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Theanine Production by Coupled Fermentation with Energy Transfer Using γ-Glutamylmethylamide Synthetase of *Methylovorus mays* No. 9

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γ-Glutamylmetylamide synthetase (GMAS) of *Methylovorus mays* No. 9, produced by *Eschericia coli* AD494 (DE3) harboring pET21aGM, formed theanine from glutamic acid and ethylamine with coupling of the reaction with sugar fermentation of baker's yeast cells as an ATP-regeneration system. Theanine formation was stimulated by the addition of Mn^{2+} to the reaction mixture, whereas Mg^{2+} was less effective. Increases to a certain level in the concentrations of GMAS and the substrates in the mixture were effective in increasing theanine formation, but high concentrations of ethylamine (900 mM or more) inhibited yeast sugar fermentation, and eventually decreased theanine formation. The inhibitory effect of ethylamine was restored by increasing the concentration of potassium phosphate buffer in the mixture. Approximately 600 mM (110 mg/ml) theanine was formed in 48 h in an improved reaction mixture containing 600 mM sodium glutamate, 600 mM ethylamine·HCl, 300 mM glucose, 200 mM potassium phosphate buffer (pH 7.0), 30 mM $MgCl_2$, 5 mM $MnCl_2$, 5 mM AMP, 30 units/ml of GMAS, and 40 mg/ml of yeast cells. The yield of theanine was 100% on the substrates (glutamic acid and ethylamine) and also on the energy source (glucose consumed).

Key words: theanine; γ-glutamylmethylamide synthetase; coupled fermentation with energy transfer

Theanine (γ-glutamylethylamide) is an unique amino acid found in Japanese green tea leaves.[1] It is synthesized in the root of the tea plant by theanine synthetase (L-glutamate:ethylamine ligase; ADP-forming, E.C. 6.3.1.6) with glutamic acid, ethylamine, and ATP as substrates.[2] Theanine greatly contributes to the taste of green tea,[3] and the quality of the tea leaves is dependent on its contents. In addition, theanine has received much attention due to its many favorable physiological functions in several animals, including human beings: significant reductions in blood pressure,[4] relief from convulsions induced by caffeine,[5] generation of α-brain waves, indicating relaxation,[6] and a favorable influence on the levels of several bioactive substances in the brain.[7–9] Hence the demand for theanine has increased as a supplement for maintaining human health.

Recently, enzymatic production of theanine with glutamine and ethylamine as substrates was accomplished by γ-glutamyl transfer reaction of bacterial glutaminase[10] and γ-glutamyl transpeptidase,[11] but this method has certain shortcomings with respect to the supply of glutamine, the hydrolysis of glutamine, and the formation of other γ-glutamyl derivatives.[10,11]

This indicates a need for another efficient method, and we have investigated a process of theanine production from glutamic acid and ethylamine as substrates using a bacterial theanine synthetase-like enzyme.[12–15] In this process, sugar fermentation of dried baker's yeast cells was coupled as an ATP-regenerating system (coupled fermentation with energy transfer).

In earlier studies,[12,13] we demonstrated the principle of theanine production by coupled fermentation with energy transfer using glutamine synthetase (GS; L-glutamate:ammonia ligase, ADP-forming, E.C. 6.3.1.2) of *Pseudomonas taetrolens* Y-30, which has a certain reactivity toward ethylamine. The results suggested the possibility of theanine production in high concentrations by the use of large amounts of GS in the reaction mixture,[13] but this possibility could not be confirmed because of the difficulty of solving large amounts of *P. taetrolens* Y-30 GS with low reactivity to ethylamine.[14]

