Purification and Characterization of C-S Lyase from Fusobacterium varium

A C-S CLEAVAGE ENZYME OF CYSTEINE CONJUGATES AND SOME S-CONTAINING AMINO ACIDS*

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An enzyme responsible for the carbon-sulfur bond cleavage of various S-aryl, S-aralkyl, and S-alkyl cysteines has been purified about 270-fold from Fusobacterium varium. Incubation of a cysteine conjugate of p-bromobenzene with the enzyme yielded equimolar amounts of p-bromobenzenethiol, pyruvic acid, and ammonia, indicating that the carbon-sulfur bond cleavage proceeds via an \( \alpha,\beta \)-elimination reaction. The enzyme activity was inhibited either by hydroxylamine or KCN and stabilized by pyridoxal phosphate, which probably acted as cofactor. The broad substrate spectrum of this enzyme suggested an important role in the intestinal metabolism of xenobiotics.

During the past few years, in vivo formation of methylthio-containing metabolites has been reported for a wide variety of compounds including drugs, herbicides, aromatic hydrocarbons, and environmental contaminants (1-4).

Two mechanisms have been proposed for the formation of such methylthio metabolites so far. The first mechanism proposed by Miller and his associates (5) was a nucleophilic attachment of a methylthio group of methionine or \( N \)-acetyl-methionine to reactive carbon compounds. A second mechanism has been proposed by Tateishi et al. (6) and Tateishi and Shimizu (7, 8) in which cysteine conjugates were converted to the corresponding methylthiolated metabolites according to the reaction pathway shown in Scheme 1.

\[
\begin{align*}
\text{Deacylase} & \quad \text{Deacylase} \\
R-S-\text{CH}_2-\text{CH} & \quad R-S-\text{CH}_2-\text{CH} \\
| & | \\
N\text{HCO}_3^- & \quad N\text{H}_2 \\
\beta-\text{Lyase} & \quad S-\text{Methyltransferase} \\
\rightarrow R-SH & \quad \rightarrow R-SCH_3 \\
\end{align*}
\]

**Scheme 1**

Among the above three enzymes, both deacylase and cysteine-conjugate \( \beta \)-lyase (EC 4.4.1.13) have been purified and characterized by Tateishi et al. (6) Tateishi and Shimizu (7, 8) and Suzuki and Tateishi (9).

In a previous paper (10), we described the presence of a C-S bond cleavage enzyme in intestinal microorganism(s); the enzyme exhibited high activity toward cysteine conjugates but virtually no activity toward other sulfur-containing conjugates such as mercapturic acids or glutathione conjugates. This observation raised the possibility that not only the rat liver \( \beta \)-lyase but also the C-S lyase of intestinal microorganism(s) might play an important role in the in vivo formation of methylthio-containing metabolites according to a mechanism similar to that shown in Scheme 1. In the present report, we describe the purification and characterization of the C-S lyase from Fusobacterium varium (ATCC 8501), one of the large intestinal microorganisms in man, and discuss the participation of the enzyme in methylthiolation reactions of xenobiotics.

