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Synthesis of *S*-(Carboxymethyl)-D-cysteine by 3-Chloro-D-alanine Chloride-Lyase of *Pseudomonas putida* CR 1-1

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Abstract

S-(Carboxymethyl)-D-cysteine, which is an important component of semisynthetic cephalosporin, MT-141, was enzymatically synthesized. *S*-(Ethoxy-carbonyl-methyl)-D-cystein was synthesized from 3-chloro- D-alanine and ethyl thioglycolate by the β-replacement reaction of 3-chloro-D-alanine chloride-lyase from *Pseudomonas putida* CR 1-1 and subsequently hydrolyzed by alkali. The synthesized *S*-(carboxymethyl)-D-cysteine was isolated from a large scale reaction mixture and identified physicochemically. The reaction conditions for the synthesis of *S*-(ethoxycarbonylmethyl)-D-cysteine were optimized using resting cells of *P. putida* CR 1-1.

Index Entries: Synthesis, of *S*-(carboxymethyl)-D-cysteine, synthesis of; 3-chloro-D-alanine chlorie lyase; *Pseudaanonas putida* Cr 1-1, in synthesis of *S*-(carboxymethyl)-D-cysteine.

Introduction

3-Chloro-D-alanine chloride-lyase has been found in *Pseudomonas putida* CR 1-1, which is resistant to 3-chloro-D-alanine (*1*). The enzyme catalyzes the α,β-elimination reaction of 3-chloro-D-alanine to form pyruvate, ammonia, and chloride ion [Eq. (1)]. The enzyme also catalyzes the β-replacement reaction of

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3-chloro-D-alanine in the presence of a high concentration of sodium hydrosulfide to form D-cysteine [Eq. (2)] (*2*).

$$\text{D-ClCH}_2\text{CH(NH}_2\text{)COOH} + \text{H}_2\text{O} \rightarrow \text{CH}_3\text{COCOOH} + \text{HCl} + \text{NH}_3 \quad (1)$$

$$\text{D-ClCH}_2\text{CH(NH}_2\text{)COOH} + \text{NaHS} \rightarrow \text{D-HSCH}_2\text{CH(NH}_2\text{)COOH} + \text{NaCl} \quad (2)$$

We have already reported the production of D-cysteine by using intact cells of *P. putida* CR 1-1 (*3,4*). In the course of further study on the substrate specificity of the β-replacement reaction, we found that various *S*-substituted D-cysteine derivatives could be synthesized by this enzyme (*5*).

In this paper, we have attempted to synthesize *S*-(carboxymethyl)-D-cysteine, an important component of a semisynthetic cephalosporin, MT-141 (*6*).

Materials and Methods

Materials

3-Chloro-D-alanine was synthesized from D-serine according to the method of Walsh et al. (*7*). Lactate dehydrogenase (EC 1.1.1.27) from pig heart was obtained from Oriental Yeast (Japan). D-Amino acid oxidase (EC 1.4.3.3) from pig kidney and catalase (EC 1.11.1.6) from bovine liver were purchased from Boehringer. All other chemicals were commercially available and of analytical grade.

Enzyme Preparation and Enzyme Assay

3-Chloro-D-alanine chloride-lyase was prepared from the cells of *P. putida* CR 1-1 grown on the 3-chloro-D-alanine-supplemented medium, as previously described (*1*). The enzymatic α,β-elimination reaction was assayed by measuring the amount of pyruvate liberated from 3-chloro-D-alanine using a spectrophotomeric method with pig heart lactate dehydrogenase and NADH; one unit of the activity was defined as the amount of enzyme catalyzing the formation of 1 μmol pyruvate/min under the standard conditions (*1*). In some cases, the amount of pyruvate formed was determined according to the method of Friedemann and Haugen (*8*). Cysteine was determined by Gaitonde's acid ninhydrin method (*9*).

Preparation of Resting Cells

Cultivation of *P. putida* CR 1-1 and the preparation of resting cells were carried out as described in the previous papers (*3,4*).

