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A Novel Alliinase from Onion Roots. Biochemical Characterization and cDNA Cloning[1]

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We have purified a novel alliinase (EC 4.4.1.4) from roots of onion (*Allium cepa* L.). Two isoforms with alliinase activity (I and II) were separated by concanavalin A-Sepharose and had molecular masses of 52.7 (I) and 50.5 (II) kD on sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and 51 (I) and 57.5 (II) kD by gel filtration fast-protein liquid chromatography. Isoform I had an isoelectric point of 9.3, while isoform II had isoelectric points of 7.6, 7.9, 8.1, and 8.3. The isoforms differed in their glycosylation. Both contained xylose/fucose containing complex-type *N*-linked glycans, and isoform II also contained terminal mannose structures. Both isoforms had activity with *S*-alk(en)yl-L-cysteine sulfoxides. Unlike other allium alliinases, *A. cepa* root isoforms had cystine lyase activity. We cloned a gene from *A. cepa* root cDNA and show that it codes for *A. cepa* root alliinase protein. Homology to other reported allium alliinase genes is 50%. The gene coded for a protein of mass 51.2 kD, with two regions of deduced amino acid sequence identical to a 25- and a 40-amino acid region, as determined experimentally. The *A. cepa* root alliinase cDNA was expressed mainly in *A. cepa* roots. The structure and function of the alliinase gene family is discussed.

The characteristic flavor of onion (*Allium cepa* L.), garlic (*Allium sativum* L.), and related alliums occurs when the enzyme alliinase (EC 4.4.1.4) hydrolyzes the *S*-alk(en)yl-L-Cys sulfoxides (ACSOs) to form pyruvate, ammonia, and sulfur-containing volatiles. The ACSO substrates are located in the cytoplasm, while alliinase is compartmentalized in the vacuole until cell rupture (Lancaster and Collin, 1981). The production of volatile sulfur products by this reaction is central to the characteristic and commercially important flavor of these plants.

Alliinase (synonyms: alliin alkyl-sulfonate-lyase, alliin lyase, alkyl Cys sulfoxide lyase, Cys sulfoxide lyase, and C-S lyase) is present in *A. cepa* bulbs at high levels, representing up to 6% by weight of the soluble protein in the bulb (Nock and Mazelis, 1987). Alliinase enzymes from garlic cloves (Mazelis and Crews, 1968), *Allium tuberosum* (chinese chives) (Manabe et al., 1998), *Allium ursinum* (ramson or wild garlic) (Landshuter et al., 1994), and *Allium porrum* (leek) (Lohmüller et al., 1994) have also been purified to homogeneity and characterized. Related enzymes have also been identified in *Acacia farnesiana* seedlings (Mazelis and Creveling, 1975), *Brassica oleracea* buds (Hamamoto and Mazelis, 1968), bacteria (Nomura et al., 1963; Kamitani et al., 1990, 1991), and fungi (Iwami and Yasumoto, 1980).

Alliinase is a glycoprotein with an estimated carbohydrate content of 4.6% in onion and 5.5% in garlic (Nock and Mazelis, 1987). Pyridoxal 5′-P (PyrP) has been demonstrated as an essential co-factor with empirical measurements predicting one very tightly bound PyrP per subunit (Tobkin and Mazelis, 1979) at Lys-285 in the amino acid sequence deduced from *A. cepa* cDNA (Kitamura et al., 1997).

Genes encoding alliinase have been isolated from *A. cepa* (Van Damme et al., 1992; Clark, 1993; Gilpin et al., 1995; King et al., 1998), *A. tuberosum* (Chinese chives) (Manabe et al., 1998), *Allium ascalonicum* (shallot) (Van Damme et al., 1992), and *A. sativum* (Van Damme et al., 1992; Rabinkov et al., 1994).

Northern analysis in *A. sativum* tissues showed that alliinase cloned from clove cDNA was expressed in cloves and leaves but not in roots (Rabinkov et al., 1994), although they were shown to contain high alliinase activity. This indicated the existence in *A. sativum* roots of a non-homologous alliinase gene.

In this paper we report the purification and characterization of *A. cepa* root alliinase protein and the cloning of a gene from *A. cepa* root cDNA encoding this protein. The gene is substantially different in sequence, and the protein has novel structure and function compared with other allium alliinases. The role of this novel alliinase in roots is discussed.

MATERIALS AND METHODS

Protein Purification and Sequencing

Alliinase was purified from onion (*Allium cepa* cv Pukekohe Longkeeper) roots grown in hydroponic solution culture (Randle et al., 1995). Roots (typically 300 g) were harvested, washed in tap water, and blotted dry before being frozen in liquid N_2 and homogenized in an equivalent volume (w/v) of buffer A (0.02 M sodium phosphate, 30% [v/v] ethylene glycol, and 2.5 μg mL^{-1} PyrP, pH 6.5) containing 0.05% (w/v) 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 mM 6-amino-*n*-hexanoic acid, and 5 mM EDTA. All purification and column steps were performed at 4°C.

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The homogenate was squeezed through two layers of cheesecloth and the cell debris removed by centrifugation. The supernatant was applied to a CM-Sepharose CL6B (11.0- × 3.0-cm) column equilibrated with buffer A. After washing off unbound material, active fractions were eluted with buffer A containing 0.5 M NaCl and pooled. Pooled fractions were adjusted to 1 mM $MnCl_2$ and 1 mM $CaCl_2$ and applied to a concanavalin A (ConA)-Sepharose 4B column (9 × 1.5 cm) previously equilibrated in buffer B (0.05 M Tris, 0.5 M NaCl, 30% [v/v] ethylene glycol, 2.5 μg mL^{-1} PyrP, 1 mM $MnCl_2$, and 1 mM $CaCl_2$, pH 7.5). After washing off unbound material with buffer B, the column was then eluted with buffer B containing 5 mM methyl α-D-glucopyranoside and fractions pooled and concentrated. Further elution was with buffer B containing 0.2 M methyl α-D-mannopyranoside. Both concentrated fractions (about 5 mL) were dialyzed against buffer A. Each was applied to its own CM-Sepharose CL6B (4.5- × 1.5-cm) column equilibrated in buffer A, and, after washing off unbound material, active enzyme was eluted with buffer A containing 0.5 M NaCl.

Sample purity was established by SDS-PAGE (Laemmli, 1970) followed by visualization with silver staining (Giulian et al., 1983). Subunit sizes were determined using a 10-kD molecular mass ladder (Gibco-BRL, Cleveland).

Synthesis of Potential Alliinase Substrates

The substrates *S*-methyl-L-Cys sulfoxide (MCSO), *S*-ethyl-L-Cys sulfoxide (ECSO), *S*-propyl-L-Cys sulfoxide (PCSO), and *S*-butyl-L-Cys sulfoxide (BCSO) were synthesized by the method of Lancaster and Kelly (1983). *S*-Propenyl-L-Cys sulfoxide (PrenCSO) was prepared by hydrolyzing γ-glutamyl-propenyl-L-Cys sulfoxide with γ-glutamyl transpeptidase (Kuttan et al., 1974). *S*-Allyl-L-Cys sulfoxide (AllylCSO) was obtained from Extrasynthese (Genay, France), and L-Cys, L-cystine, methyl Cys, and cystathionine were purchased from Sigma (St. Louis).

Measurement of Alliinase Activity

Alliinase activity was measured by a coupled NADH/lactate dehydrogenase (LDH) assay in 0.2 M *N*-[2-hydroxy-1,1-Bis(hydroxymethyl)ethyl]glycine (Tricine)-KOH, pH 8.0, 0.1 mM NADH, 12.5 units of LDH, 0.1% (v/v) alliinase preparation, depending on the stage of preparation and activity of sample, and, 0.01 M ECSO at 20°C. Activity was calculated from the conversion of NADH to NAD^+ as measured by a decrease in A_{340} (Schwimmer and Mazelis, 1963) and is reported as nanokatals (1 nkat = 1 nmol s^{-1}). The protein concentration of eluants was determined using a refinement of the Bradford dye binding assay (Spector, 1978).

Molecular Mass Determination by Gel Filtration Fast-Protein Liquid Chromatography (FPLC)

Protein samples (1–2 mg) eluted with 5 mM methyl α-D-glucopyranoside (alliinase isoform I) and 0.20 M methyl α-D-mannopyranoside (alliinase isoform II) from the Con A-Sepharose 4B column were dissolved in 0.05 M Tris-HCl buffer (pH 7.5) containing 0.15 M NaCl and 15% (v/v) ethylene glycol, (1 mL), and centrifuged at 15,000*g* for 10 min. The samples were analyzed by FPLC on a 1- × 30-cm Sepharose 12 column (Pharmacia LKB Biotechnology, Uppsala) at 20°C. Samples (100 μL) were injected onto the column and eluted with the above buffer at 0.4 mL min^{-1} and 224 psi. Eluting proteins were detected at 280 nm, and fractions (0.2 mL) were collected at 4°C. Alliinase activity was measured in each fraction to determine the elution times, and the molecular mass of alliinase was estimated from a calibration graph of markers (from Sigma): apoferritin (443 kD), β-amylase (200 kD), bovine serum albumin (66 kD), ovalbumin (43 kD), and carbonic anhydrase (29 kD).

