ORGANIC LETTERS

2000
Vol. 2, No. 14
2027−2030

Substrate Mimetics and Freezing Strategy: A Useful Combination That Broadens the Scope of Proteases for Synthesis

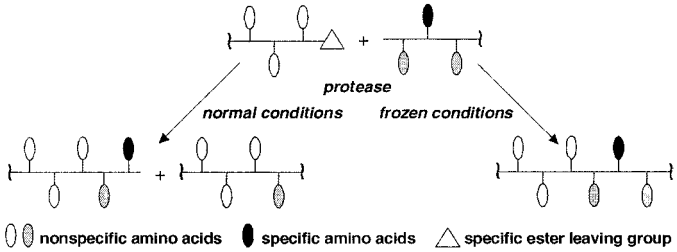
Nicole Wehofsky,[†] Sebastian W. Kirbach,[†] Marion Haensler,[†] Johannes-Dieter Wissmann,[†] and Frank Bordusa*[,†,‡]

Max-Planck-Society, Research Unit "Enzymology of Protein Folding", Weinbergweg 22, D-06120 Halle/Saale, Germany, and Department of Biochemistry, University of Leipzig, D-04103 Leipzig, Germany

bordusa@uni-leipzig.de

Received March 28, 2000

ABSTRACT



We present an irreversible and efficient protease-based method for peptide synthesis which occurs independently of the primary specificity of proteases and also without proteolytic side reactions. The key feature of this approach is the combination of the substrate mimetics strategy with frozen state enzymology. Model reactions catalyzed by several proteases qualify this approach as a powerful concept in the direction of a more universal application of proteases as biocatalysts for peptide ligation.

Solution- and especially solid-phase peptide synthesis has been well developed as a powerful tool for the step-by-step synthesis of various sizes of peptides. Because of the statistical accumulation of low-level resin-bond byproducts, however, traditional stepwise solid-phase synthesis is limited to the routine preparation of peptides of around 50−60 amino acids.[1] Generally the coupling of peptide segments, in particular by native chemical ligation, is being widely used to provide access to larger peptides up to proteins.[2] Because of the mechanism, that is the reaction of a peptide thioester with a C-terminal Cys of the second nucleophilic peptide segment, this ligation strategy is limited to the coupling of Xaa−Cys bonds. Thus, peptides with sequences devoid of suitably spaced Cys residues are generally not targets of this technique. Enzymatic methods based on proteases combined with chemical synthetic methods are attractive alternatives because enzymatic reactions are regio- and stereospecific, free from racemization, and only require minimal protective group chemistry.[3] With these advantages, however, come the disadvantages that the native amidase activity of proteases causes proteolytic side reactions and that the substrate specificity of these enzymes limits the choice of amino acids between which a peptide bond can be synthesized. A powerful concept to overcome this limitation of the classical

[†] Max-Plank Society.
[‡] University of Leipzig.

(1) (a) Bodansky, M.; Bodansky, A. *The Practice of Peptide Synthesis*; Springer-Verlag: New York, 1984. (b) Kaiser, E. T. *Acc. Chem. Res.* **1989**, *22*, 957.

(2) (a) Kemp, D. S.; Grattan, J. A.; Reczek, J. *J. Org. Chem.* **1975**, *40*, 3465−3466. (b) Fotouhi, N.; Galakatos, N. G.; Kemp, D. S. *J. Org. Chem.* **1989**, *54*, 2803−2809. (c) Schnalzer, M.; Kent, S. B. H. *Science* **1992**, *256*, 221−225. (d) Dawson, P. E.; Muir, T. M.; Clark-Lewis, I.; Kent, S. B. H. *Science* **1994**, *266*, 776−779. (e) Liu, C.-F.; Tam, J. P. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 6584−6588.

(3) (a) Schellenberger, V.; Jakubke, H.-D. *Angew. Chem., Int. Ed. Engl.* **1991**, *30*, 1437−1449. (b) Wong, C.-H. *Science* **1989**, *244*, 1145−1152. (c) Jackson, D. Y.; Burnier, J.; Quan, C.; Stanley, M.; Tom, J.; Wells, J. A. *Science* **1994**, *266*, 243−247.

