Biochemical Characterization of Cystine Lyase from Broccoli (*Brassica oleracea* Var. *italica*)

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Cystine lyase is the enzyme responsible for off-aroma deterioration in fresh unblanched broccoli. In this research, cystine lyase purification from broccoli has been optimized. Only one protein peak with cystine lyase activity was found during purification. Broccoli cystine lyase was purified 100-fold to homogeneity. L-Cystine, L-cysteine-*S*-sulfate, L-djenkolic acid, and some *S*-alkyl-L-cysteines and their sulfoxides are substrates, but the enzyme had negligible activity with L-cystathionine. A K_m value of 81.2 μM was found for L-cystine. Inhibition and K_i determinations indicated that L-cysteine is a linear noncompetitive inhibitor with a K_i of 5 mM and DL-homocysteine is a competitive inhibitor with a K_i of 1.5 mM. The molecular weight of cystine lyase was determined to be 100 kDa by three methods, with two subunits of 48 kDa each and a carbohydrate content of 3%. Further characterization included cofactor quantification, the effects of temperature and pH on activity and stability, and amino acid composition.

Keywords: *Purification; cystine lyase; β-cystathionase; broccoli; Brassica oleracea var. italica*

INTRODUCTION

Cystine lyase (cystathionine L-homocysteine-lyase; EC 4.4.1.8) activity in broccoli (*Brassica oleracea* var. *italica*) was first reported by Hamamoto and Mazelis (1986). Previously, activity of an alkyl-cysteine sulfoxide lyase was reported for broccoli and several species of *Brassica* (Mazelis, 1963). It is possible this lyase could also use cystine as substrate, but this was not investigated at the time. Cystine lyase activity has been found in several members of the *Brassica* genus such as cabbage, cauliflower, kale, mustard, turnip, and rutabaga, but cystine lyase activity in broccoli was not reported (Mazelis et al., 1967).

Hamamoto and Mazelis (1986) purified cystine lyase from broccoli buds. The presence of two different chromatographic peaks with cystine lyase activity, presumably isozymes, was observed. One of those proteins (cystine lyase I) was purified to homogeneity and partially characterized. The primary substrates were L-cystine, *S*-methyl-L-cysteine sulfoxide, and *S*-allyl-L-cysteine sulfoxide (alliin); however, it did not have activity toward L-cystathionine. The possibility that cystine lyase II may have β-cystathionase activity was suggested, but not tested.

Cystine lyase was determined to be the principal enzyme responsible for the off-aroma deterioration of unblanched broccoli (Lim et al., 1989). Its use as a blanching indicator has been proposed to improve the quality of frozen broccoli (Ramírez and Whitaker, 1998).

The main objective of this project was the further characterization of cystine lyase from broccoli to obtain a better understanding of its mechanism of action. Results obtained from this study help resolve the controversy of the cystine lyase substrate specificity. Is broccoli cystine lyase a true β-cystathionase, or should this enzyme and other cystine lyases from higher plants be given a different nomenclature by the Enzyme Commission? This study also investigated the molecular weight of broccoli cystine lyase by three different methods and addresses the question of true isozymes.

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MATERIALS AND METHODS

Materials. Broccoli was purchased in local markets. *S*-Alkyl-L-cysteine sulfoxides were donated by Dr. Mendel Mazelis and were prepared according to the method of Stoll and Seebeck (1951). All other chemicals were of analytical grade.

Solubilization of Cystine. L-Cystine (96 mg) was dissolved in 0.60 mL of 1.5 M NaOH and diluted to 10 mL with water (40 mM). Cystine undergoes β-elimination in the presence of high concentrations of NaOH (Nashef et al., 1977), so only the minimum amount of NaOH (2 mol of NaOH/mol of cystine) was used for solubilization.

Enzymatic Activity. Activity of cystine lyase was measured by the pyruvate produced after a fixed time or by continuously following thiocysteine production. Pyruvate quantitation was done according to the method of Friedemann and Haugen (1943) by measuring dinitrophenylhydrazone (DNPH) formation in the supernatant of the enzymatic reaction. Thiocysteine measurement was done using 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) monitoring the formation of colored aryl mercaptan by the increase in absorbance at 412 nm (Ellman, 1959). One unit of activity corresponds to the production of 1 μmol of pyruvate (or thiocysteine)/min.

Enzyme Purification. All purification steps were monitored with activity determination (thiocysteine measurement using DTNB) and protein quantification [absorbance at 280 or 214 nm, biuret (Gornall et al., 1949) or BCA (Smith et al., 1985) methods]. Temperature was controlled at 4 °C unless otherwise indicated.

Broccoli florets were blended with buffer (0.05 M phosphate/0.05 M citric acid/5% NaCl, pH 6.4), 1:1 (w/v), and 5% insoluble polyvinylpolypyrrolidone (PVPP) for 30 s, and the homogenate was filtered through two layers of cheesecloth. The homogenate pH was then adjusted to 4.0 by slow addition of HCl (6 N) with thorough stirring continued for 15 min at 25 °C. The homogenate was centrifuged (1 h, 10000*g*), and the supernatant was decanted and saturated to 40% with solid $(NH_4)_2$-

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Table 1. Purification Table of Cystine Lyase from Broccoli

step	activity (units/mL)	protein (mg/mL)	specific activity (unit/mg)	volume (mL)	total activity (units)	recovery (%)	purification (fold)
extract[a]	1.39	17.1	0.08	1200	1668	100	1.00
acid precip[b]	1.26	8.0	0.16	1100	1386	83	2.00
40% AS[c]	0.93	6.9	0.13	1200	1116	67	1.63
40−60% AS[d]	20.14	28.0	0.72	33	664	40	9.00
GF[e]	6.60	1.4	4.71	53	350	21	58.88
DEAE[f]	10.76	1.4	7.69	22	237	14	96.13

[a] Fresh broccoli florets (1000 g) were blended with buffer (0.05 M phosphate/0.05 M citric acid/5% NaCl, pH 6.4), 1:1 (w/v), and 5% insoluble PVPP for 30 s, and the homogenate was filtered through two layers of cheesecloth. [b] pH was adjusted to 4.0 by slow addition of HCl (6 N) with thorough stirring continued for 15 min at 25 °C. [c] Saturation to 40% with solid $(NH_4)_2SO_4$, constantly stirred for 30 min. [d] Saturation to 60% $(NH_4)_2SO_4$, constantly stirred for 30 min. [e] Gel filtration using a Fractogel TSK HW 55(S) column (L = 95 cm/d = 2.6 cm) with buffer (0.02 M phosphate/20 μM PALP/0.1 M NaCl/0.02% NaN_3, pH 6.4) and peristaltic pump. [f] Enzyme was eluted in a 0.02 M phosphate buffer, pH 6.4, using a linear NaCl gradient from 0 to 1.0 M. An LKB DEAE-Trisacryl M column connected to an FPLC system was used.

