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Industrial Production of L-2-Amino-4-phenylbutyric Acid from 2-Oxo-4-phenylbutyric Acid by *Paracoccus denitrificans* Containing Aminotransferase Activity

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ABSTRACT

An industrial production method of L-2-amino-4-phenylbutyric acid from 2-oxo-4-phenylbutyric acid by microbial cells containing aminotransferase activity was investigated. By using *Paracoccus denitrificans* pFPr-1, 0.19 *M* L-2-amino-4-phenylbutyric acid was produced with a 95% conversion yield. Accumulated L-2-amino-4-phenylbutyric acid was readily isolated in pure form. Overall yield from 2-oxo-4-phenylbutyric acid was 83.7%.

Index Entries: L-2-Amino-4-phenylbutyric acid, enzyme production of and conversion of 2-oxo-4-phenylbutyric acid to; 2-oxo-4-phenylbutyric acid, L-2-amino-4-phenylbutyric acid production from; aminotransferase activity, in *Paracoccus denitrificans; Paracoccus denitrificans,* useful aminotransferase source for L-2-amino-4-phenylbutyric acid production.

INTRODUCTION

It is said that inhibition of angiotensin-converting enzyme (ACE) has been demonstrated to be an effective means of controlling hypertension

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in humans, and a new class of oral ACE inhibitors is now available for investigational use as medicines. L-2-Amino-4-phenylbutyric acid can be easily derived to *N*-[(1S)-1-ethoxycarbonyl-3-phenylpropyl]-L-alanine that is a portion of the molecule of some of a new class of oral ACE inhibitors (*1–3*), which have attracted great therapeutic interest in the cardiovascular field. There are a few reports of production of L-2-amino-4-phenylbutyric acid by resolution of chemically synthesized DL-2-amino-4-phenylbutyric acid (*4–6*), but there is no report of microbial production of L-2-amino-4-phenylbutyric acid. To establish a new process for the production of L-2-amino-4-phenylbutyric acid, we tried to utilize a microbial enzyme for the conversion of 2-oxo-4-phenylbutyric acid to L-2-amino-4-phenylbutyric acid. Previously, we reported that *P. denitrificans* pFPr-1 had high aminotransferase activity and produced L-phenylalanine from phenylpyruvic acid (*7*). In succeeding studies, we found that the aminotransferase in *P. denitrificans* pFPr-1 catalyzed an amination of 2-oxo-4-phenylbutyric acid and formed L-2-amino-4-phenylbutyric acid. In this study, we determined the optimum conditions for the conversion of 2-oxo-4-phenylbutyric acid to L-2-amino-4-phenylbutyric acid, and investigated production of L-2-amino-4-phenylbutyric acid in pilot plant scale to establish a workable enzymatic method for the commercial production of L-2-amino-4-phenylbutyric acid.

MATERIALS AND METHODS

Microorganism and Culture Methods

The strain used in this study was *Paracoccus denitrificans* pFPr-1 (p-fluoro-DL-phenylalanine resistant mutant) derived from *P. denitrificans* IFO 12442, which has been reported to show high aminotransferase activity (*8*). *P. denitrificans* pFPr-1 was cultured by the method described previously (*7*). Under these conditions, maximum aminotransferase activity was 0.23 U/mL of culture broth and 0.028 U/mg of dried cells. For production in pilot plant scale, the culture was carried out using 2000-L fermenter under conditions as follows: volume of medium, 1100 L; agitation, 160 rpm.; aeration, 0.55 vvm.; pressure, 0.5 kg/cm^2.

Assay Methods

Aminotransferase activity was measured by monitoring L-2-amino-4-phenylbutyric acid formation from a reaction mixture (pH 8.0) containing 0.2 *M* 2-oxo-4-phenylbutyric acid, 0.3 *M* L-aspartic acid, 0.1 m*M* pyridoxal-5′-phosphate, and microbial cells at 30°C for 1 h. One unit of enzyme activity was defined as the activity that produces 1 μmol of product per

min. Specific activity was expressed as units per mg of dried cells. Total activity was expressed as units per mL of culture broth.

Production of L-2-Amino-4-phenylbutyric Acid

Unless otherwise noted, production reaction of L-2-amino-4-phenylbutyric acid was carried out as follows: After cultivation, the cells were harvested from 100 mL of the culture broth, and then the cells were suspended in a 100 mL of substrate solution (pH 8.0) containing 0.2 *M* 2-oxo-4-phenylbutyric acid, and 0.3 *M* L-aspartic acid, and the reaction mixture was incubated at 30°C.

