L-Glutamic Acid Fermentation with Molasses

Part VI. Effect of the Saturated- Unsaturated Fatty Acid Ratio in the Cell Membrane Fraction on the Extracellular Accumulation of \( L \)-Glutamate

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Conditions suitable for the cell wall lysis of a \( L \)-glutamate-producing bacterium, \textit{Microbacterium ammoniaphilum}, by egg white lysozyme were studied, in order to make clear the correlation of the fatty acid composition of the cellular fractions and the extracellular accumulation of \( L \)-glutamate.

The cell wall of a phage-resistant strain was recognized to be almost completely lyzed by the lysozyme.

Using this result, the relationship between the fatty acid composition of each fraction and extracellular accumulation of \( L \)-glutamate was investigated, and the following thesis was proposed: The extracellular accumulation of \( L \)-glutamate in large quantity took place when the molar ratio of saturated/unsaturated fatty acid in the cell membrane fraction was above 1.

**INTRODUCTION**

The extracellular accumulation of \( L \)-glutamate (\( L \)-GA) in large quantity was shown in the beet molasses medium, only with addition of polyoxyethylene fatty acid esters (POEFE).\(^1\)

As the result of the investigation of the reason for this phenomenon, it was found that a large amount of \( L \)-GA was accumulated in the medium under aeration and agitation when the molar ratio of the saturated fatty acid (Sat. FA.)/unsaturated fatty acid (Unsat. FA.) in the hydrolyzate of the whole cell of \( L \)-GA-producing bacteria was above 1, whether the carbon source was beet molasses or glucose. In the case of beet molasses media, POEFE was confirmed to affect the ratio, and in glucose media, the concentration of added biotin was confirmed to have a close relation to the ratio.\(^2,3\)

The purpose of the present paper is to make clear the composition of the fatty acids in the hydrolyzate of the cell wall of \( L \)-GA-producing bacteria, their protoplasm, and especially their cell membrane, which is thought to play an important role in the cellular permeability, and to discuss the mechanism of the extracellular accumulation of \( L \)-GA from a view of exit transport.

To this purpose, first of all, the conditions suitable for the bacterial cell wall lysis by lysozyme were studied. As there were no previous reports on the conditions leading to the cell wall lysis of \( L \)-GA-producing bacteria by the enzyme, the factors that would probably be effective were selected from the reports.


that had treated other bacteria, such as *Escherichia coli*, *Pseudomonas aeruginosa* and *Achromobacter fischeri* among the gram-negative bacteria, *Sarcina lutea*, *S. flava*, *Bacillus megaterium*, *B. subtilis*, *Micrococcus lysodeikticus* and *Streptococcus faecalis* among the gram-positive bacteria.

After the confirmation of the cell wall lysis by electron microscope photography, the cell fractionation of the L-GA-producing bacteria was carried out with the protoplast prepared by lysozyme treatment. The composition of the fatty acids in each fraction was determined by gas chromatography, and it was found that a large amount of L-GA was accumulated when the ratio of Sat. FA./Unsat. FA. of the cell membrane was above 1. It is considered that the fatty acids in the cell wall fraction and the protoplasm fraction do not play important roles in the mechanism of the extracellular accumulation of L-GA.

**MATERIALS**

**Lysozyme.** Grade I egg white lysozyme of Sigma Chemical Co. (U.S.A.) was used.

**POEFE.** In this report, only one kind of polyoxyethylene fatty acid mono ester was used, in which the polyoxyethylene part has the molecular weight of 600 and the fatty acids are consisted of 70% of palmitate and 30% of stearate.

**Strains.** *Microbacterium ammoniaphilum A* strain and *C* strain, the latter is resistant to the phages (P-1~5) of the former.

**METHODS**

1. **Assay**

L-GA. L-GA was assayed by Marburg's manometric method using acetone powder of *E. coli* Crooks.

**Fatty Acids.** Fatty acids in each fraction and whole cell were analyzed by Shimazu's GC-1B type gas chromatography after being submitted to hydrolysis with 15 ml of 2 N HCl per g of the lyophilized samples and to methylation by the method of Marr and Ingraham.

