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

JYOJI KATO, MASAHIKO KISUMI, AND ICHIRO CHIBATA

Department of Applied Biochemistry, Chemical Research Laboratory, Tanabe Seiyaku Co., Ltd., Kashimacho, Higashiyodogawa-ku, Osaka, Japan

Received for publication 4 January 1972

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.

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% NH₄Cl, 0.7% corn steep liquor, 1.1% casein hydrolysate, 2% K₂HPO₄, and 0.1% MgSO₄·7H₂O 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 (pH 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 lysis 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 (pH 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 (pH 7.6), 200 µmoles; monooammonium 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 aspartase β-decarboxylyase (L-aspartate 4-carboxylyase, EC 4.1.1.12), CO₂ liberated from aspartic acid was measured manometrically. The main compartment of a Warburg vessel contained: sodium acetate (pH 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 aspartase kinase (adenosine triphosphate: L-aspartate 4-phosphotransferase, EC 2.7.2.4), the incubation mixture contained: Tris-hydrochloride (pH 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 FeCl₃ solution. Hydroxamate formed was measured by the procedure of Lipmann and Tuttle (11).

For glutamate kinase (1, 22), the method of Reberio 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.

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

---

**Table 1. Consumption rates of aspartic acid and glutamic acid in microorganisms**

| Microorganism          | Consumption rate*                  |
|------------------------|-----------------------------------|
|                        | Aspartic acid | Glutamic acid |
| Bacillus subtilis OUT 8103 | 0.68          | 0.52          |
| Brevibacterium ammonienes IAM 1641 | 0.82          | 1.36          |
| Escherichia coli ATCC 11303 | 0.34          | 0             |
| Kuruha catenaforma No. 45 | 3.30          | 0             |
| Micrococcus flaus OUT 8276 | 0.57          | 0.16          |
| Pseudomonas fluorescens IFO 3081 | 0.45          | 0.13          |
| Serrata marcescens OUT 8259 | 0.70          | 0.04          |

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

*Expressed as milligrams of amino acid consumed per milligram of dry cells.
amino acids by \textit{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, \textit{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.

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

| Microorganism* | Enzyme activity* |
|----------------|-----------------|
|                | Aspartate aminotransferase | Aspartase | Aspartate \(\beta\)-decarboxylase | Aspartate kinase | Glutamate kinase |
| \textit{B. subtilis} | 12 | 89 | 2.8 | 6.3 | 52 |
| \textit{B. ammoniagenes} | 105 | 211 | 6.6 | 4.0 | 4 |
| \textit{E. coli} | 59 | 75 | 12.3 | 9.7 | 52 |
| \textit{K. catenaforma} | 85 | 167 | 3.0 | 6.0 | 75 |
| \textit{M. flauus} | 114 | 151 | 4.7 | 2.0 | 2 |
| \textit{P. fluorescens} | 15 | 99 | 1.8 | 13.5 | 26 |
| \textit{S. marcescens} | 49 | 112 | 3.7 | 9.2 | 6 |

* 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.

* 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* | Transport rate (A/B × 100) | Glutamate kinase* | 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 |

* 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 |
|---------------------------------------------|-----------------|-----------------|-----------------|
| Medium | Detergent* | Growth† | 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 |

* The cultures were incubated for 72 hr.
† CTAB = cetyltrimethylammonium bromide; SLP = sodium laurylphosphate; PESL = polyoxyethylene sorbitan monolaurate.

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

| Addition to medium* | Glutamate kinase* | Aspartic kinase* |
|---------------------|-------------------|------------------|
|                     | Intact cells (A)  | Transport rate (A/B x 100) |
|                     | Extract (B)       | Intact (A)       | Extract (B)   |
| CTAB, 0.0003%       | 0.98              | 3.2              | 0.73          | 1.05  |
| SLP, 0.004%         | 4.50              | 14.6             | 0.82          | 1.09  |
| PESL, 0.008%        | 0.92              | 3.0              | 0.55          | 0.85  |
| None                | 1.07              | 3.3              | 0.70          | 0.97  |

* CTAB = cetyltrimethylammonium bromide; SLP = sodium laurylphosphate; PESL = polyoxyethylene sorbitan monolaurate.

† 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 | Glutamic acid | Aspartic acid |
|-----------------------------|---------------|---------------|
|                            | Glutamate kinase | Transport rate | Aspartate kinase | Transport rate |
|                            | Intact cells (A) | Extract (B) (A/B x 100) | Intact cells (A) | Extract (B) (A/B x 100) |
| 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            |

* CTAB = cetyltrimethylammonium bromide; SLP = sodium laurylphosphate; PESL = polyoxyethylene sorbitan monolaurate.

