0273–2289/86/1302–0147$03.80

REVIEW

Enzymatic Transformations of 3-Chloroalanine into Useful Amino Acids

TORU NAGASAWA* AND HIDEAKI YAMADA

Department of Agricultural Chemistry, Kyoto University, Sakyo-Ku Kyoto 606 Japan

Received October 3, 1985; Accepted December 3, 1985

ABSTRACT

The investigation of the combination of enzymatic and chemical synthetic processes for the production of useful compounds has been carried out. This review focuses on the enzymatic transformation of chemically synthesized 3-chloroalanine into useful amino acids.

Index Entries: 3-Chloroalanine, enzymatic transformation of into useful amino acids; amino acids, production of by microbial reactions; enzymatic process, of microbial production.

INTRODUCTION

Nowadays, antibiotics, nucleic acids, amino acids, organic acids, and other various useful compounds are produced by means of microbial reactions. The microbial production processes for useful compounds can be divided broadly into two categories, fermentation and enzymatic processes. In fermentation production, cheap carbon and nitrogen sources can be used for the medium, but many steps of microbial metabolism, of which some are complicated, are involved in the production processes. The microbial productions of alcohols and organic acids are representative examples of fermentation production processes. On the other hand, in the enzymatic production processes, a microorganism is regarded as a bag containing only an enzyme that catalyzes the desired reaction. Namely, only the catalytic activity of the microorganism is considered and applied for the production processes, i.e., a catalyst in organic synthesis.

*Author to whom all correspondence and reprint requests should be addressed.

We have tried to find and establish new enzymatic production processes. Particularly, we have investigated the combination of enzymatic and chemical synthetic processes for the production of useful compounds. In the present review we have focused on the enzymatic transformation of chemically synthesized 3-chloroalanine into useful amino acids.

REACTIONS OF 3-CHLOROALANINE WITH PYRIDOXAL-P-DEPENDENT ENZYMES

Most pyridoxal 5′-phosphate (pyridoxal-P) -dependent enzymes are involved in the biosynthesis or biodegradation of amino acids in an organism. These enzymes catalyze a variety of reactions, including transamination, racemization, α,β-elimination, α,γ-elimination, decarboxylation, and aldol-type condensation and retrocondensation reactions (Fig. 1). The removal of an α-proton of an amino acid is the essential first step of the transamination, racemization, and α,β- and α,γ-elimination reactions. 3-Chloroalanine, an artificial amino acid, is easily attacked by pyridoxal-P-dependent enzymes. As soon as its α proton is removed by the pyridoxal-P-dependent enzymes, a good leaving group, Cl^-, is easily eliminated. Thus, the α,β-elimination reaction proceeds. For example, 3-chloro-L-alanine is degraded by glutamate-oxaloacetic acid trans-

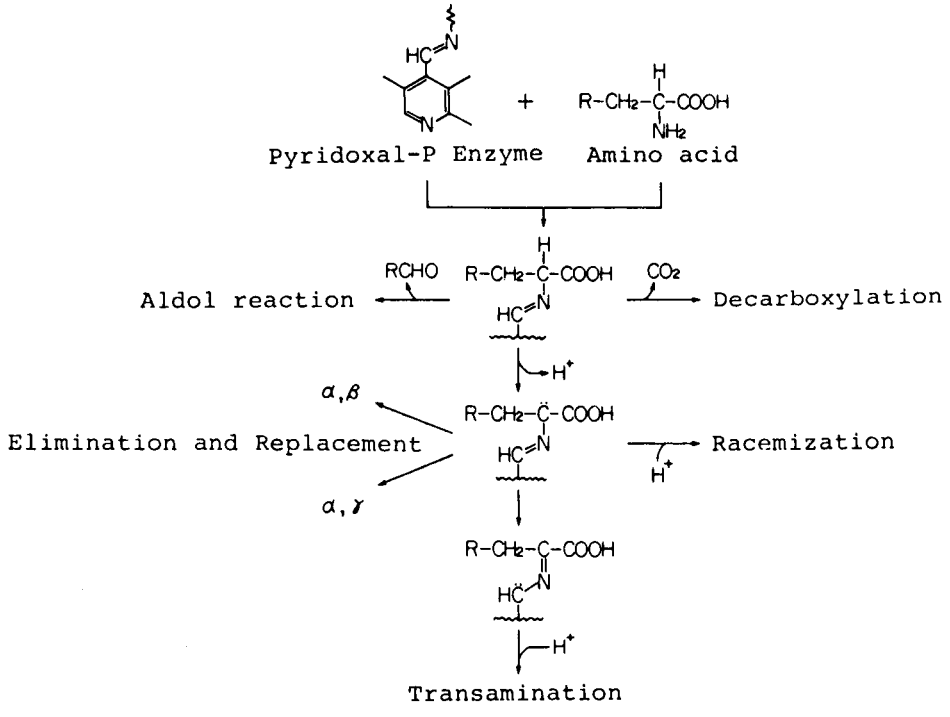


Fig. 1. Reactions catalyzed by pyridoxal-P-dependent enzymes.

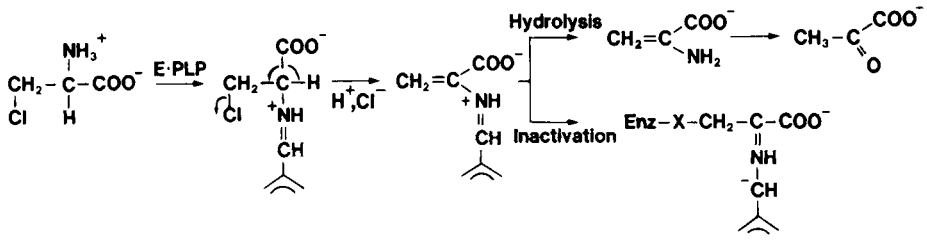


Fig. 2. Reaction mechanism of 3-chloroalanine with pyridoxal-P-dependent enzymes.

aminiase (GOT) (E.C. 2.6.1.1)(*1*), glutamate-pyruvic acid transaminase (GPT) (E.C. 2.6.1.2)(*2*), and amino acid racemase (*3*) to produce pyruvic acid, ammonia, and HCl. However, during the course of these reactions, the enzymes are concomitantly inactivated. The inactivation phenomenon is called suicide of the enzyme. The suicide phenomenon is a result of the reaction-intermediate (enzyme-bound α-aminoacrylate intermediate) reacting with an amino acid residue at or near the active site of the enzyme and causing the inactivation (Fig. 2). Since the highly reactive intermediate derived from 3-chloroalanine reacts with a specific amino acid residue, which is essential for the catalytic activity of the enzyme, 3-chloroalanine has been used as an affinity-labeling reagent for the determination of amino acid residues near the active site or for the primary structure of the active site of an enzyme.

On the other hand, some pyridoxal-P-dependent enzymes that catalyze the α,β-elimination or β-replacement reaction are insensitive to the suicidal function of 3-chloroalanine. 3-Chloroalanine serves as a preferable substrate for such pyridoxal-P-dependent enzymes. Therefore, the enzymatic transformation of 3-chloroalanine into useful amino acids is possible. Although a chemical synthetic method for obtaining 3-chloroalanine from serine was reported (*4*), the economical production of 3-chloroalanine on a large scale has not been accomplished. However, the recent development of chemical synthesis techniques has made it possible to produce 3-chloroalanine economically on a large scale (*5,6*). Therefore, we review here the studies on the transformation of 3-chloroalanine into useful amino acids using pyridoxal-P-dependent enzymes.

