L-Glutamic Acid Fermentation

Part IV. The Relation between the Cellular Fatty Acid Contents and the Productivity of L-Glutamic Acid*

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The effect of biotin and oleic acid on the cellular fatty acid contents, and the relation between the cellular fatty acid contents and the productivity of L-glutamic acid were investigated using *Brevibacterium thiogenitalis* No. 653 and its oleic acid-requiring mutant, D-248.

While the synthesis of palmitic acid in D-248 was stimulated by biotin and competitively reversed by oleic acid added to the culture medium, the level of cellular oleic acid was scarcely affected by biotin but regulated by oleic acid in the medium.

For the productivity of L-glutamic acid, the most important factor was the level of cellular oleic acid, and the effect of cellular palmitic acid was considerably weak. This relation was subjected to a figuration and able to be expressed on the whole as one exponential-like curve. An amount of over 70 per cent of cellular fatty acids was distributed in the phospholipid fraction and its fatty acid composition was almost the same as that of whole cells.

INTRODUCTION

In the previous paper,¹⁾ the authors discussed about reasons why the oleic acid-requiring mutant of *Brevibacterium thiogenitalis* could accumulate a large amount of L-glutamic acid, even if, in the excess biotin-containing media, and speculated that a main reason would be in the independency of concentration of biotin in the medium for cellular oleic acid content of the mutant. The relation between cellular fatty acid contents and the productivity of L-glutamic acid has been studied by several groups of investigators^{2,3)} and it has been

known that cellular fatty acids of the L-glutamic acid-producing bacteria are almost distributed in the membrane fractions,⁴⁾ particularly in phospholipid fractions.⁵⁾

Recently, J. J. Gavin and W. W. Umbreit⁶⁾ presented a reasonable hypothesis which might explain the relation between the nature of cellular fatty acids and the change of permeability in *Escherichia coli*.

But there may be still some uncertainty as to the exact mechanism by which L-glutamic acid is accumulated.

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1) H. Okazaki, T. Kanzaki, A. Sugawara and H. Fukuda, *This Journal*, **31**, 1410 (1967).

2) T. Ôtani, S. Ôtsuka, R. Tsugawa and S. Okumura, *Amino Acid and Nucleic Acid*, **11**, 89 (1965); *ibid.*, **12**, 33 (1965).

3) M. Shibukawa, H. Takahashi and T. Ohsawa, *This Journal*, **29**, 813 (1965).

4) M. Shibukawa and T. Ohsawa, *This Journal*, **30**, 750 (1966).

5) K. Takinami, K. Yamada, I. Kinoshita and H. Okada, The symposium of Amino Acid and Nucleic Acid, held at Nagoya, November 11, 1966.

6) J. J. Gavin and W. W. Umbreit, *J. Bact.*, **89**, 437 (1965).

The present paper deals with the effect of biotin and oleic acid on the cellular fatty acid contents of D-248 and the relation of the cellular fatty acid contents to the productivity of L-glutamic acid.

MATERIALS AND METHODS

Organisms. *Brevibacterium thiogenitalis* No. 653 and its oleic acid-requiring mutant, D-248, were used through this study.

Culture. The culture conditions are shown in Table I.

Analysis of Fatty Acids. The method was the same as reported¹⁾ previously.

Separation of Lipids. Lipids were extracted by a modified method of T. Kaneshiro and A. G. Marr⁷⁾ as shown in Fig. 1. Freeze-dried cells were treated with lysozyme (0.025%, pH 6.8, 60 min.) and followed with Trametes enzyme⁸⁾* (0.1%, pH 5.0, 60 min.) at 45°C.

In these treatments, the optical density of the cell suspension was scarcely changed. The sediment fraction (3 to 5 g as dry weight) was extracted twice with 75 ml of 95% ethanol for ten minutes at 60°C and twice with 75 ml of chloroform-methanol (7:3

by volume) for ten minutes at room temperature. The supernatant fraction of enzyme-treated cell suspension was evaporated to dryness under N₂ gas

TABLE I. CULTURE CONDITIONS

Kind of medium	Seed (%)	Main (%)
Glucose	2.0	10.0
Urea	0.5	0.5
CSL	0.5	0.3
KH ₂ PO ₄	0.1	0.1
K ₂ HPO ₄	—	0.1
MgSO ₄ ·7 aq	0.04	0.04
FeSO ₄ ·7 aq	—	0.001
MnSO ₄ ·7 aq	—	0.001
Na-oleate	100 µg/ml	200~1000 µg/ml
Biotin	—	0~30 µg/l
Phenol red	—	10 mg/l
Temperature	28°C	32°C
Volume/200-ml flask	20 ml	15 ml
r.p.m.	200	200
Inoculum size	1 loopful	5%
Culture time	16 hr.	24 hr.
pH	7.0 (start)	7~8*

* Controlled with urea during the fermentation.

