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Alliinase (alliin lyase) from garlic (*Alliium sativum*) is glycosylated at ASN^{146} and forms a complex with a garlic mannose-specific lectin

AARON RABINKOV, MEIR WILCHEK and DAVID MIRELMAN*

Department of Membrane Research and Biophysics, The Weizmann Institute of Science, Rehovot 76100, Israel

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Alliinase (EC 4.4.1.4) catalyses the production of allicin (thio-2-propene-1-sulfinic acid S-allyl ester), a biologically active compound which is also responsible for the characteristic smell of garlic. It was demonstrated that alliinase which contains 5.5–6% of neutral sugars, gives clear PAS-staining, binds to Con A and can form a complex with garlic mannose-specific lectin (ASA). Evidence that the formation of such a complex is mediated by the interaction of the carbohydrate of the glycoprotein enzyme with the lectin was obtained from a radioligand assay which demonstrated the binding of alliinase to ASA and competitive inhibition of this binding by methyl α-D-mannoside. ASA I was shown as the lectin mainly present in the complex with alliinase. The results of this study also demonstrate that alliinase is glycosylated at Asn^{146} in the sequence Asn^{146}-Met^{147}-Thr^{148}.

Keywords: garlic, alliinase, lectin, site of glycosylation

Abbreviations: ASA (*Allium sativum* agglutinin). Garlic mannose specific lectin(s); PMSF, Phenyl methyl sulfonyl fluoride; HPLC, High performance liquid chromatography; SDS-PAGE, Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; PAS, Periodic acid-Schiff reagent stain; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; TFA, Trifluoracetic acid; HEPES, *N*-2-Hydroxyethylpiperazine *N'*-2-ethanesulfonic acid; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)]-ethyl glycine; PVDF; poly-(vinylene difluoride).

Introduction

Garlic, *Allium sativum L.*, has been known as a folk medicine since ancient times. Fresh garlic and preparations of garlic extract possess antimicrobial and cancer preventing activity, and were shown to increase fibrinolytic activity, to inhibit platelet aggregation and to lower the levels of cholesterol in serum [1–3]. One of the known reactions which occurs following crushing of the garlic clove is the generation of allicin (thio-2-propene-1-sulfinic acid *S*-allyl ester), a volatile compound responsible for the pungent smell, which has been shown to possess a variety of biological activities [4–7]. The intact garlic clove does not contain allicin but rather its precursor, the non-protein amino acid alliin (*S*-allyl-L-cysteine sulfoxide) [8]. Alliin is converted to allicin, pyruvate, and ammonia by the enzyme alliinase (cysteine sulfoxide lyase, alliin lyase) (EC 4.4.1.4), which apparently resides in another compartment of the clove and was first described by Stoll and Seebeck [9, 10]. Study of the enzymatic distribution in the various garlic plant tissues showed that the specific activity of alliinase increased from the leaves to the bulb. The total activity in the bulb of a mature plant is approximately 10 times higher than that in the leaves [11].

Alliinase has been isolated and purified to homogeneity [11–16]. Molecular and biochemical studies showed that the enzyme is a dimer of two subunits of MW 51.5 kDa [11]. Each subunit contains one pyridoxal 5'-phosphate as coenzyme [14, 17]. The pH optimum of alliinase has been shown to be 6.5, and isoelectric point between 6.0–7.0 [8, 11]. Recently, cloning and characterization of the cDNAs encoding alliinase were achieved [11, 12, 18]. The alliinase gene, according to the full length sequence, consists of 1344 bp encoding a protein of 448 amino acid

*To whom correspondence should be addressed.

residues. The nucleotide coding sequence in two independent studies was closely related except for five amino acid substitutions [11, 18]. This is in accord with an earlier assumption [18] on the presence of a family of closely-related alliinase genes in garlic which encode for different isozymes. Nock and Mazelis have previously shown that alliinase is a glycoprotein containing 5.5% carbohydrate, and they succeeded in purifying the enzyme on a Con A-Sepharose column [14]. We have confirmed that alliinase contains about 6% of neutral sugars and binds tightly to Con A-Sepharose, from which it can be eluted by methyl α-D-mannoside [11]. According to the amino acid sequence, four putative N-glycosylation sites were identified in alliinase at positions 19, 146, 191 and 328, respectively. Taking into consideration that alliinase contains 5–6% of sugars, only one or two of these sites can be occupied. The purpose of the present study was to further characterize the glycoprotein nature of alliinase, to identify alliinase site(s) of glycosylation, and to demonstrate that the enzyme exists at least partly in a complex with recently described garlic mannose specific lectin(s) (ASA), the second most abundant protein present in garlic clove [19, 20]. Since there are two closely-related but different lectins, ASA I and ASA II, we also wished to establish which of the two lectins forms the complex with alliinase.

