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The C-S Lyases of Higher Plants[1]

ISOLATION AND PROPERTIES OF HOMOGENEOUS CYSTINE LYASE FROM BROCCOLI (*BRASSICA OLERACEA* VAR *BOTRYTIS*) BUDS

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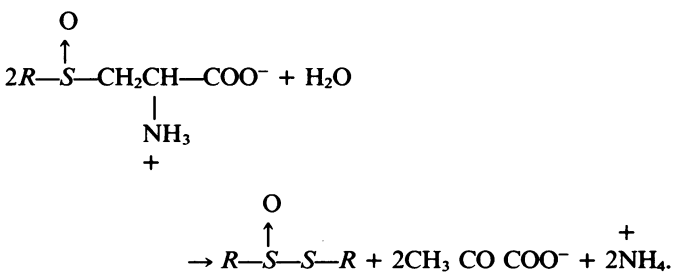
ARLENE HAMAMOTO[2] AND MENDEL MAZELIS*
Department of Food Science and Technology, University of California, Davis 95616

ABSTRACT

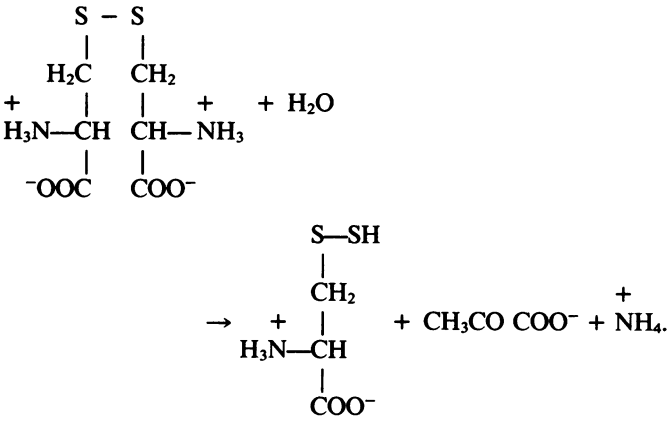
Cystine lyase degrades L-cystine by a β-elimination to form cysteine persulfide, pyruvate, and ammonia. This enzyme is common in *Brassica* sp. and has been purified to homogeneity from extracts of broccoli (*Brassica oleracea* var *botrytis*) buds. Two isozymes were separated on DEAE-Fractogel columns and the first peak, cystine lyase I further purified to homogeneity. The purified enzyme had a narrow range of substrate specificity with L-cystine and *S*-alkyl-L-cysteine sulfoxides being the primary substrates. The K_m for L-cystine was 1.9 millimolar and for *S*-ethyl-L-cysteine sulfoxide was 15.6 millimolar, suggesting that L-cystine would be preferred *in vivo*. Using gel filtration and sodium dodecyl sulfate-polyacrylamide gel electrophoresis the molecular weight of the holoenzyme was estimated as 152,000 composed of subunits of approximately 49,000. This strongly suggests the native enzyme is a trimer. The presence of carbohydrate in the native enzyme was detected at the level of 5.8% on a weight basis. Except for the ability to utilize L-cystine as a substrate there are many similarities between cystine lyase I and the alliin lyase of onion (*Allium cepa*).

S-Alkyl-L-cysteines and their sulfoxides are prominent constituents of the nonprotein amino acid pool in a number of species of the Amaryllidaceae, Cruciferae, and Leguminosae (4, 18). Many of these plants are of considerable economic importance as vegetables. Much of the characteristic odor and flavor associated with these plants is due to the degradation of the cysteine derivatives by a C-S lyase present in the tissues but separated from the substrate until the tissue is ruptured (9).

The first of these enzymes to be described was alliin lyase (EC 4.4.1.4) found in garlic cloves (16). This enzyme catalyzes reaction 1 below:



This enzyme has been purified to homogeneity from garlic (8, 14) and onion (17) and found in a number of *Brassica* sp. (10). The garlic and onion enzymes are specific for *S*-alkyl-L-cysteine sulfoxides, however the *Brassica* preparations studied to date (1, 6, 11, 12) also can cleave L-cystine according to reaction 2:



The question as to whether *Brassica* sp. contain two enzymes or one enzyme with two activities required the preparation of homogeneous enzyme from a member of this genus. The present report describes the purification of a cystine lyase from broccoli (*Brassica oleracea* var *botrytis*) buds to homogeneity and some of its characteristics.