Analytical Methods

Cell concentration was determined turbidimetrically at 660 nm in a Hitachi electric photometer EPO-B and was expressed as dry cell weight (mg/mL) calculated from a standard curve.

L-2-Amino-4-phenylbutyric acid was determined using a Shimadzu LC-3A high-performance liquid chromatograph (column, Nucleocil 10C18; carrier, 30% methanol).

2-Oxo-4-phenylbutyric acid was determined using a Shimadzu LC-3A high-performance liquid chromatograph (column, Nucleocil 10C18; carrier, 40% methanol containing 0.5% acetic acid).

Optical rotation was determined with Perkin-Elmer 141 polarimeter. Infrared spectrum was determined in Nujol using a Shimadzu IR-27G infrared spectrophotometer. Elemental analysis was performed with Perkin-Elmer 240 element analyzer.

Chemicals

2-Oxo-4-phenylbutyric acid was prepared as follows: To a suspension of sodium methylate and methyl 3-phenylpropionate in tetrahydrofuran, dimethyl oxalate was added, and stood at 35°C for 4 h. After cooling, the reaction mixture was poured into cold 3 *N*-hydrochloric acid. The upper layer was separated from water and was washed with sodium bicarbonate solution and saturated sodium chloride solution to remove the acid. The organic layer was evaporated to dryness. Then, the resulting syrupy residue was added to 7 *N*-hydrochloric acid and gradually heated to 110°C. After 6 h, the solution was cooled to 10°C and stood overnight. The precipitated crystals were collected by centrifugation and washed with cold water. Thus the obtained 2-oxo-4-phenylbutyric acid was used to nest step without drying (yield; 81% from methyl 3-phenylpropionate).

L-Aspartic acid of analytical standard grade produced by Tanabe Seiyaku Co., Ltd. (Osaka, Japan) was used. The other chemicals were analytical grade.

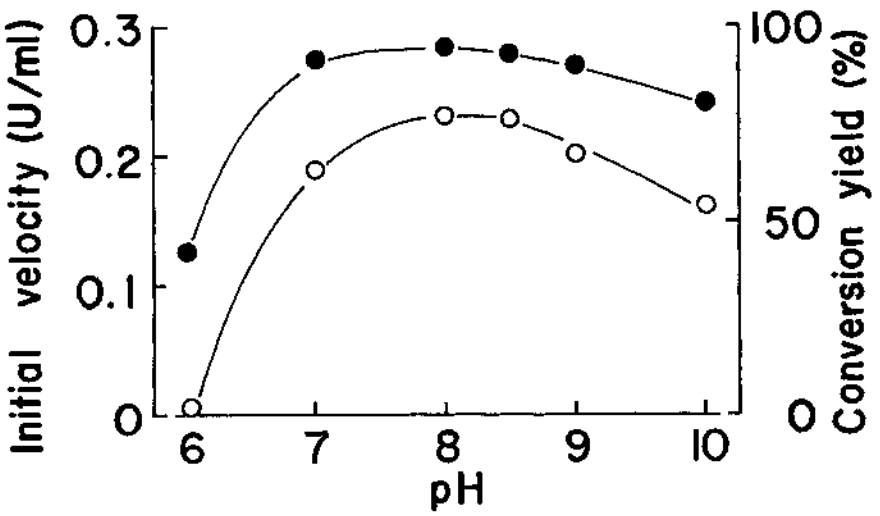

Fig. 1. Effect of pH on initial velocity and conversion yield. The reaction mixture containing 0.2 *M* 2-oxo-4-phenylbutyric acid, 0.3 *M* L-aspartic acid, 0.1 m*M* pyridoxal-5′-phosphate, and *P. denitrificans* pFPr-1 cells was adjusted to the indicated pH with NaOH and incubated at 30°C. Symbols: ○, Initial velocity; ●, Conversion yield.

RESULTS AND DISCUSSION

Conditions for Production of L-2-Amino-4-phenylbutyric Acid from 2-Oxo-4-phenylbutyric Acid by Intact Cells

To establish the most advantageous conditions for enzymatic production of L-2-amino-4-phenylbutyric acid from 2-oxo-4-phenylbutyric acid by aminotransferase activity of intact cells, the following points were investigated.

Effect of pH on Initial Velocity and Conversion Yield

The effect of pH on initial velocity and conversion yield of production of L-2-amino-4-phenylbutyric acid by intact cells was investigated at the standard condition, except for the pH. As shown in Fig. 1, maximum activity was observed at pH 8.0–8.5, and the maximum conversion yield was observed at pH 8.0.