Materials and methods

Purification of alliinase from garlic cloves using chromatography on ConA-Sepharose

Peeled garlic cloves (60 g) were homogenized in the cold in a mincing machine in Hepes (90 ml, 0.05 M, pH 7.2) containing glycerol (10%), PMSF (1 mM), 1 mM $CaCl_2$, 1 mM $MnCl_2$, 1 mM $MgCl_2$ and 0.02 mM pyridoxal 5′-phosphate (buffer A). The homogenate was filtered through two layers of cheesecloth and the filtrate was centrifuged at 20 000 × **g** for 30 min at 4 °C. Polyethylene glycol-8000 was added to the supernatant (to 25% w/v) and the mixture was stirred slowly for 20 min at 4 °C. The slurry was then sedimented at 20 000 × **g** for 15 min at 4 °C. The pellet was resuspended in 120 ml of buffer A, and subjected to centrifugation again at 20 000 × **g** for 20 min at 4 °C. The supernatant was placed on a ConA-Sepharose (Pharmacia) column (2.2 × 50 cm). The column was washed with buffer A containing 0.5 M NaCl (buffer B). Elution of alliinase was carried out with 0.1 M methyl α-D-mannoside in buffer B. Fractions of the eluate were assayed for alliinase activity (see below). Specific activity of the alliinase preparation obtained was 115.4 μmol pyruvate per min per mg protein.

Enzymatic assay

The assay was based on the method of Schwimmer and Mazelis [21] as modified by us [11].

Protein assay

Protein was assayed by the procedure of Lowry *et al.* [22] with ovalbumin as a standard. Protein concentration of purified alliinase was verified by amino acid analysis data [11]. Amino acid analyses were performed on a Dionex D-500 amino acid analyser (Durrum Instrument Corp., Palo Alto, CA, USA). Protein samples were hydrolysed in vacuum with 6 M HCL at 110 °C for 22 h.

Gel electrophoresis and electroblotting onto PVDF paper

Proteins were separated on 15% SDS-PAGE according to Laemmli *et al.* [23]. Peptide fragments were separated by gradient (10–25% acrylamide) SDS-PAGE as described by Fling and Gregerson [24], or by Tricine/SDS-PAGE essentially according to Schagger and von Jagow [25].

In the latter 1 mm 16.5% gels (20 cm), a 10% spacing gel (2 cm) and 4% stacking gel (1.5 cm) were used. The following modifications were introduced to minimize radical-induced modification of polypeptide [26]. (1) Samples were dissolved in stock buffer (diluted five-fold) containing 0.313 M Tris, 10% SDS, 50% sucrose, 0.025% Serva blue G, 10% β-mercaptoethanol, and 50 mM glutathione. (2) Gels were aged for at least 24 h. (3) A prerun was performed for 1 h at 15 mA using a five-fold diluted sample buffer and cathode buffer containing 1 M Tris, pH 8.45, 0.1% SDS, and 0.1 mM thioglycolate. The 16.5% gels were run for 24 h at 30 mA. After staining, identical bands (from five lanes) were cut out of the gel and placed horizontally in wells of a 10% gel; gel buffer was added, the gel was run overnight at 12 mA, fixed and stained as described [23] and then transferred to PVDF paper. Electroblotting from unfixed and unstained 10% gels onto PVDF paper followed the procedure of Matsudaira [27] using a Semi-Phor TE70 semi-dry transfer apparatus (Hoefer Scientific Instruments). Transfer was essentially complete after 1 h at 12 V. The PVDF paper was stained as described by Xu and Shively [28], washed in water, and air-dried.

Deglycosylation

One mg of alliinase in 0.1 ml 20 mM Na phosphate buffer pH 7.4 was treated with 10 U of recombinant N-endoglycosidase F (Boeringer-Mannheim) for 24 h at 37 °C. The enzyme-treated, as well as a sample of untreated material, was analysed by SDS-PAGE.

Synthesis of mannose-sepharose column

Mannose-Sepharose-4B was synthesized by the divinyl sulfone method [29].

Isolation of ASA

Garlic lectin was purified according to the procedure of Van Damme *et al.* [19] with some modification. Peeled

garlic cloves (50 g) were homogenized in the cold in a mincing machine in 1 M $(NH_4)_2SO_4$. The homogenate was filtered through two layers of cheesecloth and the filtrate was centrifuged at 20000 × **g** for 30 min at 4 °C. After storing overnight at −20 °C, the homogenate was thawed, centrifuged at 20000 × **g** for 30 min and the supernatant was loaded onto a mannose-Sepharose 4B column with a flow rate of 30 ml h^{-1}. After washing with 1 M $(NH_4)_2SO_4$ the lectin was eluted with 0.5 M of methyl α-D-mannoside in the same solvent.