MATERIALS AND METHODS

Enzyme Assay. The standard reaction mixture consisted of the following constituents in a final volume of 1 ml: Bicine pH 8.4, 150 μmol; PALP[3], 0.025 μmol; L-cystine, 12 μmol; and enzyme protein 10 to 100 μg. A standard cystine solution was prepared by dissolving 96 mg of L-cystine in 0.5 ml of 2.5 M NaOH and diluting to 10 ml making a 0.04 M solution. The reaction mixture was incubated at 30°C for 8 min, then terminated by the addition of 1 ml of 10% TCA. After centrifugation to remove the precipitated protein, an aliquot of the supernatant was assayed for pyruvate colorimetrically (11).

Protein Determination. Protein concentrations were estimated by the method of Bradford (2).

Gel Electrophoresis. PAGE was carried out as described by Hames (7). The stacking gel was 2.5% acrylamide at pH 6.8 and the running gel was 7% at pH 8.8. SDS-PAGE was done as described previously (7) using 2.5% stacking gels at pH 6.8 and 12% running gels at pH 8.8.

Molecular Weight Estimations. The enzyme mol wt was

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[2] Present address: Calreco, 8015 Van Nuys Blvd., Van Nuys, CA 91412.

[3] Abbreviation: PALP, pyridoxal-5'-phosphate.

estimated by the use of exclusion gel chromatography on a Fractogel TSK HW-55F column (2.5 × 100 cm) at 4°C. Standard proteins used as markers were fibrinogen (mol wt 470,000), β-amylase (152,000), and conalbumin (86,180). The elution volume of the enzyme compared to the elution volume of the marker proteins was treated graphically by the method of Whitaker (19) to obtain the lyase mol wt.

Subunit mol wt was determined by SDS-PAGE using slab gels and a set of marker proteins obtained from Bio-Rad. The low mol wt set consisted of lysozyme (14,400), soybean trypsin inhibitor (21,500), carbonic anhydrase (31,000), ovalbumin (45,000), BSA (66,200), and phosphorylase *b* (92,500); the high mol wt standard consisted of ovalbumin, BSA, phosphorylase *b*, β-galactosidase (116,250), and myosin (200,000).

Chemicals. *S*-Methyl-L-cysteine, L-djenkolic acid, PALP, and Bicine were products of Sigma Chemical Co. *S*-Allyl-L-cysteine was obtained from Nutritional Biochemical Corp. The sulfoxides were prepared from the thioethers by oxidation with acid H_2O_2 (16) and recrystallized from ethanol-water solutions. Fractogel TSK DEAE-650M and Fractogel TSK HW-55(F) were obtained from EM Science. Hydroxylapatite, fast flow, dry powder was a product of Calbiochem-Behring Corp., and Sephadex products were purchased from Pharmacia. All other chemicals were of the highest grade available and purchased from commercial sources.

RESULTS

Enzyme Purification. Fresh broccoli was purchased from local markets. The buds were separated and used as the enzyme source. All purification procedures were performed in the cold. A typical isolation was carried out as follows:

Step 1. Broccoli buds (2500 g) were homogenized in a chilled Waring Blendor for 30 s in a solution of 0.05 M K-phosphate-0.05 M citric acid (pH 6.4) containing 5% NaCl and 5% PVP. One ml of solution per g of buds was used. The homogenate was strained through several layers of cheesecloth.

Step 2. HCl (6 N) was added dropwise to the supernatant solution with stirring until the pH was 4.0. The acidified solution was brought to a temperature of 25°C, stirred for 15 min, then centrifuged at 4°C for 1 h at 10,000*g*. The precipitate was discarded.

Step 3. Enzyme grade $(NH_4)_2SO_4$ was added to the supernatant with constant stirring until 40% saturation was obtained. The precipitate was removed by centrifugation and discarded. The supernatant solution was then brought to 60% saturation with further $(NH_4)_2SO_4$ addition. The resulting precipitate was sedimented by centrifugation and dissolved in a minimal amount of 0.02 M K-phosphate-20 μM PALP buffer at pH 6.4. The enzyme solution was then dialyzed for a total of 6 h against a 50-fold excess volume of the suspending buffer with two changes of external buffer. The dialysate was clarified by centrifugation.

