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Production of aromatic D-amino acids from α-keto acids and ammonia by coupling of four enzyme reactions [1]

Hee-Sung Bae [a], Seung-Goo Lee [a], Seung-Pyo Hong [a], Mi-Sun Kwak [a], Nobuyoshi Esaki [b], Kenji Soda [c], Moon-Hee Sung [a,*]

[a] *Microbial conversion Research Unit, Korea Research Institute of Bioscience and Biotechnology (KRIBB), P.O. Box 115, Yusong, Taejon 305-600, South Korea*

[b] *Laboratory of Microbial Biochemistry, Institute for Chemical Research, Kyoto University, Uji, Kyoto 611, Japan*

[c] *Department of Biotechnology, Kansai University, 3-3-35, Yamate-cho, Suita-shi, Osaka 564, Japan*

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Abstract

A multi-enzyme system composed of glutamate racemase, thermostable D-amino acid aminotransferase, glutamate dehydrogenase and formate dehydrogenase was employed for the production of aromatic D-amino acids, D-phenylalanine and D-tyrosine, from the corresponding α-keto acids, phenylpyruvate and hydroxyphenylpyruvate, respectively. The optimal concentration of ammonium formate for the production of these D-amino acids was found in the range of 0.25–1.0 M. The optimal concentration of α-keto acid was determined to be 50 mM, above which the productivity greatly decreased. To keep the concentration of α-keto acid around this concentration, α-keto acid was intermittently fed into the multi-enzyme system during the production period. By running the multi-enzyme system for 35 h, 48 $g l^{-1}$ of D-phenylalanine and 60 $g l^{-1}$ of D-tyrosine were produced with 100% of optical purity from the equimolar amounts of phenylpyruvate and hydroxyphenylpyruvate, respectively. The production levels of both aromatic D-amino acids were demonstrated to be dependent on the stability of glutamate racemase.

Keywords: Aromatic D-amino acid; D-Amino acid aminotransferase; Glutamate racemase; α-Keto acid; Multi-enzyme system

1. Introduction

Aromatic D-amino acids are widely used in the pharmaceutical field as precursors for the synthesis of antibiotics, bioactive peptides, and other physiologically active compounds. Among them, D-phenylalanine is used as an intermediate for the synthesis of optically active peptides such as dextroamphetamine sulfate, methamphetamine hydrochloride [1] and *l*-norephedrine hydrochloride [2], and is an important component of antibiotics (gramicidin), neuropeptides (achatin) and peptide sweeteners. D-Phenylalanine, itself, has also been shown to play a physiologically important role in animal cells [3,4].

Some feasible methods are known for the industrial production of aromatic D-amino acids,

* Corresponding author. Tel.: +82-42-860-4372; fax: +82-42-860-4595; E-mail: smoonhee@kribb4680.kribb.re.kr

[1] Dedicated to Professor Hideaki Yamada in honor of his 70th birthday.

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which include the purification of D-phenylalanine by the optical resolution of *N*-acetyl-D,L-phenylalanine with an amidase (EC 3.5.1.4) [5], as well as the treatment of substituted hydantions or a D,L-phenylalanine racemic mixture with a culture broth of microorganisms [6]. Takahashi et al. [7] have developed a chemo-enzymatic route for the production of various D-amino acids including aromatic D-amino acids from hydantoin derivatives with hydantoinase.

Recently, multi-enzyme systems have been developed for the production of various D-amino acids by Nakajima et al. [8] and Galkin et al. [9], in which aromatic D-amino acids, D-phenylalanine and D-tyrosine, can be directly synthesized from phenylpyruvate and hydroxyphenylpyruvate, respectively. However, the large-scale production of D-phenylalanine and D-tyrosine by the multi-enzyme systems is limited because the two of the substrates, phenylpyruvate and hydroxyphenylpyruvate, are poor substrates for D-amino acid aminotransferase (D-AAT) [10,11].

In the present study, we report the reaction conditions necessary to maximize the production of aromatic D-amino acids, D-phenylalanine and D-tyrosine, with a multi-enzyme system composed of glutamate racemase (GluRA), D-AAT, glutamate dehydrogenase (GDH), and formate dehydrogenase (FDH). We also discuss the stability of the 4 enzymes for the production of the aromatic D-amino acids.

