γ-GLUTAMYL TRANSFER REACTIONS BY GLUTAMINASE FROM *PSEUDOMONAS NITROREDUCENS* IFO 12694 AND THEIR APPLICATION FOR THE SYNTHESSES OF THEANINE AND γ-GLUTAMYL METHYLMALAMIDE

Takashi Tachiki, Takeshi Yamada, Katsushige Mizuno, Masashi Ueda, Ju-ichi Shioe, and Hiroshi Fukami

Department of Bioscience and Biotechnology, Faculty of Science and Engineering, Ritsumeikan University, Nojihigashi 1-1-1, Kusatsu 525-8577, Japan

Received September 16, 1997

In a mixture containing γ-glutamyl donor (donor) and γ-glutamyl acceptor (acceptor), the glutaminase of *Pseudomonas nitroreducens* IFO 12694 simultaneously catalyzed a γ-glutamyl transfer reaction and hydrolysis of the donor. The variation of the activities responding to the concentration of glutathione and glycylglycine indicated that the enzyme might be classified in a group of glutaminases that shows hydrolysis prior to transfer reaction. On the other hand, the results with glutamine and ethylamine or methylamine indicated that the enzyme was active in the transfer reaction with suppressed hydrolysis of glutamine, and suggested the possibility of using the reaction for producing γ-glutamylethylamide (theanine) or γ-glutamylmethyalmide (γ-GMA). In fact, in a mixture containing high concentrations of substrates (0.7 M glutamine, 1.5 M ethylamine or methylamine) and 0.5 unit/ml glutaminase (borate buffer pH 11), 270 mM (47 g/L) theanine or 250 mM (38 g/L) γ-GMA was formed in 7 h of incubation at 30°C.

**Key words:** theanine; γ-glutamylmethyalmide; glutaminase; γ-glutamyl transfer reaction; *Pseudomonas nitroreducens*

Glutaminase (EC 3.5.1.2) is defined as an enzyme that dehydroglycine glutamine to glutamic acid and ammonia. However, a lot of glutaminases catalyze γ-glutamyl transfer from such γ-glutamyl donor (donor) as glutamine or glutathione to γ-glutamyl acceptor (acceptor) like D-glutamyltransferase (EC 2.3.2.1) and γ-glutamyltransferase (EC 2.3.2.2). On the other hand, many of the glutamyltransferases hydrolyze donors. Such similarity necessarily obscures the distinction between glutaminase and the glutamyltransferases. In a review on glutaminases and glutamyltransferases, Hartman classified these enzymes into 4 groups on the bases of catalytic properties:11 (A) only hydrolysis, (B) hydrolysis prior to transfer reaction with some acceptors, (C) transfer reaction prior to hydrolysis (hydrolysis is largely suppressed by suitable acceptor in favor of transfer reaction), and (D) only transfer reaction.

In our previous paper,21 we found that the glutaminase of *Pseudomonas nitroreducens* IFO 12694 catalyzed, to a certain degree, γ-glutamyl transfer from glutamine to ethylamine or methylamine to form γ-glutamylethylamide (theanine) or γ-glutamylmethyalmide (γ-GMA). We also indicated that glutathione could serve as a γ-glutamyl donor to hydroxylamine. From these findings, further investigation on the transfer reactions became necessary from the following two viewpoints.

The first is understanding of the relationship of *P. nitroreducens* glutaminase to other enzymes catalyzing γ-glutamyl transfer reaction and hydrolysis of donor. The second is application of the transfer reaction for production of theanine or γ-GMA: the former, a taste-enhancing substance of infused green tea,3 has been reported to have physiologically favorable effects on animals,4-6 and the latter, with a taste similar to theanine,7 occurs as an intermediate in metabolism of methyalmine in several bacteria8-13 or plants.14,15

Theanine or γ-GMA is synthesized in vivo by theanine synthetase,16,17 (EC 6.3.1.6) or γ-GMA synthetase,18 (EC 6.3.4.12) from glutamic acid and ethylamine or methylamine with consumption of ATP as glutamine is formed by glutamine synthetase (EC 6.3.1.2). We have tried to produce theanine or γ-GMA by using a reaction of bacterial glutamine synthetase with ethylamine or methylamine coupled with a sugar fermentation reaction of baker’s yeast as an ATP-regenerating system.19 By this method, though some amount of γ-GMA was produced, the production of theanine was unsatisfactory because of the low reactivity of glutamine synthetase toward ethylamine and of difficulty in pH control of the reaction mixture.19 After that, no attempt to produce theanine or γ-GMA with microbial enzyme has been made.