10.1021/ol005867g CCC: $19.00
Published on Web 06/10/2000

enzymatic approach is the concept of substrate mimetics.[4] Contrary to commonly used acyl donor esters, in substrate mimetics the site-specific amino acid moiety is transferred from the C-terminal of the peptide residue to the ester leaving group. This shift in the location of the specific moiety is accompanied by a shift in the enzyme activity, enabling proteases to react with nonspecific coded and noncoded amino acids and even with non-amino acid-derived carboxylic acid derivatives.[5] Moreover, because of the coupling of nonspecific sequences the newly formed peptide bond cannot be attacked by the enzyme and therefore is generally stable toward secondary hydrolysis.

However, as revealed by trypsin- and chymotrypsin-catalyzed model reactions (Figure 1), the application of the substrate mimetics approach to the ligation of peptides is seriously limited when using specific amino acid-containing segments. In such cases, unwanted cleavages of the peptide reactants or products after the specific amino acid moieties (Arg for trypsin and Tyr for chymotrypsin) are favored over their coupling with the appropriate substrate mimetics. As a result, the product yields of intact peptides significantly decrease from about 80 to 20%. Moreover, the use of the Glu-specific BL-GSE as the catalyst and amino acid carboxymethyl thioester derivatives as the substrate mimetics led to an even faster, and therefore complete, cleavage of the peptide reactants at the sensitive Glu bonds (data not shown). Consequently, formation of intact peptide products could not be obtained. These results clearly demonstrate that the broad use of the substrate mimetics approach to peptide synthesis requires further efforts to minimize the inherent native amidase activity of proteases responsible for these unwanted cleavage reactions.

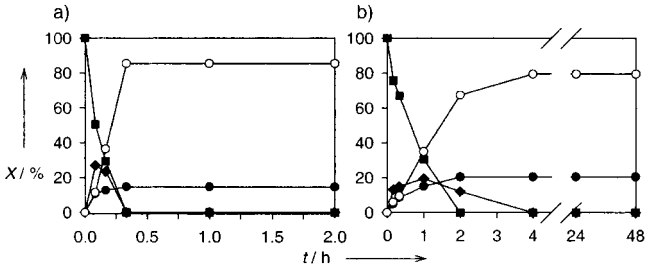


Figure 1. Course of the trypsin- and chymotrypsin-catalyzed coupling of substrate mimetics with specific amino acid-containing peptides at 25 °C: (a) Bz-Gly-OGp[7] and H−Ala-Ala-Arg-Ala-Gly-OH using trypsin; (b) Bz-Gly-OPh and H−Ala-Ala-Tyr-Ala-Gly-OH using chymotrypsin. (-■-) Bz-Gly-OGp/-OPh, (-●-) Bz-Gly-OH, (-♦-) Bz-Gly-Ala-Ala-(Arg/Tyr)-Ala-Gly-OH, (−○−) Bz-Gly-Ala-Ala-(Arg/Tyr)-OH. Conditions: 0.1 M Hepes buffer (pH 8.0), 0.2 M NaCl, 0.02 M $CaCl_2$, [acyl donor] = 2 mM, [acyl acceptor] = 15 mM, [trypsin] = 1.0×10^{-7} M, [chymotrypsin] = 1.0×10^{-6} M, X = product yield.

It is known from the literature that lowering the reaction temperature usually decreases the activity of proteases for cleaving peptide bonds.[6,7] Freezing of the aqueous reaction medium finally results in enzyme species with only minimal amidase activity. On the contrary, the intrinsic esterase activity of most serine and cysteine proteases is obviously less affected, enabling these enzymes to react with analogous peptide esters under frozen-state conditions.[8] Owing to these distinct effects on esterase and amidase activity, freezing was found to be useful to repress unwanted proteolytic cleavages of the enzymatically formed peptide products.[9] Although this effect of freezing could be verified for numerous proteases, up to now there are only reports on using this approach for the coupling of common acyl donor esters of highly specific amino acids with amino acid amides or small peptides lacking specific amino acid moieties. Since common acyl donor esters are generally orders of magnitude more highly specific than the appropriate peptide products formed, it is questionable whether this freezing effect also holds for the coupling of the less specific substrate mimetics[10] with peptide segments containing highly specific amino acid moieties. Furthermore, it remains open as to whether the differences in the mechanism of catalysis which holds for substrate mimetics and common acyl donor esters (**4f**) may affect the enzyme activity toward substrate mimetics under frozen-state conditions.

