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Alliin Lyase (Alliinase) from Garlic (*Allium sativum*)

Biochemical Characterization and cDNA Cloning

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ABSTRACT

The garlic plant (*Allium sativum*) alliinase (EC 4.4.1.4), which catalyzes the synthesis of allicin, was purified to homogeneity from bulbs using various steps, including hydrophobic chromatography. Molecular and biochemical studies showed that the enzyme is a dimer of two subunits of MW 51.5 kDa each. Its K_m using synthetic S-allylcysteine sulfoxide (+isomer) as substrate was 1.1 m*M*, its pH optimum 6.5, and its isoelectric point 6.35. The enzyme is a glycoprotein containing 6% carbohydrate. *N*-terminal sequences of the intact polypeptide chain as well as of a number of peptides obtained after cyanogen bromide cleavage were obtained. Cloning of the cDNAs encoding alliinase was performed by a two-step strategy. In the first, a cDNA fragment (*pAli-1*-450 bp) was obtained by PCR using a mixed oligonucleotide primer synthesized according to a 6-amino acid segment near the *N*-terminal of the intact polypeptide. The second step involved screening of garlic λgt11 and λZAPII cDNA libraries with *pAli-1*, which yielded two clones; one was nearly full length and the second was full length. These clones exhibited some degree of DNA sequence divergence, especially in their 3′ noncoding regions, suggesting that they were encoded by separate genes. The nearly full length cDNA was fused in frame to a DNA encoding a signal peptide

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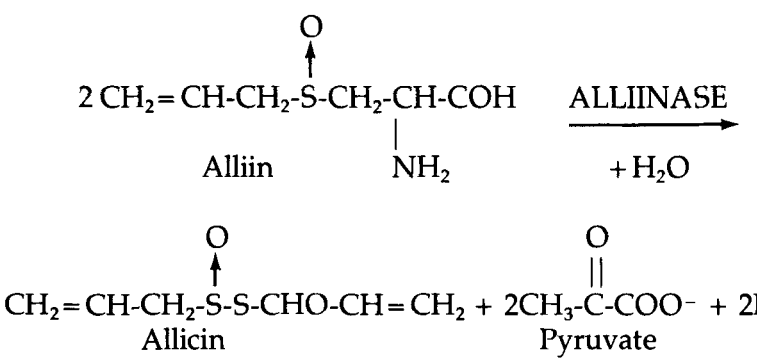
from α wheat gliadin, and expressed in *Xenopus* oocytes. This yielded a 50 kDa protein that interacted with the antibodies against natural bulb alliinase. Northern and Western blot analyses showed that the bulb alliinase was highly expressed in bulbs, whereas a lower expression level was found in leaves, and no expression was detected in roots. Strikingly, the roots exhibited an abundant alliinase activity, suggesting that this tissue expressed a distinct alliinase isozyme with very low homology to the bulb enzyme.

Index Entries: Alliinase; cDNA sequence; alliin; allicin; garlic.

Abbreviations: PMSF, Phenyl methyl sulfonyl fluoride; HPLC, high performance liquid chromatography; SDS-PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; DTNB, 5,5′dithiobis-(2-nitrobenzoic acid); CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid); PCR, polymerase chain reaction.

INTRODUCTION

Garlic, *Allium sativum L.*, has been known for generations to be a source of compounds that were prescribed for a variety of maladies. During the last two decades there has been a renewed research interest in the therapeutic uses of garlic. Fresh garlic and preparations of garlic extract were shown to increase fibrinolitic activity, inhibit platelet aggregation, lower the levels of cholesterol in serum, and possess antitumor activity (*1*). One of the known reactions that occurs extremely fast following crushing of the garlic clove is the generation of allicin (thio-2-propene-1-sulfinic acid *S*-allyl ester) (*2*). Allicin is a labile compound that easily decomposes. It is responsible for the pungent smell and has been shown to possess a variety of biological activities, such as antifungal, antiamebic, and antithrombotic activities, and is the molecule that is most likely responsible for lowering cholesterol levels (*3–5*). The intact garlic clove does not contain allicin but rather its precursor, the nonprotein amino acid alliin (*S*-allyl-L-cysteine sulfoxide) (*6*). Alliin is converted to allicin, pyruvate, and ammonia by the enzyme alliinase (Alliin lyase) (EC 4.4.1.4), which apparently resides in another compartment of the clove and was first described by Stoll and Seebeck (*7,8*).



Alliinase has been isolated and purified (*9–12*), but conflicting results were reported with regard to its molecular mass and biochemical characteristics. In the present investigation, we describe an improved method for the purification of alliinase and assays to study its biochemical properties. In addition, we have cloned and sequenced the cDNA and expressed the recombinant enzyme in the heterologous system of *Xenopus laevis* oocytes. Preliminary results of this study were described (*13–16*). After completion of this study, a paper that describes the sequence of an alliinase that has considerable homology to the one described in this report was published (*17*).

MATERIALS AND METHODS

Synthesis of Alliin

Alliin was synthesized from L-cysteine and allyl bromide following oxidation by H_2O_2 by the procedure of Stoll and Seebeck (*8*). the stereospecific product obtained, (+)*S*-allyl-L-cysteine sulfoxide (MP = 164°, $[\alpha]_D^{20}$ in H_2O + 62.1°), was identical to the natural substrate, alliin. Its yield was 15.7%.

Purification of Alliinase from Garlic Cloves

Peeled garlic cloves (60 g) were homogenized in the cold in a mincing machine in Na-phosphate buffer (90 mL, 0.02*M*, pH 7.2) containing glycerol (10%), PMSF (1 m*M*), and pyridoxal 5′-phosphate (0.02 m*M*) (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%) 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 solution was placed on a hydroxylapatite (DNA-grade bio-gel HTP, Bio-Rad, Richmond, CA) column (2.2 × 50 cm). The column was washed with Na-phosphate buffer (0.05*M*, pH 7.2) and protein containing enzymatic activity (*see below*) was eluted with Na-phosphate buffer (0.3*M*, pH 7.2). The eluate was brought to 50% saturation with $(NH_4)_2SO_4$, and stirred slowly for 30 min; the slurry was then centrifuged at 20,000*g* for 15 min at 4°C. The pellet was dissolved in 2 mL of K-phosphate buffer (0.1*M*, pH 7.2), diluted with the same volume of Na_2SO_4 (2*M*), and placed on an HPLC hydrophobic interaction chromatography column (7.5 × 75 mm, Ultrapac TSK Phenyl 5PW, LKB, Sweden). Elution of the enzyme was carried out with a gradient of Na_2SO_4 (from 1.0 to 0*M*) in K-phosphate buffer (0.1*M*, pH 7.2). Fractions of 1 mL were collected and aliquots assayed for alliinase activity.

Enzymatic Assay

The assay was based on the method of Schwimmer and Mazelis (*18*). The standard reaction mixture contained Na-phosphate buffer (0.1*M*, pH 6.5); pyridoxal 5′-phosphate (0.025 m*M*), NADH (0.2 m*M*), lactic dehydrogenase (10 U), alliin (6 m*M*), and an alliinase sample in a total vol of 1 mL. Enzymatic activity was monitored spectrophotometrically by the decrease in absorbance at 340 n*M* using cuvets of 1 cm in path length. A unit of activity was defined as the amount of enzyme required to release 1 μmol of pyruvate per min.

SDS-PAGE and Protein Analysis

Proteins were separated on 10% SDS-PAGE according to Laemmli et al. (*19*), or on gradient (7.5–20%) SDS-PAGE. Protein was assayed by the procedure of Lowry et al.(*20*) with ovalbumin as a standard. Protein concentration of purified alliinase was verified by amino acid analysis data.