SO_4, constantly stirred for 30 min, followed by centrifugation (30 min, 10000g). Supernatant was then saturated to 60% $(NH_4)_2SO_4$, constantly stirred for 30 min, and centrifuged (30 min, 10000g). The precipitate was redissolved in a minimal amount of buffer [0.02 M phosphate/20 μM pyridoxal 5′-phosphate (PALP) buffer, pH 6.4] and the suspension dialyzed against the same buffer (Spectrapor membrane, MW cutoff 12000−14000). The solution was clarified by centrifugation and concentrated by ultrafiltration (Amicon microconcentrator, Diaflo PM10 membrane).

The enzyme solution was fractionated on the basis of size through a Fractogel TSK HW 55(S) column (L = 95 cm/d = 2.6 cm) with buffer (0.02 M phosphate/20 μM PALP/0.1 M NaCl/0.02% NaN_3, pH 6.4) using a peristaltic pump. Active fractions were pooled, concentrated, and dialyzed. The dialysate was applied to an LKB DEAE-Trisacryl M column (L = 13.5 cm/d = 4.6 cm) connected to an FPLC system. The enzyme was eluted from the column with 0.02 M phosphate buffer, pH 6.4, using a linear NaCl gradient (0−1 M). Active fractions were pooled. The purification steps are presented in Table 1.

Kinetic Determinations and Nature of Active Site. K_m and V_{max} were obtained by measuring activity using different initial substrate concentrations ([So]). Due to differences in assay sensitivity the [So] was 2×10^{-5}−1×10^{-3} M when thiocysteine production was measured and 2.8×10^{-4}−5.0×10^{-3} M when pyruvate formation was followed.

Substrate specificity was investigated by measuring thiocysteine or pyruvate formation or both, depending on the products expected. Possible substrates (Table 2) were investigated. Reaction conditions were kept constant, and in all cases [So] = 12 mM.

PALP was quantitated by measuring fluorescence according to the procedure of Adams (1979) to determine the molar ratio of this cofactor to the enzyme. Enzyme was dialyzed in buffer for removal of any unbound PALP, prior to the determination.

Inhibition determinations were done by measuring pyruvate or thiocysteine production, depending on the nature of the inhibitor. Potential inhibitors investigated included sulfhydryl enzyme inhibitors, thiol compounds, inhibitors of PALP-dependent enzymes, products of the cystine lyase reaction, and pseudosubstrates (Table 3). Reaction conditions were kept constant, and [So] = 12 mM. Some pseudosubstrates were also assayed at a lower [So] of 1 mM. K_i values for L-cysteine and DL-homocysteine were determined by measuring pyruvate formation.

pH and Temperature Effects. The effect of pH on enzyme stability was determined by incubation of the enzyme in a series of buffers (phosphate−citrate, pH 3−8; borate, pH 8−10; μ = 436 mM) at 30 °C for 24 h. After incubation, activity was determined by thiocysteine formation at pH 8.4. The pH optimum was determined by following the production of thiocysteine at different pH values (phosphate−citrate buffers, pH 6−8.5; borate buffers, pH 8−11; μ = 2.23 M). All other reaction conditions were kept constant.

The effect of temperature on enzyme stability was determined by incubation of the enzyme in 0.5 M borate buffer, pH 8.4, for 10 min at intervals of temperatures from 20−70 °C. An aliquot of the incubation reaction was used for determination of enzymatic activity left at 30 °C by production of thiocysteine. Temperature optimum was determined by following thiocysteine production from 5 to 65 °C while all other reaction conditions remained constant.

Structural Characterization: Molecular Weight. *Fractogel Column.* Molecular weight was determined according to the method of Whitaker (1963), using the gel filtration system described under enzyme purification. Standard proteins used were horse spleen apoferritin, sweet potato β-amylase, yeast

Table 2. Substrate Specificity of Cystine Lyase

DTNB method[a]		DNPH method[b]	
compound	relative activity[c]	compound	relative activity[c]
L-cystine	100	L-cystine	100
D-cystine	2	D-cystine	2
L-homocystine	1	L-cystathionine	4
L-cystathionine	3	L-cysteine	0
L-cysteine-*S*-sulfate	11	L-djenkolic acid	18
L-djenkolic acid	24	*S*-methyl-L-cysteine	13
S-methyl-L-cysteine	29	*S*-ethyl-L-cysteine	18
		S-benzyl-L-cysteine	0
		S-ethyl-L-cysteine sulfoxide	71
		S-propyl-L-cysteine sulfoxide	29
		S-carboxymethyl-L-cysteine	4
		DL-homocysteine	0
		N-acetyl-L-cysteine	0
		aminoethyl-L-cysteine	2

[a] The reaction mixture contained 12.00 mM substrate, 312.5 mM borate, 25.00 μM PALP, 250.0 μM DTNB, and 227.3 nM enzyme. Temperature of the reaction was kept at 30 °C and pH at 8.4. [b] The reaction mixture contained 12.0 mM substrate, 150 mM bicine, 25.0 μM PALP, and 227.3 nM enzyme. Temperature of the reaction was kept at 30 °C and pH at 8.4. [c] Expressed as percentage relative to the activity using L-cystine as substrate.

