SYNTHESIS OF S-ALKYL-L-CYSTEINE FROM PYRUVATE, AMMONIA AND ALKYL-MERCAPTAN BY CYSTEINE DESULFHYDRASE OF *AEROBACTER AEROGENES*

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Summary

Synthesis of S-methyl-L-cysteine from pyruvate, ammonia and methylmercaptan is catalyzed by highly purified cysteine desulfhydrase from *Aerobacter aerogenes*. The synthetic reaction proceeds optimally at pH 10, as a function of enzyme concentration and incubation time. Methylmercaptan is replaced by ethylmercaptan and propylmercaptan to synthesize S-ethyl-L-cysteine and S-propyl-L-cysteine, respectively. The enzymatically synthesized S-methyl-L-cysteine and S-ethyl-L-cysteine were purified and identified by physicochemical meanings.

In recent studies about bacterial enzymes such as tyrosine phenol-lyase* and tryptophanase**, it was found that the α,β-elimination (Equation 1 for tyrosine phenol-lyase, Equation 2 for tryptophanase) reaction catalyzed by these enzymes is readily reversible at high concentrations of pyruvate and ammonia (1, 2, 3). The enzymatic preparation of L-tyrosine, L-tryptophan and their derivatives by the reversal reaction has been reported in previous papers (4, 5, 6).

$$\text{L-Tyrosine} + H_2O \longrightarrow \text{phenol} + \text{pyruvic acid} + NH_3 \quad (1)$$

$$\text{L-Tryptophan} + H_2O \longrightarrow \text{indole} + \text{pyruvic acid} + NH_3 \quad (2)$$

$$\text{L-Cysteine} + H_2O \longrightarrow \text{hydrogen sulfide} + \text{pyruvic acid} + NH_3 \quad (3)$$

Cysteine desulfhydrase*** is an enzyme which catalyzes degradation of

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* L-Tyrosine phenol-lyase (deaminating) E. C. 4. I. 99.2 formerly known as β-tyrosinase

** L-Tryptophan indole-lyase (deaminating) E. C. 4. I. 99.1

*** L-Cysteine hydrogen sulfide-lyase (deaminating) E. C. 44. 1. 1

L-cysteine into pyruvate, ammonia and hydrogen sulfide, through the similar α,β-elimination reaction as tyrosine phenol-lyase and tryptophanase (Equation 3). Some properties of the inducible cysteine desulfhydrase of *Salmonella typhimurium* and *Escherichia coli* have been described by Guarneros and Ortega (7). The effects of cryolysis on the enzyme from *S. typhimurium* have been examined by Collins and Monty (8), using preparations purified several-fold. They reported about the kinetics and catalytic properties of the enzyme (9). Recently, Kredich *et al.* have purified the enzyme from *S. typhimurium* to a state of near homogeneity and reported its physical and chemical properties (10, 11). The reversibility of the cysteine degradation by the enzyme has not been observed yet with any enzyme preparations from various sources (12). In recent studies, we proved that S-methyl-, S-ethyl- and S-propyl-L-cysteines were synthesized from pyruvate, ammonia and methyl-, ethyl- and propyl-mercaptans, respectively, with a highly purified cysteine desulfhydrase from *Aerobacter aerogenes*. The formation and identification of these S-alkyl-cysteine derivatives will be described in this paper.

Materials and Methods

Cysteine desulfhydrase activity has been found in growing cells of various bacteria belonging to genera of *Escherichia*, *Aerobacter*, *Serratia*, *Proteus*, *Alcaligenes*, *Agrobacterium*, *Micrococcus* and *Sarcina*. The cells of *Aerobacter aerogenes* isolated from soil were selected as a likely source of enzyme for the present investigation. The cells with higher enzyme activity were prepared by growing them in a medium containing 0.2% L-cysteine HCl H_2O, 0.1% glycerol, 0.5% meat extract, 0.5% polypeptone, 0.3% yeast extract, 0.2% NaCl and 0.2% $CaCl_2$ in tap water. The pH of the medium was adjusted to 7.5 by the addition of 4N NaOH. Fifty milliliters of the culture cultivated for 6 hr in 500 ml shaking flasks at 28°C was inoculated to the main culture. The cultivation was carried out at 28°C for 10 hr in 2 liter shaking flasks containing 500 ml of the medium under shaking. Harvested cells were suspended

in 0.05 M potassium phosphate buffer, pH 7.0, containing 10^{-5} M pyridoxal phosphate, 10^{-3} M β-mercaptoethanol, 10^{-4} M EDTA and 20% of glycerol, and disrupted with a DYNO-MILL KDL (W. A. Bachofen, Switzerland). Crude extract was fractionated by 0.6 ammonium sulfate saturation and subjected to DEAE-Sephadex column chromatography, followed by Sephadex G-200 gel filtration. The purified enzyme gave a single band on acrylamide gel electrophoresis carried out in Tris-glycine buffer, pH 8.3 (13), containing 10^{-4} M pyridoxal phosphate. The report about the formation and purification of the enzyme is under preparation.