Isoelectric Focusing (IEF)

IEF of alliinase isoforms I and II was performed on 0.2-mm-thick 5% (w/v) T, 0.75% (w/v) C (Bis) acrylamide gels, containing 5% (w/v) glycerol, 2% (w/v) Bio-Lyte 3/10 Ampholyte cast onto Gel Bond support film and run on a Multiphor flat bed gel apparatus (Pharmacia, Piscataway, NJ). Typically, a 124- × 110-mm gel was run at 8 W of constant power for 3,300 V h^{-1} using a cathode buffer of 0.02 M Lys, 0.02 M Arg, and 2 M ethylene diamine, and an anode buffer of 0.02 M Asp and 0.02 M Glu. PIs were estimated from IEF standard markers (Bio-Rad, Hercules, CA): cytochrome *c*, pI 9.60; lentil lectin, pI 7.8, 8.0, and 8.2; human hemoglobin C, pI, 7.50; and human hemoglobin A, pI 7.1. Gels were visualized with silver staining (Giulian et al., 1983) and also for alliinase activity on the substrate ECSO by staining for thiol reaction products (see below) (Ukai and Sekiya, 1997).

Determination of Substrate Specificity

Alliinase isoforms I and II were separated by IEF as above, and subsequently reacted with 1, 5, and 10 mM of each of the substrates PrenCSO, MCSO, and ECSO in situ. Alliinase activity in situ in IEF gels was demonstrated by staining for thiol reaction products (Ukai and Sekiya, 1997). The gel was soaked in 0.05 M Tris-HCl, pH 8.0, containing 2.5 μg mL^{-1} PyrP with shaking for 10 min, and put into a 15-mL Falcon tube. Into each tube was added the following solution: 0.5 mL of 1 M Tris-HCl, pH 8.0, 2.5 mg of 3-(4′,5′-dimethyl-2-thiazol)-2,5-diphenyl-2H-tetrazolium bromide, 2.5 mg of phenazine methosulfate, and 10 μL of 10 mM PyrP made up to 9 mL with water. The reaction was started and timed by the introduction of each of the above substrates in a total volume of 1 mL. After incubation, the solution was poured off and the strips were washed in water until no color was left. The strips were then removed and dried.

Kinetic analysis of alliinase isoforms I and II was carried out in vitro in solution with the substrates MCSO, ECSO, PrenCSO, PCSO, AllylCSO, cystine, methyl Cys, Cys, and cystathione. Approximately 0.05 to 0.33 μg of protein, purified as in Table I, was used for each incubation. Substrate concentrations ranged between 1 and 25 mM. Alliinase and substrates were incubated at 30°C in 0.2 M Tricine-KOH

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Table I. *Purification of alliinase from A. cepa roots*

Fraction	Total Protein	Total Activity	Specific Activity	Purification	Recovery	
	mg	*nkat*	*nkat mg^{-1}*	*fold*	*%*	
Crude extract	193.2	6,000	31	1.0	100	
CM-Sepharose CL6B	13.05	5,000	383.2	12.4	83	
Con A-Sepharose, 5 mM methyl-D-glucopyranoside (I)	0.243	513	2,109.8	68.1	8.6	}57.5
Con A-Sepharose, 200 mM methyl-D-mannopyranoside (II)	0.940	2,937	3,124.2	100.8	48.9	
CM-Sepharose CL6B, 5 mM methyl-D-glucopyranoside (I)	0.062	210	3,380.7	109.0	3.5	}28.8
CM-Sepharose CL6B, 200 mM methyl-D-mannopyranoside (II)	0.761	1,519	1,995.6	64.4	25.3	

(pH 8.0) buffer containing 0.01 mM PyrP in a total volume of 200 μL. Incubation was for 1 min for the ACSO substrates and for 20 min (with 0.2 M Tris-HCl, pH 8.8) for cystine. Methyl Cys, Cys, and cystathionine were incubated for 60 min. The reaction was stopped by adding 200 μL of 2,4 dinitrophenylhydrazine (0.0125% [w/v] in 2 N HCl). The reaction was incubated at 37°C for 10 min, 1.0 mL of 0.6 N NaOH was added, and A_{420} determined after 5 min. A pyruvic acid (0.1–0.8 μmol mL^{-1}) standard curve was generated and used for calibration of the enzymically developed pyruvate (Randle and Bussard, 1993). Activity was calculated as nanokatals per milligram of protein (1 nkat = 1 nmol pyruvate s^{-1}). Calculation of K_m and V_{max} was carried out using a computerized least-squares-fit method.

Western Blotting of IEF Gels

Alliinase isoform I (0.128 μg) and alliinase isoform II (1.313 μg) were loaded into lanes and focused by IEF as above. The isoforms were blotted onto a polyvinylidene difluoride (PVDF) membrane. The gel was washed in 0.01 M 3-(cyclohexylamino)propanesulfonic acid (CAPS), 10% (w/v) MeOH, pH 11.0, transfer buffer for 15 min with a change every 5 min. The gel was removed from the backing film by placing a dry piece of blotting paper on the surface and carefully peeling it off. Blotting was done at 0.8 mA cm^{-2} for 30 min in a semi-dry apparatus. Bands were visualized by Coomassie staining and excised. N-Terminal sequences were determined using an on-line gas phase sequencer (model 470A, Applied Biosystems, Foster City, CA) using the standard protocol of the manufacturer.

Determination of *N*-Linked Glycosylation of Alliinase Isoforms

To probe each alliinase with glycan-specific antibodies, isoforms I and II were separated by IEF and blotted onto PVDF membrane as described above. Antibody staining with monoclonal antibody 2.23 (McManus et al., 1988) was performed at a dilution of 1:1,000, and immune recognition was visualized using alkaline phosphatase secondary antibodies. Blots with digoxigenin-labeled *Galanthus nivalis* agglutinin (GNA) lectin (Boehringer Mannheim, Mannheim, Germany) and alkaline-phosphatase conjugated anti-digoxigenin (Boehringer Mannheim) were performed according to the manufacturer's instructions. For digestion with endoglycosidase H (endo H) (Boehringer Mannheim), 10 μg of alliinase isoform II or the soybean agglutinin lectin (L1395, Sigma) was denatured in 0.1 M 2-mercaptoethanol and 0.05% (w/v) SDS by heating at 100°C for 5 min. After cooling, the denatured protein mixture was buffered with 50 mM sodium citrate, pH 5.6, and PMSF was added to a final concentration of 0.4% (w/v). Endo H (0.4 unit) was then added and the reaction incubated at 25°C for 24 h. The reduction in molecular mass at the conclusion of the endo H treatment was determined by SDS-PAGE (Laemmli, 1970).

Cyanogen Bromide Cleavage of Alliinase

Alliinase isoform II, eluted with 0.2 M methyl α-D-mannopyranoside from Con A-Sepharose, was further purified on CM-Sepharose CL6B. Cyanogen bromide peptides for amino acid sequencing were produced by dissolving 40 to 50 μg of alliinase isoform II in 25 μL of 70% (w/v) formic acid containing 1.5 mg of cyanogen bromide and incubating overnight at 4°C in the dark. The mixture was diluted 10-fold with water and vacuum-dried under centrifugation. The peptides were separated on 16.5% (w/v) Tris-Tricine polyacrylamide gels (Bio-Rad) and blotted onto PVDF membrane. The bands were excised and N-terminal sequences were determined using an on-line gas phase sequencer (model 470A, Applied Biosystems) using standard protocols.

The peptides were also separated by SDS-PAGE and transferred to PVDF membrane (Immobilon-P, Millipore, Bedford, MA) by blotting at 0.8 mA cm^{-2} for 30 min in a semi-dry apparatus. The membrane was probed for oligosaccharide side chains with Con A-horseradish peroxide conjugate and visualized with 4-chloro-l-napthol and H_2O_2 (Clegg, 1982; DeBlas and Cherwinski, 1983).

