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Stimulation of L-Aspartate β-Decarboxylase Formation by L-Glutamate in *Pseudomonas dacunhae* and Improved Production of L-Alanine

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The formation of L-aspartate β-decarboxylase by *Pseudomonas dacunhae* was compared on media containing a variety of organic acids and amino acids as a carbon source. Although the enzyme was formed constitutively when the organism was grown on basal medium or on that containing tricarboxylic acid cycle intermediates, it was induced twofold by L-glutamate and repressed one-tenth by L-serine. L-Glutamine, L-proline, L-leucine, glycine, and L-threonine also showed induction effects lower than that of L-glutamate. L-Glutamate derepressed the serine effect. This glutamate effect was observed with other microorganisms, e.g., *Achromobacter pestifer* and *Achromobacter liquidum*. Since the intermediates from L-glutamate metabolism had no effect, this induction effect was specific to L-glutamate. The formation of some glutamate-related enzymes was measured and is discussed in relation to the formation of L-aspartate β-decarboxylase. L-Aspartate β-decarboxylase was purified to an electrophoretically homogeneous state from L-glutamate-grown cells of *P. dacunhae*, and some properties were compared with those of the enzyme from fumarate-grown cells. The two enzymes were identical in disc electrophoresis, molecular weight, and some enzymatic properties. The industrial production of L-alanine from L-aspartic acid was improved by using the culture broth with highly induced L-aspartate β-decarboxylase (9.4 U/ml of broth).

Enzymatic conversion of L-aspartic acid to L-alanine by L-aspartate β-decarboxylase (L-aspartate 4-carboxy-lyase [EC 4.1.1.12]) is a highly efficient method for industrial production of L-alanine (4). The occurrence and enzymatic properties of L-aspartate β-decarboxylase have been reported in a number of microorganisms, such as *Pseudomycobacterium* (13), *Clostridium perfringens* (14, 15), *Desulfovibrio desulfuricans* (3, 21), *Nocardia globerula* (7), *Pseudomonas reptilivora* (20), *Acetobacter* sp. (6), *Achromobacter* d-15 (29, 30), *Alcaligenes faecalis* (16, 17), *Xanthomonas oryzae* (28), and *Pseudomonas dacunhae* (4), and in invertebrates such as silk worms (2), crayfish, and lobsters (8).

L-Aspartate β-decarboxylase is a pyridoxal phosphate-dependent enzyme, and α-keto acids operate as allosteric activators of this enzyme (5, 22, 24). The reaction mechanism has been extensively studied (5, 16, 17, 22, 24), but no report has appeared on the mechanism of synthesis of this enzyme.

This report describes the stimulation of L-aspartate β-decarboxylase formation by L-glutamate and its application to improved industrial production of L-alanine.

MATERIALS AND METHODS

Materials. All chemicals, unless otherwise specified, were Katayama (Osaka) certified reagent grade. Pyridoxal phosphate, α-ketoglutaric acid (Nakarai, Kyoto), bovine serum albumin (Armour), α-hydroxyglutaric acid, γ-aminobutyric acid (Sigma), and hydroxypropyl cellulose (molecular weight, 3×10^4 to 4×10^4) (Nippon Soda, Tokyo) were obtained from the sources given in parentheses. Nikkol OP-10, polyoxyethylene-octylphenyl ether, was purchased from Nikko Chemicals (Tokyo). Diethylaminoethyl-Sephadex A-50 was purchased from Pharmacia (Uppsala, Sweden), and hydroxyapatite was from Bio-Rad Laboratories, Richmond, Calif.

Organisms and growth conditions. *P. dacunhae* IAM 1152 was grown in 100 ml of medium containing the following components in a 500-ml shaken flask: sodium L-glutamate, 2 g; peptone, 0.9 g; casein hydrolysate, 0.2 g; KH_2PO_4, 0.05 g; $MgSO_4 \cdot 7H_2O$, 0.01 g; and distilled water. The final pH of the growth medium was adjusted to 7.2 with 1 N NH_4OH. For the experiments relating to the effect of carbon source, various organic acids or amino acids were added to the medium

instead of L-glutamate at a concentration of 1%. The basal medium refers to the medium from which only L-glutamate was omitted. Serine medium refers to the medium to which 1% L-serine was added instead of L-glutamate. The inoculated media were incubated at 30°C for 24 h with reciprocal shaking (140 rpm, 7-cm stroke). Cell growth was monitored by measuring absorbance at 660 nm in a Hitachi model 101 spectrophotometer, using appropriate dilutions made with 0.9% saline. Cell concentrations were calculated from the standard curve correlating absorbance at 660 nm to dry cell weight. For comparative studies *Achromobacter pestifer* IAM 1446 and *Achromobacter liquidum* IAM 1667 were also grown by the above method.