2. Experimental

2.1. Enzyme preparation and assay

GluRA was produced with *Escherichia coli* JM109 carrying the plasmid pGR3 encoding the *E. coli* GluRA gene [12]. The cell-free extract of the recombinant cells was used without further purification in this study. GluRA was assayed by the determination of L-glutamate formed from the D-counterpart by an HPLC. The assay mixture contained 10 μmol of D-glutamate, 100 μmol of Tris–HCl buffer (pH 8.5), and crude enzyme in a final volume of 1 ml. D-AAT was also produced with *E. coli* HB101 harboring the plasmid pICT113, which carries the thermostable D-AAT gene of *Bacillus* sp. YM-1 [11]. The cell extract was incubated at 60°C for 20 min, and centrifuged to remove the heat-denatured proteins derived from the *E. coli* host cells. The clear supernatant solution was used as the D-AAT preparation. The assay mixture contained 50 μmol of D-alanine, 5 nmol of pyridoxal 5′-phosphate, 100 μmol of Tris–HCl buffer (pH 8.5), 50 μmol of α-ketoglutarate and enzyme in a final volume of 1 ml. Pyruvate formed in the mixture was determined by the salicylaldehyde method [13].

GDH from bovine liver and FDH from *Candida boidinii* were purchased from Boehringer Mannheim (Mannheim, Germany). Both enzymes were assayed spectrophotometrically as described previously [14,15].

One unit of enzyme was defined as the amount of enzyme catalyzing the formation of 1 μmol of product per min under the conditions used. Specific activity was expressed as units per mg of protein. Protein was determined by Bradford's method [16] with bovine serum albumin as a standard protein.

2.2. Production of aromatic D-amino acids

Unless otherwise stated, the standard reaction mixture for the production of aromatic D-amino acids contained 10 mM of L-glutamate, 1 mM of NAD^+, 50 μM of pyridoxal 5′-phosphate (PLP), 100 mM of Tris–HCl buffer (pH 8.5), 1 M of ammonium formate, an appropriate amount of α-keto acid, and 4 enzymes (GluRA, D-AAT, GDH, FDH). The ratio for the 4 enzymes was adjusted as follows: GluRA: D-AAT: GDH: FDH = 1: 5: 10: 1 in units each enzyme activity as described previously [8]. The reaction was started by the addition of α-keto acid. One hundred microliters aliquot of the reaction mixture was sampled after the appropriate period of

incubation, and then supplemented with 5 μl of 12 N HCl to stop the reaction.

2.3. Analytical methods

The concentration of D-phenylalanine and phenylpyruvate in the reaction mixture was determined by an HPLC with a reverse-phase C_{18} column (25 cm $\times$ 4.4 mm) and a UV detector at 254 nm. The mobile phase consisted of 50 mM of acetate buffer (pH 6.8)-methanol (95:5, v:v), and its flow rate was adjusted to 1 ml min^{-1}. The amount of D-tyrosine and hydroxyphenylpyruvate was determined at 280 nm.

Enantiomers of amino acids (glutamate, phenylalanine and tyrosine) were clearly separated from each other and detected after the reaction with the mixture of *o*-phthalaldehyde (Sigma) and *N*-acetyl-L-cystein (Sigma) in borate buffer (pH 9.5), by an HPLC with a fluorescence detector (excitation at 342 nm and emission at 452 nm) and a C_{18} column. The mobile phase consisted of 50 mM of acetate buffer (pH 6.8) and methanol (90:10, v:v).

3. Results and discussion

3.1. A multi-enzyme system for production of aromatic D-amino acids

For the production of 2 aromatic D-amino acids (D-phenylalanine and D-tyrosine), a multi-