They were determined by the peak area-measuring method. In this paper, regarding to the results of gas chromatography, C<sub>16</sub> represents methyl palmitate, C<sub>18</sub> represents methyl octadecylmonoenoate (possibly oleate) and so on.

**Optical density (O.D.).** The reaction mixture was sampled and diluted 20 times with distilled water and its optical density was measured at 660 m<sub>λ</sub> in 10 mm cells by Hitachi's photoelectric meter.

**Weight of cells.** Cells were weighed after lyophilization.

2. **Culture of the Bacteria.**

*M. ammoniaphilum-A* strain and *C* strain were cultured in 20 l jar fermenters, under the conditions shown in Table I. During the cultivation, pH was kept in the range of 7~8 by feeding NH₃ gas, and temperatures at 30°C and 33~35°C for *A* strain and *C* strain, respectively.

The amount of L-GA formed at the sampling time and the final time is listed on Table II.

3. **Preparation of the Bacterial Cell.**

The broth was sampled after twelve hours of culture in the case of beet molasses media and fourteen hours of culture in the case of glucose media, and cells were harvested by refrigerating centrifuge (1500×g), followed by washing twice with 1/3 volume of saline.

4. **Preparation for Electron Microscope.**

The bacterial cells were collected both before and after the reaction, and the diluted water suspensions of them were dropped on the surface of collodion 10 and 19. The latter is resistant to the phages (P-1~5) of the former.

**REFERENCES**

TABLE I. CULTURAL CONDITION BY JAR FERMENTOR

<table>
<thead>
<tr>
<th>Articles</th>
<th>Medium</th>
<th>Seed Medium</th>
<th>Fermentation Medium</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Beet Molasses (as Total sugar)</td>
<td></td>
<td>B-0</td>
</tr>
<tr>
<td></td>
<td>Glucose</td>
<td></td>
<td>7.0</td>
</tr>
<tr>
<td></td>
<td>KH₂PO₄</td>
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<td>0.2</td>
</tr>
<tr>
<td></td>
<td>MgSO₄-7H₂O</td>
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</tr>
<tr>
<td></td>
<td>MnSO₄-4H₂O</td>
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</tr>
<tr>
<td></td>
<td>FeSO₄-7H₂O</td>
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<td>0.5</td>
</tr>
<tr>
<td></td>
<td>Urea</td>
<td></td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td>(NH₄)₂SO₄</td>
<td></td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>POEFE</td>
<td></td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>Biotin</td>
<td></td>
<td>2.6</td>
</tr>
<tr>
<td></td>
<td>Brothout Time</td>
<td></td>
<td>7.5</td>
</tr>
<tr>
<td></td>
<td>pH</td>
<td></td>
<td>7.2</td>
</tr>
</tbody>
</table>

* controlled with NH₃ during the fermentation.
** same as the left colomn except written item.

membrane. After drying, they were shadowed with Chromium by vacuum evaporation.

Nippon Denshi’s JEM-T₄E was used to take electron microscope photographs.

RESULTS AND DISCUSSION

I. Conditions for the Bacterial Cell Wall Lysis by the Lysozyme

To estimate the most effective conditions for the protoplast formation with L-GA-forming bacteria by lysozyme treatment, following experiments were carried out.

*M. ammoniaphilum* A strain or C strain grown in the beet-POEFE medium was suspended at the concentration of 0.45 g dry cell/100 ml in m/20 Na-phosphate buffer.

The degree of the effectiveness of the conditions was measured by the decrease of O.D. of the suspension, according to the experimental design method.

1. *M. ammoniaphilum* A strain

As shown in Fig. 1, the cell wall of *M. ammoniaphilum*-A was attacked by the lysozyme to some extent, but the lysis was not complete as can be seen by comparison with that of C strain in the decrease rate of O.D. and the final O.D. level, and so the completion of the cell wall lysis of this strain was considered to be doubtful.

2. *M. ammoniaphilum* C strain

1) Effect of Inorganic Salts. As shown in Fig. 2, the presence of NaCl was very important for the wall lysis, and the maximum effect was obtained when the concentration of......
NaCl was more than 0.3 mM/ml. The effectiveness of KCl was less than that of NaCl, though it was fairly large. On the contrary, MgCl₂ and CaCl₂ did not show any effect. To certify the effectiveness of Na⁺, the effects of NaCl and NaNO₃ were compared at the conditions shown in Fig. 3. The result showed that the Na⁺ of both NaCl and NaNO₃ were effective, but NaNO₃ was consistently more effective than NaCl, though the difference was not very large.