Step 4. The dialysate was applied to a Fractogel TSK DEAE-650M column (3 × 27 cm) equilibrated with 0.02 M K-phosphate pH 6.4. The enzyme was eluted with a linear gradient of the buffer containing 0 to 0.35 M NaCl at a flow rate of 28 ml/h collecting 13 ml fractions. Two partially resolved cystine lyase peaks were eluted between 0.1 and 0.2 M NaCl. Figure 1 shows the elution pattern from this column. The fractions containing each peak were pooled and concentrated to approximately 60 ml by ultrafiltration. The subsequent steps were performed on each peak separately.

Step 5. The concentrated enzyme solution was applied to a hydroxylapatite column (2.1 × 21 cm) equilibrated with 0.02 M K-phosphate-20 μM PALP pH 6.4. Two bed volumes of 0.15 M K-phosphate-20 μM PALP pH 6.4 were passed through the column at a flow rate of 15 ml/h and the eluant discarded. A solution of 0.4 M K-phosphate-20 μM PALP pH 6.4 at the same flow rate collecting 3.5 ml fractions eluted the enzyme. The active fractions were pooled and concentrated by ultrafiltration to a final protein concentration of about 1 mg/ml. The elution patterns are shown in Figure 2.

Step 6. The concentrated enzyme solution was applied to a Fractogel TSK HW-55F column (2.1 × 115 cm) which had been equilibrated with a 0.02 M K-phosphate-20 μM PALP-0.1 M NaCl pH 6.4 buffer. The enzyme was eluted using the same buffer at a flow rate of 15 ml/h collecting 3.5 ml fractions. The fractions containing the lyase activity were pooled.

The results of a typical isolation are summarized in Table I. The final purification of DEAE peak 1 was 176-fold with a recovery of 4% of the total starting activity. DEAE peak 2 gave a final 179-fold purification and a recovery of 4%. The enzyme activity of the final fraction was stable under these conditions for at least 2 months stored at −10°C.

Electrophoretic Analysis for Purity. The homogeneity of the enzyme from DEAE peaks 1 and 2 after the step 6 gel filtration was examined by PAGE. A densitometer scan of the tube gel using 20 μg for each enzyme fraction is shown in Figure 3. There

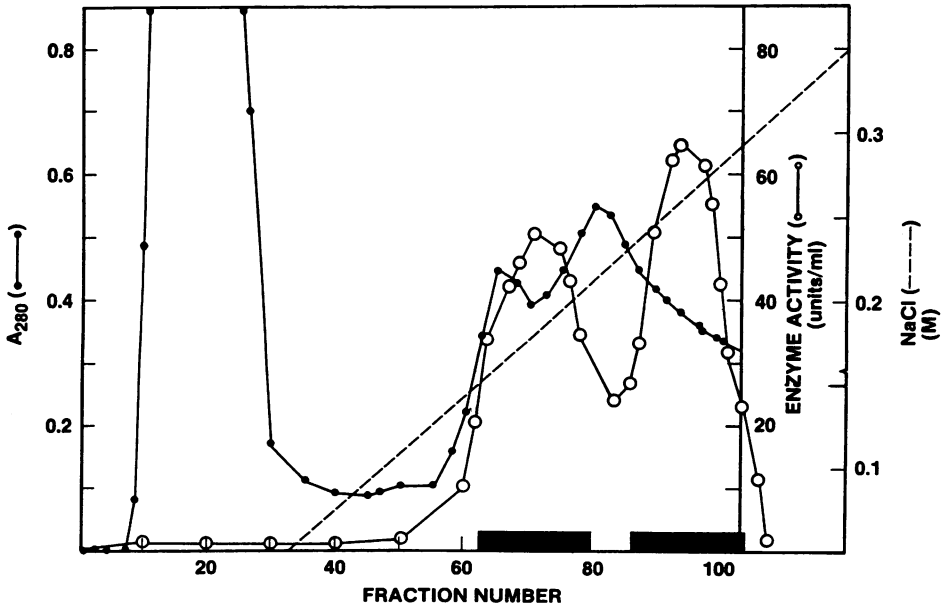

FIG. 1. The elution pattern of the step 3 fraction from the DEAE-Fractogel column obtained as described in the text. Activity in each fraction was assayed by the standard procedure described in "Materials and Methods". The solid bars at the bottom of the graph represent the combined test tube fractions which were subjected to the succeeding isolation steps. Fractions 62 to 80 were combined and called cystine lyase I. Fractions 86 to 105 were combined for further purification as cystine lyase II.

