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Substrate Mimetics-Specific Peptide Ligases: Studies on the Synthetic Utility of a Zymogen and Zymogen-Like Enzymes

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Abstract: Although proteases are capable of synthesizing peptide bonds, they are not proficient at peptide fragment ligation. Further manipulations are needed to shift the native enzyme activity from the cleavage to the synthesis of peptides. This account reports on the synthetic potential of nonactivatable trypsinogen and zymogen-like enzymes designed to minimize proteolytic side reactions during peptide synthesis.

Chemical synthesis is the most powerful method for assembling selectively modified proteins, providing a freedom for protein engineering inaccessible by standard site-directed mutagenesis. Presently, there are several feasible strategies of chemical protein synthesis, each with its own individual advantages and disadvantages. Step-by-step solid-phase synthesis, although reaching a high degree of sophistication, is restricted by the cumulative effects of synthetic inefficiencies. This inevitably results in the accumulation of low-level resin-bound byproducts that usually limits step-by-step synthesis to the preparation of peptides of about 50 amino acids.[1] Convergent synthetic approaches, which apply protected peptide fragments as reactants, allow for several proteins to be assembled, but they have been mainly handicapped by the insolubility of larger protected peptides in solvents required for purification or subsequent ligation.[2] To solve this problem, several chemoselective ligation strategies have been developed enabling unprotected peptides to be coupled. Presently, "native chemical ligation" is the most successful and most commonly used synthetic ligation approach.[3] Because of a mechanism that is based on the reaction of a peptide thioester with an acceptor peptide bearing an *N*-terminal Cys, this strategy, however, is highly limited in the choice of residues at the ligation site. Proteases used as the ligation catalyst tolerate a higher structural diversity at this position, although the substrate specificity of these enzymes primarily narrows the scope of these enzymes for synthesis.[4] Substantial improvements have been attained by the development of substrate mimetics used as donor components that eliminate the specificity problem.[5] Nonspecificity toward the peptide sequence of substrate mimetics is reached by attaching a site-specific ester leaving group at the *C*-terminus of the originally nonspecific peptide moiety. This concept achieves protease-mediated peptide coupling at nonspecific and even artificial ligation sites, regardless of the specificity of the enzyme.[6] Moreover, as nonspecific sequences are coupled, the newly formed peptide bond is stable against secondary cleavage. On the other hand, the risk of undesired cleavages of sensitive peptide bonds by the protease remains a serious limitation of this approach.[7]

This paper reports on the synthetic utility of a nonactivatable zymogen, i.e., mutant trypsinogen K15A, for the substrate mimetics-mediated coupling of specific amino acid-containing peptides. The study has been expanded to a zymogen-like enzyme species (mutant trypsin D194N) with a partly destabilized activation domain. Further optimization of the biocatalyst yielded the mutant trypsins D189S,D194N and K60E,D189S,D194N.

Originally, zymogens were known as catalytically inactive precursors of active enzymes. Activation of the precursor enzyme to the active species is usually a result of limited proteolytic cleavage. In the case of trypsinogen/trypsin conversion, the peptide bond between Lys15 and Ile16 of the zymogen becomes specifically cleaved either by enterokinase or autocatalytically by trypsin itself. Thus, the exchange of Lys15 with Ala protects trypsinogen against activation. Most zymogens have practically no or only negligible proteolytic activity. In contrast, activity vis-à-vis ester bonds, although reduced significantly, could be detected.[8] Decrease in the zymogen's esterase activity was found to be mainly the result of

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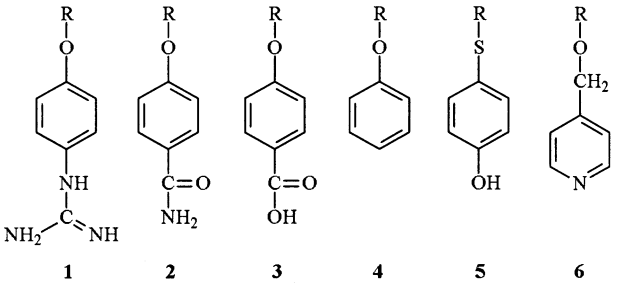
SCHEME 1. Structures of Substrate Mimetics[a]



