Enzyme-Catalyzed Peptide Segment Condensation Using 5(4H)-Oxazolones as Acyl Donors

Byung Keun Hwang, Qu-Ming Gu, and Charles J. Sih*

School of Pharmacy, University of Wisconsin Madison, Wisconsin 53706

Received May 26, 1993

Although proteins as many as 99 and 140 amino acids in length have been constructed using the stepwise solid-phase technique,[1] the convergent strategy of segment synthesis–condensation remains an attractive approach for several reasons, not the least of which being its versatility for the semisynthesis of analogs of larger natural peptides and proteins.[2]

Although peptides containing 10–15 amino acids may now be readily prepared using commercial peptide synthesizers,[2,3] the chemical coupling procedures of such peptide segments often suffer from problems of racemization and low yields. Consequently, enzyme-catalyzed peptide syntheses[4] have attracted considerable interest because of their well-documented advantages such as stereoselectivity, lack of racemization, and minimal side-chain protection in the coupling steps. While several oligopeptides have successfully been prepared via segment coupling,[5] most of the attention has been focused on the optimization of reaction conditions for the stepwise assembly of amino acids to form small peptides.[6]

Generally, a kinetically-controlled approach[4d,g] using activated acyl esters as donors and acyl amides as nucleophiles is preferred in enzymatic syntheses. However, the chemoselective esterification of the C-terminal amino acid in peptides containing acidic amino acids is still an unresolved problem, thereby limiting the usefulness of this methodology. We now report that this limitation can largely be avoided by the use of 5(4H)-oxazolones as activated acyl donors in protease-catalyzed peptide segment condensations.

α-Chymotrypsin is one of the most intensively studied enzymes.[7] It is an endopeptidase with a primary specificity for peptide substrates containing aromatic amino acids in the P_1 position.[8] Because the 5(4H)-oxazolones undergo spontaneous hydrolysis in water and they racemize with ease,[9] they have not been exploited for use in peptide synthesis until now, although chymotrypsin had been shown to hydrolyze oxazolones derived from glycine, dimethylglycine, and the aromatic amino acids with modest enantioselectivities.[10]

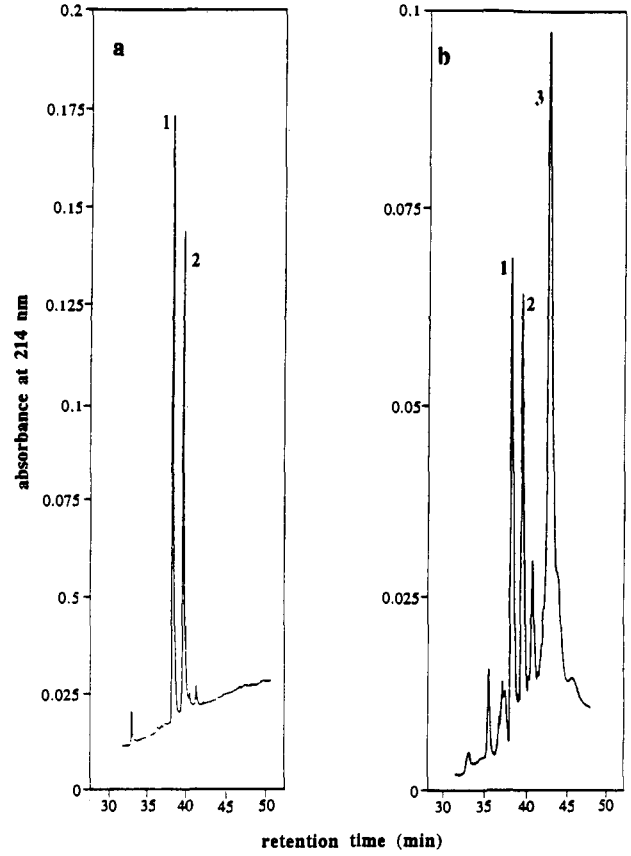
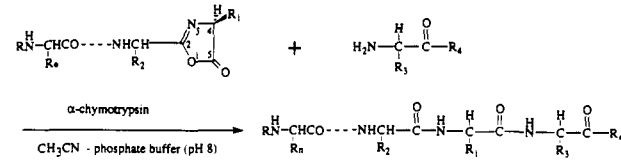


Figure 1. HPLC elution profile of the α-chymotrypsin-catalyzed synthesis of pELYENK(*N*-TFA)PRRPYILRPKPQQFFGLM-NH_2 (**3**). Reaction conditions: **1** (0.6 mM), **2** (0.5 mM), α-chymotrypsin (80 μg), 0.2 mL of CH_3CN/0.2 M phosphate buffer, 1:1, pH 8.0. (a) RPKPQQFFGLM-NH_2 (**1**) and pELYENK(N-TFA)PRRPYIL (**2**). (b) Reaction mixture after 5 min incubation.



