Enzymatic Coupling of Specific Peptides at Nonspecific Ligation Sites: Effect of Asp189Glu Mutation in Trypsin on Substrate Mimetic-Mediated Reactions†

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Two main drawbacks seriously restrict the synthetic value of proteases as reagents in peptide fragment coupling: (i) native proteolytic activity and, thus, risk of undesired peptide cleavage; (ii) limited enzyme specificities restricting the amino acid residues between which a peptide bond can be formed. While the latter can be overcome by the use of substrate mimetics achieving peptide bond formation at nonspecific ligation sites, the risk of proteolytic cleavage still remains and hinders the wide acceptance of this powerful strategy for peptide coupling. This paper reports on the effect of the trypsin point mutant Asp189Glu on substrate mimetic-mediated reactions. The effect of this mutation on the steady-state hydrolysis of substrate mimetics of the 4-guanidinophenyl ester type and on trypsin-specific Lys- and Arg-containing peptides was investigated. The results were confirmed by enzymatic coupling reactions using substrate mimetics as the acyl donor and specific amino acid-containing peptides as the acyl acceptor. The competition assay verifies the predicted shift in substrate preference from Lys and Arg to the substrate mimetics and, thus, from cleavage to synthesis of peptide bonds. The combination of results obtained qualifies the trypsin mutant D189E as the first substrate mimetic-specific peptide ligase.

Solution- and especially solid-phase synthesis strategies have reached a high degree of sophistication and are usually the methods of choice for synthesizing peptides up to oligopeptide level. The stepwise coupling of more than 50 or 60 amino acids, however, often suffers from serious problems mainly due to the accumulation of low-level resin-bound byproducts.[1] Generally, the coupling of protected peptide fragments can be used to provide access to larger peptides. However, the solubility of fully protected peptides in solvents required for synthesis is frequently low and, thus, seriously limits the synthetic utility of this coupling method. Presently, the "native chemical ligation" is one of the most useful strategy for the synthesis of larger peptides and proteins.[2] Because of the mechanism, that involves the reaction between an N-terminus Cys residue of the amino fragment and a C-terminal thioester of the carboxy fragment, this ligation strategy occurs selectively and does not require temporary protection of trifunctional amino acid side chains. With these advantages, however, come the disadvantage that only Xaa-Cys bonds can be coupled while peptides with sequences devoid of suitably spaced Cys residues are generally not targets of this technique.

Enzymatic methods based on proteases combined with the solid-phase peptide synthesis of fragments are attractive alternatives because enzymatic reactions are regio- and stereospecific, free from racemization, and only require minimal protective group chemistry.[3] While the general function of this semisynthetic strategy could be already demonstrated, the classical enzymatic approach requires very careful planning and optimization of reaction conditions in respect to limited enzyme specificities and unwanted proteolytic side reactions which may occur. The concept of substrate mimetics is a powerful strategy to overcome this limitation achieving protease-mediated peptide coupling at nonspecific and even artificial ligation sites regardless of the primary specificity of the enzyme.[4] Moreover, as nonspecific sequences are coupled, the newly formed peptide bond is not subject to secondary

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† Abbreviations: Boc, *tert*-butyloxycarbonyl; Bz, benzoyl; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid); Mops, 3-(*N*-morpholino)propanesulfonic acid; OGp, 4-guanidinophenyl ester; SBTI, soy bean trypsin inhibitor; Z, benzyloxycarbonyl.

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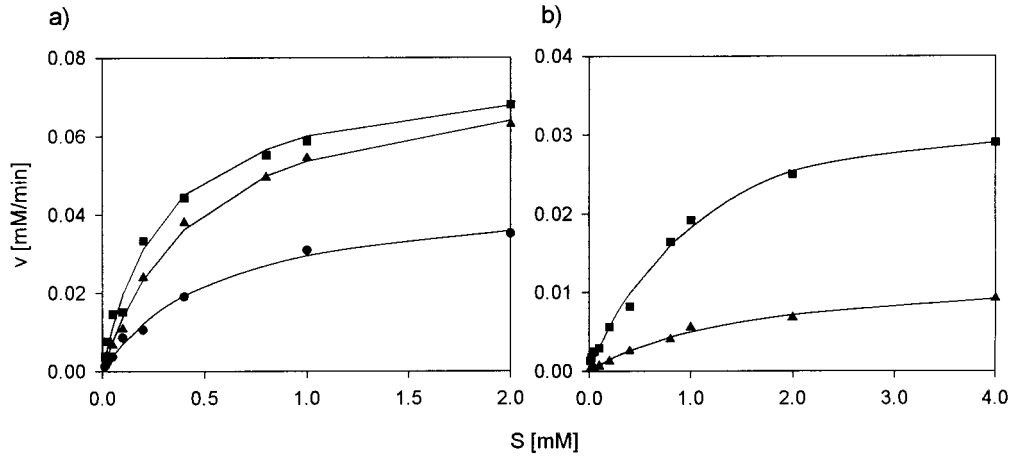