This paper deals with several properties of γ-glutamyl transfer reactions by *P. nitroreducens* glutaminase with glutamine or glutathione as a donor, and ethylamine, methylamine, or glycylglycine as an acceptor. Use of the transfer reaction to produce theanine or γ-GMA is also described.

**Materials and Methods**

Glutaminase of *P. nitroreducens*. Glutaminase was isolated from *P. nitroreducens* IFO 12694 by the

---

1 Corresponding author.

Amino acids are ε-isomers in this paper unless otherwise stated.
method described in our previous paper.\textsuperscript{21} One unit of the enzyme activity was defined as the amount of the enzyme forming 1 μmole of p-nitroaniline per min at 30°C in a mixture containing 2.5 mM γ-glutamyl p-nitroanilide, 100 mM imidazole-HCl buffer (pH 9.0) and the enzyme preparation.\textsuperscript{21}

Assay of γ-glutamyl transfer and γ-glutamyl hydrolysis activities. A mixture containing 35 mM donor, 150 mM acceptor, and 150 mM buffer, and the enzyme preparation was used for assaying two reactions that proceed simultaneously in the mixture: formation of γ-glutamyl derivative (transfer reaction) and hydrolysis of donor. Among the transfer reactions catalyzed by the enzyme, the reactions with glutamine and ethylamine, glutamine and methyamine, and glutathione and glycylglycine were mainly examined in this paper. Glutathione and glycylglycine are the usual substrates for γ-glutamyl transferring enzymes.

Another mixture with higher concentrations of glutamine and ethylamine or methyamine was used for producing theanine or γ-GMA (detailed conditions are described for each result).

Incubation was done at 30°C statically or with moderate shake. The reaction was stopped by immersing the reaction tube in boiling water for 3 min, and γ-glutamyl derivatives (transfer reaction) and glutamic acid (hydrolysis) were measured.

Analysis. Amino acids and γ-glutamyl derivatives in the reaction mixture were estimated by ninhydrin colorimetry after paper chromatography\textsuperscript{23} or by using a Hitachi automated amino acid analyzer L-8500 (column size, 4.6 mm × 60 mm; resin, #2622SC).

Chemicals. γ-GMA was prepared by the method described previously.\textsuperscript{23} Theanine was purchased from Tokyo Kasei Co. Ltd., and amino acid mixture solution Type H was purchased from Wako Pure Chemicals Co. Ltd. Other reagents were the highest grade commercial products.

Results and Discussion

γ-Glutamyl acceptors active toward glutamine or glutathione

The rate of γ-glutamyl transfer from glutamine or glutathione to several acceptors was measured at pH 7.0 (Table 1). The reactivity of the enzyme with these acceptors was not as high as that to hydroxylamine. However, it might be necessary to examine each reaction under its optimum conditions because the conditions for known γ-glutamyl transferring enzymes are variable with substrate and/or their source.\textsuperscript{20,28} In the following experiments, ethylamine or methyamine was chosen as an acceptor for glutamine, and glycylglycine for glutathione.

Effect of pH

Figure 1 (A, B) indicates that the optimum pH of the transfer reaction with glutamine and ethylamine or methyamine was 10–11, being different from the neutral optimum pH for the reaction with hydroxylamine.\textsuperscript{23} The alkaline optimum pH was a scarce example: many microbial γ-glutamyl transferring enzymes catalyze the reaction optimally at neutral or slightly alkaline pH range (7.5 to 9)\textsuperscript{20,22–28} except for the enzymes from \textit{Lentinus edodes} (pH 9–10 for the reaction with lentinic acid and valine),\textsuperscript{22} \textit{Proteus mirabilis} (pH 9.5–11, with glutathione and 3,4-dihydroxyphenylalanine),\textsuperscript{23} and \textit{Escherichia coli} (pH 10.8, with glutamine and 3,4-dihydroxyphenylalanine).\textsuperscript{20} The simultaneous hydrolysis of glutamine proceeded optimally at pH 9.0 as did the hydrolysis of glutamine, which was described in our