Agglutination assay

Haemagglutination activity of ASA was assayed by double dilution technique in microtitre plates [30] using a 4% suspension of human or rabbit erythrocytes in the absence and presence of D-mannose. Agglutination was monitored visually after the plates had been left at room temperature for 1 h. Specific activity of ASA was defined as the lowest concentration of lectin giving visible agglutination after 1 h.

Preparation of antibodies

Rabbits were immunized by intradermal injection of 0.25 mg of either homogeneous alliinase or ASA emulsified in complete Freund's adjuvant, followed by three biweekly booster injections with 0.125 mg protein in incomplete Freund's adjuvant. The titre of anti-alliinase and anti-lectin serum was estimated by ELISA and its specificity by Western blot analysis.

Western blot analysis

Following SDS-PAGE, proteins were transferred to nitrocellulose paper according to Towbin *et al.* [31] and immunoblotted with anti-alliinase or anti-lectin rabbit serum. The serum dilutions used were 1:200. The immuno-reactive bands were detected by the alkaline phosphatase assay using a goat anti-rabbit-alkaline phosphatase conjugate (Bio Makor, Rehovot, Israel) [32].

Radioligand assay

Alliinase was iodinated by the chloramine-T method with 1 mCi of $Na^{125}I$ per 100 μg alliinase according to the procedure of Hunter and Greenwood [33]. For the alliinase-ASA interaction study radioactive alliinase (4×10^9 cpm mg^{-1}) diluted by the unlabelled enzyme was used. Solid phase radioligand assay on Falcon 3911 Microtest III Flexible Assay plates was performed according to the procedure described by Tsu and Gezenberg [34].

Carbohydrate analysis

The neutral sugars content of alliinase was determined by the phenol-sulfuric method of Dubois *et al.* [35] using glucose as a standard.

PAS (periodic acid-Schiff stain)

Gels were fixed overnight in an aqueous solution containing 50% methanol and 7% glacial acetic acid (w/w), and stained for sugar with PAS according to Zacharius *et al.* [36].

Thin layer chromatography

TLC separation of glycopeptides was carried out on precoated cellulose plates (Merck) using a solvent of 50% n-propanol containing 0.5% TFA. After drying, the plates were sprayed with either ninhydrin (0.25%) or orcinol reagents (0.2% in 1 M H_2SO_4) and placed in an oven at 110 °C for 10 min.

CNBr cleavage of alliinase

(A) In the presence of formic acid: 10 mg of lyophilized pure alliinase was dissolved in 340 μl of 70% formic acid. Solid CNBr was added to a final concentration of 0.7 M. Cleavage proceeded in the dark, at room temperature, for 20 h as described by Steers *et al.* [37]. (B) In the presence of TFA: 2 mg of pure alliinase was dissolved in 100 μl of 75% TFA. Following this step the procedure was the same as described for cleavage in the presence of formic acid. (C) Consecutive cleavage in the presence of formic acid and TFA: 2 mg of lyophilized CNBr peptides obtained in the presence of formic acid were treated with 0.7 M CNBr in 75% TFA as described above. All peptide preparations were analysed by SDS-PAGE.

S-*pyridylethylation and chymotryptic digestion*

Pure lyophylized alliinase (20 mg) was denaturated at room temperature in 4 ml of 0.5 M Tris HCL, pH 9.2 containing 2.5 mM EDTA, 4 M guanidine hydrochloride and 6 mM dithiotreitol for 3 h. For S-alkylation 20 μl of 4-vinylpyridine (Aldrige) was added to a 4 ml sample of the denaturated enzyme. After 1 h incubation at room temperature under argon, the same treatment was repeated with additional 20 μl of 4-vinylpyridine. The sample was dialysed against water in the dark overnight, the precipitate formed was suspended in 2 ml of 0.1 M ammonium bicarbonate and digested with 2 mg of chymotrypsin at 37 °C for 4 h.

HPLC separation of proteins and peptides

The HPLC assembly consisted of an LKB 2150 pump and a 2152 controller, a Jasco 875 UV variable wavelength monitor, and a Spectra-physics SP4290 Integrator. The profile of Con A-Sepharose purified alliinase was analysed by size exclusion chromatography on Superose-12 HR 10/30 (Pharmacia), equilibrated with 0.1 M Na-phosphate buffer, pH 7.4, containing 0.15 M NaCl, at a flow rate of 0.4 ml min^{-1}. The eluate from the column was monitored by absorbance at 280 nm. All protein containing fractions were analysed on SDS-PAGE.

Size exclusion chromatography of peptides obtained after CNBr treatment of alliinase was carried out on Superdex-75 HR 10/30 column (Pharmacia) equilibrated with 0.05 M HCl at a flow rate of 1 ml min^{-1}. The eluate was monitored by absorbance at 225 nm.

