Superiority of the Carbamoylmethyl Ester as an Acyl Donor for the Protease-catalyzed Kinetically Controlled Peptide Synthesis in Organic Media: Application to Segment Condensations

Toshifumi Miyazawa,* Eiichi Ensatsu, Kayoko Tanaka, Ryoji Yanagihara, and Takashi Yamada
Department of Chemistry, Faculty of Science, Konan University, Higashinada-ku, Kobe 658-8501

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The superiority of the carbamoylmethyl ester as an acyl donor for the α-chymotrypsin-catalyzed kinetically controlled peptide synthesis was demonstrated in several segment condensations carried out in organic media with low water content. Then this approach was successfully applied to the construction of the Leu-enkephalin sequence via the 4 + 1 segment condensation.

Enzymatic peptide synthesis using proteases[1] is becoming recognized as an alternative or complement to chemical synthesis of biologically active peptides. The advantages of enzymatic methodologies are, among others, freedom from racemization, high regio- and stereoselectivity, and minimal side-chain protection. On the other hand, the following are counted as major drawbacks: narrow substrate specificity and the secondary hydrolysis of a growing peptide. In a previous paper,[2] we have reported on the broadening of substrate specificity of a serine protease, α-chymotrypsin, by using such activated esters as the 2,2,2-trifluoroethyl or carbamoylmethyl ester as acyl donors in the kinetically controlled approach of peptide bond formation. In the present work, the method employing the carbamoylmethyl ester as the acyl donor was further applied to several model segment condensations and also to the synthesis of the Leu-enkephalin sequence.

Table 1 shows the results of the α-chymotrypsin-catalyzed couplings of several kinds of fragments with L-Leu[3] amide[4] employing the carbamoylmethyl esters as acyl donors conducted in acetonitrile containing 4% Tris buffer (pH 7.8).[5] The table also includes the results obtained using the methyl and trifluoroethyl esters for the purpose of comparison. The couplings of fragments bearing a *C*-terminal Ala residue as carboxyl components were examined first (Entries 1-4). When the methyl esters were used as acyl donors, the yields of the desired peptides were low. The use of the carbamoylmethyl ester once again resulted in a marked increase in the peptide yield. In addition, no racemization of the L-Ala residue accompanied the couplings, which was ascertained by reversed-phase HPLC analysis. During the coupling of L-Phe-L-Ala, the bond between Phe and Ala was expected to be susceptible to cleavage by α-chymotrypsin on account of its substrate specificity.[6] In fact, when the methyl ester was used as the acyl donor, the production of 1.4% of Z-L-Phe-L-Leu-NH_2 was observed besides 8.7% of the desired peptide after 5 h of incubation. In this case no production of Z-L-Phe-OH was detected, which indicates that the Leu derivative served as a much better nucleophile than water under the present conditions. In the case of the carbamoylmethyl ester as the acyl donor, the production of the defective peptide was not observed after 1 h of incubation, but it became detectable (4.1%) after 2 h together with the maximum yield of the desired peptide (79.4%) and 10.7% of the hydrolysis product of the donor ester. The couplings of fragments bearing a *C*-terminal Phe residue were next examined (Entries 5-10). As expected, these couplings were rather fast even when the methyl ester was used as an acyl donor. The trifluoroethyl ester moderately increased the coupling yield,

Table 1. α-Chymotrypsin-catalyzed fragment couplings with L-Leu-NH_2 as an amine component[a]

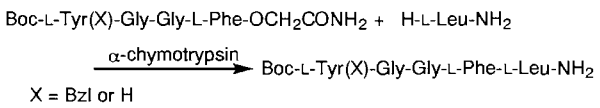
Entry	*C*-Component	Time (min)	Yield / %[b] Peptide[c]	Hydrolysis product[d]	Other by-products[e]
1	Z-Gly-L-Ala-OMe	30	8.3	1.1	
2	Z-Gly-L-Ala-OCam	30	86.0	12.6	
3	Z-L-Phe-L-Ala-OMe	60	1.7	0	A
4	Z-L-Phe-L-Ala-OCam	60	70.8	9.8	B
5	Z-Gly-L-Phe-OMe	5	14.3	1.1	
6	Z-Gly-L-Phe-OTfe	5	56.2	1.2	
7	Z-Gly-L-Phe-OCam	5	95.3	4.7	
8	Z-Gly-Gly-L-Phe-OMe	5	14.9	1.0	
9	Z-Gly-Gly-L-Phe-OTfe	5	62.3	2.4	
10	Z-Gly-Gly-L-Phe-OCam	5	88.3	4.5	
11	Z-L-Phe-L-Phe-OMe	60	12.8	0.9	C
12	Z-L-Phe-L-Phe-OTfe	60	50.6	4.2	
13	Z-L-Phe-L-Phe-OCam	60	83.4	8.1	
14	Z-Gly-DL-Phe(2Br)-OMe[f]	30	4.8[g]	1.6	
15	Z-Gly-DL-Phe(2Br)-OCam[f]	30	39.6[g]	10.6	

[a]A mixture of 0.05 mmol of a carboxyl component, 0.2 mmol of L-Leu-NH_2 · HCl, 0.2 mmol of TEA, and 150 mg (corresponding to 4.6 mg of α-chymotrypsin) of the immobilized α-chymotrypsin on Celite was incubated with shaking in a solvent composed of 2 ml of acetonitrile and 80 μl of Tris buffer (pH 7.8) at 30 °C. [b]Quantified by reversed-phase HPLC analysis. [c]Desired peptide. [d]Hydrolysis product of the donor ester. [e]A, 1.4% of Z-L-Phe-L-Leu-NH_2 after 5 h; B, 4.1% of Z-L-Phe-L-Leu-NH_2 after 2 h; C, 0.5% of Z-L-Phe-L-Leu-NH_2 after 1 h. [f]Using 0.1 mmol of the carboxyl component. [g]L-L Peptide.