2) Optimal pH. The optimal pH was found to be 6.2 as shown in Fig. 4. In Figs. 4 and 5, -ΔO.D. represents (O.D. at 360° - O.D. at 0°)

3) Effect of lysozyme concentration. The value of -ΔO.D. increased as the concentration of the enzyme increased, but, when the concentration was above 0.33 mg/ml, the value of -ΔO.D. did not increase any further. In other words the concentration of 0.33 mg/ml
was enough to cause the complete cell wall lysis.

4) Effect of the Reaction Temperature. As shown in Fig. 6, the higher the temperature, the larger the initial reaction rate, but the final value of O.D. is independent on the initial rate. Finally the reaction at 30°C was considered to be most favorable.

5) Effect of EDTA. The addition of EDTA had no effect at optimal pH. Summing up the results described above, the following conditions were considered to be the most adequate for the cell wall lysis of *M. ammoniaphilum* C strain:

6) Sensitivities of Various Cell Preparations toward Lysozyme. In order to study whether it is necessary to modify the conditions for bacteria obtained in different medium, the sensitivity of the cells grown in beet medium was compared with that of the cells grown in beet-POEFE medium. And it was found that no modification of the conditions was necessary (Fig. 8). With the bacterial cells grown in glucose-biotin media, same conclusion was obtained from following results:

- **Glucose medium**
  - O.D. at 0' 0.54 0.17
  - O.D. at 14 hr 0.45 0.14

7) Confirmation of the Cell Wall Lysis with Phase-Contrast Microscope and Electron Microscope. The effects of lysozyme which had been evidenced by the O.D. drop were checked by phase-contrast microscope photography (photo. 1 and 2) and electron microscope photography (photo. 3 and 4). Cells grown in beet-POEFE medium were used. Photo. 1 and 3 were taken before the reaction and
L-Glutamic Acid Fermentation with Molasses. Part VI

PHOTO. 2. Phase-Contrast Microscope Photograph of *M. ammoniaphilum* after the Reaction.

PHOTO. 3. Electron Microscope Photograph of *M. ammoniaphilum* before the Reaction.

PHOTO. 4. Electron Microscope Photograph of *M. ammoniaphilum* after the Reaction.

Photo. 2 and 4 were taken after the fourteen hours of reaction. From them, it was clear that almost all of cell wall had been removed off at the time of fourteen hours reaction. Moreover, the cells were assumed to have existed as the protoplast-like body after the reaction, as can be seen in photo. 2.

II. Relationship of Fatty Acid Composition of the Cell Fraction and L-GA Accumulation

After being submitted to the reaction with the lysozyme under the conditions established above, the cells were fractionated according to the procedure shown in Diagram 1.

Diagram 1. Fractionation Procedure of the Cell Reaction Mixture 300 ml

\[
\begin{align*}
20\text{ hr} & \quad \text{Sup.} \\
\text{Cell Wall fraction} & \quad \text{Ppt.} \\
\text{Quartz} & \quad \text{Grind 45' 5ºC} \\
\text{-100 ml Na-Phosphate Buffer (pH 6.6)} & \quad \text{-20,000 x g Super-Centrifuge (S-C)} \\
\text{(Hitachi 40 P type)} & \\
\end{align*}
\]

The fatty acid compositions of hydrolyzates of the whole cell and each cell fraction were determined by gas chromatography. The results are shown in Tables II and III. Fatty acids other than C_{16}, C_{18}, C_{18} and C_{18} were not included in the consideration of the results, because they were present only in trace (less than 0.5 mg/g dry cell) or not at all.

