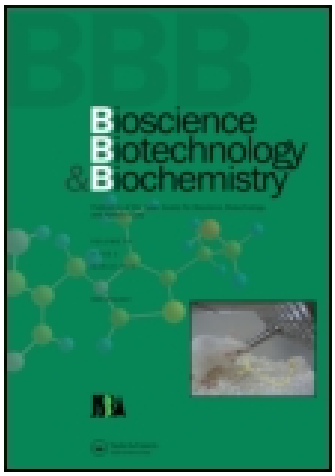

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Effective Formation of Di- and Tri-γ-glutamates by γ-Glutamyltranspeptidase

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Note

Effective Formation of Di- and Tri-γ-glutamates by γ-Glutamyltranspeptidase

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Usinhg γ-glutamyltranspeptidase, conditions for di- and tri-γ-glutamates synthesis were studied with glutamine and glutamic acid esters as substrates. The reactivity of amino acid esters was higher than for free ones. The efficient conditions were the combination of glutamine ethyl and glutamate diethyl esters with a molar ratio of 1/10 at pH 7–8.

Enzymatic synthesis of peptides has been much studied in both aqueous and non-aqueous media[1,2] because no racemization occurs during the reaction as it does with chemical synthesis, and it has stereoselectivity and easy applicability to preparative synthesis.[3] Enzymatic γ-glutamyl bond formation by γ-glutamyltranspeptidase (γ-GTP) has been reported.[4,5] In this paper, we examined enzymatic di- and tri-γ-glutamate formation by using γ-GTP as enzyme and found highly effective conditions. Here, we used crude γ-GTP from bovine kidney because it is commercially available and not expensive.

Gamma-GTP (crude, from bovine kidney, 8.2 unit/mg solid) and glutamic acid dimethyl (Glu(OMe)-OMe) and diethyl (Glu(OEt)-OEt) esters were purchased from Sigma Chem. Co. (St. Louis, MO, U.S.A.). Glutamine methyl (Gln-OMe) and ethyl esters (Gln-OEt) were synthesized by the thionyl chloride method.[6]

Standard conditions for the reaction mixture were: 20 mM Glx-OR (R = H, CH_3, C_2H_5) + 20 mM Glu-R + 1–2 U/ml of γ-GTP in 50 mM buffer. All reactions were done at 37°C. Reaction products were analyzed by the HPLC-UV (220 nm) system with an Asahipak GS-320 column (7.6 × 500 mm, Asahi Chem. Ind., Tokyo) eluted with 50 mM ammonium acetate (1 ml/min). When ester substrates were used, they were hydrolyzed with 1 N NaOH at 60°C for 15 min before analysis.[7] Polymerization degree of

Table Effects of Various Substrate Combinations on the Formation of γ-Glutamylglutamic Acid

Twenty mM carboxyl and 20 mM amine components were reacted with 1 U/ml of γ-GTP in 50 mM borate buffer, pH 9.0, for 4 h.

Carboxyl component Amine component	Gln Glu	Glu Glu	Gln–OEt Glu	Gln Glu(OEt)–OEt	Gln–OEt Glu(OEt)–OEt	Glu(OEt)–OEt Glu(OEt)–OEt
γ-Glu-Glu (mM)	0.4	0	1.3	0.7	4.06	2.54