while it was significantly enhanced by the use of the carbamoylmethyl ester. No racemization of the L-Phe residue accompanied these couplings. The coupling of L-Phe-L-Phe was next tried where the bond between the two Phe residues was susceptible to cleavage by α-chymotrypsin (Entries 11-13). In fact, when the methyl ester was used as the acyl donor, the production of a small amount (0.5%) of Z-L-Phe-L-Leu-NH_2 (and no formation of Z-L-Phe-OH) was observed after 1 h of incubation. The coupling yield was greatly improved by the use of the carbamoylmethyl ester without the formation of the defective peptide and the racemization of the *C*-terminal L-Phe residue.

Fragment couplings were also tried using a carboxyl component bearing a sterically demanding non-protein amino acid residue at the *C*-terminal position (Entries 14 and 15). The low coupling yield with the methyl ester as an acyl donor was significantly improved by the use of the carbamoylmethyl ester. Starting from the racemic substrate, the L-ester reacted specifically and the D-counterpart remained unchanged even when the carbamoylmethyl ester was employed. Thus, this approach should be very useful for the incorporation of non-protein amino acids into peptides, because racemic amino acids can be directly used for coupling without resolving them before use.

Finally, the preparation of the Leu-enkephalin sequence[7] was carried out via the 4 + 1 segment condensation (Scheme 1).[8] The



Scheme 1.

Tyr-Gly bond in the carboxyl component might be cleaved and the phenolic hydroxy group of the Tyr residue might be attacked. First, the carboxyl component carrying *O*-benzyl-Tyr was allowed to couple with L-Leu amide. The reversed-phase HPLC analysis permitted the detection of the epimers of the resulting protected pentapeptide and of the other possible by-products [*i.e.*, Boc-L-Tyr(Bzl)-Gly-Gly-L-Phe-OH, Boc-L-Tyr(Bzl)-OH and Boc-L-Tyr(Bzl)-L-Leu-NH_2] besides the desired peptide by adjusting the composition of the eluents. After 10 min of incubation, the desired peptide was obtained in 84.7% yield when the carbamoylmethyl ester was employed as the acyl donor. No epimeric peptide was formed and no products attributable to the fission of the Tyr-Gly bond were detected. However, a small amount (3.4%) of the hydrolysis product of the donor ester was inevitable. Next, the carboxyl component carrying Tyr with a free hydroxy group was used for the coupling with the same amine component. In this case also, the desired pentapeptide was produced in high yield (94.7%) after only 10 min of incubation.[9] Neither the epimerization of the L-Phe residue nor the cleavage of the Tyr-Gly bond occurred during the coupling. Moreover, the chromatogram of the reaction mixture on HPLC showed no indication of the occurrence of side reactions at the hydroxy group of the Tyr residue.

The results obtained so far indicate the usefulness of the carbamoylmethyl ester as an acyl donor in the α-chymotrypsin-catalyzed segment condensations. The application of this approach to peptide synthesis using other proteases is now under investigation in our laboratory.

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References and Notes

1 For reviews, see: C.-H. Wong and G. M. Whitesides, "Enzymes in Synthetic Organic Chemistry," Pergamon, Oxford (1994), p. 46; H.-D. Jakubke, "Enzyme Catalysis in Organic Synthesis," ed by K. Drauz and H. Waldmann, VCH, Weinheim (1995), B.2.5, p. 431. For a recent example, see: F. Bordusa, C. Dahl, H.-D. Jakubke, K. Burger, and B. Koksch, *Tetrahedron: Asymmetry*, **10**, 307 (1999).

2 T. Miyazawa, K. Tanaka, E. Ensatsu, R. Yanagihara, and T. Yamada, *Tetrahedron Lett.*, **39**, 997 (1998).

3 The abbreviations given by the IUPAC-IUB Commission are used throughout. Additional abbreviations: Tris, tris(hydroxymethyl)aminomethane; Z, benzyloxycarbonyl; Cam, carbamoylmethyl; Tfe, 2,2,2-trifluoroethyl; Phe(2Br), 2-bromophenylalanine; TEA, triethylamine; Boc, *t*-butoxycarbonyl; Bzl, benzyl.

4 The ameliorating effect of the carbamoylmethyl ester was verified in the couplings with a number of L-amino acid amides.

5 The amounts of the donor ester, the desired peptide and its epimer, and other possible by-products were determined by HPLC analysis on an ODS column using aqueous methanol containing 0.01 M H_3PO_4 as a mobile phase.

6 W. Kullmann, "Enzymatic Peptide Synthesis," CRC Press, Boca Raton (1987), p. 41.

7 *Cf.* T. Miyazawa, T. Otomatsu, Y. Fukui, T. Yamada, and S. Kuwata, *Int. J. Peptide Protein Res.*, **39**, 308 (1992).

8 Using Boc-L-Tyr(Bzl or H)-Gly-Gly-L-Phe-OCam (0.05 mmol), L-Leu-NH_2·HCl (0.2 mmol), TEA (0.2 mmol), and the immobilized α-chymotrypsin (150 mg) in the same manner as described in the footnote to Table 1.

9 The yield of the hydrolysis product of the donor ester was 5.0%.