Separation of alliinase fragments after chymotryptic digestion was performed on a Superose-12 column, equilibrated with 0.1% TFA at a flow rate of 0.4 ml min^{-1}. The eluate was monitored by absorbance at 225 nm. Low MW fractions (less than 5 kDa) were tested by orcinol and orcinol positive fractions were collected and dried in a Speed Vac.

Isolation of glycopeptides from chymotryptic digest of alliinase

Lyophilized orcinol positive low MW glycopeptides obtained on size exclusion chromatography were dissolved in 0.1 M acetic acid and placed on a 5 × 10 mm Silica gel column (Silica gel 63-100 Woelm Pharma) equilibrated with n-butanol-acetic acid-H_2O (4:1:1). All material eluted with this solvent was orcinol negative when analysed on TLC. A carbohydrate containing fraction was eluted by 50% n-propanol containing 0.1% TFA. After drying in Speed Vac, it was redissolved in 0.5% TFA and separated by HPLC reverse phase chromatography using a Protein & Peptide Vydac C_{18} column and a linear gradient of 0–5% of acetonitrile in 0.5% TFA. The eluate was monitored by absorbance at 220 nm. A fraction of the major orcinol-positive peak was dried, redissolved in 0.5% TFA, rechromatographed under the same conditions, and sequenced.

N-terminal sequencing

N-terminal sequencing of protein and peptide samples was done with an Applied Biosystems 475 A sequencer.

Results

PAS staining of garlic proteins

Different garlic samples have been analysed for the presence of glycoproteins. Following gel electrophoresis of a garlic extract, it was shown that alliinase was the only protein that stained both with Coomassie blue and PAS. In addition to the alliinase band, several very high and low MW (less than 10 kDa) PAS-positive bands could be seen. These may be polysaccharides not corresponding to Coomassie blue stained protein bands. Alliinase was also the only PAS-positive protein in the precipitate after fractionation of a crude extract with $(NH_4)_2SO_4$ (50% saturation) (Fig. 1). Treatment of native alliinase with N-glycosidase F failed to change its migration on SDS-PAGE and its reaction with PAS, indicating that the oligosaccharide moieties were not removed from the native enzyme.

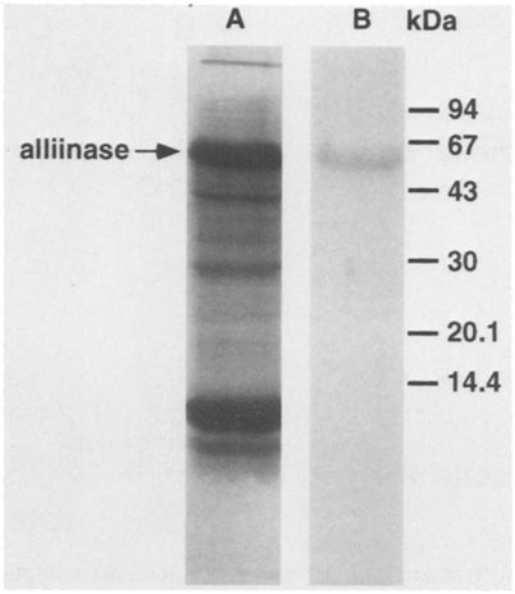

Figure 1. SDS-PAGE of garlic extract after $(NH_4)_2SO_4$ precipitation (50% of saturation). Lane A: stained with Coomassie blue. Lane B: the same preparation PAS-stained.

Alliinase purification on Con A-Sepharose

A minor contamination of less than 14 kDa, which contains at least two protein bands, was observed on SDS-PAGE in alliinase preparations after purification on Con A Sepharose (Fig. 2, Lane D). Western blot of this preparation with rabbit polyclonal antibodies against purified alliinase showed no interaction with these low molecular weight protein bands. (Fig. 3B).

Size exclusion chromatography on a Superose-12 column

Separation of Con A-Sepharose purified alliinase on a Superose-12 column showed that a small peak preceded the main one (Fig. 4, Lanes a, b). Analysis of the two

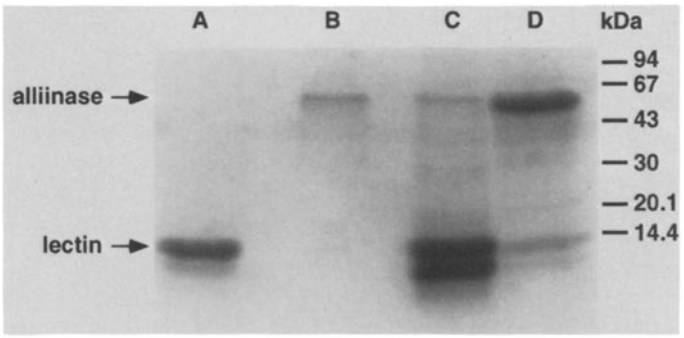

Figure 2. Evidence for the natural complex occuring between alliinase and garlic mannose-specific lectin(s). A and B: SDS-PAGE of ASA and alliinase preparations respectively which were finally purified on HPLC size exclusion chromatography (Superose-12 column). C: lectin purified on mannose-Sepharose; D: alliinase purified on Con A-Sepharose. Proteins were stained with Coomassie blue.

