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Improvement of the Bitter Taste of Amino Acids through the Transpeptidation Reaction of Bacterial γ-Glutamyltranspeptidase

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The tastes of several bitter amino acids and their γ-glutamyl derivatives were compared. The bitterness of Phe, Val, Leu, and His was reduced, sourness was produced, and preferences were increased by γ-glutamylization. Because the effect of γ-glutamylization of bitter amino acids was most obvious for Phe, which is an atypical bitter amino acid, an enzymatic method for the synthesis of γ-glutamylphenylalanine (γ-Glu-Phe) involving bacterial γ-glutamyltranspeptidase was developed. The optimum reaction conditions were 200 mM Gln, 200 mM Phe, and 0.5 unit/mL GGT, pH 10.4. After 1.5-h of incubation at 37 °C, 140 mM γ-Glu-Phe was obtained, the yield being 70%. γ-Glu-Phe was purified on a Dowex 1x8 column and then identified by NMR.

KEYWORDS: **γ-Glutamyltranspeptidase; γ-glutamyl amino acid; γ-glutamylphenylalanine; bitter taste; enzymatic synthesis**

INTRODUCTION

The water solubility of a γ-glutamyl amino acid is better than that of the original amino acid (*1*). It is also known that a γ-glutamyl amino acid is resistant to the peptidase action in plasma (*1*). Because the γ-glutamyl linkage is cleaved and L-DOPA is released from γ-Glu-L-DOPA mainly at organs with high γ-glutamyltranspeptidase (GGT; EC 2.3.2.2) activity, γ-Glu-L-DOPA is a kidney and brain specific precursor of dopamine and can be used as a prodrug (*2*, *3*). A similar effect has been observed for γ-Glu-dermorphin (*4*). GGT catalyzes not only the hydrolysis of the γ-glutamyl linkages of γ-glutamyl compounds but also the transfer of their γ-glutamyl moieties to other amino acids or peptides (*5*). We developed an enzymatic method for synthesizing γ-Glu-L-DOPA involving the transpeptidation reaction of GGT (*6*, *7*), and γ-Glu-L-DOPA thus synthesized was shown to be useful not only as a prodrug (*8*) but also as a substrate for a highly sensitive assay method for GGT (*9*).

Among amino acids some L-amino acids are known to be bitter ones. Aromatic amino acids, basic amino acids, and branched-chain amino acids are bitter amino acids. Several bitter amino acids are essential for humans. Therefore, when an amino acid mixture is administrated orally, the bitterness of these amino acids is a crucial problem. A long time ago, Kirimura et al. reported that γ-Glu-Phe was not bitter, but sour and astringent (*10*). All γ-glutamyl amino acids they tested were astringent, and no one has tried to use γ-glutamylization to improve the taste of bitter amino acids.

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In this paper, we report improvement of the bitter taste of amino acids by γ-glutamylization and an enzymatic method for synthesizing γ-Glu-Phe from Gln and Phe involving bacterial GGT.

MATERIALS AND METHODS

Reagents and Enzyme. The amino acids and γ-glutamyl amino acids used were all free of salts. All amino acids were of the L-form, and γ-glutamyl amino acids consisted of L-amino acids. All amino acids were purchased from Nacalai Tesque (Kyoto, Japan); γ-Glu-Phe, γ-Glu-Val, and γ-Glu-Leu were from Bachem (Bubendorf, Switzerland); and γ-Glu-His was from Sigma Chemical Co. (St. Louis, MO). *Escherichia coli* K-12 strain SH642, which harbors pUC18 with the *E. coli* GGT gene, was grown at 20 °C in LB broth containing 100 μg/mL ampicillin, and the GGT overproduced was purified as described previously (*11*).