FIG. 1. Separation of Lipid Fractions.

7) T. Kaneshiro and A. G. Marr, *J. Biol. Chem.*, **236**, 2615 (1961); *J. Lipid Research*, **3**, 184 (1962).

8) T. Fugono, Personal Communication.

* An enzyme having lytic activity to Gram positive bacteria and showing a more broader lytic spectrum than that of lysozyme.

and the residue was extracted with chloroform-methanol (7:3 by volume). These extracts were combined and evaporated to dryness under N_2 gas, re-extracted with chloroform-methanol-0.1 M potassium chloride (10:5:3 by volume) and the chloroform layer was separated into neutral and phospholipid fractions by silica-gel chromatography.

Chemicals. Oleic acid was the product of Nutritional Biochemicals Corporation, and its purity analyzed by gas-chromatography was 99%. Wakogel C-200 was obtained from Wako Pure Chemical Industries, Ltd.

RESULTS

Effect of Biotin and Oleic Acid Concentration on Cellular Fatty Acid Contents

As reported previously,¹⁾ oleic acid and palmitic acid were main components of cellular fatty acids of *Brevibacterium thiogenitalis* and these acids occupied about 90% of the total amount of cellular fatty acids and the synthesis of these fatty acids was stimulated by biotin in No. 653.

In the case of D-248, the synthesis of palmitic acid was stimulated by biotin as in No. 653, but the levels of oleic acid were scarcely changed by biotin concentration in the medium as shown Figs. 2 and 3.

As regards to the effect of oleic acid concentration in the medium, content of cellular palmitic acid had a tendency of reducing with

FIG. 2. Effect of Biotin Concentration on Cellular Fatty Acid Contents.

○—○ Oleic acid concentration 300 μg/ml
 ●—● " 600 μg/ml
 ▲—▲ " 1000 μg/ml

FIG. 3. Effect of Oleic Acid Concentration on Cellular Fatty Acid Contents.

○—○ 30 μg/l Biotin added
 ●—● 15 μg/l "
 ▲—▲ 6 μg/l "

increase of the oleic acid concentration, so it seemed that the synthesis of palmitic acid was competitively reversed in the presence of oleic acid. However, the content of cellular oleic acid was increased in proportion to the oleic acid added to the medium, and it is evident that cellular oleic acid content of D-248 was able to be controlled by the amount of oleic acid in the medium.

The Relation of Cellular Fatty Acid Contents to the Amount of L-Glutamic Acid Produced

As described above, it was found that cellular fatty acid contents of D-248 were artificially able to be controlled to a certain degree by the amount of biotin and oleic acid which were added to the medium.

Making use of this character, the relation between cellular fatty acid contents and the productivity of L-glutamic acid was studied.

The relation of cellular palmitic acid content to the yield of L-glutamic acid is shown in Fig. 4. Each spot was classified into four parallel lines (A to D) when figured on the basis of cellular oleic acid content.

The results obtained from this experiment make following explanations possible; (i) the yield of L-glutamic acid is increased linearly in proportion to the amount of cellular palmitic acid, if the cellular oleic acid content

FIG. 4. Relation of Cellular Palmitic Acid to Amount of L-Glutamic Acid Produced.

* Calculated on fermented sugar basis.

Cellular Oleic Acid
(mg/gdc)
A: 5.6 ~ 5.9
B: 7.2 ~ 7.6
C: 8.8 ~ 9.0
D: 13.0 ~ 17.0

is almost the same level, (ii) this relation is shown in the parallel lines, if the levels of cellular oleic acid are different, that is, the effect of cellular palmitic acid on the productivity of L-glutamic acid was independent of the level of oleic acid, (iii) considering this relation with the molar ratio of palmitic acid and oleic acid, the relation of the molar ratio (palmitic acid to oleic acid) to the yield of L-glutamic acid was shown in the parallel lines similar to those in Fig. 4, that is, the yield was changed at the same molar ratio when the levels of cellular oleic acid were different.

The relation of cellular oleic acid to the yield of L-glutamic acid is shown in Fig. 5. Each spot was figured on one exponential-like curve with some fluctuations probably having been caused by the effect of palmitic acid as shown in Fig. 4 and experimental errors. That is, this result suggests that the primary effect on

FIG. 5. Relation of Cellular Oleic Acid to Amount of L-Glutamic Acid Produced.

