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One-pot chemo-enzymatic enantiomerization of racemates

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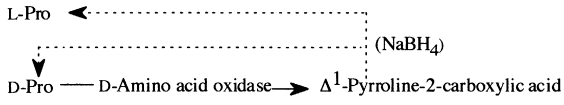
Abstract

A new one-pot chemo-enzymatic procedure was developed for enantiomerization of racemates based on enzymatic enantiospecific oxidation of a substrate and chemical non-enantiospecific reduction of the product. The principle is shown as follows for the L-proline production.



L-Proline and L-pipecolate were produced from racemic proline and pipecolate by means of D-amino acid oxidase and sodium borohydride in high yield in this reaction system [J.W. Huh, K. Yokoigawa, N. Esaki, K. Soda, Biosci., Biotechnol., Biochem. 56 (1992) 2081]. DL- and L-Lactate were DL-enantiomerized in a one-pot reaction system containing L-lactate oxidase and sodium borohydride in the similar manner [S. Mukoyama, K. Yamanaka, T. Oikawa, K. Soda, Nippon Nogei Kagaku Kaishi 73 (1999) 62]. Pyruvate was also converted to an equimolar amount of D-lactate in the same system. D-α-Hydroxybutyrate can be produced from the DL- and L-isomers, and α-ketobutyrate in the same manner though slowly. This method is applicable to production of other chiral compounds from the corresponding racemates.

Keywords: Chemo-enzymatic enantiomerization; One-pot; Racemates

1. Introduction

Stereospecificity is one of the most salient characteristics of enzymes. Enzymes catalyze a variety of reactions with only one of an enantiomeric on diastereomeric pair of chiral compounds. Most of the important physiological compounds that occur in nature and are produced chemically are chiral such as amino acids, hydroxy acids and sugars. They are widely used for the synthesis of pharmaceutical compounds, agrochemicals, cosmetics and others. Though asymmetric syntheses of chiral compounds are possible in some cases, they are generally very difficult, and in particular, their large-scale production is substantially impossible. Accordingly, chiral compounds are usually produced with growing, intact, resting and dried cells, and enzymes. Almost all of L-amino acids are now produced by fermentation, and some

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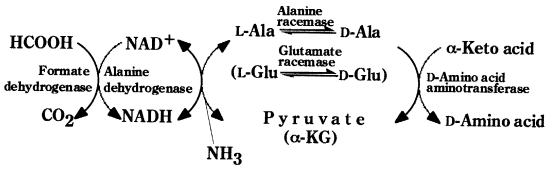

Fig. 1. Enzymatic synthesis of D-amino acids by coupling of reactions of four enzymes: formate dehydrogenase, L-alanine dehydrogenase or L-glutamate dehydrogenase, alanine racemase or glutamate racemase and D-amino acid aminotransferase.

are manufactured with enzymes [1]. Various enzymatic procedures have been reported for the enantiomer synthesis of amino acids from racemic starting materials [2–6]. Although many have dealt with kinetic resolution of the racemate, the coupled conversion of a racemate into a single enantiomer is much more convenient. We developed, for instance, an enzymatic method for the total conversion of racemic methionine to the L-enantiomer by a multienzyme system [7]. The conversion of DL-alanine and DL-leucine to their L-enantiomers was also carried out by a closely similar method [7]. In another report, we showed a new procedure for the stereospecific conversion of various α-keto acids into the corresponding D-amino acids with four thermostable enzymes as shown in Fig. 1 [8,9]. In the reaction system, D-amino acid aminotransferase catalyzes transamination of D-alanine with α-keto acids such as phenylpyruvate, which are chemically produced in industry to produce pyruvate and corresponding D-amino acids such as D-phenylalanine. Alanine dehydrogenase catalyzes formation of L-alanine from pyruvate produced, and ammonia in the presence of NADH, and L-alanine is racemized to supply D-alanine. NADH is regenerated from NAD^+ though the reaction of formate dehydrogenase, in which formate added is irreversibly oxidized to carbon dioxide. D-Alanine, pyruvate and L-alanine, and also NADH and NAD^+ are circulated in the system, and accordingly, only a small amount of them are required. Alanine racemase, L-alanine dehydrogenase, and D-alanine can be replaced by glutamate racemase, L-glutamate dehydrogenase, and D-glutamate in another reaction system forming D-amino acids from their keto analogues. The pH optima of the four enzymes were similar to one another (about pH 7.6). For example, D-enantiomers of glutamate, leucine, norleucine, methionine, and valine with high optical purity were produced at high conversion rates, when the reaction system containing L-glutamate dehydrogenase, glutamate racemase, and glutamate was incubated at 37°C for 8 h. Recently, we developed chemo-enzymatic methods for production of an enantiomer from the racemates, and here describe outlines of these new methods based on the strict stereospecificity and low structural specificity of D-amino acid oxidase [10], and efficient and non-stereospecific chemical reduction with sodium borohydride.