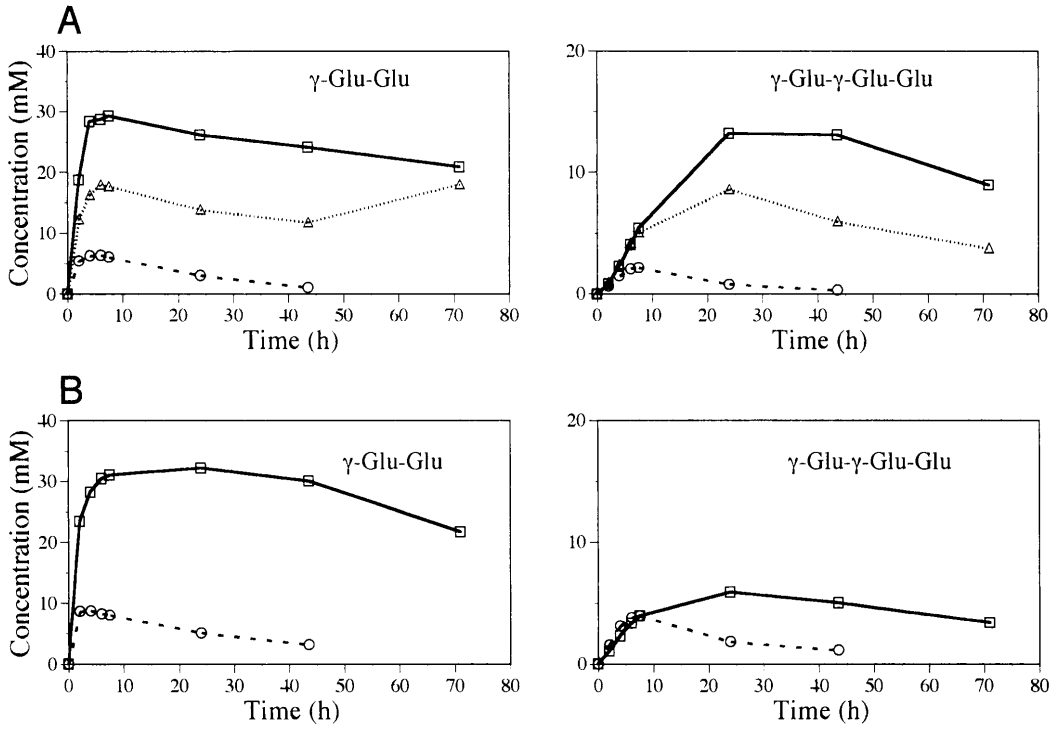

Fig. Effects of Molar Ratio of Substrates and pH on the Formation of Di- and Tri-γ-glutamate.

Twenty mM Glu(OEt)-OEt and 20–200 mM Gln-OEt were reacted with 2 U/ml of γ-GTP at pH 7.0 or 8.0. Left and right panels: γ-Glu-Glu and γ-Glu-γ-Glu-Glu concentrations, respectively. Gln-OEt concentration: ○, 20 mM; △, 100 mM; □, 200 mM. A: in 1 M sodium phosphate buffer, pH 7.0. B: in 0.5 M Tris–HCl, pH 8.0.

Abbreviation: γ-GTP, γ-glutamyltranspeptidase.

products was measured by comparing the increment ratio of the HPLC fraction after complete hydrolysis with 6 N HCl for 24 h at 110°C. The nature of the peptide bond formed, *i.e.*, α- or γ-bond, was judged from the NMR spectrum.[8]

At first, the reactivity of the substrates was compared (Table). The pH was kept at 9.0 because the transpeptidation activity of γ-GTP of bovine kidney was optimum at pH 8.8–9.0.[9] The results for methylated substrates are not shown, but all the reactivities of methylated glutamine and glutamic acid were lower than ethyl esters. The most reactive combination was Gln-OEt and Glu(OEt)-OEt.

Next, the pH profile was evaluated. The pH was varied from 6.0 to 9.5 at 0.5 pH intervals. At all pHs, γ-glutamyl di- and tripeptides were synthesized. Moreover, tetrapeptides was synthetized at pH 7.0–9.0, though the concentration of tetrapeptide was low (< 0.2 mM). Most efficient formations of di- and tripeptides were found at pH 8.0 and 7.0, respectively.

Then, the molar ratio of Gln-OEt and Glu(OEt)-OEt was examined at pH 7.0 and 8.0 for effective reactivity (Fig.). Preliminary experiments showed that the optimal buffer concentrations for tri- and tetra-γ-Glu formation were 1.0 M at pH 7.0 and 0.5 M at pH 8.0, respectively. Thus these conditions were used. Among the ratios used, the combination of 10 Gln-OEt and 1 Glu(OEt)-OEt was most effective and the concentrations of di- and tripeptides were as much as 32 and 13 mM at pH 8.0 and 7.0, respectively. The yield of dipeptide exceeded the concentration of initial amine component (Glu(OEt)-OEt, 20 mM) of peptide bond formation. This might be due to the conversion of Gln-OEt to Glu-OEt during the reaction because γ-GTP from bovine kidney has glutaminase activity.[9]

Tomita *et al.*[10] and Hasegawa and Matsubara[11] reported γ-Glu-Glu could be formed by γ-GTP activity of a glutaminase from *Aspergillus oryzae*, and a γ-glutamylpeptide hydrolytic enzyme of *Corynebacterium glutamicum*. But the yields are too low to the preparative application. On the other hand, during the purification procedure of γ-GTP, Szewczuk and Baranowski,[9] and Orlowski and Meister[12] reported oligo-γ-glutamates (di- to tetrapeptides) formation by γ-GTP from bovine and hog kidney with γ-glutamyl-*p*-nitroanilide and γ-glutamyl-α-naphthylamide as substrates. In this study, di- and tri-γ-glutamates could be synthesized effectively by using cheaper ethyl ester substrates than *p*-nitroanilide or α-naphthylamide. Furthermore, we showed here a method by which a high yield of di- and tri-γ-glutamates could be obtained by using a specific ratio of Gln-OEt and Glu(OEt)-OEt as substrates and crude γ-GTP of bovine kidney as the enzyme.

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