Figure 1. Plots of the initial rates of the mutant trypsin D189E-catalyzed hydrolysis of Boc-L/D-Xaa-OGp esters. (a) Boc-L-Xaa-OGp. (b) Boc-D-Xaa-OGp. (-●-) Xaa: Gly, (-■-) Xaa: L/D-Leu, (-▲-) Xaa: L/D-Ala. Conditions: 0.025 M Mops buffer, pH 7.6, 0.1 M NaCl, 5 mM $CaCl_2$, 25 °C, 20% methanol, [enzyme]: $3.1 \times 10^{-6} - 6.2 \times 10^{-7}$ M, [substrate]: 0.006−4.0 mM.

cleavage. However, due to the use of native proteases as the catalyst, the synthetic utility of this approach is limited to reactions with nonspecific amino acid-containing peptide fragments while the coupling of fragments bearing specific ones usually leads to undesirable cleavages.[5] Consequently, the broad use of the substrate mimetic-based semisynthetic approach to peptide ligation requires further efforts to minimize the native amidase activity of proteases responsible for unwanted peptide cleavages.

This paper reports on the effect of the exchange of Asp 189 of trypsin (EC 3.4.21.4) with Glu on substrate mimetic-mediated coupling of specific amino acid-containing peptide fragments. Asp 189, positioned at the base of the S_1 binding pocket, was found to be largely responsible for the high activity of trypsin for cleaving arginyl- and lysyl-peptide bonds.[6] The additional methylene group separating the D189E carboxylate from the protein backbone generally decreases the activity of trypsin toward Arg and Lys while reactions with Arg, which has the longer side chain, are more severely affected.[7] The effect of this mutation on the specificity of 4-guanidinophenyl esters (OGp)—the most specific substrate mimetic for trypsin—was investigated by hydrolysis kinetic studies using esters of the general structure Boc-L/D-Xaa-OGp. Owing to the increase in relative substrate mimetic specificity, model reactions were performed using specific amino acid-containing peptides as the acyl acceptor and various Bz-L-Xaa-OGp esters as the acyl donor.

Results and Discussion

Hydrolysis Studies. The extent of proteolytic side reactions during enzymatic peptide ligations can be estimated from the relative specificity of the protease toward the acyl donor ester on the one side and the specific amino acid-containing peptide on the other. Generally, accumulation of intact peptide product requires an enhanced specificity toward the acyl donor while the amidase activity for cleaving peptide bonds should be reduced. *Wt*-trypsin-catalyzed hydrolysis studies indicate, however, a similar or even lower specificity of Boc-L-*Xaa*-OGp (*Xaa* = Gly, Ala, Leu, Gln) substrate mimetics compared to specific Lys- and Arg-containing peptides (data in the supplement). Thus, enzymatic coupling reactions of acyl-4-guanidinophenyl esters with specific amino acid-containing peptide fragments using *wt*-trypsin as the catalyst should be accompanied by simultaneous peptide cleavages. The one to 2 orders of magnitude decreased specificity of Boc-D-*Xaa*-OGp (*Xaa* = D-Ala, D-Leu, D-Gln) indicates that the coupling of D-amino acid-derived substrate mimetics should exclusively lead to the cleavage of the higher specific peptide reactants making the formation of intact peptide products unlikely.