In the initial phases of our study, we used 2-phenyl-4-benzyloxazolin-5-one as the acyl donor and various acyl acceptors in the α-chymotrypsin-catalyzed syntheses of small peptides. Several interesting features of the reaction are worthy of note. (a) The enzyme utilized 2-phenyl-L-4-benzyloxazolin-5-one[11] as the acyl donor to form the acyl–enzyme intermediate, which was effectively deacylated by various acyl acceptors (entries 3–6, Table I). Since only one peptide product was isolated in each case, one could surmise that little nonenzymatic coupling or racemization of the L-oxazolone occurred under these reaction conditions. (b) When 2-phenyl-DL-4-benzyloxazolin-5-one was used, instead, as the acyl donor and L-Phe-NH_2 as the acyl acceptor, the enzyme

(1) (a) Clark-Lewis, I.; Aebersolf, R.; Ziltener, H.; Schrader, J. W.; Hood, L. E.; Kent, S. B. H. *Science* **1986**, *231*, 134–139. (b) de L. Milton, R. C.; Milton, S. C. F.; Kent, S. B. H. *Science* **1992**, *256*, 1445–1448.

(2) For example, a 60 amino acid homeo domain of the *Antennapedia* gene regulatory protein was recently prepared by this approach: Mihara, H.; Chmielewski, J. A.; Kaiser, E. T. *J. Org. Chem.* **1993**, *58*, 2209–2215. See also: Chaiken, I. M. *CRC Crit. Rev. Biochem.* **1981**, *11*, 255–301. See also: Bongers, J.; Lambros, T.; Liu, W.; Ahmad, M.; Campbell, R. M.; Felix, A. M.; Heimer, E. P. *J. Med. Chem.* **1992**, *35*, 3934–3941.

(3) DeGrado, W. F.; Kaiser, E. T. *J. Org. Chem.* **1982**, *47*, 3258–3261.

(4) (a) Fruton, J. S. *Adv. Enzymol.* **1982**, *53*, 239–306. (b) Mitin, Y. V.; Zapevalova, N. P.; Gorbunova, E. Y. *Int. J. Peptide Protein Res.* **1984**, *23*, 528–534. (c) Kullman, W. *Enzymatic Peptide Synthesis*; CRC Press: Boca Raton, FL, 1987. (d) Schellenberger, V.; Jakubke, H. D. *Angew. Chem., Int. Ed. Engl.* **1991**, *30*, 1437–1449. (e) Widmer, F.; Breddam, K.; Johansen, J. T. *Carlsberg Res. Commun.* **1981**, *46*, 97–106. (f) Kasche, V.; Michaelis, G. *Biochemistry of Peptide Antibiotics*; H. Kleinkauf and H. Von Döhren: New York, 1990; pp 81–101. (g) See also: Wong, C. H. *Trends Biotechnol.* **1992**, *10*, 378–381, and references cited therein.

(5) (a) Kaufmann, W. *Proc. Natl. Acad. Sci. U.S.A.* **1982**, *79*, 2840–2844. (b) Nakatsuka, T.; Sasaki, T.; Kaiser, E. T. *J. Am. Chem. Soc.* **1987**, *109*, 3808–3811. (c) Kitaguchi, H.; Klibanov, A. M. *J. Am. Chem. Soc.* **1989**, *111*, 9272–9273. (d) Isowa, Y.; Ohmori, M.; Sato, M.; Mori, K. *Bull. Chem. Soc. Jpn.* **1977**, *50*, 2766–2772.

(6) (a) Ricca, J. M.; Crout, D. H. G. *J. Chem. Soc., Chem. Commun.* **1989**, 2126–2127. (b) Gill, I.; Vulfson, E. N. *J. Am. Chem. Soc.* **1993**, *115*, 3348–3349. (c) Gaertner, H.; Watanabe, T.; Sinisterra, J. V.; Puigserver, A. *J. Org. Chem.* **1991**, *56*, 3149–3153. (d) West, J. B.; Hennen, W. J.; Lalonde, J. L.; Bibbs, J. A.; Zhong, Z.; Meyer, E. F.; Wong, C. H. *J. Am. Chem. Soc.* **1990**, *112*, 5313–5320. (e) Shellenberger, V.; Schellenberger, U.; Mitin, Y. V.; Jakubke, H. D. *Eur. J. Biochem.* **1990**, *187*, 163–167.