**EXPERIMENTAL PROCEDURES**

**Materials**

| S-(p-Bromophenyl)-L-cysteine, S-(p-bromophenyl)-D-cysteine, N-acetyl-(p-bromophenyl)-L-cysteine, S-(p-bromophenyl)-L-glutathione, and S-(p-nitrobenzoyl)-L-cysteine were prepared by the method of Saunders (11). S-(p-Bromophenyl)-cysteamine was synthesized by a reaction of diazotized p-bromobenzoic acid and cuprous mercaptide of cysteamine (12). S-(p-Bromophenyl)-3-thiopyruvic acid was prepared by a reaction of p-bromobenzenethiol with 3-fluoropyruvic acid (Aldrich) according to the procedure described by Farrod (13). S-(1-Propyl-L-cysteine, S-(2-propyl)-L-cysteine, S-(1-butyl)-L-cysteine, S-(tert-butyl)-L-cysteine, and S-cyclohexyl-L-cysteine were synthesized by a reaction of L-cysteine with the corresponding alkyl iodides (Tokyo Kasei Kogyo Co., Tokyo) by the following method. To the ethanolic solution (2 ml) of the alkyl iodides (1 mmol) was added cysteine (1 mmol) dissolved in 2 N NaOH solution. The mixture was heated at 100 °C for 2 min followed by standing at room temperature to yield colorless crystals. After filtration, the crystals were washed with ice-chilled water and recrystallized in ethanol (yield, 60-70%). The structure of each compound was confirmed by the analyses of the mass and nmr spectrum. S-Methyl-L-cysteine, S-ethyl-L-cysteine, S-benzyl-L-cysteine, S-djenkolic acid, S-carboxymethyl-L-cysteine, \( S \)-cysteic acid, \( S \)-phenyl-L-cysteine, and \( S \)-cystathionine were obtained from Tokyo Kasei Kogyo Co., p-Bromobenzenethiol and p-fluorobenzenethiol were purchased from Aldrich, p-Bromophenylmethyl sulfide was synthesized from p-bromobenzenethiol by the method previously described (10). p-Nitrobenzylmethyl sulfide and cyclohexylmethyl sulfide were prepared by the reaction of methanethiol with \( p \)-nitrobenzylchloride and bromocyclobexane, respectively, by the usual method. S-(p-Bromophenyl)-L-[U-\( ^{14} \)C]cysteine was prepared from L-[U-\( ^{14} \)C]cysteine (Amersham Corp.) and \( p \)-bromobenzoic acid (11). S-Adenosyl[Me-\( ^{35} \)S]methionine with a radiopurity of 97% was purchased from Amersham Corp. All other amino acids and reagents used in the present study were purchased commercially and were of analytical special grade. DEAE-cellulose (DE52) and hydroxyapatite were obtained from Whatman and Sephadex G-150 superfine and Sephadex G-200 from Pharmacia Fine Chemicals (Sweden). Liquid Blood Culture Bottle Columbia Roche was obtained from Nippon Roche (Tokyo).

**Assay Methods**

**Assay of C-S Lyase**—For routine assay of C-S lyase activity, S-(p-bromophenyl)-L-cysteine was used as the substrate and the amount of the p-bromobenzenethiol formed was determined with a high pressure liquid chromatograph by the method described in the previous report (10). The assay mixture contained in a final volume of
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0.40 ml: S-(p-bromophenyl)-L-cysteine, 0.20 μmol; diithiothreitol, 2 μmol; pyridoxal phosphate, 0.02 μmol; potassium phosphate buffer, pH 7.4, 20 μmol; and enzyme solution (0.02-0.2 ml). The reaction mixture was incubated at 37°C for 20 min under anaerobic conditions. The reaction was terminated by addition of 0.4 ml of acetonitrile containing p-fluorobenzzenethiol (60 μg) as internal standard.

After pH 7.4, the C-S lyase activity was determined as the quantity of enzyme that catalyzes the formation of 1 pmol of the thiol compound/minute. The reaction mixture contained 0.40 ml: S-(p-bromophenyl)-L-cysteine, 0.20 μmol; pyridoxal phosphate, 0.02 pmol; potassium phosphate buffer, pH 7.4, 20 μmol; and enzyme solution (0.02-0.2 ml). The reaction mixture was incubated at 37°C for 20 min under anaerobic conditions. The reaction was terminated by addition of 0.4 ml of acetonitrile containing p-fluorobenzzenethiol (60 μg) as internal standard.

Assay of α-Ketocarboxylic Acids—A spectrophotometric method with 3-methyl-2-benzothiazoline hydrazine hydrochloride was used for the quantitative determination of α-ketocarboxylic acid according to the procedure of Soda (14). Pyridoxal 5'-phosphate in the incubation mixture, which was reported to react with the reagent to yield the corresponding azine derivative, did not interfere with the assay of α-ketocarboxylic acid at a wavelength of 320 nm.

Assay of Ammonia—Ammonia in reaction mixtures was determined with Nessler's reagent (6).

