Pergamon

Tetrahedron Letters, Vol. 37, No. 20, pp. 3609-3612, 1996

0040-4039/96 $15.00 + 0.00

S0040-4039(96)00635-1

Enzymatic Synthesis of Carboxyglutamic Acid Containing Peptides in Organic Media

Elena Pera, Josep Lluís Torres and Pere Clapés*

Unit for Protein Chemistry & Biochemistry, C.I.D.-C.S.I.C., Jordi Girona 18-26, 08034-Barcelona, Spain.

Abstract: Kinetically-controlled papain-catalyzed synthesis of carboxyglutamic acid containing peptides is reported. The stereoselectivity of papain allowed to use the racemic Z-γ,γ'-di-*tert*-butyl-D,L-Gla-OMe intermediate as acyl donor ester to obtain diasteromerically pure peptide derivatives. As examples, several carboxyglutamic acid containing dipeptide derivatives were synthesized enzymatically in high chemical and enantiomeric yields. Copyright © 1996 Elsevier Science Ltd

There is an increasing interest in peptides containing γ-carboxyglutamic acid. γ-Carboxyglutamic acid (Gla) is a vitamin K-dependent post-translational modification of glutamate present in proteins of the coagulation cascade, osteocalcins and conantokins.[1-5] Particularly, the conantokins are potent antagonists of the *N*-methyl-D-aspartate (NMDA) receptor with potential application in both fundamental studies on cognitive function and drug design of neuroprotective agents.[6, 7]

The solid-phase synthesis of Gla containing peptides is usually performed using Fmoc-γ,γ'-di-*tert*-butyl-L-Gla-OH as building block under the Fmoc/*tert*-butyl protocol.[2, 5, 7] Most of the syntheses of Gla derivatives are based on the condensation of Z-*O*-tosyl-Ser-OMe with dialkyl-malonate.[5, 8-10] The intermediate obtained after the condensation reaction is Z-γ,γ'-di-*tert*-butyl-D,L-Gla-OMe. This intermediate must be resolved, the Z group removed and the Fmoc introduced. We have recently developed an advantageous enzymatic method to resolve the enantiomers of Z-γ,γ'-di-*tert*-butyl-D,L-Gla-OMe in quantitative yields and enantiomeric excess higher than 99.5% using the endoprotease papain as catalyst.[11] In this paper, we explore the ability of papain to synthesize peptides directly from the racemic mixture of the intermediate. Under kinetic control, Z-γ,γ'-di-*tert*-butyl-D,L-Gla-OMe was used as acyl donor ester and different amino acid amide and ester derivatives were tested as nucleophiles using papain deposited on Celite as catalyst.[12] When enzymes are used in organic media, both solvent and water content have to be optimized regarding the product yield and enzymatic activity.[13,14] In first preliminary experiments acetonitrile and ethyl acetate were tested because they have been proved to be suitable solvents for enzymatic peptide synthesis.[15, 16] Using the synthesis of Z-γ,γ'-di-*tert*-butyl-L-Gla-L-Leu-NH_2 as model reaction[17], several experiments were performed in these two solvents containing different amounts (0% to 4%) of buffer. It is important, in this case, to carry out the reactions under inert atmosphere to avoid deactivation of the papain that occurred even in the presence of β-mercaptoethanol. A typical time-course reaction[18] for the synthesis of Z-γ,γ'-di-*tert*-butyl-L-Gla-L-Leu-NH_2 is presented in Figure 1. The enzyme only recognized the enantiomer L of the carboxyglutamic acid derivative while the Z-γ,γ'-di-*tert*-butyl-D-Gla-

OMe remained in the reaction medium. The stereoselectivity of the enzymatic reaction was determined following a method described previously[11] and it was found to be higher than 99.5%.

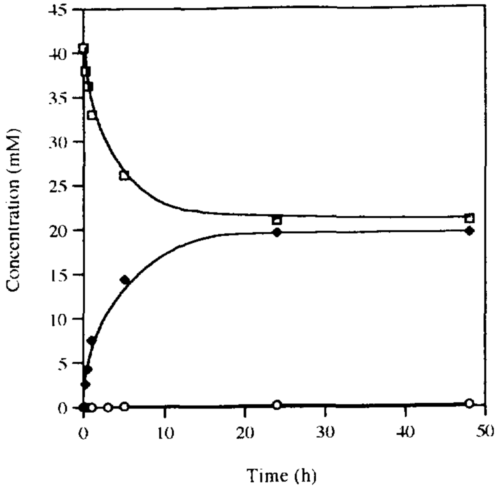

Figure 1. Time-course reaction of the enzymatic synthesis of Z-γ,γ'-di-*tert*-butyl-L-Gla-L-Leu-NH_2. Donor ester Z-γ,γ'-di-*tert*-butyl-D,L-Gla-OMe (□) the hydrolysis product Z-γ,γ'-di-*tert*-butyl-L-Gla-OH (○) and the dipeptide (◆) were analyzed by HPLC.