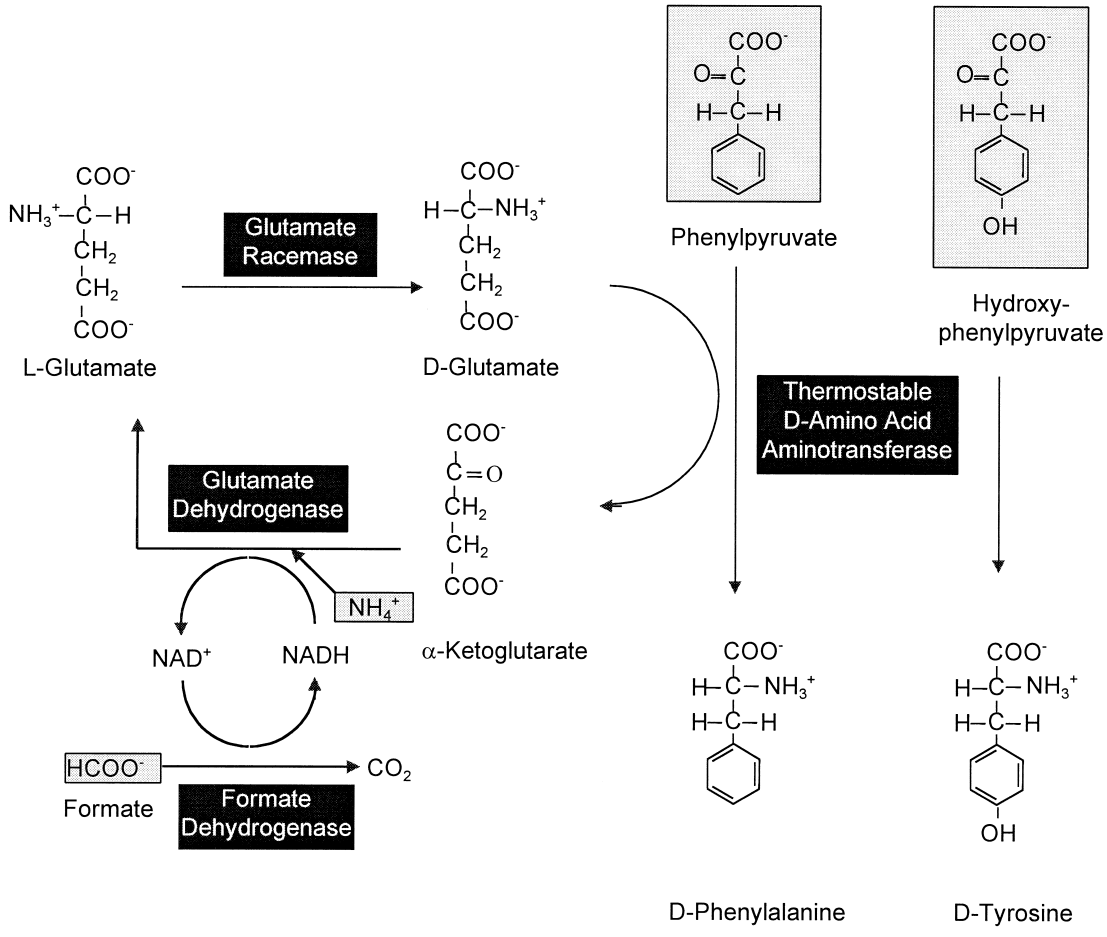

Fig. 1. A multi-enzyme system for the production of D-phenylalanine and D-tyrosine from the corresponding α-keto acids.

enzyme system composed of 4 enzymes (GluRA, D-AAT, GDH, FDH) was constructed (Fig. 1). Each aromatic D-amino acid was produced from the corresponding α-keto acid by a D-AAT reaction with the consumption of D-glutamate. D-glutamate is continuously regenerated by the coupled reactions of GDH and GluRA from α-ketoglutarate, NADH and ammonia. NADH is also regenerated by the FDH reaction from formate. Thus, only small amounts of L-glutamate and NAD^+ are required for the production of aromatic D-amino acids.

3.2. Production of D-phenylalanine in the multi-enzyme system

3.2.1. Effects of substrate concentrations on the production of D-phenylalanine

In order to determine the optimal concentrations of phenylpyruvate and ammonium formate for the production of D-phenylalanine, the reaction was carried out at various concentrations of each substrate, and the activity was calculated by measuring the amount of D-phenylalaine produced in 1 h. As shown in Fig. 2A, the optimal phenylpyruvate concentration was 50 mM, above which D-phenylalanine productivity greatly decreased. When the concentration of phenylpyruvate in the reaction mixture increased up to 300 mM, a relative activity of only 40% was observed. The decreased productivity may be due to the inactivation of enzymes by the high concentration of phenolic substrate, phenylpyruvate.

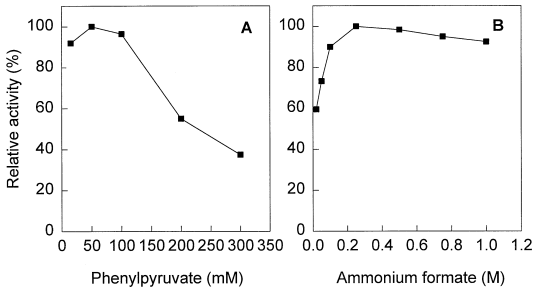

Fig. 2. Effects of the concentrations of (A) phenylpyruvate and (B) ammonium formate on the production of D-phenylalanine. The reaction was performed with the standard reaction mixture (1 ml) except for the concentrations of phenylpyruvate and ammonium formate.