Preparation of cell-free extracts. Cells from culture broth were collected, sonicated at 10 kcycles/s for 10 min, and centrifuged at 10,000 × *g* for 15 min. The supernatants were used as cell-free extracts.

Enzyme assay. The L-aspartate β-decarboxylase activity was assayed by manometric measurements of CO_2 liberated from L-aspartic acid at 30°C under the following conditions. The reaction mixture, with intact cells in a Gilson respirometer (differential manometer) vessel, contained 4 μmol of pyridoxal phosphate, 5 μmol of α-ketoglutarate, 600 μmol of sodium acetate buffer (pH 5.3), 1 mg of Nikkol OP-10, 0.05 to 0.2 ml of culture broth, and 300 μmol of sodium L-aspartate in a final volume of 3 ml. For the assays with cell-free extracts, sonicated supernatant or purified enzyme was used instead of culture broth, 0.1 mg of hydroxypropyl cellulose was added as a stabilizer (22), and Nikkol OP-10 was omitted. One unit of enzyme activity was defined as the amount of enzyme forming 1 μmol of carbon dioxide per min. Total activity was expressed in terms of units per milliliter of culture broth, and specific activity was expressed in units per milligram of dry cell weight. For solubilized enzyme, specific activity was expressed in terms of units per milligram of protein, which was determined by the procedure of Lowry et al. (12).

L-γ-Glutamyl transpeptidase activity was assayed by spectrophotometric measurement of *p*-nitroanilide liberated from L-γ-glutamyl-*p*-nitroanilide at 410 nm by the method of Tate and Meister (25). The activities of L-glutamate-oxalacetate transaminase, L-glutamate dehydrogenase, and L-glutamate decarboxylase were measured by the methods of Bergmeyer and Bernt (1), Veronese et al. (26), and Shukuya and Schwert (23), respectively. The activity of L-glutaminase was assayed in the reaction mixture of Hartman (10), and liberated ammonium was determined by the indophenol method (19).

Determination of L-alanine and L-aspartic acid. L-Alanine was assayed by microbioassay with *Leuconostoc citrovorum* ATCC 9081 (27), and L-aspartic acid was assayed by manometric measurement of CO_2 with L-aspartate β-decarboxylase.

RESULTS

P. dacunhae has the most active L-aspartate β-decarboxylase among the organisms in which we found L-alanine formation (4), and this organism was used in the following experiments.

Effects of organic acids and amino acids on L-aspartate β-decarboxylase formation. As described previously (4), *P. dacunhae* could not utilize ordinary sugars for growth, and fumarate has been the most effective carbon source for growth and L-aspartate β-decarboxylase formation.

Table 1 presents the results of representative experiments in which a wide variety of organic acids and amino acids was used to determine the most effective carbon source. Monobasic organic acids, such as formate, acetate, propionate, butyrate, *iso*-butyrate, and caprylate, were not utilized by *P. dacunhae*. Among di- and tribasic acids, tricarboxylic acid cycle intermediates were efficiently utilized for growth and L-aspartate β-decarboxylase formation, but the specific activities of the cells grown on these acids were 1.0 to 1.1 U/mg of dry cells, which was the same as that of the control cells. Among the 25 amino acids tested, L-glutamate, L-glutamine, L-proline, L-leucine, glycine, and L-threonine produced higher specific activities than tricarboxylic acid cycle intermediates. L-Serine was utilized for growth, but L-aspartate β-decarboxylase formation was repressed to one-tenth that in basal medium. L-Glutamate, the most effective

TABLE 1. *Effect of organic acids and amino acids on L-aspartate β-decarboxylase formation by P. dacunhae*