Tasting of Amino Acids and Their γ-Glutamyl Derivatives. Several bitter amino acids and their γ-glutamyl derivatives were tasted by eight panel members to evaluate their taste characteristics. The members were told about the purpose of the test but were not told what each sample was. At the beginning of the test, and between samples, the panel members rinsed their mouths with mineral water. Amino acids and peptides were dissolved in deionized water, and 0.7 mL of each sample was pipetted onto the center of the panel members' tongues. Bitterness, sourness, and preferences were rated according to a five-point category scale. When the members detected bitterness or sourness, they were asked to name a kind of food with similar bitterness or sourness.

Measurement of GGT Activity. GGT activity was measured as described previously (*12*). One unit of enzyme was defined as the amount of enzyme that released 1 μmol of *p*-nitroaniline per minute from γ-glutamyl-*p*-nitroanilide in the transpeptidation reaction.

Measurement of γ-Glu-Phe and Gln. The concentrations of γ-Glu-Phe and Gln were measured with a high-performance liquid chromatograph (HPLC) equipped with a Shim-pack Amino-Na column and a

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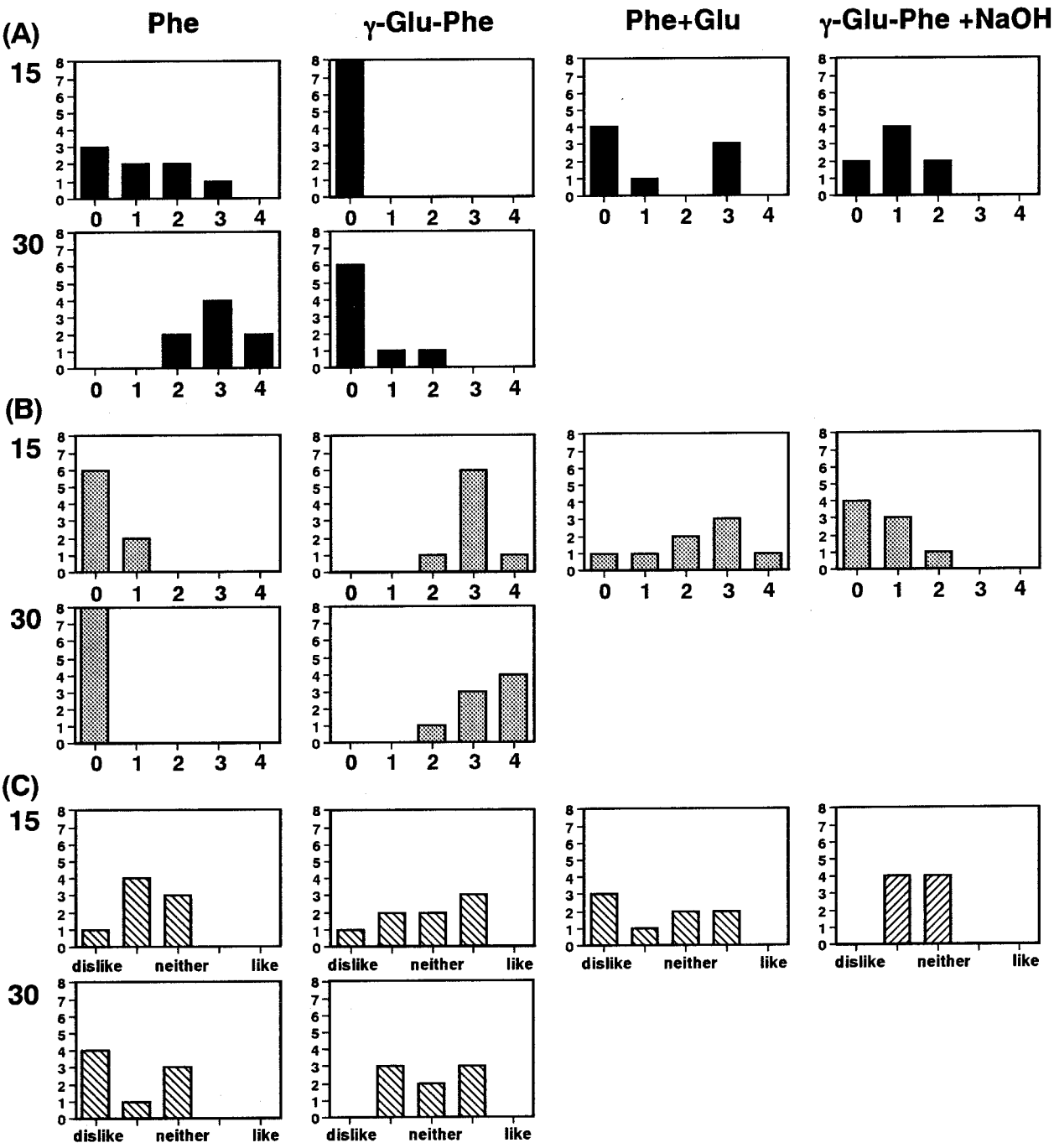

