Figure 9. Schematic representation of lectin-alliinase complexes from garlic and ramsons. The models A and C give a schematic representation of the structure of the lectin-alliinase complexes from garlic and ramsons, respectively, in the presence of excess free mannose. Aggregated forms of the garlic and ramsons lectin-alliinase complexes (i.e. in the absence of excess free mannose) are depicted in B and D, respectively. The models presented here imply that the alliinases contain two different types of glycan chains (represented by stalked circles and squares) with a different affinity for the lectins.

oligosaccharides on the alliinases whereby stable complexes (Aas_2Las_2, Aau_2Lau_4) are formed. In the absence of excess free mannose, both binding sites of the lectins are capable of binding both types of oligosaccharides on the respective alliinases whereby large complexes (with the overall structure $(Aas_2Las_2)_n$ and $(Aau_2Lau_4)_n$ are formed.

Most of the alliinase and the lectins in garlic and ramsons bulbs occur as free, uncomplexed proteins, which implies that only a subpopulation of the alliinase molecules is bound by the heterodimeric lectins. Since virtually all alliinase molecules are glycosylated the selective binding of this subpopulation is due most probably to some peculiarities in the structure of their glycan chain(s). The alliinases present in the complexes we isolated contain twice as much carbohydrate as their free counterparts. Since the cyanogen bromide cleavage products of the free and complexed aliinases definitely differ, the alliinases involved in the complexes may be encoded by separate gene(s) of the alliinase gene family [22]. Evidently, the possible involvement of different genes does not explain the presumed differences in the structure of the glycan chain(s) of the free and complexed alliinases. Most probably these differences are due to a differential modification of the original glycan chains during the maturation of the oligosaccharides. One can speculate that the alliinase molecules involved in the formation of lectin-alliinase complexes are derived from a subset of bulb cells, which specifically express a subset of alliinase genes and possess or lack one or more particular glycosyltransferases.

At present it is not clear whether the lectin-alliinase complexes fulfil a specific physiological role. However, since they are most likely formed immediately upon disruption of the bulb tissue they may be involved in wound protection. The high stability of the lectin-alliinase complexes in comparison to the instability of the naked alliinases possibly enhances the conversion of alliin into allicin, which by virtue of its antimicrobial activity can prevent or reduce microbial infection of the wounded surfaces.

The isolation of stable lectin-alliinase complexes from garlic and ramsons demonstrates that some lectins are capable of binding to glycoproteins of the same plant. In addition, the fact that the complexes cannot be disrupted with an excess free mannose indicates that the lectins have a high affinity for the glycan chains on the alliinase. However, irrespective of the high affinity of the heterodimeric garlic and ramsons lectins for their respective alliinases, the occurrence of lectin-alliinase complexes does not necessarily imply that the alliinases are the natural receptors or lectins binders of ASAI and AUAI since they may be formed after disruption of the tissue. Evidently, the same reasoning holds true for the previously reported complexes between lectins and glycoproteins in soybean (*Glycine max*) and jackbean (*Canavalia ensiformis*) [32], and between two different lectins from *Vicia cracca* [33]. Although there is no doubt that some plant lectins are capable of interacting with glycoproteins from the same tissue, it remains to be proven that stable lectin-glycoprotein complexes of a well defined structure (rather than aggregates) occur *in situ*. A recent study of a (glycosylated) mannose/glucose self-aggregatable lectin from the bark of yellow wood (*Cladrastis lutea*) suggests the opposite. In contrast to the purified lectin, which forms large aggregates by carbohydrate-mediated lectin-lectin interactions, the lectin in the crude extracts remains fully soluble because of the excess free sugar [34].

Acknowledgements

This work was supported in part by grants from the Catholic University of Leuven (OT/90/19) and the National Fund for Scientific Research (Belgium, Fonds voor Geneeskundig Wetenschappelijk Onderzoek grant 2.0046.93). W.P. is Research Director and E.V.D. Postdoctoral Fellow of this fund. Furthermore we want to acknowledge grant 7.0047.90 from the Nationaal Fonds voor Wetenschappelijk Onderzoek-Levenslijn fund.