Table 3. Inhibitors of Cystine Lyase

inhibitor	[inhibitor] (M)	% activity left[c]	% activity left[d]
iodoacetamide[a]	1.25E−02	116	111
sodium tetrathionate[a]	3.13E−03	108	98
DTNB[a]	5.00E−04	100	104
N-ethylmaleimide[a]	1.25E−03	57	54
p-hydroxymercuribenzoate[a]	3.43E−06	120	48
hydroxylamine HCl[a]	1.25E−02	3	3
sodium cyanide[a]	1.25E−02	5	5
L-cysteine[a]	1.25E−02	35	
S-methyl-L-cysteine[a]	1.25E−02	99	
S-ethyl-L-cysteine[a]	6.25E−03	96	
L-homocystine[a]	2.50E−03	96	
L-cystathionine[a]	2.50E−03	94	
S-benzyl-L-cysteine[a]	6.25E−03	95	
DL-homocysteine[a]	1.25E−02	30	
pyruvic acid[a]	1.25E−02	75	
pyridoxal[a]	1.25E−03	88	
L-cysteine[b]	2.5E−03	71	
S-methyl-L-cysteine[b]	2.5E−03	116	
S-ethyl-L-cysteine[b]	2.5E−03	120	
L-homocystine[b]	2.5E−03	136	
L-cystathionine[b]	2.5E−03	122	
S-benzyl-L-cysteine[b]	2.5E−03	103	
DL-homocysteine[b]	2.5E−03	59	
pyridoxal[b]	2.5E−03	63	

[a] [L-cystine] = 12 mM, T = 30 °C, pH 8.4, and 5 min reaction time. [b] [L-cystine] = 1 mM, T = 30 °C, pH 8.4, and 10 min reaction time. [c] Inhibitor added to reaction mixture. [d] Enzyme incubated with inhibitor.

alcohol dehydrogenase, mushroom polyphenol oxidase, bovine serum albumin, and chicken ovalbumin. Five milligrams of each protein was passed independently through the Fractogel TSK HW 55(S) column and eluted with buffer (0.02 M phosphate/20 μM PALP/0.1 M NaCl/0.02% NaN_3 at pH 6.4) at a flow rate of 1 mL/min. The ratio V_e/V_o was plotted against the log MW of the standard proteins.

HPLC System. Molecular weight was determined according to the method of Whitaker (1963). Standards were Blue Dextran, horse spleen apoferritin, sweet potato β-amylase, yeast alcohol dehydrogenase, mushroom polyphenol oxidase, and bovine serum albumin. Amounts of 10−50 μg of each protein were passed independently through a TSK-GS3000 column (L = 30 cm/d = 0.78 cm, particle size = 10 μm) linked to an HPLC system and eluted with buffer (0.02 M phosphate/0.1 M NaCl, pH 6.4) at a flow rate of 1 mL/min.

Mass Spectral Analysis. Confirmation of molecular weight of cystine lyase and subunits was performed by Ciphergen (Palo Alto, CA), using a matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrum (Chait and Kent, 1992). A surface-enhanced laser desorption/ionization (SELDI) probe was used to clean up the sample.

The presence (and number) of subunits was determined by SDS−PAGE under denaturing conditions (Sambrook et al., 1987). Low molecular weight standards were run in the gel to determine subunit molecular weight. Purity of the enzyme was determined by native PAGE using a modified protocol of Sambrook et al. (1987) by which the gel is poured without SDS and the loading buffer contains only nondenaturing reagents.

Amino acid composition was determined according to the procedure of Hare (1977). Cystine lyase was hydrolyzed with 6 N hydrochloric acid for 24 h and analyzed using a Beckman 6300 amino acid analyzer. Cysteine and methionine residues were determined by oxidizing the enzyme with performic acid prior to hydrolysis. Tryptophan was determined after mercaptoethane sulfonic acid (MES) hydrolysis.

Carbohydrate content was determined according to the method of Dubois et al. (1956). Glucose and mannose were used as standards. PAGE gels were stained for glycoprotein using the periodic acid−Schiff base reaction (Holden et al., 1971).

RESULTS AND DISCUSSION

Purification. Cystine lyase was purified 100-fold to homogeneity with a 14% recovery as shown in Table 1. Automation of the chromatographies (FPLC system and usage of a peristaltic pump), along with appropriate column sizes, allowed purification of cystine lyase to homogeneity using only two types of chromatography after extraction and differential precipitations.

In the past, the presence of two proteins with cystine lyase activity, presumably isozymes, had been reported in broccoli (Hamamoto and Mazelis, 1986). Reports of possible cystine lyase isozymes in turnip roots have been published (Mazelis et al., 1982; Hall and Smith, 1983). Characterization of the two purified apparent turnip cystine lyase isozymes was done (Wongpaibool and Mazelis, 1994). Similar characteristics of the two apparent isozymes in molecular weights, number of subunits, N-terminal analyses, pH optima, thermal stabilities, substrate specificities, and inhibitors were reported. The main differences between the two proteins were the specific activity (0.38 unit/mg for cystine lyase I and 3.3 units/mg for cystine lyase II) and the degree of glycosylation (0.3 and 1.1%, respectively).

During our initial purification, two peaks with enzymatic activity, not separated to baseline, were found during gravity DEAE-Trisacryl M chromatography (results not shown). All peak fractions with activity were pooled, analyzed, and submitted to Mono Q [$-CH_2-N^+-(CH_3)_3$] chromatography independently. The fractions with activity behaved exactly the same on this stronger ion exchanger, eluting from the column at the same NaCl concentration.