Determination of S-(Carboxymethyl)-cysteine and S-(Ethoxycarbonyl-methyl)-cysteine

S-(Carboxymethyl)-cysteine was estimated by cochromatography with identified *S*-(carboxymethyl)-D-cysteine on a standard amino acid analyzer (Kyowa Seimitsu model K-101) (*10*). *S*-(Ethoxycarbonylmethyl)-cysteine was hydrolyzed completely to *S*-(carboxymethyl)-cysteine by incubating it in 4*M* HCl at 70°C for 2 h

and determined by using identified S-(carboxymethyl)-D-cysteine on an amino acid analyzer.

Analytical Measurement

The ^{1}H-NMR spectrum was recorded with a Hitachi Perkin Elmer (60 MHz) spectrometer in deuterium oxide with sodium 2,2-dimethyl-D-silapentane-5-sulfonate as an internal standard. The mass spectrum was recorded in a Hitachi M-80. The infrared spectrum was recorded on a Shimazu IR 27G. Optical activity was measured with a Hitachi Perkin Elmer 241 polarimeter. Elemental analysis was carried out on a Yanagimoto C.H.N. recorder MT-1. Sulfur was determined by the method of Wagner (*11*).

Results and Discussion

Enzymatic Synthesis of S-(Carboxymethyl)- D-cysteine

Various S-alkyl-D-cysteine could be synthesized from 3-chloro-D-alanine and alkyl mercaptans (*5*). However, some thiol compounds having free carboxyl group such as thioglycolic acid, 2-thiomalic acid, thiosalycylic acid, D-cysteine, L-cysteine, *N*-acetyl-L-cys teine, and DL-homocysteine could not act as a substrate in the β-replacement reaction of 3-chloro-D-alani ne chloride-lyase from *P. putida* CR 1-1. Namely, it was impossible to synthesize directly S-(carboxymethyl)-D-cysteine from 3-chloro-D-alanine and thioglycolic acid by the enzyme. On the contrary, we found that the β-replacement reaction proceeded efficiently when the carboxyl group of thioglycolic acid was esterified with ethanol or methanol. Therefore, we have devised a synthetic method for S-carboxymethyl-D-cysteine, as shown in Scheme I.

$$ClCH_2CH(NH_2)COOH + HSCH_2COOCH_2CH_3$$

$$\xrightarrow{\text{3-Chloro-D-alanine chloride-lyase}} CH_3CH_2OOCCH_2SCH_2CH(NH_2)COOH$$

$$\xrightarrow{\text{Hydrolysis by acid or alkali}} HOOCCH_2SCH_2CH(NH_2)COOH + CH_3CH_2OH$$

Scheme I

Synthesis of S-(carboxymethyl)-D-cysteine was carried out at 30°C in 100 mL of reaction mixture containing 10 mmol of 3-chloro-D-alanine, 50 mmol of ethyl thioglycolate, 10 mmol of potassium phosphate buffer (pH 7.5), and 100 units of 3-chloro-D-alanine chloride-lyase. After incubation for 2 h, the formation of S-(ethoxycarbonylmethyl)-cysteine was found with an amino acid analyzer and on thin-layer chromatography. The reaction was stopped by adding 20 mL of 30% trichloroacetic acid, and the denatured protein was removed by centrifugation at 10,000*g* for 10 min. It was difficult to isolate S-(ethoxycarbonylmethyl)-cysteine from the reaction mixture because it was partially hydrolyzed to S-(carboxymethyl)-cysteine in accordance with the progress of the synthetic reaction; to hydrolyze it completely to S-(carboxymethyl)-cysteine, the reaction mixture was

incubated with 0.4*M* ammonia solution at room temperature for 24 h. The reaction mixture was diluted 10-fold with distilled water and subjected to Dowex 50 × 8 (4 × 30 cm, H^+ form). After washing the column with distilled water, *S*-(carboxymethyl)-cysteine was subsequently eluted with 0.4*M* ammonia solution. The eluate was spotted on a silica gel plate and *S*-(carboxymethyl)-cysteine was detected by spraying ninhydrin solution. The eluates containing *S*-(carboxymethyl)-cysteine were pooled and then subjected to Dowex 1 × 2 (4 × 30 cm, OH^- form). After washing the column with distilled water, *S*-(carboxymethyl)-cysteine was eluated with 0.7*M* acetic acid. The eluate containing *S*-(carboxymethyl)-cysteine was combined and concentrated to dryness *in vacuo*. The dried compound was dissolved in small amount of water and cooled on ice. Crystallization was induced by adding ethanol. After the solution was left to stand overnight, precipitated crystals were collected by filtration. Recrystallization was carried out in a similar manner. *S*-(Carboxymethyl)-cysteine (982 mg) was obtained as white crystals; the yield was 54.9% of the added 3-chloro-D-alanine.

