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Effect of L-Aspartic Acid and L-Glutamic Acid on Production of L-Proline

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To elucidate the effect of aspartic acid on growth of *Kurthia catenaforma* during the proline fermentation, this organism was compared with other bacteria with respect to the rate of consumption of aspartic acid, and to the activities of enzymes concerned in the metabolism of aspartic acid. Although no marked difference in enzyme activities was observed, the aspartic acid consumption rate of *K. catenaforma* was markedly higher than that of other organisms. The consumption of glutamic acid by *K. catenaforma* was not detected at 24 hr of culture. The difference between the consumption of aspartic acid and glutamic acid in this strain might result from a difference in permeability to the amino acids. We considered that L-glutamic acid might substitute for L-aspartic acid if the uptake of glutamic acid could be increased. A number of detergents were screened for their effect on consumption of glutamic acid. Cetyltrimethylammonium bromide, sodium laurylphosphate, and polyoxyethylene sorbitan monolaurate were found to increase the transport rate of glutamic acid, but not of aspartic acid. A method of producing L-proline from glutamic acid was established with the aid of detergents.

We previously reported on the production of L-proline by *Kurthia catenaforma*, which requires a high concentration of aspartic acid for maximal growth (8). The following results were found in the earlier study: (i) neither amino acids nor organic acids concerned in the metabolism of aspartic acid could substitute for aspartic acid, and (ii) L-aspartic acid promoted growth during the later stages of the fermentation. These observations suggested that the transport rate of aspartic acid in *K. catenaforma* is higher than that of glutamic acid. Accordingly, if the transport rate of glutamic acid in this strain could be increased by detergents, the organism might utilize glutamic acid and produce proline from glutamic as well as aspartic acid.

To investigate the effect of aspartic acid on this fermentation, the consumption rates and permeabilities of aspartic and glutamic acids were compared in the present work. The effect of aspartic acid was found to be due to efficient transport of aspartic acid in this organism. The acceleration of glutamic acid uptake by some detergents supports this hypothesis and resulted in a process for proline production from glutamic acid.

MATERIALS AND METHODS

Organisms and growth conditions. The organisms used in this study are described in Table 1. A serine-requiring mutant, strain 45, of *K. catenaforma* IAM 1996 was mainly used.

Unless indicated otherwise, cells were grown with reciprocal shaking (140 rev/min, 8-cm stroke) at 30 C. A medium containing 6% glucose, 0.5% urea, 0.3% NH_4Cl, 0.7% corn steep liquor, 1.1% casein hydrolysate, 2% K_2HPO_4, and 0.1% $MgSO_4 \cdot 7H_2O$ was used as a standard medium. The standard medium supplemented with 2% aspartic acid or 2.2% glutamic acid was employed as an aspartic acid medium or a glutamic acid medium, respectively. Glucose was autoclaved separately and added aseptically.

Analytical methods. L-Proline and L-aspartic acid were measured microbiologically by using *Leuconostoc mesenteroides* p-60. L-Glutamic acid was determined by glutamic decarboxylase (L-glutamate 1-carboxy-lyase, EC 4.1.1.15) from *Escherichia coli* Crooks (19). Glucose was estimated by use of the Somogyi-Nelson method (17). For the estimation of growth, the culture broth was diluted 1:10 with saline, and optical density was measured at 660 nm with a Hitachi photoelectric photometer (EPO-B type). Protein concentrations were determined according to the method of Lowry et al. (12).

The consumption rates of aspartic and glutamic acids calculated from data at 24 hr are expressed as

milligrams of the amino acid consumed per milligram of dry cells.

Preparation of crude extracts and enzyme assays. Unless otherwise stated, cells were grown for 20 hr, harvested, washed twice with saline, and suspended in 0.02 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride (*p*H 7.4) containing 1 mM disodium ethylenediaminetetraacetic acid and 1 mM 2-mercaptoethanol. The cell suspensions were used for enzyme assay as intact cells. For the preparation of cell-free extracts, cells were lysed with lysozyme (400 μg/ml) at 37 C for 15 min. The lysate was centrifuged at 30,000 × *g* for 30 min, and the sediments were discarded. The supernatant fluids were employed for enzyme assay as crude extracts.