Amino Acid Composition

Amino acid analyses were performed on a Dionex D-500 amino acid analyzer (Durrum Instrument Corp., Palo Alto, CA). Protein samples were hydrolyzed in vacuum in 6*M* HCL at 110°C for 22 h.

Preparation of Antibodies

A rabbit was immunized by intradermal injection of 0.25 mg of purified alliinase emulsified in complete Freund's adjuvant followed by three biweekly booster injections with 0.125 mg protein in incomplete Freund's adjuvant. The titer of anti-alliinase serum was estimated by ELISA and its specificity by Western blot analysis.

Western Blot Analysis

Following SDS-PAGE, proteins were transferred to a nitrocellulose paper according to Towbin et al. (*21*) and immunoblotted and antialliinase rabbit serum. The serum dilutions used were 1:200. The immunoreactive bands were detected by the alkaline phosphatase assay using a goat anti-rabbit–alkaline phosphatase conjugate (Bio Makor, Rehovot, Israel) as a secondary antibody (*22*).

Molecular Mass Estimation

The molecular mass of garlic alliinase was determined by HPLC analysis on a Varian TSK 3000 SW size exclusion chromatography column equilibrated with 0.1*M* imidazole-HCL buffer, pH 6.8, containing 0.15*M* NaCl. For calibration, marker proteins of known molecular mass (Sigma [St. Louis, MO] kit MW-GF-1000) were used. Molecular mass of the sub-

units was estimated by SDS-PAGE 7.5–20% gradient system using markers for molecular mass calibration (Pharmacia, Uppsala, Sweden).

Determination of pI

The pI value of alliinase was determined by analytical IEF on Phast Gel IEF 3.5–10 media, and a pI-marker protein kit for pI-value estimation from 3.5–9.3.

K_m Value

The K_m value for the garlic alliinase was calculated from Lineweaver-Burk double-reciprocal plots derived from enzymatic assays at different concentrations of synthetic alliin as substrate.

Carbohydrate Analysis

The carbohydrate content of alliinase was determined by the phenol-sulfuric method of Dubois et al. (23) using glucose as a standard. The glycoprotein nature of alliinase was also confirmed by its interaction with Concanavalin A.

Determination of SH-Groups

Free SH-groups content was analyzed with DTNB (Ellman's reagent) (24) and 4,4′ dithiodipyridine (25).

CNBr Cleavage of Alliinase

Purified alliinase (2 mg/mL) was desalted on a Sephadex G-25 column (0.5 × 25 cm), concentrated by acetone precipitation (acetone:protein solution, 10:1) and redissolved in 70% formic acid (3 mg/mL). Solid CNBr was added to a final concentration of 0.7–1.0*M*. Cleavage proceeded in the dark, at room temperature, for 20 h as described by Steers et al. (*26*). Peptide fragments were separated by gradient SDS-PAGE as described by Fling and Gregerson (*27*) with a high-molarity Tris-HCl buffer (3*M*, pH 8.8). The resolving gel contained 10–25% acrylamide and the stacking gel 2.5% acrylamide.

N-Terminal Sequencing

N-terminal sequencing of protein and peptide samples was done with an Applied Biosystems 475 A sequencer. Analysis was performed after SDS-PAGE separation and transfer of the bands of intact alliinase and its CNBr fragments by electroblotting (300 mA, 3–5 h) onto an Immobilon poly(vinylene difluoride) (PVDF) membrane in 10 m*M* CAPS acid buffer, pH 11.0, containing 10% methanol (*28*). The membrane was washed with water, stained with Coomassie blue (0.25% in 50% methanol/10% acetic acid) and the stained bands were cut off from the membrane after drying.

Isolation of Allicin

The procedure for the isolation of allicin was carried out according to Jansen et al. (*29*). Alliin (300 mg) was dissolved in 300 mL of 0.1*M* Na-phosphate buffer, pH 6.5, and incubated at 37°C together with purified alliinase (30 U/mg). After 2 h of incubation the solution was extracted twice with ether and dried over Na_2SO_4. Ether was removed with a stream of dry air at room temperature. Allicin (92 mg) was dried in a refrigerated dessicator over sulfuric acid (98%).

Thin Layer Chromatography

TLC identification of *S*-allyl-L-cysteine and alliin was carried out on precoated cellulose plates (Merck, Darmstadt, Germany) using as solvent *n*-butanol-acetic acid-H_2O (4:1:1 v/v). After drying, the plates were sprayed with ninhydrin (0.25%) reagent and placed in an oven at 100°C for 10 min. Allicin (R_f = 0.375) was identified by chromatography on silica gel plates (Merck) using a solvent system consisting of benzene-ethyl acetate (90:10), allicin was detected on the plates by free iodine vapor (*3*).

Quantitative Determination of Alliin and Allicin

Quantitative determinations of alliin and allicin were obtained using an LKB HPLC system with an SP 4290 integrator (Spectraphysics). The separation was achieved on a LiChrosorb RP-18 column using as eluant methanol (60%) in water containing 0.1% formic acid. Alliin emerged after 6.4 min and Allicin after 12.6 min.

Nucleic Acid Labeling

Plasmid DNAs were labeled by using a mixture of random hexanucleotides based on the method developed by Feinberg and Vogelstein (*30,31*). The reaction mixture as well as [α-^{32}P]dATP and Klenow enzyme (Promega, Madison, WI) were incubated at room temperature for 1 h followed by ethanol precipitation. RNA probes were prepared essentially by the above procedures except that the cap structure was omitted and [α-^{32}P] UTP (50 mCi, 400 Ci/*M*) was added.

Preparation of Poly(A)$^+$ RNA

Total RNA was extracted from various parts of the garlic plant using the guanidium/CsCl procedure (*32*). Poly(A)$^+$ RNA was isolated by fractionating the total RNA on a column of oligo(dT)-cellulose (Boehringer Mannheim GmbH, Mannheim, Germany), as previously reported (*33*).

Synthesis and Cloning of a cDNA Fragment Coding for Alliinase by MOPAC (Mixed Oligonucleotide Primed Amplification of cDNA) Strategy

Oligonucleotide primers were prepared based on the *N*-terminal amino acid sequence with an Applied Biosystems 480A DNA synthesizer. The mixed oligonucleotides together with oligo $d(T)_{30}$ were used as primers in the polymerase chain reaction (PCR) according to Lee et al. (*34*). The template used was the first strand cDNA generated from poly(A)$^+$ mRNA by reverse transcriptase with oligo d(T) primers. PCR products were obtained after 30 cycles of amplification (20 s at 94°C, 40 s at 58°C, and 40 s at 72°C), and directly subcloned into PCR1000 using the TA cloning kit (Invitrogen Corp., San Diego, CA) according to the manufacturer's protocol.

Construction of cDNA Libraries

An oligo (dT)-primed double-stranded cDNA was synthesized from 5 μg of poly(A)$^+$ RNA obtained from mature garlic bulbs essentially as described (*35*) using a cDNA synthesis kit (Promega Corp.) and *Eco*RI-cut dephosphorylated λgt11 vector according to the manufacturer's instructions. The resulting garlic cDNA library (7.5×10^4 primary recombinant phages) was screened with ^{32}P-labeled probes as described (*32*).

Screening of the cDNA Libraries

Recombinant phages were plated with *Escherichia coli* Y1090, and then blotted on Hybond-N membranes (Amersham, Arlington Heights, IL) in duplicates. Membranes were hybridized with oligonucleotides or PCR fragments labeled with [α-^{32}P]dATP; hybridization was carried out in 5X SSC, 5X Denhardt's solution and 1% SDS at 65°C for 12–16 h. The membranes were washed twice for 10 min each at room temperature with 2X SSC and 0.1% SDS, and then for 15 min at 65°C with 2X SSC and 0.1% SDS. Autoradiography signals were obtained after 12–16 h of exposure at −70°C using Kodak X-Omat AR film with a Cronex Lightning Plus intensifying screen.