SYNTHESIS OF L-TRYPTOPHAN AND OTHER AMINO ACIDS WITH TRYPTOPHAN SYNTHASE

It has been found that 3-chloro-L-alanine serves as a substrate for the β-replacement reaction of tryptophan synthase (*7*). *Escherichia coli* tryptophan synthase (E.C. 4.2.1.20) has an $\alpha_2\beta_2$-subunit composition. Two α subunits combine with one β_2 dimer to form an $\alpha_2\beta_2$ complex. Each α and β_2 subunit has its own catalytic activity (Table 1). The pyridoxal-P is com-

TABLE 1
Reactions Catalyzed by *Escherichia coli* Tryptophan Synthase and Its Subunits

Reactions			Catalyzed by
(1) L-Serine	→ Pyruvic acid	+ ammonia	β_2
(2) 3-Chloro-L-alanine + H_2O	→ Pyruvic acid	+ NH_4Cl	β_2
(3) Indole + L-serine	→ L-Tryptophan	+ H_2O	β_2, $\alpha_2\beta_2$
(4) Indole + 3-chloro-L-alanine	→ L-Tryptophan	+ HCl	β_2, $\alpha_2\beta_2$

bined with β_2, and the β_2 subunit catalyzes the α,β-elimination of L-serine and the formation of tryptophan from L-serine and indole. The $\alpha_2\beta_2$ form cannot catalyze the α,β-elimination reaction, catalyzing only the β-replacement reaction at a higher rate than the β_2 subunit does. The β_2 subunit catalyzes the α,β-elimination reaction of 3-chloro-L-alanine without the concomitant suicidal inactivation. The β_2 subunit catalyzes the β-replacement reaction of 3-chloro-L-alanine in the presence of indole to form L-tryptophan, and the synthetic rate is almost the same as in the case of L-serine.

The α,β-elimination reaction of 3-chloro-L-alanine proceeds through five steps, shown in Fig. 3.

(1) Schiff's base formation between the aldehyde carbonyl of pyridoxal-P and the amino group of the substrate (I → II).
(2) Removal of the α-proton of 3-chloro-L-alanine (II → III).
(3) Elimination of Cl^- (a good leaving group) from the β-carbon position and the formation of an α-aminoacrylate–pyridoxal-P Schiff's base intermediate (III → IV).
(4) Reprotonation at the β-carbon position and formation of a ketimine intermediate (IV → VII).
(5) Hydrolysis of the ketimine intermediate to ammonia and pyruvic acid (VII → I).

The β-replacement reaction of 3-chloroalanine also proceeds through the same reaction mechanisms, and an enzyme-bound α-aminoacrylate–pyridoxal-P Schiff's base intermediate is formed (I → II → III → IV). R (Indole) attacks the β-position carbon, followed by immediate reprotonation to form L-tryptophan and the pyridoxal-form enzyme (V → VI → I).

The α,β-elimination and β-replacement reactions of L-serine, *S*-methyl-L-cysteine, and *O*-methyl-L-serine also possibly proceed through the same reaction mechanisms. Various thiol compounds serve as preferable substrates instead of indole for the β-replacement reaction of tryptophan synthase. Particularly, thiobenzylalcohol, 1-propanethiol, and 1-butanethiol are more preferable than indole for the production of the corresponding *S*-substituted L-cysteine derivatives (*8*). Recently, using cells of *E. coli* that contained a high amount of tryptophan synthase, the

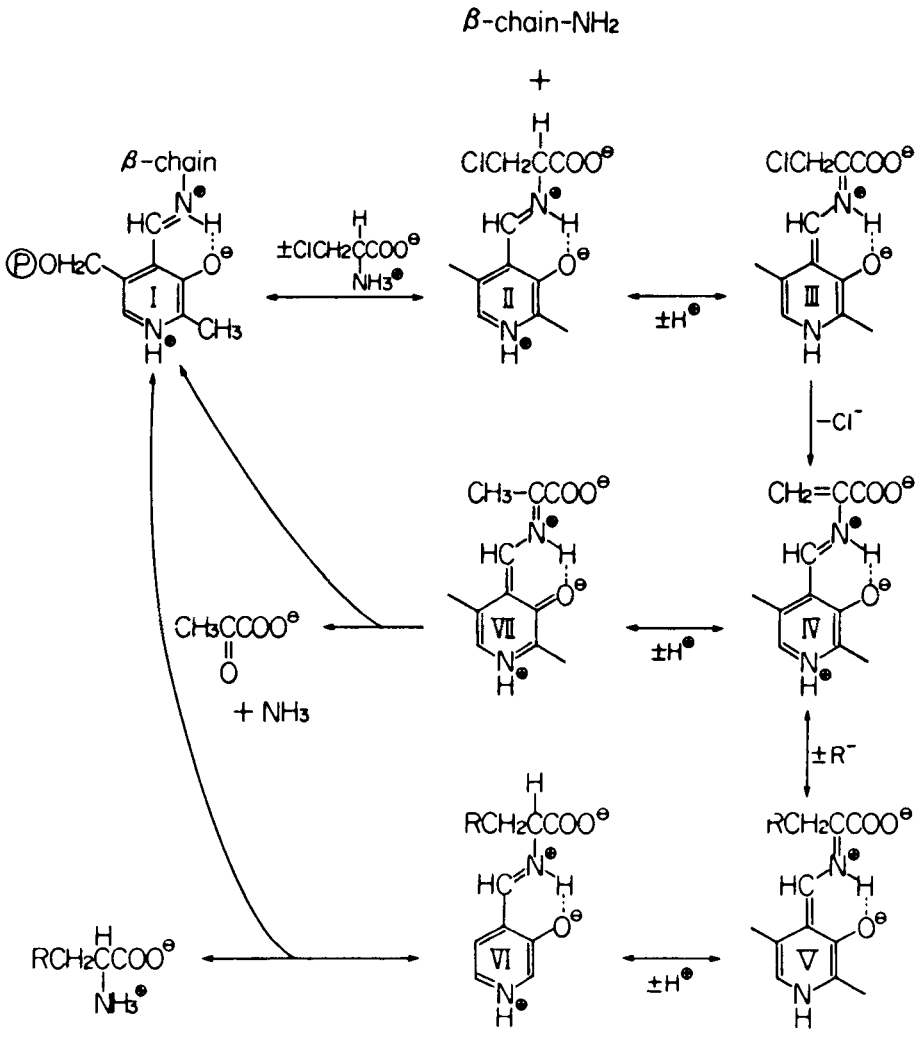


Fig. 3. Proposed reaction mechanisms of the α,β-elimination and the β-replacement reactions catalyzed by pyridoxal-P-dependent enzymes.

enzymatic synthesis of *S*-carboxymethyl-L-cysteine from 3-chloro-L-alanine and thioglycolate was studied. Under the optimal reaction conditions, the enzymatic synthesis of *S*-carboxymethyl-L-cysteine (about 1.6 g/100 mL of the reaction mixture) was accomplished with a conversion yield from 3-chloro-L-alanine of 81% (*9*).