* Calculated on fermented sugar basis.

the productivity of L-glutamic acid is the cellular oleic acid content and the palmitic acid content is secondary one.

Fatty Acid Contents of Whole Cells and Lipid Fractions

Neutral- and phospho-lipid of D-248 were extracted and separated by the method shown in Fig. 1.

As shown in Table II, almost all of cellular fatty acids were contained in phospholipid fraction and only a trace amount of fatty acids was found in the neutral lipid fraction.

The recovery of fatty acids contained in the whole cells was about 70% as a form of phospholipids and the remaining fatty acids were almost found in the cell debris, so they seem to be contained in the form of unextractable bound lipids. That is, an intracellular pool of free oleic acid is presumed to be negligible in these cells.

Therefore, it seems reasonable to consider that the same relations as shown in Figs. 4 and 5 may exist between the fatty acid contents in the phospholipid fraction and the yield of L-glutamic acid.

TABLE II. FATTY ACID CONTENTS OF WHOLE CELLS AND LIPID FRACTIONS

Exp. No.	1	2	3	4
Medium	C ₁₈ (μ g/ml) 300	B(μ g/l) 0	C ₁₈ 300 30	B 600 0
	(mg, methyl ester/g, dry cells)			
Whole cells				
C ₁₄	0.51	0.17	0.21	0.28
C ₁₆	3.70	6.64	2.21	6.39
C ₁₈	7.32	6.40	10.52	9.70
Phospholipid fraction				
C ₁₄	0.46	0.08	0.14	0.19
C ₁₆	2.68	4.13	1.55	4.32
C ₁₈	5.14	4.46	7.53	6.88
Neutral-lipid fraction				
C ₁₄	trace	trace	trace	trace
C ₁₆	0.04	trace	0.03	0.04
C ₁₈	0.03	trace	0.05	0.04

*C₁₄: myristic acid, C₁₆: palmitic acid, C₁₈: oleic acid, B: biotin.

DISCUSSION

The present study demonstrated that the level of cellular oleic acid in the mutant, D-248, was able to be controlled by the amount of oleic acid which was added to the medium, and that the productivity of L-glutamic acid was closely related to the level of cellular oleic acid. D-248 was able to grow in the presence of palmitoleic acid in place of oleic acid.^{1,2)} In another experiment, the fatty acid composition of cells grown in the medium containing palmitoleic acid (300 μ g/ml) as a substitute for oleic acid was shown as follows: palmitic acid, 3.79; palmitoleic acid, 17.87; oleic acid, trace as methylester mg/g dry cells.

Therefore, the role of cellular oleic acid seems to be replaced generally by one of unsaturated fatty acids. The relation between cellular unsaturated fatty acid and the mechanism of L-glutamic acid accumulation cannot yet be explained completely.

T. Ôtani, S. Ôtsuka, R. Tsugawa and S. Okumura²⁾ suggested that the cells which accumulated a large amount of L-glutamic acid had always a low oleic acid content and so

oleic acid would play a very important role in the production of L-glutamic acid using *Brevibacterium lactofermentum* No. 2256.

However, fatty acid spectra of cells grown in the medium containing oleic acid as a substitute for biotin showed a very particular pattern, and cellular oleic acid content of cells grown in the medium containing 1000 μ g/ml of oleic acid was considerably low but L-glutamic acid was hardly accumulated.

M. Shibukawa, H. Takashashi and T. Ohsawa³⁾ proposed a hypothesis about the relation between biotin and oleic acid to the extracellular accumulation of L-glutamic acid by *Microbacterium ammoniaphilum*.

They have reported that the molar ratio of fatty acids, saturated fatty acids to unsaturated fatty acids, in cells would have an important role for the accumulation of L-glutamic acid and a large amount of L-glutamic acid was accumulated when the ratio was over one.

Such an assumption, however, cannot be applied to the cells grown in the medium containing oleic acid as a substitute for biotin and so the function of oleic acid was supposed to be dualistic. Namely, the one was the moiety which played an important role on the cellular permeability of L-glutamic acid and the other was the moiety which did not play such a role, and this moiety took a larger part of cellular oleic acid.

In the case of D-248, about 70% of cellular fatty acids was distributed into the phospholipid fraction which was one of the components of cell membrane when cultured in the presence of oleic acid. Besides, the relation between the level of cellular oleic acid and the productivity of L-glutamic acid was shown in one exponential-like curve. But the role of phospholipid fraction for the permeability of L-glutamic acid is still unknown.

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