Analysis of ACSOs

ACSOs from *A. cepa* roots were extracted in methanol: chloroform:water (12:5:3; v/v) and ethanol (80%; v/v) according to the method of Lancaster and Kelly (1983). BCSO, which is not normally found in allium species, was added as internal standard at 1 mg g^{-1} fresh weight to determine

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the percentage recovery through the extraction, derivatization, and analysis procedures. The onion aqueous extract (1 g fresh weight equivalent) was lyophilized at −36°C in vacuo and then redissolved in 1 mL of deionized water. Sample fractionation was performed by ion-exchange chromatography with Dowex 1×8 (200–400 mesh, 10- × 40-mm column) resin in the acetate form. A 0.5-mL sample of extract was loaded onto the column and eluted using acetic acid (HOAc) at 0.1, 1.0, and 2.0 N. The 0.1 N HOAc fraction contained the ACSOs. The 1.0 and 2.0 N fractions contained the γ-glutamyl peptide biosynthetic intermediates. Samples were lyophilized and derivatized with phenyl isothiocyanate and analyzed by reverse-phase HPLC (Randle et al., 1995).

Cloning of a Partial Alliinase cDNA from *A. cepa* Root for Library Screening

Oligonucleotide primers were designed from a 438-bp *A. cepa* leaf cDNA AOB249 (accession no. AA451570; King et al., 1998). Primers were synthesized by Gibco-BRL: AOB249-1 (5′-GGCTGGTAGCGGCAGTCTACT-3′) situated at the 5′ end, and AOB249-R (5′-TGTCGTAGTTGTACCCA-GACG-3′) situated at the 3′ end. Total RNA was extracted from root tips of hydroponically grown *A. cepa* using TRIZOL (Gibco-BRL). First-strand cDNA was generated with a *Not*I-d(T)18 primer using Ready-To-Go T-Primed First Strand Beads (Amersham-Pharmacia Biotech, Uppsala). The first-strand cDNA was used as template to amplify a 570-bp product by PCR with the 5′ primer AOB249-1. Amplification conditions were 30 s at 95°C, 30 s at 60°C, and 30 s at 72°C for 40 cycles. PCR products were purified with the Prep-A-Gene kit (Bio-Rad) and then cloned into the pGEM-T vector using the pGEM-T vector system (Promega, Madison, WI). Plasmid DNA was isolated using a plasmid isolation kit (High Pure, Roche, Basel) from colonies that had the correct insert size, as determined by PCR with M13 forward and reverse primers. Sequencing reactions were performed using a terminator cycle sequencing kit (ABI PRISM Big Dye kit, Perkin-Elmer Applied Biosystems, Foster City, CA) and analyzed on an automated sequencer (model 377, Perkin-Elmer-Applied Biosystems). A 300-bp fragment (rootAOB) was PCR amplified from the vector for probe with the primers AOB249-1 and AOB249-R (conditions as above) and purified with a PCR product purification kit (High Pure, Roche).

Construction and Screening of a cDNA Library

Total RNA was extracted from root tips of hydroponically grown *A. cepa* as described above. The mRNA fraction was isolated from total RNA by double selection using a mRNA isolation kit (Messagemaker, Gibco-BRL). A cDNA library was constructed from 5 μg of mRNA using the ZAP cDNA synthesis kit (Stratagene, La Jolla, CA), and packaged in vitro with Gigapack II Gold packaging extract (Stratagene). The resulting cDNA library (1.8×10^6 primary recombinant phages) was excised in vivo.

A total of 5×10^4 excised phagemids were plated with host strain SOLR at a density of 5×10^3 colonies per 10 cm^2 plate and transferred to nitrocellulose membranes (Hybond-C Extra, Amersham-Pharmacia Biotech). Membranes were placed colony side up onto 3MM filter paper (Whatman, Clifton, NJ) prewetted with a 5% (w/v) SDS, 2× SSC solution and heated for 3 min on medium-high in a 850 W microwave, followed by vacuum drying at 80°C for 30 min.

Membranes were hybridized with ^{32}P-labeled root AOB fragment randomly primed with the Megaprime labeling kit (Amersham-Pharmacia Biotech). Hybridization was carried out in 6× SSC, 10% (w/v) dextran sulfate, 0.1% (w/v) $Na_2H_2P_2O_7$, 1% (w/v) SDS, and 0.3 mg mL^{-1} denatured herring sperm DNA at 65°C for 16 h. Post-hybridization washes were carried out at 65°C for 30 min each: 2× SSC, 0.1% (w/v) SDS, 1× SSC, 0.1% (w/v) SDS, and 0.1× SSC, 0.1% (w/v) SDS. Autoradiography signals were obtained after 16 h of exposure at −80°C using Kodak X-Omat AR film (Eastman Kodak, Rochester, NY) with special intensifying screens (Agfa Curix CX, Agfa Gevaert, Mortsel, Belgium). Colonies that gave a positive signal from hybridization were checked for insert size by PCR using M13 forward and reverse primers.

Sequence Analysis of the Full-Length Root Alliinase cDNA

Plasmid DNA was isolated from colonies that had an insert size of approximately 1,600 bp as determined by PCR. Restriction digests were also carried out on the plasmid DNA with *Eco*RI and *Xho*I to check insert size. Sequencing reactions were performed as previously described. Sequencing primers used were: SK (Stratagene), M13 reverse, and AOB249-1. Further primers were designed to sequence the complete gene: Alliroot1 (5′-TACGAATG-GAAGGGAAATGC-3′) at position 686, and Alliroot2 (5′-GCTGATGCCACTACTGGTGA-3′) at position 329.

Northern Hybridization

Total RNA was extracted from root, leaf, and bulb tissues of *A. cepa* as described above. Approximately 10 μg of RNA was separated by electrophoresis through a 1% (w/v) agarose gel containing formaldehyde and transferred to Zeta-Probe GT nylon membrane (Bio-Rad) (Sambrook et al., 1989). Three duplicate membranes were hybridized with separate probes as previously described: the full-length alliinase *A. cepa* root cDNA, a full-length alliinase *A. cepa* leaf cDNA (Clark, 1993), and a pea ubiquitin cDNA (Watts and Moore, 1989).

RESULTS

Purification and Characterization of Alliinase Protein

Molecular Mass of Purified Alliinase

Alliinase was purified from onion roots using ion-exchange and affinity chromatography. The results of a typical purification of alliinase are summarized in Table I. The separation of alliinase into two peaks of activity on the Con A-Sepharose column indicated the presence of iso-

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forms (Fig. 1a). Alliinase activity eluted at 5 mM methyl α-D-glucopyranoside (isoform I) and 0.2 M methyl α-D-mannopyranoside (isoform II). Further purification on CM-Sepharose resulted in isoform I being 109-fold purified, with a specific activity of 3,380 nkat mg^{-1}, and isoform II being 64-fold purified, with a specific activity of 1,995 nkat mg^{-1}. The specific activity of isoform II appeared to decrease after this final purification step. However, visualization after SDS-PAGE showed that contaminating proteins had been removed by the final CM-Sepharose purification. It is possible that factors important to activity may also be removed by this step. On gel filtration FPLC, alliinase isoforms I and II eluted at a molecular mass of 51 and 57.5 kD, respectively (Fig. 1b). Isoforms I and II were separated by SDS-PAGE (Fig. 1c). Each isoform was homogeneous on SDS-PAGE. Isoforms I and II gave submit masses of 52.7 and 50.5 kD, respectively.

IEF

The alliinase isoforms I and II were separated by IEF and silver stained (Fig. 1d). Isoform I gave one band at pI 9.3.