SYNTHESIS OF L-DOPA AND L-TYROSINE-RELATED AMINO ACIDS WITH TYROSINE PHENOL-LYASE

Tyrosine phenol-lyase (E.C. 4.1.99.2) catalyzes the α,β-elimination reaction of L-tyrosine. We have purified and crystallized the enzyme from *Citrobacter intermedius* (*10*), *Erwinia herbicola* (*11*), and *Proteus*

morganni (*12*). The enzyme is pyridoxal-P dependent, has a molecular weight of approximately 200,000, and consists of four subunits identical in molecular weight. The enzyme also catalyzes the α,β-elimination reactions of L-serine, *S*-methyl-L-cysteine, and 3-chloro-L-alanine. Particularly, 3-chloro-L-alanine is degraded rapidly and its V_{max} value is ninefold higher than that of L-tyrosine (Table 2) (*13*). We have revealed that the degradation of tyrosine by tyrosine phenol-lyase, which was previously thought to be an irreversible reaction, is readily reversible with high concentrations of pyruvate and ammonia and with an adequate concentration of phenol (Eq. 1) (*14*).

$$\text{HO—}\Phi\text{—CH}_2\text{CH(NH}_2\text{)COOH} + \text{H}_2\text{O} \rightarrow \text{CH}_3\text{COCOOH} + \text{NH}_3 + \text{HO—}\Phi \quad (1)$$

Since the solubility of L-tyrosine in water is very low, the L-tyrosine synthesized precipitates in the reaction mixture. Therefore, the production of L-tyrosine can be accomplished with a high yield. The finding of the new catalytic ability of tyrosine phenol-lyase has allowed the enzymatic synthesis of L-tyrosine on an industrial scale. This new process for L-tyrosine production has been the subject of keen interest.

Alkylphenol, alkoxyphenol, halophenol, and hydroxyphenol can be substituted for phenol, which results in the formation of various tyrosine related amino acids. When pyrocatechol is added instead of phenol, L-DOPA [3-(3,4-dihydroxyphenyl)-L-alanine], which is useful in the medical treatment of Parkinson disease, is produced (*15*). However, when a high concentration of pyruvate is added to the reaction mixture, a nonenzymatic condensation reaction occurs between the L-DOPA formed and pyruvic acid (formation of X-1 and X-2) (Fig. 4), which causes

TABLE 2
Catalytic Properties of Tyrosine Phenol-lyase

Compound	Role	Product	K_M, mM	V_{max}, μmol/min/mg
α,β-Elimination				
L-Tyrosine	Substrate	Pyruvate	0.23	1.9
L-Serine	Substrate	Pyruvate	34	0.35
S-methyl L-cysteine	Substrate	Pyruvate	1.8	1.2
3-Chloro-L-alanine	Substrate	Pyruvate	4.5	18.2
β-Replacement				
L-Serine	Cosubstrate	L-Tyrosine	35	0.33
S-methyl-L-cysteine	Cosubstrate	L- Tyrosine	1.8	0.82
3-Chloro-L-alanine	Cosubstrate	L-Tyrosine	4.5	18.2
Phenol	Cosubstrate	L-Tyrosine	1.2	

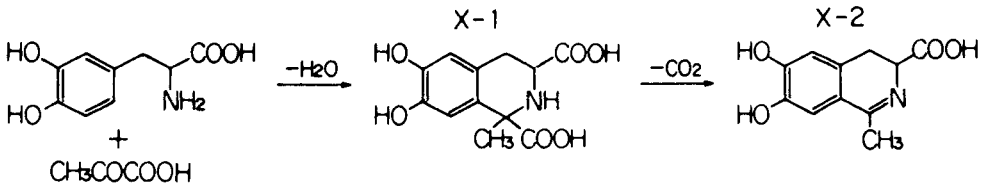


Fig. 4. Proposed mechanism of byproducts formation from pyruvic acid and L-DOPA.

a marked decrease in the production of L-DOPA. For the production of L-DOPA, the use of the β-replacement reaction of tyrosine phenol-lyase seems to be superior to the reverse reaction of α,β-elimination. 3-Chloro-L-alanine and L-serine serve as preferable substrates for both the α,β-elimination and β-replacement reactions (Eq. 2).

$$\text{L-RCH}_2\text{CH(NH}_2\text{)COOH} + \text{HO—}\Phi \rightarrow \text{L-HO—}\Phi \text{—CH}_2\text{CH(NH}_2\text{)COOH} + \text{RH} \quad (2)$$

(R: Cl, OH)

Figure 5 shows the effect of the concentration of pyrocatechol on the formation of L-DOPA from 3-chloro-L-alanine. 3-Chloro-L-alanine is degraded in the absence of pyrocatechol (α,β-elimination), whereas the

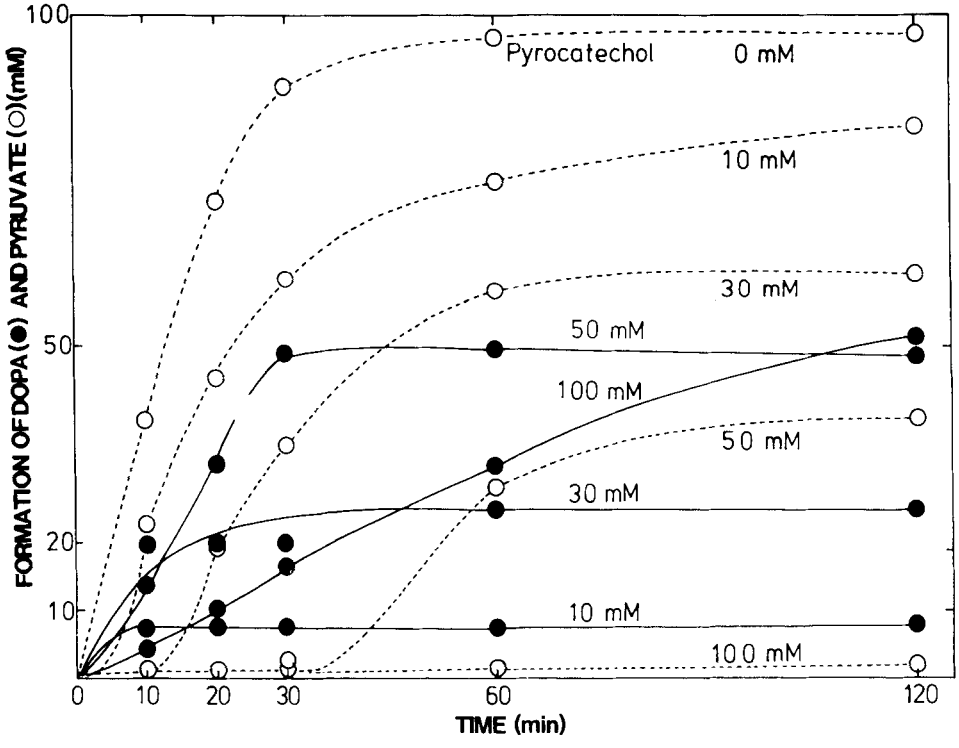


Fig. 5. Enzymatic synthesis of L-DOPA from 3-chloro-L-alanine and pyrocatechol by tyrosine phenol-lyase.

β-replacement reaction becomes progressively more dominant with an increase in pyrocatechol, and the α,β-elimination reaction is depressed. About 100% of 3-chloro-L-alanine is converted to L-DOPA in the presence of 50 m*M* pyrocatechol with a 30-min incubation period. When the incubation is prolonged, the remaining 3-chloro-L-alanine begins to be degraded (Fig. 5). However, the high concentration of pyrocatechol denatures the enzyme, which results in a decrease in the yield of L-DOPA. Accordingly, it is very important to control the concentration of pyrocatechol in the reaction mixture. Compared with L-serine, 3-chloro-L-alanine has a higher affinity for the enzyme and is a more preferable substrate for the β-replacement reaction. The V_{max} value of 3-chloro-L-alanine is 12-fold higher than that of L-serine. The mechanism of the β-replacement reaction of tyrosine phenol-lyase also proceeds through the steps shown in Fig. 3. R corresponds to hydroxyphenol or various phenol derivatives and reacts with 3-chloro-L-alanine to produce the corresponding L-tyrosine-related amino acids (*16,17*).

