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# Enzymatic Synthesis of γ-Glutamylvaline to Improve the Bitter Taste of Valine

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The taste of several bitter amino acids is reduced, sourness produced, and preference increased by γ-glutamylization. An enzymatic method for synthesizing γ-Glu-Val involving bacterial γ-glutamyltranspeptidase (GGT) was developed. The optimum reaction conditions for the synthesis of γ-Glu-Val were 20 mM Gln, 300 mM Val, and 0.04 U/ml GGT, pH 10. After 3-hr incubation at 37 °C, 17.6 mM γ-Glu-Val was obtained, with the yield being 88%. γ-Glu-Val was purified on a Dowex 1 × 8 column and then identified by NMR.

**KEYWORDS:** **γ-glutamyltranspeptidase; γ-glutamyl amino acid; γ-glutamylvaline; bitter taste; enzymatic synthesis**

## INTRODUCTION

Some L-amino acids are known as bitter amino acids. Branched-chain amino acids, basic amino acids, and aromatic amino acids are bitter (*1*). Several essential amino acids for human beings are bitter. Therefore, when an amino acid mixture is administered orally, the bitterness of these amino acids is a crucial problem. These days, various kinds and large amounts of beverages called "supplements" are sold by several companies in Japan. These beverages contain large amounts of branched-chain amino acids, and some aromatic and/or basic amino acids. Without the addition of sweeteners and flavoring, their taste would be terrible. We discovered and reported that the γ-glutamylization of bitter amino acids abolished or at least reduced their bitterness and improved preference of the panel members of the taste test (*2*). We have developed an enzymatic method involving bacterial γ-glutamyltranspeptidase (GGT, EC 2.3.2.2) and various amino acids as substrates for synthesizing γ-glutamyl amino acids, including γ-glutamylphenylalanine, γ-glutamyltyrosine methylester, and γ-glutamylhistidine (*2*−*4*). The method is superior because (i) glutamine, which is less expensive than glutathione, can be used as a γ-glutamyl donor efficiently; (ii) GGT does not require any energy source such as ATP and product inhibition by ADP is not a problem; (iii) a great deal of GGT is readily available because both *E. coli* and *B. subtilis* GGTs can be purified by simple two-step purification procedures from overexpressing strains (*5*, *6*); (iv) the protection and deblocking of reactive groups of substrates are not required, unlike for a chemical synthetic method; and (v) the substrate specificity of GGT for γ-glutamyl acceptors is broad and thus various γ-glutamyl compounds can be synthesized. In fact, aromatic amino acids and basic amino acids are good acceptors. Unfortunately, however, branched-chain amino acids are poor ones, as described previously (*7*), and we have to overcome this problem to synthesize γ-glutamyl branched-chain amino acids using bacterial GGT.

## MATERIALS AND METHODS

**Reagents and the Enzyme.** The amino acids and γ-glutamyl amino acids used were all of the L-form. Amino acids were purchased from Nacalai Tesque (Kyoto, Japan), γ-Glu-Gln was from Sigma Chemical (St. Louis, MO), and γ-Glu-Val and γ-Glu-Leu were from Bachem (Bubendorf, Switzerland). *Escherichia coli* K-12 strain SH642, which harbors pUC18 with the *E. coli* GGT gene, was grown at 20 °C (*8*) in LB broth containing 100 μg/mL ampicillin, and the GGT over-produced was purified from the periplasmic fraction (*9*) as described previously (*5*).

**Measurement of GGT Activity.** GGT activity was measured as described previously (*7*). One unit of enzyme was defined as the amount of enzyme that released 1 μmole of *p*-nitroaniline per min from γ-glutamyl-*p*-nitroanilide through the transpeptidation reaction.

**Measurement of γ-Glutamyl Amino Acids and Gln.** The concentrations of γ-Glu-Val, γ-Glu-Ile, γ-Glu-Gln, Gln, and Val were measured with a high-performance liquid chromatograph (HPLC) equipped with a Shim-pack Amino-Na column and a fluorescence detector (model LC-9A; Shimadzu, Kyoto, Japan), with *o*-phthalaldehyde as the detection reagent, as described previously (*10*). That of γ-Glu-Leu was measured by a reverse phase HPLC as described previously (*11*).