Figure 1. Comparison of the taste of Phe and γ-Glu-Phe: (A) bitterness; (B) sourness; (C) preferences. The vertical axes indicate the number of people. The horizontal axes in (A) and (B) indicate the intensity of the taste: 0, did not perceive; 1, perceived slightly; 2, perceived weakly; 3, perceived; 4, perceived strongly. The horizontal axes in (C) indicate the preferences on a five-point scale. The numbers, 15 and 30, on the left side of the figures indicate the concentrations (mM) of Phe and γ-Glu-Phe.

fluorescence detector (model LC-9A; Shimadzu, Kyoto, Japan), with *o*-phthalaldehyde as the detection reagent.

NMR Analysis. Five milligrams of γ-Glu-Phe was dissolved in 0.8 mL of D_2O and then analyzed with a Bruker 500 MHz spectrometer, and the spectrum was compared with that obtained with γ-Glu-Phe purchased from a commercial source.

RESULTS

Comparison of the Taste of Phe with That of γ-Glu-Phe. The bitterness of Phe was obvious at 15 mM and higher concentrations, so the tastes of Phe and γ-Glu-Phe were compared at 15, 30, and 45 mM (data obtained with 45 mM samples are not shown). All members of the panel thought 30 and 45 mM Phe were bitter; however, most did not think γ-Glu-Phe was bitter (Figure 1A). Phe was practically not sour, but the sourness of γ-Glu-Phe was obvious (Figure 1B). Also, the preference for γ-Glu-Phe was better than that for Phe (Figure 1C).

The effect of the addition of 15 mM Glu to 15 mM Phe was investigated (Figure 1). When Glu was added to Phe, the mixture was sourer than Phe alone, but the bitterness did not change and improvement of the preference was not observed.