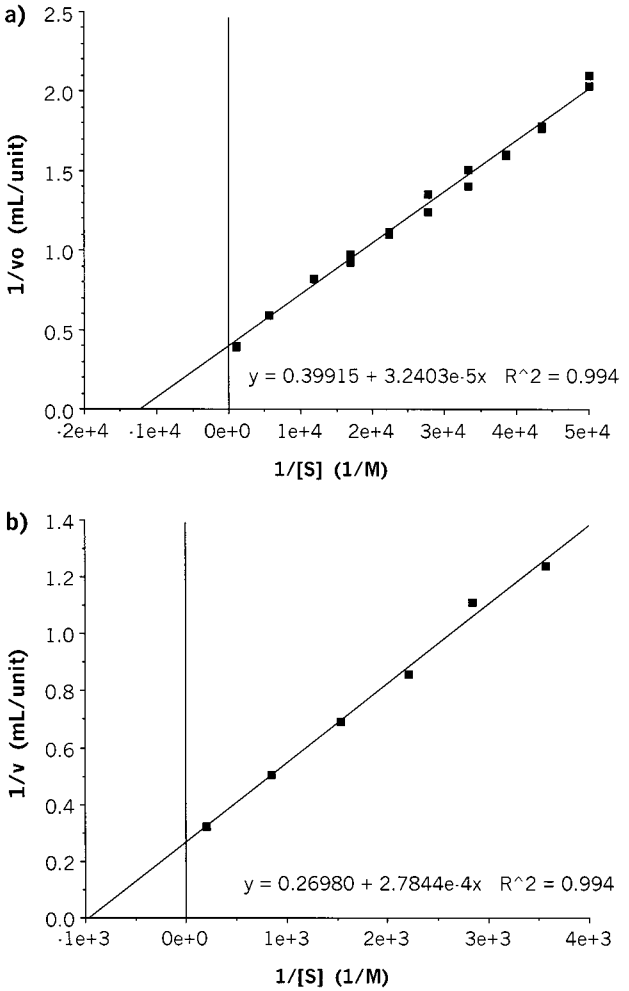

Figure 1. Lineweaver−Burk plot for determination of K_m and V_{max} for cystine lyase and L-cystine measuring (a) thiocysteine production and (b) pyruvate production. Initial formation of thiocysteine was measured in reaction mixtures containing 20−1000 μM L-cystine, 412.5 mM borate buffer, 25.00 μM PALP, 250.0 μM DTNB, and 90.9 nM enzyme. Reaction mixture for pyruvate production contained 0.28−5.00 mM L-cystine, 190 mM bicine, 25 μM PALP, and 90.9 nM enzyme.

Only one peak with enzymatic activity during the DEAE-Trisacryl M step was obtained with automatization. This peak showed only one protein band using native electrophoretic analysis at two different pH values. The apparent presence of two forms of cystine lyase reported previously (Hamamoto and Mazelis, 1986) could be attributed to chromatographic artifacts. Our results show the presence of only one protein with cystine lyase activity in broccoli.

K_m and V_{max}. K_m values for L-cystine, determined by measuring initial velocity for thiocysteine and pyruvate production, were 81.2 μM and 1.00 mM, respectively (Figure 1). The results for maximum velocity were 2.5 and 3.7 units/mL depending on the method used (DTNB versus DNPH formation). Hamamoto and Mazelis (1986) reported a K_m value of 1.9 mM measuring pyruvate by DNPH formation. The DTNB method measures initial velocity of the thiocysteine formation, whereas the pyruvate is measured as DNPH after a fixed period of time. Due to the greater sensitivity of the DTNB method, it was possible to measure initial velocities

using initial substrate concentrations as low as 2×10^{-5} M. This method probably gives a more accurate K_m value.

PALP as a Cofactor. PALP as a cofactor of cystine lyase has been reported previously (Tishel and Mazelis, 1966; Mazelis et al., 1967, 1982; Anderson and Thompson, 1979), and this is recognized by the Enzyme Commission. Cystine lyase showed a spectrum typical of enzymes bound to PALP. Spectral analysis of the holoenzyme after dialysis to remove all unbound PALP showed a ratio of A_{280nm} over A_{330nm} of 4.7 (results not shown), which correponds to the spectra of enzymes with bound PALP (Yang and Metzler, 1979). PALP was determined to be a prosthetic group for cystine lyase in broccoli because the enzyme remains 100% active after dialysis against buffer.

Experiments measuring the fluorescence emitted from the reaction of bound PALP with cyanide after denaturation and precipitation of cystine lyase showed a ratio of 2 mol of PALP/mol of enzyme, using the determined molecular weight of cystine lyase of 110000. This indicates there is a mole of PALP per mole of subunit. The molar ratio of PALP with respect to protein has been investigated in alliin lyase from onion (Tobkin and Mazelis, 1979) and in β-cystathionase from *Escherichia coli* (Dwivedi et al., 1982). Alliin lyase has (with three subunits) 3 mol of PALP/mol of enzyme, whereas β-cystathionase has (with six subunits) 6 mol of PALP/mol of enzyme.

Substrate Specificity. Results expressed as relative activities with respect to L-cystine as a substrate are shown in Table 2. Cystine lyase is stereospecific for L-cystine, with a relative activity of 100 for L-cystine versus 2 for D-cystine. Cystine lyase used as substrates L-cystine, L-cysteine-*S*-sulfate, L-djenkolic acid, and some *S*-alkyl-L-cysteines and their sulfoxides.

Broccoli cystine lyase is not a cystathionine β-lyase, because it showed negligible activity (3−4% as maximum) toward L-cystathionine. Enzymes with β-cystathionase activity show a higher specificity for L-cystathionine than for L-cystine (Dwivedi et al., 1982; Giovanelli, 1987; Uren, 1987; Stanton and Mazelis, 1991). In all higher plant materials investigated to date, cystine lyase activity is not found together with β-cystathionase activity. According to these results and those of previous researchers (Tishel and Mazelis, 1966; Mazelis et al., 1967, 1982; Hamamoto and Mazelis, 1986; Wongpaibool and Mazelis, 1994), higher plant cystine lyases are different enzymes from β-cystathionase; therefore, these cystine lyases should receive a systematic name and classification number different from that given presently by the Enzyme Commission (cystathionine L-homocysteine-lyase, EC 4.4.1.8) (International Union of Biochemistry and Molecular Biology. Nomenclature Committee. Enzyme Nomenclature, 1992). The proposed systematic name for the higher plant enzymes is L-cystine L-cysteine persulfide-lyase, or L-cystine L-thiocysteine-lyase (EC 4.4.1.X).

Analysis of the substrate specificities of cystine lyase gives us some information about its active site. The following conclusions can be made from our results: (a) Having a disulfide bond in the substrate is not a requirement for β-elimination; broccoli cystine lyase did not act on L-homocystine but could use L-djenkolic acid and L-cysteine-*S*-sulfate as poor substrates. L-Homocystine might be too large for the active site of the enzyme, whereas the other two substrates might fit in it. (b) Cystine lyase is active on some substituted L-cysteines, such as *S*-methyl-L-cysteine and *S*-ethyl-L-cysteine, but not on others, such as L-cystathionine, *S*-benzyl-L-cysteine, aminoethyl-L-cysteine, *S*-carboxymethyl-L-cysteine, and *N*-acetyl-L-cysteine; this indicates bulky and charged groups do not fit the active site of cystine lyase. (c) Cystine lyase is not active on SH-amino acids, such as L-cysteine and L-homocysteine. It is required that the sulfur or thiol amino acids be substituted in order to serve as substrates. (d) The enzyme is active on the *S*-alkyl-L-cysteine sulfoxides investigated (*S*-ethyl-L-cysteine sulfoxide and *S*-propyl-L-cysteine sulfoxide). Therefore, compounds with either substituted S(2+) or S(4+) can be substrates.