For aspartate aminotransferase (L-aspartate:2-oxoglutarate aminotransferase, EC 2.6.1.1), the reaction mixture contained: Tris-hydrochloride (*p*H 7.6), 300 μmoles; L-aspartic acid, 100 μmoles; α-ketoglutaric acid, 400 μmoles; pyridoxal phosphate, 0.2 μmole; and crude extracts (1 to 3 mg of protein) in a final volume of 1.5 ml. Incubation was for 30 min at 37 C. The reaction was stopped by placing the tubes into boiling water for 5 min. Glutamic acid formed was measured as described in the preceding section.

For aspartase (L-aspartate ammonia-lyase, EC 4.3.1.1), the reaction mixture contained: Tris-hydrochloride (*p*H 7.6), 200 μmoles; monoammonium fumarate, 100 μmoles; crude extracts (1 to 3 mg of protein), and water in a final volume of 1.0 ml. Incubations were for 30 min at 37 C. The reaction was stopped by placing the tubes into boiling water for 5 min. Aspartic acid formed was measured as described in the preceding section.

For aspartate β-decarboxylase (L-aspartate 4-carboxylyase, EC 4.1.1.12), CO_2 liberated from aspartic acid was measured manometrically. The main compartment of a Warburg vessel contained: sodium acetate (*p*H 5.3), 600 μmoles; L-aspartic acid, 320 μmoles; pyridoxal phosphate, 0.4 μmole; and water in a final volume of 2.7 ml. The side arm contained crude enzyme (2 to 5 mg of protein) in 0.5 ml. After equilibration at 30 C, the contents of the vessel were mixed and readings were made at 5-min intervals.

For aspartate kinase (adenosine triphosphate: L-aspartate 4-phosphotransferase, EC 2.7.2.4), the incubation mixture contained: Tris-hydrochloride (*p*H 8.0), 200 μmoles; L-aspartic acid, 100 μmoles; hydroxylamine hydrochloride, 600 μmoles; adenosine triphosphate, 20 μmoles; magnesium chloride, 10 μmoles; and crude extracts in a final volume of 1.0 ml. Incubation was for 30 or 60 min at 37 C. The reaction was stopped by addition of 1.5 ml of $FeCl_3$ solution. Hydroxamate formed was measured by the procedure of Lipmann and Tuttle (11).

For glutamate kinase (1, 22), the method of Rebello and Strauss (13) was used.

In all cases, specific activities are expressed as nanomoles of products formed per minute per milligram of dry cells or protein.

TABLE 1. *Consumption rates of aspartic acid and glutamic acid in microorganisms*[a]

Microorganism	Consumption rate[b]	
	Aspartic acid	Glutamic acid
Bacillus subtilis OUT 8103	0.68	0.52
Brevibacterium ammoniagenes IAM 1641	0.82	1.36
Escherichia coli ATCC 11303	0.34	0
Kurthia catenaforma No. 45	3.30	0
Micrococcus flavus OUT 8276	0.57	0.15
Pseudomonas fluorescens IFO 3081	0.45	0.13
Serratia marcescens OUT 8259	0.70	0.04

[a] The cultures were incubated for 24 hr in the medium containing aspartic acid or glutamic acid.

[b] Expressed as milligrams of amino acid consumed per milligram of dry cells.

RESULTS

Consumption of aspartic and glutamic acids. To elucidate whether a marked difference between the consumption rates of aspartic acid and glutamic acid is a characteristic only of *K. catenaforma*, uptake rates of these amino acids were compared in a series of microorganisms. As shown in Table 1, aspartic acid is generally more readily utilized than glutamic acid. The consumption rate of aspartic acid in *K. catenaforma* was about 4 to 10 times those in other microorganisms. *E. coli* and *K. catenaforma* did not utilize any glutamic acid within 24 hr of culture.

It was thought that the high consumption rate of aspartic acid in *K. catenaforma* might result from high activities of enzymes concerned in the metabolism of aspartic acid. We determined the activities of these enzymes (Table 2) and found that the levels of enzymes in *K. catenaforma* are not very different from those in other microorganisms. Furthermore, there was no marked difference between the activities of glutamate kinase and the enzymes concerned in the metabolism of aspartic acid. Consequently, the high rate of aspartic acid consumption did not depend on the levels of the enzymes in *K. catenaforma*.

These results supported the theory that the difference between consumption of aspartic acid and glutamic acid could be caused by a difference in the relative uptake of these amino acids.