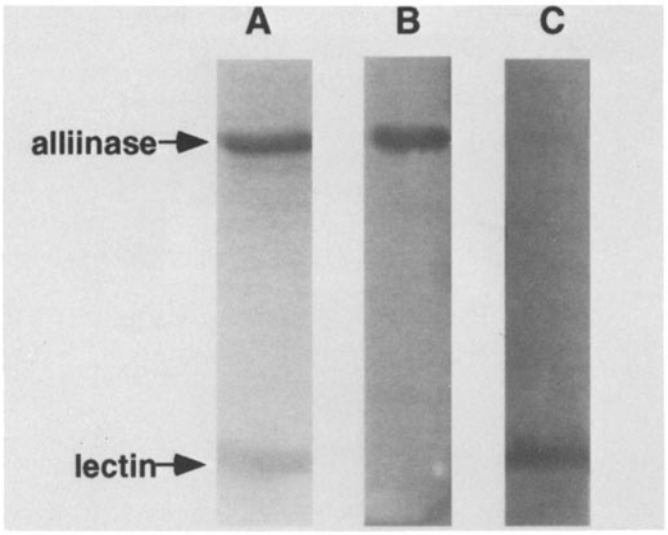

Figure 3. Western blot of alliinase after chromatography on ConA-Sepharose. Lane A: Coomassie stained alliinase. Lane B: blot of alliinase developed with antialliinase antibodies. Lane C: blot of alliinase developed with antiASA antibodies.

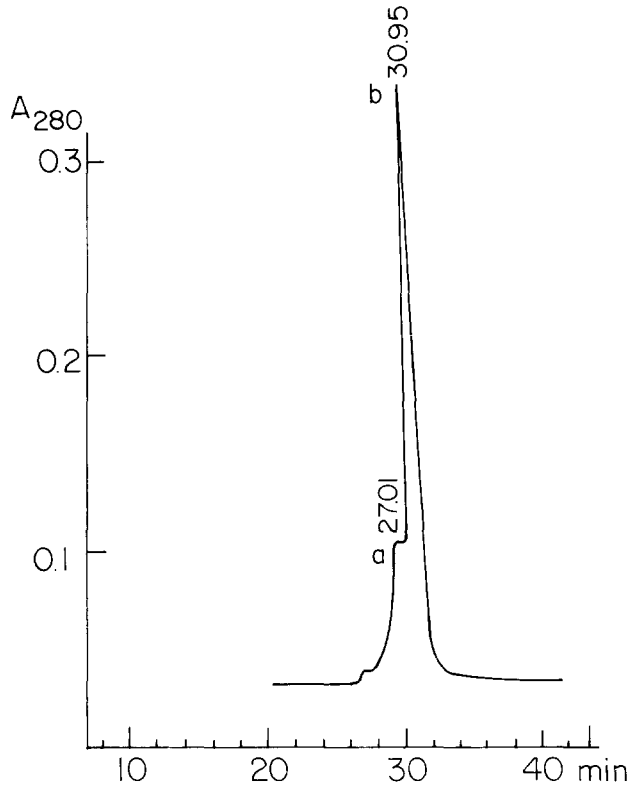

Figure 4. Size exclusion chromatography of Con A-Sepharose purified alliinase on a column of Superose-12. Buffer, PBS, pH 7.4. Flow rate: 0.4 ml min^{-1}. Elution times for alliinase-lectin complex (a) and pure alliinase (b) were 27 and 31 min respectively.

peaks by gel electrophoresis revealed that both contained alliinase, but whereas the second peak contained only alliinase, the first one (Fig. 4, Lane a) contained the enzyme with the same contaminants as seen in the starting material. Size exclusion chromatography of crude garlic extract also confirmed the presence in alliinase fractions of the same proteins (data not shown). Recently, low MW proteins were identified in garlic bulbs as isoforms of a mannose-specific lectin (ASA) [19]. Western blot with anti-ASA antiserum indeed revealed the identity between ASA and the low MW proteins coeluted with alliinase from Con A-Sepharose (Fig. 3, Lane C). Therefore we concluded that ASA forms a complex with alliinase through the sugar moiety of the enzyme.