The physicochemical properties of the product are summarized in Table 1. The agreement between the calculated and found value was within the usual limit of a variation of elemental analysis. The presence of a molecular ion peak was barely observed in the mass spectrum. A main peak, which was ascribed to intramolecular dehydration during measurement, was observed. The infrared absorption spectrum agreed very closely with the authentic *S*-(carboxymethyl)-D-cysteine (Fig. 1). The $[\alpha]_D^{25}$ was measured and found to be −1.0° with 3.65% solution of this product in 1*M* HCl. To confirm the configuration of the product, the synthesized *S*-(carboxymethyl)-D-cysteine (1 μmol) was incubated in 2.0 mL of the reaction mixture containing 7.5 units of D-amino acid oxidase, 0.3 μmol of flavin adenine dinucleotide, 400 units of catalase, and 0.15 mmol of potassium phosphate buffer (pH 8.0) at 30°C for 1 h with shaking. This treatment with D-amino acid oxidase

TABLE 1
Summary of the Physicochemical Properties of *S*-(Carboxymethyl-D-cysteine)[a]

Molecular weight	179.19				
Molecular ion peak in the mass spectrum	161($-H_2O$)				
		C%	H%	N%	S%
Elemental analysis	calcd:	33.51	5.06	7.82	17.90
	found:	33.18	5.17	7.80	17.94
Melting point (decomposed)	196–198°C				
$[\alpha]_D^{25°}$	−1.0° (c = 3.65)				
^{1}H-NMR spectra (ppm)	3.87^{α} 3.05^{β} 3.03^{β} 3.27 singlet				

[a]Values marked $^{\alpha}$ and $^{\beta}$ correspond to α-methine proton (a double doublet of *X*) and β-methylene proton (two doublets of the *ABX* type), respectively, of the cysteine moiety.