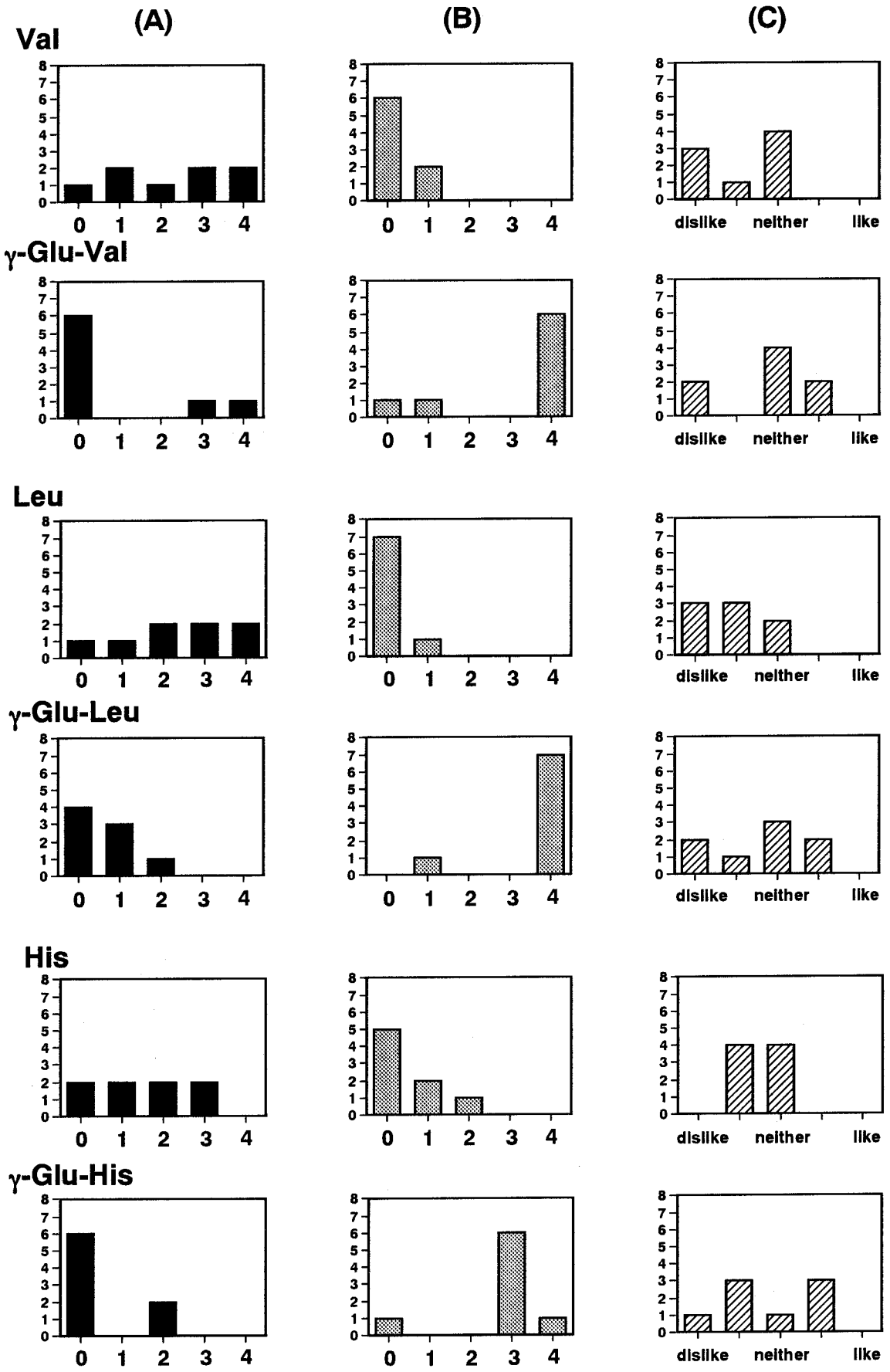

Figure 2. Effect of γ-glutamylization of Val, Leu, and His: (A) bitterness; (B) sourness; (C) preferences. The axes are the same as in Figure 1. The concentrations used were 50 mM for Val, Leu, and their γ-glutamyl derivatives and 70 mM for His and γ-Glu-His.

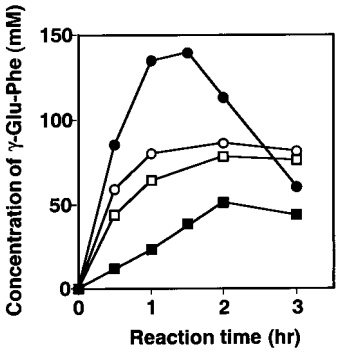

Figure 3. Effect of GGT concentration on γ-Glu-Phe synthesis. The reaction was carried out with 200 mM Gln and 200 mM Phe at pH 10.4 and 37 °C. The GGT concentrations were 0.1 (■), 0.3 (□), 0.4 (○), and 0.5 unit/mL (●). γ-Glu-Phe was assayed by HPLC as described under Materials and Methods.

The taste of 15 mM γ-Glu-Phe neutralized with NaOH was also examined (Figure 1). There was no significant difference in taste between γ-Glu-Phe neutralized with NaOH and Phe.