References

1 Van Damme EJM, Allen AK, Peumans WJ (1988) *Physiol Plant* **73**: 52–57.
2 Van Damme EJM, Goldstein IJ, Peumans WJ (1991) *Phytochem* **30**: 509–14.
3 Van Damme EJM, Goldstein IJ, Vercammen G, Vuylsteke J, Peumans WJ (1992) *Physiol Plant* **86**: 245–52.
4 Van Damme EJM, Smeets K, Torrekens S, Van Leuven F, Goldstein IJ, Peumans WJ (1992) *Eur J Biochem* **206**: 413–20.
5 Van Damme EJM, Smeets K, Torrekens S, Van Leuven F, Peumans WJ (1993) *Eur J Biochem* **217**: 123–29.
6 Van Damme EJM, Smeets K, Balzarini J, Pusztai A, Van Leuven F, Goldstein IJ, Peumans WJ (1993) *Plant Mol Biol* **23**: 365–76.
7 Van Damme EJM, Smeets K, Torrekens S, Van Leuven F, Peumans WJ (1994) *Eur J Biochem* **221**: 769–77.
8 Van Damme EJM, Goossens K, Smeets K, Van Leuven F, Verhaert P, Peumans WJ (1995) *Plant Physiol* **107**: 1147–58.
9 Koike T, Titani K, Suzuki M, Beppu H, Kuzuya H, Maruta K, Shimpo K, Fujita K (1995) *Biochem Biophys Res Commun* **214**: 163–70.
10 Van Damme EJM, Briké F, Winter HC, Van Leuven F, Goldstein IJ, Peumans WJ (1996) *Eur J Biochem* **236**: 419–27.
11 Shibuya N, Goldstein IJ, Van Damme EJM, Peumans WJ (1988) *J Biol Chem* **263**: 728–34.
12 Saito K, Komae A, Kakuta M, Van Damme EJM, Peumans WJ, Goldstein IJ, Misaki A (1993) *Eur J Biochem* **217**: 677–81.
13 Kaku H, Van Damme EJM, Peumans WJ, Goldstein IJ (1992) *Carbohydrate Res* **229**: 347–53.
14 Balzarini J, Schols D, Neyts J , Van Damme E, Peumans W, De Clercq E (1991) *Antimicrob Agents Chemother* **35**: 410–16.
15 Balzarini J, Neyts J, Schols D, Hosoya M, Van Damme E, Peumans W, De Clercq E (1992) *Antiviral Res* **18**: 191–207.

16 Hilder VA, Powell KS, Gatehouse AMR, Gatehouse JA, Gatehouse LN, Shi Y, Hamilton WDO, Merryweather A, Newell C, Timans JC, Peumans WJ, Van Damme EJM, Boulter D (1995) *Transgenic Res* **4**: 18–25.
17. Pusztai A, Grant G, Spencer RJ, Duguid TJ, Brown DS, Ewen SWB, Peumans WJ, Van Damme EJM, Bardocz S (1993) *J Appl Bact* **75**: 360–68.
18 Smeets K, Van Damme EJM, Peumans WJ (1994) *Glycoconjugate J* **11**: 309–20.
19 Nock PL, Mazelis M (1986) *Arch Biochem Biophys* **249**: 27–33.
20 Jansen H, Müller B, Knobloch K (1989) *Planta Med* **55**: 434–39.
21 Landshuter J, Lohmüller E, Knobloch K (1994) *Planta Med* **60**: 343–47.
22 Van Damme EJM, Smeets K, Torrekens S, Van Leuven F, Peumans WJ (1992) *Eur J Biochem* **209**: 751–57.
23 Rabinkov A, Zhu X, Grafi G, Galili G, Mirelman D (1994) *Appl Biochem Biotech* **48**: 149–71.
24 Rabinkov A, Wilchek M, Mirelman D (1995) *Glycoconjugate J* **12**: 690–98.
25 Laemmli UK (1970) *Nature* **227**: 680–85.
26 Bradford MM (1976) *Anal Biochem* **72**: 248–54.
27 Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F (1956) *Anal Chem* **28**: 350–56.
28 Schwimmer S, Guadagni DG (1962) *J Food Sci* **27**: 94–97.
29 Tobkin HE Jr, Mazelis M (1979) *Arch Biochem Biophys* **193**: 150–57.
30 Ellmore GS, Feldberg RS (1994) *Am J Bot* **81**: 89–94.
31 Hester G, Kaku H, Goldstein IJ, Wright CS (1995) *Nature Struct Biol* **2**: 472–79.
32 Bowles DJ, Marcus S (1981) *FEBS Lett* **129**: 135–38.
33 Baumann C, Rüdiger H (1981) *FEBS Lett* **136**: 279–83.
34 Van Damme EJM, Barre A, Bemer V, Rougé P, Van Leuven F, Peumans WJ (1995) *Plant Mol Biol* **29**: 579–98.

Received 26 June 1996 and accepted 28 August 1996