Effect of Concentration of 2-Oxo-4-phenylbutyric Acid on Initial Velocity and Conversion Yield

The effect of concentration of 2-oxo-4-phenylbutyric acid on initial velocity and conversion yield of production of L-2-amino-4-phenylbutyric

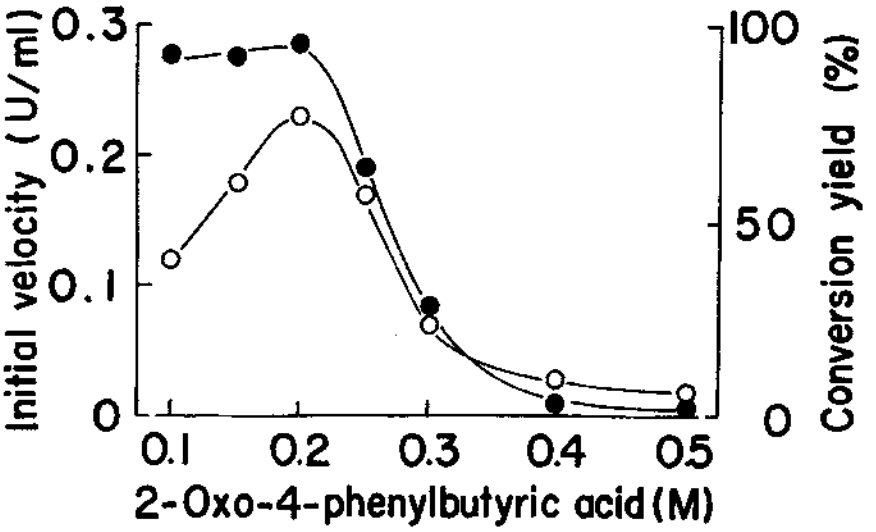

Fig. 2. Effect of concentration of 2-oxo-4-phenylbutyric acid on initial velocity and conversion yield. The reaction mixture (pH 8.0) containing the indicated concentration of 2-oxo-4-phenylbutyric acid, L-aspartic acid, 0.1 m*M* pyridoxal-5'-phosphate, and *P. denitrificans* pFPr-1 cells was incubated at 30°C. The concentration of L-aspartic acid was 1.5-folds mol of 2-oxo-4-phenylbutyric acid. Symbols: ○, Initial velocity; ●, Conversion yield.

acid by intact cells was investigated. As shown in Fig. 2, the initial velocity of production of L-2-amino-4-phenylbutyric acid was varied with the concentration of 2-oxo-4-phenylbutyric acid. It was found that 2-oxo-4-phenylbutyric acid markedly inhibited the aminotransferase activity at more than 0.2 *M*. The conversion yield also markedly decreased at a high concentration of 2-oxo-4-phenylbutyric acid. The conversion yield did not increase, even when enzyme preparations were freshly added. From these results, the substrate concentration appropriate for the production of L-2-amino-4-phenylbutyric acid was 0.2 M.

Effect of Amino Donor on Production of L-2-Amino-4-phenylbutyric Acid

The initial velocity and conversion yield of production of L-2-amino-4-phenylbutyric acid with various amino acids and 2-oxo-4-phenylbutyric acid as the amino acceptor are presented in Table 1. Among amino acids tested, transamination of L-aspartic acid and L-glutamic acid were significantly higher than those observed for the other amino donors. When the reaction was carried out using L-glutamic acid as an amino donor, the initial velocity was two times higher than that using L-aspartic acid. However, on the point of conversion yield, the reaction was stopped at about

Table 1
Effect of Amino Donor of Production of L-2-Amino-4-phenylbutyric Acid[a]

Amino donor	Initial velocity, U/mL of broth	Conversion yield, %
L-Aspartic acid	0.23	95
L-Glutamic acid	0.52	85
L-Alanine	0.11	7.5
Others[b]	0–0.1	5

[a]The reaction mixture containing 0.2 *M* 2-oxo-4-phenylbutyric acid, 0.3 *M* each amino acid, 0.1 m*M* pyridoxal-5′-phosphate, and *P. denitrificans* pFPr-1 cells as enzyme preparation (pH 8.0), were incubated at 30°C.
[b]L-Arg, L-Cys, Gly, L-His, L-Ile, L-Leu, L-Lys, L-Met, L-Pro, L-Ser, L-Thr, L-Trp, L-Val.

85% of theoretical conversion and further conversion was hardly attained, even when aminotransferase was added and the incubation time sufficiently prolonged. On the other hand, when L-aspartic acid was used as an amino donor, 95% of 2-oxo-4-phenylbutyric acid was converted to L-2-amino-4-phenylbutyric acid. L-Aspartic acid showed higher conversion yield than L-glutamic acid. Oxaloacetic acid is not a stable product and will decompose chemically and/or enzymatically to pyruvic acid. Thereby, the reaction may be driven to completion by removal of one of the products, oxaloacetic acid. On the basis of these data, L-aspartic acid was used as the amino donor for production of L-2-amino-4-phenylbutyric acid.