2. Chemo-enzymatic production of L-proline from the racemate

We, in the first place, describe a simple and convenient procedure for the one-pot synthesis of L-proline from the racemate by coupling of the reactions of D-amino acid oxidase and sodium borohydride [11] as shown in Fig. 2. In this reaction system, D-proline is enantiospecifically oxidized to form an achiral heterocyclic compound, Δ^1-pyrroline-2-carboxylate, and this is simultaneously reduced to DL-proline with the chemical reductant. Consequently, almost all of DL-proline (and D-proline also) are converted to the L-enantiomer. This chemo-enzymatic L-enantiomerization is based on a simple, but novel principle.

The standard reaction mixture contained 500 mM potassium phosphate buffer (pH 8.0), 30 μM FAD,

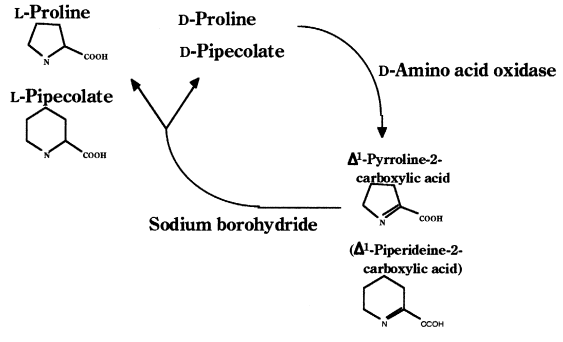

Fig. 2. Chemo-enzymatic synthesis of L-proline and L-pipecolate from their racemates by a coupled reaction of D-amino acid oxidase and sodium borohydride.

2.5 mM DL-proline, 1 mg of bovine serum albumin (as a stabilization of the enzyme) and 0.3 unit of D-amino acid oxidase (0.09 unit/mg) in a total volume of 1.0 ml. During incubation at 37°C for 60 min with shaking, the enzyme and sodium borohydride were added at 10-min intervals, and the reaction was terminated by addition of 0.5 ml of 2N HCl. Each enantiomer of proline was analyzed by enantioselective high-performance liquid column chromatography (HPLC) with 0.25 mM $CuSO_4$ solution as an eluent. D-Proline was determined with D-amino acid oxidase as well. The product isolated was purified by Dowex 50 (X8) column chromatography, and identified as L-proline by enantioselective HPLC, but D-proline was not detected. Fig. 3 shows the time course of D-proline consumption and L-proline production: D-proline was quantitatively converted to the L-enantiomer. Addition of larger amounts of the enzyme led to the same results, and an increase in the amount of sodium borohydride caused inhibition of the enzyme. This is probably the first example of a one-pot chemo-enzymatic conversion of racemic compounds to their enantiomers through an enantiospecific oxidation of the starting material and non-enantiospecific reduction of the achiral intermediate.

3. Chemo-enzymatic production of L-pipecolate from the racemate

L-Pipecolate is a non-proteinous N-replaced amino acid occurring in plants, animals and fungi, and a homologue of L-proline. We applied the chemo-enzymatic procedure for the L-enantiomerization of DL-proline to the production of L-pipecolate from the racemate. The standard reaction mixture contained 500 μmol of potassium phosphate buffer (pH 8.0), 30 nmol of FAD, 2.5 μmol of DL-pipecolate, 1 mg of bovine serum albumin and 0.3 unit of D-amino acid oxidase in a total volume of 1.0 ml. The reaction was carried out at 37°C with shaking. D-Pipecolate was oxidized to an achiral heterocyclic compound, Δ^1-piperideine-2-carboxylate by catalysis of D-amino acid oxidase, and Δ^1-piperideine-2-carboxylate was immediately reduced to DL-pipecolate (Fig. 3). D-Pipecolate was fully converted to the L-enantiomer. Both Δ^1-pyrroline 2-carboxylate and Δ^1-piperideine-2-carboxylate enzymatically produced are relatively stable even in an aqueous solution, although they are in equilibrium with α-keto-δ-imino valerate and α-keto-ε-amino caproate, respectively. They are effectively reduced to racemic proline and pipecolate, and consequently L-enantiomers of proline and pipecolate are produced in a very high yield. Other similar cyclic DL- and L-N-replaced amino acids and their derivatives that are oxidized by the enzyme are also chemo-enzymatically L-enantiomerized in the same way.

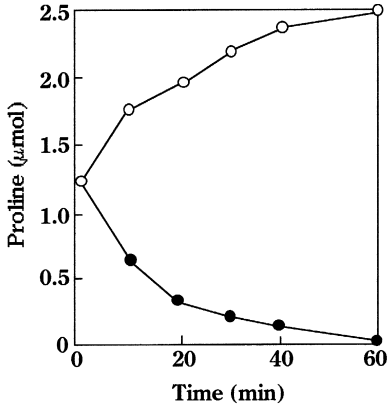

Fig. 3. Conversion of D-proline to L-proline.