Purification of C-S Lyase from F. varium

F. varium (ATCC 8501) was grown at 37°C in Liquid Blood Culture Bottle Columbia Roche (which is suitable for the culture of aerobic and anaerobic microorganisms) containing 10.9 g of pantone, 10.0 g of biotine, 3.0 g of tryptic digest of beef heart, 0.1 g of L-cysteine, 2.5 g of dextrose, 5.8 g of NaCl, 0.1 g of MgSO4, 0.02 g of FeSO4, 0.6 g of Na2CO3, 0.83 g of tris(hydroxymethyl)aminomethane, 286 g of tris(hydroxymethyl)aminomethane hydrochloride, and 0.5 g of Liquoid (sodium polyanetbolesulfonate) per liter. Cells were harvested at 16 h in post-log phase with an absorbance of about 0.8 at 660 nm. A typical growth curve for F. varium and for its C-S lyase activity at respective time points is shown in Fig. 1. C-S lyase was purified from F. varium by the following procedure. All steps of the purification were carried out at 0-4°C. A typical example of purification of the enzyme is shown in Table I.

Step 1: Preparation of Crude Extracts—Twenty six g (wet weight) of cells were suspended in 220 ml of 10 mM potassium phosphate buffer, pH 7.4, and centrifuged at 20,000 x g for 20 min. The supernatant solution was used in the following enzyme purification.

Step 2: Ammonium Sulfate Fractionation—To 210 ml of the crude extract was added 140 ml of a saturated solution of ammonium sulfate to attain 40% saturation. The mixture was stirred for 20 min followed by centrifugation at 20,000 x g for 30 min. To the supernatant (333 ml) was added 68.3 g of powdered ammonium sulfate to result in 70% saturation. After stirring for 40 min, the suspension was centrifuged at 20,000 x g for 30 min to precipitate the active enzyme pellet. The pellet was resuspended in a minimum amount of potassium phosphate buffer (10 mM, pH 7.4) and dialyzed against 6 liter (total volume) of the same buffer for 24 h to yield 39 ml of 40-70% ammonium-sulfate fraction.

Step 3: First DEAE-cellulose—The ammonium-sulfate fraction was applied on a DEAE-cellulose column (4.0 x 13 cm) equilibrated with 10 mM potassium phosphate buffer, pH 7.4, containing 100 mM KCl. Proteins were first eluted with 1200 ml of the same buffer and then with 900 ml of the phosphate buffer containing a linear gradient concentration of 100-500 mM KCl at a flow rate of 1 ml/min. Eluate fractions of 15 ml were collected. The enzyme activity emerged in both the void volume of the initial washing and approximately 150-250 mM KCl (Fig. 2). The total enzyme activity in the void volume was less than 20% of that in the latter fractions. Hence, only fractions 90-106 were combined, concentrated with a Diaflo ultrafiltration apparatus (Amicon), and dialyzed against 10 mM potassium phosphate buffer.

Step 4: Second DEAE-cellulose—The dialyzed first DEAE-cellulose fraction was chromatographed on a second DEAE-cellulose column (4.0 x 17.5 cm) equilibrated with 10 mM potassium phosphate buffer containing 100 mM KCl. Proteins were eluted with the phosphate buffer containing a linear gradient concentration of 100-300 mM KCl. Fractions of 15.7 ml were collected. The enzyme was eluted at a KCl concentration of about 190-240 mM (Fig. 3). Fractions 64-70 were combined, concentrated (to about 5.0 ml), and desalted as described in Step 3.

Step 5: Hydroxypatite—The enzyme fraction obtained in Step 4 was applied to a hydroxyapatite column (2.0 x 12.5 cm) equilibrated with 100 mM potassium phosphate buffer, pH 6.25, and first washed with 275 ml of the same buffer. The enzyme was then eluted at a flow rate of 0.7 ml/min with potassium phosphate buffer with a linear gradient concentration of 100-350 mM. Fractions of 10 ml were collected in fraction tubes, each containing 100 μl of an aqueous solution of pyridoxal 5'-phosphate (0.5 μmol) for stabilization of the enzyme. The enzyme activity emerged at a buffer concentration of about 150-220 mM. Fractions 45-48 were pooled, and concentrated with a Diaflo membrane as described above.