SYNTHESIS OF L-CYSTEINE AND *S*-SUBSTITUTED L-CYSTEINE DERIVATIVES WITH CYSTEINE DESULFHYDRASE

Cysteine desulfhydrase (E.C. 4.4.1.1) is a pyridoxal-P-dependent enzyme that catalyzes the degradation of L-cysteine to hydrogen sulfide, pyruvic acid, and ammonia (Eq. 3).

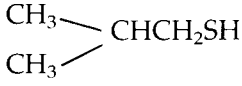
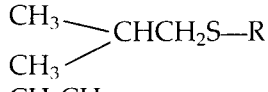
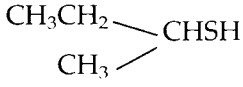
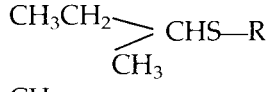
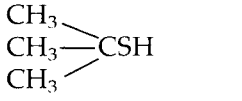
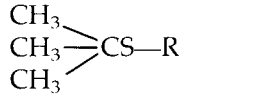
$$\text{L-HSCH}_2\text{CH(NH}_2\text{)COOH} + \text{H}_2\text{O} \rightarrow \text{CH}_2\text{COCOOH} + \text{NH}_3 + \text{H}_2\text{S} \quad (3)$$

Kredich et al. and our group have purified and characterized the enzymes from cells of *Salmonella typhimurium* (*18*) and *Enterobacter aerogenes* (*19*), respectively, and we have crystallized the *E. aerogenes* enzyme. In addition to L-cysteine (V_{max} 958 μmol/min/mg), 3-chloro-L-alanine serves as a preferable substrate (V_{max} 1600 μmol/min/mg) (*20*). The enzyme also catalyzes the β-replacement reaction of 3-chloro-L-alanine in the presence of sulfide or thiol compounds to form L-cysteine or *S*-substituted L-cysteine derivatives, respectively. Using this β-replacement reaction, we have synthesized and isolated L-cysteine and various *S*-substituted L-cysteine derivatives (Table 3).

The mechanisms of the α,β-elimination and β-replacement reactions catalyzed by the enzyme probably include the formation of enzyme-bound α-aminoacrylate as an intermediate, shown in Fig. 3. The enzyme also catalyzes the reverse reaction of α,β-elimination, although the reaction velocity is very low (*21*).

The enzymatic synthesis of L-cysteine by resting cells of *Enterobacter cloacae* has been investigated (*22*). When the reaction is carried out at 30°C and at pH 9.5 for 2 h in a reaction mixture containing cells of *E. cloacae*

TABLE 3
Relative Synthetic Rates of L-Cysteine-Related Amino Acids From 3-Chloro-L-Alanine and Mercaptans by Cysteine Desulfhydrase

Mercaptan	Synthesized L-amino acids	Relative synthetic rate
H_2S	HS—R[a]	100
CH_3SH	CH_3S—R	0.6
CH_3CH_2SH	CH_3CH_2S—R	2.0
$CH_3(CH_2)_2SH$	$CH_3(CH_2)_2S$—R	0.49
CH_2=CH—CH_2SH	CH_2=CH—CH_2S—R	7.2
$CH_3(CH_2)_3SH$	$CH_3(CH_2)_3S$—R	0.13
		0.09
		0.05
		0.04
Φ—SH	Φ—S—R	0.02
Φ—CH_2SH	Φ—CH_2S—R	0.17

[a]R: Alanyl residue.

harvested from 100 mL of cultured broth, 485 m*M* 3-chloro-L-alanine, 50 m*M* $Na_2S{\cdot}9H_2O$, 8% (v/v) acetone, 784 m*M* NH_4Cl, 121 μ*M* pyridoxal-P, and 1.73 m*M* sodium dodecyl sulfate, more than 80% of the added 3-chloro-L-alanine is transformd to L-cysteine, and about 5 g L-cysteine is synthesized per 100 mL of the reaction mixture.

For high productivity of L-cysteine, the addition of acetone is required. Previously, Kredich et al. (*18*) indicated that during the cysteine desulfhydrase reaction, part of an intermediate, enzyme-bound α-aminoacrylate, reacted with additional cysteine to form thiohemiketamine or 2-methyl-2,4-thiazolidine-dicarboxylate, shown in Fig. 6. It is known that acetone reacts with cysteine nonenzymatically to form 2,2-dimethyl-4-thiazolidine-carboxylic acid, and that when the compound is treated with hot, 1*N* HCl, the reverse reaction occurs and cysteine is regenerated. Actually, the addition of 8% (v/v) acetone, which corresponds to 3.6 times the concentration of added 3-chloro-L-alanine, enhanced the productivity of L-cysteine. We assume the mechanism of the effect of acetone to be as follows; overproduced L-cysteine is trapped by the acetone as 2,2-dimethyl-4-thiazolidine-carboxylic acid. The removal of L-cysteine

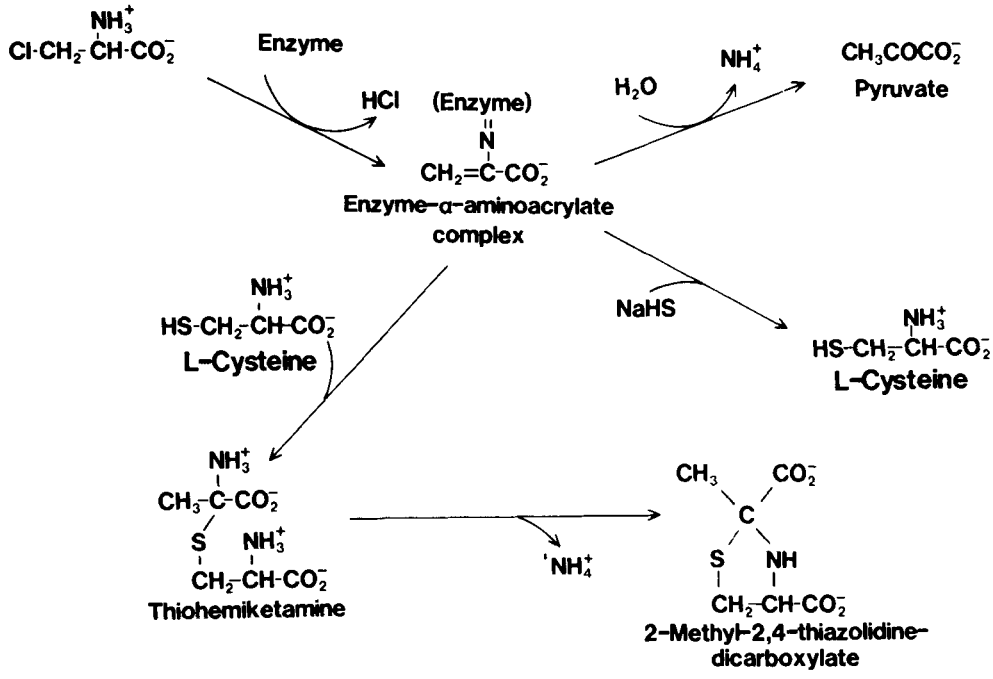


Fig. 6. Proposed reaction mechanisms of the α,β-elimination and the β-replacement reactions catalyzed by cysteine desulfhydrase.

then depresses the formation of thiohemiketamine or 2-methyl-2,4,-thiazolidine-dicarboxylate, which results in the efficient conversion of 3-chloro-L-alanine to L-cysteine.