In conclusion, substrates for cystine lyase need to be substituted L-cysteines or substituted L-cysteine sulfoxides. The substitution can be an alkyl group, but the substituent cannot be too long (L-cystathionine) or bulky (*S*-benzyl-L-cysteine). The substituent can also be charged, but the position of the charges may be important for the enzymatic activity.

Inhibitors. Results expressed as percentage of activity left when compared to activity of cystine lyase without inhibitor are shown in Table 3. Cystine lyase was inhibited up to 50% by some sulfhydryl reacting compounds such as *N*-ethylmaleimide and *p*-hydroxymercuribenzoate and not by others (DTNB, iodoacetamide, and sodium tetrathionate). PALP is bound to PALP-requiring enzymes as a Schiff base formed through reaction of the carbonyl group of the cofactor with the ϵ-amino group of a lysyl residue of the enzyme (Johnson and Metzler, 1970). *N*-Ethylmaleimide and *p*-hydroxymercuribenzoate could be acting as modifiers of the ϵ-amino group of cystine lyase that binds PALP (Vallee and Riordan, 1969), and this could explain the inhibition of the enzymatic reaction by these compounds after incubation of the enzyme with them. It was inhibited almost completely by hydroxylamine and sodium cyanide, as expected for enzymes with PALP as a cofactor. It is also inhibited by L-cysteine and DL-homocysteine, but not by alkylcysteines and other substrate-like compounds (*S*-methyl-L-cysteine, *S*-ethyl-L-cysteine, L-homocystine, L-cystathionine, and *S*-benzyl-L-cysteine). Inhibition and K_i determinations indicated that L-cysteine is a linear noncompetitive inhibitor with a K_i of 5 mM (Figure 2a) and that DL-homocysteine is a competitive inhibitor with a K_i of 1.5 mM (Figure 2b).

Prior to experimentation, it was predicted that L-cysteine would be a competitive inhibitor for cystine lyase; the experimental data, however, showed a linear noncompetitive inhibition (Figure 2a). As concluded from experiments on substrate specificity, L-cysteine should fit into the active site of cystine lyase. One could speculate that L-cysteine does bind to the active site of cystine lyase and, because of the proximity, forms a covalent bond at the catalytic site, either a disulfide bond with an essential −SH or a thiazolidine ring with PALP (Mazelis et al., 1967). If this is the case, inhibition of cystine lyase by L-cysteine should be analyzed by methods for irreversible inhibition.

Effect of pH on Enzyme Stability and Activity. The broccoli cystine lyase is completely stable from pH 4 to 8 at 30 °C after incubation for 24 h. Figure 3 shows a sharp decrease in stability at pH <4; the activity was completely lost when the enzyme was incubated at pH 3. The decrease in stability at pH >8 seems to be less dramatic, requiring at least a 2 pH unit interval for complete loss of activity. When the enzyme was incu-

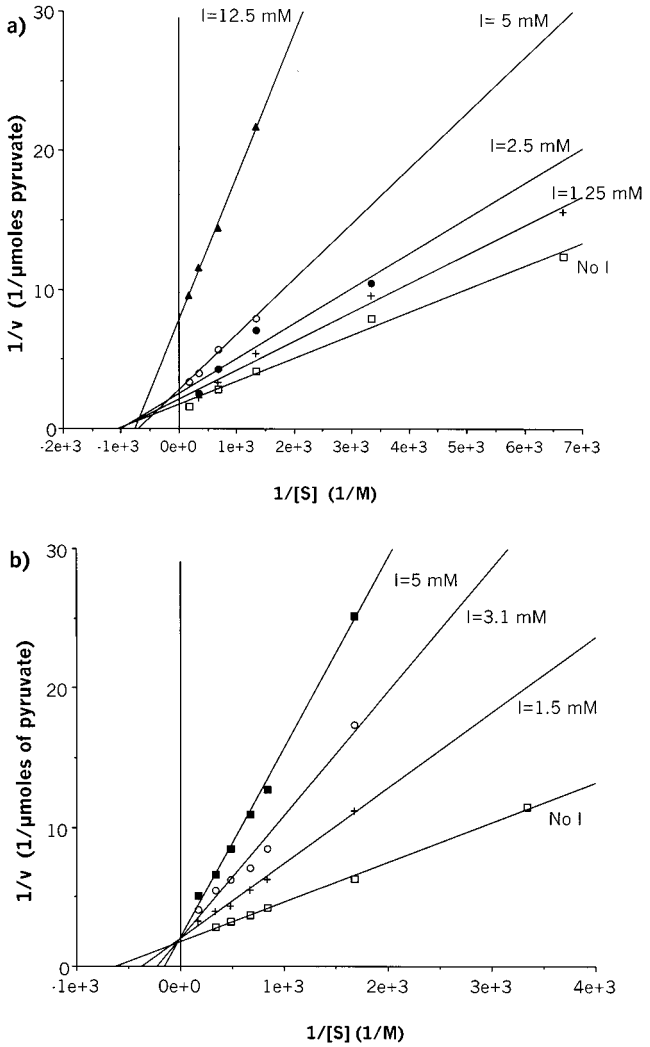

Figure 2. Determination of K_i for (a) L-cysteine and (b) DL-homocysteine. The L-cysteine reaction mixtures contained 0.150−6.000 mM L-cystine, 172.5 mM bicine, 0−12.50 mM L-cysteine, 25.00 μM PALP, and 17.00 μg (protein) of purified enzyme in a total volume of 1 mL. The DL-homocysteine reaction mixtures contained 0.300−6.000 mM L-cystine, 172.5 mM bicine, 0−5.000 mM DL-homocysteine, 25.00 μM PALP, and 17.00 μg (protein) of purified enzyme in a total volume of 1 mL. The incubations were done for 10 min at 30 °C.