Production of L-2-Amino-4-phenylbutyric Acid from 2-Oxo-4-phenylbutyric Acid Using *P. denitrificans* pFPr-1 Cells

The production reaction of L-2-amino-4-phenylbutyric acid from 2-oxo-4-phenylbutyric acid using *P. denitrificans* pFPr-1 was carried out under the conditions described in Materials and Methods. A typical time course of L-2-amino-4-phenylbutyric acid production from 2-oxo-4-phenylbutyric acid is illustrated in Fig. 3. L-2-Amino-4-phenylbutyric acid increased in proportion to the consumption of 2-oxo-4-phenylbutyric acid. A solubility of L-2-amino-4-phenylbutyaric acid was very low (about 1 mg/mL), so accumulated L-2-amino-4-phenylbutyric acid crystallized in the reaction mixture. After 72 h, the reaction finished and 0.19 *M* (34.0 mg/mL) L-2-amino-4-phenylbutyric acid was accumulated in the reaction mixture from 0.2 *M* 2-oxo-4-phenylbutyric acid (conversion yield; 95%). The accumulated L-2-amino-4-phenylbutyric acid did not decompose even with prolonged incubation.

Isolation and Purification of L-2-Amino-4-phenylbutyric Acid

The L-2-amino-4-phenylbutyric acid was readily isolated and purified as follows: After the reaction, the crystals of accumulated L-2-amino-4-

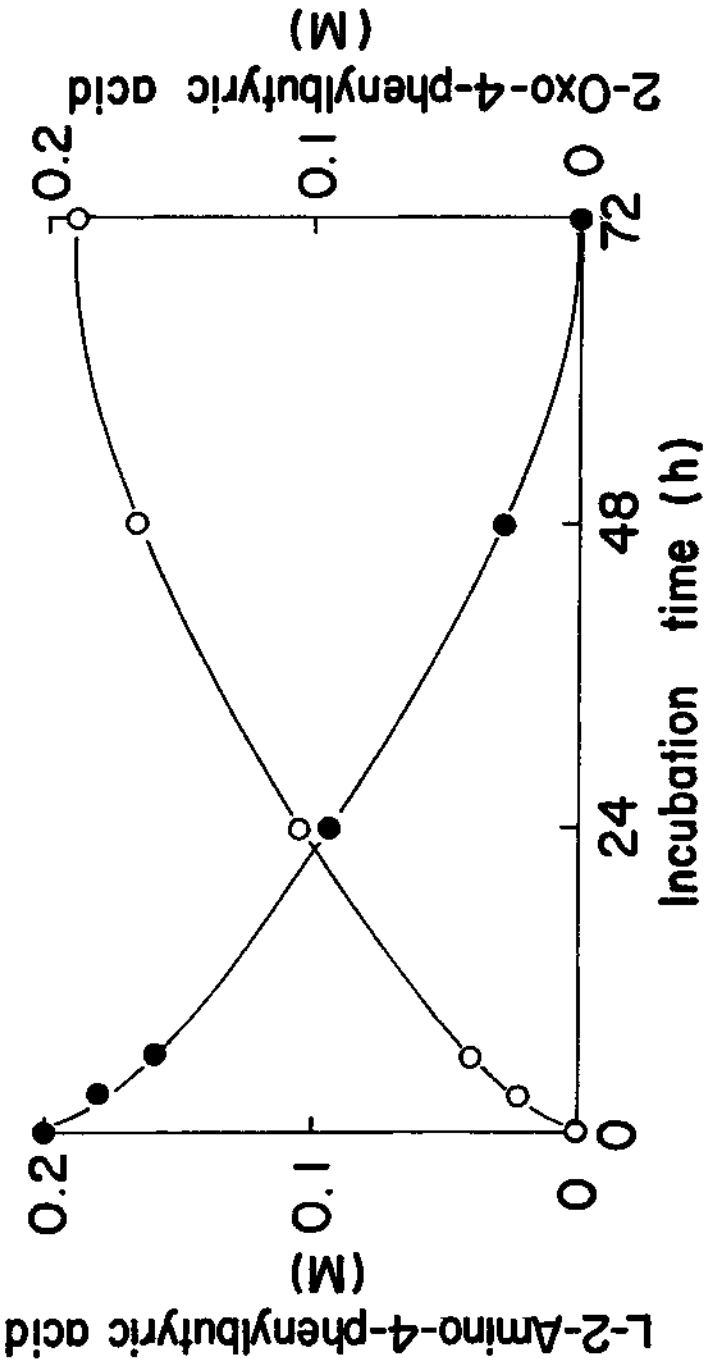

Fig. 3. Production of L-2-amino-4-phenylbutyric acid from 2-oxo-4-phenylbutyric acid using *P. denitrificans* pFPr-1 cells. Symbols: ○, L-2-Amino-4-phenylbutyric acid; ●, 2-Oxo-4-phenylbutyric acid.