Subsequently, we found a new enzyme in *Methylovorus mays* No. 9, that has high theanine-forming activity.[15] The isolated enzyme predominantly catalyzed the γ-glutamylmethylamide-forming reaction, with glutamic acid, methylamine, and ATP as the substrates. It also had high reactivity to ethylamine to form theanine, but hardly catalyzed the glutamine-
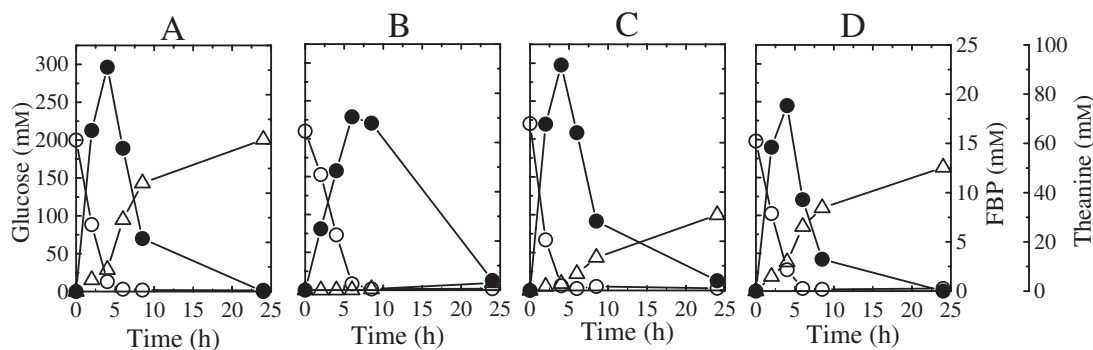
Fig. 1. Theanine Formation and Effect of Divalent Cations.

A, The initial reaction mixture (see "Materials and Methods") containing 1 unit/ml of recombinant GMAS was incubated at 30 °C for 24 h with shaking (200 rpm). Mg^{2+} and Mn^{2+} (B), Mn^{2+} (C), or Mg^{2+} (D) was omitted from the mixture for (A). Incubation was carried out at 30 °C for 24 h with shaking (200 rpm). Symbols: ○, glucose; ●, FBP; △, theanine.

forming reaction (a GS-like reaction).

The reactivity of the *M. mays* No. 9 enzyme toward methylamine, ethylamine, and ammonia was very similar to that of known γ-glutamylmethylamide synthetase[16] (GMAS; L-glutamate:methylamine ligase, ADP-forming, E.C. 6.3.4.12), indicating that the enzyme was GMAS. In addition, its reactivity to ethylamine was realized in a mixture of coupled fermentation with energy transfer,[16] which suggested theanine production in high concentration using GMAS.

Since an overexpression system of the gene of *M. mays* No. 9 GMAS has been constructed,[17] this paper deals with theanine production by coupled fermentation with energy transfer using recombinant GMAS.

Materials and Methods

Enzyme preparation. Pressed baker's yeast supplied by Oriental Yeast (Tokyo) was dried as described previously.[18]

Recombinant GMAS produced by *E. coli* AD494 (DE3) harboring pET21aGM (an expression vector for the GMAS gene of *M. mays* No. 9) was prepared by the method described previously.[17] One unit of GMAS was defined as the amount of enzyme forming 1 μmol of γ-glutamylhydroxamate per min at 30 °C in a reaction mixture consisting of 50 mM sodium glutamate, 15 mM hydroxylamine, 7.5 mM ATP, 30 mM MgCl₂, 100 mM imidazole buffer (pH 8.0), and recombinant GMAS preparation.[15,17]

To examine optimum pH for the theanine-forming reaction, the reaction mixture was modified by replacing hydroxylamine with ethylamine, and the amount of inorganic phosphate formed was determined.