Compound	Growth (mg/ml)	Enzyme activity	
		Total (U/ml)	Specific (U/mg)
None	1.1	1.1	1.0
Dibasic acids			
Malonate	1.2	1.1	0.9
Succinate	3.2	3.2	1.0
Fumarate	4.1	4.5	1.1
Maleate	1.1	1.1	1.0
Malate	3.2	3.2	1.0
Tartrate	1.9	1.0	0.5
Tribasic acids			
Citrate	3.0	3.3	1.1
iso-Citrate	3.7	4.1	1.1
cis-Aconitate	3.8	4.6	1.2
Amino acids			
L-Alanine	3.9	4.7	1.0
L-Aspartate	4.2	4.2	1.0
L-Asparagine	3.8	3.8	1.0
L-Glutamate	4.2	9.2	2.2
L-Glutamine	3.8	7.2	2.0
Glycine	4.1	7.4	1.8
L-Leucine	3.6	6.5	1.8
L-Proline	4.4	8.4	1.9
L-Serine	3.3	0.3	0.1
L-Threonine	4.2	6.3	1.5

amino acid, had a specific activity of 2.2 U/mg of dry cells and total activity of 9.2 U/ml of broth.

When 0.01 to 1 M L-glutamate was added to the assay mixture for L-aspartate β-decarboxylase of culture broth grown on basal medium, fumarate, aspartate, or glutamate, the enzyme activity was not affected at all. From this point of view, the effect of glutamate is not on enzyme activity but rather on enzyme synthesis.

The optimum concentration of sodium L-glutamate for induction of the enzyme was 2% (Fig. 1).

Effect of addition of L-glutamate during fermentation. The inductive effect of L-glutamate was followed during fermentation. L-Glutamate induced enzyme synthesis at all stages of cell growth, but the addition from zero time to early log phase was most effective (Table 2). L-Glutamate derepressed the synthesis of the enzyme when it was added to repressed cells grown on L-serine. This situation is more clearly shown in Fig. 2. In both basal and serine media growth reached late log phase at 16 to 18 h. At this time L-glutamate was added and culture was continued until 40 h. Even at late log phase L-glutamate addition was markedly effective in stimulating the synthesis of L-aspartate β-decarboxylase.

Effect of glutamate on other microorganisms. Table 3 shows the stimulation of L-aspartate β-decarboxylase formation by L-glutamate in a variety of microorganisms.

Effect of L-glutamate-related compounds. Among the glutamate-related compounds, γ-aminobutyrate, α-aminobutyrate, glutarate, hydroxyglutarate, DL-norvaline, and D-glutamate were ineffective, and only α-ketoglutarate had a favorable effect.

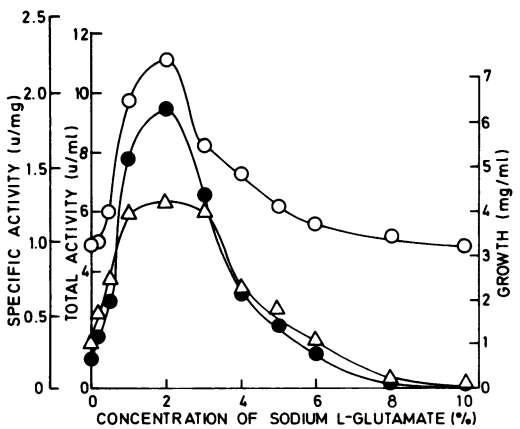

FIG. 1. *Effect of L-glutamate concentration on L-aspartate β-decarboxylase formation. Symbols: △, growth; ●, total activity; ○, specific activity.*

TABLE 2. *Effect of time of L-glutamate addition on L-aspartate β-decarboxylase formation*[a]

Time of L-glutamate addition (h)	Enzyme activity (U/mg)	
	Basal medium	Serine medium
No addition	1.0	0.2
0 (initial)	2.2	2.1
4 (lag)	2.2	2.1
8 (early log)	2.1	1.9
16 (late log)	1.8	1.5
24 (stationary)	1.3	0.5

[a] Twelve flasks of *P. dacunhae* were cultured on basal and L-serine media for 0 to 24 h. At the indicated culture time, 1% sodium L-glutamate was added to each flask, and the culture was continued. The enzyme activity was measured at 40 h.