Isolation of a Full Length cDNA Clone from a λZAPII cDNA Library

Total RNA from garlic bulbs was extracted as previously described above and a garlic cDNA library was constructed in λZAPII vectors (Clonetech, Palo Alto, CA). The number of recombinant phages was ca 1.6×10^6/mL. A total of 7.2×10^4 plaques were screened using the 450 bp DNA fragment (*see below*) *pAli-1* as a probe. The phagemids were excised from the positive clones by the automatic excision protocol according to the manufacturers' instructions.

Subcloning and DNA Sequencing

The DNA insert was excised out of the phage with *EcoRI* and then subcloned into the dephosphorylated *EcoRI* site of pBluescript™ KS(+) (Stratagene, La Jolla, CA). The nucleotide sequence was determined on both strands by the dideoxy chain termination method (Sequenase Version 2; US Biochemical Corp., Cleveland, OH) (*35,36*).

Northern Blot Hybridization

Total RNA was prepared from root, leaf, and bulb tissues of garlic plants by the guanidium isothiocyanate method (*31*). Approximately 7 μg of RNA was separated by electrophoresis on agarose gels (1%) using a buffer containing formaldehyde, MOPS (50 m*M*, pH 7.0), and transferred to Hybond-N nylon membrane and hybridized with the ^{32}P labeled random primed alliinase cDNA probe as described above. mRNA size was estimated according to the migration of 28S and 18S ribosomal RNA in Methylene blue-stained membranes prior to hybridization.

In Vitro Transcription

The plasmid construct containing the alliinase cDNA was linearized at its 3′ end downstream to the poly(A) sequence by digestion with *HindIII*. Capped Poly(A)$^+$ RNA was transcribed in vitro as previously described (*37*) using T7 RNA polymerase and the cap analog G(5′)ppp(5′)G.

Expression in *Xenopus* Oocytes

Xenopus laevis females were anesthetized in ice cold water. Stage 6 oocytes were dissected manually, microinjected with 50 nL of a mRNA solution, and then incubated at 20°C for 14 h in excess OR2 medium (*38*). Oocytes were extracted with a solution of Tris (50 m*M*, pH 7.5), PMSF (1%), and NP-40 (0.5%) and analyzed on SDS-PAGE followed by immunodetection.

RESULTS

Distribution of Alliinase Activity in the Garlic Plant

Study of the enzymatic distribution in the various garlic plant tissues showed that specific activity of alliinase increases from the leaves to the bulb (Fig. 1). The total activity in the bulb of a mature plant is approx 10 times higher than that in the leaves. The specific activity of alliinase in the different sections of the garlic plant correlated with the level of the enzyme protein, as detected on Western blots by antibodies prepared against

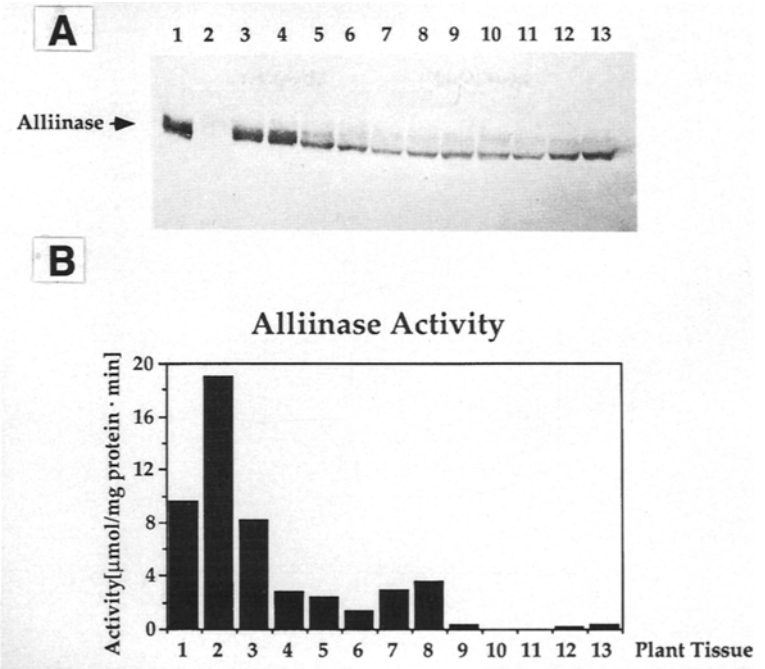


Fig. 1. Distribution of alliinase in garlic plant. Protein samples were extracted from mature garlic bulb (lane 1), root (lane 2), and consecutive 4-cm sections from the bulb to the green leaves along the garlic plant (lanes 3–13). Equal amounts of total protein from each extraction were loaded on SDS-PAGE and then subjected to a western blot analysis with antialliinase (**A**). Each sample was subjected to an activity assay as described in Materials and Methods and the data was plotted as shown (**B**). Arrow indicates the position of alliinase on the western blot.

purified alliinase (Fig. 1). Interestingly, a very high lyase activity was detected also in the roots (Fig. 1A,B; lane 2), although no immunological cross reaction material was detected in the Western blots. This activity was apparently caused by a different alliin lyase enzyme.

Purification of Alliinase

The enzyme was purified to homogeneity from bulbs as described in Methods. A remarkably high degree of purification was achieved by the inclusion of a hydrophobic interaction chromatography step (Fig. 2). Results of purification are summarized in Table 1. The yield of purified alliinase was approx 3.2 mg/10 g cloves. The yield of alliinase differed significantly with the source of garlic and the length of time it had been in storage. Freshly harvested young green garlic cloves obtained straight from the field in spring were the best source.

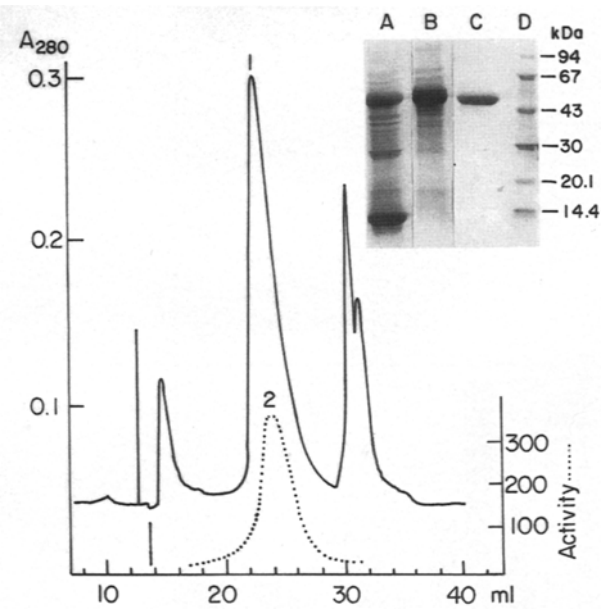


Fig. 2. HPLC hydrophobic interaction chromatography. Alliinase preparation after hydroxylapatite column was loaded onto a TSK phenyl 5PW column (7.5 × 75 cm). The gradient used was: Solution A: Na_2SO_4 (1*M*) in K phosphate buffer (0.1*M*, pH 7.2); Solution B: K-phosphate buffer (0.1*M*, pH 7.2). Time intervals were: 0–10 min, 40% B; 10–20 min, 40–46% B; 20–40 min, 46–100% B. The flowrate was 1 mL/min. Fraction volume was 1.0 mL. Curve 1: absorption at 280 nm; Curve 2: alliinase activity (mmol/min^{-1}/mL^{-1}). Insert: Coomassie stained SDS-PAGE of alliinase fractions. A, Original crude garlic extract; B, Alliinase fraction from hydroxylapatite colume (*see* Methods); C, Alliinase fraction from hydrophobic interaction column; D, molecular mass markers (Pharmacia LMW Kit E) (phosphorylase b, 94 kDa; bovine serum albumin, 67 kDa; ovalbumin, 45 kDa; carbonic Anhydrase, 30 kDa; trypsin inhibitor, 20.1 kDa; α-lactalbumin, 14.4 kDa).