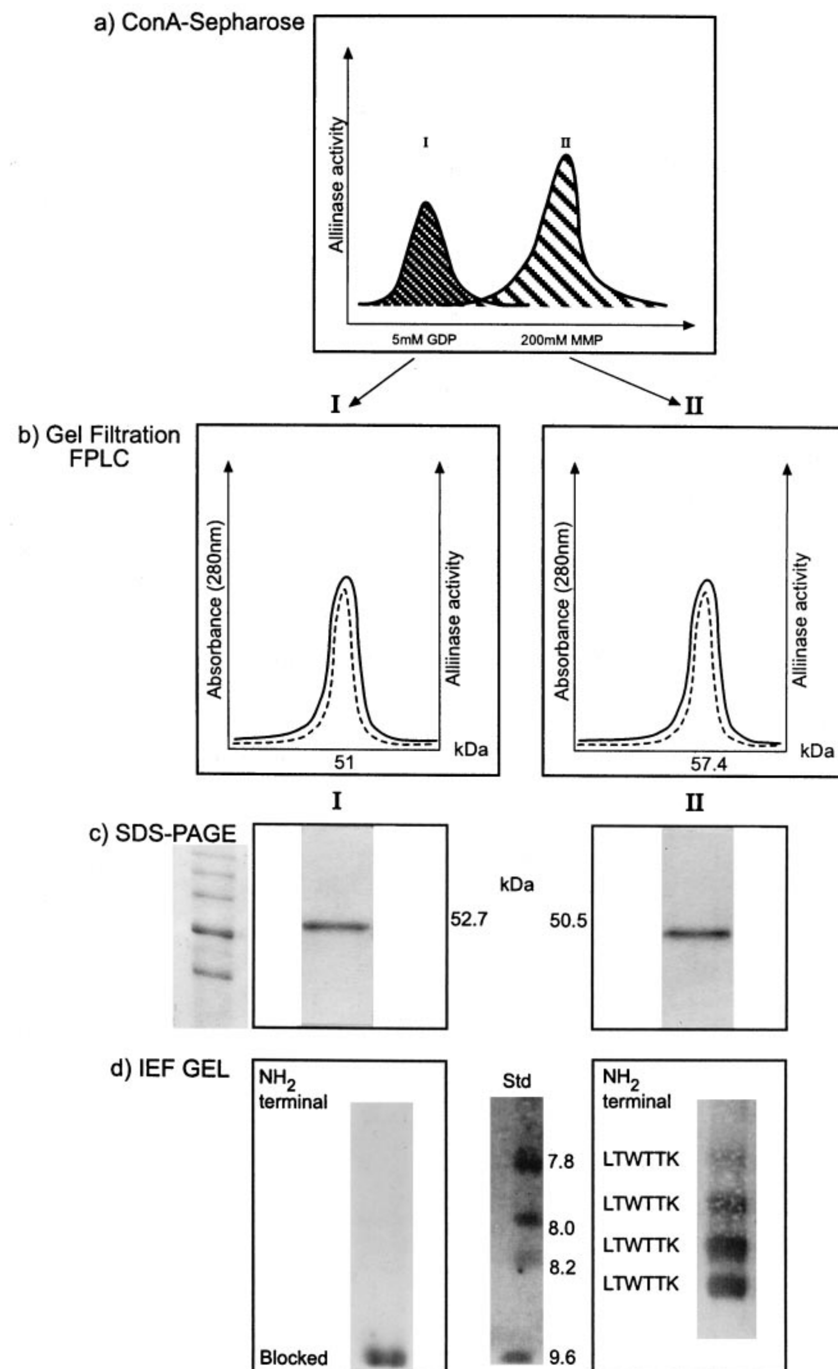

Figure 1. Physical characterization of alliinase. Analysis of purified alliinase isoforms I and II by Con A-Sepharose (a), gel-filtration FPLC (solid line, protein at 280 nm; broken line, alliinase activity) (b), SDS-PAGE and silver staining (c), and IEF gel stained with silver and N-terminal sequence (d). Panel I is 5 mM methyl-α-D-glucopyranoside-eluted alliinase activity, and panel II is 0.2 M methyl-α-D mannopyranoside-eluted alliinase activity. A 10-kD ladder was used for SDS-PAGE, and all molecular mass calculations are in kD.

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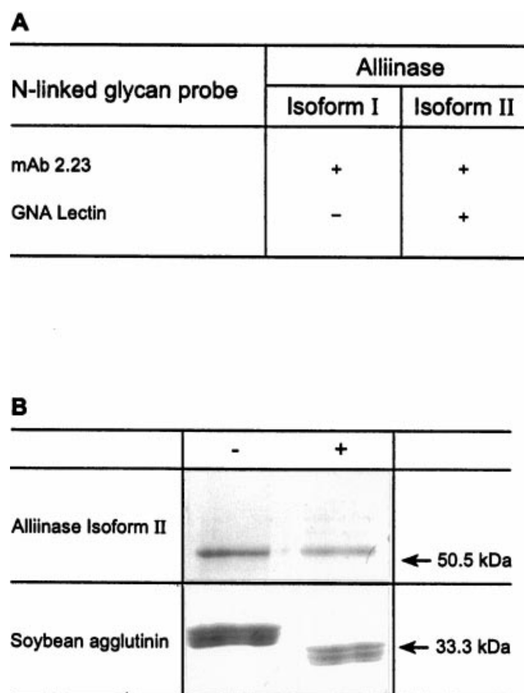
Isoform II gave four bands at pIs 7.6, 7.9, 8.1, and 8.3. The isoform II bands with alliinase activity were N-terminal sequenced, and all gave the sequence LTWTTK (Fig. 1d). The 9.3-pI band from isoform I could not be sequenced, possibly due to blockage at the N terminus.

Alliinase Glycosylation

Western blotting using glycan-specific antibodies was used to determine the nature of *N*-linked glycosylation of each alliinase isoform. Isoform I was recognized by monoclonal antibody 2.23 but not by the GNA lectin, while isoform II was recognized by both probes (Fig. 2a). Isoform II was subject to endo H digestion, but no reduction in molecular mass of the protein was observed (Fig. 2b). In contrast, a decrease in molecular mass of the endo H-digested soybean agglutinin lectin was observed, a protein known to be glycosylated with an oligo-Man *N*-linked glycan structure (Dorland et al., 1981).

Internal Amino Acid Sequence

Alliinase isoform II was digested with cyanogen bromide and the amino acid sequence of the resultant peptides was determined. There were two main peptides of 25 and 12 kD. The 12-kD peptide gave the amino acid sequence LTWTTKAATEAEKVAAIYCSDHGRA. This band gave a negative response when western blotted and probed with Con A, indicating an absence of oligosaccharide side chains. The 25-kD peptide gave the amino acid sequence SDELDRHIRLLHNAVGNAKVDD KFLVFGNG VT QLL NGVII. This peptide gave a positive response when western blotted and probed with Con A, indicating the presence of oligosaccharide side chains.


A

N-linked glycan probe	Alliinase	
	Isoform I	Isoform II
mAb 2.23	+	+
GNA Lectin	-	+

Figure 2. A, Summary of recognition, by western blotting, of alliinase isoform I and II by monoclonal antibody 2.23 and GNA lectin. +, Recognition; −, non-recognition. B, Coomassie blue staining after SDS-PAGE of denatured alliinase isoform II and the soybean agglutinin lectin after incubation without (−) and with (+) endo H.

Alliinase Activity and Substrate Specificity

Alliinase isoform II was separated by IEF gel into four bands and reacted in situ with each of MCSO, ECSO, and PrenCSO. All four bands, which were of equal loading, gave comparable activity staining with each of the substrates. Alliinase isoform I, separated by IEF gel, was inactive in situ with each of the same substrates. Purified alliinase isoforms were then reacted in vitro in solution, with each of the substrates MCSO, ECSO, PCSO, AllylCSO, PrenCSO, cystine, methyl Cys, Cys, and cystathionine (Table II). K_m, V_{max}, and K_m/V_{max} were calculated from the enzyme reaction data. Both alliinase isoforms showed a similar order of activity with the substrates. They were most active with PrenCSO and showed decreasing activity through the sequence Allyl CSO, PCSO, ECSO, and MCSO. Cystine was the least active. Both isoforms were inactive with methyl Cys, Cys, and cystathionine.

Root Flavor Precursors

Roots contained the flavor precursors MCS and PrenCSO at concentrations of 0.04 and 0.11 mg g^{-1} fresh weight, respectively. PCSO was below detection. There was an absence of γ-glutamyl sulfur peptides.

Cloning, Sequencing, and Expression of Alliinase cDNA

Alliinase cDNA Cloning

A partial *A. cepa* leaf cDNA clone (AOB249) encoding a putative alliinase gene with approximately 60% homology to *A. cepa* bulb alliinases was previously identified during sequencing of seedling leaf cDNA clones used to develop the onion linkage map (King et al., 1998). This sequence was used to design a primer for reverse transcripase PCR that would not anneal with *A. cepa* bulb alliinases. When used in reverse transcripase PCR reactions with root cDNA, this gave a product that was cloned and found to be homologous with AOB249. A Uni-ZAP XR cDNA library was constructed containing 1.8×10^7 plaque forming units. Approximately 1.8×10^6 plaque forming units were mass excised, and 5×10^4 phagemids were screened using the 300-bp root-derived AOB probe. Thirty-one positive signals were detected from colony hybridization. Of the 31 clones, three had an expected insert size of approximately 1,600 bp, as determined by PCR with M 13 forward and reverse primers and restriction digest with *Eco*RI and *Xho*I.