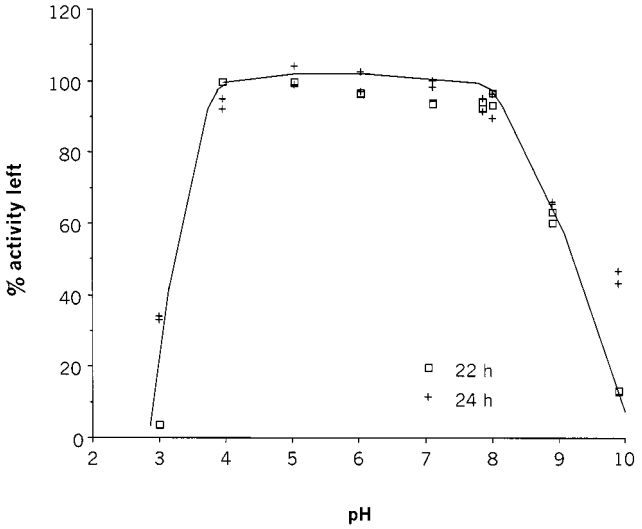

Figure 3. pH stability of cystine lyase. Enzyme (3.6 μM) was incubated with appropriate buffer at different pH values for 22 and 24 h at 30 °C. An aliquot of 50 μL was used for the determination of enzymatic activity left. Reaction mixture contained 1 mM L-cystine, 412.5 mM borate buffer, pH 8.4, 25 μM PALP, 250 μM DTNB, and the aliquot from the pH incubation in a total volume of 1 mL. Reaction was followed by thiocysteine measurement using DTNB.

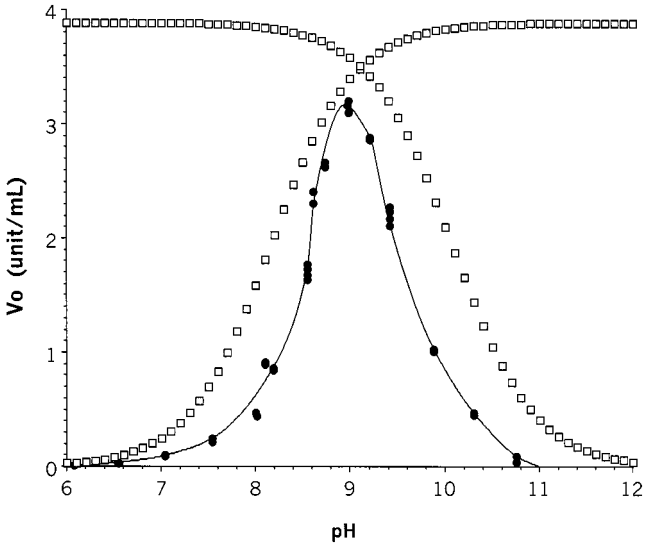

Figure 4. pH activity of cystine lyase. Solid circles show experimental values. Open squares represent theoretical data calculated by using the Michaelis pH function. Reaction mixture contained 1 mM L-cystine, 412.5 mM phosphate−citrate (pH 6−8.5) or borate (pH 8−11) buffer, 25 μM PALP, 250 μM DTNB, and 90.9 nM enzyme. Reaction was done at 30 °C and followed by thiocysteine measurement using DTNB. Reaction pH of the mixtures was measured.

bated for shorter times (10 and 30 min), no pH effect on enzyme stability was observed (results not shown).

The plot of activity versus pH shows a somewhat narrower typical bell shape curve because the two pK_a values are <3 units apart (Figure 4, solid circles). The pH optimum was determined to be 9.1, with experimental pK_{a_1} = 8.53 and pK_{a_2} = 9.70. Experimental height of the peak was determined by the Michaelis pH function to be 0.813 times the true V_{max} (Michaelis, 1922; Dixon and Webb,1964). V_{max} was calculated to be 3.89 units/mL of enzyme (43.2 units/nmol); this theoretical value can never be achieved due to the close proximity of the cystine lyase pK_a values. The theoretical pH curve was calculated (Figure 4, open squares); the true pK_a values were determined as 8.16 and 10.07. pK_{a_1} may correspond to the ionization of a sulfhydryl group, whereas pK_{a_2} may correspond to the ionization of an ϵ-amino group.

The accepted mechanism of the β-elimination reaction by PALP-dependent enzymes involves several steps (Miles, 1986). After the Schiff base is formed between the enzyme and the PALP, the first step of the reaction is the formation of the Schiff base between the substrate and the PALP (enzyme·substrate complex). Next, the extraction of the α-hydrogen of the substrate by a basic group on the enzyme is facilitated through the tautomerization of the Schiff base linkage. According to the results of the present study, it is postulated that it is the ionized sulfhydryl group in the catalytic site of broccoli cystine lyase that is responsible for the extraction of the proton from the α-carbon of the substrate. The role of the amino group may be assigned to formation of the Schiff base with the carbonyl group of PALP.

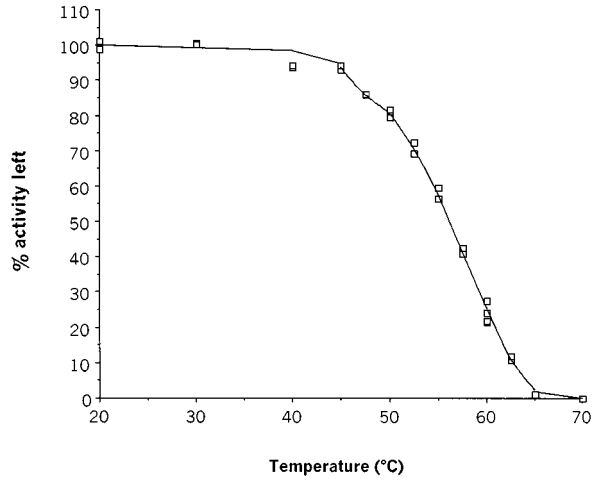

Figure 5. Temperature stability of cystine lyase. Enzyme (3.6 μM) was incubated in borate buffer, pH 8.4, at different temperatures for 10 min. An aliquot of 50 μL was used for the determination of enzymatic activity at 30 °C. Reaction mixture contained 4 mM L-cystine, 412.5 mM borate buffer, 25 μM PALP, 250 μM DTNB, and the aliquot from the temperature incubation in a total volume of 1 mL. Buffers were adjusted to pH 8.4 at the temperature to be used, and the reaction was followed by thiocysteine measurement using DTNB.