### Table 1. γ-Glutamyl Transfer from Glutamine or Glutathione to Several Acceptors by Glutaminase of \textit{P. nitroreducens}

<table>
<thead>
<tr>
<th>Donor</th>
<th>Acceptor</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>glutamine</td>
<td>Hydroxylamine</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Ethylamine</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>Methylamine</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Methionine</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Glycylglycine</td>
<td>4</td>
</tr>
<tr>
<td>glutathione</td>
<td>Hydroxylamine</td>
<td>83</td>
</tr>
<tr>
<td></td>
<td>Ethylamine</td>
<td>0.7</td>
</tr>
<tr>
<td></td>
<td>Methylamine</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>Methionine</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Glycylglycine</td>
<td>5</td>
</tr>
</tbody>
</table>

The mixture was defined on the bases of the previous finding\textsuperscript{22} on the transfer reaction with glutamine and hydroxylamine, and contained 35 mM donor, 150 mM acceptor, 150 mM imidazole buffer (pH 7.0) and appropriate amounts of glutaminase. γ-Glutamylhydroxamate was measured by the method described previously,\textsuperscript{22} and other reaction products such as theanine, γ-GMA, γ-glutamylmethionine, and γ-glutamylglycylglycine were by ninhydrin colorimetry or with an automated amino acid analyzer as described in Materials and Methods. Under these conditions, 1 unit of glutaminase formed 2.3 μmole of γ-glutamylhydroxamate/min, and the activities were expressed relative to that of the transfer reaction with glutamine and hydroxylamine.

Theanine, γ-GMA, γ-glutamylmethionine, or γ-glutamylglycylglycine was isolated from each reaction mixture by ion exchange column chromatography on Dowex 1 × 8 (H\textsuperscript{+}) and/or Dowex 1 × 2 (Cl\textsuperscript{−}), and identified by its behaviors in paper chromatography and/or HPLC-mass spectrometer, and by analysis of chemical components of acid-and enzymatic-hydrolysates.

![Fig. 1. Effects of pH on the Transfer and the Hydrolysis Activities.](image-url)

The reaction mixture contained 35 mM glutamine (A, B) or glutathione (C), 150 mM ethylamine (A), methyamine (B) or glycylglycine (C), 150 mM buffer, and 0.6 unit/ml glutaminase. Imidazole buffer (○, ●) was used for pH 7.5–9.5, and borate buffer (○, ●) for pH 9.0–12.0. Incubation was done for 1 h. Symbols: ○ and ●, theanine (A), γ-GMA (B) or γ-glutamylglycylglycine (C); ○ and ●, glutamic acid (A, B, C).
previous paper. 2)

On the other hand, the transfer reaction and the hydrolysis in the mixture with glutathione and glycyglycine proceeded optimally at neutral pH (Fig. 1C), and the transfer activity was lower than that with glutamine and ethylamime or methylamine.

The activity ratio of the transfer reaction to the hydrolysis (T/H ratio) was 0.2-0.3 in the reaction with glutamine and ethylamine or methylamine (at pH 11.0), and 0.1 with glutathione and glycyglycine (at pH 7.0), which were much lower than those (1 to 13) observed with γ-glutamyl transferring enzymes from other microorganisms. 30,21,23-26

The optimum pH for the transfer reaction with glutamine and methionine, glutamine and glycyglycine, or glutathione and methionine was also pH 7, and the activity was almost the same as or less than that with glutathione and glycyglycine (data not shown).

Effects of substrate concentration

Figure 2 compares the transfer and the hydrolysis activities in the mixtures containing various concentrations of acceptor at a fixed concentration of donor. As shown in Fig. 2A or 2B, the transfer activity with glutamine and ethylamine or methylamine became higher with increase of the acceptor, and the hydrolysis activity was lowered. The T/H ratio was about 0.4 with 200 mM ethylamine or methylamine, and further addition of the acceptor brought about certain increases of the transfer activity together with a decrease of the hydrolysis activity (data not shown). In case of the reaction with glutathione and glycyglycine (Fig. 2C), the transfer activity was not so varied (T/H ratio, 0.2 with 200 mM glycyglycine).