† 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 |
|---------------------------|----------|------------------|
| Intact cells              | 0        | 0.9              |
|                           | 0.003    | 8.5              |
| Extract                   | 0        | 31.5             |
|                           | 0.003    | 32.4             |
| CTAB-treated cells        | 0        | 8.0              |
|                           | 0.003    | 7.7              |
| CTAB-treated cell supernatant | 0.003  | 0.09             |

* Specific activity is expressed as nanomoles per minute per milligram of dry cells.

† 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

![Chemical changes in glutamic acid medium](image-url)
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 Camen 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.

ACKNOWLEDGMENTS

We are indebted to T. Suzuki and S. Iwanaga of the Institute for Protein Research, Osaka University, to T. Takayama of Research and Development and K. Fujii of the Product Control Laboratory for their encouragement during the course of this investigation, and to K. Matsumoto for technical assistance.

LITERATURE CITED

1. Baich, A. 1969. Proline synthesis in Escherichia coli. A proline-inhibitable glutamic acid kinase. Biochim. Biophys. Acta 182:463-467.
2. Camen, M. N., and M. S. Dunn. 1957. Saturated fatty acids as bacterial antimetabolites. Arch. Biochem. Biophys. 76:327-345.
3. Chibata, I., T. Kakimoto, and J. Kato. 1965. Enzymatic production of L-proline by Pseudomonas denitrificans. Appl. Microbiol. 13:638-645.
4. Gilby, A. R., and A. V. Few. 1960. Lysis of protoplasts of Micrococcus lysodeikticus by ionic detergents. J. Gen. Microbiol. 23:19-26.
5. Halpern, Y. S., and A. Even-Shoshan. 1967. Properties of the glutamate transport system in Escherichia coli. J. Bacteriol. 93:1009-1016.
6. Halpern, Y. S., and M. Lupo. 1965. Glutamate transport in wild-type and mutant strains of Escherichia coli. J. Bacteriol. 96:1288-1296.
7. Halpern, Y. S., and H. E. Umbarger. 1961. Utilization of L-glutamic and 2-oxoglutaric acid as sole sources of carbon by Escherichia coli. J. Gen. Microbiol. 26:175-182.
8. Kato, J., S. Horie, S. Komatsubara, M. Kisumi, and I. Chibata. 1968. Production of L-proline by Kurthia catenaforma. Appl. Microbiol. 16:1200-1206.
9. Kitahara, K., S. Fukui, and M. Misawa. 1960. Preparation of L-aspartic acid by bacterial asparaginase. J. Agr. Chem. Soc. Jap. 34:44-48.
10. Leive, L. 1965. A nonspecific increase in permeability in Escherichia coli produced by EDTA. Proc. Nat. Acad. Sci. U.S.A. 53:745-750.
11. Lipmann, F., and L. C. Tuttle. 1945. A specific micromethod for the determination of acyl phosphates. J. Biol. Chem. 159:21-28.
12. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1961. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265-275.
13. Rebello, J. L., and N. Strauss. 1969. Regulation of synthesis of glutamine synthase in Bacillus subtilis. J. Bacteriol. 98:683-688.
14. Reid, K. G., N. M. Utech, and J. T. Holden. 1970. Multiple transport components for dicarboxylic amino acids in Streptococcus faecalis. J. Biol. Chem. 245:5261-5272.
15. Roseman, S. 1969. The transport of carbohydrates by a bacterial phosphotransferase system. J. Gen. Physiol. 54:1336s-180s.
16. Shioi, I., S. Otaka, and N. Kataya. 1963. Cellular permeability and extracellular formation of glutamic acid in Brevibacterium flauus. J. Biochem. 53:333-340.
17. Somogyi, M. 1962. Notes on sugar determination. J. Biol. Chem. 196:19-23.
18. Takinami, K., H. Okada, and T. Tsuunoda. 1964. Biochemical effects of fatty acid and its derivatives on L-glutamic acid fermentation. II. Effective chemical structure of fatty acid derivatives on the accumulation of L-glutamic acid in biotin sufficient medium. Agr. Biol. Chem. 28:114-119.
19. Tsunoda, T., T. Tsuunoda, H. Okada, K. Kinosita, and A. Kawamoto. 1961. Studies on the accumulation of L-glutamine in L-glutamic acid fermentation. I. Confirmation and determination of L-glutamine. J. Agr. Chem. Soc. Jap. 35:260-274.
20. Udagawa, K., S. Abe, and S. Kinosita. 1962. Effects of
surface active agents on L-glutamic acid fermentation. J. Ferment. Technol. 40:614-619.

21. Ulitzur, S. 1970. The transport of β-galactosides across the membrane of permeaseless Escherichia coli ML4 cells after treatment with cetyltrimethylammonium bromide. Biochim. Biophys. Acta 211:533-541.

22. Yoshinaga, F., Y. Takeda, and S. Okumura. 1967. Glutamate kinase activity in Brevibacterium flavum: relationship between L-proline and L-glutamine biosynthesis. Biochem. Biophys. Res. Commun. 27:143-149.