Identification of Enzymatic Reaction Products

Reaction of purified alliinase and synthetic alliin yielded allicin as detected by TLC and HPLC analysis (3). Significant amounts of alliinase (ratio of three units of purified enzyme to 1 mg substrate) were required to yield a satisfactory conversion (>70%) of alliin into allicin, as determined by HPLC. Further incubation of the enzymatic reaction did not increase the yield of allicin. Neither did the addition of substrate to the reaction mixture. Reisolation of the alliinase protein from the reaction mixture by Sephadex G-25 revealed that the enzyme lost about 50% of its activity. Rabbit serum containing antialliinase antibodies inhibited the enzymatic activity of alliinase at dilutions of 1:100 in a typical enzymatic assay.

Table 1
Purification of Alliinase from Garlic Cloves (60 g)[a]

Stage of purification	Total activity, μmol/min	Protein content, mg	Specific activity, μmol/min × mg of protein	Purification
Crude Extract	16,500	2200	7.5	—
PEG-8000	14,400	306.0	47.0	6.27
Hydroxylapatite	7300	104	70.0	9.33
Ammonium sulfate, 314 g/L	6500	59	105.0	14.0
HPLC hydrophobic interaction, phenyl TSK column	5000	19.2	260.0	34.7

[a]Purification was carried out as described in Materials and Methods. The enzyme was assayed by method 2 (*see* Materials and Methods). As substrate, synthetic alliin was used. Polyethylene Glycol (PEG-8000) was added dropwise to the protein preparation at 4°C as a 50% w/v solution. Purified alliinase was kept at −70°C in the presence of 10% glycerol for months without apparent decreasing of the enzymatic activity.

Molecular Mass Estimation

The molecular mass of garlic alliinase estimated by HPLC size exclusion chromatography was approx 90 kDa. After denaturation and SDS-PAGE, the alliinase subunit mol mass was about 51.4 kDa (Fig. 2), suggesting that the enzyme is a monodimer consisting of two identical subunits.

Properties and Characteristics of Purified Alliinase

Alliinase, which contains a 5′ pyridoxal phosphate cofactor (*11,39*), had the expected absorbance spectrum in the visible region with maximum at 430 nm. The K_m value estimated using synthetic alliin as a substrate was 1.1 m*M*. The pH optimum of the enzyme was 6.5, and the isoelectric point was determined to be 6.35. The amino acid composition of natural garlic alliinase is presented in Table 2. Alliinase is a glycoprotein, as shown by direct carbohydrate colorimetric analysis of purified enzyme. Using glucose as a standard, it was estimated that the enzyme contains 6% carbohydrate (*23*). Alliinase was bound tightly to a Sepharose Con A column and was eluted as a semipurified protein (>95%) by methyl-α-D-mannoside (0.2*M*) (data not shown).

Table 2
Amino Acid Composition of Garlic Alliinase (Residues/mol)[a]

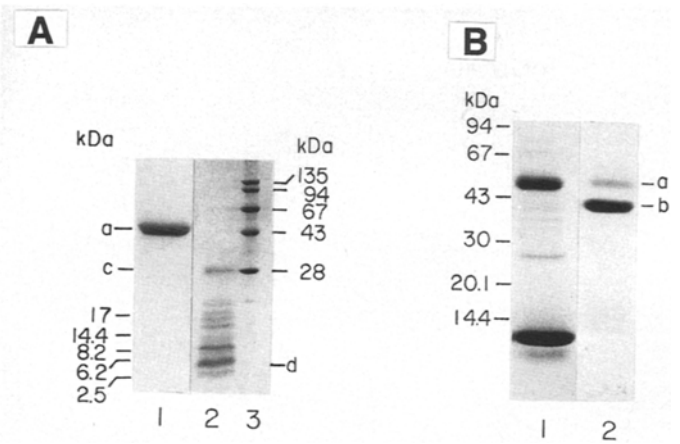
Amino acid	Alipep[b]	Purified alliinase
Ala	29	34
Cys	10	3
Asp+Asn	44	48
Glu+Gln	48	43
Phe	23	23
Gly	25	24
His	9	10
Ile	21	21
Lys	33	29
Leu	30	32
Met	13	12
Pro	21	26
Arg	21	22
Ser	32	36
Thr	28	31
Val	29	27
Trp	8	6
Tyr	24	24

[a]The deduced amino acid sequence of the full length alliinase gene, Alipep, was obtained by using the computer program GCG Package and the amino acid composition was summarized (Column "Alipep"). Amino acid analysis of alliinase protein was performed on a Dionex D-500 amino acid analyzer (Durram) after protein samples were hydrolyzed with 6*M* HCl at 110°C for 22 h (Column "Alliinase"). Tryptophane contents were estimated spectrophotometrically. Evaporation was carried out with a Speed-Vac concentrator.

[b]Amino acid composition deduced from translation of cDNA clones *pAli-1* and *pAli-2*.

Amino Acid Sequence Analysis of Alliinase

The sequence of the first 25 *N*-terminal amino acids was determined (Fig. 3A, bottom, peptide (a)). During incubation (24 h) of purified alliinase preparations at room temperature, a minor contaminant containing protease activity caused the degradation of the enzyme yielding a truncated component of 40 kDa ((b) in Fig. 3B). Sequencing of this component revealed the 19 amino acid *N*-terminal sequence shown in Fig. 3A, bottom, peptide (b). Additional information on the primary structure of alliinase was obtained, by treatment of the intact polypeptide chain



(a) Intact alliinase polypeptide

NH_2-KMTETMKAAEEAEAVANINCSEHGR

(b) Truncated 40 kDa fragment

NH_2 - FNPVSNFISFELEKTIKEL

(c) CNBr - 30 kDa fragment

NH_2 - KAAEEAEAVANINCSEHGRAFL

(d) CNBr -5 kDa fragment

NH_2 - RPPSPSYAWVNCEWEEDKDCYQTFQNGRI

Mixed primer

5'- GCT CTA GAA GAA GCI GAA GCA GTI GC -3'

Xba I G G G C G T

Fig. 3. SDS-PAGE of alliinase and its degradation products. (**A**) Purified alliinase (lane 1), alliinase reacted with CNBr (lane 2), and molecular mass markers (lane 3). (**B**) Crude garlic extract (lane 1) and truncated fragment of alliinase that was obtained after overnight incubation of the purified enzyme at room temperature (lane 2). The N-terminal sequences of intact alliinase (a), the 40-kDa truncated fragment (b), and two fragments obtained by reaction with CNBr (c and d) are shown in the lower panel along with the nucleotide sequence of the mixed primer corresponding to the underlined amino acid sequence in (a).

with CNBr that cleaves at methionine residues (*26*). Since at least 12 methionine residues are present in alliinase (Table 2), a number of peptides were obtained. These were separated on SDS PAGE (Fig. 3A) and then blotted on PVDF membranes. Sequencing of the 30 and 5 kDa peptides gave the *N*-terminal sequence shown in Fig. 3A, bottom, peptides (c) and (b). The 19-amino acid *N*-terminal sequence of the 30 kDa peptide exactly coincided with a cleavage at the second methionine (met-7) residue, close to the *N*-terminal end of the intact polypeptide chain (*see* (a) and (c) in Fig. 3).

