Relationship between the Glutamate Production and the Activity of 2-Oxoglutarate Dehydrogenase in *Brevibacterium lactofermentum*

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Enzyme activities of 2-oxoglutarate dehydrogenase complex and glutamate dehydrogenase of wild type *Brevibacterium lactofermentum*, one of the typical glutamate-producing coryneform bacteria, were investigated by using cells cultured under glutamate-productive and glutamate-non-productive conditions.

Significant reduction of the former enzyme activity was observed in the cells under the several glutamate-productive conditions, namely, in the cells cultured in media containing a) limited concentrations of biotin, b) sub-lethal amounts of penicillin, and c) sub-optimal amounts of a surface-active agent, as compared with those under the non-productive conditions. The activity of the latter enzyme was essentially unchanged in every condition.

The relationship between glutamate production and the enzyme activities as well as permeability of glutamate through cell membrane was discussed from the results obtained.

Key words: glutamate production; *Brevibacterium lactofermentum*; 2-oxoglutarate dehydrogenase

Fermentation processes for glutamate production by using coryneform bacteria such as those belonging to the genera *Brevibacterium* and *Corynebacterium* have been industrialized worldwide, and approximately 800,000 metric tons of monosodium glutamate are estimated to be produced annually today.1,2)

Only minor differences have been found in microbiological characteristics among these coryneform bacteria. They are characterized by a requirement of biotin for growth, which is important in biosynthesis of lipids for the cell membrane.

Production of large quantities of glutamate is observed when the bacteria are cultured in media containing a) limited amounts (ordinarily 2–5 μg/liter) of biotin, b) sub-lethal concentrations of penicillin or c) sub-optimal amounts of surface-active agents (surfactant). Among them, b) and c) are effective methods for practical glutamate production since biotin-rich materials such as cane molasses are used as carbon sources. These conditions would lead to altered physical properties of the membrane, which becomes permeable to glutamate.

During 1960s–1970s, many studies had focussed on regulation of membrane permeability and biosynthesis of glutamate. Consequently a “leak model” had been proposed for the mechanism of glutamate production in coryneform bacteria.1,3)

The model implied the leakage of overproduced glutamate through injured cell membrane by the treatments a)–c) above.

On the other hand, recently, Kraemer et al. reported an “active efflux model,” in which the presence of a specific glutamate carrier system in the membrane has been proposed,4) although substantial identification and characterization of the system has not been obtained.

In our previous report,5) we found that large amounts of lysine and glutamate were simultaneously produced by cultivating a lysine-producing mutant of *Brevibacterium lactofermentum* with the addition of surface-active agents or penicillin. The results indicated that glutamate production of coryneform bacteria could be explained with difficulty only using the regulation of glutamate permeability. For example, the specific increase of glutamate production and reduction of lysine production in this simultaneous fermentation could not be accounted for by the leak model.

2-Oxoglutarate dehydrogenase complex (ODHC) (EC 1.2.4.2), which catalyzes the first-step reaction for the conversion of 2-oxoglutarate to succinyl-CoA at a branch point of glutamate formation from the TCA cycle, and glutamate dehydrogenase (GDH) (EC 1.4.1.2), which catalyzes the reaction for the conversion of 2-oxoglutarate to glutamate, were presumed to be important in the production of glutamate.

Thus, the activities of these enzymes in the cells cultured in various glutamate-productive conditions were examined and were compared with those in the cells of non-productive conditions.

This paper deals with the findings that the ODHC activity was inversely related to the glutamate production in *B. lactofermentum*.

Materials and Methods

Organisms. The microorganism used in this work was *B. lactofermentum* ATCC 13869.

Chemicals. Penicillin G and polyoxyethylene sorbitan monopalmitate (PESP) were purchased from Tokyo Kasei Co., and Wako Chemicals Co., respectively. Other chemicals were obtained from Junsei Kagaku Co.

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Abbreviations: ODHC, 2-oxoglutarate dehydrogenase complex; GDH, glutamate dehydrogenase; APDPN, 3-acylpyridine adenine dinucleotide; PESP, polyoxyethylene sorbitan monopalmitate.
Medium and cultivation. The medium (S-1) used for seed preparations contained in 1 liter of distilled water, 40 g of glucose, 4 g of urea, 1 g of KH₂PO₄, 0.6 g of MgSO₄·7H₂O, 10 mg of FeSO₄·7H₂O, 10 mg of MnCl₂·4H₂O, 55 ml of soybean protein hydrolysate, 200 µg of thiamine monohydrochloride, and 50 µg of biotin, and the pH was adjusted to 7.0 with KOH. The medium (G-1) for glutamate production contained in 1 liter of distilled water, 100 g of glucose, 45 g of (NH₄)₂SO₄, 1 g of KH₂PO₄, 0.4 g of MgSO₄·7H₂O, 10 mg of FeSO₄·7H₂O, 10 mg of MnCl₂·4H₂O, 55 ml of soybean protein hydrolysate, 200 µg of thiamine monohydrochloride, and 50 g of CaCO₃, and the pH was adjusted to 7.0 with KOH. CaCO₃ was sterilized separately.