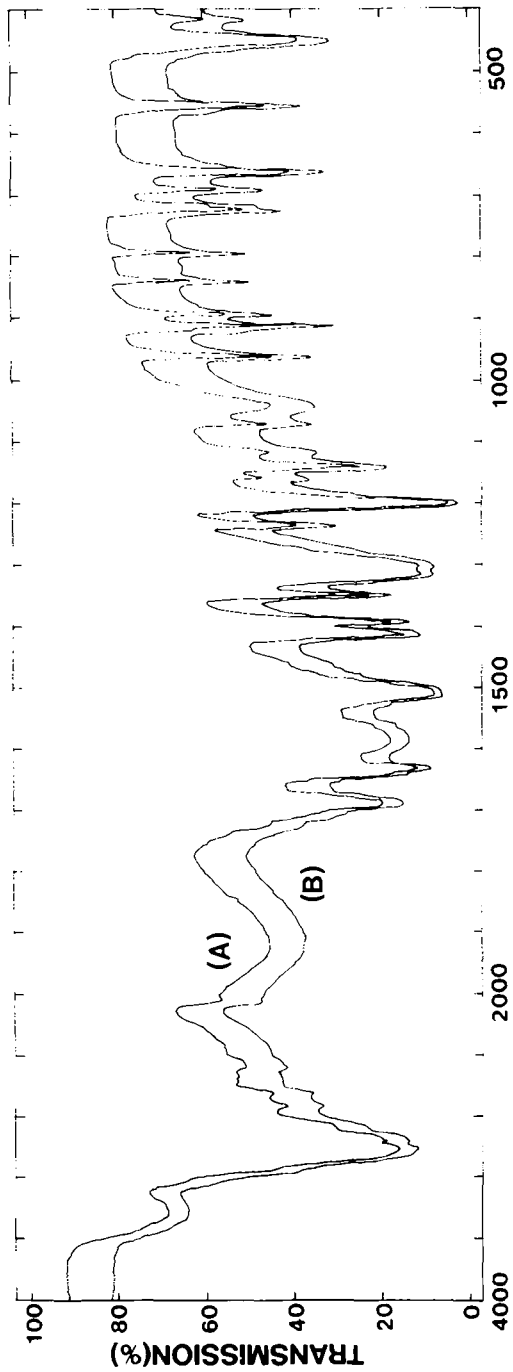

Fig. 1. Infrared absorption spectra of the isolated *S*-(carboxymethyl)-D-cysteine (A) and the authentic *S*-(carboxymethyl)-D-cysteine (B). All spectra were measured by the KBr pellet method.

resulted in the disappearance of the peak corresponding to synthesized *S*-(carboxymethyl)-cysteine on an amino acid analyzer. These results show that the enzymatically synthesized *S*-substituted cysteine derivative is D-isomer.

The synthetic rate of *S*-(ethoxycarbonylmethyl)-D-cysteine from 3-chloro-D-alanine and ethyl thioglycolate was examined. The reaction was carried out at 30°C for 10 min in a reaction mixture (1.0 mL) containing 100 μmol of 3-chloro-D-alanine, 500 μmol of ethyl thioglycolate, 100 μmol of potassium phosphate buffer (pH 7.5), 0.1 μmol of pyridoxal 5′-phosphate, and 0.5 units of 3-chloro-D-alanine chloride-lyase. The reaction was stopped by adding 0.2 mL of 30% trichloroacetic acid and the denatured protein was removed by brief centrifugation. The *S*-(ethoxycarbonylmethyl)-D-cysteine formed was calculated to be 2313 μmol/min/mg protein using an amino acid analyzer. In the previous study (*1*), the maximum velocity, V_{max}, of the synthesis of D-cysteine catalyzed by the highly purified 3-chloro-D-alanine chloride-lyase was calculated to 1620 μmol/min/mg protein. Therefore, ethyl thioglycolate functioned as a better substrate than sodium hydrosulfide in the β-replacement reaction.

The α,β-elimination of *S*-(carboxymethyl)- D-cysteine by 3-chloro-D-alanine chloride-lyase was examined. The synthesized *S*-(carboxymethyl)-D-cysteine (100 μmol) was incubated with 5.6 units of 3-chloro-D-alanine chloride-lyase, 0.1 μmol of pyridoxal 5′-phosphate, 100 μmol of potassium phosphate buffer (pH 8.0), 2.6 μmol of NADH, and 5 units of lactate dehydrogenase in a total volume of 2 mL at 30°C. The absorbance at 340 nm was followed, but the formation of pyruvate was not detected. We concluded that 3-chloro-D-alanine chloride-lyase cannot catalyze the α,β-elimination reaction of *S*-(carboxymethyl)-D-cysteine.

Reaction Conditions for the Synthesis of S-(Carboxymethyl)-D-cysteine by Resting Cells

Reaction conditions to synthesize *S*-(ethoxycarbonylmethyl)-D-cysteine efficiently were studied by using the resting cells of *P. putida* CR 1-1. The effect of pH on the synthesis of *S*-(ethoxycarbonylmethyl)-D- cysteine by resting cells was examined using potassium phosphate buffer and HEPES (*N*-2-hydroxyethyl piperazine-*N*-2-ethansulfonic acid) buffer (Fig. 2). The optimal pH was found to be 8.0.

Figure 3 shows the effect of the concentration of ethyl thioglycolate on the synthesis of *S*-(carboxymethyl)-D- cysteine; each reaction was carried out in the presence of 100 mM 3-chloro-D-alanine. When 100 m*M* ethyl thioglycolate was added to the reaction mixture, only 60% of the added 3-chloro-D-alanine wasd converted to *S*-(ethoxycarbonylmethyl)-cysteine and about 11% of the added 3-chloro-D-alanine was degraded to pyruvate by the α,β-elimination reaction after a 2-h incubation. At higher concentrations of ethyl thioglycolate (<300 m*M*), about 85% of the added 3-chloro-D-alanine could be converted to *S*-(ethoxycarbonylmethyl)-D -cysteine after a 1.5- to 2-h incubation and, under such conditions, the α,β-elimination reaction was almost completely depressed.