It is already known that Asp 189 of trypsin is extremely sensitive for high-level catalysis. Exchange of Asp 189 with most other amino acids generally leads to a drastic decrease up to a complete loss of enzyme activity.[6] On the contrary, the replacement for Asp with Glu affects the activity of the enzyme to a lesser extent while the decrease in relative Arg specificity is more pronounced than that toward Lys.[7] The effect of this mutation on the acceptance of substrate mimetics was investigated by analogous hydrolysis kinetic studies. Plots of the initial rates of hydrolysis for some substrate mimetics are shown in Figure 1. Generally, all plots show the typical Michealis−Menten kinetics without evidence of substrate inhibition or activation indicating that the exchange of Asp 189 with Glu does not disturb the activity of trypsin toward the substrate mimetics. The resulting kinetic data are summarized in Table 1 and verify that all substrate mimetics with L- and even those with D-amino acids are specifically accepted by the trypsin mutant. However, analyzing the specificity constants k_{cat}/K_M, the additional methylene group of trypsin D189E leads to a decrease in specificity by one to 2 orders of magnitude compared to the *wt*-enzyme. This effect can be mainly addressed to a decrease of the corresponding k_{cat}-values while the values for K_M are affected to a lesser extend. Assuming the deacylation to be the rate-limiting

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Table 1. Steady-state Kinetic Parameters for the Hydrolysis of Boc-L/D-Xaa-OGp and Lys- and Arg-containing Peptides by Mutant Trypsin D189E[a]

substrate	K_M [mM]	k_{cat} [s^{-1}]	k_{cat}/K_M [M^{-1} s^{-1}]
Boc-Gly-OGp	0.541	1.23	2.3×10^3
Boc-L-Ala-OGp	0.472	2.13	4.5×10^3
Boc-L-Gln-OGp	0.522	2.21	4.2×10^3
Boc-L-Leu-OGp	0.290	2.07	7.1×10^3
Boc-D-Ala-OGp	1.56	0.067	4.3×10^1
Boc-D-Gln-OGp	1.37	0.110	8.0×10^1
Boc-D-Leu-OGp	1.31	0.227	1.7×10^2
Bz-Ala-Ala-Lys-Ala-Gly-OH	4.85	5.98	1.2×10^3
Bz-Ala-Ala-Arg-Ala-Gly-OH	4.38	1.52	3.0×10^2

[a] Conditions: 0.025 M Mops buffer, pH 7.6, 0.1 M NaCl, 5 mM $CaCl_2$, 25 °C, 20% methanol, [enzyme]: $3.1 \times 10^{-6} - 6.2 \times 10^{-7}$ M, [substrate]: 0.006−4.0 mM. All errors are less than 15%.

step of hydrolysis of substrate mimetics as known for the *wt*-enzyme,[4b,d] this fact indicates a similar binding and acylation rate while the deacylation may be hindered by Glu 189. According to *wt*-trypsin,[4d] D-amino acid-derived substrate mimetics were found to be less specific by one to 2 orders of magnitude compared to the L-counterparts. Interestingly, in the case of the D-amino acid derivatives the effect of the lowering of specificity results from both an increase in K_M and a decrease in k_{cat}. The highest effect of mutation on the specificity, however, was found for the Arg-containing peptide. According to the data reported in the literature,[7] the mutation D189E decreases the k_{cat}/K_M-value for the Arg substrate by about 3 orders of magnitude. Since the specificity constant for the Lys-derived peptide is only decreased by a factor of about 100, mutant trypsin D189E is 4-fold more specific for lysyl- than for cleaving analogous arginyl-bonds. Most importantly, the specificity constants of both the Lys- and Arg-peptides are lower than those found for the L-amino acid-derived substrate mimetics. Thus, the rate of proteolytic side reactions during trypsin D189E-catalyzed coupling of specific amino acid-containing peptides should be equally decreased compared to that with the parent enzyme. In the case of the D-configured counterparts, the hydrolysis of the specific peptides still leads to about 2- to 28-fold higher k_{cat}/K_M-values, indicating that the usefulness of this trypsin variant for coupling of specific amino acid-containing peptides should be restricted to L-amino acid-derived substrate mimetics.