Step 6: Gel Filtration—The solution (0.5 ml) obtained from the hydroxyapatite column chromatography was applied on a Sephadex G-200 column (2.0 x 66 cm) previously equilibrated with 10 mM phosphate buffer, pH 7.4. Elution was effected with the same buffer at a flow rate of 0.01 ml/min. For Step 7, fractions (4.5 ml each) of 27-32 were combined, concentrated, and then applied on a Sephadex
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Fig. 3. Second DEAE-cellulose column chromatography (purification Step 4). After purification through Step 3 and dialysis, the C-S lyase fractions were rechromatographed on a second DEAE-cellulose column (4.0 X 17.5 cm) equilibrated with 10 mM K+-phosphate buffer containing 100 mM KCI. Proteins were eluted with the phosphate buffer containing a linear gradient concentration of 100-300 mM KCI. Fractions of 15.7 ml were collected. Protein (■) and C-S lyase activity (○) were assayed as described in the text.

Table I. Purification of C-S lyase from Fusobacterium varium (ATCC 8501)

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume</th>
<th>Total Protein</th>
<th>Total Activity</th>
<th>Specific Activity</th>
<th>Purification</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Extract</td>
<td>210</td>
<td>3570</td>
<td>532</td>
<td>0.149</td>
<td>1.0</td>
<td>100</td>
</tr>
<tr>
<td>2. Ammonium sulfate fractionation</td>
<td>39</td>
<td>2964</td>
<td>374</td>
<td>0.126</td>
<td>0.85</td>
<td>70</td>
</tr>
<tr>
<td>3. 1st DEAE-cellulose</td>
<td>180</td>
<td>554</td>
<td>189</td>
<td>0.341</td>
<td>2.29</td>
<td>35.5</td>
</tr>
<tr>
<td>4. 2nd DEAE-cellulose</td>
<td>110</td>
<td>88</td>
<td>119</td>
<td>1.35</td>
<td>9.1</td>
<td>22.4</td>
</tr>
<tr>
<td>5. Hydroxyapatite</td>
<td>5.0</td>
<td>8.7</td>
<td>109</td>
<td>12.6</td>
<td>84.6</td>
<td>20.5</td>
</tr>
<tr>
<td>6. Sephadex G-200</td>
<td>16.3</td>
<td>0.82</td>
<td>33</td>
<td>40.5</td>
<td>272</td>
<td>6.2</td>
</tr>
</tbody>
</table>

G-150 superfine column (2.0 X 74 cm) equilibrated with the same buffer. Upon elution with the buffer (0.04 ml/min), the initial 187 ml of the eluate was discarded and the following 16.3 ml was collected (Fig. 4).

Fig. 4. Sephadex G-150 superfine column chromatography (purification Step 7). C-S Lyase fractions obtained from Step 6 were applied to a Sephadex G-150 superfine column (2 × 74 cm) equilibrated with 10 mM K⁺-phosphate buffer, pH 7.4. Elution was conducted with the same buffer at a flow rate of 0.07 ml/min. Fractions of 4.5 ml each were collected, and protein (■) and C-S lyase activity (○) were assayed as described in the text.

RESULTS

Purification of C-S Lyase—Among intestinal microorganisms (F. varium, Fusobacterium mortiferum, Bacteroides fragilis, Lactobacillus casei, Lactobacillus plantarum, Lactobacillus leichmannii, Lactobacillus fermenti, Lactobacillus brevis, and Escherichia coli) examined for C-S lyase activity, only F. varium was found to possess significant activity toward S-(p-bromophenyl)-L-cysteine. Hence, the bacterial C-S lyase was purified about 270-fold over crude extract of F. varium by the six-step procedure (Table I). Disc electrophoretic monitoring of the enzyme preparation at several steps of the purification procedure is shown in Fig. 5. The enzyme solution at Step 7 produced one main band in the disc electrophoresis. During storage of the purified enzyme for 7 months at -20 °C, the enzyme activity did not decrease to any significant extent.