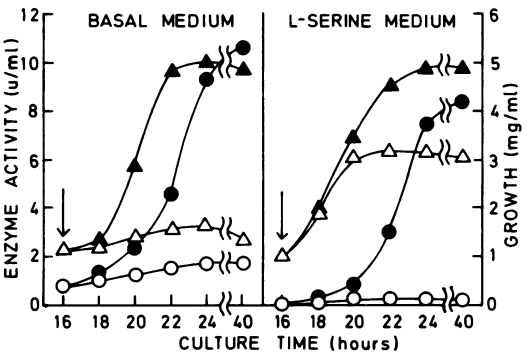

FIG. 2. *Effect of addition of L-glutamate on L-aspartate β-decarboxylase formation. Basal medium refers to the medium which lacks carbon source, and serine medium refers to that which contains 1% L-serine as carbon source. At 16 h of culture 1% sodium L-glutamate was added to one of the two running flasks, and culture was continued until 40 h. Symbols: ○, ●, enzyme activity; △, ▲, growth; open symbols, cultures to which no glutamate was added; closed symbols, cultures to which glutamate was added.*

Levels of glutamate-related enzymes. Activities of L-aspartate β-decarboxylase and glutamate-related enzymes were monitored in various media. γ-Glutamyl transpeptidase activity of the cells grown on L-glutamate or L-aspartate was half that of those grown on basal medium or fumarate. γ-Glutamyl transpeptidase activity of the cells grown on L-serine was the same as that of those grown on basal medium. But inhibition of γ-glutamyl transpeptidase activity by serine occurred (60% by 0.01 M); thus, the γ-glutamyl cycle may become low in serine medium. The level of L-glutamate dehydrogenase activity of the cells grown on basal medium, fumarate, or L-serine was 0.025 to 0.028 U/mg of

TABLE 3. *Effect of L-glutamate on L-aspartate β-decarboxylase formation by various microorganisms*

Microorganism	Carbon source	Growth (A_{660},[a] ×20)	Enzyme activity	
			Total (U/ml of broth)	Specific (U/mg of protein)
P. dacunhae IAM 1152	Fumarate	0.305	4.02	1.00
	L-Glutamate	0.340	7.25	1.95
A. liquidum IAM 1446	Fumarate	0.106	0.102	0.085
	L-Glutamate	0.127	0.185	0.152
A. pestifer IAM 1667	Fumarate	0.347	0.255	0.082
	L-Glutamate	0.365	0.520	0.172

[a] A_{660}, Absorbancy at 660 nm.

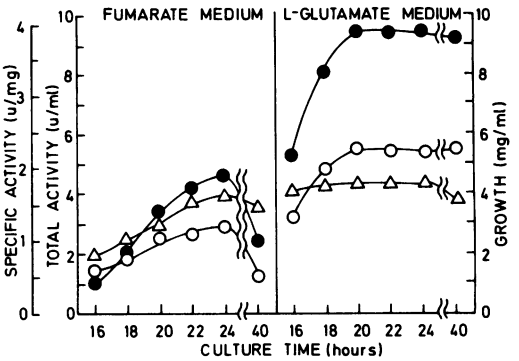

FIG. 3. *Effect of fumarate and L-glutamate on L-aspartate β-decarboxylase formation. Symbols: △, growth; ●, total activity; ○, specific activity.*

protein, but the cells grown on L-aspartate and L-glutamate produced this enzyme two- and fourfold, respectively. L-Glutaminase activity of cell-free extracts prepared from L-glutamine-grown cells was 0.010 U/mg of protein. So L-glutamine was probably converted to L-glutamate during cultivation. L-Glutamate-oxalacetate transaminase activity was equal in all the media tested (2.0×10^{-3} U/mg of protein). Glutamate decarboxylase was not detected, suggesting that the γ-aminobutyric acid pathway is not operative in this organism.