DISCUSSION

1. Some characteristics of each cellular fraction were identified as follows.

"Cell wall fraction" was assumed to be almost cell wall from the observation by electron microscope photographs and phase-con-
TABLE II. FATTY ACID COMPOSITION IN EACH FRACTION OF THE CELLS GROWN IN BEET MOLASSES MEDIA

<table>
<thead>
<tr>
<th>Medium</th>
<th>l-GA formed mg/ml</th>
<th>Fraction</th>
<th>Amounts of Esters mg/g Dry Cell</th>
<th>Sat./Unsat. F.A. Molar Ratio</th>
<th>Sum of the Esters</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>12 hr</td>
<td>Brothout</td>
<td>C₁₈</td>
<td>C₁₉ω</td>
<td>C₁₈</td>
<td>C₁₉ω</td>
</tr>
<tr>
<td>POEFE</td>
<td>0.15 g/dl</td>
<td>20.5</td>
<td>78.7</td>
<td>Wall</td>
<td>1.27</td>
<td>Tr.</td>
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<tr>
<td></td>
<td></td>
<td>Membrane</td>
<td>5.85</td>
<td>0.75</td>
<td>1.06</td>
<td>4.61</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Plasm</td>
<td>0.52</td>
<td>0.11</td>
<td>0.21</td>
<td>0.21</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Total</td>
<td>7.64</td>
<td>0.86</td>
<td>1.48</td>
<td>5.83</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Whole Cell</td>
<td>8.74</td>
<td>0.95</td>
<td>1.60</td>
<td>7.05</td>
</tr>
<tr>
<td>POEFE</td>
<td>4 g/dl</td>
<td>3.8</td>
<td>5.2</td>
<td>Wall</td>
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<td>Tr.</td>
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<tr>
<td></td>
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<td>Membrane</td>
<td>7.37</td>
<td>1.21</td>
<td>0.45</td>
<td>8.79</td>
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<td>Plasm</td>
<td>1.34</td>
<td>0.21</td>
<td>0.17</td>
<td>1.08</td>
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<tr>
<td></td>
<td></td>
<td>Total</td>
<td>10.81</td>
<td>1.42</td>
<td>0.62</td>
<td>11.48</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Whole Cell</td>
<td>11.71</td>
<td>1.62</td>
<td>0.66</td>
<td>13.20</td>
</tr>
</tbody>
</table>

TABLE III. FATTY ACID COMPOSITION IN EACH FRACTION OF THE CELLS GROWN IN GLUCOSE-BIOTIN MEDIA

<table>
<thead>
<tr>
<th>Medium</th>
<th>l-GA formed mg/ml</th>
<th>Fraction</th>
<th>Amounts of Esters mg/g Dry Cell</th>
<th>Sat./Unsat. F.A. Molar Ratio</th>
<th>Sum of the Esters</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>14 hr</td>
<td>Brothout</td>
<td>C₁₈</td>
<td>C₁₉ω</td>
<td>C₁₈</td>
<td>C₁₉ω</td>
</tr>
<tr>
<td>Biotin</td>
<td>2.5 γ/l</td>
<td>16.3</td>
<td>66.2</td>
<td>Wall</td>
<td>0.72</td>
<td>0.10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Membrane</td>
<td>5.01</td>
<td>0.45</td>
<td>0.26</td>
<td>4.76</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Plasm</td>
<td>1.02</td>
<td>0.22</td>
<td>0.26</td>
<td>0.65</td>
</tr>
<tr>
<td></td>
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<td>Total</td>
<td>6.75</td>
<td>0.77</td>
<td>0.62</td>
<td>5.76</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Whole Cell</td>
<td>7.80</td>
<td>1.01</td>
<td>0.71</td>
<td>7.02</td>
</tr>
<tr>
<td>Biotin</td>
<td>20 γ/l</td>
<td>3.9</td>
<td>5.2</td>
<td>Wall</td>
<td>1.44</td>
<td>0.44</td>
</tr>
<tr>
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<td>1.03</td>
<td>0.31</td>
<td>11.20</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Plasm</td>
<td>2.02</td>
<td>0.33</td>
<td>0.48</td>
<td>1.56</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Total</td>
<td>10.57</td>
<td>1.80</td>
<td>1.13</td>
<td>13.29</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Whole Cell</td>
<td>11.70</td>
<td>1.87</td>
<td>1.29</td>
<td>15.69</td>
</tr>
</tbody>
</table>

Contrast microscope photographs, in which ghost cells were usually observed to be only about 10% of protoplast-like bodies.