(7) Fersht, A. R. *Enzyme Structure and Mechanism*; Freeman, New York, 1985.

(8) Keil, B. *Protein Seq. Data Anal.* **1987**, *1*, 13–20.

(9) (a) Benoiton, N. L. *The Peptides* **1983**, *5*, 217–284. (b) Crich, J. Z.; Brieva, R.; Marquart, P.; Gu, R. L.; Flemming, S.; Sih, C. J. *J. Org. Chem.* **1993**, *58*, 1068–1075.

(10) (a) DeJersey, J.; Zerner, B. *Biochemistry* **1969**, *8*, 1967–1974. (b) Daffe, V.; Fastrez, J. *J. Am. Chem. Soc.* **1980**, *102*, 3601–3605.

(11) Goodman, M.; Levine, L. *J. Am. Chem. Soc.* **1964**, *86*, 2918–2922.

0002-7863/93/1515-7912$04.00/0

Table I. Coupling Yields in the Synthesis of Peptides Catalyzed by α-Chymotrypsin[a]

entry	acyl donor	acyl acceptor	time (min)	yield,[b] %	FAB-MS calcd	FAB-MS found
1	*N*-Bz-DL-Phe	Phe-NH_2	30	59 (LL) 13[c] (DL)	415.5	416
2	*N*-Bz-DL-Phe	Arg-NH_2	5	70 (LL) 17[c] (DL)	*d*	
3	*N*-Bz-Phe	Phe-NH_2	30	64	415.5	416
4	*N*-Bz-Phe	Arg-NH_2	30	85		
5	*N*-Bz-Phe	Arg-Phe-NH_2	5	70	*d*	
6	*N*-Bz-Phe	Ala-Ala-Ala[e]	20	42	335.4	336
7	*N*-TFA–cholecystokinin (26–27) (3.8 mM)	cholecystokinin (28–33) (3.8 mM)	30	64	1158.3	1189[f]
8	*N*-Ac–substance P (1–7) (1.7 mM)	substance P (8–11) (3.4 mM)	30	57	1431.8	1432
9	*N*-TFA–neurotensin (0.6 mM)	angiotensin III (1.0 mM)	5	97		
10	*N*-TFA–neurotensin-(DL-Leu[13]) (0.74 mM)	angiotensin III (1.3 mM)	30	[g]		
11	*N*-TFA–neurotensin (0.5 mM)	substance P (0.6 mM)	5	73		

[a] Abbreviations: Ac, acetyl; Bz, benzoyl; TFA, trifluoroacetyl. All amino acids are of the L configuration unless otherwise stated. The peptides used were as follows: *N*-TFA–cholecystokinin (26–27), *N*-TFA-DY; cholecystokinin (28–33), MGWMDF-NH_2; *N*-Ac-substance P (1–7), *N*-Ac-RPK(*N*-Ac)PQQF; substance P (8–11), FGLM-NH_2; TFA–neurotensin, pE-LYENK(*N*-TFA)PRRPYIL; angiotensin III, TVYlHPF; substance P, RPKPQQFFGLM-NM_2. Experimental conditions (unless otherwise stated) were acyl donor, 10 mM:acyl acceptor, 50 mM; reaction media, CH_3CN/0.2 M phosphate buffer (1:1), pH 8.0–8.5; α-chymotrypsin, 0.4–1.0 mg/mL; 24 °C. The progress of reactions was monitored by HPLC analysis [(Waters C_{18} [4 μm] Nova-PAK (8 × 100 mm^2) or Zorbax 300 SB-C8 column); gradient (0.1% TFA in H_2O to 0.1% TFA in CH_3CN in 40 min at a flow rate of 1 mL/min)]. Amino acid analyses were performed using the Waters Pico-Tag system. [b] Yields were estimated from HPLC analyses and were based on the acyl donor. [c] Yields of Bz-D-Phe-L-Phe-NH_2 and Bz-D-Phe-L-Arg-NH_2. [d] The amino acid analyses of **2** and **5** were as follows: Arg(1) 1, Phe(1) 1; and Arg(1) 1, Phe(2) 1.7 respectively. [e] The reaction medium was CH_3CN/0.05M phosphate buffer = 1/1, v/v, pH 8.5, and 2 M KCl. [f] Two Mets were oxidized. [g] L-oxazolone of *N*-TFA–neurotensin disappeared, whereas *N*-TFA–(D-Leu[13])neurotensin remained. The HPLC retention times of the corresponding acids derived from the L- and D-oxazolones were 21 and 21.5 min, respectively.