Transport of aspartic and glutamic acids. The transport system for glutamic acid was investigated in *E. coli* by Halpern (5–7). With regard to the transport of aspartic acid in bacteria, there exists one report showing that *Streptococcus faecalis* takes up aspartic acid by the same transport system as glutamic acid (14). We investigated the transport of both

amino acids by *K. catenaforma* in media containing 2.0% L-aspartic acid, 2.2% L-glutamic acid, or no additive. As shown in Table 3, the transport rate of aspartic acid is expressed as the ratio of aspartate kinase activity in intact cells to that in crude extract. The transport rate of glutamic acid is expressed in the same manner. No difference among the media was observed in the transport rate of either aspartic acid or glutamic acid. Accordingly, *K. catenaforma* grown on aspartic acid was not induced for the aspartic acid transport system. Assuming that both kinases were extracted from the cells with equal yield, the transport rate of aspartic acid was about 20 times that of glutamic acid. The result suggests that the difference between the consumption of the two acidic amino acids may be attributed to the difference in transport activity.

Screening of detergents. It appeared that the effect of aspartic acid on this fermentation is due to its rapid uptake. Therefore, if the consumption rate of glutamic acid could be increased to that of aspartic acid, glutamic acid might accelerate growth and be efficiently converted to proline.

It has been shown that treatment of *E. coli* with a low concentration of detergents alters the permeability of these cells without affecting their viability (10, 21). Some detergents have been used in the fermentative production of amino acids, such as glutamic acid (16, 18, 20), aspartic acid (9), and alanine (3). Thus, we investigated the effect of detergents on consumption of glutamic acid.

Various concentrations of 5 amphoteric detergents, 3 cationic detergents, 5 anionic detergents, and 25 nonionic detergents were added to the glutamic acid medium. After 72 hr of shaking culture, the consumption of glutamic acid was determined. Of the detergents tested, cetyltrimethylammonium bromide (CTAB), sodium laurylphosphate (SLP), and polyoxyethylene sorbitan monolaurate (PESL) were effective (Table 4). In the glutamic acid medium supplemented with detergents, growth was slightly inferior to that in the aspartic acid medium, but the production of proline significantly increased to the same level as that in the medium with aspartic acid. These results suggest that the uptake of glutamic acid was accelerated by the action of detergents.

TABLE 2. *Enzyme activities responsible for the consumption of* L-*aspartic acid and* L-*glutamic acid*

Microorganism[a]	Enzyme activity[b]				
	Aspartate aminotransferase	Aspartase	Asparate β-decarboxylase	Aspartate kinase	Glutamate kinase
B. subtilis	12	89	2.8	6.3	52
B. ammoniagenes	105	211	6.6	4.0	4
E. coli	59	75	12.3	9.7	52
K. catenaforma	85	167	3.0	6.0	75
M. flavus	114	151	4.7	2.0	2
P. fluorescens	15	99	1.8	13.5	26
S. marcescens	49	112	3.7	9.2	6

[a] Cells incubated for 20 hr in the medium containing aspartic acid were harvested by centrifugation, washed with saline, sonically treated (10 kc, 5 min), and centrifuged. The supernatant fluids were used as enzyme solutions.

[b] Expressed as nanomoles per minute per milligram of protein.

TABLE 3. *Transport rates of* L-*aspartic acid and* L-*glutamic acid*

Addition to medium	Aspartic acid			Glutamic acid		
	Aspartate kinase[a]		Transport rate (A/B × 100)	Glutamate kinase[a]		Transport rate (A/B × 100)
	Intact cells (A)	Extract (B)		Intact cells (A)	Extract (B)	
Aspartic acid	0.77	1.07	72	0.90	32.5	2.8
Glutamic acid	0.70	0.97	72	1.07	32.2	3.3
None	0.65	1.00	65	1.00	30.7	3.3

[a] Specific activity in intact cells or extract is expressed as nanomoles per minute per milligram of dry cells.

Effect of detergents on cells. Changes in the consumption of glutamic acid can be brought about by the following mechanisms: (i) an increase of cell permeability, or (ii) a stimulation of metabolism in cells. To study these points, the effect of detergents on growing cells and on resting cells was investigated.

The transport rate of glutamic acid in the cells grown on SLP increased about fourfold, but no effect of CTAB and PESL was observed (Table 5). The activity of glutamate kinase in extracts was the same with or without detergents, and the enzyme activity in intact cells was increased only by SLP. On the other hand, the activity of aspartate kinase in intact cells and in extracts was not affected by detergents, even by SLP. These results indicate that SLP alters permeability to glutamic acid rather than its metabolism in cells.

