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Kinetic Study on the Enzymatic Production of D-Alanine from D-Aspartic Acid

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An enzymatic reaction for the production of D-alanine from D-aspartic acid and pyruvate as substrates by a thermostable D-amino acid aminotransferase (D-AAT) was investigated at various conditions in the temperature range of 40-70°C and pH range of 6.0-9.5. The D-AAT was produced with recombinant *E. coli* BL21, which hosted the chimeric plasmid pTLK2 harboring the D-AAT from the novel thermophilic *Bacillus* sp. LK-2. The enzyme reaction was shown to follow the Ping Pong Bi Bi mechanism. The K_m values for D-aspartic acid and pyruvate were 4.38 mM and 0.72 mM, respectively. It was observed that competitive inhibition by D-alanine, the product of this reaction, was evident with the inhibition constant K_i value of 0.1 mM. A unique feature of this reaction scheme is that the decarboxylation of oxaloacetic acid, one of the products, spontaneously produces pyruvate. Therefore, only a catalytic amount of pyruvate is necessary for the enzyme conversion reaction to proceed. A typical time-course kinetic study showed that D-alanine up to 88 mM could be produced from 100 mM of D-aspartic acid with a molar yield of 1.0.

Key words: D-alanine, D-amino acid aminotransferase, D-aspartic acid, competitive inhibition, ping pong mechanism

D-amino acids play increasingly important roles as intermediates for the production of medicines, food additives, and agricultural chemicals (Takahashi, 1986; Kwak *et al.*, 1999). Among D-amino acids, D-alanine has a high potential in the market as it can be used as a raw material for a new sweetener or medicinal peptides (Stoineva and Petkov, 1985; Kakushima *et al.*, 1999).

D-alanine can be prepared by various methods: (a) direct fermentation using glucose as a carbon source, (b) selective digestion of L-isomer by yeast in the racemic mixture of DL-alanine, (c) enzymatic resolution of a racemic mixture synthesized chemically, or (d) a separation and crystallization method from DL-alanine (Kamphuis *et al.*, 1990; Ozaki *et al.*, 1992; Umemura *et al.*, 1992; Galkin *et al.*, 1997; Kwak *et al.*, 1999). Although the direct fermentation method which has been commercialized is advantageous in that it uses cheap glucose as a carbon source, it has its own drawback in that the productivity is considerably lower than enzymatic methods because of the low product concentration together with long fermentation time. Therefore, the development of an efficient enzymatic method which can produce D-alanine from a relatively cheap substrate is of great commercial importance. Tanizawa *et al.* (1989) have suggested a method to synthesize D-amino acids by a multi-enzymatic system from the corresponding keto acids and ammonia. Three to four different kinds of enzymes including D-amino acid aminotransferase (D-AAT) (D-alanine:2-oxoglutarate aminotransferase : EC 2.6.1.21) from *Bacillus* sp. were used in this system. Another more efficient way of synthesizing D-alanine using D-AAT has been suggested (Nakajima *et al.*, 1998; Kwak *et al.*, 1999). In this system, DL-aspartic acid and pyruvate were used as the substrates. The amino group of D-aspartic acid is transferred to pyruvate to produce oxaloacetic acid and D-alanine. A unique and economically attractive feature of this system is that the oxaloacetic acid thus formed degrades spontaneously to pyruvate (Fig. 1). Therefore, only a catalytic amount of pyruvate is required to produce D-alanine. As shown in other transamination reactions (Sugio *et al.*, 1995), pyridoxal phosphate (PLP) is required for this system as a coenzyme. DL-Aspartic acid is used as a substrate instead of D-aspartic acid because of the economic advantage. The L-aspartic acid, which is inert to the enzymatic reaction, can be readily separated from D-alanine because of the big difference in the isoelectric points of the two amino acids.

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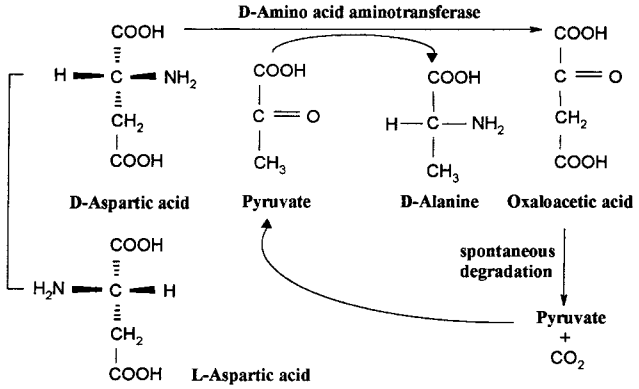

Fig. 1. A reaction scheme of D-alanine production from D-aspartic acid and pyruvate using a D-amino acid aminotransferase.