**NMR Analysis.** Purified γ-Glu-Val (10 mg) was dissolved in $D_2O$ and then analyzed with a Bruker 500 MHz spectrometer, the spectrum obtained being compared with that obtained with γ-Glu-Val purchased from a commercial source.

## RESULTS AND DISCUSSION

**Enzymatic Synthesis of γ-Glu-Val with GGT.** The synthesis of γ-Glu-Val from Gln and Val with GGT as the catalyst was

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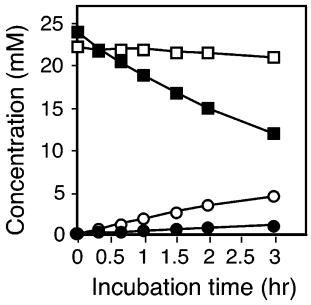


**Figure 1.** Enzymatic synthesis of γ-Glu-Val involving bacterial GGT. The reaction was carried out with 20 mM Gln, 20 mM Val, and 0.04 U/ml GGT at pH 10 and 37 °C. A part of the reaction mixture was withdrawn at the times indicated and the concentrations of amino acids, γ-Glu-Val, and γ-Glu-Gln were determined by HPLC as described under Materials and Methods. Gln (closed square), Val (open square), γ-Glu-Val (closed circle), and γ-Glu-Gln (open circle).

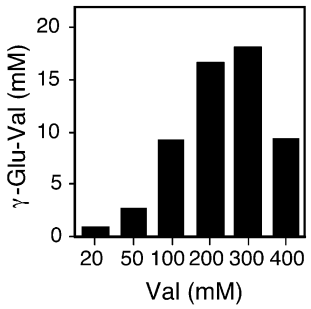


**Figure 2.** Effect of the Val concentration on γ-Glu-Val synthesis. The reaction was carried out with 20 mM Gln and 0.04 units/ml GGT at pH 10 and 37 °C for 3 h, the concentration of Val being varied as indicated in the figure. γ-Glu-Val was determined by HPLC as described under Materials and Methods.

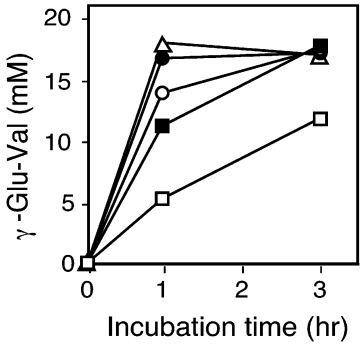


**Figure 3.** Effect of the GGT concentration on γ-Glu-Val synthesis. The reaction was carried out with 20 mM Gln and 300 mM Val at pH 10 and 37 °C. The GGT concentrations were 0.02, 0.04, 0.08, 0.14, and 0.16 U/ml (open squares, closed squares, open circles, closed circles, and open triangles, respectively). γ-Glu-Val was determined by HPLC as described under Materials and Methods.

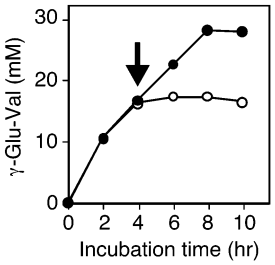


**Figure 4.** Effect of the feeding 20 mM Gln after 4 h of incubation on the yield of γ-Glu-Val. The reaction was carried out with 20 mM Gln, 300 mM Val, and 0.04 U/ml GGT at pH 10 and 37 °C. γ-Glu-Val was determined by HPLC as described under Materials and Methods.

confirmed. Gln and Val were dissolved in water and then the pH was adjusted to 10 with NaOH. Their concentrations in the reaction mixture were 20 mM. GGT was added to this solution (final, 0.04 U/ml) and then the reaction mixture (total volume, 2 mL) was incubated at 37 °C for 3 h. The reaction was terminated by the addition of a 1/10 volume of 100% trichloroacetic acid, and then the filtrate through the membrane filter (Millex-LH, Millipore) was subjected to HPLC analysis. Synthesis of γ-Glu-Val was observed; however, its concentration was only 1.0 mM after 3 h of incubation, and more γ-Glu-Gln was formed (**Figure 1**).