Additions of biotin, penicillin, and PESP were varied (refer to the text). A loopful of cells inoculated into 20 ml of medium S-1 in flasks were cultured at 31.5°C for 24 h with shaking. Each one ml of seed culture was inoculated into 19 ml of medium G-1, and cultured at 31.5°C with shaking. Samples were taken, and cell growth, quantities of glutamate, and enzyme activity were measured.

Analytical methods. Bacterial growth was measured by optical density at 562 nm (OD₅₆₂) after 24-fold dilution with 0.1 N HCl to dissolve CaCO₃. Glutamate was measured by an autoanalyzer using glutamate decarboxylase prepared from pumpkins.

Enzyme assays. Bacterial cells harvested and washed twice with 0.2% KCl were disrupted sonically after suspending in 0.1 M TES buffer (pH 7.0) containing 30% glycerol. Crude enzymes were prepared from the supernatants of sonic extracts by centrifugation at 8900 x g for 30 min at 4°C, followed by gel filtration with Sephadex G-25. The filtration was done with 0.1 M TES-NaOH buffer containing 30% glycerol to remove endogenous substrates and inhibitors for the enzyme such as succinate, oxaloacetate, ATP, etc. Activity of 2-oxoglutarate dehydrogenase complex (ODHC) was assayed by the method of Shio and Ujigawa-Takeda by measuring the initial rate of absorption at 365 nm in a reaction mixture containing, 100 mM TES-NaOH (pH 7.0), 0.2 mM coenzyme A, 0.3 mM thiamine pyrophosphate, 1 mM 2-oxoglutarate, 3 mM cysteine, 5 mM MgCl₂, and 1 mM 3-acytylpipridine adenine dinucleotide (ADPDPN). ADPDPN was used in place of NAD to avoid the effect of concomitant oxidase. Enzyme activity was expressed as absorbance/min/mg protein. Glutamate dehydrogenase (GDH) was assayed by the method of Shio and Ozaki. The activity was expressed as µmol NADP formed/min/mg protein.

Protein concentration of the crude enzymes was measured by using the "Protein Assay Kit" of Bio-Rad Co., with bovine serum albumin as the standard.

Results
Effects of biotin limitation on growth, glutamate production, and activities of 2-oxoglutarate dehydrogenase (ODHC) and glutamate dehydrogenase (GDH)
B. lactofermentum ATCC 13869 was cultured in medium G-1 with 3 and 30 µg/liter of biotin, respectively. A sample was taken at 5, 9, 24, and 32 h, and cell growth, glutamate production, and the activities of ODHC and GDH were measured. As expected, glutamate production (approximately 230 mM), accompanied with reduced cell growth, was observed in cultures of 3 µg/liter of biotin, but not (less than 5 mM) in cultures of 30 µg/liter, by 32 h cultivation (Fig. 1(A)).

Reduction of the ODHC activity was observed after 9 h cultivation, and the low activity was kept constantly during the glutamate production period in the biotin-limited cultures as shown in Fig. 1(B). While, activities of GDH were essentially unchanged in both biotin-limited and biotin-rich conditions (Fig. 1(C)).

Fig. 1. Effects of Biotin on the Growth and Glutamate Production (A), ODHC Activity (B), and GDH Activity (C) of B. lactofermentum ATCC 13869.
Symbols are as follows: (A) growth of cells cultured in G-1 medium with 3 (●) and 30 (○) µg/liter of biotin, and amounts of glutamate cultured in 3 (●) and 30 (○) µg/liter of biotin, (B) ODHC activities of cells cultured in 3 (●) and 30 (○) µg/liter of biotin, and (C) GDH activities in 3 (●) and 30 (○) µg/liter of biotin.

Fig. 2. Effects of Penicillin G Addition on the Growth and Glutamate Production (A), ODHC Activity (B), and GDH Activity (C) of B. lactofermentum ATCC 13869.
Symbols are as follows: (A) growth of cells cultured in G-1 medium with 30 µg/liter of biotin plus 1.0 unit/ml of penicillin G (●) and that with 30 µg/liter of biotin (○), amounts of glutamate in the culture with 30 µg/liter of biotin plus 1.0 unit/ml of penicillin G (●) and with 30 µg/liter of biotin (○), (B) ODHC activities of cells cultured with 30 µg/liter of biotin plus 1.0 unit/ml of penicillin G (●) and that with 30 µg/liter of biotin (○), and (C) GDH activities of cells cultured in 30 µg/liter of biotin plus 1.0 unit/ml of penicillin G (●) and that in 30 µg/liter of biotin (○). Arrows in the figures indicate the time of penicillin G addition.
Effects of penicillin on growth, glutamate production, and activities of ODHC and GDH

