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Engineering β-lactamase zymogens for use in protease activity assays

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Supporting Information

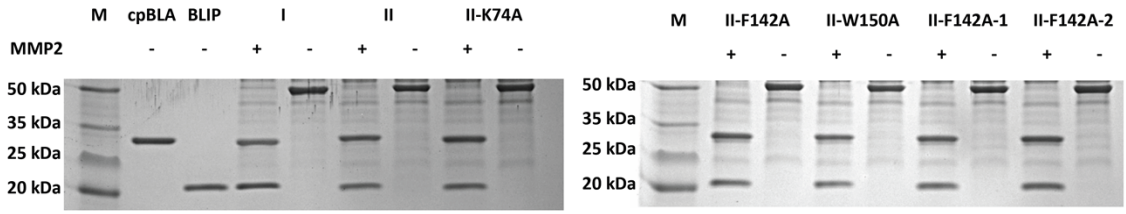

Fig. S1. Specific and complete cleavage of zymogens by MMP2. Each zymogen (construct I, II, II-K74A, II-F142A, II-W150A, II-F142A-1, or II-F142A-2) was incubated with MMP2 (5 μg/mL) for 4 hours, after which the resulting reaction mixtures were analyzed by SDS-PAGE.

Materials and Methods

Plasmid construction

The gene encoding TEM-1 including the signal sequence was amplified from pET-22b and cloned into pET-28a by using the *Nco*I and *Xho*I sites. The resulting expression vector for TEM-1 was named pSPEL104. A synthetic double-stranded oligonucleotide encoding only the TEM-1 signal sequence was ligated into pET28a digested with *Nco*I and *Nde*I (pTHY41), and a synthetic gene for mature cpBLA with an N-terminal

His_6-tag was cloned into pTHY41 by using the *Nde*I and *Xho*I sites. The resulting expression vector for cpBLA was named pTHY47. The gene encoding mature BLIP amplified from pGR32[1] was cloned into pTHY41 by using the *Nde*I and *Bam*HI sites, after which the gene encoding mature cpBLA was additionally cloned into the BLIP-encoding pTHY41 construct by using the *Hind*III and *Xho*I sites. The resulting expression vector was named pSPEL019. A synthetic double-stranded oligonucleotide encoding a linker containing an MMP2 cleavage site (GGSGGSGPLGVRGGSGGS) was ligated into pSPEL019 digested with *Bam*HI and *Hin*dIII to yield the pSEPL050 expression vector encoding construct I. For construct II, the gene encoding mature cpBLA was cloned into pTHY41 by using the *Nde*I and *Bam*HI sites, after which the gene encoding mature BLIP was additionally cloned into the cpBLA-encoding pTHY41 construct by using the *Hin*dIII and *Xho*I sites. The resulting expression vector was named pSPEL017. The double-stranded oligonucleotide encoding the linker (GGSGGSGPLGVRGGSGGS) was cloned in pSPEL017 by using the *Bam*HI and *Hin*dIII sites to yield the pSPEL051 expression vector encoding construct II. The plasmids encoding constructs II-F74A (pSPEL074), II-F142A (pSPEL075), and II-W150A (pSPEL076) were generated using a site-directed mutagenesis kit (QuikChange II, Agilent Technologies, Santa Clara, CA, USA). For different linkers, synthetic double-stranded oligonucleotides encoding GGSGPLGVRGGS and GGGSGPLGVRGGGS were individually ligated into pSPEL75 digested with *Bam*HI and *Hin*dIII to yield the pSPEL165 and pSPEL166 vectors encoding constructs II(F142A)-1 and II(F142A)-2, respectively.

Expression and purification of proteins

The expression of all recombinant proteins was induced with IPTG (0.1 mM) at 37°C overnight in *Escherichia coli* BL21 (DE3). The lactamase enzymes and zymogens were expressed in the periplasmic space and were purified after periplasmic fractionation[2]. The cell pellets obtained from the centrifugation of 400 mL 2xYT media cultures were resuspended in 11 mL sucrose solution (0.75 M in 0.1M Tris, pH 8) containing lysozyme (50 mg/mL), after which 21 mL of EDTA solution (1 mM) was added and the suspension was gently mixed. After incubation on ice for 10 min, 1.5 mL $MgCl_2$ solution (0.5 M) was added, and the suspension was incubated on ice for an additional 10 min. The periplasmic fraction was obtained as a supernatant following centrifugation of the cell suspension (10,000 × *g,* 20 min), and the protein containing a C-terminal His_6-tag was purified under native conditions by affinity chromatography using Nickel resin (His60, Clontech, Mountain View, CA, USA) according to the manufacturer's instructions. The concentrations of the purified proteins were determined by measuring the absorbance at 280 nm, using extinction coefficients calculated from the ProtParam site (http://web.expasy.org/protparam/).

Enzyme kinetics

The specific activities of TEM-1, cpBLA, and the engineered zymogens were determined by measuring the rates of CENTA (CENTATM β-lactamase substrate, EMD Millipore, Billerica, MA, USA)[3] hydrolysis in PBSB (10 mM phosphate, pH 7.0, 150 mM NaCl, 0.2% BSA). The hydrolysis of CENTA was measured by monitoring absorbance at 405 nm ($\Delta\varepsilon$ = 6,400 $M^{-1}cm^{-1}$) by using a plate reader (Eon microplate spectrophotometer, BioTek, Winooski, VT, USA). Reactions were initiated by the addition of 50 µL CENTA solution (100–2000 µM for TEM-1 and cpBLA, and 200–4000 µM for zymogens) to 50 µL protein solution (4 nM for TEM-1 and cpBLA, and 400 nM for zymogens). Kinetic constants were determined by fitting the Michaelis–Menten model to the initial velocities measured for various concentration of CENTA by using a software (Origin, Origin Lab, Northampton, MA, USA).

MMP2 activity assay

MMP2 (Recombinant Human MMP-2, R&D systems, Minneapolis, MN, USA) was diluted to 100 µg/mL and was activated in MMP2 activation buffer (100 mM Tris, 10 mM $CaCl_2$, 150 mM NaCl, 0.05% (w/v) Brij-35, pH 8.0, 1 mM *p*-aminophenylmercuric acetate) for 1 hour at 37°C. Equal volumes of zymogen (4 µM) and activated MMP2 in MMP2 buffer (50 mM Tris, 10 mM $CaCl_2$, 150 mM NaCl, 0.05% (w/v) Brij-35, 0.2% BSA, pH 7.5) were combined and incubated at room temperature for 4 hours. The reaction mixture was diluted and mixed with PBSB containing CENTA (final concentration 200 µM). The appropriate dilutions were determined based on the specific activity of the zymogens. The velocity of CENTA hydrolysis was measured by monitoring the resulting absorbance at 405 nm.

References

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