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S-Carboxymethylcysteine Synthase from *Escherichia coli*

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An enzyme that catalyzes the synthesis of *S*-carboxymethyl-L-cysteine from 3-chloro-L-alanine (3-Cl-Ala) and thioglycolic acid was found in *Escherichia coli* W3110 and was designated as *S*-carboxymethyl-L-cysteine synthase. It was purified from the cell-free extract to electrophoretic homogeneity and was crystallized. The enzyme has a molecular weight of 84,000 and gave one band corresponding to a molecular weight of 37,000 on SDS-polyacrylamide gel electrophoresis. The purified enzyme catalyzed the β-replacement reactions between 3-Cl-Ala and various thiol compounds. The apparent *Km* values for 3-Cl-Ala and thioglycolic acid were 40 mM and 15.4 mM. The enzyme showed very low activity as to the α,β-elimination reaction with 3-Cl-Ala and L-serine. It was not inactivated on the incubation with 3-Cl-Ala. The absorption spectrum of the enzyme shows a maximum at 412 nm, indicating that it contains pyridoxal phosphate as a cofactor. The N-terminal amino acid sequence was determined and the corresponding sequence was detected in the protein sequence data bank, but no homogeneous sequence was found.

3-Chloro-L-alanine (3-Cl-Ala) acts as a so-called suicide substrate for some pyridoxal-phosphate (PLP) dependent enzymes, such as glutamate-oxaloacetic acid transminase,[1] glutamate-pyruvic acid transaminase[2] and amino acid racemase.[3] On the other hand, some other PLP enzymes that catalyze the α,β-elimination and β-replacement reactions were found to be insensitive to 3-Cl-Ala and to utilize it as a preferable substrate.[4～7] Furthermore, the development of chemical synthesis techniques has made it possible to produce 3-Cl-Ala economically on a large scale.[8] These observations prompted us to investigate and to develop an enzymatic method for the synthesis of useful amino acids from 3-Cl-Ala.

This paper reports the purification and properties of the enzyme responsible for the synthesis of S-carboxymethyl-L-cysteine (SC-Cys) from 3-Cl-Ala and thioglycolic acid (TGA). SC-Cys exhibits mucolytic activity and is useful for loosening mucus in the trachea.[9]

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Materials and Methods

Reagents. Casamino acids and Tryptone were obtained from Difco Laboratories, yeast extract from Oriental Yeast Co., amido black 10B from E. Merck AG and phenylmethylsulfonyl fluoride from Calbiochem-Behring. Sephadex G-150 and G-100, and blue dextran 2000 and molecular weight markers were from Pharmacia. DEAE-Cellulofine AH and Cellulofine GC-700-m were from Chisso Co. SC-Cys was from Tokyo Kasei Co., 3-Cl-Ala was a generous gift from Showa Denko Co., and PLP was kindly provided by Dainippon Pharmaceutical Co. Other chemicals were purchased from commercial sources.

Bacterial strain and culture. Escherichia coli W3110 was obtained from the stock culture of the Laboratory of Industrial Microbiology, Department of Food Science and Technology, Faculty of Agriculture, Kyoto University.

Cells were picked up from an agar plate containing LB medium,[10] inoculated into a tube containing 5 ml of LB broth and then grown at 37°C overnight with reciprocal shaking. This preculture was transferred to a 2-l Sakaguchi flask containing 500 ml of modified Vogel and Bonner medium[11] containing 0.5% glucose, 0.2% citric acid·H_2O, 0.35% $NaNH_4HPO_4 \cdot 4H_2O$, 1% K_2HPO_4, 0.02% $MgSO_4 \cdot 7H_2O$ and 0.05% Casamino acids, and then grown at 28°C for 24 hr with reciprocal shaking. Two such sub-

cultures were inoculated into a 30-l jar fermentor (type MSJ-U 301; Marubishi Co.) containing 20 l of the modified Vogel and Bonner medium. Cultivation was carried out at 30°C for 24 hr with aeration (0.5 l/l of medium per min) and agitation (150 rpm). The grown cells were harvested with a refrigerated continuous flow centrifuge (type GLE; Carl Padberg GmbH).