Typical changes during fermentation. The data for typical fermentation for enzyme formation on fumarate and L-glutamate media are given in Fig. 3. On fumarate medium maximal enzyme formation and highest specific activity were attained at 24 h, when growth reached maximum. On L-glutamate medium, however, enzyme formation continued after growth reached maximum at 18 h, and maximal enzyme formation (9.4 U/ml of broth) and highest specific activity (2.3 U/mg of dry cells) were attained at 20 h. Moreover, the enzyme formed on L-glutamate medium was more stable than that formed on fumarate medium.

Purification of L-aspartate β-decarboxylase from glutamate-grown cells. L-Aspartate β-decarboxylase was purified to electrophoretically homogeneous crystals from L-glutamate-grown cells of *P. dacunhae* according to the previously reported procedure (11). The purified enzyme had the same specific activity and yield as that from fumarate-grown cells (11) (Table 4). The crystalline form of the enzyme from glutamate-grown cells was rhombic plate, and the enzyme had 16 mol of pyridoxal phosphate as coenzyme per 800,000 g of the enzyme. The optimum pH for decarboxylation of L-aspartate was pH 5.3; the K_m for L-aspartate was 1 mM, and the V_{max} was 89.0 U/mg of protein. DL-*erythro-* and *-threo-β*-hydroxyaspartic acid competitively inhibited the decarboxylation of L-aspartate (22). The purified enzymes from glutamate- and fumarate-grown cells migrated with the same velocity in disc electrophoresis, and the two enzymes had the same molecular weight of 800,000 in gel filtration.

Improved production of L-alanine. Typical alanine formation is illustrated in Fig. 4. The experiment was conducted by incubating a mixture of 100 ml of the broth cultured under optimal conditions on L-glutamate medium as enzyme source, 100 g of L-aspartic acid, 7 ml of 28% NH_4OH, and 0.05 g of Nikkol OP-10 at 37°C. L-Alanine increased linearly with the consumption of L-aspartic acid. At the beginning of the reaction there were crystals of L-aspartic acid in the reaction mixture, since the substrate was fed over its solubility. But these crystals were continuously solubilized into the solution during the enzyme reaction. When L-alanine accumulated over its solubility, crystals of L-alanine emerged. The final amount of accumulated L-alanine was 66 g/100 ml of broth. During the reaction, the pH was maintained at 5.3, which was optimal for the enzymatic reaction, and it rapidly rose to 9.2 at the end of the reaction. Neither formation of amino acids other than alanine nor decomposition and racemization of accumulated L-alanine occurred during prolonged incubation. The L-alanine formed was easily isolated from the reaction mixture as described below. Three grams of charcoal was added to the reaction mixture, and this mixture was adjusted to pH 4.0, boiled, and filtered at >60°C. To the supernatant solution an equal volume of methanol was added and cooled to 4°C. Crystallized L-alanine was collected by filtration and recrystallized from aqueous methanol to yield colorless needles of L-alanine: yield, 61.4 g (93%); $[\alpha]_{25}^{D} = +14.3°$ (c = 4, 6 N HCl).

TABLE 4. *Purification of L-aspartate β-decarboxylase from P. dacunhae grown on L-glutamate medium*[a]

Step	Fraction	Vol (ml)	Total protein (mg)	Total activity (U)	Sp act (U/mg)	Yield (%)
1	Crude extract	500	9,250	23,865	2.58	100.0
2	$(NH_4)_2SO_4$, 0.3–0.5 saturated	340	3,832	15,750	4.11	70.0
3	Heat treatment	320	1,003	14,600	14.56	61.2
4	DEAE-Sephadex[b]	700	315	12,437	39.48	52.1
5	Sephadex G-150	40	168	11,051	65.78	46.3
6	Hydroxyapatite	280	117	10,332	88.31	43.3
7	Crystals	10	90	8,010	89.0	33.6

[a] Purification was performed from 5 liters of broth (24.5 g of dry cells).
[b] DEAE, Diethylaminoethyl.