Alliinase Nucleotide Sequence

Two of the cDNA clones were sequenced and found to be identical. The complete nucleotide sequence of the root alliinase cDNA is shown in Figure 3 (accession no. gbAF126049). The clone is 1,700 bp with an open reading

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Table II. *Substrate specificity of A. cepa root alliinase isoforms I and II, with MCSO, ECSO, PCSO, AllylCSO, PrenCSO, cystine, Cys, methyl-L-Cys, and cystathionine*

Reactions were carried out in vitro in solution. K_m and V_{max} were calculated using a least-squares-fit method.

Substrate	Isoform I		V_{max}/K_m	Isoform II		V_{max}/K_m
	K_m	V_{max}		K_m	V_{max}	
	mM	*nkat/mg*		*mM*	*nkat/mg*	
MCSO	47.0 ± 12.7	5,412 ± 564	115	96.6 ± 16.6	5,251 ± 569	54
ECSO	45.7 ± 15.7	25,013 ± 3,693	547	68.4 ± 9.5	28,568 ± 2,730	418
PCSO	22.0 ± 5.7	24,824 ± 3,281	1,128	16.3 ± 3.3	9,384 ± 797	576
AllylCSO	18.3 ± 4.1	64,471 ± 7,569	3,523	24.1 ± 2.4	46,463 ± 2,724	1,928
PrenCSO	2.6 ± 0.5	25,085 ± 1,856	9,648	3.0 ± 0.7	21,524 ± 1,867	7,175
Cystine	2.2 ± 0.9	30.1 ± 3.0	14	9.7 ± 3.2	35.6 ± 5.9	4
Methyl Cys	No activity			No activity		
Cys	No activity			No activity		
Cystathionine	No activity			No activity		

frame of 1,493 bp. There is a possible translation termination codon at 1,517 bp and a 3′ untranslated region of 180 bp. Deduced amino acid sequence gave a mature protein of 453 amino acids and 51.2 kD with a predicted pI of 7.23. Two regions of deduced amino acid sequence are identical to the 25 and 40 amino acids determined experimentally from the purified protein, indicating that the cDNA cloned codes for *A. cepa* root alliinase isoform II (Fig. 3). Upstream of the N terminus (LTWTTK) is a leader sequence of 132 bp and 44 amino acids. The *A. cepa* root-alliinase-deduced amino acid sequence contains four potential glycosylation sites of the Asn-X-Ser/Thr (NXS/T) conformation at amino acid positions 55, 143, 182, and 188. A Lys residue at position 248 from the N terminus indicates a possible PyrP-binding residue within the PyrP consensus sequence [GD]-X-D-[LIV]-X(3)-[STAGCM](2)-[TS]-K-[FYW]-[LI]-X-G-[HQ]-[SGN] (Manabe et al., 1998).

Alignment with Other Alliinases

The deduced alliinase isoform II amino acid sequence was aligned with other alliinase amino acid sequences from alliums (Fig. 4). Homology with *A. cepa* alliinases are 52.8% for leaf shoots (Clark, 1993) and 53.4% for bulb (Van Damme et al., 1992). Homology with garlic (*Allium sativum*) clove alliinase is 51.4% (Rabinkov et al., 1994) and 52.5% (Van Damme et al., 1992). Homology with shallot (*Allium ascalonicum*) is 51.8% (Van Damme et al., 1992), and with chinese chives (*Allium tuberosum*) homology is 49.4% (Manabe et al., 1998). In contrast, the sequences (amino acids 238–253) around the predicted PyrP Lys-248 binding residue of the *A. cepa* root alliinase show strong agreement with the predicted PyrP binding motif of other alliinases (Fig. 4). *A. cepa* root alliinase cDNA has four predicted glycosylation NXS/T sites (amino acids 55, 143, 182, and 188), of which two are in common with glycosylation sites of the other allium alliinase cDNA sequences (143 and 188) (Fig. 4). The site at amino acid 327 in *A. cepa*, *A. sativum*, and *A. ascalonicum*, is absent from *A. cepa* root alliinase cDNA. The *A. cepa* root alliinase leader sequence is 44 amino acids long. There is little amino acid sequence homology between the *A. cepa* root alliinase leader and the leader sequences of *A. tuberosum*, *A. cepa*, *A. sativum*, and *A. ascalonicum* alliinases, although the latter three have high sequence homology with each other. However, allium alliinase leader sequences reported so far have the hydrophobic regions indicative of glycoproteins, and the *A. cepa* root alliinase is similar in this feature. *A. cepa* root alliinase cDNA also has a C-terminal extension of 11 amino acids, YGGDEGSYEST, which is absent in the other allium alliinase sequences.

Expression of Root Alliinase

A. cepa root alliinase cDNA was highly expressed in *A. cepa* roots, but expressed to a very low extent in *A. cepa* leaf and bulb tissue (Fig. 5A). Alliinase cDNA from *A. cepa* leaf tissue was expressed in leaf and bulb tissue, but not in roots (Fig. 5B). Loadings of RNA were similar for both root and leaf tissue, but lesser for bulb tissue (Fig. 5C).

DISCUSSION

We report here the purification and characterization of an allium protein and show, for the first time to our knowledge, that the enzyme has both alliinase and cystine lyase activity. The protein from *A. cepa* roots is coded for by a cDNA with low sequence homology to other allium alliinases. The existence of a protein with alliinase activity, but with low sequence homology to *A. sativum* alliinase cDNA was postulated by Rabinkov et al. (1994) for roots of *A. sativum*.

A. cepa root alliinase was purified in three steps into two isoforms of alliinase specific activities 3,380 (isoform I) and 1,995 (isoform II) nkat mg^{-1} protein. The isoform specific activities are considerably greater than that reported for bulb alliinase of approximately 750 nkat mg^{-1} protein (Nock and Mazelis, 1987; Hanum et al., 1995; Clark et al., 1998). On SDS-PAGE the *A. cepa* root alliinase isoforms I and II separated at 52.7 and 50.5 kD, respectively. The *A. cepa* bulb alliinase is a doublet on SDS-PAGE with masses of 53.3 and 51.6 kD (Clark et al., 1998). *A. cepa* root alliinase isoforms I and II gave activity at 51 and 57.4 kD on gel filtration FPLC, indicating that root alliinase is active as a monomer. Alliinase in *A. cepa* has previously been reported as a trimer or tetramer (Nock and Mazelis, 1987;

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```
1                                    M  D  S  D  K  S  V  M  S  R  N  K
1      AAGTTCATAGTTGTAAAGTTAAACTATGGATTCAGACAAATCAGTCATGAGTAGAAATAA

13      L  W  L  I  T  F  L  F  L  T  S  L  V  V  N  L  Y  F  Y  Y
61     GCTTTGGCTAATTACATTTCTCTTCTTAACATCCCTTGTAGTAAACCTATACTTCTACTA

33      D  N  S  F  A  L  S  S  P  F  L  R  L  T  W  T  T  K  A  A
121    TGACAACAGTTTCGCTTTGAGCAGCCCTTTCCTCAGACTGACTTGGACTACCAAGGCAGC

53      T  E  A  E  K  V  A  A  I  Y  C  S  D  H  G  R  A  Y  L  D
181    AACTGAGGCTGAGAAAGTGGCTGCTATATACTGTTCTGATCATGGCAGAGCTTATTTGGA

73      G  I  P  V  N  G  V  P  I  C  E  C  H  N  C  Y  S  G  P  T
241    TGGAATACCAGTTAATGGTGTCCCTATATGTGAGTGTCATAATTGCTACAGCGGTCCTAC

93      C  S  V  L  A  S [N  C  T] A  D  A  T  T  G  D  A  M  F  L
301    TTGCTCCGTCTTGGCCTCAAATTGCACTGCTGATGCCACTACTGGTGATGCAATGTTTCT

113     E  K  Y  W  L  H  H  R  V  N  T  A  M  L  E  S  G  W  H  R
361    AGAAAAGTACTGGCTCCATCACAGAGTAAACACTGCAATGCTGGAATCCGGCTGGCACAG

133     M  S  Y  F  I  G  H  N  F  M  S  D  E  L  D  R  H  I  R  L
421    AATGAGCTACTTTATTGGCCATAATTTCATGTCTGATGAACTAGACAGACACATTAGACT

153     L  H  N  A  V  G  N  A  K  V  D  D  K  F  L  V  F  G  N  G
481    TCTACACAATGCAGTTGGCAATGCTAAAGTCGATGATAAATTTCTCGTGTTTGGTAACGG

173     V  T  Q  L  L  N  G  V  I  I  S  L  S  P [N  V  T] A  T  P
541    AGTTACGCAACTTCTCAATGGAGTTATTATCTCACTGTCGCCAAATGTTACGGCTACACC

193     T  A  P  I  K  K  V  V  A  Y  V  P  Y  Y  P  V  F  K  S  Q
601    AACTGCTCCTATAAAGAAAGTAGTTGCCTATGTTCCTTATTACCCTGTGTTCAAGAGTCA