phenylbutyric acid were harvested by the centrifugal separator using cotton filter cloth from 100 mL of the reaction mixture. The cells and other components in the reaction mixture passed through the filter cloth, and were removed from the crystals by this operation. The crystals were washed with water and harvested again by the centrifugal separator. Then the crystals were suspended in water, and dissolved by addition of concentrated hydrochloric acid. The solution was treated with active charcoal and filtrated, 5 *N*-NaOH was slowly added to the filtrate, adjusted pH to 5.5, and L-2-amino-4-phenylbutyric acid was crystallized out from the filtrate. After filtration and desiccation, 3 g of pure L-2-amino-4-phenylbutyric acid was obtained. Overall yield from 2-oxo-4-phenylbutyric acid was 83.7%. The crystals were identical in their infrared spectrum with authentic L-2-amino-4-phenylbutyric acid. The optical rotation was $[\alpha]^{20}_{D} = +48.0°$ ($c = 1$, 1 *N*-HCl). Elemental analysis corresponds to $C_{10}H_{13}NO_2$; calculated: *C*, 67.02%; *H*, 7.31%; *N*, 7.82%; found: *C*, 67.09%; *H*, 7.27%; *N*, 7.76%.

Scaleup of Production of L-2-Amino-4-phenylbutyric Acid

On the basis of the data described above, we investigated pilot plant scale production of L-2-amino-4-phenylbutyric acid from 2-oxo-4-phenylbutyric acid. The culture of *P. denitrificans* pFPr-1 was carried out using 2000-L fermenter under the conditions described in Materials and Methods, and the cells were harvested from the culture broth by ultrafiltration. The production reaction of L-2-amino-4-phenylbutyric acid was carried out at both 300- and 1000-L scale. The results were summarized in Table 2. As shown in Table 2, the conversion yield and overall yield from 2-oxo-4-phenylbutyric acid were nearly same as those of small scale experiments. From these results, it was thought that the scale of reaction did not affect the production of L-2-amino-4-phenylbutyric acid.

CONCLUSIONS

By the production methods of L-2-amino-4-phenylbutyric acid by resolution of chemically synthesized DL-2-amino-4-phenylbutyric acid described previously (*4–6*), the yields of L-2-amino-4-phenylbutyric acid are low and racemization of remained D-2-amino-4-phenylbutyric acid is necessary for the complete conversion to L-2-amino-4-phenylbutyric acid from DL-2-amino-4-phenylbutyric acid. On the other hand, by the production method described in this report, the conversion yield and overall yield from 2-oxo-4-phenylbutyric acid are very high. The production process of L-2-amino-4-phenylbutyric acid in this report is very promising as a commercial process because 2-oxo-4-phenylbutyric acid can be obtained at low cost, and the accumulated L-2-amino-4-phenylbutyric acid can be easily isolated from the reaction mixture.

Table 2
Pilot Plant Scale Production of L-2-Amino-4-phenylbutyric Acid from 2-Oxo-4-phenylbutyric Acid[a]

	OPB[b]		Enzyme reaction[c]			L-APB[d]		
Exp.	Wet weight kg	Content %	Activity[e] U	Time h	Conversion yield %	Dry weight kg	Purity %	Overall yield %
1	12.1	68.0	60,000	67	92	6.5	97.0	76.5
2	37.1	67.7	200,000	64	90	20.2	95.2	75.7

[a]The reaction mixture (pH 8.0) containing 0.15 *M* 2-oxo-4-phenylbutyric acid, 0.3 *M* L-Asp, and *P. denitrificans* pFPr-1 cells was incubated at 30–32°C.
[b]OPB; 2-oxo-4-phenylbutyric acid.
[c]The scale of reactions were 300 L (Exp. 1), and 1000 L (Exp. 2).
[d]L-APB; L-2-amino-4-phenylbutyric acid.
[e]The cells harvested from 300 L broth (Exp. 1) and 1000 L broth (Exp. 2) were used for the production. The aminotransferase activity of culture broth was 0.2 U/mL.

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