[a] (**1**) 4-Guanidinophenyl (OGp), (**2**) 4-carboxamidophenyl (OCap), (**3**) 4-carboxyphenyl (OCp), (**4**) Phenyl (OPh), (**5**) 4-hydroxythiophenyl (SPhOH), (**6**) picolyl (OPic), R = Boc-alanyl.

reduced acylation rates, while the rates of deacylation of the zymogen and the enzyme appear to be very similar. Thus, improvements in the acylation rate could make zymogens interesting peptide ligases without any proteolytic activity. This, together with the discovery that substrate mimetics of the 4-guanidinophenyl ester (OGp) type act as highly efficient acylating agents for active trypsin,[5d] prompted us to study the behavior of trypsinogen toward these donor components. Taking into account the differences in the substrate binding sites and, hence, in the specificities of trypsinogen and trypsin,[9] several other substrate mimetics have been included in the study (Scheme 1). The acceptance of the esters by the zymogen has been checked by hydrolysis studies and peptide synthesis reactions as well. Unexpectedly, in neither case could a conversion of the esters be detected, indicating that trypsinogen does not accept substrate mimetics as donor components.

To improve the catalytic power of trypsinogen, we exchanged Asp194 with Asn keeping Lys15 intact. The side chain carboxylate of Asp194 forms originally a salt bridge to the N^{α}-amino moiety of Ile16, which triggers a conformational change in the activation domain and creates the fully active enzyme.[10] Thus, exchange of Asp194 with Asn prevents the formation of the salt bridge leading to a trypsinogen-like enzyme structure. Studies in Hedstrom's laboratory showed that this mutation causes a ~100-fold decrease in the specificity for cleaving specific Arg/Lys-containing peptides, while the specificity for hydrolyzing analogous ester bonds was decreased only by a factor of 10.[11] Hydrolysis studies with the substrate mimetics listed in Scheme 1 revealed a well-defined preference of trypsin D194N toward the OGp ester, while the remaining analogues showed similar but ~10-fold decreased hydrolysis rates (cf. Supporting Information, Figure S1). Compared to wild-type (wt) trypsin, a decrease by a factor of ~60 is evident.[5d] Peptide synthesis reactions using Bz-Gly-OGp as the donor component and Ala-Ala-(Lys/Arg)-Ala-Gly as the acceptor peptides displayed a pronounced cleavage activity toward the specific Lys and Arg moieties, a situation similar to that found for wt-trypsin[7] (cf. Supporting Information, Figure S2).

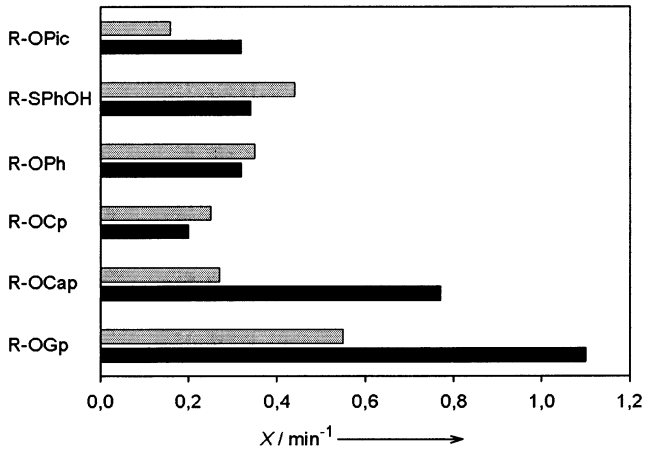


FIGURE 1. Initial rates of hydrolysis of Boc-Ala-OR esters catalyzed by trypsin D189S,D194N (gray) and K60E,D189S,-D194N (black). Conditions: 0.1 M Hepes buffer (pH 8.0), 0.1 M NaCl, 0.01 M $CaCl_2$, 8% (v/v) DMF, [Boc-Ala-OR] = 2 mM, [enzymes] = 1.5×10^{-5} M, X = hydrolysis rate, R = Boc-alanyl.

Consequently, the exchange of Asp194 with Asn, although leading to an active enzyme species, does not shift the activity of trypsin from cleavage to synthesis.