Reaction conditions for theanine production. A temporary reaction mixture (dubbed the initial reaction mixture) for coupled fermentation with energy transfer was defined according to previous findings with *P. taetrolens* Y-30 GS.[13] The mixture (1 ml in a test tube)

contained (i) 200 mM sodium glutamate and 300 mM ethylamine·HCl (substrates for theanine synthesis by GMAS), (ii) 200 mM glucose and 50 mM potassium phosphate buffer, pH 7.0 (substrates for yeast sugar fermentation), (iii) 30 mM MgCl₂ and 5 mM MnCl₂ (cofactors for GMAS and several enzymes in yeast sugar fermentation), (iv) 5 mM AMP (ATP source), and (v) enzyme preparations (recombinant GMAS and 40 mg/ ml of dried yeast cells; GMAS concentration is indicated in the legend to each experiment). The reaction was carried out at 30 °C with shaking (200 rpm), and terminated by immersing the reaction tube in boiling water for 3 min. The supernatant obtained by centrifugation at 1,500 × g for 10 min at room temperature was submitted to assay.

The yield of theanine on the energy source (glucose) in coupled fermentation with energy transfer was calculated on the basis of the amount of ATP regenerated by the glucose consumed; yield (%) = {(theanine formed, mM)/(2 × glucose consumed, mM)} × 100.[13]

Assay. Theanine, inorganic phosphate, protein, glucose, and fructose 1,6-bisphosphate (FBP) were determined by methods described previously.[12,13]

Reagent. Glucose oxidase (glucose CII-test Wako) was supplied by Wako Pure Chemical (Osaka), and theanine was by Tokyo Kasei (Tokyo). The other reagents were highest-grade commercial products.

Results and Discussion

Theanine formation and effect of divalent cation

As Fig. 1A indicates, theanine was formed with a decrease in glucose and a temporary accumulation of FBP in the initial reaction mixture for coupled fermentation with energy transfer, and that the amounts reached to about 60 mM. When GMAS or the yeast cell was removed from the reaction mixture, theanine formation did not occur (data not shown), indicating that the formation in Fig. 1A was produced by coupling of the

theanine-forming reaction by recombinant GMAS and sugar fermentation by the yeast cells.

These results were nearly the same as those for theanine formation using *P. taetrolens* Y-30 GS,[13] but the amount of theanine formed with GMAS (Fig. 1A, about 60 mM) was much larger than that (8 mM) with *P. taetrolens* Y-30 GS[13] under the same conditions, due to the high reactivity of GMAS to ethylamine.

The theanine formation in Fig. 1A also did not occur when the divalent cations were removed from the reaction mixture (Fig. 1B), whereas sugar fermentation proceeded slowly but steadily, suggesting that divalent cations contained in the yeast cells were effective for sugar fermentation but not for the theanine-forming reaction by GMAS. A similar observation was made in the case of theanine production with *P. taetrolens* Y-30 GS.[13]

The addition of Mg^{2+} (Fig. 1C) or Mn^{2+} (Fig. 1D) stimulated theatnine formation, and Mn^{2+} was more effective than Mg^{2+}. Theanine formation with Mn^{2+} (Fig. 1D) was stimulated to some extent by the addition of Mg^{2+} to the mixture (see Fig. 1A).

The effect of Mg^{2+} or Mn^{2+}, observed in Fig. 1C and D, appeared inconsistent with the divalent cation dependence of GMAS with ethylamine as a substrate, which was observed previously[15] in the enzyme reaction: the reactivity with Mg^{2+} was about 3 times higher than that with Mn^{2+} at optimum pH (7.8) for the Mg^{2+}-dependent reaction. However, detailed repeat experiment of the enzyme reaction with Mn^{2+} revealed that the Mn^{2+}-dependent reaction proceeded optimally at pH 7.0 (Table 1), and the reactivity with Mn^{2+} at pH 7.0 was almost the same as that of the Mg^{2+}-dependent reaction.

Table 1 might explain the findings in Fig. 1C and D, because the pH of the reaction mixture of the coupled fermentation was maintained at neutral or slightly acidic pH due to the buffer-like action of large amounts of

yeast cells, and also to CO_2 generated by yeast sugar fermentation.[19,20]

Several divalent cations have been reported to induce unique structural changes in GS protein,[21,22] and to vary its enzymatic properties such as the pH-activity profile, reactivity and affinity to substrates, *etc.*[21,22] GMAS has a certain homology with GS with respect to amino acid sequence and subunit structure.[17] The data in Table 1 are probably due to cause similar to that in the case of GS.