Enzyme assay. SC-Cys synthase activity was determined with an automatic amino acid analyzer (type K-101-AS; Kyowa Seimitsu Co.; with Kyowa Gel 62210-S) by measuring the SC-Cys synthesized. The assay solution contained 50 μmol of 3-Cl-Ala, 50 μmol of TGA, 0.1 mmol of potassium phosphate (pH 7.8), 20 nmol of PLP, 4.0 μmol of EDTA and the enzyme in a final volume of 1.0 ml. The reaction was started by the addition of 3-Cl-Ala, and incubation was carried out at 37°C and stopped by the addition of 0.5 ml of 0.3 N HCl.

O-Acetylserine sulfhydrylase activity was determined according to Kredick and Becker.[12]

The enzymatic α,β-elimination reaction was assayed by measuring the amount of pyruvate by a spectrophotometric method with lactate dehydrogenase and NADH.[13]

One unit of an enzyme is defined as the amount of enzyme that catalyzed the formation of 1 μmol of the product under the assay conditions. Specific activity is expressed as units per mg protein.

Protein determination. Protein concentrations were determined by the method of Lowry *et al.*[14] with bovine serum albumin as a standard.

Electrophoresis. Electrophoresis and protein staining were carried out according to the guide book from Pharmacia (*Polyacrylamide Gel Electrophoresis-Laboratory Techniques*). Proteins on native gel electrophoresis were stained with amido black 10B and those on SDS polyacrylamide gel electrophoresis were stained with Coomassie blue R-250.

Molecular weight estimation. The molecular weight of the enzyme was determined by gel filtration on a high performance liquid chromatograph (HPLC) equipped with a TSK-GEL 3,000 SW column. The molecular weight of a subunit was estimated by SDS polyacrylamide electrophoresis, using an LMW molecular weight calibration kit purchased from Pharmacia. It was also determined by SDS gel filtration on a HPLC equipped with a TSK-GEL 2,000 SW column.

Ion-exchange column chromatographies. Ion exchange column chromatography was performed for the purification of SC-Cys produced through the enzyme reaction. Cation-exchange column chromatography was performed on a Dowex 50×8 (H^+ form) column (3.6 by 25 cm) and elution was carried out with a 0.25 N NH_4OH solution. Anion-exchange chromatography was performed on a Dowex 1×2 (OH^- form) column (3.6 by 34 cm) and elution was carried out with 0.2 N acetic acid solution.

HPLC. HPLC was performed with an M6000A pump, a U6K injector and an M440 UV detector (wavelength fixed at 280 nm) (Waters Assoc., Milford, MA, U.S.A.).

Paper chromatography. Some products of the enzyme reaction were detected and determined by paper chromatography. Ten μl of the reaction mixture was applied on a filter pater (Toyo No. 51A), followed by development in the descending mode with a solvent system of *n*-butanol–acetic acid–water (4 : 1 : 1). The development was repeated to obtain a good separation. The products were detected by spraying a 0.3% ninhydrin solution in a mixture of acetone and ethanol (3 : 1). For determination of the product, the spot was cut out and extracted with 5 ml of a 75% ethanol solution in water containing one drop of 5% $CuSO_4$. The amount of the product was estimated spectrophotometrically by measuring the absorbance at 500 nm.

Spectrophotometric measurement. Spectrophotometric determination and measurement of absorption spectra were carried out with a Hitachi 150-20 spectrophotometer.

^{1}H-NMR spectrometric analysis. The ^{1}H-NMR spectrum was measured in D_2O with a Hitachi Model R-22 (90 MHz) spectrometer and chemical shifts were expressed relative to DSS as an internal standard.

Mass spectrometric analysis. The mass spectrum was measured with a Hitachi Model M-80 mass spectrometer with electron impact ion at an ionizing voltage of 20 eV.

Optical rotatory dispersion analysis. Optical rotatory dispersion was measured with a JASCO J-5 ORD recorder. SC-Cys was dissolved in 1 N HCl at the concentration of 20 mg/ml.

Amino acid sequence analysis. Amino acid sequence analysis was carried out by automated Edman degradation with an Applied Biosystems gas phase sequencer (Model 470A) equipped online with a PTH-amino acid analyzer (Model 120A).

Purification of SC-Cys synthase from E. coli W3110. All operation were carried out at 0 to 5°C unless otherwise stated. The pH of the enzyme solution was adjusted to 7.0 with a 2.5% ammonium hydroxide solution after each addition of ammonium sulfate.