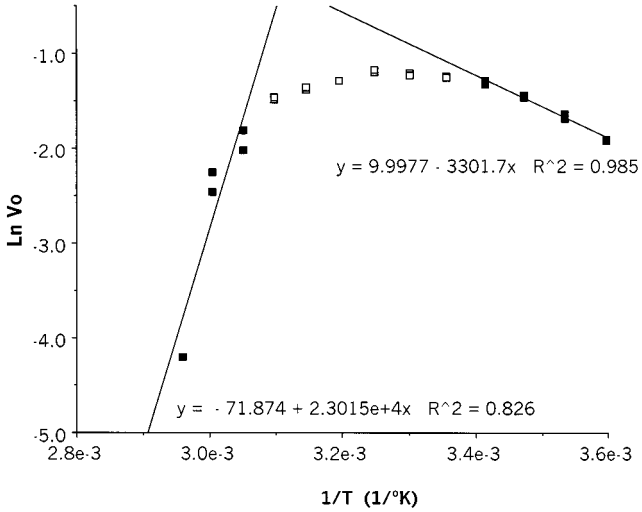

Figure 6. Effect of temperature on activity for cystine lyase. Open squares represent the natural logarithm of initial velocities at different temperatures. Points on either end (solid squares) were selected to calculate the slopes for the energies of activation and denaturation. Reaction mixture contained 4 mM L-cystine, 412.5 mM borate buffer, pH 8.4, 25 μM PALP, 250 μM DTNB, and 90.9 nM enzyme. Buffers were adjusted to pH 8.4 at the temperature to be used, and the reaction was followed by thiocysteine measurement using DTNB from 5 to 65 °C (at 5 °C intervals).

Effect of Temperature on Enzyme Stability and Activity. Broccoli cystine lyase was completely stable up to 45 °C for 10 min (Figure 5). The temperature optimum of cystine lyase was determined to be 35 °C; by plotting the natural logarithm of initial velocities versus the inverse temperatures, an energy of activation of 6500 cal/mol and an energy of denaturation of 46000 cal/mol were calculated (Figure 6). It is difficult to compare these values with those of other cystine lyases because very little information about the effect of temperature on cystine lyase is available in the literature and no previous information is available about the thermal stability and activity properties of broccoli cystine lyase.

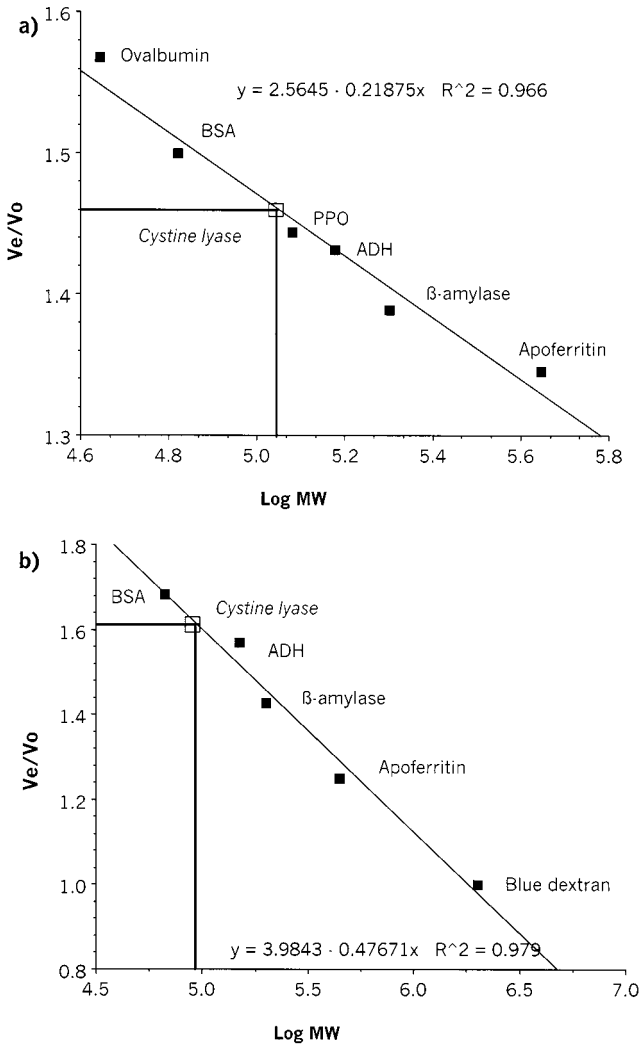

Figure 7. Molecular weight determination of cystine lyase using (a) a Fractogel TSK HW 55(S) column and (b) a GS3000 column linked to an HPLC system. Standards (5 mg/each for Fractogel and 10−50 μg for GS3000) were eluted independently, and the elution volume was recorded. For Fractogel column standard proteins used were horse spleen apoferritin (MW 443 kDa), sweet potato β-amylase (MW 200 kDa), yeast alcohol dehydrogenase (MW 150 kDa), mushroom polyphenol oxidase (MW 120 kDa), bovine serum albumin (MW 66 kDa), and chicken ovalbumin (MW 44 kDa). For GS3000 standards were Blue Dextran (MW 2000 kDa), horse spleen apoferritin (MW 443 kDa), sweet potato β-amylase (MW 200 kDa), yeast alcohol dehydrogenase (MW 150 kDa), mushroom polyphenol oxidase (MW 120 kDa), and bovine serum albumin (MW 66 kDa).

Structural Characterization. The molecular weights of broccoli cystine lyase and its subunits were determined using different systems. Total molecular weight was found to be 110000 Da using the Fractogel column (Figure 7a), 100000 Da using a TSK-GS3000 column linked to the HPLC system (Figure 7b), and 94000 Da with a subunit molecular weight of 47000 when laser-assisted time-of-flight mass spectroscopy was used (Figure 8). Two subunits of the same size (48 kDa) were found using SDS−PAGE.