The effect of the concentration of 3-chloro-D- alanine on the synthesis of *S*-(ethoxycarbonylmethyl)-D-cysteine was examined in the presence of 500 m*M*

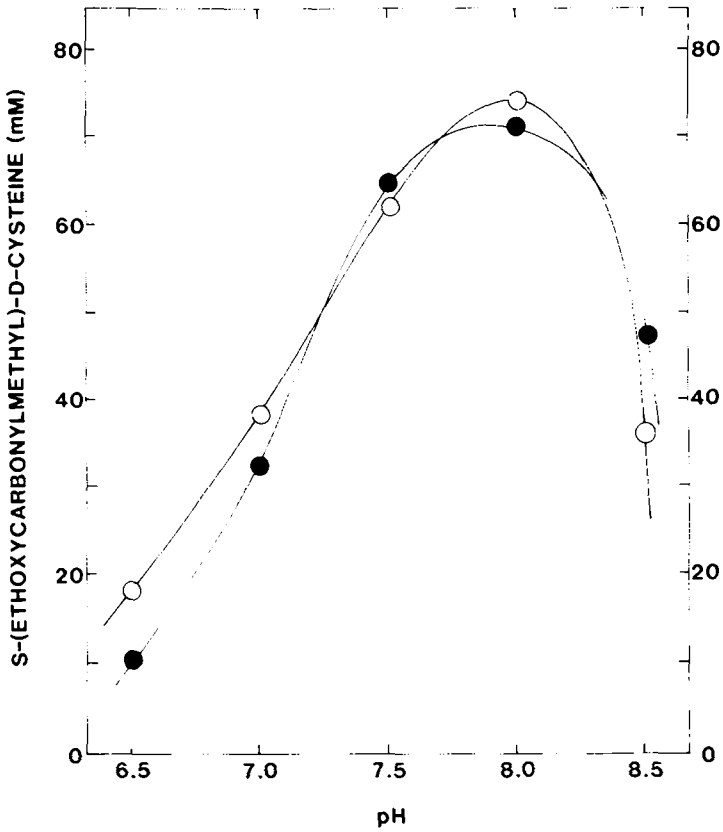

Fig. 2. Effect of pH on the synthesis of *S*-(ethoxycarbonylmethyl)-D-cysteine by resting cells. The reaction mixture contained 100 μmol of 3-chloro-D-alanine, 500 μmol of ethyl thioglycolate, 0.1 μmol of pyridoxal 5′-phosphate, resting cells from 1 mL of culture broth and 100 μmol each of the following buffer in a total volume of 1 mL: HEPES buffer (●), pH 6.5, 7.0, 7.5, 8.0, and 8.5; potassium phosphate buffer (○), pH 6.5, 7.0, 7.5, 8.0, and 8.5. The reaction was carried out at 30°C for 2 h with shaking.

ethyl thioglycolate (Fig. 4). When 200 m*M* 3-chloro-D-alanine was added, about 50% of it was converted to *S*-(ethoxycarbonylmethyl)-D-cysteine. With the increase in the added 3-chloro-D-alanine, the yield of *S*-(carbonylmethyl)-D-cysteine decreased.

Based on the above results, we found that the synthetic reaction can proceed efficiently in the reaction mixture containing 100 m*M* 3-chloro-D-alanine, 300 m*M* ethyl thioglycolate, 100 m*M* potassium phosphate buffer or HEPES buffer (pH 8.0), and the resting cells from the same volume of culture broth as the reaction mixture.