Enzymatic Syntheses. The synthetic utility of trypsin D189E to couple acyl-4-guanidinophenyl esters and specific amino acid-containing peptide fragments was investigated by a competition assay using Bz-L-*Xaa*-OGp esters (*Xaa* = Gly, Ala, Leu, Gln) as the acyl donor and pentapeptides of the general structure Ala-Ala-*Xbb*-Ala-Gly (*Xbb* = Lys, Arg, Phe, Tyr) as the acyl acceptor. The use of Bz-protected acyl donors rather than the Boc-protected counterparts was favored because of the higher absorption coefficient of the benzoyl-group allowing a more sensitive HPLC analysis of the synthesis reactions. Due to the lower specificity constants relative to those found for the lysyl- and arginyl-peptides, the analogous D-configured esters were excluded from this study. As a point of reference reactions with *wt*-trypsin as the catalyst were performed. To control for spontaneous hydrolysis and aminolysis of the acyl donor esters, the reactions were analyzed without enzyme. On the basis of these experiments, nonenzymatic aminolysis could be ruled out and the extent of spontaneous hydrolysis was found to be less than 5%. The courses of the *wt*- and mutant trypsin-catalyzed coupling of Bz-Gly-OGp with the specific peptides Ala-Ala-*Lys*-Ala-Gly and Ala-Ala-*Arg*-Ala-Gly are illustrated in Figures 2a−d. On analyzing the reactions catalyzed by the *wt*-enzyme, in both cases the enzymatically formed peptide products are rapidly degraded due to proteolysis of the appropriate sensitive bonds within the nucleophilic peptides, as was predicted by the hydrolysis kinetic studies. If applying an exact time control of synthesis reaction, intact Bz-Gly-Ala-Ala-*Lys*-Ala-Gly-OH can still be obtained with a yield of about 50%, the maximum yield of the higher specific Arg-containing product was more strongly reduced from about 80% to less than 25%. The time-courses of syntheses revealed in both cases that the cleavage of the peptide products occurs simultaneously with the coupling reaction and, thus, cannot be avoided by a simple time control of synthesis. From a synthetic point of view, the result clearly demonstrates the limitation of the substrate mimetic-mediated synthesis approach using *wt*-trypsin as the catalyst. Contrary to this, trypsin D189E was much better suited for the coupling of Lys- and Arg-containing peptides. As illustrated by the Figures 2c and 2d, yields of about 65% of both specific amino acid-containing peptide products could be obtained almost independently of the individual specific amino acid moiety. The distinct specificities of trypsin D189E for Lys and Arg (cf. Table 1) only affect the stability of the peptide products. Due to higher specificity, the Lys-derived product is subject to a somewhat faster cleavage. Nevertheless, the significant lower rates of product degradation drastically reduces the risk of undesired proteolytic side reactions.

Considering the increase of chymotrypsin-like activity of most trypsin variants lacking Asp 189,[6c,8] analogous model reactions using the Phe- and Tyr-containing peptides Ala-Ala-*Phe*-Ala-Gly and Ala-Ala-*Tyr*-Ala-Gly were performed. In both cases there is no evidence of any proteolytic cleavage of the Phe- as well as Tyr-peptide bond by the mutant trypsin (data in the supplement). Furthermore, a comparison of the product yields found for reactions catalyzed by the mutant enzyme with those obtained for the *wt*-enzyme does not show significant differences. Hence, the time-course of synthesis as well as the efficiency of the coupling of chymotrypsin-specific peptide fragments seem to be practically unaffected by the exchange of Asp 189 with Glu.

The synthetic utility of mutant trypsin D189E was further investigated by variation of the C-terminal amino acid moiety of the acyl donor component. The results observed for reactions with Bz-L-Leu-OGp, Bz-L-Ala-OGp, and Bz-L-Gln-OGp are summarized in Table 2. As already found for the coupling of Bz-Gly-OGp, only reactions using the Lys- and Arg-containing peptides lead to competitive product cleavages. Since the individual amino acid residue at position 3 of the nucleophilic pentapeptides did not affect the ratio between the formation of Bz-*Xaa*-OH and Bz-*Xaa*-NHR (R = pentapeptides and partly cleaved peptides), the differences in the product yields between the Phe/Tyr- and Lys/Arg-containing peptides reflect the extent of competitive cleavages of the Lys/Arg-derived products. Thus, in case of the most specific Bz-L-Leu-OGp the relative proportion of partly

