Supporting Information

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

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Materials and enzymes

N^{α}-protected amino acid derivatives, coupling reagents, and ester components were products of Bachem, Sigma, Fluka (Switzerland) or Merck (Germany). All reagents were of the highest purity commercially available. Solvents were purified and dried by the usual methods.

Enzymes and buffers for DNA manipulation came from NEW ENGLAND BIOLABS. Oligonucleotide primers were obtained from MWG-BIOTECH. Plasmid DNA was isolated with the S.N.A.P. isolation kit (INVITROGENE). The quick change site-directed mutagenesis kit came from STRATAGENE. The QiaQuick Gel Extraction Kit (QIAGEN) was employed for purification of DNA fragments from agarose gels. The pYT plasmid of trypsin K60E and pST plasmid of the mutant D189S were friendly gifts of Prof. L. Hedstrom (Brandeis University) and Dr. T. Kurth (University of Leipzig).

Chemical syntheses

Bz-Gly-OGp[1] was prepared according to our previously described protocols.[2] Similarly, Boc-Ala-OPh and Boc-Ala-OPic were synthesized by DCC coupling of the appropriate Boc-protected amino

acids with phenol and 4-hydroxymethyl pyridine, respectively. Boc-Ala-SPhOH was prepared by using the mixed anhydride method (isobutylchloroformiate/4-ethylmorpholine) while Boc-Ala-OCap and Boc-Ala-OCp were synthesized by coupling the ester bond using TBTU. Pentapeptides have been synthesized with a semiautomatic batch peptide synthesizer SP 650 (LABORTECH AG) using *p*-alkoxybenzylalcohol resin, synthesized according to Wang and standard Fmoc chemistry.[3] The peptides were precipitated with dry diethyl-, diisopropylether or mixtures of hexane/ethylacetate. The identity and purity of all final products were checked by analytical HPLC (220 nm), NMR, thermospray mass spectroscopy, and elemental analysis. In all cases, satisfactory analytical data were found (± 0.4% for C, H, N).

Enzymatic syntheses

Enzymatic reactions were performed in a total volume of 450 µl at 25 °C. Stock solutions of Bz-Gly-OGp (4 mM) were prepared in distilled water containing 8% (v/v) DMF while the pentapeptides (30 mM) were dissolved in 0.2 M Hepes buffer (pH 8.0), 0.2 M NaCl, and 20 mM $CaCl_2$ containing 8% (v/v) DMF as cosolvent. If required, the pH was readjusted to 8.0 using 1 N NaOH. The final concentrations of acyl donors and acyl acceptors were 2 mM and 15 mM, respectively. The latter were calculated as free, N^{α}-unprotonated nucleophile concentration $[HN]_0$ according to the formalism of Henderson–Hasselbalch $[HN]_0=[N]_0/(1+10^{pK-pH})$.[4] After thermal equilibration of assay mixtures, the reactions were initiated by addition of the appropriate enzyme stock solutions. Subsequently, the mixtures were rapidly shaken and transferred into a thermomixer adjusted to 25 °C. Finally, the reactions were analyzed by RP-HPLC. For this purpose, at defined time intervals, 50 µl aliquots were withdrawn and diluted with a quenching solution of 50% methanol containing 5% TFA. To control for spontaneous hydrolysis and aminolysis of the acyl donor esters, parallel reactions without enzyme were analyzed. On the basis of these experiments non-enzymatic aminolysis could be ruled out and the extent of spontaneous hydrolysis was found to be less than 5%. The data reported are the average of at least three independent experiments. The identity of the formed peptide products has been established by thermospray mass spectroscopy.

Enzymatic ester hydrolyses

Hydrolysis reactions were performed in a total volume of 450 µl at 25 °C. Stock solutions of Boc-Ala-OR esters were prepared in 0.1 M Hepes buffer (pH 8.0), 0.1 M NaCl, and 10 mM $CaCl_2$ containing 8% (v/v) DMF as cosolvent. After thermal equilibration of assay mixtures, the reactions were initiated by addition of the appropriate enzyme stock solutions. Subsequently, the mixtures were rapidly shaken

and transferred into a thermomixer adjusted to 25 °C. Finally, the reactions were analyzed by RP-HPLC. For this purpose, at defined time intervals, 50 µl aliquots were withdrawn and diluted with a quenching solution of 50% methanol containing 5% TFA. To control for spontaneous hydrolysis of the acyl donor esters, parallel reactions without enzyme were analyzed. On the basis of these the extent of spontaneous hydrolysis was found to be less than 5%. The data reported are the average of at least three independent experiments.