ASA purification on mannose-Sepharose

In order to study the interaction between alliinase and ASA, we have purified the lectin(s) on a mannose-Sepharose column as described in the Methods. The isolated lectin composed of subunits of MW 11.5 and 12.5 kDa (Fig. 2, Lane C) did not agglutinate human erythrocytes but caused the agglutination of rabbit erythrocytes at a concentration about 100 $\mu g\,ml^{-1}$, and was selectively inhibited by 20–50 mM of D-mannose. Van Damme *et al.* [19] also reported rather low specific activity of ASA. The specific activity could be increased (up to 1 $\mu g\,ml^{-1}$) if biotinylated ASA in the presence of avidin was used (B. Tchernishev, private communication). As can be seen in Fig. 2 (Lane C) the ASA preparation after elution from the mannose-Sepharose column contained some alliinase. This is analogous to the presence of small amounts of lectin in alliinase purified on Con A-Sepharose column (Fig. 2, Lane D).

Study of the nature of alliinase – ASA interaction

In order to study the complex formation between garlic alliinase and ASA we used a radioligand assay. Addition of increasing amounts of [^{125}I]-alliinase to a plate coated with ASA led to increasing but saturable binding of the radioactivity, indicating a complex formation (Fig. 5A). Addition of methyl α-D-mannoside together with alliinase prevented enzyme binding (Fig. 5B). The competition was concentration-dependent. The calculated value of I_{50} for methyl α-D-mannoside was 3×10^{-2} M. These results confirm the existence of carbohydrate mediated alliinase-lectin interactions.

Identification of the lectin type present in the complex with alliinase

To find out which of the two lectins ASA I and ASA II, present in the garlic bulbs, forms the complex with alliinase, the isolated complex was dissociated by SDS-PAGE [24]. The N-terminal sequence analysis of the first 10 amino acid residues of the lectin bands revealed the sequences TLARNILRND and RNLLTNCYAG. These

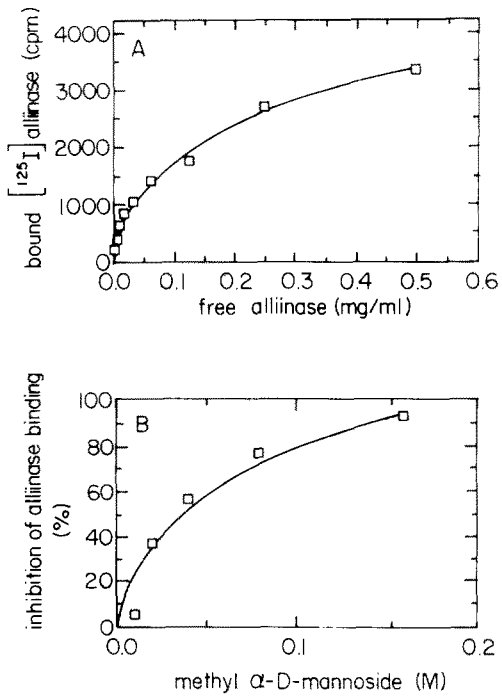

Figure 5. Competition between methyl α-D-mannoside and [I^{125}]-alliinase for binding to garlic lectin. Binding of [I^{125}]-alliinase to ASA in the absence (A) and in the presence (B) of methyl α-D-mannoside.

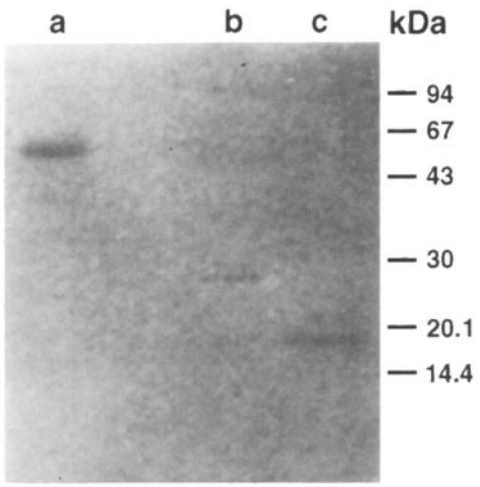

Figure 6. PAS-staining of alliinase and its degradation products after CNBr cleavage, using gradient SDS-PAGE according to Fling and Gregerson [22]. Lane a: purified alliinase. Lane b: CNBr treated alliinase in the presence of 70% formic acid. Lane c: CNBr treated alliinase in the presence of 70% trifluoroacetic acid.

sequences correspond to the 12.5 and 11.5 kDa subunits of ASA I. An insignificant amount (less than 10%) of ASA II was found to contaminate the 12.5 kDa band of ASA I subunit.

Identification of glycosylation site of alliinase

Taking into consideration that alliinase contains 5.5–6% of sugar [11, 13], only one or two of the four putative glycosylation sites could be occupied. Their identification was achieved from analysis of a number of peptides obtained after CNBr and chymotryptic digestion.