The use of D-amino acid has become increasingly important in medicine. In particular, D-amino acids in cephalosporins play an important role in their efficacy. Recently, it has been reported that *S*-(carboxymethyl)-D-cysteine is an important

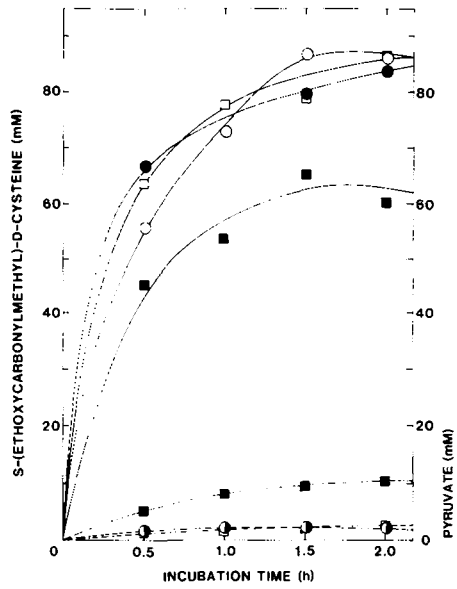

Fig. 3. Effect of the concentration of ethyl thioglycolate on the synthesis of *S*-(ethoxycarbonylmethyl)-D -cysteine. The reaction was carried out at pH 8.0 and at 30°C using 1 mL of the reaction mixture containing 100 μmol of 3-chloro-D-alanine, 0.1 μmol of pyridoxal 5′-phosphate, 100 μmol of potassium phosphate buffer (pH 8.0), resting cells from 1 mL of the culture broth and the following amounts of ethyl thioglycolate: ■ 100 m*M*, □ 300 m*M*, ● 500 m*M*, ○ 1000 m*M*.

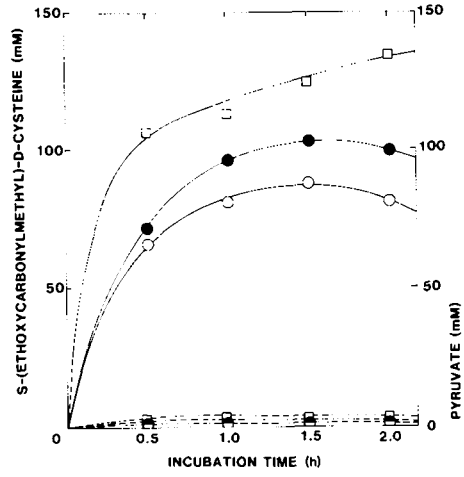

Fig. 4. Effect of the concentration of 3-chloro-D-alanine on the synthesis of *S*-(ethoxycarbonylmethyl)-D-cysteine. The reaction was carried out at pH 8.0 and at 30°C using 1 mL of the reaction mixture containing 500 μmol of ethyl thioglycolate, 0.1 μmol of pyridoxal 5′-phosphate, 100 μmol of potassium phosphate buffer (pH 8.0), and the following amounts of 3-chloro-D-alanine: ○ 100 m*M*, ● 200 m*M*, □ 300 m*M*.

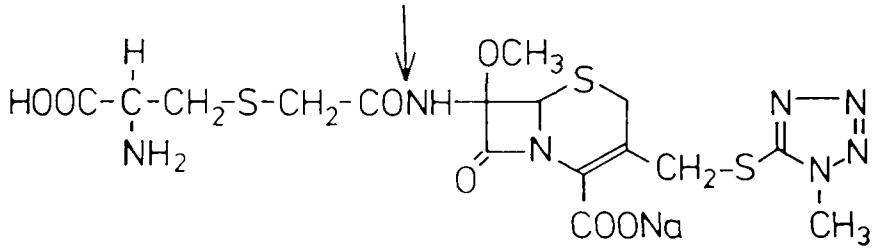

Fig. 5. Structure of MT-141. 7β-(2-D-Amino-2-carboxyethylthioacetamido)-7α-methoxy-3-(1-methyl-1H-tetrazol-5-yl)thiomethyl-3-cephem-4-carboxylic acid.

constituent of a new semisynthetic β-lactam antibiotic, MT-141 (Fig. 5), which as a marked antibacterial activity *in vivo* and causes a potent lysis of bacterial cells (6). In the present study, a previously unknown *S*-substituted D-cysteine derivative, *S*-(carboxymethyl)-D-cysteine, is synthesized by taking advantage of the β-replacement reaction of the multifunctional enzyme.

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