Effect of γ-Glutamylization on Other Bitter Amino Acids. The effect of γ-glutamylization on other bitter amino acids, Val, Leu, and His, was examined. Because Val, Leu, and His are not as bitter as Phe, they were tested at much higher concentrations than Phe. As shown in Figure 2, the effect of γ-glutamylization on Val, Leu, and His was similar to that on Phe.

Enzymatic Synthesis of γ-Glu-Phe with GGT. Whether or not γ-Glu-Phe could be synthesized from Gln and Phe with GGT as a catalyst was examined. Gln and Phe were dissolved in water, and then the pH was adjusted to 8.3 with NaOH. Their concentrations in the reaction mixture were 200 mM. GGT was added to this solution (final = 0.2 unit/mL), and then the reaction mixture (total volume = 3 mL) was incubated at 37 °C for 2 h. The reaction was terminated by the addition of the same volume of 10% trichloroacetic acid. The supernatant was diluted with 0.5 M sodium citrate buffer (pH 2.2) and then subjected to HPLC analysis. Synthesis of γ-Glu-Phe was observed. Its concentration was 71 mM, and the yield was 36%.

Reaction Conditions for the Enzymatic Synthesis of γ-Glu-Phe. Although γ-Glu-Phe was synthesized from Gln and Phe using GGT, the yield was fairly low. Therefore, the reaction conditions for the synthesis of γ-Glu-Phe were investigated to improve the yield. The optimum concentrations of Gln and Phe were 200 mM, and the optimum reaction pH was 10.4 (data not shown). The concentration of GGT in the reaction mixture was varied, and its effect was measured (Figure 3). When 0.5 unit/mL of GGT was used, 140 mM γ-Glu-Phe was synthesized after 1.5-h of incubation and the yield was 70%.

Isolation and Identification of γ-Glu-Phe. The optimum conditions for the synthesis of γ-Glu-Phe were determined to be 200 mM Gln, 200 mM Phe, 0.5 unit/mL GGT, pH 10.4, and incubation at 37 °C for 1.5 h. A 100 mL reaction mixture was prepared, and γ-Glu-Phe was synthesized. The reaction mixture was applied to a Dowex 1x8 column that was prepared as CH_3COO^- form. The column was washed with water and 0.05 N CH_3COOH, and then γ-Glu-Phe was eluted with 0.5 N CH_3COOH. The fractions containing only γ-Glu-Phe were collected and lyophilized. The purification yield was 19%.

The HPLC chromatograms of synthesized and commercial γ-Glu-Phe were compared, their retention times being found to match well (data not shown). The NMR spectra of synthesized and commercial γ-Glu-Phe were identical except that an intense peak at 1.95 ppm was observed only for the synthesized sample (Figure 4).

DISCUSSION

The effect of γ-glutamylization of Phe on its taste was evaluated. The results clearly indicate that γ-glutamylization of Phe can abolish its bitterness and improve the preference. Several panel members said γ-Glu-Phe had a lemon-like refreshing sourness, and we think this was the reason they preferred γ-Glu-Phe. It was also shown that the γ-glutamyl linkage is necessary for the effect and that the existence of Glu is not enough (Figure 1). The sourness of a mixture of Phe and Glu was not refreshing. Moreover, when Glu was added, the taste became harsh. This might be why the addition of Glu was not effective. The taste of γ-Glu-Phe neutralized with NaOH and Phe was compared, the results clearly indicating that the sourness of γ-Glu-Phe is critical for the effect.

The effect of γ-glutamylization on the taste of several other bitter amino acids that we tested was the same as that on the taste of Phe (Figure 2). Kirimura et al. compared the taste of various dipeptides (*10*) and reported that α-Glu-Phe was bitter and sour, whereas γ-Glu-Phe was not bitter, but sour. They also reported that all γ-glutamyl amino acids they tested were astringent. As far as we tested, γ-Glu-Phe, γ-Glu-Val, γ-Glu-Leu, and γ-Glu-His were not astringent at all, which is inconsistent with their results. In conclusion, the γ-glutamylization of bitter amino acids will become a powerful method for improving the taste of bitter amino acids.