The molecular weight of the C-S lyase as determined by gel filtration on Sephadex G-200 and polyacrylamide gel electrophoresis was approximately 70,000.

Identification of Thiol-containing Metabolites—S-(p-Bromophenyl)-L-cysteine, S-(p-nitrobenzyl)-L-cysteine, and S-cyclohexyl-L-cysteine, 2 µmol of each, were incubated separately with the enzyme from Step 6. Incubation conditions were the same as those used for the enzyme assay except that the scale was five times as large. Each thiol compound in the reaction mixture was extracted with 4 ml of ether containing 0.2 ml of acetonitrile. After evaporation of ether, an excess amount of ethereal diazomethane was added to the residue and the mixture was kept at room temperature for 5 min for methylation of the thiol group. After evaporation of diazomethane, a portion of the solution was injected into the gas chromatog-
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Aromatic-mass spectrometer. Under the conditions described under “Experimental Procedures,” p-bromophenylmethyl sulfide, p-nitrobenzylmethyl sulfide, and cyclohexylmethyl sulfide appeared at 5.0, 4.3, and 4.2 min, respectively.

In the mass spectrum, p-bromophenylmethyl sulfide showed a molecular peak at \( m/z \) 202 (base peak) with its isotope peak at \( m/z \) 204, \( m/z \) 181 (M\(^+\) - CH\(_3\), relative intensity 20\%), isotope peak at \( m/z \) 189, \( m/z \) 169 (M\(^+\) - SH, 7\%), isotope peak at \( m/z \) 171, \( m/z \) 156 (M\(^+\) - SCH\(_3\), 7\%), isotope peak at \( m/z \) 158, \( m/z \) 123 (M\(^+\) - Br, 10\%), and \( m/z \) 108 (M\(^+\) - Br - CH\(_3\), 8\%) (Fig. 6).

The mass spectrum of p-nitrobenzylmethyl sulfide showed a molecular peak at \( m/z \) 183 (base peak) together with major fragment peaks at \( m/z \) 136 (M\(^+\) - SCH\(_3\), relative intensity 80\%), \( m/z \) 106 (M\(^+\) - CH\(_2\)SCH\(_2\) - NO, 38\%), and \( m/z \) 78 (M\(^+\) - NO - CH\(_3\)SCH\(_2\), 20\%) (Fig. 6).

The fragmentation pattern of the cyclohexylmethyl sulfide in the mass spectrum was as follows: a molecular peak at \( m/z \) 130 (relative intensity 72\%), \( m/z \) 116 (M\(^+\) - CH\(_3\) + I, 7\%), \( m/z \) 115 (M\(^+\) - CH\(_3\), 4\%), \( m/z \) 91 (M\(^+\) - CH\(_3\)H, 22\%), \( m/z \) 87 (M\(^+\) - CH\(_2\), 12\%), \( m/z \) 83 (M\(^+\) - SCH\(_3\), 29\%), \( m/z \) 82 (M\(^+\) - SCH\(_3\) - H, base peak), and \( m/z \) 67 (C\(_6\)H\(_7\)), 90\%) (Fig. 6).

Each fragmentation pattern of these derivatives was identical with that of the respective authentic samples.

In a control incubation mixture, from which the enzyme was omitted, formation of the thiol compounds was not observed.

The results indicate that thiol compounds were formed from the corresponding S-alkyl, S-aralkyl, and S-aryl cysteine conjugate by the action of the C-S lyase.