Isolation of glycopeptides after CNBr cleavage of alliinase

Upon CNBr cleavage of alliinase in the presence of 70% formic acid a set of peptides was obtained. PAS-staining of SDS-PAGE gels revealed a major positive band of 28 kDa (Fig. 6, Lane b). The first 10 amino acid residues from its N-terminal had the sequence: SYFFNPVSNF. Based on the known protein sequence of alliinase it was concluded that this large fragment starts at Ser^{91} and contains three out of the four putative glycosylation sites. Analysis of potential sites of CNBr cleavage of alliinase showed that there are at least three methionine residues followed by Ser or Thr. It is known that Met-Ser and Met-Thr are resistant to CNBr cleavage in the presence of 70% formic acid but cleavable in the presence of TFA [38, 39]. Accordingly CNBr treatment of alliinase in the presence of 75% TFA yielded a set of peptides, among which was a PAS-positive one of 18 kDa (Fig. 6, Lane c). N-terminal sequence analysis revealed the same 10 amino acids as above, and comparison with the protein sequence showed that this 18 kDa fragment contains two potential glycosylation sites.

In an attempt to further reduce the size of the carbohydrate-containing CNBr peptide, we performed a CNBr cleavage, in the presence of 75% TFA, of the peptides obtained from CNBr treatment in the presence of 70% formic acid. SDS-PAGE revealed that only one major peptide from this CNBr digest was stained by PAS (data not shown). For its isolation, the CNBr peptide mixture was lyophilized and separated on a Superdex-75 column. It resolved into three main peaks (Fig. 7a). Only the first peak was PAS-positive. Tricine SDS-PAGE (16.5% gel) of this peak (Fig. 7b) resulted in several peptides of which only one at 6 kDa was PAS positive. The N-terminal sequence of this glycopeptide was the same as in both previous cases. Simple calculation revealed that the 6 kDa peptide obtained by CNBr cleavage starts from Ser^{91} and ends after the next methionine residue. Therefore this peptide consists of 56 amino acids, which is in good agreement with its experimentally estimated MW and contains only one glycosylation site at its C-terminal sequence: Asn^{146}-Met^{147}-Thr^{148}. It could thus be concluded that Asn^{146} is the site of alliinase glycosylation. To further support this conclusion we analysed the peptides obtained after chymotrypsin cleavage of alliinase. The presence of sugars in the peptides was detected with orcinol on TLC. Due to the high hydrophilicity of the orcinol positive peptides they were poorly resolved by HPLC on a reverse phase

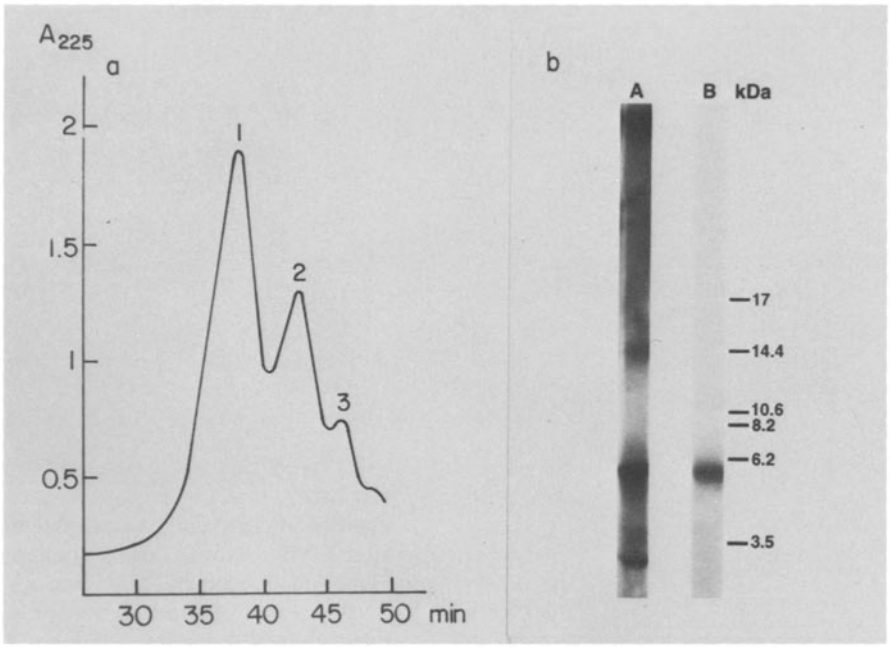

Figure 7. (a) Size exclusion chromatography of alliinase after sequential CNBr cleavage, first in the presence of 70% formic acid, followed by cleavage in the presence of 70% trifluoroacetic acid. Column, Superdex-75; flow rate, 0.5 ml min^{-1}. (b) Tricine SDS-PAGE of the peak 1 fraction from (a). Lanes A and B: Coomassie and PAS-stained gels, respectively.

column. Therefore we used a column of Superose-12 for the isolation of low MW orcinol positive fractions. Further purification was carried out on a silica gel column, where enriched carbohydrate containing fractions were obtained. Final isolation of the glycopeptide was performed on HPLC reverse phase as described in Methods. One major peptide peak which was orcinol positive was obtained (data not shown). N-terminal analysis of the first five residues revealed the following sequence: LSP*MT, in which(*) should be ASN^{146} according to the alliinase sequence.