4. Chemo-enzymatic production of D-lactate from racemic and L-lactates, and pyruvate

L-Lactate is industrially produced by fermentation, and widely used in food industry, chemical industry and so forth. In contrast, D-lactate is useful as a starting material of agrochemicals, angiotensin-converting enzyme inhibitors and others, but very expensive. D-Lactate is also produced by fermentation with *Lactobacillus delbrueckii* and several other lactic acid bacteria [12]. We developed D-lactate production from racemic 2-chloropropionate (2-CPA) with bacterial L-2-haloacid dehalogenase as shown in Fig. 4 [13]. L-2-CPA of the racemate is dehalogenated to D-lactate with inversion of the configuration at the carbon-2 of substrate by L-2-haloacid dehalogenase from *Pseudomonas putida*. D-2-CPA unreacted is chemically dehalogenated to D-lactate

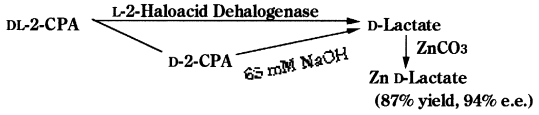

Fig. 4. Chemo-enzymatic D-lactate production from DL-2-CPA.

with retention of the C-2 configuration in the alkaline conditions.

We recently developed the one-pot chemo-enzymatic method for the D-enantiomerization of DL-lactate (Fig. 5) [14]. L-Lactate oxidase from *Aerococcus viridans* is produced by Asahi Chemicals, Tokyo. We used the enzyme in order to specifically oxidize L-lactate to pyruvate. Pyruvate is reduced to racemic lactate with sodium borohydride. Consequently, L-lactate is eventually converted to D-lactate.

The reaction mixture contained 0.5 M potassium phosphate buffer (pH 8.0), 0.05 mg of sodium borohydride, 5 mM DL-lactate, 1 mg of bovine serum albumin, 0.05 unit of L-lactate oxidase, and 0.01 unit of catalase in a final volume of 1.0 ml. The reaction was carried out at 37°C for 90 min with shaking. After termination of the reaction with HCl (a final concentration of 0.7 M), D- and L-lactates were determined with D- and L-lactate oxidase, respectively, and pyruvate was analyzed with salicylaldehyde at 480 nm [15]. The reaction product was analyzed by HPLC with a Chiral Pak MA (+) (Daicel Chemical, Tokyo, the eluent: 2 mM $CuSO_4$, 0.5 ml/min, at 30°C), and we identified it as D-lactate by determination of the retention time (6 min) and co-chromatography with the authentic D-lactate.

We optimized the reaction conditions. The highest yield was obtained at pH 8.0 and 8.5, and also at 37°C. Sodium borohydride was much more effective

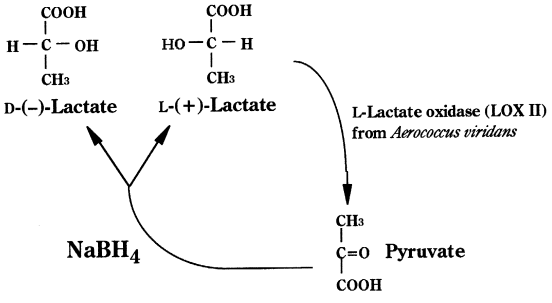

Fig. 5. One-pot chemo-enzymatic D-enantiomerization of DL-lactate.

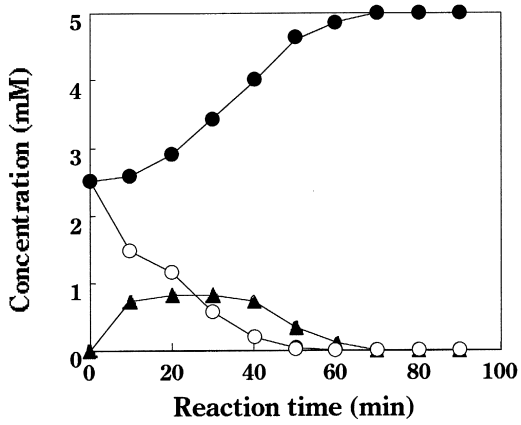

Fig. 6. Time course of D-enantiomerization of DL-lactate. ●: D-Lactate, = ○: L-Lactate, = △: Pyruvate.

than sodium cyanoborohydride as a reductant, and the highest yield was obtained when the reaction was carried out for 90 min with 0.05 mg of sodium borohydride. The addition of a larger amount of sodium borohydride such as 0.4 mg decreased the yield.