The question may arise as to how γ-glutamyl amino acids are taken up in the intestine of humans and metabolized. One possibility is that the γ-glutamyl linkage is cleaved by GGT existing on the brush border of the columnar epithelial cells of the tips of the villi of the small intestine (*13*, *14*), and the released Glu and amino acids are taken up through regular transport systems for amino acids. This is similar to the mechanism of glutathione transport that Inoue suggested (*15*). Another possibility is that γ-glutamyl amino acids are taken up intact and the γ-glutamyl linkage is cleaved by GGT existing in the kidney. This is similar to the transport system suggested for theanine (γ-glutamylethylamide) (*16*).

Among the bitter amino acids we tested, the effect of γ-glutamylization was most remarkable for Phe. It is an atypical bitter amino acid and also an essential one. Therefore, an enzymatic method for the synthesis of γ-Glu-Phe involving bacterial GGT was developed. Bacterial GGTs are either periplasmic (*17*) or extracellular (*18*) enzymes and can be purified as soluble enzyme preparations from tank culture, whereas eukaryotic GGTs are membrane-bound enzymes (*5*). Therefore, bacteria are superior sources of GGT. At first we confirmed that γ-Glu-Phe could be synthesized with GGT from Gln and Phe. Then, the reaction conditions were optimized. When 0.5 unit/mL of GGT was used (Figure 4), the concentration of γ-Glu-Phe decreased rapidly after 1.5 h, and more Phe was observed after 2 h of incubation than at 1.5 h in the reaction mixture (data not shown). This indicates that the synthesized γ-Glu-Phe was hydrolyzed by GGT after Gln had been consumed. This phenomenon was also observed when we used a high concentration of GGT for the synthesis of γ-Glu-DOPA with GGT (*7*), and γ-Glu-Phe would decrease more rapidly if more GGT were used. Therefore, we decided to use 0.5 unit/mL. Because we did not optimize the purification method, the purification yield in this study was only 19%. This is because Phe and γ-Glu-Phe were not separated efficiently on the ion exchange column; however, the yield could be improved by using reversed phase HPLC.

The NMR spectrum of synthesized γ-Glu-Phe showed a peak at 19.5 ppm. We think that this peak was due to CH_3COOH