Earlier attempts to repress the proteolytic activity of trypsin led to the enzyme variant D189S.[7c] The mutant was found to have significantly reduced cleavage activity toward trypsin-sensitive bonds, achieving the efficient coupling of OGp esters with both Lys- and Arg-containing peptides. Similar syntheses with Tyr-derived peptides, however, exhibited a remarkable cleavage activity, reflecting the inherent chymotrypsin-like specifity of trypsin D189S for cleaving Tyr-Xaa bonds. In contrast, trypsin D194N does not possess chymotrypsin activity. We expected that the combination of both mutations could result in an enzyme variant with neither trypsin- nor chymotrypsin-like cleavage activity. Initially, the effect of the concurrent D194N and D189S exchange on the acceptance of substrate mimetics was checked by hydrolysis studies using the esters listed in Scheme 1. Accordingly, the additional D189S mutation, although reducing the preference for the OGp ester, did not disturb the activity of the enzyme toward substrate mimetics (Figure 1). The small differences in the rate of reaction indicate a broad specificity of trypsin D189S,D194N, a behavior that was also found for trypsin D189S.[7c] The synthetic utility of the double mutant trypsin was finally investigated by coupling of Arg/Lys- and Phe/Tyr-containing peptides with Bz-Gly-OGp as the donor (Figure 2a–d). Remarkably, as illustrated by the syntheses timelines, the mutant enzyme catalyzes exclusively the coupling of the substrate mimetic with the appropriate peptides, remaining free of practically any proteolytic side reactions. Only a small amount of peptide cleavage can be detected after longer incubation times after completion of syntheses. Obviously, the combination of the two mutations leads to a combination of the behavior of the appropriate single enzyme variants, resulting in an enzyme species with reduced trypsin- and chymotrypsin-like cleavage activity as well. From a synthetic point of view, this offers practical, irreversible peptide bond formation without the risk of undesired proteolytic reactions at primarily specific cleavage sites within the

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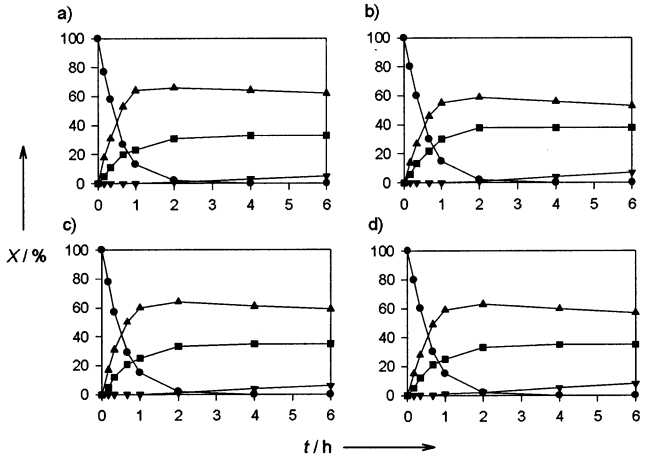

FIGURE 2. Course of the trypsin mutant D189S,D194N-catalyzed coupling of Bz-Gly-OGp with Ala-Ala-*Xaa*-Ala-Gly. *Xaa*: (a) Lys, (b) Arg, (c) Phe, (d) Tyr. (●) Bz-Gly-OGp, (■) Bz-Gly-OH, (▼) Bz-Gly-Ala-Ala-*Xaa*-OH, (▲) Bz-Gly-Ala-Ala-*Xaa*-Ala-Gly-OH. Conditions: 0.1 M Hepes buffer (pH 8.0), 0.1 M NaCl, 0.01 M $CaCl_2$, 8% (v/v) DMF, [Bz-Gly-OGp] = 2 mM, [Ala-Ala-*Xaa*-Ala-Gly] = 15 mM, [enzyme] = 1.5×10^{-4} M, *X* = product yield.