Effect of concentrations of GMAS and substrates on theanine formation

Figure 2A-1, A-2, and A-3 indicate the effect of the concentration of GMAS on theanine production in the initial reaction mixture. The amount of theanine formed in the mixture increased with increases in GMAS concentrations, as observed in the reaction using *P. taetrolens* Y-30 GS.[13] In the mixture with 7.5 units/ml of GMAS (Fig. 2A-3), sodium glutamate (200 mM) was converted completely to theanine (approximately 200 mM) in 24 h.

As Fig. 2A-3 indicates, the theanine yield was 50% based on glucose consumed, but this value is incorrect because the concentration of sodium glutamate (200 mM) was less than all the energy released from 200 mM glucose (corresponding to 400 mM ATP). In order to evaluate the yield on glucose correctly, the reaction was done again with a mixture containing 400 mM sodium glutamate and 600 mM ethylamine·HCl.

About 300 mM theanine was formed using 7.5 units/ml GMAS with the yield of 75% on glucose consumed (Fig. 2B-1), and the yield reached 100% with the formation of about 400 mM theanine (Fig. 2B-2) when the GMAS concentration was increased to 15 units/ml. The theanine formation, of 400 mM, was much larger than that by *P. taetrolens* Y-30 GS (170 mM, 28% yield on glucose consumed).[13]

Inhibitory effect of high concentrations of substrate on yeast sugar fermentation

To achieve theanine formation at higher concentrations, the concentrations of the substrates was increased to 300 mM (glucose), 600 mM (sodium glutamate), and 900 mM (ethylamine·HCl). However, as shown in Fig. 3A, yeast sugar fermentation was repressed, and eventually the final amount of theanine decreased to less than that shown in Fig. 2B-2 (with 200 mM glucose, 300 mM sodium glutamate, and 600 mM ethylamine· HCl).

Several experiments analyzing and coping with the inhibition revealed that ethylamine was the main cause of the inhibition, and that potassium phosphate buffer restored or accelerated yeast sugar fermentation. Figure 3B-1 and 3B-2 show some of the results: yeast sugar fermentation decreased with increases in the concentrations of ethylamine (Fig. 3B-1), and was restored with increases in the concentration of potassium phos-

Table 1. Effect of pH on Theanine-Forming Reaction with Mg^{2+} or Mn^{2+}

| Buffer | pH | Inorganic phosphate formed (mM/min) | |
		with Mg^{2+}	with Mn^{2+}
Acetate	4.0	0.26	0.29
	5.0	0.26	0.27
	6.0	0.29	0.67
Imidazole	6.0	0.39	0.51
	6.5	0.77	1.3
	7.0	1.6	1.5
	7.8	2.5	0.77
	8.0	2.3	0.71
Tris–HCl	8.0	1.9	0.58
	8.5	1.2	0.43
	9.0	0.81	0.40