SYNTHESIS OF L-CYSTEINE WITH *O*-ACETYLSERINE SULFHYDRYLASE

Although 3-chloro-L-alanine does not inhibit bacterial growth as much as its D-isomer, about 50% of tested bacteria cannot grow on a nutrient medium supplemented with 31.2 m*M* 3-chloro-L-alanine. Recently, we isolated a microorganism from soil that is resistant to 50 m*M* 3-chloro-L-alanine. Taxonomic studies have indicated that this organism is *Bacillus sphaericus* L-118 (*23*). The crude cell extract of this microorganisms shows high enzyme activity for the synthesis of L-cysteine from sodium hydrosulfide and 3-chloro-L-alanine (Eq. 4) when the cells are cultivated with the addition of 3-chloro-L-alanine to the nutrient medium. We have purified and crystallized the enzyme (*23*). This enzyme cannot catalyze the α,β-elimination reactions of L-cysteine and 3-chloroalanine, but catalyzes only the β-replacement reaction. The enzyme has been found to have a high-reaction velocity and a low apparent K_m value for *O*-acetyl-L-serine (Eq. 5).

$$\text{L-ClCH}_2\text{CH(NH}_2\text{)COOH} + \text{NaHS} \rightarrow \text{L-HSCH}_2\text{CH(NH}_2\text{)COOH} + \text{NaCl} \quad (4)$$

$$\text{L-CH}_2\text{COOCH}_2\text{CH}_2(\text{NH}_2)\text{COOH} + \text{NaSH} \rightarrow \text{L-HSCH}_2\text{CH}(\text{NH}_2)\text{COOH} + \text{CH}_3\text{COONa} \quad (5)$$

These results suggested that *O*-acetyl-L-serine might be the true substrate in the biological system. To explore this possibility, we compared our data with the results obtained for *O*-acetylserine sulfhydrylase of *S. typhimurium* (*24*). The reference and purified enzymes have the same molecular weights, subunit molecular weights, numbers of subunits, pyridoxal-P contents, absorption spectra, and sedimentation coefficients. These similarities provide strong evidence that the purified enzyme is *O*-acetylserine sulfhydrylase (E.C. 4.2.99.8)(*23*).

The formation of the enzyme in *B. sphaericus* L-118 is markedly enhanced by adding 3-chloro-L-alanine to the nutrient medium. Figure 7 shows that the addition of 0.3% (w/v) 3-chloro-L-alanine to the medium results in 4.2-fold enhancement of the enzyme formation, when L-cysteine (a repressive compound) is not added. The enhancement becomes less with increasing L-cysteine concentration. In other words, the repression of *O*-acetylserine sulfhydrylase formation is probably released by 3-chloro-L-alanine at a low concentration of L-cysteine. The enhancing effect of 3-chloro-L-alanine on the formation of *O*-acetylserine sulf-

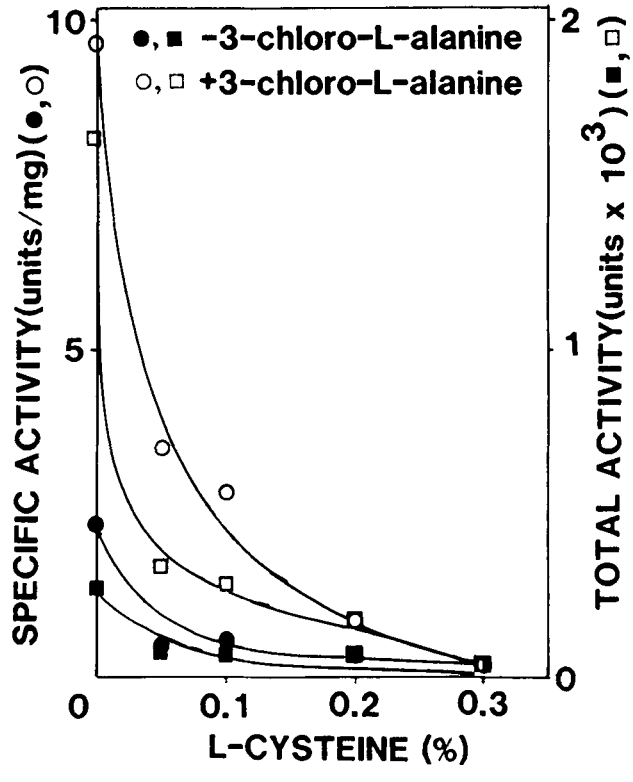


Fig. 7. Effect of 3-chloro-L-alanine and L-cysteine on the formation of *O*-acetyl-L-serine suldhydrylase.

hydrylase was also observed for some bacteria belonging to the genera *Proteus*, *Agrobacterium*, and *Bacillus* (*25*).

The enhancement of *O*-acetylserine sulfhydrylase formation by 3-chloro-L-alanine is favorable for the preparation of cells showing high *O*-acetylserine sulfhydrylase activity. In addition, *O*-acetylserine sulfhydrylase does not catalyze the α,β-elimination reactions of the 3-chloro-L-alanine and L-cysteine formed. The optimum pH for the purified *O*-acetylserine sulfhydrylase was found to be around 10.5 (*23*). However, its physiological substrate, *O*-acetyl-L-serine, is easily transformed to *N*-acetylserine under alkaline conditions (*26*). Therefore, 3-chloro-L-alanine may be more stable and suitable as a substrate than *O*-acetyl-L-serine for the production of L-cysteine. Thus, the catalytic and regulatory properties of *O*-acetylserine sulfhydrylase and the peculiar properties of the substrate, itself, prompted us to use 3-chloro-L-alanine as a substrate and resting cells of *B. sphaericus* as a likely source of the enzyme for the production of L-cysteine.

The following optimum reaction of L-cysteine from 3-chloro-L-alanine, using resting cells of *B. spaericus* L-118: A reaction mixture (100 mL) consisting of 700 m*M* 3-chloro-L-alanine, 3.5*M* sodium hydrosulfide, 200 m*M* 3-cyclohexylaminopropanesulfonic acid (CAPS)/HCl buffer (pH 9.5), 8% (v/v) acetone, and cells (174 mg as dry weight) harvested from 100 mL of cultured broth; temperature, 37°C and incubation period, 2 h. Under these conditions, about 80% of the added 3-chloro-L-alanine is converted to L-cysteine (titer, 7 g/100 mL of the reaction mixture) (*25*).

O-Acetylserine sulfhydrylase shows strict substrate specificity for sulfide, and various *S*-substituted L-cysteine derivatives cannot be synthesized as they are in the case of cysteine desulfhydrase.

SYNTHESIS OF D-CYSTEINE AND D-CYSTEINE-RELATED AMINO ACIDS WITH 3-CHLORO-D-ALANINE DEHYDROCHLORINASE

The enzymatic transformation of 3-chloro-L-alanine to useful amino acids is described above. Next, the enzymatic transformation of the D-isomer of 3-chloroalanine into D-cysteine and D-cysteine-related amino acids is described.