Site-directed mutageneses

Recombinant rat trypsinogen II was prepared from *E. coli* vector pST.[5] This plasmid encodes for the *wt*-protein from *Rattus norvegicus* fused to ADH/GAPDH promoter and α factor leader sequence. The desired mutations have been introduced by PCR using the *wt*-trypsin, trypsin mutant D194N, D189S or K60E pST plasmids as the templates. In a first step site-directed mutagenesis was performed by thermocycling using a PCR mixture (50 µl) that contained 50 – 150 ng of template pST DNA, 2.5 U *Pfu* turbo DNA polymerase, 2.5 µM of each desoxynucleotide, the reaction buffer, and 200 ng of the following oligonucleotide primers: 5'-TGATGATGACGCCATCGTTGGAG-3', 5'-CTCCAACGATGGCGTCATCATCA-3' (K15A); 5'-TGCCAGGGTAACTCTGGTGGC-3', 5'-GCCACCAGAGTTACCCTGGCA-3' (D194N); 5'-TGCCAGGGTAACTCTGGTGGC-3' and 5'-GCCACCAGAGTTACCCTGGCA-3' (D189S,D194N). After removing original pST template DNA by incubation with 20 U *Dpn*I, the PCR products were transformed into *E. coli* ultra competent cells. The isolated plasmids were then verified by sequence analysis. The processed trypsinogen constructs were 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. D189S,D194N pST plasmid was additionally digested with *Bam*HI and *Msc*I and then subcloned into the similar excised K60E pYT vector leading to the K60E,D189S,D194N mutant. Following a further amplification in *E. coli*, the EZ transformation kit (ZYMO-RESEARCH) was employed to transfer the pYT variants to *Saccharomyces cerevisiae*.

Expression and purification of trypsin variants

S. cerevisiae transformants were selected and amplified on uracil- and subsequently leucine-deficient SC media. 20 ml cultures (Leu-deficient, 3 d incubation) were used to inoculate one liter 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). Mature trypsin variants D194N, D189S,D194N, and K60E,D189S,D194N were obtained by treatment with trypsin-free enterokinase (BIOZYME). Final purification of the proteins was achieved

by perfusion chromatography on a POROS 20 HQ column (PERSEPTIVE BIOSYSTEMS). After dialysis, the proteins were concentrated to 1 – 3 mg ml^{-1} and stored in 1 mM HCl at 4 °C.

HPLC analyses

HPLC measurements were performed with a Shimadzu LC-10A HPLC system using a LiChrospher RP 18 column (250 × 4 mm, 5 µm, Merck, Germany) or a Capcell PAK C8 column (250 × 4 mm, 5 µm, Shiseido, Japan). Samples were eluted with various mixtures of water/acetonitril containing 0.1% TFA under isocratic and gradient conditions at flow rates of 1.0 ml min^{-1}. Detection was at 254 nm. The reaction rates and product yields were calculated from peak areas of the ester substrates, hydrolysis and aminolysis products, respectively.

Supporting results

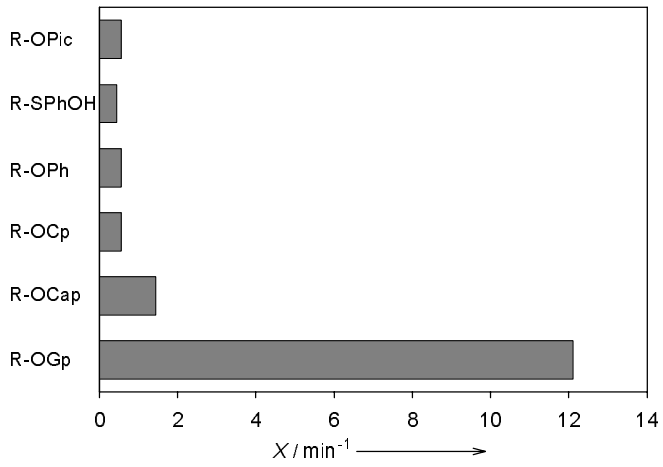

Figure S1. Initial rates of hydrolysis of Boc-Ala-OR esters catalyzed by trypsin D194N. Conditions: 0.1 M Hepes buffer (pH 8.0), 0.1 M NaCl, 0.01 M $CaCl_2$, 8% (v/v) DMF, [Boc-Ala-OR] = 2 mM, [enzymes] = 1.2×10^{-6} M, X = hydrolysis rate, R: Boc-alanyl.