The tremendous potential of enzymatic methods is displayed in this case. The estereoselectivity of papain allowed us to use the racemic intermediate Z-γ,γ'-di-*tert*-butyl-D,L-Gla-OMe directly, without any previous resolution, in the synthesis of diasteromerically pure carboxyglutamic acid containing peptide derivatives. The influence of solvent and buffer content on both yield and enzymatic activity in the synthesis of Z-γ,γ'-di-*tert*-butyl-L-Gla-L-Leu-NH_2 is presented in Figure 2. Acetonitrile always gave the best enzymatic activity and yield.[19] In this solvent, the activity of the enzyme increased until a maximum at 0.5% (v/v) of buffer and then decreased. In ethyl acetate the activity was constant until 0.5% (v/v) of buffer and then dropped. Dipeptide yields were less influenced by the buffer content between 0% and 0.5% (v/v), decreasing with higher amount of buffer added. It is noteworthy that in both solvents the enzyme was active under anhydrous conditions. The best reaction conditions considering the activity and yield were: acetonitrile with 0.5% (v/v) of buffer. They were used in the rest of experiments.

This methodology was extended to other amino acid amides and ester derivatives. The choice was made according to the sequences of natural occurring carboxyglutamic acid containing proteins[2, 20]. Papain-catalyzed synthesis of different dipeptide derivatives[21] are presented in Table I. All the dipeptides were synthesized in a high yield respect to L isomer. It is noteworthy that amino acid C-α ester derivatives were also suitable nucleophiles facilitating the direct enzymatic coupling of the subsequent amino acid or peptide fragment residues. In kinetically controlled enzymatic synthesis, the covalent complex formed between the enzyme and the donor ester (acyl-enzyme) can be deacylated by water yielding the hydrolysis of the ester or by another amino acid giving the product. The ratio between the initial reaction rate of hydrolysis and synthesis provides a criterion of nucleophile efficiency and it was calculated for each amino acid tested (Table I). The lowest values of this ratio were obtained for leucine, tyrosine and phenylalanine derivatives, in agreement with the S' subsite specificity of papain.[22] Finally, as an example of the use of this methodology in peptide synthesis we have obtained the Z-γ,γ'-di-*tert*-butyl-L-Gla-L-Leu-OEt derivative at a preparative scale.[23] We are currently preparing larger carboxyglutamic acid containing peptides using a combination of enzymatic and chemical methods.

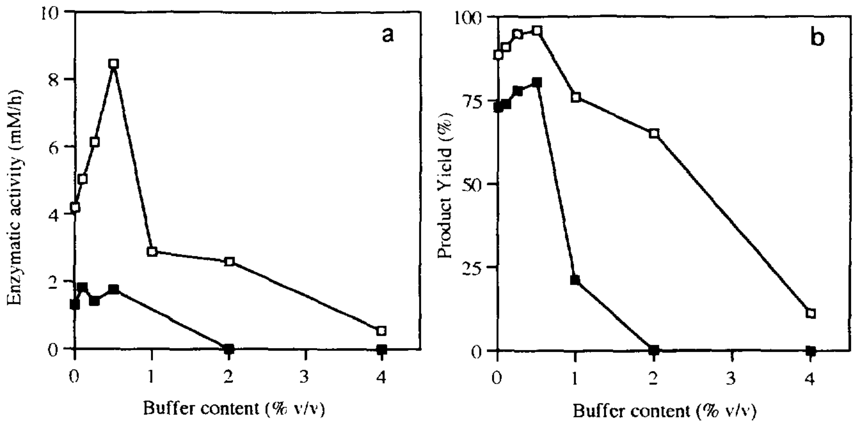

Figure 2. Influence of buffer content on both enzymatic activity (a) and dipeptide yield at 24 h of reaction time (b) in papain catalyzed synthesis of Z-γ,γ'-di-*tert*-butyl-L-Gla-L-Leu-NH_2 in acetonitrile (□) or ethyl acetate (■).

Table I. Dipeptide (Z-γ,γ'-di-*tert*-butyl-L-Gla-L-XX-OR) and donor ester hydrolysis (Z-γ,γ'-di-*tert*-butyl-L-Gla-OH) yields in papain-catalyzed synthesis of carboxyglutamic acid dipeptide derivatives in organic media.