The effect of detergents on permeability in resting cells was examined. In this case, CTAB and PESL increased the activity of glutamate kinase in intact cells (Table 6). SLP was not effective for resting cells. As the aspartate kinases in intact cells and in extracts were not affected by detergents, CTAB and PESL are considered to accelerate uptake of glutamic acid into the cells. Thus, the effect of detergents on glutamic acid transport was presumed to be due to the following two mechanisms: (i) an action on growing cells, as with SLP; and (ii) an action on resting cells, as with CTAB or PESL. The observation that the transport of aspartic acid and glutamic acid showed different behavior in the presence of detergents suggests that the transport systems are independent. There is, however, a possibility that these detergents cause leakage of glutamate kinase. Glutamate kinase activity of CTAB-treated cells was compared with that of the supernatant fluid (Table 7). The enzyme activity was observed in cells, but not in the fluid. Accordingly, we conclude that the effect of CTAB results from an increase of glutamic acid transport rather than leakage of the enzyme.

Changes during culture on medium with glutamic acid. In the proline fermentation, changes occurring during culture in the basal medium and in the medium containing L-glutamic acid were analogous, since glutamic acid was scarcely utilized by the organism (8). As the utilization of glutamic acid was facilitated by the detergents, we anticipated that the

TABLE 4. *Effect of detergents on growth, glutamic acid consumption, and proline formation*[a]

Medium	Detergent[b]	Growth[c]	L-Glutamic acid (mg/ml)	L-Proline (mg/ml)
Glutamic acid	CTAB, 0.0003%	0.360	1	30
	SLP, 0.004%	0.360	1	28
	PESL, 0.008%	0.380	2	30
	None	0.305	12	12
Aspartic acid	None	0.500	0	29

[a] The cultures were incubated for 72 hr.
[b] CTAB = cetyltrimethylammonium bromide; SLP = sodium laurylphosphate; PESL = polyoxyethylene sorbitan monolaurate.
[c] Optical density.

TABLE 5. *Effect of detergents on transport rates of glutamic acid and aspartic acid in growing cells*

Addition to medium[a]	Glutamic acid			Aspartic acid		
	Glutamate kinase[b]		Transport rate (A/B × 100)	Asparate kinase[b]		Transport rate (A/B × 100)
	Intact cells (A)	Extract (B)		Intact (A)	Extract (B)	
CTAB, 0.0003%	0.98	30.7	3.2	0.73	1.05	70
SLP, 0.004%	4.50	30.8	14.6	0.82	1.09	75
PESL, 0.008%	0.92	30.5	3.0	0.55	0.85	65
None	1.07	32.2	3.3	0.70	0.97	72

[a] CTAB = cetyltrimethylammonium bromide; SLP = sodium laurylphosphate; PESL = polyoxyethylene sorbitan monolaurate.
[b] Specific activity in intact cells or extract is expressed as nanomoles per minute per milligram of dry cells.

TABLE 6. *Effect of detergents on transport rates of glutamic acid and aspartic acid in resting cells*

Addition to reaction mixture[a]	Glutamic acid			Aspartic acid		
	Glutamate kinase[b]		Transport rate (A/B × 100)	Aspartate kinase[b]		Transport rate (A/B × 100)
	Intact cells (A)	Extract (B)		Intact cells (A)	Extract (B)	
CTAB						
0.001%	1.4	30.5	4.6	0.68	1.00	68
0.003%	8.5	31.5	27.0	0.71	1.02	70
0.01%	47.5	30.3	154.8	0.65	0.98	66
SLP						
0.01%	0.9	30.5	3.0	0.69	1.01	68
0.04%	0.9	31.0	2.9	0.70	0.98	71
0.1%	1.0	31.3	3.2	0.72	1.03	70
PESL						
0.01%	1.1	30.5	3.6	0.73	1.02	72
0.08%	2.4	29.7	8.1	0.63	0.99	69
0.12%	3.7	30.0	12.3	0.75	1.02	74
None	0.9	30.5	3.0	0.72	1.00	72

[a] CTAB = cetyltrimethylammonium bromide; SLP = sodium laurylphosphate; PESL = polyoxyethylene sorbitan monolaurate.

[b] Specific activity in intact cells or extract is expressed as nanomoles per minute per milligram of dry cells.

TABLE 7. *Acceleration of glutamic acid transport by cetyltrimethylammonium bromide (CTAB)*

Material	CTAB (%)	Glutamate kinase[a]
Intact cells	0	0.9
	0.003	8.5
Extract	0	31.5
	0.003	32.4
CTAB-treated cells[b]	0	8.0
	0.003	7.7
CTAB-treated cell supernatant[b]	0	0.09
	0.003	0.09

[a] Specific activity is expressed as nanomoles per minute per milligram of dry cells.