Figure 3A or 3B indicates that the increase of glutamine at the fixed concentration of ethylamine or methylamine caused a large increase of the transfer and the hydrolysis activities (maximum T/H ratio, 0.8-0.9), but not of the activities with glutathione and glycyglycine (Fig. 3C, maximum T/H ratio, 0.2).

The change of the activities responding to the concentrations of glutamine and ethylamine or methylamine seemed to indicate a property31 of the group C enzymes, and suggested a need for further investigation, which was done in the next section. However, the observations on the transfer reaction with glutathione and glycyglycine (low activity, and its insignificant change responding to the substrate concentration) were quite apart from those with other γ-glutamyl transferring enzymes, suggesting that P. nitroreducens glutaminase belongs to group B (hydrolysis prior to transfer reaction).

Formation of theanine or γ-GMA with higher concentrations of substrates

The transfer reaction with glutamine and ethylamine or methylamine was reexamined in respect to production of theanine or γ-GMA with higher concentrations of the substrates.

Figure 4 indicates the pH dependence of the transfer reaction and the hydrolysis. The optimum pH (pH 11) of each transfer reaction was almost the same as that in Fig. 1. The activity of the simultaneous hydrolysis was extremely low at pH 11 (T/H ratio, about 60 in Fig. 4A; 5.1 in Fig. 4B), probably being caused by higher concentrations of ethylamine or methylamine, and this might be advantageous for the production of theanine or γ-GMA.

Formation of theanine or γ-GMA in the mixtures containing various concentrations of the substrates are summarized in Table 2. Experiment A1 or A2 showed that increase of glutamine with 1.5 mM ethylamine or methylamine increased theanine or γ-GMA, and the maximum amount of the product reached 270 mM (theanine, about 47 g/L) or 250 mM (γ-GMA, about 38 g/L) in 7 h-incubation in the mixtures with 0.7 mM glutamine. The amounts were much larger than that (10.5 mM theanine...
Fig. 4. Effects of pH on the Transfer and the Hydrolysis Activities in the Mixture with Higher Concentration of Substrates.

The reaction mixture contained 0.3 mM glutamine, 1.5 mM ethylamine (A) or methylamine (B), 100 mM buffer, and 0.6 unit/ml glutaminase. Imidazole buffer (○, ●) was used for pH 8.0-9.5, and borate buffer (△, ▲) for pH 9.0-13.0. Incubation was done for 1 h. Symbols: ●, ○, theanine (A) or γ-GMA (B); ▲, △, glutamic acid (A, B).

Fig. 5. Formation of Theanine and γ-GMA with Various Amounts of Glutaminase.

The reaction mixture contained 0.3 mM glutamine, 1.5 mM ethylamine (A) or methylamine (B), 100 mM buffer (pH 11), and various amounts of glutaminase. Incubation was done for 1 h. Symbols: ●, ○, 0.5 unit/ml; ▲, △, 0.3 unit/ml; ■, ■, 0.2 unit/ml; ◇, ◇, 0.1 unit/ml; △-△, 0.05 unit/ml.