Purification steps.

(i) *Preparation of a cell extract.* Cells harvested from 40 l of culture (wet weight, 230 g) were suspended in 1,150 ml of 0.1 M potassium phosphate buffer, pH 7.8, containing 10 mM 2-mercaptoethanol, 20 μM PLP and 4.0 mM EDTA

(this solution was named Buffer A) and 1.0 mM phenylmethyl sulfonyl fluoride, and disrupted with a Dyno-Mill (W. A. Bachofen, Maschinenfabrik, Basel, Switzerland) at the agitation rate of 3,000 rpm using glass beads of 0.25 to 0.50 mm in diameter. The supernatant solution was obtained by centrifugation.

(ii) *First ammonium sulfate fractionation.* Solid ammonium sulfate was added to the enzyme solution to 35% saturation. After standing for more than 1 hr, the supernatant was collected by centrifugation and brought to 70% saturation in the same manner. After standing overnight, the precipitate was collected by centrifugation, dissolved in a small amount of 50 mM Buffer A and then dialyzed against the same buffer for 2 days.

(iii) *First DEAE-Cellulofine column chromatography.* The dialyzed enzyme solution was applied to a DEAE-Cellulofine AH column (6.0 by 53 cm) equilibrated with 50 mM Buffer A. The column was washed with the same buffer and then the enzyme was eluted in 20-ml fractions with 70, 100 and 150 mM Buffer A. The activity was eluted with 70 and 100 mM Buffer A.

(iv) *Second ammonium sulfate fractionation.* The active fractions from the DEAE-Cellulofine column were combined and fractionated by the addition of solid ammonium sulfate. The activity was found in the fractions precipitated between 50 to 90% saturation.

(v) *Cellulofine GC-700-m gel filtration.* The enzyme solution was applied to a Cellulofine column (4.0 by 90 cm) equilibrated with 50 mM Buffer A. The enzyme was eluted with the same buffer in 20-ml fractions. The active fractions were combined and concentrated by the addition of ammonium sulfate to 90% saturation. After standing overnight, the precipitate was collected by centrifugation, suspended in 50 mM Buffer A and then dialyzed against the same buffer.

(vi) *Second DEAE-Cellulofine column chromatography.* The dialyzed active fraction was applied to a DEAE-Cellulofine AH column (2.8 by 38 cm) equilibrated with 50 mM Buffer A. The column was washed with the same buffer and then the enzyme was eluted in 5-ml fractions with an ascending linear gradient of the potassium phosphate in Buffer A, from 50 to 120 mM. The activity was eluted at the concentration of about 80 mM. The active fractions were combined and concentrated by the addition of ammonium sulfate to 90% saturation.

(vii) *Sephadex G-100 gel filtration.* The enzyme solution was applied to a Sephadex G-100 column (2.0 by 102 cm) equilibrated with 50 mM Buffer A. The enzyme was eluted with the same buffer in 5-ml fractions. The active fractions were combined and concentrated by the addition of ammonium sulfate to 90% saturation.

(viii) *Sephadex G-150 gel filtration.* The enzyme solution was applied to a Sephadex G-150 column (2.0 by 100 cm) equilibrated with 50 mM Buffer A. The enzyme was eluted with the same buffer in 2-ml fractions. The active fractions were combined and precipitated by the addition of ammonium sulfate to 90% saturation.

(ix) *Crystallization.* The precipitated enzyme protein (14.1 mg) was dissolved in a small amount (about 0.6 ml) of Buffer A. Solid ammonium sulfate was added to the solution carefully until it became slightly turbid, and then the mixture was placed in a refrigerator. Crystallization of the enzyme was observed from the next day and was completed within 1 week.

Results

Formation of SC-Cys synthase by E. coli W3110

E. coli W3110 produced SC-Cys synthase constitutively in the LB broth. The activity increased exponentially during the exponential phase and then became constant at the maximum level in the stationary phase. The addition of L-tryptophan or L-cysteine to the medium did not affect the enzyme activity at all.