[b] Wet cells were suspended in 4.5 ml of 0.02 M Tris buffer, pH 7.4; 0.5 ml of 0.1% CTAB was added, and the suspension was incubated at 37 C for 30 min. The CTAB-treated cells were centrifuged in the cold and resuspended in 5 ml of the same buffer. The cells were removed by centrifugation to provide the CTAB-treated cell supernatant fluids.

chemical changes in the glutamic acid medium would become similar to the changes occurring in aspartic acid medium. To confirm this, the chemical changes during fermentation in the presence of SLP were compared in media containing L-aspartic acid, L-glutamic acid, or no additive (Fig. 1). Production of L-proline in the medium with glutamic acid reached the same level as that in medium with aspartic acid at 40 hr. In medium with glutamic acid, however, glutamic acid consumption, glucose consumption, proline production, and growth were

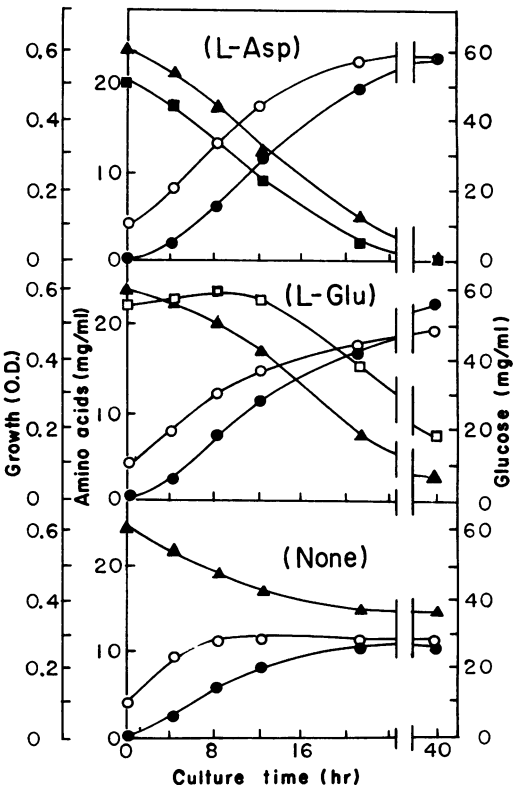

FIG. 1. *Comparison of changes during incubation in medium containing L-aspartic acid or L-glutamic acid in the presence of sodium laurylphosphate. Symbols: ●, L-proline; ■, L-aspartic acid; □, L-glutamic acid; ▲, glucose; ○, growth (optical density).*

slightly reduced compared with those in medium with aspartic acid.

These results demonstrate that the effect of aspartic acid on the proline fermentation is due to a high-velocity transport system of aspartic acid in *K. catenaforma*.

DISCUSSION

It has been shown that the growth-promoting effect of aspartic acid on this fermentation results from the efficient uptake of aspartic acid into cells. This effect was studied in comparison with transport of glutamic acid.

The microorganisms that belong to *Kurthia* (*Bergey's Manual*, 7th ed.) generally show an appreciable lag in minimal medium. *K. catenaforma* has the characteristic that aspartic acid is taken into cells much more rapidly than glutamic acid. With the aid of detergents, proline production with glutamic acid was increased up to the same degree as that with aspartic acid, but both growth and consumption of glutamic acid and glucose were inferior to those in medium with aspartic acid (Table 4, Fig. 1). These phenomena appear likely to be due to a transport rate of glutamic acid that is lower than that of aspartic acid even after it has been increased by detergents. Nevertheless, proline production with glutamic acid was as good as with aspartic acid. This probably happens because glutamic acid, a precursor of proline, is more subject to conversion to proline than aspartic acid.

Since SLP was effective for growing cells, we suspect that SLP antagonizes the effect of unsaturated fatty acids required for the synthesis of cell membrane, and alters permeability, as pointed out in lactic acid bacteria by Camien and Dunn (2). With respect to the action of CTAB on the cell membrane, it has been reported that CTAB removes one of the masked units on the outer layer of the cell membrane (21), and that cationic agents such as CTAB act on the complex phosphatidic acid lipid of the membrane (4). Accordingly, an increase of transport rate in resting cells is assumed to be due to these actions of CTAB. Transport into the cells can be considered the first step in the metabolism of an amino acid by bacteria. The increase of the glutamic acid transport rate by addition of detergents resulted in the promotion of growth and an increase of proline production, owing to the high level of glutamate kinase in *K. catenaforma*.

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