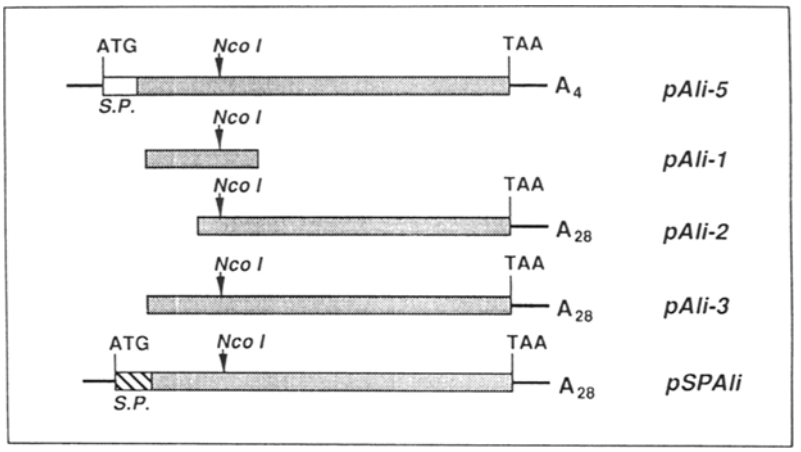


Fig. 4. Schematic representation of the various alliinase cDNA fragments obtained in this work.. (SP) The DNA encoding the signal peptide, (ATG) initiation codon, (TAA) stop codon; (NcoI) the common NcoI site used to combine *pAli-1* and *pAli-2* to form *pAli-3*. The DNA of *pAli-3* was fused to frame to a DNA encoding a signal peptide from α wheat gliadin to produce *pSPAli*.

Production of an Alliinase cDNA Probe by MOPAC

Based on the *N*-terminal amino acid sequence of purified alliinase, a mixed oligonucleotide primer was synthesized (Fig. 3B, bottom). First strand cDNA was synthesized by reverse transcriptase using Poly(A)$^+$ mRNA extracted from the garlic leaves. A PCR product (450 bp) was generated after 30 cycles of amplification, subcloned into PCR 1000 (Fig. 4; *pAli-1*) and sequenced. The sequence revealed an open reading frame that corresponded, with one mismatch (Trp-4 instead of Glu), to the NH_2-terminal sequences obtained from the alliinase protein (*see later* in Fig. 6). Identical matching was also observed with the sequence of the three additional amino acids obtained from the 30 kDa CNBr fragment (AFL in (c), Fig. 3A). This suggested that the PCR product *pAli-1* corresponded to the alliinase cDNA.

Northern blot analysis using garlic root, leaf, and bulb mRNAs revealed a single band ~1.8 kb in size (Fig. 5). Since the mol mass of alliinase is ~50 kDa, this strongly suggested that *pAli-1* hybridized to alliinase mRNA. No hybridization was observed with root mRNA (Fig. 5).

Screening of cDNA Libraries

A λgt11 cDNA library containing approx 7.5×10^4 phage plaques was screened using *pAli-1* as a probe. One sensitive clone was isolated and the insert (1.3 kb) was subcloned into the EcoRI site of pBluescript KS(+) and designated *pAli-2* (Fig. 4). This was found to be an incomplete alliinase

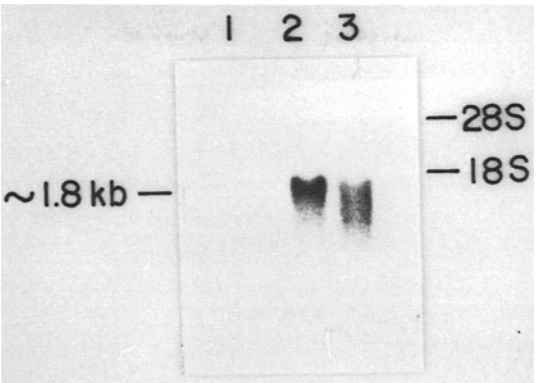
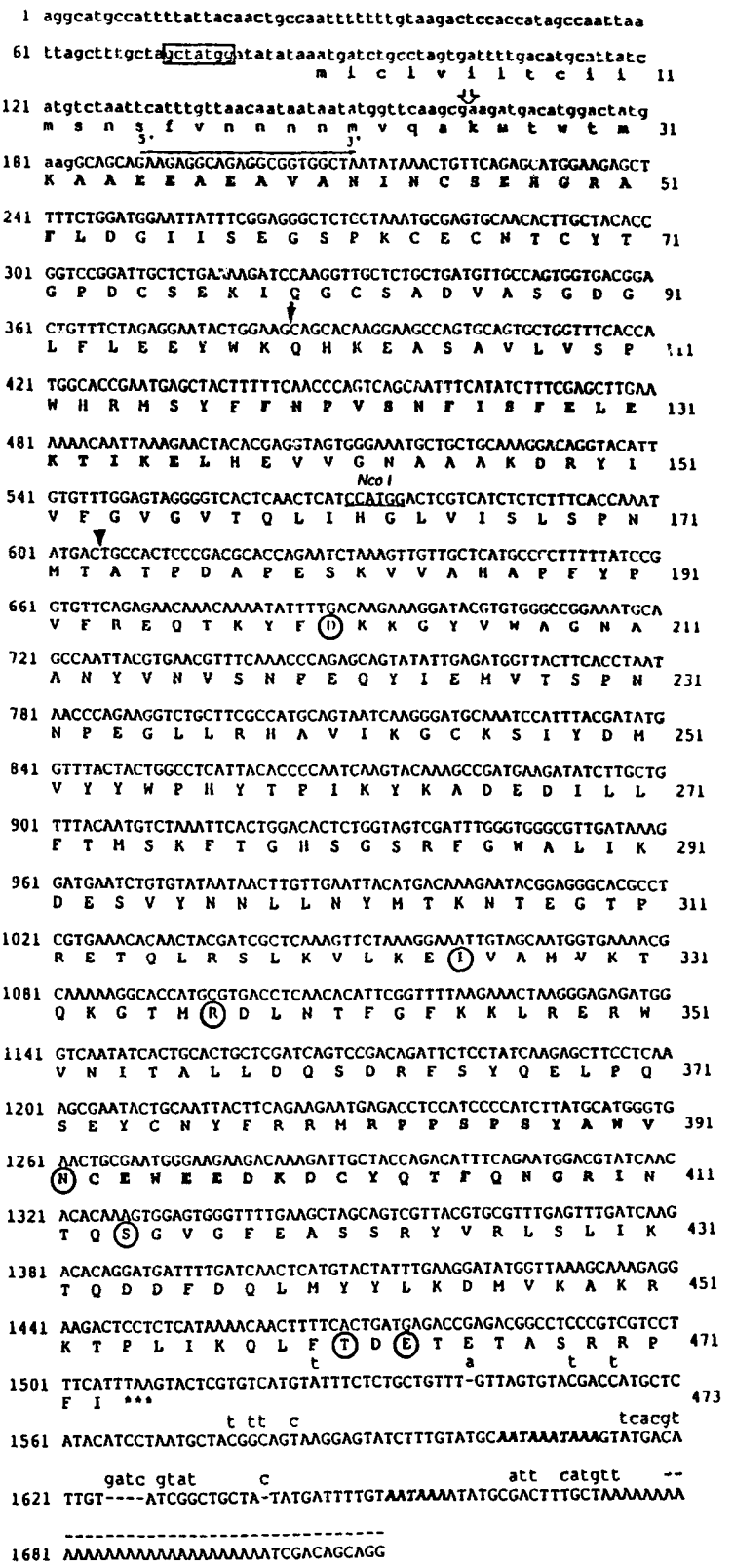


Fig. 5. Northern Blot Analysis of *pAli-1* Related mRNA. mRNA (7 mg) from (1) roots, (2) developing bulbs, and (3) garlic leaves, was analyzed on a 1.2% agarose gel in the presence of formaldehyde and then subjected to hybridization with ^{32}P random primed *pAli-1* as a probe. Positions of 28S and 18S ribosomal RNA are indicated on the right. The location of the ~1.8-kb transcript is indicated on the left, as observed in lanes 2 and 3.

cDNA sharing 200 bp overlap with the 3′ region of *pAli-1*. In addition, this cDNA contained a 3′ noncoding region and a poly(A) tail of 28 residues (Fig. 4). A λZAPII cDNA library was also screened using *pAli-1* as a probe. The largest positive clone (designated *pAli-5, see* Fig. 4) was shown by sequence analysis to contain an additional 183 bp of the 5′ sequence to that of *pAli-1*, suggesting that it represented a full length alliinase clone. The complete sequence of alliinase, as combined from *pAli-1, pAli-2,* and *pAli-5* is presented in Fig. 6. The 3′ noncoding sequence contained two potential polyadenylation sites and a poly A tail of four residues. Comparison of the 3′ regions of *pAli-2* and *pAli-5* exhibited some DNA sequence divergence between them (Fig. 6), suggesting that they were derived from two closely related but different genes.