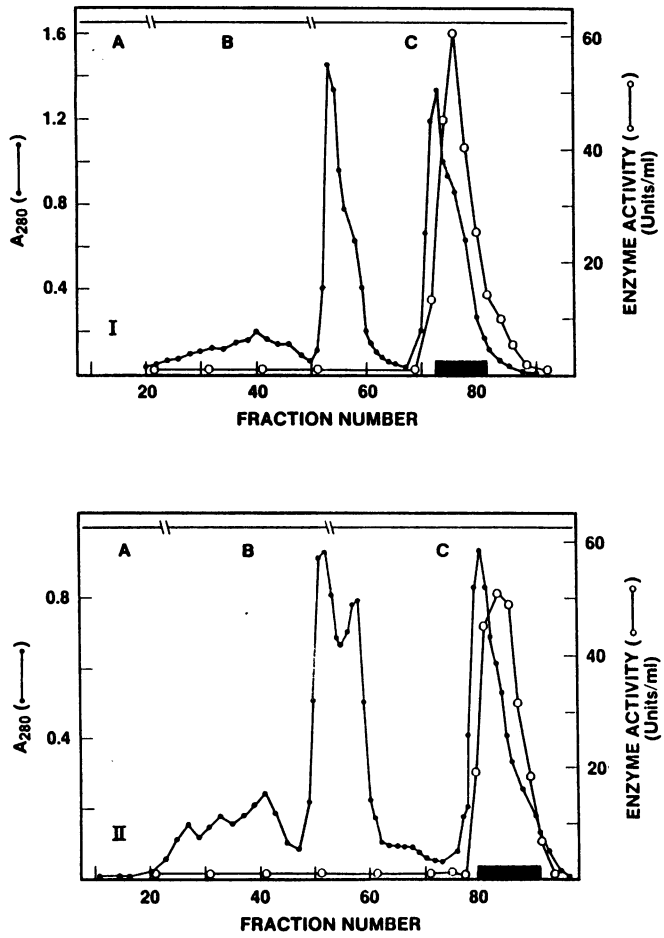

FIG. 2. The elution of cystine lyases I and II from the hydroxylapatite column using the combined fractions obtained from the DEAE column elution. The fractions in the shaded region were combined for further purification. Elution was carried out batchwise using K-phosphate pH 6.4 buffers containing 20 μM PALP at the following phosphate concentrations: A, 0.02 M; B, 0.15 M; C, 0.4 M.

is essentially only a single protein band visualized on the gel with purified peak 1, but that with peak 2 shows a number of protein bands other than the enzyme. The position of the enzyme on the gels was confirmed by the use of an activity stain for pyruvate (5). Duplicate samples of step 6 enzyme were run on a slab gel and electrophoresed. The gel was sliced in half, each portion containing identical amounts of protein sample. One-half was stained with the protein stain Coomassie blue and the other incubated with a complete assay mixture for 30 min at 25°C and stained for pyruvate. The blue protein stain and the red band of the activity stain marked the enzyme location.

Substrate Specificity and K_m. The purified peak 1 enzyme was treated with a number of *S*-substituted cysteines as substrates for the lyase activity. The results are tabulated in Table II. It is obvious that the cysteine sulfoxides and cystine are the most effective substrates. Djenkolic acid and *S*-methyl-L-cysteine do act to some extent as substrates, however L-cysteine and DL(+)-allocystathionine appear to be completely inactive. *S*-allyl-L-cysteine sulfoxide under the conditions of assay is even better as a substrate than L-cystine. However, this amino acid is the dominant non-protein amino acid in garlic and is not found in broccoli. The K_m values of *S*-ethyl-L-cysteine sulfoxide and L-cystine were obtained by the graphical use of the inverse plots of initial velocity against substrate concentration. The K_m for L-cystine was 1.9 mM and 15.6 mM for the *S*-ethyl derivative. The *S*-ethyl derivative is at least as active as the *S*-methyl derivative as a substrate.