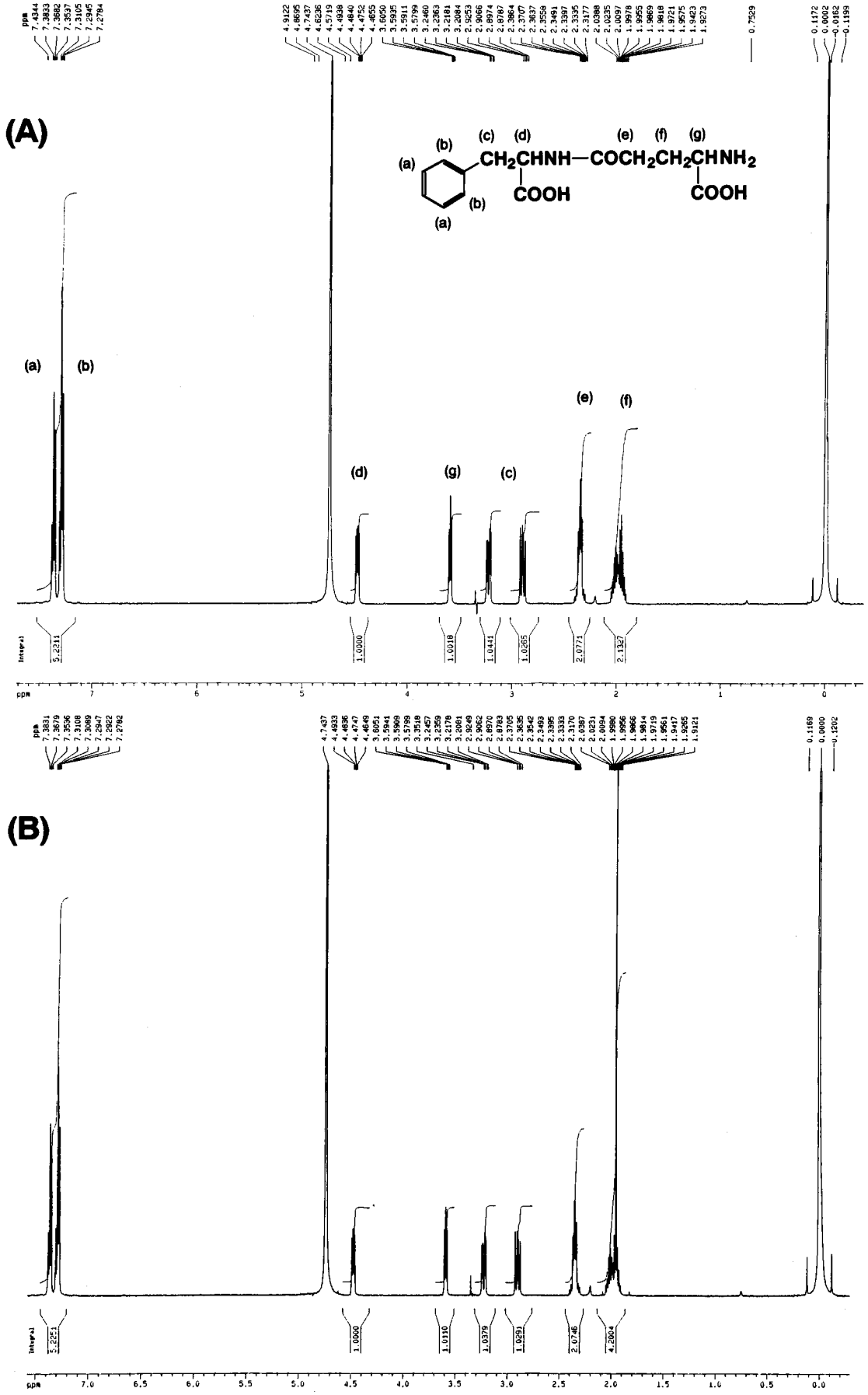

Figure 4. NMR spectra of commercial γ-Glu-Phe (A) and the isolated sample (B) measured in D_2O with a Bruker 500 MHz spectrometer.

used in the elution from the ion exchange column and remaining in our last preparation. In conclusion, the synthesized compound was identified as γ-Glu-Phe.

A chemical synthetic method is used to synthesize γ-Glu-Phe industrially, but it requires the protection and deblocking of reactive groups. Several γ-glutamyl amino acids were synthesized with γ-glutamyl cysteine synthetase (*19*, *20*). However, this enzyme requires ATP as an energy source and is inhibited by a product, ADP. Therefore, to utilize γ-glutamyl cysteine synthetase in industry, the development of an effective and economical ATP-supplying system is required. In contrast, GGT does not require any energy source. Unlike mammalian GGT, bacterial GGTs prefer a less expensive γ-glutamyl donor, Gln, as well as glutathione. Therefore, the enzymatic method for synthesizing γ-Glu-Phe from Gln and Phe involving bacterial GGT developed in this study is a superior method to synthesize γ-Glu-Phe. Because bacterial GGTs can utilize various amino acids as a γ-glutamyl acceptor (*12*), this method may be applicable to the synthesis of various γ-glutamyl compounds.

ABBREVIATIONS USED

GGT, γ-glutamyltranspeptidase; DOPA, 3,4-dihydroxyphenylalanine

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