The open reading frame of *pAli-5* started 75 nucleotides downstream of the coding region of the mature protein beginning with a methionine and a sequence possessing a hydrophobic character (Fig. 6). This is expected as alliinase is a secretary protein that apparently contains an *N*-terminal signal peptide (*40*). Yet, this open reading frame was preceded by another short open reading frame starting with a Kosak-like ATG motif (Fig. 6, boxed region), and terminating with a stop codon three codons later. The first Methionine codon of the mature alliinase open reading frame started immediately after this TAA codon. The alliinase sequence included two regions (from residue 382 to 410 and from residue 119 to 137) whose amino acid sequences were identical with the *N*-terminal sequences obtained for the 5 kDa CNBr fragment and the 40 kDa truncated fragment, respectively (Fig. 3).


Nco I

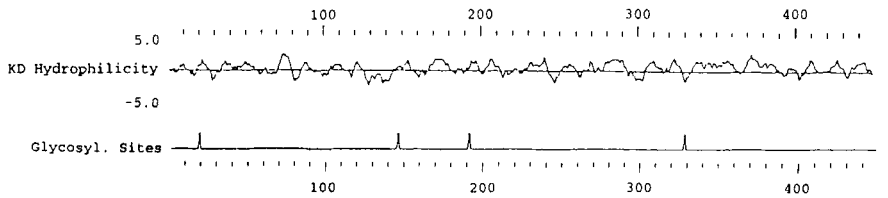


Fig. 7. Computer predictions from the sequences of alliinase. The deduced amino acid sequence of the alliinase gene was analyzed on the computer using a program Plotstructure. The hydrophilic nature and four glycosylation sites are shown.

Comparison of the deduced amino acid composition of *pAli-1* and *pAli-2* with that of the natural alliinase purified from garlic bulbs (Table 2) showed considerable similarity (±10%). Differences were found in cysteine, which is known to be sensitive to acid degradation (*41*). The deduced composition confirmed that alliinase has 10 cysteines. SH group determination showed that native and denatured (in the presence of 6*M* guanidine HCl) alliinase contained less than 0.3 free SH groups per protein subunit. Therefore the cysteine residues are potentially capable of formation of five intramolecular disulfide bonds. The position of four *N*-linked glycosylation sites and a hydrophilicity profile were predicted by the computer program (Fig. 7). This matches well with the water soluble characteristics and the carbohydrate content of the alliinase protein. A single subunit of the mature enzyme consists of 448 amino acid residues and has a mol mass of 51,500. The calculated extinction coefficient of alliinase is ($\epsilon_{280} = 7.9 \times 10^4 M^{-1}$). This value correlated well with the experimentally estimated ones, by spectrophotometry (with ovalbumin as a standard) and on the basis of amino acid analysis -$\epsilon_{280} = 7.9 \times 10^4 M^{-1}$ and $7.86 \times 10^4 M^{-1}$, respectively.

Fig. 6. (*opposite page*). cDNA and deduced amino acid sequence of alliinase. A fragment, *pAli-1*, containing nucleotide sequences (from nucleotides 184–605; the triangle indicates the end of *pAli-1*), was obtained by PCR using cDNA as a template and synthetic oligonucleotides as primers (indicated by arrowed line), as described in text. The DNA sequence of *pAli-2* (from nucleotides 385–1712, marked by a full arrowhead) was obtained after screening a λgt11 cDNA library. The overlapping region of the DNA fragments *pAli-1* and *pAli-2* was between nucleotides 385–605. The common NcoI site (which was used to combine the two clones) is underlined. The stop codon for translation is indicated by asterisks. The three transcription termination and polyadenylation signals are in boldfaced italics. The boxed region represents the Kozak-like ATG motif (*43*). The two regions sharing identity with the *N*-terminal amino acid sequences of CNBr cleavage peptides as well as that of the truncated alliinase are indicated in bold face (aa 25 open arrow, aa 118, and aa 382). The 5′ and 3′-end DNA sequences (shown in lower cases) and the deduced *N*-terminal amino acid sequence (lower case) were obtained from clone *pAli-5* after screening the λZAPII cDNA library. Amino acid differences in the deduced sequence of the mature alliinase between the cDNA cloned in this work and the cDNA cloned previously (*17*) are circled.

Construction and Expression of a Nearly Full Length Alliinase cDNA

In order to confirm that our cDNAs indeed encode alliinase we tested whether they can express a protein that would crossreact with the anti-alliinase serum. Since *pAli-5* contained at the 5′ end an additional short open reading frame whose function was not clear, a recombinant alliinase cDNA was constructed by combining the DNA sequences of *pAli-1* and *pAli-2* (Fig. 4). This was performed by fusing these cDNAs using a common NcoI site (Figs. 4 and 8). The new construct, termed *pAli-3*, lacked only the first nine *N*-terminal amino acids of the natural alliinase. The *pAli-3* construct was then subcloned into the *EcoRI* and *NotI* digested pBluescript downstream to the T7 promoter, yielding *pAli-4* (Fig. 8). In addition, since alliinase is expected to be a secretory protein, a DNA fragment (115 bp) containing the sequences coding for the signal peptide of a wheat α-gliadin storage protein (42) was fused in frame upstream of the 5′ of the coding sequences of *pAli-4* (Fig. 8). This new construct was designated as *pSPAli.* Sequencing of the recombinant plasmid verified that the signal peptide was added in frame, 5′ end to the alliinase coding sequence. Synthetic alliinase mRNA was prepared using the linearized *pSPAli* clone as template and injected into *Xenopus* oocytes. Analysis of proteins obtained from the oocytes and the incubation media by SDS-PAGE and Western blotting with the anti-alliinase serum revealed a band with a similar mol mass as alliinase. A faint band was also detected in the oocytes culture medium (Fig. 9, lane 2). No signal was visualized in the extracts of a control, consisting of noninjected oocytes (Fig. 9, lane 3).

DISCUSSION

A number of plant families that are widely divergent taxonomically are characterized by the presence of significant amounts of *S*-alkyl-L-cysteine or their sulfoxides, as well as C-S lyases that degrade these non-protein amino acids. In the garlic plant, *Allium sativum,* the presence of alliin (*S*-allyl-L-cysteine sulfoxide) as well as alliinase (EC 4.4.1.4) in the bulb and cloves have been known for many years (*7,8*). Their interaction following crushing of the clove yields the biologically active compound allicin.