To study the influence of freezing on the course of substrate mimetics-mediated syntheses, model reactions at −15 °C were perfomed using specific amino acid-containing pentapeptides as the acyl acceptors, nonspecific amino acid-derived substrate mimetics as the acyl donors, and trypsin, chymotrypsin, and BL-GSE as the biocatalysts (Scheme 1). Plots of the courses for some selected reactions are shown in Figure 2 for trypsin- and chymotrypsin-catalyzed syntheses and Figure 3 for analogous BL-GSE-mediated ones. As indicated by these plots, freezing of the reaction medium

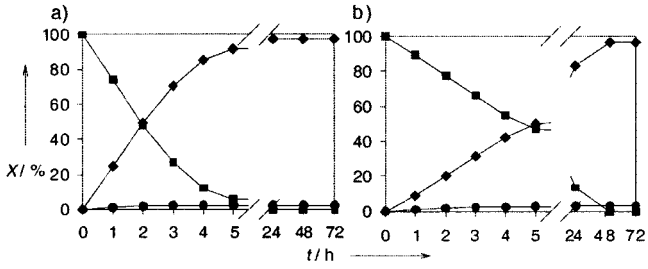
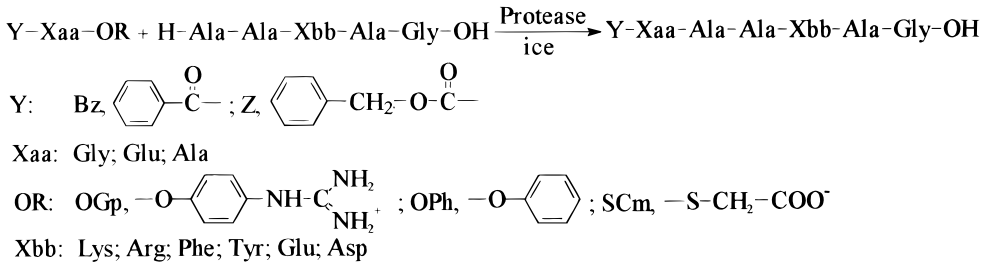


Figure 2. Course of the trypsin- and chymotrypsin-catalyzed coupling of substrate mimetics with specific amino acid-containing peptides at −15 °C: (a) Bz-Gly-OGp and H-Ala-Ala-Arg-Ala-Gly-OH using trypsin; (b) Bz-Gly-OPh and H-Ala-Ala-Tyr-Ala-Gly-OH using chymotrypsin. (-■-) Bz-Gly-OGp/-OPh, (-●-) Bz-Gly-OH, (-♦-) Bz-Gly-Ala-Ala-(Arg/Tyr)-Ala-Gly-OH. Conditions: distilled water (pH 8.0 before freezing), [acyl donor] = 2 mM, [acyl acceptor] = 15 mM, [trypsin] = 1.0×10^{-5} M, [chymotrypsin] = 1.0×10^{-5} M, X = product yield.

(4) (a) Wagner, G.; Horn, H. *Pharm.* **1973**, *28*, 428−431. (b) Tanizawa, K.; Kasaba, Y.; Kanaoka; Y. *J. Am. Chem. Soc.* **1977**, *99*, 4484−4488. (c) Mitin, Y. V.; Schellenberger, V.; Schellenberger, U.; Jakubke, H.-D.; Zapevalova, N. P. In *Peptides*; Giralt, E., Andreu, D., Eds.; ESCOM: Leiden, 1990; pp 287−288. (d) Sekizaki, H.; Itoh, K.; Toyota, E.; Tanizawa, K. *Chem. Pharm. Bull.* **1996**, *44*, 1577−1579. (e) Bordusa, F.; Ullmann, D.; Elsner, C.; Jakubke, H.-D. *Angew. Chem., Int. Ed. Engl.* **1997**, *36*, 2473−2475. (f) Thormann, M.; Thust, S.; Hofmann, H.-J.; Bordusa, F. *Biochemistry* **1999**, *38*, 6056−6062.