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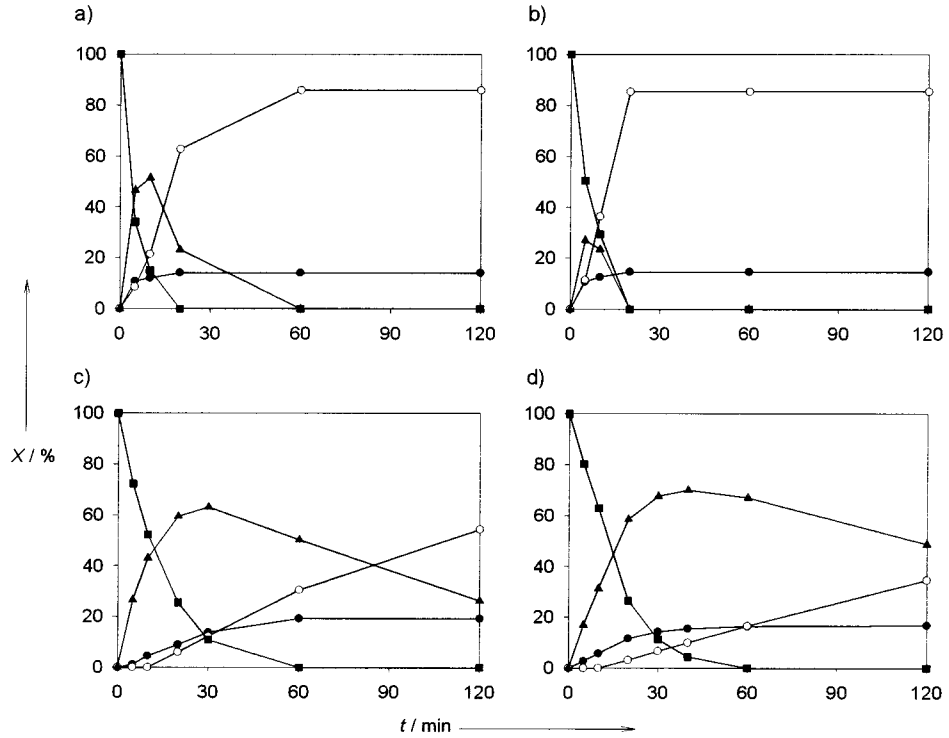

Figure 2. Time-course of the *wt*- and mutant trypsin D189E-catalyzed coupling of Bz-Gly-OGp with Lys- and Arg-containing peptides. (a) Bz-Gly-OGp and Ala-Ala-*Lys*-Ala-Gly using *wt*-trypsin. (b) Bz-Gly-OGp and Ala-Ala-*Arg*-Ala-Gly using *wt*-trypsin. (c) Bz-Gly-OGp and Ala-Ala-*Lys*-Ala-Gly using trypsin D189E. (d) Bz-Gly-OGp and Ala-Ala-*Arg*-Ala-Gly using trypsin D189E. (-■-) Bz-Gly-OGp, (-▲-) Bz-Gly-Ala-Ala-(*Lys*/*Arg*)-Ala-Gly-OH, (-○-) Bz-Gly-Ala-Ala-(*Lys*/*Arg*)-OH, (-●-) Bz-Gly-OH. Conditions: 0.2 M Hepes buffer, pH 8.0, 0.2 M NaCl, 0.02 M $CaCl_2$, 25 °C, 10% methanol, [Bz-Gly-OGp]: 2 mM, [Ala-Ala-(*Lys*/*Arg*)-Ala-Gly]: 15 mM, [*wt*-trypsin]: 1.0×10^{-7} M, [trypsin D189E]: 3.1×10^{-6} M, X = product yield.

Table 2. Yields of Intact Hexapeptide Products Synthesized by Trypsin D189E-Catalyzed Coupling of Bz-L-Xaa-OGp 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-OH	59.2
Bz-Leu-OGp	Ala-Ala-*Arg*-Ala-Gly	Bz-Leu-Ala-Ala-*Arg*-Ala-Gly-OH	68.0
Bz-Leu-OGp	Ala-Ala-*Phe*-Ala-Gly	Bz-Leu-Ala-Ala-*Phe*-Ala-Gly-OH	79.3
Bz-Leu-OGp	Ala-Ala-*Tyr*-Ala-Gly	Bz-Leu-Ala-Ala-*Tyr*-Ala-Gly-OH	81.7
Bz-Gln-OGp	Ala-Ala-*Lys*-Ala-Gly	Bz-Gln-Ala-Ala-*Lys*-Ala-Gly-OH	64.7
Bz-Gln-OGp	Ala-Ala-*Arg*-Ala-Gly	Bz-Gln-Ala-Ala-*Arg*-Ala-Gly-OH	69.1
Bz-Gln-OGp	Ala-Ala-*Phe*-Ala-Gly	Bz-Gln-Ala-Ala-*Phe*-Ala-Gly-OH	80.9
Bz-Gln-OGp	Ala-Ala-*Tyr*-Ala-Gly	Bz-Gln-Ala-Ala-*Tyr*-Ala-Gly-OH	82.0
Bz-Ala-OGp	Ala-Ala-*Lys*-Ala-Gly	Bz-Ala-Ala-Ala-*Lys*-Ala-Gly-OH	48.9
Bz-Ala-OGp	Ala-Ala-*Arg*-Ala-Gly	Bz-Ala-Ala-Ala-*Arg*-Ala-Gly-OH	45.7
Bz-Ala-OGp	Ala-Ala-*Phe*-Ala-Gly	Bz-Ala-Ala-Ala-*Phe*-Ala-Gly-OH	58.1
Bz-Ala-OGp	Ala-Ala-*Tyr*-Ala-Gly	Bz-Ala-Ala-Ala-*Tyr*-Ala-Gly-OH	59.9