Fig. 6 shows the time course of D-lactate production, L-lactate consumption and pyruvate formation during the reaction. L-Lactate was quantitatively converted to D-lactate and pyruvate. The formation of pyruvate suggests that the amount of sodium borohydride was insufficient to reduce pyruvate formed in the early stage of reaction. However, after incubation for 70 min, pyruvate disappeared, and the theoretical amount of D-lactate was produced. L-Lactate and pyruvate were also converted to an equimolar amount of D-lactate in this one-pot reaction system, though the conversion rate was relatively low when L-lactate was used as a starting material.

The concentrations of racemic lactate practically did not affect the yield. DL-Lactate was fully converted to the D-enantiomer even when a high concentration of DL-lactate was used, though a longer time was required.

5. Discussion

The one-pot chemo-enzymatic methods for enantiomerization of racemates described here consist of

(1) the enantiospecific oxidase reaction of a substrate to an achiral product, (2) the chemical non-enantiospecific reduction of the achiral product to the racemic starting material. Coupling of (1) and (2) reactions leads to the enantiomerization. Actually, L-proline and L-pipecolate were produced from racemic substrates with D-amino acid oxidase and sodium borohydride. When D-amino acids were used as substrates, achiral imino acids produced as a primary product are very labile and have to be reduced as rapidly as possible in order to avoid hydrolysis of imino acids and produce racemic amino acids. However, substrate D-replaced amino acids such as D-proline and D-pipecolate are oxidized to stable products, Δ^1-pyrroline 2-carboxylate and Δ^1-piperideine 2-carboxylate, respectively, which are not spontaneously hydrolyzed although are in equilibrium with their open forms. The products, heterocyclic compounds, are easily reduced to racemic proline and pipecolate with sodium borohydride. Consequently, D-proline and D-pipecolate were consumed, and instead, the L-enantiomers were exclusively produced with a yield of more than 98%, and optically pure.

D-Lactate was produced from racemic and L-lactate and pyruvate with a high yield in the one-pot chemo-enzymatic reaction system with L-lactate oxidase and sodium borohydride.

The enzyme reactions, in general, have to be carried out under strict conditions. For example, enzymes function in narrow pH ranges. Accordingly, when two different kinds of enzyme reactions are used in a one-pot reaction system, both are required to have similar pH optima. In contrast, chemical reactions usually do not require such a rigid condition. Therefore, chemo-enzymatic reactions are versatile in one-pot systems. This one-pot chemo-enzymatic method for enantiomerization of racemic chiral compounds is widely applicable. L-2-Hydroxybutyrate, for example, can be produced in the same way, although relatively slow. We are currently studying application of this method to the D-enantiomerization of racemic and L-amino acids such as glutamate by the use of L-glutamate oxidase and so forth.

References

[1] K. Soda, H. Tanaka, N. Esaki, in: H.J. Rehm, G. Reed (Eds.), Biotechnology, vol. 3, Verlag Chemie, Weinheim, 1983, p. 479.
[2] I. Chibata, T. Tosa, T. Sato, T. Mori, Methods Enzymol. 44 (1976) 746.
[3] H. Yamada, S. Shimizu, H. Shimada, Y. Tani, S. Takahashi, T. Ohashi, Biochimie 62 (1980) 395.
[4] T. Fukumura, Agric. Biol. Chem. 41 (1977) 1327.
[5] K. Sano, K. Mitsugi, Agric. Biol. Chem. 42 (1978) 2315.
[6] D. Rozzell, F. Wagner, Biocatalytic Production of Amino Acids and Derivatives, Hanser, New York, 1992.
[7] N. Nakajima, N. Esaki, K. Soda, J. Chem. Soc., Chem. Commun. (13) (1990) 947.
[8] N. Nakajima, K. Tanizawa, H. Tanaka, K. Soda, J. Biotechnol. 8 (1988) 243.
[9] A. Galkin, L. Kulakova, H. Yamamoto, K. Tanizawa, H. Tanaka, N. Esaki, K. Soda, J. Ferment. Bioeng. 83 (1997) 299.
[10] A. Meister, Biochemistry of the Amino Acids, vol. 1, Academic Press, New York, 1965, p. 297.
[11] J.W. Huh, K. Yokoigawa, N. Esaki, K. Soda, J. Ferment. Bioeng. 74 (1992) 189.
[12] K. Buchta, in: H.-J. Rehm, G. Reed (Eds.), Biotechnology, vol. 3, Verlag Chemie, Weinheim, 1983, p. 409.
[13] J.W. Huh, K. Yokoigawa, N. Esaki, K. Soda, Biosci., Biotechnol., Biochem. 56 (1992) 2081.
[14] S. Mukoyama, K. Yamanaka, T. Oikawa, K. Soda, Nippon Nogei Kagaku Kaishi 73 (1999) 62.
[15] M.H. Saier Jr., W.T. Jenkins, J. Biol. Chem. 242 (1967) 91.