Nucleophile H-XX-OR	Dipeptide Yield[a] % 24 h, 48h	v_h^o/v_s^o [b]	Capacity factor[c] K' (dipeptide)	FAB-MS (M+H)[d], obs.	(M+H), calc.
H-L-Ala-NH_2[e]	57, 56	0.10	2.7	508.3	508.3
H-L-Ile-NH_2	79, 89	0.11	3.0	550.4	550.3
H-L-Val-NH_2	78, 81	0.16	2.5	536.3	536.3
H-L-Phe-NH_2	89, 85	0.02	3.5	584.3	584.3
H-Gly-NH_2	69, 73	0.15	1.4	494.3	494.3
H-L-Leu-NH_2	96, 95	$\approx 10^{-4}$	3.6	550.4	550.3
H-L-Leu-OEt	93, 93	0.03	8.1	579.4	579.3
H-L-Tyr-OMe	89, 89	0.02	3.8	615.3	615.3

[a] Yields, with respect to the L isomer, were calculated from the peak areas by the external method.

[b] v_h^o is the initial donor ester hydrolysis rate; v_s^o is the initial dipeptide synthesis rate.

[c] Capacity factor defined as $(t_r-t_0)/t_0$ where t_0 is the dead time and t_r the retention time of the compound.

[d] The product was characterized by fast atom bombardment mass spectrometry (FAB-MS) on the collected fractions from analytical HPLC.

[e] In this case a new unidentified peak appeared in the HPLC chromatogram (28% of yield).

Acknowledgments

Financial support from the Spanish M.E.C., ref. PB93-0153 and C.I.C.Y.T., ref. BIO95-1083-C02-01, is acknowledged. The authors wish to thank Dra. Gloria Caminal for valuable discussions.