Molecular weights of cystine lyases vary depending on the source of the enzyme. Reported values of cystine lyase molecular weight include 150000 (Anderson and Thompson, 1979), 400000 and 440000 (Wongpaibool and Mazelis, 1994) for turnip cystine lyase, 152000 (Hamamoto and Mazelis, 1986) for the broccoli enzyme, 150000

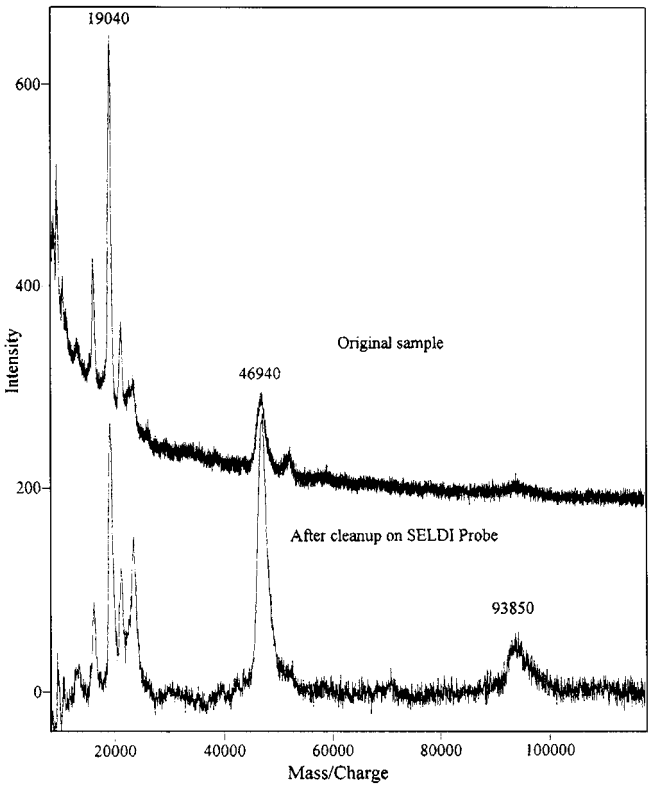

Figure 8. Molecular weight determination of cystine lyase using MALDI-TOF mass spectroscopy. A SELDI probe was used to clean up the sample (≈1 pmol). The peak at 46940 Da represents the subunit molecular weight, whereas the 93850 Da peak represents the molecular weight of the complete enzyme.

and 240000 (Smith and Hall, 1987) for cabbage cystine lyase, and 210000 (Stanton and Mazelis, 1991) for the spinach enzyme. The numbers of subunits of cystine lyase are different depending on the source, but a common characteristic among the cystine lyases is that all of the subunits are about the same size. The number of subunits (and MW) reported to date are 3 subunits (49000) for broccoli (Hamamoto and Mazelis, 1986), 4 subunits (53000) for spinach (Stanton and Mazelis, 1991), and 12 subunits (34000 and 38000) for turnip (Wongpaibool and Mazelis, 1994).

Our results for subunit molecular weight agree with those reported by Hamamoto and Mazelis (1986); the molecular weight of the active enzyme found is, however, different, and therefore the conclusion on the number of subunits. We found evidence by three different techniques that the molecular weight of cystine lyase is 100000 (± 10000) kDa, and therefore the enzyme is a dimer. Supportive evidence that the enzyme has only two subunits also comes from the results of the molar ratio of PALP to cystine lyase (2 mol of PALP/mol of protein).

Results for amino acid composition are shown in Table 4. The total number of amino acids from the amino acid composition was estimated using a cystine lyase molecular weight of 110000 and an average amino acid residue molecular weight of 113. Individual amino acid composition was calculated by multiplying the total number of amino acids by concentration in mole percent. The amino acid composition of cystine lyase showed a high percentage of aspartic and glutamic acid residues, as well as hydrophobic amino acid residues, especially alanine and leucine. No tryptophan was detected. Cystine lyase has a large number of cysteines (21), and therefore there is the possibility of disulfide bonds.

Table 4. Amino Acid Composition of Cystine Lyase

amino acid (aa)	amount (nmol/inj)[c]	mol %	no. of aa[d]	ng/inj[e]	wt %
Asp	2.737	10.4	101	315	11.0
Thr	1.739	6.6	64	176	6.2
Ser	1.868	7.1	69	163	5.7
Glu	2.407	9.1	89	311	10.9
Pro	1.630	6.2	60	158	5.5
Gly	1.832	6.9	67	104	3.7
Ala	2.730	10.3	100	194	6.8
Val	1.885	7.1	69	187	6.5
Met[a]	0.463	1.8	17	61	2.1
Ile	1.200	4.5	44	136	4.8
Leu	2.228	8.4	82	252	8.8
Tyr	0.702	2.7	26	114	4.0
Phe	1.258	4.8	46	185	6.5
His	0.467	1.8	17	64	2.2
Lys	1.721	6.5	63	220	7.7
Arg	1.011	3.8	37	158	5.5
Cys[a]	0.563	2.1	21	58	2.0
Trp[b]	0.000	0.0	0	0	0.0
totals	26.441	100.0	973	2854	100.0

[a] Determined after performic acid oxidation. [b] None detected in a 50 μg sample after MES hydrolysis. [c] As determined by peak area at A_{570nm}, except for proline at 440 nm. Injection corresponded to 2.63 μg of protein. [d] Calculated as total number of amino acids per 110000 g/mol. [e] Calculated as amount of specific amino acid (nmol/inj) by its residue molecular weight.

Carbohydrate Content. Results obtained by using the Dubois method and the periodic acid−Schiff base reaction for staining gels showed broccoli cystine lyase is a glycoprotein. This is in agreement with reports for broccoli cystine lyase (Hamamoto and Mazelis, 1986) and turnip root cystine lyase (Wongpaibool and Mazelis, 1994). Alliinases from onion (Tobkin and Mazelis, 1979), garlic (Nock and Mazelis, 1986), and leek (Won and Mazelis, 1989) also appear to be glycoproteins.

In the current research, the carbohydrate contents found according to the Dubois method were 3.1 and 0.3%, respectively, using glucose and mannose as standards. Information from glycoprotein staining in 8% native PAGE showed native cystine lyase is a glycoprotein stained by the periodic acid−Schiff base reaction.

Glycoprotein staining of 10% SDS−PAGE showed denatured monomeric broccoli cystine lyase is a glycoprotein stained by the periodic acid−Schiff base reaction.

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