**Stoichiometry Study**

S- (p-Bromophenyl) - L - \([U - ^{14}C]\) cysteine (0.2 \( \mu \)l, 2 umol) was incubated with Step 6 enzyme in 2 ml of 50 mM potassium phosphate buffer, pH 7.4, containing 5 mM dithiothreitol. Formation of thiol and ammonia was quantitatively determined in portions of the incubated mixture by the methods described under “Experimental Procedures.” Identification and quantitative analysis of formed \([^{14}C]\)pyruvic acid were performed according to the procedure described in the previous report (6). The relative rates of formation of the three products (i.e. p-bromobenzenethiol, pyruvic acid, and ammonia) were 1.0:0.97:0.97, respectively, indicating equimolar formation of three products. Neither \([^{14}C]\)serine, \([^{14}C]\)alanine, nor S-(p-bromophenyl)-3-[\(^{14}C\)]thiopropionic acid was detectable in the reaction mixture.

**Effect of pH**

The effect of pH on the activity of C-S lyase was examined with S-(p-bromophenyl)-L-cysteine as the substrate over the pH range of 3.6-9.0 using four different buffers, i.e. acetate buffer (3.6-5.4), potassium phosphate buffer (5.2-8.1), Tris-HCl buffer (7.2-8.9), and borate buffer (7.5-9.9).

Activity of the enzyme was optimal at about pH 7.5-8.0 for each buffer and absent below pH 5.0 (Fig. 7).

**Effect of Pyridoxal 5'-Phosphate and Other Reagents on Enzyme Activity**

The activity of the purified enzyme preparation (Step 6) showed a steady decrease with a half-life of about 5 min during incubation at 37°C in potassium phosphate buffer, and 97% of the initial activity was lost within 20 min. No significant loss of enzyme activity was, however, observed under similar conditions in the presence of 1 mM pyridoxal 5'-phosphate, the result suggesting that the reagent was of importance for stabilization of the enzyme.

C-S lyase was preincubated, before the enzyme assay, with N-ethylmaleimide, potassium cyanide, and hydroxylamine, each in the presence of pyridoxal 5'-phosphate. The activity was completely inhibited both by hydroxylamine and by potassium cyanide, whereas almost all the activity (97%) was retained after treatment with N-ethylmaleimide. Preincubation of the enzyme with other reagents was also investigated; pyridoxamine, pyridoxamine 5'-phosphate, pyridoxal, glutathione, dithiothreitol, EDTA, FAD, ATP, ADP, AMP, and magnesium ion were all inert as to the stabilization of the enzyme or stimulation of the activity.

**Fig. 6.** Mass spectrum of methylated derivatives of p-bromobenzenethiol, p-nitrobenzylthiol, and cyclohexylthiol. Gas-liquid chromatographic conditions are described in the text, and mass spectrometric conditions were: ionizing energy, 20 eV, and chamber temperature, 200°C.

**Fig. 7.** Effect of pH on activity of C-S lyase. Activity of C-S lyase (diluted Step 7 enzyme) was assayed in different buffers, i.e. acetate (□), K\(^+\) phosphate (○), Tris-HCl (▲), and borate (■).
TABLE II
Substrate specificity of C-S lyase