Alliinase from garlic has been purified and characterized (*9–12*). Conflicting results were reported, however, with regard to its molecular mass, and no information was available with respect to its primary structure. In this study we unequivocally demonstrate that garlic alliinase is composed of two subunits, the mol mass of each being 51.5 kDa. The amino acid sequence and composition as deduced from the nucleotide sequence of the cloned cDNA, as well as the *N*-terminal sequence of the purified alliinase protein and CNBr fragments, correlated very well. The

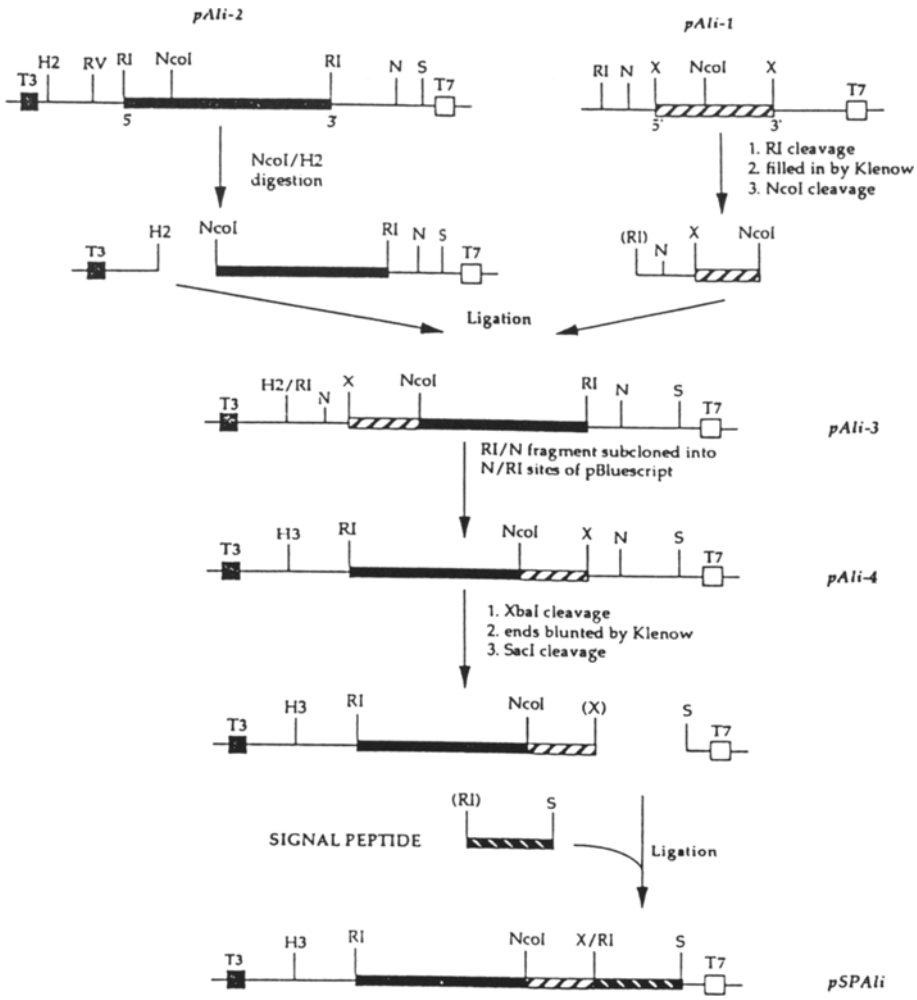


Fig. 8. Strategy for subcloning of alliinase gene and its recombination into *pSPAli*. The PCR product *pAli-1* was digested with *Nco*I, excised and then inserted into the *pAli-2* clone that was predigested with *Hinc*II and *Nco*I. The recombined construct, named *pAli-3*, was digested with *Eco*RI and *Not*I and inserted into pBluescript. The new clone, *pAli-4*, was digested with XbaI and ligated with the DNA fragment (115 bp) coding for the gliadin signal peptide as previously obtained (*42*). The resulting recombined plasmid containing also the T_7 promoter was named *pASli*. Restriction enzmes: H2, *Hinc*II; RV, *Eco*RV; RI, *Eco*RI; N, *Not*I; X, *Xba;* S, *Sac*I; *H3*, *Hind*III are indicated. For experimental details, *see text*.

only significant difference is in the number of cysteine residues that in the analysis of natural alliinase is low because of the well known acid degradation (*41*). The difference in mol mass with that previously reported by others (*10–12*) for alliinase may be owing to column elution conditions that may have resulted in enzyme aggregation.

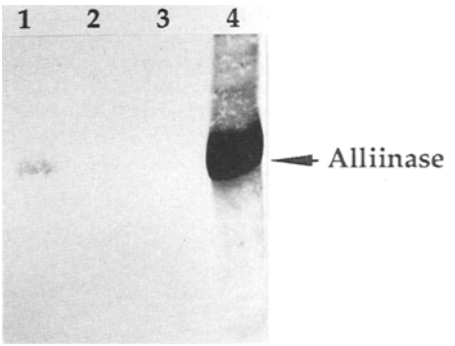


Fig. 9. Expression of alliinase gene in *Xenopus* oocytes. The plasmid DNA *pSPAli* clone was linearized by *Hind*III and synthetic mRNA was prepared by in vitro transcription followed by microinjection into *Xenopus* oocytes. Equal amounts of protein from the culture medium and extracts of the injected oocytes, as well as extracts from noninjected oocytes (as a negative control), were subjected to SDS-PAGE followed by Western blot analysis with antialliinase antibodies. Lane 1, extracts of injected oocytes; Lane 2, media; Lane 3, negative control; Lane 4, crude protein extracts from garlic bulbs. The position of alliinase is indicated by an arrow. For experimental details, *see text*.

Cloning of the garlic alliinase cDNA was achieved by a straightforward procedure involving two steps. The first step was the production of a DNA probe, *pAli-1*, by the PCR method using synthetic, mixed oligonucleotide primers which were designed on the basis of the *N*-terminal amino acid sequence of the purified protein and total garlic cDNA as the template. Sequencing of the amplified 450 bp DNA fragment revealed identity to *N*-terminal sequences obtained from the intact alliinase protein as well as to the *N*-terminal sequence of one of the CNBr cleaved peptide fragments. Northern blot analysis revealed a 1.8 kb mRNA that was the expected size for the alliinase mRNA. The *pAli-1* fragment was then used for the second step of identifying by hybridization of two alliinase clones: a 1.3 Kb partial cDNA clone, *pAli-2*, which was isolated from a λgt11 library, and a full length clone, *pAli-5* that was isolated from another λZAPII library and that contained an additional open reading frame upstream to the *N*-terminal region. In order to obtain further evidence about the identity of the mature protein encoded by these various cDNA clones, we chose to first express a recombinant alliinase clone made up only from the two clones, *pAli-1* and *pAli-2* (Fig. 4). This construct was then ligated in frame at the 5′ end to a DNA fragment containing the regulatory and secretory sequences of the previously investigated α-gliadin (42). Expression of this recombinant clone, *pSPAl* (Fig. 8) in *Xenopus* oocytes yielded a 50 kDa protein that was recognized by the antibodies raised against the purified garlic alliinase.

Recently, three closely related full length alliinase cDNA clones were isolated from garlic and closely related species (17). The coding DNA

sequence of the published alliinase cDNA was closely related to our cDNA except for five amino acid substitutions (Fig. 6). This suggests the presence of several active alliinase genes in garlic that encode different isozymes. This is also supported by the finding that *pAli-2* and *pAli-5* exhibited relatively high divergene in their 3′ noncoding sequence (Fig. 6). Interestingly, comparison of the deduced amino acid sequence of *pAli-5* to that of the recently published alliinase cDNA showed marked differences in the *N*-terminal region. The published alliinase clone was suggested to encode a precursor containing a signal peptide of 28 amino acids, which is apparently cleaved during insertion into the ER, and an additional peptide of ten amino acids, which is apparently cleaved by further processing of alliinase in the endomembrane system. The deduced amino acid sequence of the open reading frame of *pAli-5* exhibited only 25 amino acids upstream to the *N*-terminal region of the mature protein, but this was preceded by a short, in frame, small open reading frame of three amino acids. The nature of the differences between *pAli-5* and the recently published alliinase cDNA is unknown. It is possible that *pAli-5* is encoded by a pseudo gene that is still transcriptionally active. Alternatively, the short upstream open reading frame in *pAli-5* may serve as a regulatory element. Additional studies are required to solve this issue.