"Cell membrane fraction" was also almost exclusively cell membrane, because the fractionation was carried out at the lowest possible centrifugal force (12,000×g) which had been used in the fractionation of cell membrane and protoplasm, in order to prevent the contamination of the cell membrane fraction with the protoplasm. And the fact that this fraction had no succinic dehydrogenase activity supported the above-mentioned judgement.

On the other hand, the contamination with cell wall was not recognized so far as qualitative analysis of glucosamine, which was considered to be a component of cell wall, was concerned.

"Protoplasm fraction" was supposed to include some of the cell membrane fraction because of the above-mentioned reason.

2. The sum of the fatty acids of each fraction was about 85% of those of the whole cell. But the degree of the loss of each fatty acid did not differ significantly among the fatty acids, so the ratio of Sat. FA./Unsat. FA. in each fraction was not considered to be affected by the loss.
3. In the previous paper, the authors proposed the thesis that the extracellular accumulation of L-GA in large quantity depended on whether the ratio of Sat. FA./Unsat. FA. of whole cell hydrolyzate was above 1 in \textit{M. ammoniophilum}.

But when the ratio in each fraction was studied in detail, it was clear that not all the fractions had something to do with the accumulation of L-GA, because the amount of L-GA accumulation did not have any correlation with the ratio in the cell wall fraction or in the protoplasm fraction.

On the contrary, the ability of L-GA accumulation in large quantity clearly has some relation with the ratio of Sat. FA./Unsat. FA. in the cell membrane.

4. From the above-mentioned facts and discussion, the authors should like to explain more in detail the thesis proposed in the previous paper.

The extracellular accumulation of L-GA in large quantity would take place when the ratio of Sat. FA./Unsat. FA. of the cell membrane hydrolyzate was above 1, and, because the amount of the fatty acids in the cell membrane fraction is about 75\% of that in the whole cell, the relationship between the fatty acid composition of the whole cell and L-GA accumulation would be direct.

**SUMMARY**

It had already been shown that a large amount of L-GA was accumulated when the ratio of Sat. FA./Unsat. FA. was above 1, in the hydrolyzate of the whole cell of \textit{M. ammoniophilum} grown under aeration and agitation.

To make clear this relation in detail, further investigation on the correlation of the fatty acid composition of each cell fraction and the extracellular accumulation of L-GA was carried out as follows:

1. Five factors—pH, temperature, the concentration of lysozyme, the addition of inorganic ion and EDTA—were studied in order to determine the conditions suitable for the cell wall lysis of the L-GA-producing bacteria. The result was that the cell wall of \textit{M. ammoniophilum}-A was undoubtedly attacked to some extent, but was doubtful to be completely broken down by the lysozyme.

On the contrary, the cell wall lysis of \textit{M. ammoniophilum}-C was observed with electron microscope and phase-contrast microscope to be completed under the following conditions:

- Bacterial cell: 4.5 mg/ml (as dry weight)
- Lysozyme: 0.33 mg/ml
- NaNO₃: 0.3 mm/ml
- EDTA: 0
- M/20 Na-phosphate buffer (pH 6.2)
- Temp: 30°C, 14 hr.

2. Each of the C strain grown in the glucose-biotin (biotin level: 2.5 \textgamma/1 and 20 \textgamma/l) media and beet molasses-POEFE (POEFE level: free and 0.15 g/dl) media was submitted to the cell wall lysis under the above mentioned conditions.

After removal of the cell wall fraction by 6,000×g, the protoplast was ground by quartz. The cell membrane fraction and “the protoplasm fraction” were fractionated at 12,000×g, to prevent the contamination of the cell membrane fraction with the protoplasm. Each fraction was hydrolyzed and methylated before being submitted to the gas chromatography.

The correlation of the resulting fatty acid composition of each fraction and the extracellular accumulation of L-GA was discussed. From these results, following thesis was proposed: A large amount of extracellular accumulation of L-GA took place when the ratio of Sat. FA./Unsat. FA. in the cell membrane fraction was above 1 and the ratio in the cell wall and the protoplasm had no direct relation to the L-GA accumulation.

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