showed a preference for the L-oxazolone to yield two diastereomeric peptides, *N*-Bz-L-Phe-L-Phe-NH_2 and *N*-Bz-D-Phe-L-Phe-NH_2, in yields of 59% and 13%, respectively. (c) Protection of the C-terminal carboxyl group was not required[12] (entry 6). (d) The yields of the coupling reactions were not quantitative because of hydrolysis of the acyl–enzyme intermediate to yield the corresponding acid, which was isolated by HPLC; the extent of hydrolysis depends on the acyl acceptor occupying the P_1' site. No significant amount of oxazolone hydrolysis was detected in the absence of the enzyme.

The synthetic utility of this methodology was confirmed by the preparation of several oligopeptides containing 8–24 amino acid residues in overall yields of 57–97% (entries 7–11, Table I).

A representative procedure for the condensation of neurotensin (13 residues) to substance P (11 residues) was as follows: TFA–neurotensin[13] (0.1 μmol) was dissolved in 0.3 mL of a mixture consisting of acetic anhydride:dioxane (1:1). After the reaction mixture was stirred at 0 °C for 1 h, the solvents were evaporated under reduced pressure at 0 °C[14] and the residue (oxazolone) was dissolved in a mixture of acetonitrile:0.2 M phosphate buffer, pH 8 (1:1). To this solution was added substance P (0.12 μmol) and 4 μL of α-chymotrypsin (20 μg/μL), and the content was incubated at 24 °C with stirring. The progress of the reaction was monitored by removing an aliquot of the reaction mixture at regular intervals and analyzing it by HPLC on a Waters C18 (4 μm) Nova-PAK reverse-phase column. The column was eluted with a gradient of water–CH_3CN containing 0.1% TFA (0–100% in 80 min) at a flow rate of 1 mL/min. A typical profile is shown in Figure 1. The amino acid analyses of the product gave the following: Asp(1) 0.57; Glu(4) 3.67; Gly(1) 1; Arg(3) 3.20; Pro-(4) 4.36; Tyr(2) 2.31; Met(1) 0.94; Ile(1) 1.02; Leu(3) 3.23; Phe(2) 2.05; Lys(2) 3.23.

When the oxazolone of TFA–neurotensin was epimerized by treatment with pyridine, only the L-oxazolone of the TFA–neurotensin was utilized as the acyl donor (entry 10), indicating that chymotrypsin was highly diastereoselective toward cleaving this oxazolone.

In conclusion, contrary to the general notion that 5(4H)-oxazolones are too unstable for use in peptide synthesis, our results demonstrate for the first time that they can be effectively used as acyl donors in protease-catalyzed segment condensations. α-Chymotrypsin rapidly cleaved the oxazolone ring of the peptide fragment bearing a large C-2 backbone to generate the acyl–enzyme, which was deacylated by the N-terminal group of another peptide fragment at rates faster than the competing hydrolysis of the acyl–enzyme intermediate. As oxazolones of peptides can be prepared under controlled conditions without racemization and since they are relatively stable in the reaction medium, this methodology provides a new strategy for oligopeptide synthesis.

Acknowledgment. This work was supported in part by the National Institutes of Health. Q.M.G. was a recipient of a predoctoral fellowship from the Bristol-Myers-Squibb Co.

(12) The nucleophilic efficiency of Ala-Ala-Ala-CO_2^- is markedly improved by the addition of 2 M KCl to reduce the unfavorable electrostatic interaction between the CO_2^- group and the negatively charged region of the S′ subsite on Chymotrypsin. Schellenberger, V.; Aaviksaar, A.; Jakubke, H. D. *Biocatalysis* **1991**, *4*, 291–296.

(13) *N*-Trifluoroacetylations of peptides were as follows: to neurotensin (1 mg), dissolved in 0.3 mL of 0.1 M $NaHCO_3$ was added 0.1 mL of *S*-ethyl trifluorothioacetate, and the mixture was stirred vigorously at 24 °C for 2.5 h. During the reaction, the pH was maintained between 9 and 10 using 1 N NaOH. After 3 h, the solvents were evaporated to dryness under reduced pressure and the residue was dissolved in 0.2 mL of water and purified by HPLC.

(14) It is imperative that the formation of the mixed anhydride and the subsequent operations are carried out at 0 °C. At ambient temperatures, racemization of the peptide oxazolone was observed.