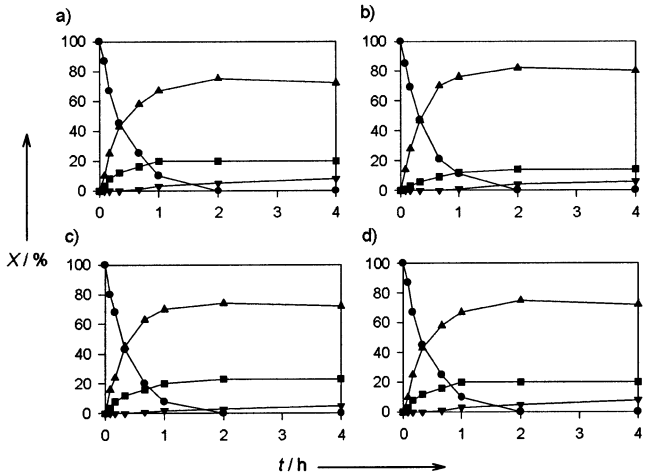

FIGURE 3. Course of the trypsin mutant K60E,D189S,-D194N-catalyzed coupling of Bz-Gly-OGp with Ala-Ala-*Xaa*-Ala-Gly. *Xaa:* (a) Lys, (b) Arg, (c) Phe, (d) Tyr. (●) Bz-Gly-OGp, (■) Bz-Gly-OH, (▼) Bz-Gly-Ala-Ala-*Xaa*-OH, (▲) Bz-Gly-Ala-Ala-*Xaa*-Ala-Gly-OH. Conditions: 0.1 M Hepes buffer (pH 8.0), 0.1 M NaCl, 0.01 M $CaCl_2$, 8% (v/v) DMF, [Bz-Gly-OGp] = 2 mM, [Ala-Ala-*Xaa*-Ala-Gly] = 15 mM, [enzyme] = 6.5×10^{-5} M, *X* = product yield.

peptide reactants or products. This qualifies mutant trypsin D189S,D194N as a useful and most suitable biocatalyst for substrate mimetics-mediated ligation of specific peptides. Critically, it must be noted, however, that for the most specific OGp esters the combination of the two mutations decreased the rate of reaction to about 10% of that of trypsin D189S and to 5% compared to the mutant D194N, which finally corresponds to a decrease in ligase activity of nearly 3 orders of magnitude when wt-trypsin is taken as the standard. Further efforts to optimize the enzyme yielded an increase in the activity of the catalyst. Preliminary results of our own studies suggest that the exchange of Lys60 with Glu may lead to a general enhancement of the catalytic power, especially for mutants lacking the native Asp189 moiety. Although the molecular basis for this behavior is completely unknown, we expected the triple mutant K60E,-D189S,D194N to be an interesting candidate. Hydrolysis studies with the esters listed in Scheme 1 showed 2-fold accelerated rates of reaction, confirming the rate increasing effect of the K60E mutation (cf. Figure 1). Comparison with the data found for the parent trypsin mutant D189S,D194N revealed that this effect appears to be specific for the OGp, OPic, and OCap ester, while the activity vis-à-vis the other esters remains practically unaffected. Because the highest reaction rate was found for the OGp ester, we also used Bz-Gly-OGp as the donor component for the synthesis reactions. The time courses of syntheses are illustrated in Figure 3a−d. Despite using less than half the amount of enzyme, the reactions proceeded with similar rates compared to those catalyzed by trypsin D189S,D194N. The degree of proteolytic side reactions does not increase significantly. Interestingly, apart from these effects, the exchange of Lys60 with Glu also reduced the rate of competing hydrolysis of the donor component. While the use of trypsin D189S,D194N leads to the formation of ∼30% of Bz-Gly-OH (cf. Figure 2), only ∼20% of hydrolysis product is formed when the triple mutant is used as the catalyst. This resulted in about 80% yield of the desired peptide products under these nonoptimized conditions.

Finally, the synthetic scope of the triple mutant was initially investigated by variation of the sequence and

TABLE 1. Yields of Intact Hexa- and Octapeptide Products Synthesized by Trypsin Mutant K60E,D189S,D194N-Catalyzed Coupling of Substrate Mimetics with Specific Amino Acid-Containing Peptides[a]