[a] Conditions: 0.2 M Hepes buffer, pH 8.0, 0.2 M NaCl, 0.02 M $CaCl_2$, 25 °C, 10% methanol; [acyl donor]: 2 mM, [acyl acceptor]: 15 mM, [enzyme]: 3.1×10^{-6} M. All errors are less than 5%.

degraded products of the whole Lys- and Arg-peptide products was about 21% and 16%, respectively. With on the average 22% and 16% practically the same values were also found for reactions using the lesser specific Bz-L-Ala-OGp and Bz-L-Gln-OGp. This finding indicates that the extent of proteolytic side reactions appear to be nearly independent of the individual C-terminal L-amino acid residue. The lower absolute yields observed for Bz-L-Ala-OGp are not typical for trypsin D189E, but were also found in previous studies for reactions catalyzed by *wt*-trypsin.[4d] According to this, a C-terminal Ala-residue within the substrate mimetics frequently leads to lower coupling yields while in most other cases the individual sequence of the acyl donor does not significantly affect the efficiency of synthesis. The very similar yields found for Bz-Gly-OGp, Bz-L-Gln-OGp, and Bz-L-Leu-OGp indicate that this behavior should also hold for trypsin D189E-catalyzed reactions.

In summary, these findings imply that mutant trypsin D189E can be used as an efficient biocatalyst for coupling specific amino acid-containing peptides with a wide variety of different nonspecific acyl components under mild reaction conditions in aqueous systems. Especially in combination with the solid-phase peptide synthesis of longer peptide esters in form of substrate mimetics[9] this designed biocatalyst should considerably extend the scope of this semisynthetic strategy for the ligation of longer peptide fragments. Studies in this direction are presently

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performed which may additionally reinforce the efforts for a further optimization of the biocatalyst.

Conclusion

Although, substrate mimetics considerably extend the synthetic scope of proteases by mediating the acceptance of nonspecific acyl moieties, the use of this beneficial strategy is limited to the coupling of peptides lacking enzyme specific amino acid residues. The presence of site-specific amino acid moieties, however, inevitably leads to proteolytic side reactions. Moreover, in the case of *wt*-trypsin used for the coupling of an Arg-containing peptide, cleavage behind the specific Arg are even favored over its coupling with the substrate mimetics. Consequently, the universal use of the substrate mimetics approach to peptide ligation requires further efforts that lead to a shift in enzyme activity from the cleavage to the synthesis reaction. Our results demonstrate the usefulness of site-directed mutagenesis to design trypsin species with an enhanced specificity for artificial substrate mimetics. Although the exchange of Asp 189 with Glu reduces the absolute enzyme specificity, the relative specificity of trypsin D189E toward acyl-4-guanidinophenyl esters is increased when compared to that of lysyl- and arginyl-bonds. Thus, compared to the *wt*-enzyme the substrate preference of the mutant trypsin is shifted from Lys and Arg to the substrate mimetics and, therefore, from the cleavage to the synthesis of peptide bonds. Similar studies using 4-guanidinophenyl esters of D-amino acid derivatives reveal, however, that this shift in specificity should be restricted to the higher specific L-counterparts. Enzymatic coupling reactions using various Bz-L-*Xaa*-OGp esters and peptides bearing trypsin- and chymotrypsin-sensitive bonds demonstrated the usefulness of trypsin D189E for peptide synthesis. While the proportions of intact peptide product of the whole peptide product in *wt*-trypsin-catalyzed coupling of Arg- and Lys-containing peptides were only about 27% and 62%, in analogous mutant trypsin-mediated syntheses relative proportions of noncleaved peptide products of 84% and 79% were found. Similar to the *wt*-enzyme, trypsin D189E also appears to be practically inactive toward chymotrypsin-sensitive peptides. In the same way there was no evidence for an influence of the individual C-terminal L-amino acid moiety of the substrate mimetics on the extent of undesired cleavage qualifying trypsin D189E as a useful and up to now only enzyme capable of the substrate mimetic-mediated ligation of specific peptides. However, to make full practical use of this approach further enzyme optimization will be required to accomplish a better differentiation between the specificity constants for the substrate mimetics and for specific peptide bonds.