213     T  S  F  F  N  F  K  G  Y  E  W  K  G [N  A  S] D  Y  V [N
661    GACCAGCTTTTTTAACTTCAAAGGATACGAATGGAAGGGAAATGCATCAGATTATGTAAA

233     T  T] N  P  Q  D  F  I  E  L  V  T  S  P  N  N  P  D  G  L
721    TACGACTAATCCACAAGATTTCATTGAGCTTGTGACTTCTCCAAATAATCCTGATGGTCT

253     L  R  K  S  I  I  P  G  S  L  A  V  Y  D  H  A  T  Y  W  P
781    ACTTCGCAAGTCAATAATCCCAGGTTCTTTAGCAGTATACGATCATGCTACATATTGGCC

273     H  Y  A  P  I  K  Y  A  S  D  E  D  I  M  L  F  A  L  S [K]
841    TCATTATGCTCCAATCAAATACGCATCCGATGAAGATATCATGTTGTTTGCTCTATCTAA

293     Y  T  G  H  S  G  S  R  F  G  W  A  F  V  R  D  K  S  V  Y
901    GTACACAGGACATTCCGGCAGTCGATTTGGGTGGGCTTTTGTAAGGGACAAGAGTGTGTA

313     D  K  L  T  T  Y  I  S  T  N  S  E  G  V  S  R  E  S  Q  L
961    TGACAAATTAACAACATACATATCAACAAACAGTGAGGGAGTTTCACGTGAATCACAACT

333     R  T  L  F  I  I  K  E  I  L  L  Q  I  K  L  N  R  G  T  I
1021   CCGTACACTCTTTATTATAAAAGAGATATTGCTACAAATAAAACTAAACCGTGGTACTAT

353     G  D  F  N  W  Y  G  H  H  T  L  R  A  R  W  V  Q  L  N  R
1081   CGGTGACTTCAACTGGTATGGCCATCACACATTGAGGGCGAGATGGGTTCAACTAAACAG

373     L  V  A  Q  S  T  R  F  S  L  Q  E  I  S  A  E  Y  C  N  Y
1141   GCTGGTAGCGCAGTCTACTCGATTCTCCCTTCAGGAAATATCTGCTGAATATTGCAACTA

393     F  Q  R  I  R  N  P  S  P  T  Y  G  W  L  K  C  E  W  E  E
1201   CTTTCAAAGAATCAGAAACCCTTCTCCAACTTATGGATGGTTGAAATGTGAGTGGGAAGA

413     D  T  D  C  A  A  V  L  S  N  G  K  I  L  T  Q  S  G  V  L
1261   AGACACAGACTGTGCAGCTGTACTTAGCAACGGAAAAATCTTAACCCAAAGTGGTGTGCT

433     F  E  A  S  S  R  Y  A  R  L  S  I  I  K  T  Q  D  D  F  N
1321   TTTTGAAGCAAGCAGCCGCTATGCACGTTTGAGTATTATCAAGACGCAAGATGATTTTAA

453     Q  L  M  E  R  L  S  V  L  V  M  A  K  R  S  T  S  G  Y  N
1381   TCAACTGATGGAACGTTTGAGTGTACTTGTTATGGCTAAAAGGAGTACGTCTGGGTACAA

473     Y  D  I  I  N  Q  E  R  S  K  R  P  F  I  Y  G  G  D  E  G
1441   CTACGACATTATTAATCAAGAACGCAGCAAACGTCCTTTCATTTATGGGGGCGATGAAGG