Molecular Weight Estimation. The mol wt of the homogeneous lyase was estimated by gel filtration chromatography as described in "Materials and Methods". The result is shown in Figure 4. The mol wt estimated at 152,000 is in good agreement with the value of 150,000 reported for the cystine lyase of turnip (1). The protein was treated with SDS and mercaptoethanol to determine the subunit structure by the method of Hames (8) using slab gel electrophoresis. A single band whose mobility corresponded to a mol wt of 49,000 was found (Fig. 5). This suggests that the native protein is a trimer of identical subunits.

Glycoprotein Nature of the Lyase. Previous work with the onion and garlic alliin lyases had disclosed that they were glycoproteins (14, 17). The purified broccoli enzyme was tested for the presence of carbohydrate after electrophoresis on a slab gel. Duplicate samples were run side by side and the gel split between the two channels. One channel was stained by the standard Coomassie blue for proteins and the other by a modified version of the periodic acid-Schiff's base method of Segrest and Jackson (15). A positive stain was observed in the same position in both samples. The presence of carbohydrate was further substantiated by assaying an aliquot of the purified enzyme for carbohydrate by the phenol-sulfuric acid method of Dubois *et al.* (3). With D-glucose as a standard, the protein was found to contain 5.8% carbohydrate on a weight basis.

DISCUSSION

There have been several studies in recent years which discuss the specificity of cystine lyase from various *Brassica* species (1, 6, 11, 13). In every instance cystine and *S*-alkyl cysteine sulfoxides were found to be the preferred substrates. However, there were some differences in the activities of the different prepara-

Table I. *Summary of Purification*

Step	Volume	Total	Protein	Specific Activity	Purification	Recovery
	ml	*units*[a]	mg	units/mg protein	−fold	%
1. Homogenate	2400	6590	19200	0.34		
2. Acid treatment	2250	4072	3375	1.2	3.5	62
3. Ammonium sulfate	75	2344	1140	2.1	6.2	36
4. DEAE peak 1	250	480	33	15	44	7
5. Hydroxylapatite	29	326	10	33	97	5
6. Gel filtration	33	239	4	60	176	4
4. DEAE peak 2	260	593	34	17	50	9
5. Hydroxylapatite	33	356	9	40	118	5
6. Gel filtration	36	246	4	61	179	4

[a] One unit of enzyme activity produces 1 μmol pyruvate/min at 30°C.

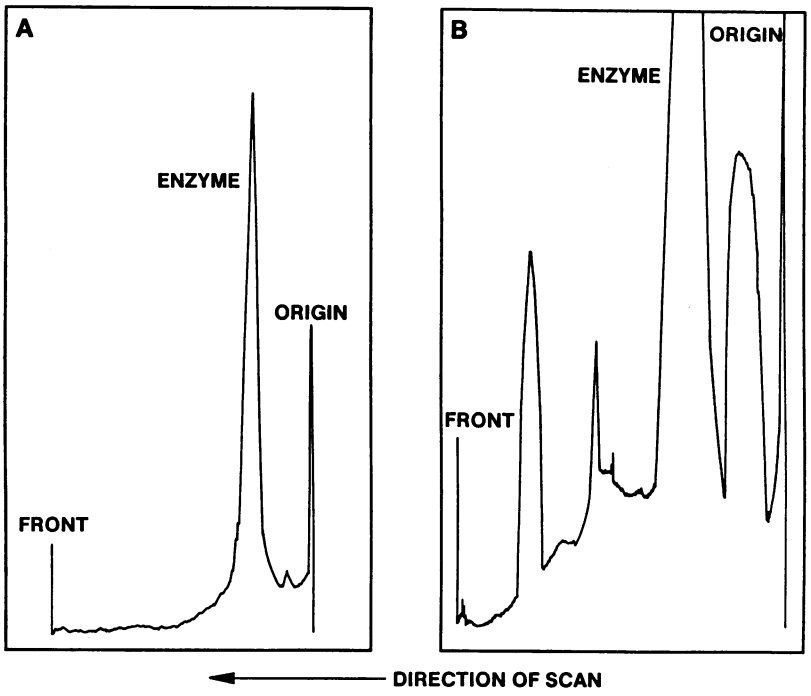

FIG. 3. Densitometer tracings of gels stained with Coomassie blue after electrophoresis of step 6 protein from cystine lyase I and II purifications. In each case 20 μg of protein were used and after destaining was scanned at 590 nm. A, Cystine lyase I; B, cystine lyase II.