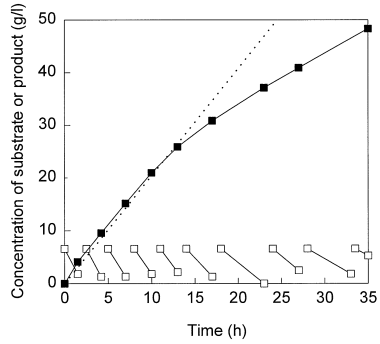

Fig. 3. Production of D-phenylalanine by successive feeding of phenylpyruvate. The reaction was carried out with the standard reaction mixture (10 ml) except for the concentration of phenylpyruvate. The initial ammonium formate concentration was 1.0 M and the phenylpyruvate was intermittently added as described in text. The dotted line indicates the expected productivity on the basis of the initial production rate. Symbols: □, phenylpyruvate; ■, D-phenylalanine.

Ammonium formate gave a maximal productivity at 0.25 M and showed no substrate inhibition (Fig. 2B).

3.2.2. Successive feeding of phenylpyruvate for the production of D-phenylalanine

Because concentrations of phenylpyruvate higher than 50 mM decreased the production of D-phenylalanine, we kept its concentration below 50 mM throughout the intermittent feeding of phenylpyruvate into the standard reaction mixture (10 ml). Under this operation conditions, phenylpyruvate was converted to D-phenylalanine at a constant level of productivity for 13 h; thereafter the initial production rate began to gradually decrease (Fig. 3). After 35 h, approximately 48 $g\,l^{-1}$ of D-phenylalanine was produced from the equimolar amount of supplemented phenylpyruvate. The optical purity of D-phenylalanine produced was found to be 100% in the analysis of the product with an HPLC-fluorescence detector system.

3.3. Production of D-tyrosine in the multi-enzyme system

3.3.1. Effects of substrate concentrations on the production of D-tyrosine

Effects of the concentration of hydroxyphenylpyruvate and ammonium formate on the production of D-tyrosine were examined in the same way described for the production of D-phenylalanine. The optimal concentration was 50 mM of hydroxyphenylpyruvate, above which D-tyrosine productivity decreased, as in the case of D-phenylalanine production (Fig. 4A).

The optimal concentration of ammonium formate for the production of D-tyrosine was 0.25 M, and no substrate inhibition was observed (Fig. 4B).

3.3.2. Successive feeding of hydroxyphenylpyruvate for the production of D-tyrosine

D-Tyrosine production was carried out in the same mode employed for the D-phenylalanine production. D-Tyrosine was stoichiometrically produced from hydroxyphenylpyruvate at a constant level of productivity for approximately 8 h; thereafter the initial production rate began to decrease (Fig. 5). After 35 h, approximately 60 $g\,l^{-1}$ of D-tyrosine with 100% optical purity was produced. As the reaction proceeded, D-tyrosine precipitated as a white precipitate, remaining soluble only up to 40 mM.

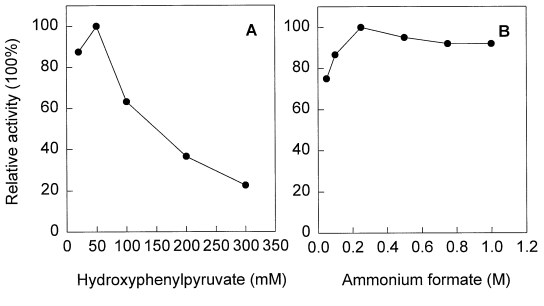

Fig. 4. Effects of the concentrations of (A) hydroxyphenylpyruvate and (B) ammonium formate on the production of D-tyrosine. The reaction was performed with the standard reaction mixture (1 ml) except for the concentrations of hydroxyphenylpyruvate and ammonium formate.