In the present study, we investigated the kinetics and reaction mechanism for the enzymatic conversion of D-aspartic acid to D-alanine using a novel thermostable D-amino acid aminotransferase. A time-course batch kinetic study was also carried out at optimum conditions in terms of pH and temperature to investigate the practical significance of this enzyme system.

Materials and Methods

Materials

DL-Aspartic acid, D-alanine and pyruvate were purchased from Sigma Chemical Co.(St. Louis, USA). All the chemicals used were reagent grade.

Enzyme preparation

D-AAT used in this study was obtained from the thermophilic *Bacillus* sp. LK-2 isolated from the soil of Korea (Kwak *et al.*, 1999). The structure gene encoding D-AAT of the *Bacillus* sp. LK-2 was cloned into pTrc99 to construct pTLK2. After transformation of the pTLK2 into *E. coli* BL21, the recombinant *E. coli* BL21 harboring the pTLK2 was cultured in a 30-liter-jar fermentor at 30°C with LB medium, harvested by centrifugation at 7,000 g for 10 min and sonicated in an ice bath for 10 min to disrupt the cells. After centrifugation at 12,000 g for 20 min, the supernatant was collected and then incubated at 50°C for 30 min to deactivate other heat labile proteins of the host cells. Then, the denatured proteins were removed by centrifugation at 15,000 g for 20 min and the enzyme solution was partially purified by chromatography using DEAE Toyopearl resin (Tosoh Corp., Japan) prior to use (see Table 1).

Enzyme assay

D-AAT activity was measured by a method similar to that described previously (Kwak *et al.*, 1999). A certain amount of the crude enzyme solution was mixed with 100 μl of 1 M Tris-HCl buffer (pH 8.3), 100 μl of 100 mM D-alanine, 100 μl of 100 mM α-ketoglutarate, and 15 μl of 10 mM PLP, and the final volume was adjusted to 1 ml with distilled water. After incubating the reaction mixture at 50°C for 10 min, the reaction was stopped by adding 200 μl of 60% KOH. D-AAT activity was determined by measuring the pyruvate produced from D-alanine (Kwak *et al.*, 1999). To measure pyruvate, 100 μl of 2% (v/v) salicyl aldehyde was added to the reacted solution and the solution was incubated further at 37°C for 30 min for color development. Finally, the reaction solution was diluted by the addition of an adequate amount of cold water and then the absorbance was measured at 480 nm using a spectrophotometer (Spectronic 21, USA). One unit of enzyme activity was defined as the amount of enzyme producing 1 μmol of pyruvate at 50°C in 1 min.

Table 1. Summary of partial purification of thermostable D-amino acid aminotransferase from *Escherichia coli* BL21 carring pTLK2

Step	Total protein (mg)	Specific activity (unit/mg)	Yield (%)
Cell-free extract	71.0	67.1	100
Heat treatment	34.2	135.2	100
DEAE-Toyopearl	15.4	222.2	74

Kinetic studies

Unless otherwise specified, enzyme reactions were carried out for 3 min at 60°C and pH 8.5 in test tubes containing 1 ml of reaction mixture. To determine the kinetic parameters and reaction mechanism in which two substrates are involved, Lineweaver-Burk plots for D-aspartic acid concentrations in the range 4-40 mM together with various pyruvate concentrations in the range 1-30 mM were employed (Segel, 1975). For kinetic studies the amount of enzyme used was 0.06 units per 1 ml reaction mixture and the enzyme reaction was stopped by adding 50 μl of 12 N HCl to the reaction mixture. The effects of temperature and pH on the stability of the enzyme were determined by measuring the relative activity after incubating the enzyme for 10 hrs at various specified conditions.

Analytical methods

To determine the amount of amino acids in the reaction solution, amino acids were first tagged with OPA (*o*-phthalaldehyde) and the derivatives were analyzed by HPLC (Waters Associate 244, USA) equipped with a Spherisorb ODS II column (Phase Separations, UK) and a fluorescence detector (Waters 420, USA). An aqueous solution (pH 4.3) containing 12.8 g/l sodium nitrate and 5 g/l sodium citrate was used as the eluent at a flow rate of 0.5 ml/sec. Pyruvate and oxaloacetic acid were analyzed by HPLC (Waters Associate 244, USA) equipped with an Aminex HPX-87H ion exclusion column (Bio-Rad, USA)

and a UV detector (Waters 420, USA) at 210 nm. A 4 mM sulfuric acid solution was used as the eluent with the flow rate of 1.0 ml/sec. The column temperature was controlled at 40°C.

Results and Discussion

Effect of temperature

The optimum temperature for the enzyme activity, determined by measuring the initial reaction rate for 3 min, was found to be 60°C as shown in Fig. 2. The stability of the enzyme in the presence of substrate was not greatly affected by temperature. However, enzyme activity without substrate declined very rapidly as the incubation temperature increased from 45 to 60°C. As a result, the operational temperature of 60°C was selected throughout the course of this work.