Scheme 1. General Scheme of Enzymatic Peptide Coupling Reactions



does not disturb the activity of the three proteases toward the substrate mimetics. Remarkably, comparison of the course of reactions under frozen conditions with that at room temperature (cf. Figure 1) reveals a radical shift in enzyme preferences. While the activity of the proteases in cleaving the sensitive peptide bonds is higher than that for coupling under usual conditions, freezing of the reaction medium generally leads to the complete opposite behavior. Accordingly, under frozen-state conditions the three enzymes exclusively catalyze the coupling of the substrate mimetics with the appropriate peptide segments, remaining devoid of any proteolytic side reactions. From a synthetic point of view, this offers practical, irreversible peptide bond formation without the risk of unwanted proteolytic reactions at specific cleavage sites within the peptide reactants or products. The lower rates of enzymatic reactions in the frozen system, which usually require a higher level of enzyme, can be easily compensated either by the elongation of the incubation time or (and) by a further increase of the enzyme concentration. Due do the high stability of the reactants under these conditions, longer reaction times do not favor the formation of nonenzymatic hydrolysis or aminolysis byproducts. The elongation of the reaction time to several days, studied for BL-GSE-catalyzed reactions (cf. Figure 3), does not affect the course of synthesis. In the same way, there is no evidence of an influence of the individual C-terminal amino acid of the substrate mimetics on the course of the reaction, as indicated by the similar results found for the appropriate Gly- and Glu-containing substrate mimetics in trypsin- and chymotrypsin-catalyzed syntheses (Table 1). In all cases, freezing completely suppresses proteolytic side reactions which finally guarantees high yields of the intact peptide

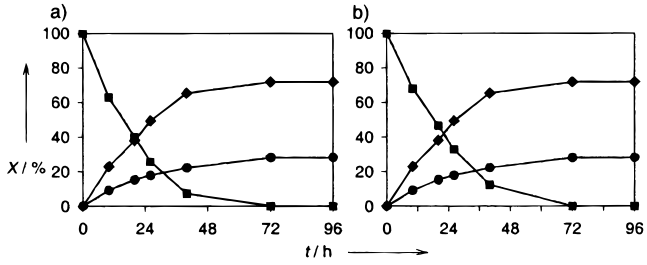


Figure 3. Course of the BL-GSE-catalyzed coupling of Z-Ala-SCm with Asp- and Glu-containing peptides at −15 °C: (a) Reaction of Z-Ala-SCm with H−Ala-Ala-Asp-Ala-Gly-OH; (b) Reaction of Z-Ala-SCm with H−Ala-Ala-Glu-Ala-Gly-OH. (-■-) Z-Ala-SCm, (-●-) Z-Ala-OH, (-♦-) Z-Ala-Ala-Ala-(Asp/Glu)-Ala-Gly-OH. Conditions: distilled water (pH 8.0 before freezing), [acyl donor] = 2 mM, [acyl acceptor] = 15 mM, [BL-GSE] = 1.0×10^{-8} M, X = product yield.

Table 1. Influence of Freezing on Substrate Mimetics-Mediated Couplings of Specific Amino Acid-Containing Peptides Catalyzed by Trypsin, Chymotrypsin, and BL-GSE[a]

Protease/acyl donor	acyl acceptor	yield [%] 25 °C	yield [%] −15 °C
trypsin			
Bz-Gly-OGp	AA*K*AG	51.5 (85.9)	97.3
Bz-Gly-OGp	AA*R*AG	27.0 (85.4)	97.6
Bz-Glu-OGp	AA*K*AG	54.9 (76.5)	90.1
Bz-Glu-OGp	AA*R*AG	30.4 (79.4)	92.1
chymotrypsin			
Bz-Gly-OPh	AA*F*AG	74.1 (84.0)	90.6
Bz-Gly-OPh	AA*Y*AG	15.0 (79.5)	96.7
Bz-Glu-OPh	AA*F*AG	73.6 (83.1)	88.0
Bz-Glu-OPh	AA*Y*AG	15.0 (84.7)	94.6
BL-GSE			
Z-Ala-SCm	AA*D*AG	31.6 (31.6)	71.8
Z-Ala-SCm	AA*E*AG	0 (34.6)	71.9

[a] The specific amino acid moieties within the nucleophilic peptides are in italics. Parentheses generally indicate competitive cleavages. The data in parentheses correspond to the whole peptide products while the data outside the parentheses give the yields of the appropriate intact hexapeptide product. Conditions at 25 °C: 0.1 M Hepes buffer (pH 8.0), 0.2 M NaCl, 0.02 M $CaCl_2$, [acyl donor] = 2 mM, [acyl acceptor] = 15 mM, [trypsin] = 1.0×10^{-7} M, [chymotrypsin] = 1.0×10^{-6} M, [BL-GSE] = 3.0×10^{-5} M. Conditions at −15 °C: distilled water (pH 8.0 before freezing), [acyl donor] = 2 mM, [acyl acceptor] = 15 mM, [trypsin] = 1.0×10^{-5} M, [chymotrypsin] = 1.0×10^{-5} M, [BL-GSE] = 1.0×10^{-8} M.