493     S  Y  E  S  T  *
1501   AAGCTACGAGTCTACTTAGTCAAGATGTTTGTTTGTGATTGTGATGTGGTGCTCTTTACT

1561   ATCTTGTTTTCTCATTTTGTCTGGGTTTGATGGTTAGCTATTATGTTTGAAAGGCGAGGA

1621   GTAAGAACTGTGATGTTGTAATGGATGGTTTTTGCAGTTTGTGCAAGTGAATGTGTTGGT

1681   TTAAAAAAAAAAAAAAAAAA
```

Figure 3. Nucleotide and derived amino acid sequence of *A. cepa* root alliinase cDNA. Bold ATG, Possible translation initiation start codon; underlines, predicted amino acid sequence aligning with that determined from pure alliinase protein; overlines, potential Asn-linked glycosylation sites; dashed overlines, potential PyrP-binding residue; underlined TAG, possible translation termination codon.

Lancaster and Boland, 1990; Hanum et al., 1995), while Clark et al. (1998) showed *A. cepa* bulb alliinase was also active as a monomer as well as a trimer and tetramer. In *A. sativum* and wild garlic (*Allium ursinum*) alliinase has been shown to aggregate with low-molecular-mass lectins into stable, active complexes (Smeets et al., 1997). Aggregation of alliinase into multimeric forms does not seem to occur in *A. cepa* roots.

The separation of isoforms of alliinase with IEF was reported for *A. cepa* bulbs by Nock and Mazelis (1987), although the pI of the bands was not specified. Our own experiments (J.E. Lancaster and M.L. Shaw, unpublished results) showed that it was difficult to focus *A. cepa* bulb alliinase into bands, although they were present. The tendency of alliinase to aggregate with lectins may have contributed to the technical difficulty of focusing *A. cepa* bulb alliinase protein. In contrast, the *A. cepa* root alliinase protein focused well into one band for alliinase isoform I and four different bands for alliinase isoform II. Leek (*Allium porrum*) alliinase protein gave bands of pI 7.5 and 7.6 (Landshuter et al., 1994), similar to the pIs of 7.6, 7.9, 8.1, and 8.3 for *A. cepa* root isoform II. In contrast, *A. ursinum* alliinase protein had a low pI of 4.7 (Lohmüller et al., 1994). This low pI for *A. ursinum* alliinase could be because of the complexing of the protein with lectins. The presence of lectins is a feature of allium cloves/bulbs. Lectins focus at acidic pIs, and lectin alliinase complexes tend to migrate toward the anode.

A. cepa root alliinase separated into two isoforms on Con A-Sepharose chromatography with 5 mM methyl-α-D-glucopyranoside, and 0.2 M methyl-α-D-mannopyranoside, indicating the existence of forms of alliinase with significantly different glycosylation. Alliinase isoform I was recognized by monoclonal antibody 2.23, a monoclonal antibody which specifically recognizes Xyl/Fuc-containing complex-type *N*-linked glycans (McManus et al., 1988) with the α-1,3 Fuc sugar a critical part of the monoclonal antibody epitope (Wilson et al., 1998). The occurrence of complex type glycans is confirmed by elution of isoform I from the Con A-Sepharose column with 5 mM methyl-α-D-glucopyranoside. Alliinase isoform II reacts both with monoclonal antibody 2.23 and the GNA lectin probe, indicating the occurrence of Xyl/Fuc containing complex-type glycans (monoclonal antibody 2.23-recognized) and oligo-Man structures in at least one of the potential glycosylation sites and/or hybrid chains with terminal Man structures. Treatment of isoform II with endo H did not cause a reduction in molecular mass, suggesting that oligo-Man structures are not present. Recognition by both monoclonal antibody 2.23 and the GNA lectin is most likely due to the presence of hybrid chains with terminal Man structures that have been characterized on other plant glycoproteins (Lerouge et al., 1998). The occurrence of accessible (terminal) Man structures is confirmed by the requirement for 0.2 M methyl-α-D-mannopyranoside to elute isoform II from ConA-Sepharose. *A. cepa* bulb alliinase eluted from Con A-Sepharose with 5 mM methyl-α-D-glucopyranoside (Clark et al., 1998).

Both *A. cepa* root alliinases (I and II) showed similar enzymatic activity with the range of substrates tested. For reasons that were not apparent, *A. cepa* root alliinase isoform I was inactive when tested for alliinase activity in situ in the IEF gel. *A. cepa* roots contain PrenCSO and MCSO, but PCSO was not detected. *A. cepa* bulb alliinase gave K_m values for PrenCSO, PCSO, and MCSO of 6, 11, and 34 mM, respectively (Schwimmer, 1969). These are of the same magnitude and order as *A. cepa* root alliinase I and II, although the K_m for AllylCSO was not reported for bulb

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                                                                  ▼
A.cepa root     -MDSDKSVMSRNKLWLITFLFLTSLVVNLYFYYDNSFALSS--PFLRLTWTTKAATEAEKVAAIYCSDHG   23
A.tuberosum     -METY-KPG-N---------KMPYLIILLCV--SFPFFNT---VQT-LSWTLKAAEEAEAVAAIKCSGHG   23
                                                                ▼
A.ascalonicum   --------------------------------------------QAKVTWSLKAAEEAEAVANINCSGHG   24
A.cepa (cl)     -MESYHKVGSN---------KMPSLLILICIIMS-SFVNNN-IAQAKVTWSLKAAEEAEAVANINCSGHG   24
A.cepa (vd)     -MESYDKVGSN---------KVPCLLILTCIIMS-SFVNNN-IVQAKVSWSLKAAEEAEAVANINCSGHG   24
A.sativum (rb)  ---------------------MICLVILTCIIMSNSFVNNNNMVQAKMTWTMKAAEEAEAVANINCSEHG   24
A.sativum (vd)  MVESYKKIGSCN--------KMPCLVILTCIIMSNSLVNNNNMVQAKMTWTMKAAEEAEAVANINCSEHG   24
                                                 *  *** *** ** * ** **

A.cepa root     RAYLDGIPVNGVPICECHNCYSGPTCSVLASNCTADATTGDAMFLEKYWLHHRVNTAMLESGWHRMSYFI   93
A.tuberosum     RAYQDGVLSKGSPICECNTCYEGSDCSTKTPNCSADVASGDALFLEEYWKDHKENTAVLVSGWHRMSYFF   93
A.ascalonicum   RAFLDGILSDGSPKCECNTCYTGADCSQKITGCSADVASGDGLFLEEYWQQHKENSAVLVSGWHRTSYFF   94
A.cepa (cl)     RAFLDGILSDGSPKCECNTCYTGADCSEKITGCSADVASGDGLFLEEYWQQHKENSAVLVSGWHRMSYFF   94
A.cepa (vd)     RAFLDGILSDGSPKCECNTCYTGADCSEKITGCSADVASGDGLFLEEYWQQHKENSAVLVSGWHRMSYFF   94
A.sativum (rb)  RAFLDGIISEGSPKCECNTCYTGPDCSEKIQGCSADVASGDGLFLEEYWKQHKEASAVLVSPWHRMSYFF   94
A.sativum (vd)  RAFLDGIISEGSPKCECNTCYTGPDCSEKIQGCSADVASGDGLFLEEYWKQHKEASAVLVSPWHRMSYFF   94
                **  **    * * ***  ** *  **     * **   **  *** **  *    * * * *** ***

A.cepa root     GHN----FMSDELDRHIRLLHNAVGNAKVDDKFLVFGNGVTQLLNGVIISLSPNVTATPTA-PIKKVVAY  158
A.tuberosum     -PEKDSDFMSAELKRTITELHEIVGNAETKGKHIVFGVGVTQLLHGLVLTISPNISNCPTAGP-AKVVAR  161
A.ascalonicum   NPV--SNFISFELEKTIKELHEIVGNAAAKDRYIVFGVGVTQLIHGLVISLSPNMTATPCA-PQSKVVAH  161
A.cepa (cl)     NPV--SNFISFELEKTIKELHEIVGNAAAKDRYIVFGVGVTQLIHGLVISLSPNMTATPCA-PQSKVVAH  161
A.cepa (vd)     NPV--SNFISFELEKTIKELHEIVGNAAAKDRYIVFGVGVTQLIHGLVISLSPNMTATPCA-PQSKVVAH  161
A.sativum (rb)  NPV--SNFISFELEKTIKELHEVVGNAAAKDRYIVFGVGVTQLIHGLVISLSPNMTATPDA-PESKVVAH  161
A.sativum (vd)  NPV--SNFISFELEKTIKELHEVVGNAAAKDRYIVFGVGVTQLIHGLVISLSPNMTATPDA-PESKVVAH  161
                       * * **   *  **  ****       *** *****  *     ***    * * *  ****

A.cepa root     VPYYPVFKSQTSFFNFKGYEWKGNASDYVNTTNPQDFIELVTSPNNPDGLLRKSIIPGSLAVYDHATYWP  228
A.tuberosum     APYYAVFRDQTSYFDNKGYEWKGNAANYVNDPNPNQFIELVTSPNNPEGNLRKAMIVGSTAIYDMVYYWP  231
A.ascalonicum   APYYPVFREQTKYFDKKGYEWKGNAADYVNTSTPEQFIEMVTSPNNPEGLLRHEVIKGCKSIYDMVYYWP  231
A.cepa (cl)     APYYPVFREQTKYFDKKGYEWKGNAADYVNTSTPEQFIEMVTSPNNPEGLLRHEVIKGCKSIYDMVYYWP  231
A.cepa (vd)     APYYPVFREQTKYFDKKGYEWKGNAADYVNTSTPEQFIEMVTSPNNPEGLLRHEVIKGCKSIYYMVYYWP  231
A.sativum (rb)  APFYPVFREQTKYFDKKGYVWAGNAANYVNVSNPEQYIEMVTSPNNPEGLLRHAVIKGCKSIYDMVYYWP  231
A.sativum (vd)  APFYPVFREQTKYFNKKGYVWAGNAANYVNVSNPEQYIEMVTSPNNPEGLLRHAVIKGCKSIYDMVYYWP  231
                 * **  **  *  *** * ***  ***   *   ** ******* * **   * *    *    ***

A.cepa root     HYAPIKYASDEDIMLFALSKYTGHSGSRFGWAFVRDKSVYDKLTTYISTNSEGVSRESQLRTLFII-KEI  297
A.tuberosum     HFTPITYKADEDIMLFTMSKYTGHSGSRFGWAIIKDENVAIKLVEFMSKNTEGTSRETQLRTL-ILLKEV  300
A.ascalonicum   HYTPIKYKADEDIMLFTMSKYTGHSGSRFGWALIKDETVYNKLLNYMTKNTEGTSRETQLRSLKIL-KEV  300
A.cepa (cl)     HYTPIKYKADEDIMLFTMSKYTGHSGSRFGWALIKDETVYNKLLNYMTKNTEGTSRETQLRSLKIL-KEV  300
A.cepa (vd)     HYTPIKYKADEDIMLFTMSKYTGHSGSRFGWALIKDETVYNKLLNYMTKNTEGTSRETQLRSLKIL-KEV  300
A.sativum (rb)  HYTPIKYKADEDILLFTMSKFTGHSGSRFGWALIKDESVYNNLLNYMTKNTEGTPRETQLRSLKVL-KEI  300
A.sativum (vd)  HYTPIKYKADEDILLFTMSKFTGHSGSRFGWALIKDESVYNNLLNYMTKNTEGTPRETQLRSLKVL-KEV  300
                *  ** *  **** **  ** ***********   *  *   *      * *   ** *** *    **

A.cepa root     LLQIKLNRGTIGDFNWYGHHTLRARWVQLNRLVAQST-RFSLQEIS-AEYCNYFQRI-RNPSPTYGWLKC  364
A.tuberosum     IAMIKTHKGTPKDINFFGFQHLRQRWEKVTELLDQSNKRFSYQHLNQSEHCNYMR-MKRPPSPSYAWVRC  369
A.ascalonicum   TAMIKTQKGTMRDLNTFGFQKLRERWVNITALLDKSD-RFSYQKLPQSEYCNYFRRM-RPPSPSYAWVKC  368
A.cepa (cl)     IAMVKTQKGTMRDLNTFGFQKLRERWVNITSLLDKSD-RFSYQKLPQSEYCNYFRRM-RPPSPSYAWVKC  368
A.cepa (vd)     IAMVKTQNGTMRDLNTFGFQKLRERWVNITALLDKSD-RFSYQKLPQSEYCNYFRRM-RPPSPSYAWVKC  368
A.sativum (rb)  VAMVKTQKGTMRDLNTFGFKKLRERWVNITALLDQSD-RFSYQELPQSEYCNYFRRM-RPPSPSYAWVNC  368
A.sativum (vd)  VAMVKTQKGTMRDLNTFGFKKLRERWVNITALLDQSD-RFSYQELPQSEYCNYFRRM-RPPSPSYAWVKC  368
                    *   **  * *  *   ** **     *   *  *** *     * ***     * *** * *  *

A.cepa root     EWE-EDTDCAAVLSNGKILTQSGVLFEASSRYARLSIIKTQDDFNQLMERLSVLVMAKRSTSGYNYDIIN  433
A.tuberosum     NWPGEE-NCSEVFKEGGIITQDGPRFEAGSQYVRLSLIKTNDDFDQMMDHLKKMIKEKRPT------VIK  432
A.ascalonicum   EWE-EDKDCYQTFQNGRINTQSGEGFEAGSRYVRLSLIKTKDDFDQLMYYLKIMVEAKRKTP-----LIK  432
A.cepa (cl)     EWE-EDKDCYQTFQNGRINTQNGEGFEAGSRYVRLSLIKTKDDFDQLMYYLKNMVEAKRKTP-----LIK  432
A.cepa (vd)     EWE-EDKDCYQTFQNGRINTQSGEGFEAGSRYVRLSLIKTKDDFDQLMYYLKIMVEAKRKTP-----LIK  432
A.sativum (rb)  EWE-EDKDCYQTFQNGRINTQSGVGFEASSRYVRLSLIKTQDDFDQLMYYLKDMVKAKRKTP-----LIK  432
A.sativum (vd)  EWE-EDKDCYQTFQNGRINTQNGVGFEASSRYVRLSLIKTQDDFDQLMYYLKDMVKAKRKTP-----LIK  432
                 *  *   *      * * ** *  *** * * *** *** *** * *  *      ** *       *

A.cepa root     ------ QER--SKRPFIYGGDEGSYEST                                          452
A.tuberosum     EISGEVDK---GSRRPFI-----------                                          447
A.ascalonicum   QLS--NDQ---ISRRPFI-----------                                          445
A.cepa (cl)     QLS--NDQ---ISRRPFI-----------                                          445
A.cepa (vd)     QLS--NDQ---ISRRPFI-----------                                          445
A.sativum (rb)  QLF--TDETETASRRPFI-----------                                          448
A.sativum (vd)  QLF--IDQTETASRRPFI-----------                                          448
                            * ****
```