Effect of pH

The effects of pH on the enzyme activity and stability with substrate are shown in Fig. 3. In order to maintain high buffering capacity, 0.1 M potassium phosphate buffer was used in the pH range below pH 7.5, while 0.1 M Tris-HCl buffer was used in the pH range above pH 7.5. At pH 7.5, the enzyme activity with Tris-HCl buffer was about 20% higher than that with potassium phosphate buffer. However, the enzyme stability was higher with potassium phosphate buffer by 20% compared with Tris-HCl buffer. The reason for the differences in activity and stability was not examined in detail in the present study. Although the stability of the enzyme varied marginally depending on pH, the enzyme was considered to be stable in the pH range 6.0-9.5. Therefore, the optimum pH for the enzyme reaction was determined to be pH 8.5 from the result shown in Fig. 3.

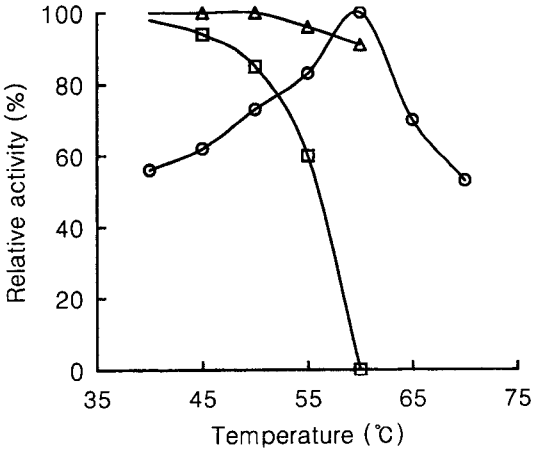

Fig. 2. Effects of temperature on the activity and stability of D-amino acid aminotransferase at pH 8.5. (○) enzyme activity; (△) stability with substrate; (□) stability without substrate

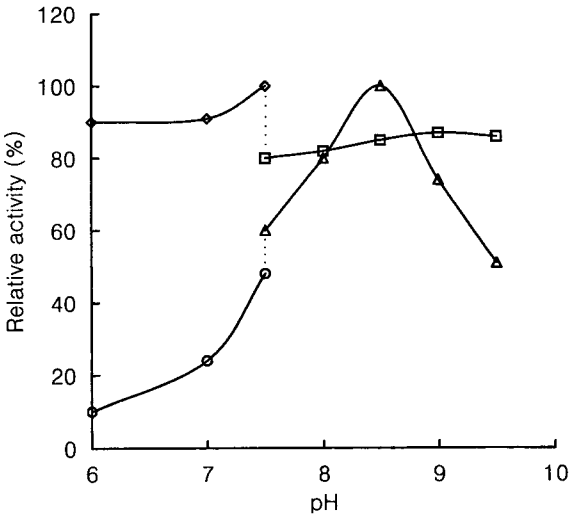

Fig. 3. Effects of pH on the activity and stability of D-amino acid aminotransferase at 60°C. (○) activity with potassium phosphate buffer; (△) activity with Tris-HCl buffer; (◇) stability with potassium buffer; (□) stability with Tris-HCl buffer

Determination of kinetic parameters

To determine the reaction mechanism together with the kinetic parameters, a double reciprocal plot of $1/V$ vs. $1/A$ with various pyruvate concentrations is shown in Fig. 4. The V indicates the reaction rate of D-alanine formation and A indicates D-aspartic acid concentration. The set of parallel lines shown in Fig. 4 confirmed that the reaction system followed the Ping Pong Bi Bi mechanism (Segel, 1975). Based on the results shown in Fig. 4, the Michaelis-Menten constants (K_m values) for D-aspartic acid and

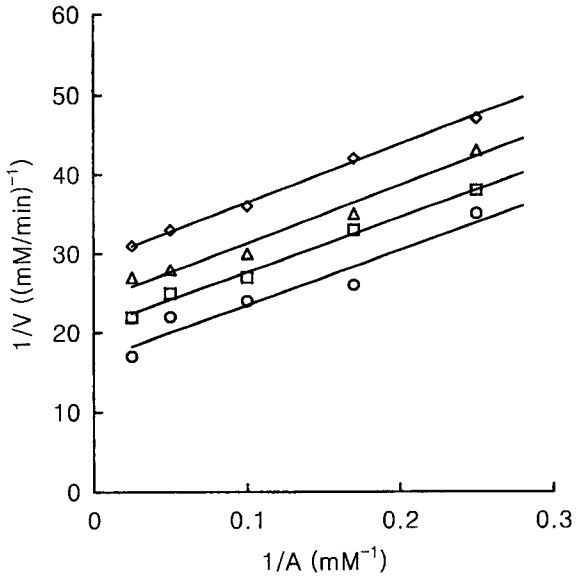

Fig. 4. Double reciprocal plots of D-amino acid aminotransferase system with various pyruvate concentrations at pH 8.5 and 60°C. (◇) 1 mM; (△) 2 mM; (□) 5 mM; (○) 30 mM