Enzyme purification

The purification process (Table I), involving ammonium sulfate fractionation and five column chromatographies, led to a 190-fold puri-

Table I. PURIFICATION OF SC-Cys SYNTHASE FROM *E. coli*

Fraction	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)
1. Cell-free	26,000	1,960	0.0755	100
2. Ammonium sulfate (1)	10,400	1,030	0.0995	53
3. DEAE-Cellulofine (1)	1,290	1,200	0.927	61
4. Ammonium sulfate (2)	572	646	1.13	33
5. Cellulofine GC-700-m	336	477	1.42	24
6. DEAE-Cellulofine (2)	111	252	2.28	13
7. Sephadex G-100	29.4	344	11.7	18
8. Sephadex G-150	14.1	200	14.2	10

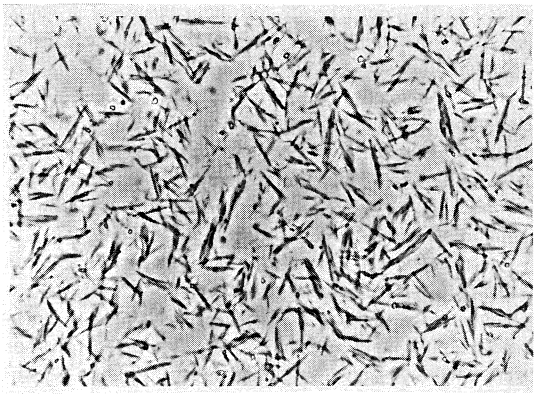

Fig. 1. Crystals of SC-Cys Synthase from *E. coli* W3110. The microphotograph was taken under a phase-contrast microscope at the magnification of ×400.

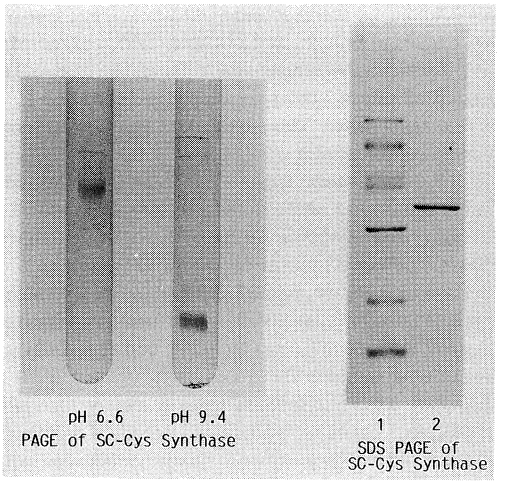

Fig. 2. Disc- and SDS-Polyacrylamide Gel Electrophoresis of SC-Cys Synthase from *E. coli* W3110.

Electrophoresis and staining were carried out as described under **Materials and Methods**. The direction of migration is from top to bottom. Disc-polyacrylamide gel electrophoresis (PAGE) was carried out with two kinds of buffer, pH 6.6 and 9.4, respectively. Fifteen μg of the enzyme protein was applied to each column. For SDS-PAGE, molecular markers (lane 1) and 15 μg (lane 2) of the enzyme were applied. The molecular markers used were phosphorylase *b* (molecular weight, 94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), trypsin inhibitor (20,100) and α-lactalbumin (14,400).

fication with a recovery of 10.2%. The purified SC-Cys synthase was crystallized as fine yellow needles by the addition of ammonium sulfate (Fig. 1). The enzyme preparation gave a single band on SDS polyacrylamide gel electrophoresis, though it showed a broad band on disk polycrylamide gel electrophoresis at pH 6.6 and 9.4 (Fig. 2).

Molecular weight and subunit structure

The molecular weight was estimated to be 84,000 by HPLC gel filtration. SDS polyacrylamide gel electrophoresis of the purified SC-Cys synthase gave a single band of 37,000 and SDS HPLC gel filtration gave a single peak of 42,000, indicating that the enzyme is a homodimer.

Catalytic properties

The apparent *Km* values for 3-Cl-Ala and TGA were 40 and 15.4 mM. The optimum pH for the SC-Cys synthase activity was 9 to 10.5 and the stable pH range was 6.5 to 7.5. The optimum temperature for SC-Cys synthesis was 50°C and the stable temperature range was up to 60°C, as judged when the enzyme was incubated at various temperatures for 10 min.