Figure 4. Alignment of *A. cepa* root alliinase predicted amino acid sequence with other alliinase sequences obtained from GenBank using DNAMAN. (cl, Clark [1993]; vd, Van Damme et al. [1992]; rb, Rabinkov et al. [1994]). Alignment and numbering are from the N terminus of each sequence (▼). **, Identical amino acids. Putative Asn glycosylation and PyrP consensus binding sequences are in bold.

alliinase. *A. cepa* bulb alliinase was reported to be inactive with cystine, Cys, and methyl Cys (Schwimmer and Mazelis, 1963). A major difference between the *A. cepa* root and bulb alliinases is the activity toward cystine. *A. cepa* root alliinase isoforms I and II were both active with cystine, and thus have both cystine lyase and Cys sulfoxide lyase activities. In contrast, only C-S lyases with Cys sulfoxide lyase activity have been reported for alliums (Ramirez and Whitaker, 1998). In *Brassica* species, C-S lyases have both Cys sulfoxide and cystine lyase activity (Ramirez and Whitaker, 1998). To our knowledge, the *A. cepa* root alliinase isoforms I and II are the first reported C-S lyases from alliums to have both functions. The *A. cepa* root alliinase did not have cystathionase activity. *A. cepa* bulb alliinase has also been shown to have activity with Cys-S conjugates such as S-chloronitrophenyl-L-Cys (Kitamura et al., 1997). *A. cepa* root alliinase was not reacted with these substrates.

A. cepa root alliinase cDNA contained an open reading frame of 1,493 bp, which translated to a mature protein of 453 amino acids, a predicted mass of 51.2 kD, and a leader sequence of 44 amino acids. The predicted mass was similar to that determined experimentally for *A. cepa* root alliinase protein, and the predicted pI was similar to those determined experimentally. The homology between the deduced amino acid sequence and the 25 and 40 amino acid sequences experimentally derived indicated that the cDNA was indeed alliinase. The *A. cepa* root cDNA also contains four potential Asn glycosylation sites at amino acid positions 55, 143, 182, and 188. *A. cepa* root alliinase protein has an actual glycosylation site at Asn-143 but not Asn-55, and possible sites at Asn-182 and Asn-188. Rabinkov et al. (1995) generated peptides from *A. sativum* alliinase with cyanogen bromide. The peptides were separated on SDS-PAGE, stained to detect carbohydrate moieties, and amino

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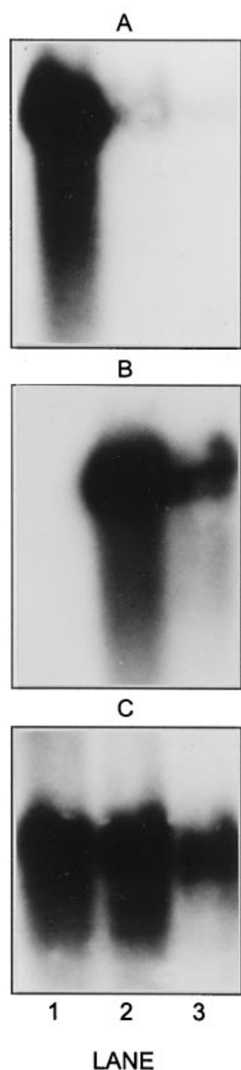

Figure 5. Expression of *A. cepa* root alliinase in *A. cepa* tissues. Total RNA from root, (lane 1), leaf (lane 2), and bulb (lane 3) tissue was separated on a formaldehyde gel, transferred to nylon membrane, and probed with full-length root alliinase cDNA (A), leaf alliinase cDNA (B), and pea ubiquitin cDNA (C).

acid sequenced. Of the four possible glycosylation sites, only one, Asn-146, was actually glycosylated in the *A. sativum* mature protein. Similarly, we have found that in *A. cepa* bulb alliinase, Asn-328 is glycosylated and Asn-146 and/or Asn-191. Asn-19 was not glycosylated, (U. Wäfler, M.L. Shaw, and J.G. Lancaster, unpublished results). The alignment of *allium* alliinase deduced amino acid sequences showed a consensus Asn glycosylation site only at Asn-146 (or Asn-143 for *A. cepa* root alliinase). It is likely that the glycosylation of site Asn-146 is necessary for alliinase activity. Glycosylation of *A. cepa* root alliinase at Asn-182 and Asn-188 is possible, but unlikely.

Site-directed mutagenesis experiments in *A. tuberosum* (Manabe et al., 1998) and PyrP-labeling studies in *A. cepa* (Kitamura et al., 1997) have shown that a Lys in the region of 280 to 285 amino acids from the N terminus is essential for alliinase activity. The allium consensus sequence around this site occurs also around Lys-248 for *A. cepa* root alliinase cDNA. Lys-248 is likely to be the PyrP-binding site for *A. cepa* root alliinase. The region around Lys-280 to -285 in allium alliinase cDNAs is also conserved in C-S lyases for the metabolism of Cys, homo-Cys, and Met in Arabidopsis cystathionine β-lyase, and cystathionine γ-synthase; *Saccharomyces cerevisiae* cystathionine γ-lyase, cystathionine β-lyase, and cystathionine γ-synthase; *Pseudomonas aeruginosa O*-succinyl homo-Ser sulfhydrylase and Met γ-lyase (Manabe et al., 1998). The reactions catalyzed by these enzymes comprise the cleavage or formation of C_β-S or Cγ-S bonds of the amino acid derivatives. Despite the conservation of this sequence for C-S lyase activity, the *A. cepa* root alliinase is the only allium protein shown to have cystine activity and Cys sulfoxide activity. The other allium alliinases are more restricted in their substrate specificity.

Homology between alliinase cDNA-deduced amino acid sequences of *A. cepa* (bulb and leaf), *A. sativum*, and *A. ascalonicum* was very high at > 90% (Van Damme et al., 1992, Clark, 1993). The *A. tuberosum* alliinase-deduced amino acid sequence was only 66% to 69% homologous to these other alliinases. *A. cepa* root cDNA deduced amino acid sequence was the most divergent at about 50%. The *A. cepa* root alliinase sequence had high homology to the AOB249 partial clone. This clone mapped to a different locus from *A. cepa* bulb alliinase (King et al., 1998). If the remainder of the AOB249 sequence is homologous to *A. cepa* root alliinase, then this clones' different locus is further evidence for the dissimilarity of *A. cepa* root alliinase from other alliinases.

Expression studies using RNA and northern analysis showed that *A. cepa* root alliinase cDNA was expressed to a much greater extent in roots than in leaves and bulb, and confirmed that alliinase leaf cDNA was not expressed in roots. As *A. cepa* alliinase bulb cDNA has a very high homology to leaf cDNA (approximately 90%), such a probe, had it been used, would not hybridize to *A. cepa* root RNA. Thus, we have confirmed in *A. cepa* what Rabinkov et al. (1994) postulated for garlic: the existence of a cDNA coding for a protein with alliinase activity that did not hybridize with alliinase bulb cDNA. As well as being divergent in sequence from the other allium alliinases, the *A. cepa* root alliinase has wider C-S lyase activity. *A. cepa* root alliinase may have a function in sulfur assimilation and remobilization in roots. It would be of interest to know if alliinase cDNAs with wide sequence divergence and wider C-S lyase activity are found in the roots of other alliums.

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