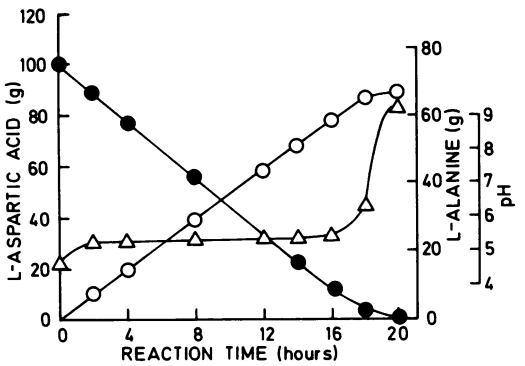

FIG. 4. *Changes during the enzymatic reaction. The experimental method is described in the text. Symbols: ●, L-aspartic acid; ○, L-alanine; △, pH.*

DISCUSSION

P. dacunhae grown on tricarboxylic acid cycle intermediates had a derepressed level of L-aspartate β-decarboxylase, and enhanced growth resulted in high total enzyme activity (4).

We have shown that L-glutamate induces L-aspartate β-decarboxylase formation twofold that of organic acid-grown cells and L-serine represses its formation. Since L-glutamate enhanced the growth of the organism, much higher total activity was obtained than on organic acid medium.

Wilson reported that tartrate is an efficient inducer for L-aspartate β-decarboxylase in *Achromobacter* sp. (29), but this acid was not an efficient inducer in *P. dacunhae*. Although L-aspartate is an efficient carbon source for the formation of L-aspartate β-decarboxylase in *Alcaligenes faecalis* (16) and *X. oryzae* (28), its effect on *P. dacunhae* is inferior to that of tricarboxylic acid cycle intermediates and is half that of L-glutamate.

The fact that an L-glutamate effect was observed with a variety of microorganisms suggested that there may be some general correlation between L-aspartate β-decarboxylase formation and L-glutamate metabolism. The experiments were performed to compare the specific activities of glutamate-metabolizing enzymes in cells grown on L-glutamate and other carbon sources during the log phase. Since the γ-glutamyl transpeptidase activity of L-glutamate-grown cells was lower than that of organic acid-grown cells, the γ-glutamyl cycle may not be a rate-limiting step in L-glutamate incorporation and may not play an important role in L-aspartate β-decarboxylase formation. L-Glutamate dehydrogenase activity of L-glutamate-grown cells was much higher than that of basal medium- or organic acid-grown cells. α-Ketoglutarate may show the effect after being converted to L-glutamate by transamination or reversible dehydrogenation. Since α-ketoglutarate had a lesser effect than L-glutamate, L-glutamate dehydrogenase may not play an important role in L-aspartate β-decarboxylase formation. L-Glutamine may be converted to L-glutamate by L-glutaminase, thus inducing the synthesis of L-aspartate β-decarboxylase. Although γ-glutamyl transpeptidase and glutamate dehydrogenase activities varied among carbon sources, these variations cannot be correlated to the L-aspartate β-decarboxylase formation. Glutamate-oxalacetate transaminase and glutamate decarboxylase were the same in all media, and glutamate-related compounds had no effect on L-aspartate β-decarboxylase formation. From these observations we conclude that L-aspartate β-decarboxylase formation is stimulated by L-glutamate itself but not by any metabolites derived from L-glutamate.

A lot of different enzymes are often reported to catalyze the same reaction. Biosynthetic and biodegradative threonine deaminases are formed in *Escherichia coli*, responding to aerobic and anaerobic culture conditions, respectively (9). In certain microorganisms different carbon sources induce different enzymes which catalyze the same reaction. Different serine transhydroxymethylases are formed responding to the carbon

source, methanol or succinate, in a facultative methylotrophic bacterium (18). These situations prompted us to investigate whether the same or different enzymes were formed in response to fumarate or L-glutamate as a carbon source (9). Our observation strongly suggests that the enzyme from fumarate-grown cells and that from glutamate-grown cells may be the same. Thus, only one L-aspartate β-decarboxylase was produced whether the organism was grown on L-glutamate or on tricarboxylic acid cycle intermediates.

Industrial production of L-alanine was performed previously from a 40-g feed of L-aspartic acid to 100 ml of culture broth (4). Now we are able to produce L-alanine from a >100-g feed of L-aspartic acid to 100 ml of culture broth. The culture broth of L-glutamate-grown *P. dacunhae* having high L-aspartate β-decarboxylase activity has been advantageously used for industrial production of L-alanine from L-aspartic acid.

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