The reaction mixture, consisting of 50 mM sodium glutamate, 15 mM ethylamine, 7.5 mM ATP, 30 mM $MgCl_2$ or 3 mM $MnCl_2$, 100 mM each buffer, and 0.17 units/ml of GMAS, was incubated at 30 °C for 10 min. The amount of inorganic phosphate formed was measured.
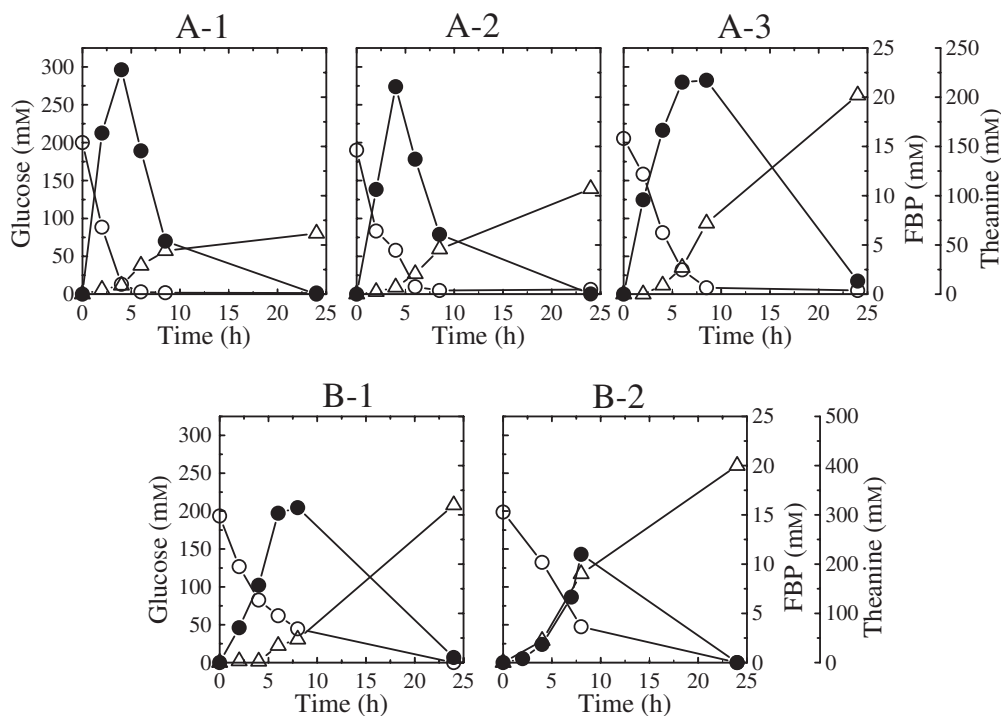
Fig. 2. Effect of Various Concentrations of GMAS and Substrates on Theanine Formation.

A, The concentration of GMAS in the initial reaction mixture with 200 mM sodium glutamate and 300 mM ethylamine·HCl was 1 unit/ml (A-1), 3 units/ml (A-2), or 7.5 (A-3) units/ml. B, The concentrations of sodium glutamate and ethylamine·HCl in the initial reaction mixture were increased to 400 mM and 600 mM respectively. The concentration of GMAS was 7.5 units/ml (B-1) or 15 (B-2) units/ml. The reaction was carried out at 30 °C for 24 h with shaking (200 rpm). Symbols: ○, glucose; ●, FBP; △, theanine.
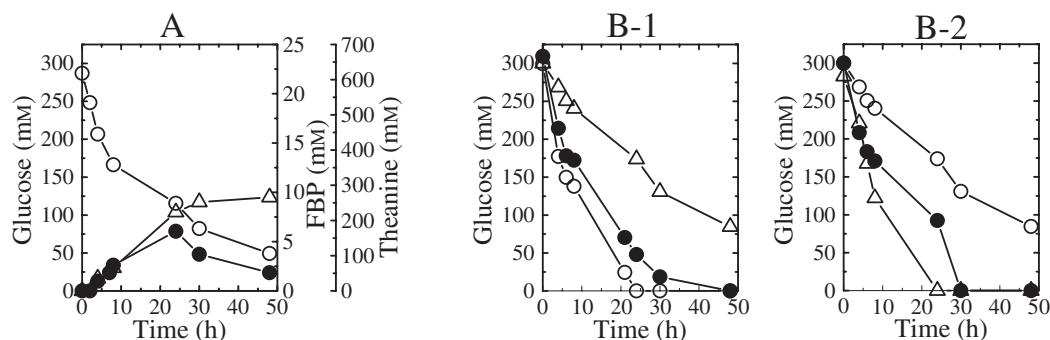

Fig. 3. Effect of Concentration of Ethylamine and Potassium Phosphate Buffer on Yeast Sugar Fermentation Inhibited by High Concentrations of Substrates.