Table II. *Substrate Specificity of Cystine Lyase*

Substrate[a]	Relative Activity[b]
L-Cystine	100
DL(+)-Allocystathionine	0
S-Methyl-L-cysteine sulfoxide	76
S-Allyl-L-cysteine sulfoxide	122
O-Acetyl serine	0
Djenkolic acid	10
S-Methyl-L-cysteine	18
L-Cysteine	0

[a] Concentration of L-cystine was 12 μmol per 1.0 ml reaction mixture, whereas the concentration of all other substrates was 60 μmol per 1.0 ml reaction mixture. pH of the reactions was 8.4. [b] Relative activity based on the pyruvate produced. Reaction time was 8 min. Activity with L-cystine was set at 100.

tions with regard to other cysteine derivatives such as L-cysteine, cystathionine, djenkolic acid, *S*-alkyl cysteines, and *O*-acetyl serine as substrates. It was suggested that this might be due to the presence of more than one enzyme in the heterogeneous preparations used as the enzyme source (13). Hall and Smith (6) found that indeed there were two cystine lyases present in cabbage leaves (*B. oleracea* var *capitata*) having somewhat different specificities. This present report shows that broccoli (*B. oleracea* var *botrytis*) buds also have two distinct cystine lyase isozymes.

By the customary criteria used, we have purified what has been termed cystine lyase I (6) to homogeneity and studied its specificity. The primary substrates utilized were L-cystine, *S*-alkyl-L-cysteine sulfoxides and to a slight extent djenkolic acid and *S*-methyl-L-cysteine. It would be likely that L-cystine would be the preferred substrate *in vivo* rather than the alkyl cysteine sulfoxides. This is based on the large difference in their K_m values. There was no activity with cystathionine, L-cysteine, and *O*-acetyl-L-serine as had been previously reported (6). However, in this latter instance the cystine lyase I preparation had a specific activity only 1% that of the enzyme reported here which suggests that other enzymic acitivities are very likely to still be present. The low K_m reported for *O*-acetyl serine (6) in comparison to