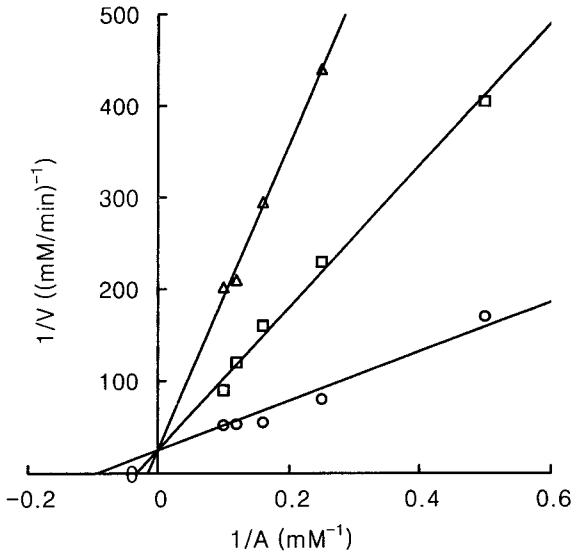

Fig. 5. Lineweaver-Burk plots of enzyme reaction rates with and without D-alanine. (○) 0 mM; (□) 0.3 mM; (△) 0.6 mM

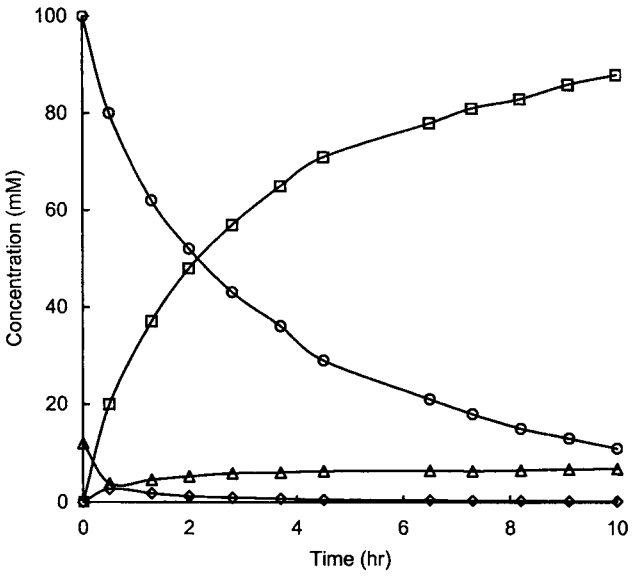

Fig. 6. A time-course kinetic study for the production of D-alanine at pH 8.5 and 60°C. (○) D-aspartic acid; (□) D-alanine; (△) pyruvate; (◇) oxaloacetic acid

pyruvate were determined to be 4.38 mM and 0.72 mM, respectively.

In order to investigate any types of enzyme inhibition that may occur in the enzyme system, D-alanine concentrations in the range of 0.3-0.6 mM were added to the reaction system with D-aspartic acid. In Fig. 5, the reaction rate with and without D-alanine is shown. The result clearly shows that D-alanine is a competitive inhibitor of this enzyme reaction with the inhibition constant K_i value of 0.1 mM.

A unique feature of this enzyme system was that oxaloacetic acid spontaneously degraded to pyruvate and carbon dioxide as illustrated in Fig. 1. It was found from this work that the reaction was first order (data not shown). Therefore, only a catalytic amount of pyruvate is required at the beginning of the enzyme reaction to convert D-aspartic acid to D-alanine. From an economic viewpoint, therefore, it makes this enzyme system more attractive.

Time-course batch reaction kinetics

In Fig. 6, a typical time-course kinetic study for the production of D-alanine from D-aspartic acid is shown. This experiment was carried out at 60°C and pH 8.5 with 100 mM D-aspartic acid and 12 mM pyruvate as substrates. At the beginning of the reaction the pyruvate concentration decreased very rapidly, and thereafter the concentration increased gradually up to 7 mM since the oxaloacetic acid, one of the products, began to degrade to pyruvate. Although the reaction rate decreased toward the end of the batch reaction probably due to a high value of K_m for D-aspartic acid and the increased competitive inhibition caused by D-alanine, the D-aspartic acid converted to D-alanine up to 88 mM within 10 h with a molar yield of 1.0 as shown in Fig. 6. It appears that the batch process with a thermostable D-AAT may well offer the potential for future commercialization.

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