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Relative activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>(S-(p\text{-Bromophenyl})\text{-L-cysteine})</td>
<td>100</td>
</tr>
<tr>
<td>(N\text{-Acetyl-S-(p\text{-Bromophenyl})\text{-L-cysteine}})</td>
<td>0</td>
</tr>
<tr>
<td>(S\text{'-(p\text{-Bromophenyl})\text{-L-glutathione}})</td>
<td>0</td>
</tr>
<tr>
<td>(S\text{-Phenyl-L-cysteine})</td>
<td>229</td>
</tr>
<tr>
<td>(S\text{-Benzy1-L-cysteine})</td>
<td>24.8</td>
</tr>
<tr>
<td>(S\text{-Benzy1-D-cysteine})</td>
<td>0</td>
</tr>
<tr>
<td>(S\text{-Cylohexyl-L-cysteine})</td>
<td>9.0</td>
</tr>
<tr>
<td>(S\text{-(\text{tert-Butyl})}\text{-L-cysteine})</td>
<td>7.8</td>
</tr>
<tr>
<td>(S\text{-(\text{n-Butyl})}\text{-L-cysteine})</td>
<td>4.5</td>
</tr>
<tr>
<td>(S\text{-Isopropyl-L-cysteine})</td>
<td>7.5</td>
</tr>
<tr>
<td>(S\text{-(\text{n-Propyl})}\text{-L-cysteine})</td>
<td>3.9</td>
</tr>
<tr>
<td>(S\text{-Ethyl-L-cysteine})</td>
<td>3.9</td>
</tr>
<tr>
<td>(S\text{-Methyl-L-cysteine})</td>
<td>3.0</td>
</tr>
<tr>
<td>(L\text{-Cysteine})</td>
<td>7.8</td>
</tr>
<tr>
<td>(D\text{-Cysteine})</td>
<td>0</td>
</tr>
<tr>
<td>(N\text{-Acetyl-L-cysteine})</td>
<td>0</td>
</tr>
<tr>
<td>(S\text{-Carboxymethyl-L-cysteine})</td>
<td>3.3</td>
</tr>
<tr>
<td>Cysteamine</td>
<td>0</td>
</tr>
<tr>
<td>(S\text{-(\text{p-Bromophenyl})}\text{-cysteamine})</td>
<td>0</td>
</tr>
<tr>
<td>(L\text{-Methionine})</td>
<td>10.1</td>
</tr>
<tr>
<td>(L\text{-Homoserine})</td>
<td>0</td>
</tr>
<tr>
<td>(L\text{-Cystine})</td>
<td>0</td>
</tr>
<tr>
<td>(L\text{-Cystathionine}^a)</td>
<td>0</td>
</tr>
<tr>
<td>(L\text{-Cysteic acid})</td>
<td>0</td>
</tr>
<tr>
<td>(L\text{-Lanthionine})</td>
<td>18.5</td>
</tr>
<tr>
<td>(L\text{-Djenkolic acid})</td>
<td>55.8</td>
</tr>
<tr>
<td>(L\text{-Serine})</td>
<td>0</td>
</tr>
<tr>
<td>(L\text{-Alanine})</td>
<td>0</td>
</tr>
<tr>
<td>(L\text{-Phenylalanine})</td>
<td>0</td>
</tr>
<tr>
<td>(p\text{-Bromo-L-phenylalanine})</td>
<td>0</td>
</tr>
<tr>
<td>(O\text{-Benzy1-L-serine})</td>
<td>0</td>
</tr>
<tr>
<td>(S\text{-(\text{p-Bromophenyl})}\text{-3-thiopyruvic acid})</td>
<td>0</td>
</tr>
</tbody>
</table>

*Generation of \(\alpha\)-keto carboxylic acid from \(L\text{-cystathionine}\) was virtually negligible under the incubation conditions described in the text, but the prolonged incubation (i.e. 1 h) yielded a small amount of the acid (relative activity, \(S\text{-\(\text{p-Bromophenyl}\)}\text{-L-cysteine-L-cystathionine}, 100/2)).

Substrate Specificity—The enzyme activity toward sulfur-containing conjugates (i.e., cysteine, glutathione, and \(N\text{-acetyl cysteine conjugates}\)) of \(p\text{-bromobenzene}\) was determined as described under “Experimental Procedures.” For other substrates, formation of pyruvic acid or \(\alpha\)-ketocarboxylic acid was assayed.

Table II shows the relative activity of C-S lyase toward various \(S\)-substituted cysteines and several amino acids. The C-S lyase cleaves the C-S bond of cysteine conjugates but not of \(N\)-acetyl cysteine or glutathione conjugates. The result is compatible with our previous finding that the large intestinal contents (10). \(S\)-Aryl-cysteine conjugates are the best substrates followed by \(S\)-aralkyl- and \(S\)-alkyl-cysteine conjugates. As distinct from cysteine conjugate \(\beta\)-lyase, the C-S lyase purified in the present study catalyzes cleavage of the C-S bond of \(L\)-djenkolic acid, \(L\)-lanthionine, L-cysteine, and \(L\)-cystine.