A, The concentrations of sodium glutamate, ethylamine·HCl, and glucose were increased to 600 mM, 900 mM ethylamine, and 300 mM respectively in the initial reaction mixture. The concentration of GMAS was 15 units/ml. Incubation was done at 30 °C with shaking (200 rpm). Symbols: ○, glucose; ●, FBP; △, theanine. B, GMAS was removed from the mixture for (A). The concentration of ethylamine·HCl (B-1) in the mixture was varied with a fixed concentration (50 mM) of potassium phosphate buffer. The concentration of potassium phosphate buffer (B-2) was varied with the fixed concentration (900 mM) of ethylamine·HCl. The glucose level in the mixture was determined. Symbols: B-1, ○, 600 mM ethylamine·HCl; ●, 750 mM; △, 900 mM; B-2, ○, 50 mM potassium phosphate buffer; ●, 100 mM; △, 200 mM.

phate buffer (Fig. 3B-2). An inhibitory effect of ethylamine on sugar fermentation was also observed in coupled fermentation with *P. taetrolens* Y-30 GS.[13] Increases in dried yeast cells were slightly effective in increasing theanine formation through acceleration of yeast sugar fermentation (data not shown). The addition of large amounts of yeast cells does not always increase product formation, due to unbalanced increases in

certain ATP-consuming reactions in the yeast cells.[13,23] Other components in the reaction mixture did not affect inhibited yeast sugar fermentation (data not shown).

Theanine formation in an improved mixture

Figure 4 shows theanine formation in an improved reaction mixture, which was designed on the basis of the above results, and contained 300 mM glucose, 600 mM
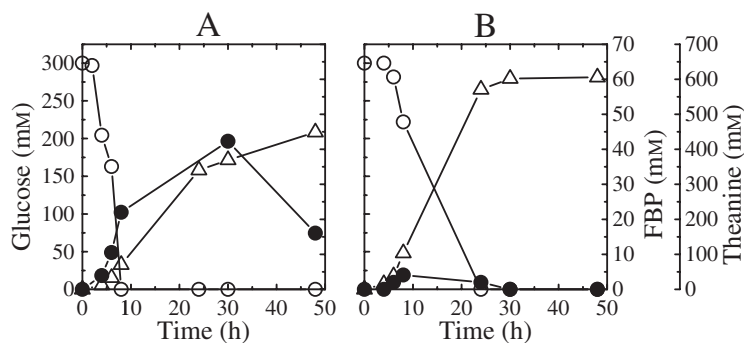
Fig. 4.   Theanine Production at High Concentrations.

   The reaction mixture contained 600 mᴍ sodium glutamate, 600 mᴍ ethylamine·HCl, 300 mᴍ glucose, 200 mᴍ potassium phosphate buffer (pH 7.0), 30 mᴍ MgCl₂, 5 mᴍ MnCl₂, 5 mᴍ AMP, 40 mg/ml of dried yeast cells, and 15 (A) or 30 (B) units/ml of GMAS. The reaction was carried out at 30 °C for 48 h with shaking (200 rpm). Symbols: ○, glucose; ●, FBP; △, theanine.

sodium glutamate, 600 mᴍ ethylamine·HCl, and 200 mᴍ potassium phosphate buffer (pH 7.0).

   Improvement of the reaction mixture restored yeast sugar fermentation, and also theanine formation (Fig. 4A). Due to increasing the GMAS concentration to 30 units/ml (Fig. 4B), approximately 600 mᴍ (110 mg/ml) theanine was formed in 48 h with 100% conversion from glutamic acid and ethylamine, and with 100% yield based on the glucose consumed. The amount of theanine formed (Fig. 4B) was about 2.5 times larger than that formed from glutamine and ethylamine by bacterial glutaminase (240 mᴍ).[10]

   The present paper describes the characteristics of theanine formation by coupled fermentation with energy transfer using recombinant GMAS from the viewpoint of production at high concentrations.

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