The purified SC-Cys synthase catalyzed pyruvate formation from 3-Cl-Ala, L-serine and L-cystathionine at the reletive rates of 1.0%, 0.02% and 0.07% of the rate of SC-Cys synthesis. Pyruvate formation was not observed with SC-Cys, L-cysteine, D-cysteine, D-serine or 3-chloro-D-alanine. Incubation of the enzyme with 3-Cl-Ala for 2 hr did not cause any loss of activity. The enzyme showed L-cysteine synthase, *O*-acetylserine sulfhydrylase, activity corresponding to 4.1% of the rate of SC-Cys synthesis.

Purification and identification of SC-Cys produced through the enzyme reaction

Enzymatic synthesis of SC-Cys was carried out under the assay conditions given under **Materials and Methods** with 50 ml of reaction mixture containing 100 mM 3-Cl-Ala and 200 mM TGA. The reaction was carried out for 24 hr and the synthesis of 680 mg (76% yield of 3-Cl-Ala) of SC-Cys was confirmed by amino acid analysis. The product was purified from

Table II. Substrate Specificity of SC-Cys Synthase for Thiol Compounds

The amounts of the products were determined as described under **Materials and Methods**. Activity is expressed relative to that found with TGA (100%).

Substrate	Product	Relative activity (%)
Thioglycolic acid	*S*-Carboxymethyl-L-Cys	100
Sodium hydrosulfide	L-Cys	—[a]
Methylmercaptan	*S*-Methyl-L-Cys	65
Ethylmercaptan	*S*-Ethyl-L-Cys	55[b]
n-Propylmercaptan	*S*-Propyl-L-Cys	4[b]
iso-Propylmercaptan	*S*-iso-Propyl-L-Cys	—
Phenylmercaptan	*S*-Phentyl-L-Cys	207[b]
Benzylmercaptan	*S*-Benzyl-L-Cys	35[b]
2-Mercaptoethanol	*S*-2-Hydroxyethyl-L-Cys	242[b]
1-Thioglycerol	*S*-2,3-Dihydroxypropyl-L-Cys	93[b]
L-Cys	L-Lanthionine	—
DL-Homocysteine	Cystathionine	—
α-Mercaptopropionic acid	*S*-1-Methylcarboxymethyl-L-Cys	—
β-Mercaptopropionic acid	*S*-2-Methylcarboxymethyl-L-Cys	—

[a] Not detected.
[b] The extinction coefficient of SC-Cys was used for the determination.

the reaction mixture by chromatographies on Dowex 50 and Dowex 1, as described under **Materials and Methods**, and crystallized from water by adjusting the pH of the solution to 3.2. Purified SC-Cys, 344.5 mg, was obtained by this procedure. The structure of *S*-carboxymethyl-L-cysteine was determined by ^{1}H-NMR, mass and ORD spectrometry, in comparison with the authentic compound.

Substrate specificity

The β-replacement reaction, SC-Cys synthesis, and the γ-replacement reaction, *S*-carboxymethyl-hormocysteine synthesis, were examined using TGA and the following amino acids as the substrates: L-cysteine, L-serine, L-threonine, L-cystine, 3-chloro-D-alanine, DL-homocysteine, L-methionine and L-homoserine. But formation of the product was not observed.

The β-replacement reaction was carried out using 3-Cl-Ala and various thiol compounds as substrates. The products were detected and determined by paper chromatography, and with an amino acid analyzer. The formation of various *S*-substituted amino acids was observed and the relative reaction rates are shown in Table II.

Absorption spectra

The enzyme solution exhibited an absorption maximum at 412 nm besides at 280 nm (Fig. 3A, B). On the addition of a substrate, 3-Cl-Ala, the absorption at 412 nm shifted to 428 nm and increased, and a new absorption peak appeared at 343 nm (Fig. 3B-a). This spectrum did not change on standing for 10 min. Further addition of TGA caused a decrease of 428 nm peak and an increase in the 343 nm peak (Fig. 3B-b, c, d, e).

Amino-terminal amino acid sequence

The amino-terminal amino acid sequence of the enzyme was determined by automated Edman degradation. Thirty-six amino acid residues, except the 33rd one from the amino-terminal end, were determined, as shown in Fig. 4. A homologous sequence was searched for in the Protein Sequence Data Base of the National Biomedical Research Foundation, but a sequence showing high homology was not found.