S-Alkyl-L-cysteines were determined by an automatic amino acid analyzer, Yanagimoto LC-5S. Samples were placed on a 70 x 0.9 cm column of Aminex A-4 resin and eluted with 0.2 M sodium citrate buffer, pH 3.25. NMR spectra were taken in deuterium oxide with internal sodium 3-(trimethylsilyl)-propanesulfonate by Varian A-60 spectrometer.

Results and Discussion

The synthesis of S-methyl-L-cysteine proceeded as a function of enzyme concentration and incubation time (Fig. 1A and 1B), when pyruvate, ammonia and methylmercaptan were incubated with cysteine desulfhydrase in the presence of added pyridoxal phosphate. With boiled enzyme, the synthesis of S-methyl-L-cysteine was not observed. Cysteine desulfhydrase, in the presence of ammonium chloride buffer, has an optimal reactivity around at pH 10.0 for the synthesis of S-methyl-L-cysteine (Fig. 2).

When methylmercaptan was replaced by ethyl- or propyl-mercaptan in the reaction mixture, S-ethyl- or S-propyl-L-cysteine was synthesized at the relative rate of 131 or 43, respectively, in comparison with S-methyl-L-cysteine, 100.

The enzymatically synthesized S-methyl-L-cysteine was isolated from a large scale incubation mixture. The incubation was carried out at 30°C for 2 hr in a reaction mixture containing sodium pyruvate (100 mmoles), ammonium

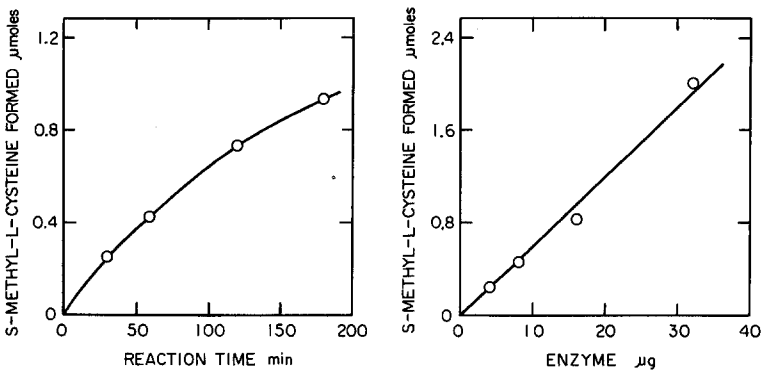


Fig. 1. Synthesis of S-methyl-L-cysteine as a function of enzyme concentration and incubation time. The reactions were carried out at 30°C in reaction mixtures containing 200 µmoles of sodium pyruvate, 300 µmoles of ammonium sulfate, 500 µmoles of methylmercaptan, 0.2 µmole of pyridoxal phosphate, 2.5 µmoles of EDTA, 100 µmoles of NH_4OH-NH_4Cl buffer, pH 10, and the enzyme in a total volume of 2 ml. In Fig. 1A, 7.5 µg of enzyme protein was used and in Fig. 1B incubation was carried out for 1 hr. The determination of protein was performed spectrophotometrically by measuring the absorbance at 280 nm. An E value of 15.0 for 10 mg per ml and for 1-cm light path was used throughout.

sulfate (150 mmoles), methylmercaptan (150 mmoles), EDTA (0.25 mmoles), pyridoxal phosphate (20 µmoles), the enzyme (2.8 mg) and NH_4Cl-NH_4OH buffer, pH 10.0 (20 mmoles), in a total volume of 200 ml. The reaction was stopped by the addition of 50 ml of 30% trichloroacetic acid and the precipitated protein was removed by centrifugation. The mixture was applied to a Dowex 50-X8 column (40 cm x 1.9 cm, H^+ form). The column was first washed with 2.5 liter of distilled water and then one liter of 0.01 M NH_4OH solution. The elution of the product was performed with 0.3 M NH_4OH solution. The elution was followed by measurement of ninhydrin reaction after development of thin-layer chromatography (Merck PF 254) with *n*-butanol-acetate-water (4 : 2 : 1). The fractions containing S-methyl-L-cysteine were collected and concentrated at 40°C *in vacuo*. The concentrated solution was treated with active charcoal after the pH was adjusted to 6.0, and evaporated to dryness *in vacuo*. The crude product was dissolved with a small amount of distilled water and crystallized by the addition of ethanol. Recrystallization was carried out from ethyl alcohol-water. Colorless crystals of the product weighing 127 mg were obtained.