### Table 2. Formation of Theanine or γ-GMA in the Mixtures with Higher Concentration of Substrates

<table>
<thead>
<tr>
<th>Glutamine (mM)</th>
<th>Ethylamine or methylamine (mM)</th>
<th>Maximum theanine or γ-GMA formed (mm)</th>
<th>Yield (%) based on glutamine</th>
<th>Time for maximum formation (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exp. A</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td>ethylamine 1.5</td>
<td>90</td>
<td>90</td>
<td>3</td>
</tr>
<tr>
<td>0.2</td>
<td>1.5</td>
<td>146</td>
<td>73</td>
<td>3</td>
</tr>
<tr>
<td>0.3</td>
<td>1.5</td>
<td>212</td>
<td>71</td>
<td>5</td>
</tr>
<tr>
<td>0.5</td>
<td>1.5</td>
<td>236</td>
<td>47</td>
<td>5</td>
</tr>
<tr>
<td>0.7</td>
<td>1.5</td>
<td>270</td>
<td>39</td>
<td>7</td>
</tr>
<tr>
<td>Exp. A2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td>methylamine 1.5</td>
<td>88</td>
<td>88</td>
<td>3</td>
</tr>
<tr>
<td>0.2</td>
<td>1.5</td>
<td>162</td>
<td>81</td>
<td>3</td>
</tr>
<tr>
<td>0.3</td>
<td>1.5</td>
<td>210</td>
<td>70</td>
<td>5</td>
</tr>
<tr>
<td>0.5</td>
<td>1.5</td>
<td>234</td>
<td>47</td>
<td>5</td>
</tr>
<tr>
<td>0.7</td>
<td>1.5</td>
<td>250</td>
<td>36</td>
<td>7</td>
</tr>
<tr>
<td>Exp. B1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.3</td>
<td>ethylamine 0.3</td>
<td>122</td>
<td>41</td>
<td>5</td>
</tr>
<tr>
<td>0.3</td>
<td>0.5</td>
<td>164</td>
<td>55</td>
<td>5</td>
</tr>
<tr>
<td>0.3</td>
<td>1.0</td>
<td>212</td>
<td>71</td>
<td>5</td>
</tr>
<tr>
<td>0.3</td>
<td>1.5</td>
<td>212</td>
<td>71</td>
<td>5</td>
</tr>
<tr>
<td>0.3</td>
<td>2.0</td>
<td>210</td>
<td>70</td>
<td>5</td>
</tr>
<tr>
<td>Exp. B2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.3</td>
<td>methylamine 0.3</td>
<td>132</td>
<td>44</td>
<td>5</td>
</tr>
<tr>
<td>0.3</td>
<td>0.5</td>
<td>162</td>
<td>54</td>
<td>5</td>
</tr>
<tr>
<td>0.3</td>
<td>1.0</td>
<td>206</td>
<td>69</td>
<td>5</td>
</tr>
<tr>
<td>0.3</td>
<td>1.5</td>
<td>210</td>
<td>70</td>
<td>5</td>
</tr>
<tr>
<td>0.3</td>
<td>2.0</td>
<td>214</td>
<td>71</td>
<td>5</td>
</tr>
</tbody>
</table>

Exp. A: The mixture contained various concentrations of glutamine, 1.5 mM ethylamine (A1), or methylamine (A2), 100 mM borate buffer (pH 11.0), and 0.5 unit/ml glutaminase.

Exp. B: The mixture contained 0.3 mM glutamine, various concentrations of ethylamine (B1) or methylamine (B2), 100 mM borate buffer (pH 11.0), and 0.5 unit/ml glutaminase.

or 110 mM γ-GMA) with bacterial glutamine synthetase and baker's yeast preparations. Experiment A1 or A2 also indicated that the product yield based on glutamine was lowered with an increase of glutamine, and that the incubation time necessary for the maximum formation was prolonged.

Considering the product yield and the incubation time, the concentration of 0.3 mM was chosen for glutamine in Experiment B1 or B2, which investigated the effects of the concentration of ethylamine or methylamine. The amount and the yield of theanine or γ-GMA increased with increases of ethylamine or methylamine, and it was indicated that 1.0 mM ethylamine or 1.5 mM methylamine was sufficient under these conditions. The time for the maximum formation of theanine or γ-GMA was almost the same (5 h) in all the mixtures.

Figure 5 shows formation of theanine or γ-GMA with various amounts of glutaminase. The finding that the reaction proceeded almost linearly for 24 h with 0.1-0.2 unit/ml enzyme indicated that the enzyme activity was maintained at least for 24 h at pH 11, and this was not inconsistent with the enzyme stability: full activity was retained after 10 min of treatment at pH 11 and at 40°C. A stabilizing effect of theanine on the enzyme was also suggested in the experiments with the immobilized *P. nitroreducens* cells which were undertaken by other authors according to our preliminary reports.

Decrease of the product in the later incubation period with larger amounts of the enzyme might be its enzymatic hydrolysis caused by decrease of glutamine.

This paper indicated that *P. nitroreducens* glutaminase might be an enzyme of group B (hydrolysis of glutathione prior to γ-glutamyl transfer to glycyglycine) while it had a property of group C (γ-glutamyl transfer to ethylamine or methylamine with repressed hydrolysis of glutamine). It also indicated that the en-
zyme was usable for a new method for producing theanine or γ-GMA with a higher concentration of substrates.

References