Experimental Section

Materials. Enzymes for DNA manipulation came from Boehringer except *Pfu* DNA polymerase being purchased from Promega. The oligonucleotide primers were obtained from MWG-Biotech. Plasmid DNA was isolated with the High Pure Plasmid Isolation Kit (Boehringer). The QiaQuick Gel Extraction Kit (Qiagen) was employed for purification of DNA fragments from agarose gels. Amino acid derivatives, 4-aminophenol, coupling reagents, benzyl chloroformate, *S*-methylisothiourea, and 4-toluenesulfonic acid were products of Bachem (Switzerland), Fluka and Merk (Germany), respectively. All reagents were of the highest commercial purity. Solvents were purified and dried by the usual methods.

Construction of Mutant Trypsin. Recombinant rat trypsinogen II was prepared from *E. coli* vector pST.[10] This plasmid codes for the *wt*-protein from *Rattus norvegicus* fused to ADH/GAPDH promoter and α factor leader sequence. Site-directed mutagenesis was performed by thermocycling in a mixture of 50 μL contained approximately 50 ng of pST, 125 ng of the appropriate primer, 1.3 units of *Pfu* DNA polymerase as well as 20 mM of each desoxynucleotide and the reaction buffer supplied with the enzyme (coupling parameters: cycle 1, 95 °C, 30 s; cycle 2−17, 95 °C, 30 s (denaturation), 55 °C, 1 min (annealing), 68 °C, 12 min (extension). The oligonucleotides GGA GGC AAG GAG TCC TGC CAG, CTG GCA GGA CTC CTT GCC TCC were used as the primers. After removing original pST template DNA by incubation with 10 units of *Dpn*I, the PCR product was transformed into *E. coli* XL2 blue ultracompetent cells. The isolated plasmid was then verified by sequence analysis. The processed trypsinogen construct was excised using *Bam*HI and *Sal*I, purified from an agarose gel and ligated into the similarly restricted and purified yeast/*E. coli* vector pYT.[10] Following a further amplification in *E. coli*, the EZ transformation kit (Zymo-Research, Orange, CA) was employed to transfer the pYT variant to *Saccharomyces cerevisiae* (DLMα101). Analogously, *wt*-trypsin was prepared using the original pST vector.

Expression and Purification of Trypsin D189E. *S. cerevisiae* transformants were selected and amplified on uracil- and subsequently leucin-deficient SC media. 20 mL cultures (Leu-deficient, 2 d incubation) were used to inoculate 1.3 l YPD medium (10 g L^{-1} yeast extract, 20 g L^{-1} peptone, 15 g L^{-1} glucose). After 4 d incubation, recombinant trypsinogen was isolated from the supernatant by cation exchange chromatography on a Toyopearl SP 650M column (Toso Haas). The mature trypsin was obtained by treatment with trypsin-free enteropeptidase (Biozyme) and subjected to affinity chromatography on SBTI agarose. Finally, after dialysis, the protein was concentrated and stored in 1 mM HCl at 4 °C.

Chemical Syntheses. Boc-Xaa-4-guanidinophenyl esters were prepared according to our previously described procedure by condensation of Boc-Xaa-OH and 4-[*N*′,*N*″-bis(Z)-guanidino]-phenol.[4d] The benzoyl-protected esters were synthesized starting with the deprotection of the N^{α}-amino group with trifluoroacetic acid and subsequent benzoylation using benzoyl chloride. Bz-Gly-OGp was prepared by direct condensation of Bz-Gly-OH with 4-[*N*′,*N*″-bis(Z)-guanidino]phenol. A final catalytic hydrogenation of the bis(Z)-protected esters resulted in the 4-*N*′,*N*″-deprotected 4-guanidinophenyl esters. Pentapeptides were synthesized with a semiautomatic batch peptide synthesizer SP 650 (Labortech AG, Switzerland) using *p*-alkoxybenzyl alcohol resin synthesized according to Wang[11] and standard Fmoc chemistry. The peptides were precipitated with dry diethyl- or diisopropyl ether or mixtures of hexane/ethyl acetate. The identity and purity of all peptides and esters were verified by analytical HPLC, NMR, thermospray mass spectroscopy, and elemental analysis. In all cases, satisfactory analytical data were found (±0.4% for C, H, N).