Manning et al. (*27*) demonstrated that 3-chloro-D-alanine is an effective antibacterial agent. They indicated that the antibacterial action of 3-chloro-D-alanine is a result of the inactivation of both D-amino acid transaminase and alanine racemase; namely, biosynthesis of the peptidoglycan layer of the bacterial cell wall is prevented. On the other hand, Kaczorowski et al. (*28*) have shown that 3-chloro-D-alanine causes rapid inactivation of the dehydrogenase-coupled transport systems in membrane vesicles.

These physiologically interesting effects of 3-chloro-D-alanine, as a suicide substrate or an inhibitor of active transport systems in membrane

TABLE 4
Physicochemical Properties of
3-Chloro-D-alanine Dehydrochlorinase

Molecular weight	
sedimentation-equilibrium	76,000
gel-filtration	73,000
Subunit molecular weight	
SDS-gel electrophoresis	38,000
Number of subunits	2
PLP content (mol/mol)	2
K_m for PLP (M)	2.3×10^{-8}
Absorption maxima (nm)	278,418
$E_{278\ nm}/E_{418\ nm}$	3.44
$E^{1cm}_{1\%}$ at 280 nm	4.89
Isoelectric point	4.8
$s^0_{20,\ w}(S)$	6.6

vesicles, prompted us to search for strains of bacteria resistant to this compound. Recently we isolated some bacteria belonging to the genus *Pseudomonas* that show resistance to 3-chloro-D-alanine (*29,30*). These resistant pseudomonads grow well in medium containing a high concentration of 3-chloro-D-alanine. In the crude cell extracts of these resistant strains, an enzyme activity, which degrades 3-chloro-D-alanine, was found. The enzyme was designated as 3-chloro-D-alanine dehydrochlorinase (E.C. 4.5.1.2). The enzyme is induced markedly only by 3-chloro-D-alanine. The inducibility increases with increasing 3-chloro-D-alanine concentration up to 0.25% (w/v). We have purified and crystallized the enzyme as small rods (*31*). Its physicochemical properties are summarized in Table 4.

The ability of the enzyme to catalyze the α,β-elimination reactions of various amino acids has been examined (*31*). The enzyme is specific for D-alanine; 3-chloro-L-alanine is not degraded. In addition to 3-chloro-alanine (V_{max}, 311 μmol/min/mg) (Table 5), D-cysteine serves as an effective

TABLE 5
Multifunctional Catalysis of 3-Chloro-D-alanine Dehydrochlorinase

	V_{max}, μmol/min/mg
α,β-Elimination reaction	
D-$ClCH_2CH(NH_2)COOH + H_2O \rightarrow CH_3COCOOH + NH_3 + HCl$	311
D-$HSCH_2CH(NH_2)COOH + H_2O \rightarrow CH_3COCOOH + NH_3 + H_2S$	106
β-Replacement reaction	
D-$ClCH_2CH(NH_2)COOH + NaHS \rightarrow$ D-$HSCH_2CH(NH_2)COOH + NaCl$	1620
Reverse α,β-elimination reaction	
$CH_3COCOOH + NH_3 + NaHS \rightarrow$ D-$HSCH_2CH(NH_2)COOH + NaOH$	0.2

substrate (V_{max}, 106 μmol/min/mg). The formation of pyruvate from D-cystine is also catalyzed, but to a lesser extent, by the enzyme (V_{max} 27 μmol/min/mg). The α,β-elimination of D-serine was barely detected (V_{max} 0.03 μmol/min/mg). With other amino acids, no activities were detected, even when a large amount of enzyme was added.

We have found that 3-chloro-D-alanine dehydrochlorinase also catalyzes the β-replacement reaction in the presence of a high concentration of sodium hydrosulfide. The product of the β-replacement reaction has been identified as D-cysteine by physiocochemical means (*30*). The ratio of the α,β-elimination and β-replacement reactions is controlled by the concentration of sodium hydrosulfide in the reaction mixture (Fig. 8). At the concentration of 0.1*M* sodium hydrosulfide, pyruvate formation (α,β-elimination reaction) is almost completely depressed. Under these conditions, in which the β-replacement reaction dominates, about 90% of added 3-chloro-D-alanine is converted to D-cysteine. According to the

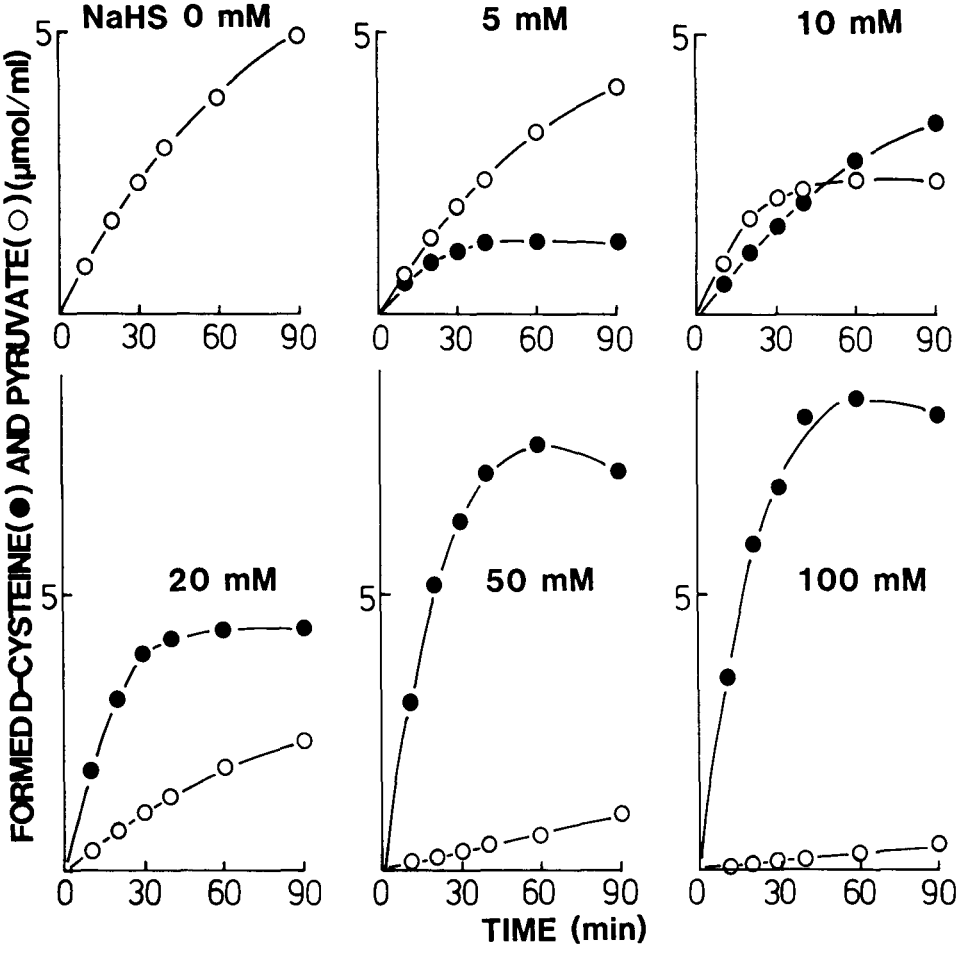


Fig. 8. Formation of D-cysteine by the β-replacement reaction catalyzed by 3-chloro-L-alanine dehydrochlorinase.

method of Lineweaver and Burk, the apparent K_m values were found to be $8.1 \times 10^{-3}M$ for 3-chloro-D-alanine and $4.5 \times 10^{-2}M$ for sodium hydrosulfide. The V_{max} value for the synthesis of D-cysteine was calculated to be 1620 μmol/min/mg (Table 5).