**Optimization of the Reaction Conditions for the Synthesis of γ-Glu-Val with GGT.** Because the yield of γ-Glu-Val was far from satifactory, the reaction conditions for its synthesis were investigated. First of all, the optimum ratio of Gln and Val was investigated. The concentration of Gln was fixed at 20 mM, while that of Val was varied. The highest amount of γ-Glu-Val was formed when 300 mM Val was used (**Figure 2**). Because the pH of the reaction mixture had a strong influence on the synthesis of γ-glutamyl compounds involving GGT, the optimum pH of the reaction mixture was investigated and determined to be 10 (data not shown). The concentration of GGT in the reaction mixture was also varied, and its effect was measured. The amount of γ-Glu-Val after 3 h of incubation was not so different when 0.04−0.16 U/ml GGT was used (**Figure 3**). To keep the amount of GGT as low as possible, 0.04 U/ml of GGT was determined to be the optimum concentration. The optimum conditions for the synthesis of γ-Glu-Val were thus determined to be 20 mM Gln, 300 mM Val, 0.04 U/ml GGT, pH 10, and incubation at 37 °C for 3 h, the yield being 17.6 mM, and the conversion rate of Gln to γ-Glu-Val being 88%. In conclusion, the yield of γ-Glu-Val synthesis involving bacterial GGT was dramatically improved. Although a large amount of Val remained after 3 h of incubation, we speculated that the conversion rate of Val could be improved by feeding Gln and/or GGT after a few hours incubation or by circulating the substrate using an immobilized enzyme. To confirm it, 20 mM of Gln was added to the reaction mixture after 4 h of incubation. As shown in **Figure 4**, the yield of γ-Glu-Val increased from 17 mM (without feeding) to 28 mM.

**Isolation and Identification of γ-Glu-Val.** A 50-mL reaction mixture was prepared to synthesize γ-Glu-Val. The reaction mixture was applied to a column (2.3 × 7.2 cm) of Dowex 1 × 8 that was prepared as the $CH_3COO^-$ form. The column was washed with water and 0.1 N $CH_3COOH$, and then γ-Glu-Val was eluted with 0.5 N $CH_3COOH$. The fractions containing only γ-Glu-Val were collected and lyophilized. The purification yield was 36.4%.

The NMR spectra of the synthesized and commercial γ-Glu-Val were identical (**Figure 5**). We concluded that the sample we had synthesized and purified was in fact γ-Glu-Val.

**γ-Glutamylization of Leu and Ile Involving Bacterial GGT.** Whether or not γ-Glu-Leu can be synthesized similarly was examined using a reaction mixture comprising 20 mM Gln, 100 mM Leu, and 0.08 U/ml GGT, pH 9.5, with incubation at 37 °C for 7 h. It was found that 3.4 mM of γ-Glu-Leu was synthesized under these reaction conditions.

Similarly, γ-Glu-Ile was synthesized using a reaction mixture comprising 20 mM Gln, 100 mM Ile, and 0.08 U/ml GGT, pH 9.5, with incubation at 37 °C. The amount of a new peak was such that we speculate γ-Glu-Ile increased along with the