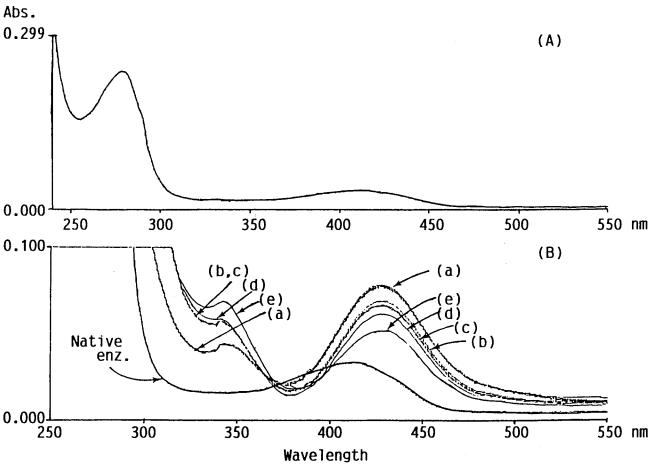

Fig. 3. Absorption Spectra of SC-Cys Synthase from *E. coli* W3110.

The crystallized enzyme was dissolved in a small amount of K_2HPO_4–KH_2PO_4 buffer, pH 7.8, containing 20 μM PLP and 4 mM EDTA, and then dialyzed against the same buffer. The spectra of the solution was read against a blank containing the dialysis buffer. The spectra of the native enzyme are shown in (A) and (B). The concentration of the enzyme protein was 0.38 mg/ml. The spectra were taken 1, 3, 5 and 10 min later after addition of 50 mM 3-Cl-Ala (a), and 1 (b), 3 (c), 5 (d) and 15 hr (e) later after the further addition of 100 mM TGA.

```
 1  2  3  4  5  6  7  8  9 10
Ser-Lys-Ile-Phe-Glu-Asp-Asn-Ser-Leu-Thr-

11 12 13 14 15 16 17 18 19 20
Ile-Gly-His-Thr-Pro-Leu-Val-Arg-Leu-Asn-

21 22 23 24 25 26 27 28 29 30
Arg-Ile-Gly-Asn-Gly-Arg-Ile-Leu-Ala-Lys-

31 32 33 34 35 36
Val-Glu- ? -Arg-Asn-Pro-
```

Fig. 4. Amino-Terminal Amino Acid Sequence of SC-Cys Synthase from *E. coli* W3110.

Sequence analysis was carried out with the lyophilized crystalline preparation, as described under **Materials and Methods**.

Discussion

We found an enzyme that synthesizes *S*-carboxymethyl-L-cysteine from 3-chloro-L-alanine and thioglycolic acid in a wild type strain of *E. coli*, and named it *S*-carboxymethyl-cysteine synthase. The purification and characterization of the enzyme are reported in this paper.

On long-term storage of the enzyme in the ammonium sulfte precipitate, its activity decreased but the activity was recovered and occasionally enhanced on dialysis of the preparation against Buffer A. This was observed during the purification of the enzyme (Table I, Steps 3 and 7).

The *E. coli* strain used in this work produced the enzyme constitutively in the LB broth and the addition of L-tryptophan or L-cysteine to the medium did not affect the enzyme activity, indicating that the enzyme is not involved in the synthesis of these amino acids, since the biosynthesis of these enzymes are known to be repressed by the final product of the synthesis pathway.

The enzyme catalyzed the α,β-elimination reaction of 3-Cl-Ala, L-serine and L-cystathionine, but at a very low rate compared with SC-Cys synthesis. The enzyme was not inactivated on incubation with 3-Cl-Ala. PLP-dependent enzymes which catalyze transamination, racemization or decarboxylation are known to catalyze the α,β-elimination and β-replacement reactions of 3-Cl-Ala as secondary reactions, but most of the enzymes are gradually inactivated during the course of reaction with 3-Cl-Ala.[15] So the catalytic properties of the enzyme so far investigated indicate that it is a β-replacement specific PLP dependent enzyme. We were unable to find an

enzyme with similar molecular and catalytic properties among other reported enzymes. So at present, the physiological reaction catalyzed by this enzyme in *E. coli* cells remains obscure.

Investigation of the reaction conditions with a cell-free extract or with *E. coli* cells is important for the practical use of this enzyme. Furthermore, it is necessary to use racemic 3-chloro-alanine as the substrate and to elucidate the fate of D-form 3-chloro-alanine in the reaction mixture with a cell-free extract or cells.

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