Various thiol compounds can be substituted for sodium hydrosulfide in the β-replacement reaction and produce the corresponding *S*-substituted D-cysteine derivatives (Table 6). We have isolated and identified physiocochemically these compounds (*32*). Bulky mercaptans cannot be used as substrates, even though they include a sulfhydryl group. Some thiol compounds with a free carboxyl group cannot act as substrates (*33*).

The probable mechanisms of the α,β-elimination and β-replacement reactions of this enzyme are shown in Fig. 3. However, the removal and reprotonation of the α-proton occur on a different side from the case of L-amino-acid-specific pyridoxal-P-dependent enzymes. The reverse of the α,β-elimination reaction is also catalyzed by the enzyme, although the V_{max} value is very low.

Using the β-replacement reaction, the enzymatic synthesis of D-cysteine by resting cells has been investigated. For production of bacterial cells showing high D-cysteine producing activity, cultivation has been carried out at 28°C for 24 h in a medium (pH 7.0) containing 0.5% (w/v) 3-chloro-D,L-alanine, 1% (w/v) glucose, 0.5% (w/v) soybean hydrolyzate, 0.5% (w/v) yeast extract, and mineral salts. The reaction conditions for D-cysteine production have been optimized (*34*). When the incubation is carried out at 30°C for 2 h in a reaction mixture (100 mL) containing 100 m*M* 3-chloro-D-alanine·HCl, 500 m*M* sodium hydrosulfide, 4% (v/v) acetone, 100 m*M* potassium phosphate buffer (pH 7.5), and cells harvested from 100 mL of cultured broth, 100% of the added 3-chloro-D-alanine is converted to D-cysteine. When 200 m*M* 3-chloro-D-alanine·HCl are added, 22.6 g of D-cysteine/100 mL of reaction mixture as the highest titer, can be synthesized (Fig. 9). The addition of 2–4% (v/v) acetone, which corresponds to 2.7–5.4 times the concentration of added 3-chloro-D-alanine, enhances the productivity of D-cysteine.

In cells of *Pseudomonas putida* CR 1-1 cultivated in a medium supplemented with a 0.25% (w/v) of 3-chloro-D-alanine·HCl, an *O*-acetylserine sulfhydrylase that forms L-cysteine from 3-chloro-L-alanine and sodium hydrosulfide was found, though the yield was low. From an economical point of view, it is important to depress the enzymatic conversion of 3-chloro-Lalanine into L-cysteine during the process of D-cysteine production. The reasons are that the D-cysteine sample produced should be free from contamination by L-cysteine, and, furthermore, the remaining 3-chloro-L-alanine can be used for the synthesis of other amino acids, for example, L-tyrosine and L-cysteine, and their derivatives. We were able to depress the synthesis of L-cysteine by treating the cells with phenylhydrazine, and the recovery of 3-chloro-L-alanine was remarkably enhanced by this treatment (*35*).

TABLE 6
Relative Synthetic Rates of D-Cysteine-Related Amino Acids from 3-Chloro-D-alanine and Mercaptans by 3-Chloro-D-alanine Chloride-Lyase

Substrate	Product	Relative rate, %
H_2S	D-Cysteine	100
CH_3SH	CH_3S—R	17.8
CH_3CH_2SH	CH_3CH_2S—R	21.4
$CH_3CH_2CH_2SH$	$CH_3CH_2CH_2S$—R	14.5
$(CH_3)_2CHSH$	$(CH_3)_2CHS$—R	4.6
$CH_3CH_2CH_2CH_2SH$	$CH_3CH_2CH_2CH_2CH_2S$—R	21.6
$(CH_3)_2CHCH_2SH$	$(CH_3)_2CHCH_2S$—R	2.0
$CH_3CH_2CH(CH_3)SH$	$CH_3CH_2CH(CH_3)S$—R	3.3
$(CH_3)_3CSH$	$(CH_3)_3CS$—R	0.4
CH_2=$CHCH_2SH$	CH_2=$CHCH_2S$—R	36.9
Φ—SH	Φ—S—R	21.8
Φ—CH_2SH	Φ—CH_2S—R	4.5
CH_3 Φ—SH	C Φ—S—R	0.4
$_3HC$ Φ—SH	$_3HC$ Φ—S—R	14.1
CH_3—Φ—SH	CH_3—Φ—S—R	7.1
$COOCH_2CH_3$ | CH_2SH	$COOCH_2CH_3$ | CH_2S—R	140
CH_2OH | CH_2SH	CH_2OH | CH_2S—R	71.0
CH_2OH | CHOH | CH_2SH	CH_2OH | CHOH | CH_2S—R	34.4
EtOOCCHCH_2SH | NH_2	EtOOCCHCH_2S—R | NH_2	9.5

[a]R: D-alanyl moiety.

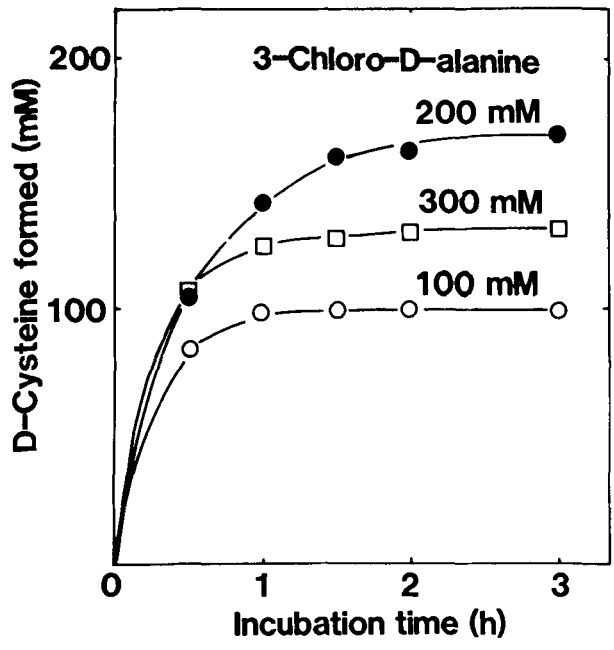


Fig. 9. Enzymatic synthesis of D-cysteine by the cells of *Pseudomonas putida* CR 1-1.

Recently, the use of D-amino acids has become increasingly important in medicine because of their peculiar properties. In particular, cephalosporins contain D-amino acids as important components for their efficacy. Recently, it has been reported that D-cysteine is an important constituent of a new semisynthetic β-lactam antibiotic, MT-141 (Fig. 10), which has a marked antibacterial activity in vivo and causes potent lysis of bacterial cells (*36*). Previously unknown *S*-substituted D-cysteine derivatives could be synthesized by taking advantage of the β-replacement reaction of a multifunctional enzyme. The pharmaceutical application of these D-cysteine derivatives seems to be promising.