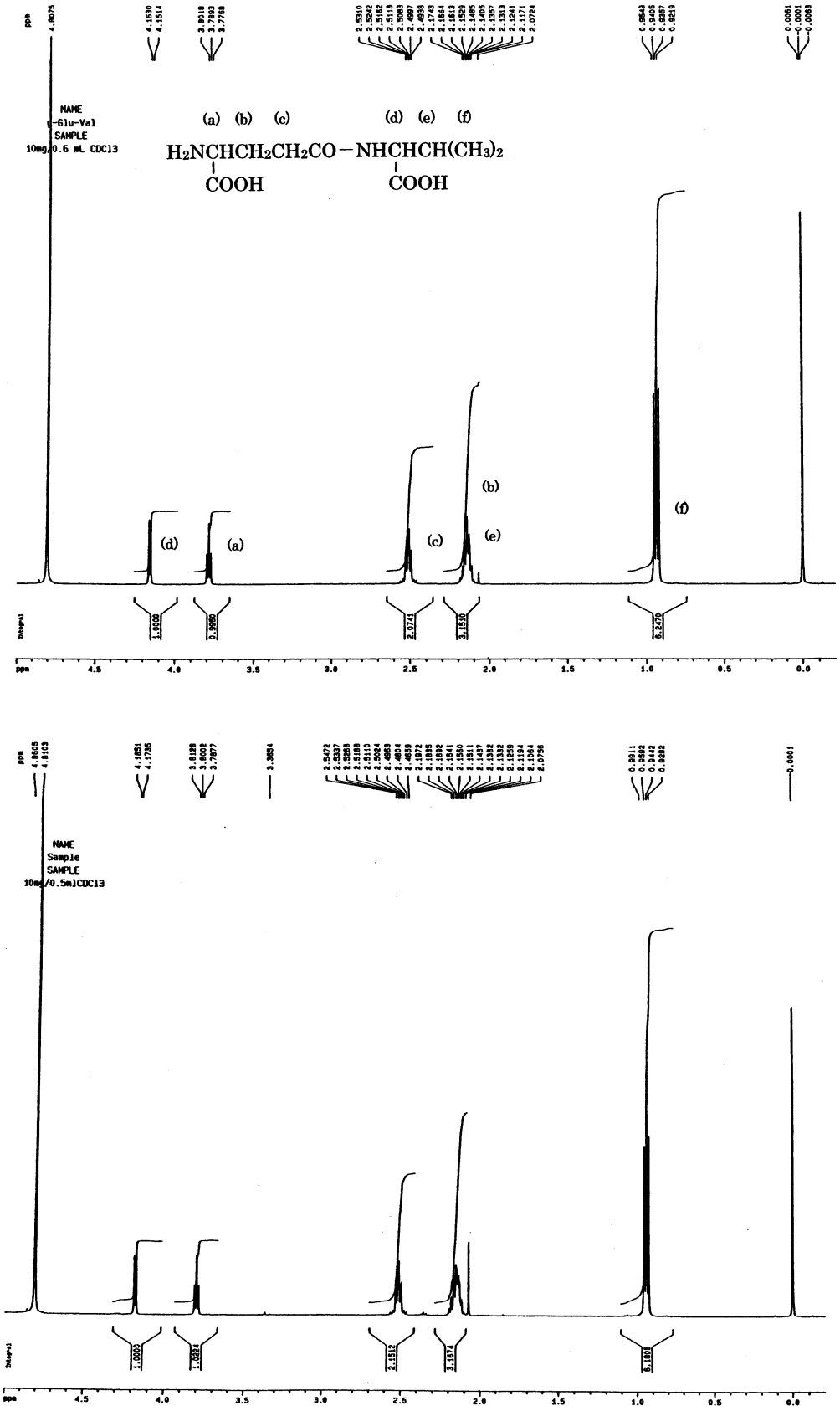


**Figure 5.** NMR spectra of commercial $\gamma$-Glu-Val (top) and the isolated sample (bottom). The NMR spectra were measured in $D_2O$ with a Bruker 500 MHz spectrometer.

incubation time (data not shown). However, we could not identify the peak as $\gamma$-Glu-Ile because $\gamma$-Glu-Ile, which can be used as an authentic sample, is not available from a commercial source. Because the structures of $\gamma$-Glu-Val, $\gamma$-Glu-Leu, and $\gamma$-Glu-Ile are quite similar, it is reasonable that GGT can catalyze the synthesis of $\gamma$-Glu-Ile.

The results presented in this report indicate that the enzymatic synthesis of γ-glutamyl branched-chain amino acids involving GGT are promising.

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