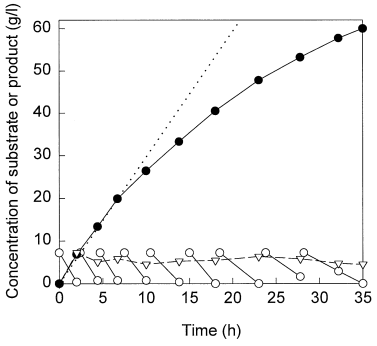

Fig. 5. Production of D-tyrosine by successive feeding of hydroxyphenylpyruvate. The reaction was carried out with the standard reaction mixture (10 ml) except for the concentration of phenylpyruvate. The initial ammonium formate concentration was 1.0 M and the hydroxyphenylpyruvate was intermittently added as described in text. The dotted line indicates the expected productivity on the basis of the initial production rate. Symbols: ○, hydroxyphenylpyruvate; ∇, soluble D-tyrosine; ●, total D-tyrosine.

3.4. Stability of the 4 enzymes in the multi-enzyme system

By keeping the concentration of phenylpyruvate and hydroxyphenylpyruvate below 50 mM, we could maintain the initial production levels of D-phenylalanine and D-tyrosine for 13 h and 8 h, respectively. After that, however, productivity levels of both gradually decreased. To find the reason for this decrease in productivity, we proposed the following possible reasons: (i) product inhibition due to the accumulation of product; (ii) depletion of the PLP for D-AAT or regeneration components (NAD^+ and L-glutamate); (iii) inactivation of an enzyme or enzymes during the 4 enzyme reactions. To determine whether the accumulation of products reduced the productivity, the reaction was performed in the presence of various concentrations of each aromatic D-amino acid (0.05–1.0 M). Initial production rates were not affected by concentrations of aromatic D-amino acids in the tested ranges. These results indicate that the accumulation of product is not the major reason for the decreased productivity. This conclusion was also supported by the decrease in productiv-

Table 1
Recovery of the productivity for aromatic D-amino acids by the supplementation of GluRA

Enzymes[a] supplemented	Aromatic D-amino acids produced[b]	
	D-Phenylalanine (mM)	D-Tyrosine (mM)
GluRA + D-AAT + GDH	30	23
GluRA + D-AAT + FDH	26	22
GluRA + FDH + GDH	30	30
D-AAT + FDH + GDH	7	8
No enzyme supplemented	6	5

[a] Three enzymes were added to the reaction mixture when the production rate decreased to approximately 25–30% of the initial level.
[b] The amount of D-amino acids produced in 2 h were determined after the supplementation.

ity which was also observed in the D-tyrosine production (Fig. 5), for which only a small portion of produced D-tyrosine was soluble.

To determine whether the decreased productivity is caused by the depletion of PLP or regeneration components (NAD^+ and L-glutamate), we added a mixture of PLP, NAD^+ and L-glutamate to the reaction mixture after 35 h. The productivity level did not recover, indicating this factor is also not the reason for the decreased productivity.

To examine whether the decreased productivity was caused by the inactivation of enzymes, we supplemented the reaction mixture with 3 enzymes in different combinations when the productivity decreased to about 25–30% of the initial level (after 35 h) and measured the production of aromatic D-amino acids for 2 h. The addition of enzyme combinations containing GluRA allowed the productivity level for both aromatic D-amino acids to recover to nearly the initial production rate, while the addition of the enzyme combination omitting GluRA did not (Table 1). These results obviously indicate that the decreased productivity is caused by the inactivation of GluRA.

The 4-enzyme reaction system used in this study was a very effective method for producing aromatic D-amino acids (D-phenylalanine and D-tyrosine) in respect to providing optically pure products without the resolution step for racemic mixtures and without yielding by-products. By operating the multi-enzyme system below 50 mM of phenolic substrates, we could achieve a 100% conversion yield. In previous studies [8,9], the conversion yield has remained below 50% in the batch operations of multi-enzyme systems with the high concentrations of phenolic substrates (100 mM).

With optimal reaction conditions, the decrease in productivity was shown to be caused by the instability of *E. coli* GluRA. Even when most of the activity of GluRA was lost, the activities of other enzymes remained at their initial levels. This finding obviously suggests what should be done to improve the productivity of aromatic D-amino acids. Our plan is to replace *E. coli* GluRA with a new thermostable GluRA to improve the multi-enzyme system. To implement this plan, we have studied the occurrence of thermostable GluRA and have found that thermophilic *Bacillus* species including *Bacillus* sp. SK-1 and *Bacillus* sp. KLS-01 isolated from the soil of Korea, exhibit thermostable GluRA activity. Details of gene cloning, sequence determination, overproduction, and application of thermostable GluRA will be reported in our following paper.

Acknowledgements

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