acyl donor	acyl acceptor	product	% yield
Bz-Leu-OGp	Ala-Ala-*Lys*-Ala-Gly	Bz-Leu-Ala-Ala-*Lys*-Ala-Gly	55.9
Bz-Leu-OGp	Ala-Ala-*Arg*-Ala-Gly	Bz-Leu-Ala-Ala-*Arg*-Ala-Gly	56.1
Bz-Leu-OGp	Ala-Ala-*Phe*-Ala-Gly	Bz-Leu-Ala-Ala-*Phe*-Ala-Gly	61.5
Bz-Leu-OGp	Ala-Ala-*Tyr*-Ala-Gly	Bz-Leu-Ala-Ala-*Tyr*-Ala-Gly	60.2
Bz-Phe-OGp	Ala-Ala-*Lys*-Ala-Gly	Bz-Phe-Ala-Ala-*Lys*-Ala-Gly	75.1
Bz-Phe-OGp	Ala-Ala-*Arg*-Ala-Gly	Bz-Phe-Ala-Ala-*Arg*-Ala-Gly	75.7
Bz-Phe-OGp	Ala-Ala-*Phe*-Ala-Gly	Bz-Phe-Ala-Ala-*Phe*-Ala-Gly	69.3
Bz-Phe-OGp	Ala-Ala-*Tyr*-Ala-Gly	Bz-Phe-Ala-Ala-*Tyr*-Ala-Gly	67.6
Boc-Phe-Gly-Gly-OGp	Ala-Ala-*Lys*-Ala-Gly	Boc-Phe-Gly-Gly-Ala-Ala-*Lys*-Ala-Gly	77.8
Boc-Phe-Gly-Gly-OGp	Ala-Ala-*Arg*-Ala-Gly	Boc-Phe-Gly-Gly-Ala-Ala-*Arg*-Ala-Gly	85.3
Boc-Phe-Gly-Gly-OGp	Ala-Ala-*Phe*-Ala-Gly	Boc-Phe-Gly-Gly-Ala-Ala-*Phe*-Ala-Gly	81.9
Boc-Phe-Gly-Gly-OGp	Ala-Ala-*Tyr*-Ala-Gly	Boc-Phe-Gly-Gly-Ala-Ala-*Tyr*-Ala-Gly	83.7

[a] 0.1 M Hepes buffer (pH 8.0), 0.1 M NaCl, 0.01 M $CaCl_2$, 8% (v/v) DMF, [Bz-Gly-OGp] = 2 mM, [Ala-Ala-*Xaa*-Ala-Gly] = 15 mM, [enzyme] = 6.5×10^{-5} M.

length of the acyl donor moiety. The results obtained for reactions with Bz-Leu-OGp, Bz-Phe-OGp, and Boc-Phe-Gly-Gly-OGp are summarized in Table 1. As already found for the coupling of Bz-Gly-OGp, a strong preference of the enzyme for catalyzing the synthesis reactions became evident. In fact, from the once formed peptide products more than 95% could be identified as intact and noncleaved peptide species. Accordingly, the degree of proteolytic side reactions accounts for less than 5%, indicating that the synthetic utility of the enzyme is neither limited to the coupling of *C*-terminal glycine nor acyl donor components containing only a single amino acid moiety. Differences in product yields of about 30% caused by the individual *C*-terminal amino acid moiety and of less than 10% caused by the acyl acceptors used are not typical for the triple mutant but were also found in previous studies for reactions catalyzed by wild type and other mutant trypsins.[5d,7b]

In summary, these findings imply that rational protein engineering starting from zymogen-like enzymes can lead to novel and powerful biocatalysts for peptide synthesis. While trypsinogen itself appears to be inactive for substrate mimetics-mediated peptide synthesis, the zymogen-like trypsin mutant D194N catalyzes the formation of peptide bonds only ~60-fold less efficiently than the wt-trypsin. However, this mutation alone does not repress the degree of proteolytic side reactions vis-à-vis trypsin-specific Lys- and Arg-containing peptides significantly. Only in combination with additional mutations in the active site of the enzyme by exchanging Asp189 with Ser is the proteolytic activity of the enzyme reduced, achieving irreversible peptide synthesis practically without undesired proteolytic reactions. This also holds true for the coupling of lysine-containing peptides, which remained as cleavage-sensitive reactants for our recently reported trypsin D189E ligase.[7b] The accompanying decrease of the total enzyme activity can be partly compensated for by the exchange of Lys60 with Glu, but remains a challenging task. Furthermore, this mutation minimizes the competing hydrolysis of the substrate mimetics as the second important side reaction of enzymatic peptide ligation. These characteristics qualify trypsin mutant K60E,D189S,D194N as a useful and most suitable biocatalyst for substrate mimetics-mediated ligation of specific amino acid-containing peptides. This designed biocatalyst should considerably extend the scope of this semisynthetic ligation approach, especially in combination with the solid-phase peptide synthesis of longer peptide esters in the form of substrate mimetics.[12] Studies in this direction are presently underway, paralleled by further attempts to increase the ligation rates of the biocatalyst.

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

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