This confirms the previous conclusion that this residue is the site of glycosylation of alliinase.

Discussion

In many proteins there are several putative N-glycosylation sites, not all of which are glycosylated. This is also the case of the glycoprotein alliinase which contains 5.5–6% of neutral sugars. From DNA sequence analysis there are four possible sites of N-glycosylation: Asn^{19}-Cys^{20}-Ser^{21}, Asn^{146}-Met^{147}-Thr^{148}, Asn^{191}-Val^{192}-Ser^{193}, and Asn^{328}-Ile^{329}-Thr^{330}, but from the sugar content only one or two sites can be occupied. In this study the site of glycosylation was established by two independent methods, using CNBr and chymotryptic cleavages. It was demonstrated that only one of the four putative glycosylation sites is glycosylated and is located on Asn^{146}. Why nature prefers this site over the others is not yet known. Determination of more sites of glycosylation on different proteins may give an answer to this important question. Our study is only a small contribution in this direction. Interestingly another glycoprotein we are working on, avidin, also contains the sugar at the same sequence [40].

The two major protein components of garlic are the enzyme alliinase, which is a mannose rich glycoprotein, and the lectin, which is specific for mannose, indicating possible interaction between these two proteins. We found only a few indications of the existence of interactions between lectins and glycoproteins in the same plant. Gansera *et al.* [41] described the interaction of Leguminosae lectins with proteins (lectin binders). Bowles and Marcus [42] showed the existence of complexes between lectins and glycoproteins in the crude extracts of soybean and jackbean. Baumann and Rüdiger [43] demonstrated the presence of a complex between two lectins from *Vicia cracca*. One of them was a mannose rich glycoprotein and the other one was a mannose binding lectin. A similar interaction was demonstrated in the present study. In all the methods used to purify either the enzyme or the lectin, the purified protein was contaminated with the other. Furthermore, gel filtration of purified alliinase showed clearly the presence of the complex. More interesting is the fact that even after purification of alliinase on a Con A column, which is specific for mannose, a part of ASA remained bound to the enzyme. The same is true with ASA purified on a mannose column, where the enzyme remained partially associated with the lectin. A possible explanation for this phenomenon is illustrated in Fig. 8, which shows that if

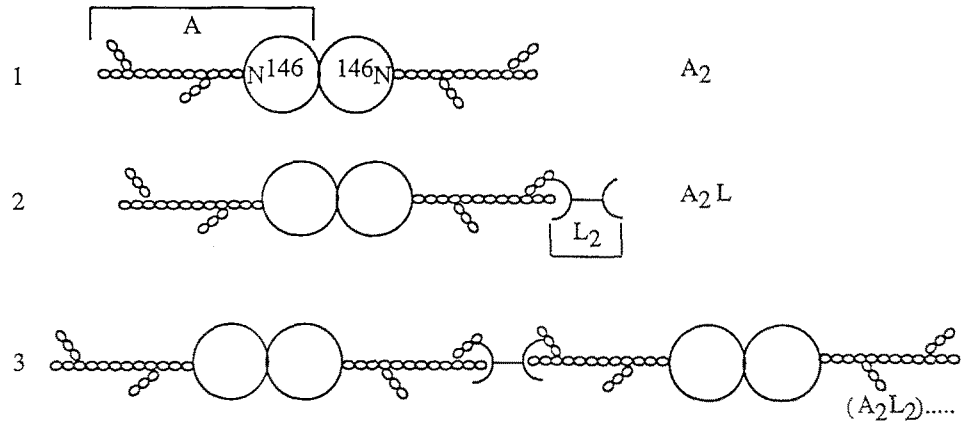

Figure 8. Some possible configurations of the alliinase-lectin complex. A: alliinase. L: lectin. (1) alliinase (dimer) with free oligosaccharide moieties. (2, 3) Some probable forms of alliinase-ASA complexes with partly occupied oligosaccharide moieties, still capable of binding to Con A-Sepharose.

one considers probable interactions between the ASA dimer and enzyme dimer, there is still room for binding to the affinity columns without disruption of the complexes. Based on the calculated apparent MW of 240 kDa for the alliinase-ASA complex (peak a in Fig. 4), the existence of both A_2L and A_2L_2 configurations seems to be possible (Fig. 8). The presence of ASA I in the complex with the alliinase is not surprising since it is known to bind mannose more effectively [44]. The benefits of such an interaction or complex formation is not yet known. But in view of the fact that the biological role of plant lectins is still unknown, any interaction between a lectin and a constituent of the same plant deserves attention.

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