Hydrolysis Studies. All reactions were performed at 25 °C using an assay mixture containing 25 mM Mops buffer, pH 7.6, 0.1 M NaCl, 5 mM $CaCl_2$, and 20% methanol to realize complete solubility of the substrates. The substrate concentrations were between 0.006 and 4.0 mM and the enzyme concentrations between $3.1 \times 10^{-6} - 6.2 \times 10^{-7}$ M. The active enzyme concentration was determined by active site titration using 4-nitrophenyl-4′-guanidinobenzoate.[12] After thermal equilibration of the assay mixtures, the reactions were initiated by addition of the enzyme. The rate of reaction was analyzed by RP-HPLC determining the disappearance of the substrates for at least 10 different concentrations. For this

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purpose, aliquots were withdrawn at defined time intervals and diluted with a quenching solution of 50% methanol containing 1% trifluoroacetic acid. The reaction rates were calculated from the peak area of the substrate with 4-toluenesulfonic acid as an internal standard. For each reaction, the extent of spontaneous hydrolysis was determined in an experiment without enzyme and found to be strictly less than 5%. The kinetic parameters were analyzed by iterative nonlinear curve fitting of the untransformed data according to the Michaelis−Menten formalism using the software SigmaPlot Scientific Graphic System (Vers. 5.0, Jandel Corp., Chicago, IL). The values reported are the average of at least three independent experiments.

Enzymatic Syntheses. The enzymatic coupling reactions were performed in 0.2 M Hepes buffer, pH 8.0, 0.2 M NaCl, 20 mM $CaCl_2$, and 10% methanol at 25 °C. Stock solutions of acyl donor esters (4 mM) were prepared in water containing 20% methanol as cosolvent. Acyl acceptor peptides (stock solution 30 mM) were dissolved in 0.4 M Hepes buffer, pH 8.0, 0.4 M NaCl, and 40 mM $CaCl_2$. The final concentrations of acyl donor and acyl acceptor were 2 mM and 15 mM, respectively. The latter was calculated as free, N^{α}-unprotonated nucleophilic concentration $[HN]_0$ according to the Henderson−Hasselbalch equation $[HN]_0 = [N]_0/(1 + 10^{pK-pH})$. The p*K* values of the α-amino group of the peptides were determined by inflection point titration on a Video Titrator VIT 90 (Radiometer, Denmark). Following thermal equilibration of assay mixtures, reactions were initiated by addition of *wt*- and mutant trypsin variant at final concentrations of 1.0×10^{-7} M and 3.1×10^{-6} M, respectively. Reaction times between 10 and 60 min led to a complete ester consumption. For the HPLC analysis, aliquots were withdrawn and diluted with a quenching solution of 50% aqueous methanol containing 1% trifluoroacetic acid. For each reaction, the extent of spontaneous ester hydrolysis was determined in an experiment without enzyme and found to be strictly less than 5%. On the basis of the same control experiments, nonenzymatic aminolysis of the acyl donor esters was investigated and could be ruled out. The values reported are the average of at least three separate experiments. The identity of the formed peptide products was established by thermospray mass spectroscopy.

HPLC Analyses. Samples were analyzed by analytical reversed phase HPLC using RP C18 and C8 columns (Vydac 218TP54, 10 μm, 300 Å, 25 × 0.4 cm, Grom Capcell SG 120, 5 μm, 5 Å, 25 × 0.4 cm, and Grom Capcell SG 300, 5 μm, 300 Å, 25 × 0.4 cm) and eluted with various mixtures of acetonitrile/water containing 0.1% trifluoroacetic acid under isocratic and gradient conditions at flow rates of 1.0 mL min^{-1}. Detection was at 254 nm. Reaction rates and product yields were calculated from peak areas of the substrate esters and the hydrolysis and aminolysis products, respectively. In the case of the pentapeptides containing the chromophoric amino acid residues Phe and Tyr the product yields were calculated from the lack of hydrolysis products using *p*-toluenesulfonic acid as an internal standard by at least five independent experiments.

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Supporting Information Available: Steady-state kinetic parameters for the hydrolysis of Boc-L/D-Xaa-OGp and Lys- and Arg-containing peptides by *wt*-trypsin and mutant trypsin D189E-catalyzed coupling of Bz-Gly-OGp with Phe- and Tyr-containing peptides, and data of the thermospray mass analysis of the enzymatically synthesized peptides. This material is available free of charge via the Internet at http://pubs.acs.org.

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