The same strain was cultured in medium G-1 with 30 µg/liter of biotin. One unit per ml of penicillin G was added at 3 h of the cultivation. Similarly as in Fig. 1, with reduced growth, high glutamate production (270 mm) was observed in the penicillin-added culture, but not (less than 5 mm) in the control without penicillin G (Fig. 2(A)). The ODHC activity was lower than the control after the addition of penicillin (Fig. 2(B)). The activities of GDH in both penicillin-added cultures and the control were at essentially the same levels (Fig. 2(C)).

Effects of a surface-active agent on growth, glutamate production, and activities of ODHC and GDH

Polyoxyethylene sorbitan monopalmitate (PESP) was selected as a typical surface-active agent, and finally 4 mg/ml of the substance was added at 3 h of the cultivation in medium G-1 with 30 µg/liter of biotin.

Glutamate production (105 mm) was observed in the PESP-added culture, but not (less than 5 mm) in the culture of the control (Fig. 3(A)). The activities of ODHC were significantly lower than that of the control after the addition of PESP and following glutamate production phase (Fig. 3(B)), while those of GDH were unchanged through the cultivation period (Fig. 3(C)).

Discussion

The mechanism of glutamate production in coryneform bacteria has been so far explained mainly not by the control of glutamate biosynthesis, but by the control of permeability of glutamate through the cell membrane. It had been believed for a long time that coryneform bacteria had essentially poor ODHC activities, because many studies of the 1960s–1970s on the glutamate production mechanisms had failed to detect the activity. However, Shio and Ujigawa-Takeda demonstrated the presence of ODHC activity in B. flavum. Shingu and Terui showed a 40–60% decrease of ODHC activity in a biotin-limited condition as compared with that in a biotin-rich condition by using Brevibacterium sp. From this result, they presumed that the decrease of ODHC contributed partly to the glutamate production.

We have found that a lysine-producing mutant of B. lactofermentum produced simultaneously a large amount of glutamate as well as lysine by culture with the addition of penicillin and a surface-active agent. This suggested that control of glutamate biosynthetic systems would be concerned deeply with the glutamate overproduction, in addition to the glutamate permeability system. On the basis of these results, the activities of ODHC and GDH, two key enzymes for the glutamate production, were examined using cells of wild type B. lactofermentum cultured under suitable conditions for glutamate productions.

The results showed that the activities of ODHC were reduced in all cells cultured under the glutamate-productive conditions; not only in limited-addition of biotin as in Shingu and Terui, but also with additions of penicillin or surface-active agents, as compared with those in glutamate production.

![Fig. 3. Effects of PESP Addition on the Growth and Glutamate Production (A), ODHC Activity (B), and GDH Activity (C) of B. lactofermentum ATCC 13869. Symbols are as follows: (A) growth of cells cultured in G-1 medium with 30 µg/liter of biotin plus 4.0 mg/ml of PESP (● and ) and that with 30 µg/liter of biotin (● ● ● ●). Amounts of glutamate in the culture with 30 µg/liter of biotin plus 4.0 mg/ml of PESP (● ●) and that with 30 µg/liter of biotin (● ● ● ●). (B) ODHC activities of cells cultured with 30 µg/liter of biotin plus 4.0 mg/ml of PESP (● ●) and that with 30 µg/liter of biotin (● ● ● ●). (C) GDH activities of cells cultured with 30 µg/liter of biotin plus 4.0 mg/ml of PESP (● ●) and that with 30 µg/liter of biotin (● ● ● ●). Arrows in the figures indicate the time of PESP addition.]

![Fig. 4. Simplified Scheme of Glutamate Biosynthesis in Coryneform Bacteria.](image-url)
non-productive conditions, while the GDH activities were essentially unchanged among these conditions. Further, the level of ODHC seemed to be inversely proportional to the yield of glutamate.

Summarizing these results, a simple scheme indicating metabolic relations of glutamate production in coryneform bacteria is shown in Fig. 4. It will be understandable from this scheme that a carbon flow of TCA cycle would be easily convertible to glutamate, which would be excreted extracellularly, when the bacteria were cultured under the conditions causing the reduction of ODHC.

It is very interesting how reduction of the ODHC activity and alterations in the cell membrane are correlated. These correlations would be explained biochemically and genetically by investigations using cells of mutants having genetically altered ODHC. We have succeeded in molecular cloning of genes for the E1α subunit of ODHC, and also in constructing its deletion mutants from *B. lactofermentum*.\(^1(10)\) We are expecting that subsequent investigations will clarify the detailed mechanism of glutamate production in coryneform bacteria.

References