Alliinase becomes rapidly inactivated following its interaction with alliin. Reisolation of the protein from the reaction product yielded a partially inactive enzyme. The mechanism of inactivation is currently being investigated and appears to involve a reaction with alliin or the product allicin.

Alliinase is known to be mostly present in garlic bulbs (cloves). The large amount of enzyme found in the cloves is more than ten times the activity found in the plant leaves and stem (Fig. 1 and data not shown). This was supported by our Northern and Western blot analyses using *pAli-1* and the anti-bulb alliinase serum. Interestingly, although the Northern and Western blot analyses did not detect any mRNA or protein that is related to the bulb alliinase in roots, this tissue exhibited the highest alliin lyase activity. This suggests that garlic roots may contain another alliinase isozyme that is structurally distinct from the bulb alliinase. The identity and possible function of this root enzyme awaits further studies.

Direct evidence for the numerous medical indications reported with respect to allicin, the product of allinase and alliin, is still very limited (*1*). This is mainly owing to the fact that allicin is an unstable compound (*2*) that is difficult to isolate from garlic preparations or to prepare by chemical synthesis, and has a short half life upon storage. The development of a quantitative assay system as well as the availability of recombinant alliinase that could be produced in relatively large amounts in a heterologous system, offers new possibilities for the controlled *in situ* generation of allicin from synthetic alliin and hence for the detailed study of its medicinal properties in various model systems. Moreover, since

alliinase is not a very efficient enzyme and is rapidly inactivated by its product, the production of recombinant alliinase in a heterologous system in the absence of substrate could facilitate improvements of its biochemical characteristics by genetic engineering techniques.

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REFERENCES

1. Block, E. (1992), *Angew, Chem. Int. Ed. Engl.* **31,** 1135–1178.
2. Cavallito, C. J. and Bailey, J. H. (1944), *J. Am. Chem. Soc.* **66,** 1950,1951.
3. Barone, F. E. and Tansey, M. R. (1977), *Micologya* **69,** 793–825.
4. Mirelman, D., Monheit, D. J., and Varon, S. (1987), *J. Infect. Dis.* **156,** 243,244.
5. Augusty, K. T. and Mathew, R. T. (1974), *Experientia* **30,** 468–470.
6. Stoll, A. and Seebeck, E. (1947), *Experientia* **III/3,** 114,115.
7. Stoll, A. and Seebeck, E. (1949), *Helv. Chim. Acta* **32,** 197–205.
8. Stoll, A. and Seebeck, E. (1951), *Adv. Enzymol.* **11,** 377–400.
9. Mazelis, M. and Crews, L. (1968), *Biochem. J.* **108,** 725–730.
10. Nock, L. P. and Mazelis, M. (1986), *Arch. Biochem. Biophys.* **249,** 27–33.
11. Kazarian, R. A. and Goryachenkova, E. V. (1978), *Biokhimya* (*Engl. transl.*) **43,** 1502–1508.
12. Jansen, H., Müller, B., and Knobloch, K. (1989), *Planta Medica* **55,** 434–439.
13. Rabinkov, A., Wilchek, M., and Mirelman, D. (1991), *FASEB J.* Abst. 4509.
14. Rabinkov, A., Wilchek, M., and Mirelman, D. (1991), *15th Int. Congress Biochem., Jerusalem, Israel.* Abst. p. 64.
15. Rabinkov, A., Wilchek, M., and Mirelman, D. (1992), *21st Annual FEBS Meeting, Dublin, Ireland.* Abst. **Tu-86.**
16. Rabinkov, A., Xiao-zhu, Z., Grafi, G., Wilchek, M., Galili, G., and Mirelman, D. (1993), *FASEB J.* **7,** LB 60.
17. Van Damme, E. J. M., Smeets, K., Torrekens, S., Van Leuven, F., and Peumans, W. J. (1992), *Eur. J. Biochem.* **209,** 751–757.
18. Shwimmer, S. and Mazelis, M. (1963), *Arch. Biochem. Biophys.* **100,** 66–73.
19. Laemmli, U. K. (1970), *Nature* **227,** 680–685.
20. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randell, R. J. (1951), *J. Biol. Chem.* **193,** 265–275.
21. Towbin, H., Staehelin, T., and Gordon, J. (1979), *Proc. Natl. Acad. Sci. USA* **76,** 4350–4354.
22. Blake, M. S., Johnston, K. H., Russel-Jones, G. J., and Gotschlich, E. C. (1984), *Anal. Biochem.* **136,** 175–179.

23. Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., and Smith, F. (1956), *Anal. Chem.* **28,** 350–356.
24. Riddles, P. W., Blakeley, R. L., and Zerner, B. (1979), *Anal. Biochem.* **94,** 75–85.
25. Grassetti, D. R. and Murray, J. J. F. (1967), *Arch. Biochem. Biophys.* **119,** 41–49.
26. Steers, E., Craven, G., Anfinsen, C. B., and Bethune, J. L. (1965), *J. Biol. Chem.* **240,** 2478–2484.
27. Fling, S. P. and Gregerson, D. S. (1986), *Anal. Biochem.* **15,** 83–88.
28. Matsudiara, P. (1987), *J. Biol. Chem.* **262,** 10035–10038.
29. Jansen, H., Müller, B., and Knobloch, K. (1987), *Planta medica* **53,** 559–562.
30. Feinberg, A. P. and Vogelstein, B. (1983), *Anal. Biochem.* **132,** 6–13.
31. Feinberg, A. P. and Vogelstein, B. (1984), *Anal. Biochem.* **137,** 266,267.
32. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989), *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
33. Kouchi, H., Tsukamoto, M., and Tajima, S. (1989), *J. Plant Physiol.* **135,** 608–617.
34. Lee, C. C., Wu, X., Gibbs, R. A., Cook, R. G., Muzny, D. M., and Caskey, C. T. (1988), *Science* **239,** 1288–1291.
35. Sanger, F., Nicklen, S., and Coulson, A. R. (1977), *Proc. Natl. Acad. Sci. USA* **74,** 5463–5467.
36. Tabor, S. and Richardson, C. C. (1987), *Proc. Natl. Acad. Sci. USA* **84,** 4767–4771.
37. Nielsen, D. A. and Shapiro, D. J. (1986), *Nucleic Acids Res.* **14,** 5936.
38. Kawata, E. E., Galili, G., Smith, L. D., and Larkins, B. A. (1988), in *Plant Molecular Biology Manual,* Gelvin, S. B. and Schilperoort, R. A., eds., Martinus Nijhof, Belgium, pp. 1–22.
39. Goryachenkova, E. V. (1952), *Dokl. Akad. Nauk USSR (Russ.)* **87,** 457–460.
40. Lancaster, J. E. and Collin, H. A. (1981), *Plant Sci. Lett.* **22,** 169–176.
41. Inglis, A. S., Rivett, D. E., and McMahon, D. T. W. (1979), *FEBS Lett.* **104,** 115–118.
42. Rafalski, J. A., Scheets, K., Metzler, M., Peterson, J. M., Hedgcoth, C., and Soll, D. (1984), *EMBO J.* **3,** 1409–1415.
43. Kozak, M. (1981), *Nucleic Acids Res.* **9,** 5233–5253.