DISCUSSION

A microorganism populating mammalian gastrointestinal tract, \(F\text{. varium}\), was found to possess an enzyme which mediates cleavage of the thioether linkage in cysteine conjugates of various xenobiotics. The general equation for the action of C-S lyase purified in the present study is represented in Scheme 2.

\[
\text{R-S-CH}_2\text{-CH-CO}_2\text{H} \rightarrow \text{RSH} + \text{CH}_2\text{-CO}_2\text{H} + \text{NH}_3
\]

\text{SCHEME 2}

Neither serine, alanine, nor \(S\)-substituted thiopyruvic acids could be detected as intermediates, although these compounds were stable under the assay conditions. Therefore, it is unlikely that the \(\alpha\)-\(\beta\)-elimination reaction catalyzed by the enzyme proceeds via a two-step mechanism involving the above mentioned amino acids or thiopyruvic acid as intermediates. The reaction probably proceeds with the formation of the pyridoxal phosphate Schiff base of the cysteine conjugate because 1) pyridoxal phosphate is required for the enzyme activity probably as a cofactor, 2) carbonyl reagents such as hydroxylamine or potassium cyanide cause a strong inhibition of the enzyme activity, and 3) a free amino group on a cysteinyl moiety is obligatory for substrate activity (Table II).

The reaction (Scheme 2) is quite similar to that catalyzed by bacterial \(L\)-methionine \(\gamma\)-lyase (EC 4.4.1.11) (16) and by cystathionase (EC 4.4.1.1) (17). Both enzymes cleave a C-S linkage of several derivatives of \(L\)-homocysteine and \(L\)-cysteine by \(\alpha\)-\(\gamma\)- and \(\alpha\)-\(\beta\)-elimination reactions, respectively, in the presence of pyridoxal phosphate, whereby the corresponding thiol-product, \(\alpha\)-ketocarboxylic acid and ammonia are stoichiometrically produced. Several lines of evidence, however, clearly indicate that the present C-S lyase is distinct from these two enzymes: 1) thiol reagents are without any inhibitory effect on the activity of the present enzyme, whereas methionine \(\gamma\)-lyase contains essential sulfhydryl groups in the catalytic site (16), and 2) the activity of the present enzyme is completely abolished by heat treatment (60 °C for 2 min), while a salient feature of the other two enzymes is their higher thermostability, 3) the substrate specificity of the C-S lyase was different from that of methionine \(\gamma\)-lyase and cystathionase. Thus, methionine \(\gamma\)-lyase attacks \(S\)-aryl and \(S\)-alkyl homocysteine by an \(\alpha\)-\(\gamma\)-elimination reaction while C-S lyase catalyzes only \(\alpha\)-\(\beta\)-elimination reactions. Cystathionase acts on \(L\)-homoserine and \(L\)-cystathionine; both showed little or no activity with the present enzyme.

In our previous reports (6, 7), we postulated that cysteine-conjugate \(\beta\)-lyase (mostly distributed in liver and kidney in several mammalian species) might play an important role in the formation of methylthio-containing metabolites of aromatic compounds (Scheme I). However, numerous alkyl or aralkyl cysteine conjugates which were not active with \(\beta\)-lyase, were found to be biotransformed \textit{in vivo} to the corresponding methylthio metabolites (18–20). These findings suggested the participation of other mechanisms in the formation of these methylthio-containing metabolites. On the basis of the substrate specificity of C-S lyase purified in the present study, it becomes evident that methylthio metabolites of alkyl and aralkyl compounds might be also formed from the corresponding cysteine conjugates via thiol intermediates (see Scheme I). Thus, at least, a part of cysteine conjugates excreted into or formed in the intestinal tract may be converted to thiol compounds by the action of the bacterial C-S lyase. Virtually no S-methylation activity was found in the intestinal contents (21); the thiol compounds thus formed are subsequently reabsorbed from the gastrointestinal tract and methylated with \(S\)-methyl transferase in the intestinal mucosa or liver, or in both. The present results are compatible with those observed \textit{in vivo} by Bakke et al. (22) who described the contribution of intestinal microorganisms to the formation of methylthio metabolites.
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These findings suggest that most, if not all, of the methylthio metabolites reported so far are formed from the corresponding conjugates.

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