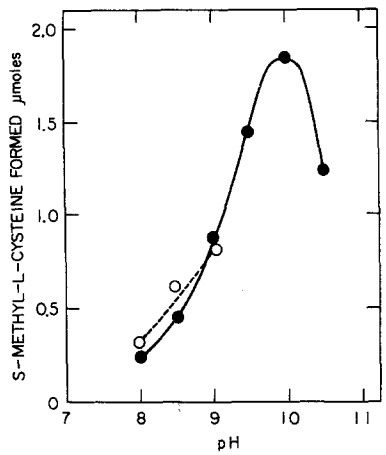


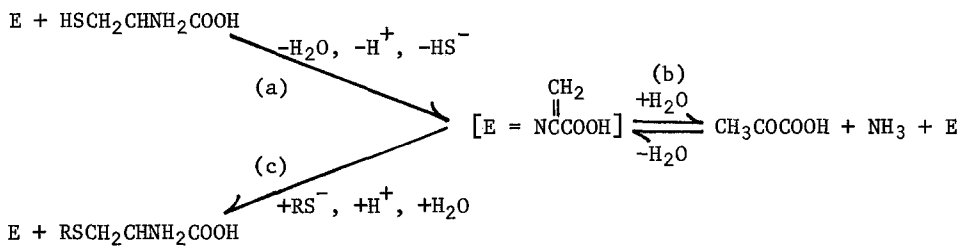
Fig. 2. Effect of pH on synthesis of S-methyl-L-cysteine by cysteine desulfhydrase. The reaction was carried out for 60 min with 10 μg of the enzyme, under the same conditions described in Fig. 1, except that the buffer was replaced as follows: Tris-HCl pH 8.0 to 9.0 (o---o), NH_4OH-NH_4Cl, pH 8.0 to 10.5 (•---•). Potassium chloride, 100 μmoles, was added when Tris-HCl was the buffer.

The enzymatically synthesized product was designated as S-methyl-L-cysteine based on the following physicochemical examination. The NMR spectrum of the product in $[^2H]H_2O$ revealed an singlet of A_3 protons at δ2.2 (PPM from the internal reference), an octet of AB protons of the ABX type at δ2.7 - 3.4 and a quartet of X proton (J_{AB} = 15.1, J_{AX} = 5.1 and J_{BX} = 7.0 cycles/sec), at δ3.8 - 4.1, suggesting the presence of nonequivalent protons. The presence of a molecular ion peak at m/e 135 was definitely observed in the mass spectrum of the product. The formation of S-methyl-L-cysteine was further confirmed by the elemental analyses and by the measurement of melting point (Table I). The $[\alpha]_D^{25}$ was measured to be -29.7°C with 1% solution of this product in H_2O. This result shows the enzymatic synthesized S-methyl-cysteine is the L-isomer.

The enzymatic synthesis of S-ethyl-L-cysteine was carried out in the same manner described above except the replacement of methylmercaptan by ethylmercaptan. The product was purified in the same way as S-methyl-L-cysteine and 195 mg of crystals were obtained. The NMR spectrum of the product in $[^2H]H_2O$ revealed a triplet of methyl group in S-ethyl group at

Table I. Summary of the physicochemical properties of the enzymatic synthesized S-methyl-L-cysteine and S-ethyl-L-cysteine.

	S-Methyl-L-cysteine			S-Ethyl-L-cysteine		
Molecular formula	CH_3-S-CH_2CHNH_2COOH			C_2H_5-S-CH_2CHNH_2COOH		
Molecular weight	135.19			149.21		
Elemental analysis	C%	H%	N%	C%	H%	N%
calcd:	35.54	6.71	10.36	40.21	7.37	9.38
found:	35.52	6.93	10.25	40.56	7.61	9.29
Molecular ion peak in the mass spectrum	135			149		
Melting point	254 - 247°C			258 - 260°C		



(E: Enzyme, R: $-CH_3$, $-C_2H_5$, $-C_3H_7$)

Scheme I

δ1.2 - 1.5 and a quartet of methine at δ2.5 - 2.9 (J_{AX} = 7.0 cycles/sec), an octet of AB protons of the ABX type at δ2.8 - 3.4 and a quartet of X proton at δ3.9 - 4.1 (J_{AB} = 14.5, J_{AX} = 5.0 and J_{BX} = 7.8 cycles/sec). A molecular ion peak at m/e 149 was observed in the mass spectrum. The results of the elemental analyses and the measurement of melting point are shown in Table I.

The mechanism of the degradation of L-cysteine by cysteine desulfhydrase (Equation 3) is explainable by adopting the general mechanism for pyridoxal phosphate-dependent reactions proposed by Braunstein and Shemyakin (14) and by Metzler (15) *et al.* In catalysis of pyruvate formation (Equation 3), L-cysteine interacts with the enzyme to form enzyme-bound α-amino-acrylate by elimination H^+ and HS^- [Scheme I, step (a)], which is hydrolyzed to form pyruvate and ammonia. The results described in this report suggest that the step (b), the degradation of the enzyme α-aminoacrylate complex into pyruvate, ammonia and the enzyme, is reversible and that the synthesis of S-alkyl-cysteine takes place *via* the reversal sequence of the α,β-elimination reaction [step (b), (c)].

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