3-Chloroalanine has been produced recently as a racemate through chemical synthesis, and it is regarded as a promising starting material for

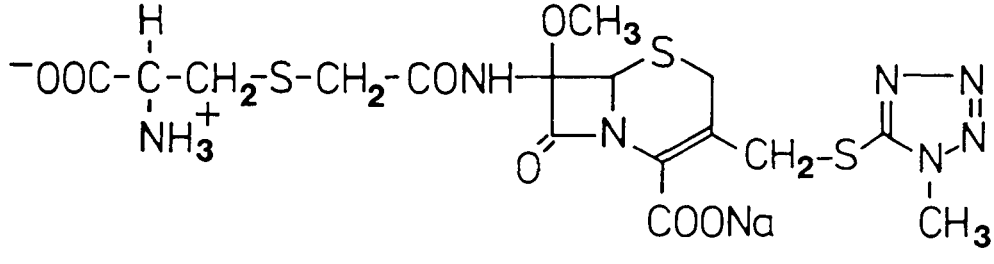


Fig. 10. Structure of MT-141. 7β-(2-D-Amino-2-carboxyethylthioacetoamide)-7α-methoxy-3-(1-methyl-1H-tetrazol-5-yl)thiomethyl-3-cephem-4-carboxylic acid.

the production of various amino acids. However, as 3-chloroalanine markedly inhibits racemase activity, the enzymatic racemization of 3-chloroalanine is impossible. That is, only the L- or D-isomer of 3-chloroalanine should be transformed to the desired product, respectively. Therefore, at present, only the independent application of the L- and D-isomers can only be considered.

In this review we have presented some examples of enzymatic transformations of chemically synthesized 3-chloroalanine into useful amino acids. In the enzymatic processes, the chemically synthesized compounds or their derivatives are used as substrates for the production of useful compounds. Accordingly unnatural compounds can also be synthesized enzymatically as long as the substrate specificity of the enzyme allows it. Thus, the successful combination of the enzymatic process with a chemical synthesis process will become more significant and promising as a new means of producing various useful compounds. In the future, new substrates for enzymatic processes will be designed and synthesized along with the development of new chemical synthesis techniques. Then, new enzymes and new functions of already known enzymes that act on new substrates will be found. Thus, the microbial catalytic ability will be used widely in various fields of chemical industry. Enzymatic process are expected to contribute to the microbial production of useful compounds.

REFERENCES

1. Morino, Y. (1973), *Seikagaku* **45,** 993.
2. Morino, Y., and Watanabe, T. (1970), *Seikagaku* **42,** 556.
3. Tanase, S., Morino, Y., Ohosumi, T., and Soda, K. (1973), *Seikagaku* **45,** 443.
4. Walsh, C., Schonbrumm, A., and Abeles, R. T. (1971), *J. Biol. Chem.* **246,** 6855.
5. Inoue, C., and Moriguchi, M. (1983), *Japanese Patent Kokoku,* 58-22140.
6. Ooka, S., Kato, T., Mita, R., Kawashima, N., Higuchi, C., Kawashima, N., Yamada, A., Nagai, H., and Takano, T. (1983), *Japanese Patent Kokai* 58-8049.
7. Kumagai, H., and Miles, E. W. (1971), *Biochem. Biophys. Res. Commun.* **44,** 1271.
8. Esaki, N., Tanaka, H., Miles, E. W., and Soda, K. (1983), *Agric. Biol. Chem.* **47,** 2861.
9. Kumagai, H., Nakayama, R., Nishikawa, K., Nishida, A., and Tochikura, T. (1983), *Symposium on Japanese Agricultural Chemistry.* (Nishinippon and Kansai Combined Meeting), p. 22. (1983).
10. Kumagai, H., Yamada, H., Matsui, H., Ohkishi, H., and Ogata, K. (1970), *J. Biol. Chem.* **245,** 1767.
11. Kumagai, H., Kashima, N., Yamada, H., Enei, H., and Okumura, S. (1972), *Agric. Biol. Chem.* **36,** 472.
12. Nagasawa, T., Yamano, H., Tani, Y., and Yamada, H. (1980), *Seikagaku,* **52,** 618.
13. Yamada, H., and Kumagai, H. (1975), *Adv. Appl. Microbiol.* **19,** 249.
14. Yamada, H., Kumagai, H., Kashima, H., and Torii, H. (1972), *Biochem. Biophys. Res. Commun.* **46,** 370.

15. Nagasawa, T., Utagawa, T., Goto, J., Kim, C. J., Tani, Y., Kumagai, H., and Yamada, H. (1981), *Eur. J. Biochem.* **117,** 32.
16. Enei, H., Nakazawa, H., Okumura, S., and Yamada, H. (1973), *Agric. Biol. Chem.* **37,** 725.
17. Yamada, H., and Kumagai, H., (1978), *Pure Appl. Chem.* **50,** 1117.
18. Kredich, N. M., Keenen, B. S., and Foote, L. J., (1972), *J. Biol. Chem.* **247,** 7157.
19. Kumagai, H., Sejima, S., Choi, Y., and Yamada, H. (1974), *FEBS Lett.* **52,** 304.
20. Kumagai, H., Tanaka, H., Sejima, S., and Yamada, H. (1977), *Agric. Biol. Chem.* **41,** 2071.
21. Ohkishi, H., Nishikawa, D., Kumagai, H., and Yamada, H. (1981), *Agric. Biol. Chem.* **45,** 259.
22. Kumagai, H., Choi, Y., Sejima, S., and Yamada, H. (1975), *Agric. Biol. Chem.* **39,** 387.
23. Dhillon, G. S., Nagasawa, T., and Yamada, H. (1984), *J. Biotechnol.* **1,** 47.
24. Kredich, N.M., and Tomkins, G. M. (1969), *J. Biol. Chem.* **241,** 4955.
25. Nagasawa, T., Dhillon, G. S., and Yamada, H. (1985), *J. Biotechnol.* **2,** 365.
26. Flavin, M., and Slaughter, C. (1963), *Biochemistry,* **4,** 1370.
27. Manning, J. M., Merrifield, N. E., Jones, W. M., and Gotschlich, E. C. (1974), *Proc. Natl. Acad. Sci. USA* **71,** 417.
28. Kaczorowski, G., and Walsh, C. (1975), *J. Biol. Chem.* **250,** 8931.
29. Ohkishi, H., Nishikawa, D., Kumagai, H., and Yamada, H. (1981), *Agric. Biol. Chem.* **45,** 2397.
30. Yamada, H., Nagasawa, T., Ohkishi, H., Kawakami, B., and Tani, Y. (1981), *Biochem. Biophys. Res. Commun.* **100,** 1104.
31. Nagasawa, T., Ohkishi, H., Kawakami, B., Yamano, H., Hosono, H., Tani, Y., and Yamada, H. (1982), *J. Biol. Chem.* **257,** 13749.
32. Nagasawa, T., Hosono, H., Ohkishi, H., Tani, Y., and Yamada, H. (1983), *Biochem. Biophys. Res. Commun.* **111,** 809.
33. Nagasawa, T., Hosono, H., Ohkishi, H., and Yamada, H., (1983), *Appl. Biochem. Biotech.* **8,** 481.
34. Nagasawa, T., Yamano, H., Hosono, H., Ohkishi, H., Tani, Y., and Yamada, H. (1982), *Agric. Biol. Chem.* **46,** 13003.
35. Nagasawa, T., Hosono, H., Yamano, H., Ohkishi, H., Tani, Y., and Yamada, H. (1983), *Agric. Biol. Chem.* **47,** 861.
36. Iwamatsu, K., Inouye, S., Tsuruoka, T., Mizutani, K., Omoto, S., Ogino, H., Miyauchi, K., Watanabe, T., and Niida, T. (1983), *J. Antibiot.* **36,** 229.