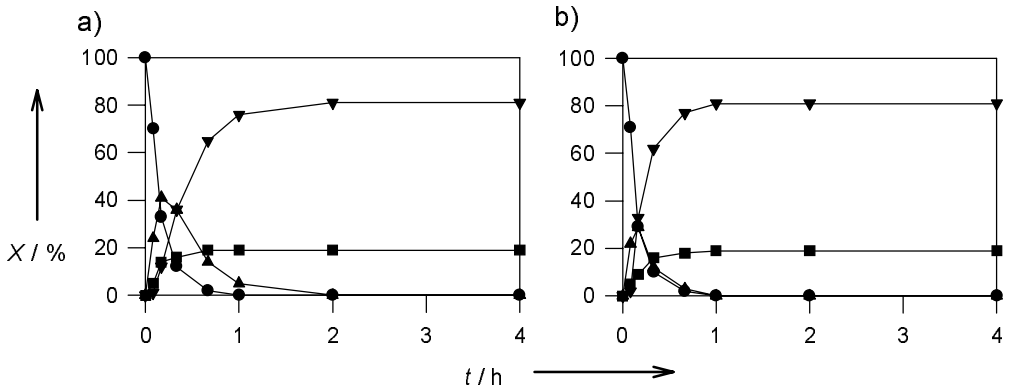

Figure S2. Course of the trypsin mutant D194N-catalyzed coupling of Bz-Gly-OGp with Ala-Ala-*Xaa*-Ala-Gly. *Xaa*: (a) Lys; (b) Arg. (–●–) Bz-Gly-OGp, (–■–) Bz-Gly-OH, (–▼–) Bz-Gly-Ala-Ala-*Xaa*-OH, (–▲–) Bz-Gly-Ala-Ala-*Xaa*-Ala-Gly-OH. Conditions: 0.1 M Hepes buffer (pH 8.0), 0.1 M NaCl, 0.01 M $CaCl_2$, 8% (v/v) DMF, [Bz-Gly-OGp] = 2 mM, [Ala-Ala-*Xaa*-Ala-Gly] = 15 mM, [enzyme] = 6.0×10^{-6} M, *X* = product yield.

Abbreviations and references

(1) *Abbreviations:* Boc, *tert*-butoxycarbonyl; Bz, benzoyl; DCC, *N*,*N*‘-dicyclohexylcarbodiimide; DMF, *N*,*N*-dimethylformamide; Fmoc, 9-fluorenylmethoxycarbonyl; Hepes, *N*-[2-hydroxyethyl]-piperazine-*N*‘-[2-ethanesulfonic acid]; OCap, 4-carboxyamidophenyl ester; OCp, 4-carboxyphenyl ester; OGp, 4-guanidinophenyl ester; OPh, phenyl ester; OPic, picolyl ester; SPh, 4-hydroxythiophenyl ester; TBTU, 2-(1*H*-benzotriazol-1-yl)-1,2,3,3-tetramethyluronium tetrafluoroborate; TFA, trifluoroacetic acid.

(2) (a) Bordusa, F.; Ullmann, D.; Elsner, C.; Jakubke, H.-D. *Angew. Chem. Int. Ed. Engl.* **1997**, *36*, 2473-2475.

(3) Wang, S. S. *J. Am. Chem. Soc.* **1973**, *95*, 1328-1333.

(4) Schellenberger, V.; Jakubke, H.-D. *Angew. Chem. Int. Ed. Engl.* **1991**, *30*, 1437-1449.

(5) Hedstrom, L.; Szilagyi, L.; Rutter, W. J. *Science* **1991**, *255*, 1249-1253.