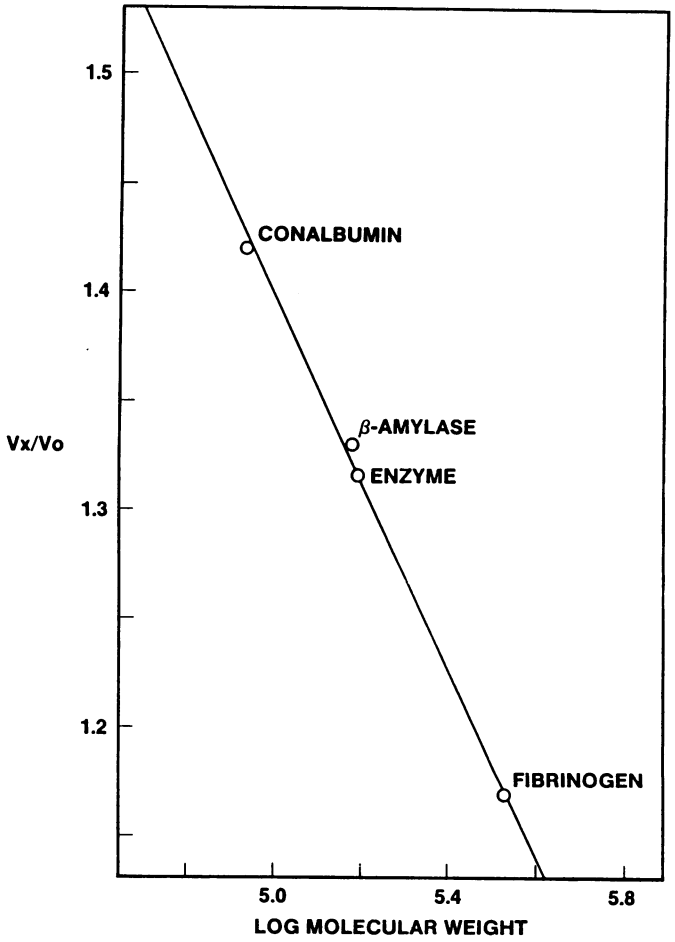

FIG. 4. The mol wt of cystine lyase I by use of gel filtration. The elution volumes of the standard proteins were determined by their *A* at 280 nm. The enzyme elution volumes were located by assaying the eluted fractions for cystine lyase activity.

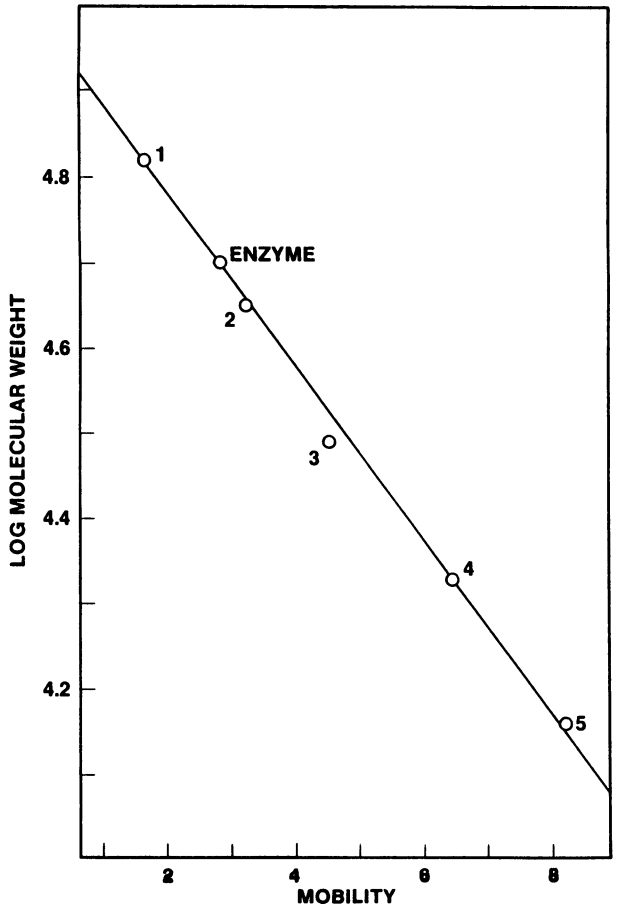

FIG. 5. Determination of subunit mol wt by SDS-PAGE. Marker proteins used: 1, BSA; 2, ovalbumin; 3, carbonic anhydrase; 4, soybean trypsin inhibitor; 5, lysozyme. The protein subunits were visualized by staining with Coomassie blue.

that previously reported in broccoli buds (12) is somewhat disturbing since at the pH used for assay it is known that the *O-N* acetyl shift does occur and therefore the concentration of the *O*-acetyl serine is being changed nonenzymically in the course of the assay.

Brassica is far removed taxonomically from the genus *Allium* which includes garlic and onion. They have in common however that *S*-alkyl-L-cysteine sulfoxides are present as major nonprotein amino acids. Recently the alliin lyase from onion (*Allium cepa*) and garlic (*Allium sativum*) has been purified to homogeneity (8, 14, 17). The cystine lyase and alliin lyase have the ability to cleave *S*-alkyl-L-cysteine sulfoxides, however the alliin lyases can not utilize L-cystine as a substrate. The onion enzyme and the broccoli enzyme have similar pH optima for activity of 8.0 to 8.5, and the garlic enzyme has a pH optimum of 6.5. The mol wt of the onion and broccoli enzyme are quite similar and each appears to consist of a trimer with a subunit mol wt of approximately 50,000. All of these enzymes have one striking similarity in that they are all glycoproteins consisting of 5.8 to 6.0% carbohydrate by weight. Further studies of each of these proteins and the purification of cystine lyase II are currently in progress.

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