Repeated Batch Production of Theanine by Coupled Fermentation with Energy Transfer Using Membrane-Enclosed γ-Glutamylmethylamide Synthetase and Dried Yeast Cells

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Theanine, γ-glutamylethylamidine, is an unique amino acid found in Japanese green tea leaves. It greatly contributes to the taste of green tea. In addition, it has received much attention due to its many favorable physiological functions in several animals, including humans. The demand for theanine has increased as a supplement to maintain human health. Among the several methods studied for theanine production, an enzymatic method with glutamine and ethylamine as substrates was recently accomplished using the γ-glutamyl transfer reaction of bacterial glutaminase or γ-glutamyl transpeptidase. However, the method has certain shortcomings with respect to the supply of glutamine, the hydrolysis of glutamine, and the formation of other γ-glutamyl derivatives.

In the previous study, we demonstrated theanine production from glutamic acid and ethylamine at high concentrations using γ-glutamylmethylamidine synthetase (GMAS) of Methylovorus mays No. 9, in which a catalytic amount of another substrate, ATP, is regenerated by sugar fermentation of dried baker’s yeast cells (coupled fermentation with energy transfer, Fig. 1). The amount of theanine formed was about 2.5 times larger than that formed by bacterial glutaminase, and the yield of theanine was 100% on the substrates (glutamic acid and ethylamine) and also on the energy source (glucose consumed). This paper deals with repeated batch production of theanine with GMAS and dried baker’s yeast cells, which were enclosed together in a dialysis membrane tube.

γ-Glutamylmethylamide synthetase and dried baker’s yeast cells were enclosed together in a dialysis membrane tube to produce theanine repeatedly by coupled fermentation with energy transfer. The membrane-enclosed enzyme preparation (M-EEP) formed approximately 600 mM theanine from glutamic acid and ethylamine at a 100% conversion rate. M-EEP maintained its productivity of theanine during six consecutive reactions in a mixture containing NAD+.

Note

Amino acid is l-isomer unless otherwise stated.

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cells were placed in the substrate solution, theanine formation did not occur (data not shown), indicating that enclosing both enzymes together was necessary to produce theanine by energy transfer.

As Fig. 2A2 and A3 show, increasing the concentration of GMAS in M-EEP and the substrates (sodium glutamate and ethylamine·HCl) increased the amount of theanine formed, as in the case of free GMAS and yeast cells (Fig. 2B2 and B3).9) The final concentration of theanine formed in 48 h (Fig. 2A3) was about 600 mM with 100% yield based on the substrates (sodium glutamate and ethylamine·HCl), and also on the glucose consumed, which were almost the same as in the mixture with the free enzyme preparations (Fig. 2B3). On the other hand, a retardation of glucose consumption and theanine formation in the mixture with M-EEP was found (Fig. 2A3 and B3), probably due to a low diffusion rate of the substrates and the other components through the dialysis membrane.

Table 1 summarizes experiments to examine the reusability of M-EEP in theanine production. The first reaction was carried out for 24 h or 48 h in a 50-ml test tube containing 1 ml of M-EEP and 4 ml of the substrate solution. After incubation, M-EEP was transferred to 4 ml of a fresh substrate solution and incubated again for 24 h or 48 h (second reaction). This procedure was repeated 6–7 times. In these experiments, sodium azide was added to the substrate solution to prevent bacterial contamination of the reaction mixture.

Experiment A (Table 1) indicated that the amount of theanine formed in the second reaction decreased to

![Glucose](image1)

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77% of that in the first reaction, and productivity declined further along with repeated reactions. However, productivity was maintained by the addition of 1 mM NAD$^+$, a cofactor for sugar fermentation of yeast cells, into the substrate solution (exp. B), and MEEP produced about 400 mM theanine during six consecutive reactions (exp. C). The addition of NAD$^+$ might have compensated for the loss of endogeneous NAD$^+$, which was washed out from MEEP during repeated batch reactions, as reported by Asada et al.13) Our preliminary experiment also confirmed that the addition of NAD$^+$ to the substrate solution.

Table 1. Theanine Productivity in Repeated Batch Reactions Using MEEP

<table>
<thead>
<tr>
<th>Exp.</th>
<th>Time (h)</th>
<th>NAD$^+$</th>
<th>1st reaction</th>
<th>2nd reaction</th>
<th>3rd reaction</th>
<th>4th reaction</th>
<th>5th reaction</th>
<th>6th reaction</th>
<th>7th reaction</th>
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<tbody>
<tr>
<td>A</td>
<td>24</td>
<td>−</td>
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<td>77</td>
<td>52</td>
<td>15</td>
<td>0</td>
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<td>B</td>
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<td>+</td>
<td>100</td>
<td>103</td>
<td>107</td>
<td>110</td>
<td>37</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>C</td>
<td>24</td>
<td>+</td>
<td>100</td>
<td>107</td>
<td>103</td>
<td>99</td>
<td>98</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>48</td>
<td>+</td>
<td>100</td>
<td>99</td>
<td>100</td>
<td>68</td>
<td>21</td>
<td>4</td>
<td>0</td>
</tr>
</tbody>
</table>

$^1$MEEP (total volume, 1 ml) contained 200 mg of dried yeast cells and 5 units of GMAS (exp. A and B), 75 units of GMAS (C), and 150 units of GMAS (D). The substrate solutions (total volume, 4 ml) contained 250 mM potassium phosphate buffer (pH 7.0), 37.5 mM MgCl$_2$, 3.75 mM MnCl$_2$, 6.25 mM AMP, and 0.2 mg/ml of sodium azide. The other components in the substrate solution (glucose, sodium glutamate, ethylamine-HCl, and NAD$^+$) were varied in each experiment, as follows: glucose, 250 mM (A, B, and C), and 750 mM (D); sodium glutamate, 250 mM (A and B), 500 mM (C), and 750 mM (D); ethylamine-HCl, 375 mM (A and B) and 750 mM (C and D); 1 mM NAD$^+$ was added to the substrate solutions of B, C, and D. The reaction conditions in exp. A were the same as those described in the legend to Fig. 2A1. The reaction conditions in exps. B, C, and D were the same as those in Fig. 2A1, A2, and A3 respectively, except for the addition of 1 mM NAD$^+$ to the substrate solution.

$^2$Incubation times for the various reactions.

$^3$Without NAD$^+$ (−) and with NAD$^+$ (+) in the reaction mixture (see above).

$^4$The amount of theanine formed was expressed relative to that in the first reaction; 100% = 65 mM (A), 68 mM (B), and 390 mM (C) over 24 h of reaction, and 590 mM (D) over 48 h of reaction.

This study indicates that MEE-P (the membrane-enclosed enzyme preparation) is more effective and practical than free enzymes in a batch reactor.

References