(5) (a) Sekizaki, H.; Itoh, K.; Toyota, E.; Tanizawa, K. *Chem. Pharm. Bull.* **1998**, *46*, 846−849. (b) Wehofsky, N.; Bordusa, F. *FEBS Lett.* **1999**, *443*, 220−224. (c) Günther, R.; Stein, A.; Bordusa, F. *J. Org. Chem.* **2000**, *65*, 1672−1679. (d) Günther, R.; Bordusa, F. *Chem. Eur. J.* **2000**, *6*, 463−467. (e) Cerovsky, V.; Bordusa, F. *J. Pept. Res.* **2000**, *55*, 325−329.

(6) Lineweaver, H. *J. Am. Chem. Soc.* **1939**, *61*, 403.

(7) Abbreviations: Bz, benzoyl; BL-GSE, *Bacillus licheniformis* Glu-specific endopeptidase; Hepes, *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid]; OGp, 4-guanidinophenyl ester; OPh, phenyl ester, SCm, carboxymethyl thioester; Z, benzyloxycarbonyl.

(8) (a) Grant, N. H.; Allburn, H. E. *Nature* **1966**, *212*, 194. (b) Schuster, M.; Aaviksaar, A.; Jakubke, H.-D. *Tetrahedron* **1990**, *46*, 8093. (c) Schuster, M.; Ullmann, G.; Ullmann, D.; Jakubke, H.-D. *Tetrahedron Lett.* **1993**, *34*, 5701−5702.

products. Reactions using other specific amino acid-containing pentapeptides such as Lys in the case of trypsin, Phe for chymotrypsin, and Asp for BL-GSE confirm these results (cf. Table 1), indicating that the effect of freezing on the course of substrate mimetics-mediated syntheses can be generalized.

Further analysis of Table 1 reveals that freezing not only represses the proteolytic activity of the proteases but also affects the ratio between hydrolysis and aminolysis of the substrate mimetics as indicated by the distinct yields of whole amide products of similar reactions. In detail, the data generally indicate higher acylation rates under frozen-state conditions than at normal room temperature. This effect becomes particularly evident in BL-GSE-catalyzed reactions, while trypsin- and chymotrypsin-mediated syntheses show smaller differences in the acylation rates because of the already high efficiency of these enzymes at normal conditions. Nevertheless, these results indicate that freezing simultaneously represses the hydrolysis of the substrate mimetics as the second important side reaction of enzymatic peptide synthesis which finally favors the formation of the peptide product.

In summary, the results demonstrate that the combination of the substrate mimetics and freezing strategy significantly broadens the scope of proteases for peptide synthesis. This achieves irreversible and efficient peptide bond formation which occurs independently of the primary specificity of proteases and without proteolytic side reactions. These characteristics qualify the approach presented as a powerful concept in the direction of a more universal application of proteases as biocatalysts for the ligation of peptide segments.

Acknowledgment. This work was supported by the Deutsche Forschungsgemeinschaft (Innovationskolleg Chemisches Signal und biologische Antwort) and Fonds der Chemischen Industrie (Liebig Scholarship, F.B.). The authors are grateful to Fluka AG for special chemicals and Mrs. Jacinta Lodge for reading and correcting the manuscript.

Supporting Information Available: Experimental procedures of enzymatic reactions and BL-GSE purification. This material is available free of charge via the Internet at http://pubs.acs.org.

OL005867G

(9) (a)Haensler, M.; Jakubke, H.-D. *Amino Acids* **1996**, *11*, 379−395. (b) Jakubke, H.-D. *J. Chin. Chem. Soc.* **1994**, *41*, 355−370.
(10) Bordusa, F. *J. Med. Bio. Res. Braz.* **2000**, *33*, 469−485.