References

1. Chenu, C.; Colucci, S.; Grano, M.; Zigrino, P.; Barattolo, R.; Zambonin, G.; Baldini, N.; Vergnaud, P.; Delmas, P. D.; Zallone, A. Z. *J.Cell.Biol.* **1994**, 127, 1149.
2. Colpitts, T. L.; Castellino, F. J. *Int. J. Peptide Protein Res.* **1993**, 41, 567.
3. Esmon, C. T. *J. Biol. Chem.* **1989**, 264, 4743.
4. Olivera, B. M.; Rivier, J.; Clark, C.; Ramilo, C. A.; Corpuz, G. P.; Abogadie, F. C.; Mena, E. E.; Woodward, S. R.; Hillyard, D. R.; Cruz, L. J. *Science* **1990**, 249, 257.
5. Rivier, J.; Galyean, R.; Simon, L.; Cruz, L. J.; Olivera, B. M.; Gray, W. R. *Biochemistry* **1987**, 26, 8508.
6. Hammerland, L. G.; Olivera, B. M.; Yoshikami, D. *Eur. J. Pharmacol.-Molecular Pharmacology Section* **1992**, 226, 239.
7. Haack, J. A.; Rivier, J.; Parks, T. N.; Mena, E. E.; Cruz, L. J.; Olivera, B. M. *J. Biol. Chem.* **1990**, 265, 6025.
8. Märki, W.; Oppliger, M.; Thanei, P.; Schwyzer, R. *Helv. Chim. Acta.* **1977**, 60, 798.
9. Boggs III, N. T.; Goldsmith, B.; Gawley, R. E.; Koehler, K. A.; Hiskey, R. G. *J. Org. Chem.* **1979**, 44, 2262.
10. Märki, W.; Schwyzer, R. *Helv. Chim. Acta* **1975**, 58, 1471.
11. Clapés, P. Valverde, I., Jaime, C., Torres, J.L. *Tetrahedron Lett.* **1996**, 37, 417.
12. The deposition of papain onto Celite was carried out as follows. Papain (50 mg) and dithiotreithol (50 mg) were dissolved in Boric/NaOH 0.1 M pH 8.2 buffer (1 ml). To this solution Celite-545 (1 g) was added. After mixing thoroughly the preparation was dried under vacuum overnight.
13. Reslow, M., Adlercreutz, P., Mattiasson, B. Appl. Microb. Biotechnol. **1987**, 26, 1.
14. Reslow, M., Adlercreutz, P., Mattiasson, B. Eur. J. Biochem. **1988**, 177, 313.
15. Gill, I., López-Fandiño, R., Vulfson, E. J. Am. Chem. Soc. **1995**, 117, 6175.
16. Clapés, P., Torres, J.L., Adlercreutz, P. Bioorgan. Med. Chem. **1995**, 3, 245.
17. The reactions at test level were carried out as follows. Z-γ,γ'-di-*tert*-butyl-D,L-Gla-OMe (34.8 mg, 0.08 mmol) and the nucleophile (H-AA-OR) (0.06 mmol, 50% excess respect to the L isomer of the donor ester) were dissolved in oxygen free acetonitrile or ethyl acetate (2 ml) containing 0.1 M Boric/NaOH pH 8.5 buffer (0 to 4% v/v, depending on the experiment) and β-mercaptoethanol (1% v/v). To this solution papain deposited on Celite (200 mg) was then added and the mixture kept under argon atmosphere.
18. The time-evolution of reactants and products was followed by high performance liquid chromatography (HPLC). Samples (50 μl) were withdrawn from the reaction medium, mixed with acetic acid (10 μl), diluted with water/CH_3CN (3:7) 0.1 % TFA (200 μl) and analyzed. The elution conditions were: solvent A, aqueous 0.1% (v/v) TFA; B, H_2O/CH_3CN (1:4) 0.1% (v/v) TFA, gradient elution 70 to 90% B in 20 min. For the reaction between Z-γ,γ'-di-*tert*-butyl-D,L-Gla-OMe and H-Ile-NH_2 the elution conditions were the following: solvent A, aqueous 0.1% (v/v) H_3PO_4; B, H_2O/CH_3CN (1:4) 0.1% (v/v) H_3PO_4, gradient elution 65 to 85% B in 30 min. In both cases: detection 254 nm flow rate 1 ml/min.
19. Yields are given respect to L isomer.
20. Fernlund, P., Stenflo, J. *J. Biol. Chem.* **1982**, 257, 1270-1279.
21. When amino acid C-α amide or ester derivatives were in hydrochloride form, 1.1 equivalent of triethylamine was added. This addition did not affect the product yield nor the enzymatic activity in comparison when the nucleophile was used as a free base.
22. Schuster,M., Kasche, V., Jakubke, H.-D. *Biochim. Biophys. Acta* **1992**, 1121, 207.
23. For the synthesis of Z-γ,γ'-di-*tert*-butyl-L-Gla-L-Leu-OEt dipeptide derivative at preparative scale, a crude inexpensive papain from Fluka was used. Z-γ,γ'-di-*tert*-butyl-D,L-Gla-OMe (2.25 g, 5 mmol) and H-Leu-OEt (0.73 g, 3.7 mmol) were dissolved in acetonitrile (100 ml) containing 0.1 M Boric/NaOH pH 8.2 buffer (0.5% v/v) and triethylamine (0.5 ml, 3.7 mmol). This solution was degassed with N_2 for about 3 min and kept under argon atmosphere. To this solution a papain-celite preparation (10 g, 100 mg of crude papain/g of celite-545) was added. The mixture was placed on a reciprocal shaker (130 rpm) at 25° C. After 24 h all the Z-γ,γ'-di-*tert*-butyl-L-Gla-OMe was consumed and the yield by HPLC was 99 % with respect to L isomer. The reaction mixture was filtered to remove the immobilized preparation and the solvent was evaporated under vacuum. The residue was dissolved with ethyl acetate and washed successively with 5% citric acid, 5% bicarbonate and saturated NaCl solution. The organic layer was dried over anhydrous sodium sulfate and evaporated to dryness. The residue was then further purified by preparative HPLC on a Delta-Pack C-4, 15 μm (5x30 cm) cartridge. Elution with a CH_3CN gradient in aqueous TFA, 52 to 68% CH_3CN over 30 min at a flow rate 100 ml/min with U.V. detection at 225 nm. The pure fractions were pooled and freeze dried to give an oil (0.89 g, 60%) with a purity of 97% by HPLC. Fast atom bombardment mass spectrometry (FAB-MS) m/z 579.4 $[M+H]^+$ $C_{30}H_{46}N_2O_9$ requires 578.7; $[\alpha]_D^{23} = -18.6$; $[\alpha]_{578}^{23} = -19.8$; ^{1}HNMR (300 MHz)($d_6$-DMSO) 8.26 d (1H), 7.39 d (1H), 7.34 m (5H), 5.00 s (2H), 4.20 br m (1H), 4.05 br m (2H), 3.33 q (2H), 2.11 br m (1H), 1.38 s (18H), 1.16 t 3H), 0.87 d (3H), 0.81 d (3H).

(*Received in UK* 14 *March* 1996; *accepted* 3 *April* 1996)