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Substrate turnover and inhibitor binding as selection parameters in directed evolution of blood coagulation factor X_a

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A library of blood coagulation factor X_a (FX_a)–trypsin hybrid proteases was generated and displayed on phage for selection of derivatives with the domain "architecture" of trypsin and the specificity of FX_a. Selection based on binding to soybean trypsin inhibitor only provided enzymatically inactive derivatives, due to a specific mutation of serine 195 of the catalytic triad to a glycine, revealing a significant selection pressure for proteolytic inactive derivatives. By including a FX_a peptide substrate in the selection mixture, the majority of the clones had retained serine at position 195 and were enzymatically active after selection. Further, with the inclusion of bovine pancreatic trypsin inhibitor, in addition to the peptide substrate, the selected clones also retained FX_a specificity after selection. This demonstrates that affinity selection combined with appropriate deselection provides a simple strategy for selection of enzyme derivatives that catalyse a specific reaction.

Introduction

Phage-display technology is a powerful tool for engineering and selecting proteins with improved or altered properties, although the outcome is dependent on the method chosen to select for the desired function. The most commonly used and robust selection strategy is to capture phage on the basis of the affinity of the displayed proteins for an immobilised target and to wash away unbound phage.[1] This strategy works well for proteins involved in ligand binding such as phage-displayed antibodies binding to an antigen, but for selection of new enzyme derivatives from libraries of phage-displayed enzymes, the strategy must focus on catalytic turnover rather than only binding affinity and specificity.

This paper describes a simple and robust selection strategy where catalytic turnover is linked to a selective advantage by deselecting displayed derivatives with low or no catalytic activity (Fig. 1). Free substrate for the desired enzyme is added, before capturing of catalytically active derivatives with a substrate-like inhibitor tethered to a solid support. Due to the catalytic turnover only the most active enzyme derivatives reach and bind the immobilised inhibitor, whereas inhibitor-binding derivatives with low or no catalytic activity are trapped in solution by the substrate.

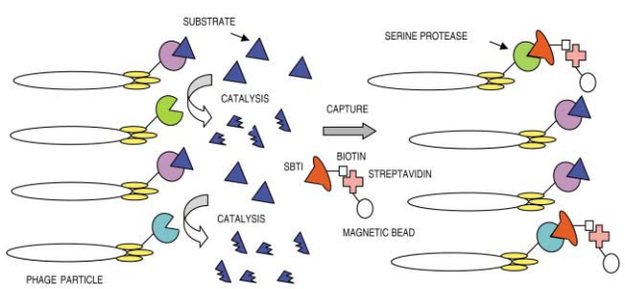

Fig. 1 Schematic representation of the presented selection strategy. A library of phage-displayed serine protease derivatives are incubated with a peptide substrate optimal for the desired reaction. Proteolytically active derivatives cleave the substrate and release the product whereas the proteolytically inactive derivatives although otherwise functional are trapped on the substrate. Eventually the proteolytically active phage-displayed serine protease derivatives are captured by biotinylated soybean trypsin inhibitor (SBTI) on streptavidin-coated magnetic beads.

The high specificity of cleavage by blood coagulation factor X_a (FX_a) makes it a widely used protease in processing of recombinant proteins (*e.g.* cleaving off a fusion partner [2]). Currently the only available FX_a is purified from bovine blood, whereas a recombinant FX_a produced in *Escherichia coli* would be of great advantage for many biotechnological applications for quantity and biosafety reasons.

The mature form of FX_a is a multi-domain protein, consisting of a light chain (composed of one Gla domain and two epidermal growth factor-like domains) linked to the serine protease domain by a single disulfide bond.[3] In comparison, trypsin is a single-chain protein, comprising the serine protease domain.[3] For the purpose of large-scale production of recombinant FX_a, it would be desirable to combine the single-domain "architecture" of trypsin and the specificity of FX_a for creation of a single-domain FX_a.

The residues in the serine protease domain of FX_a involved in contact with the light chain, are mostly located in the *C*-terminal β-barrel subdomain [4] and to decouple the serine protease domain of FX_a from the light-chain, it is tempting to exchange this subdomain with the corresponding subdomain from trypsin. Hopfner *et al.* constructed such a single-domain chimeric protein, in which the *C*-terminal subdomain of FX_a was changed to that of trypsin.[5] The hybrid protein was found to be enzymatically active, although the kinetic parameters were a combination of FX_a, trypsin, and novel properties. This demonstrates that it is possible to combine subdomains from different serine proteases and obtain an active single-domain enzyme, but further engineering has to be applied to retain the desired FX_a specificity. Therefore, we decided to use DNA shuffling to recombine the *C*-terminal subdomains of FX_a and trypsin and to use library phage display to select single-domain derivatives with the specificity of FX_a.

Results and discussion

Library construction

The hybrid library was prepared by DNA shuffling of the sequence encoding the *C*-terminal subdomain of bovine FX_a

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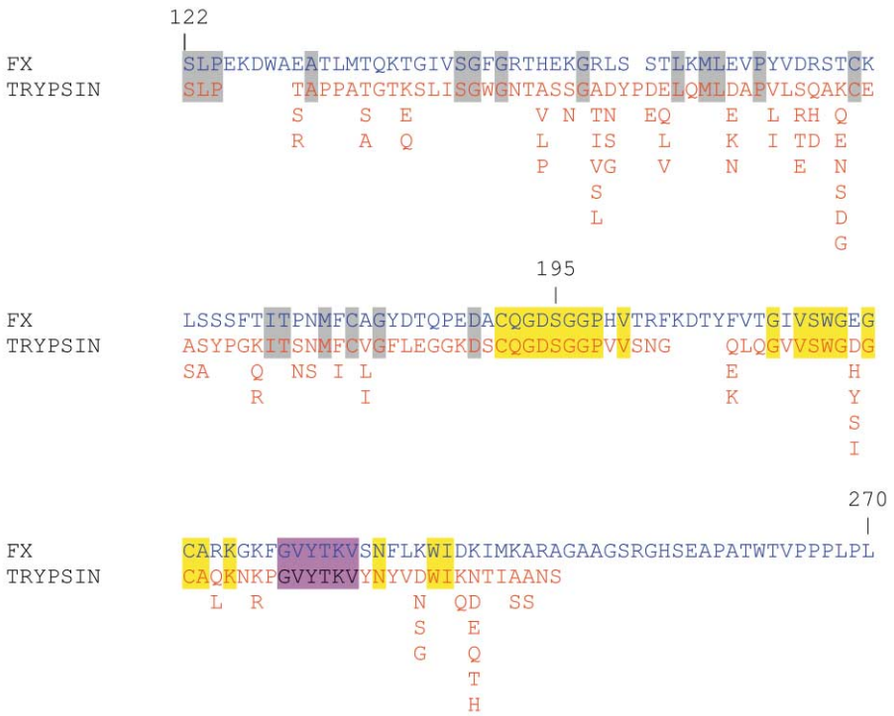

Fig. 2 Alignment of the sequence of the *C*-terminal subdomain of bovine FX_a (blue) and the designed trypsin sequence (red), in which some positions are represented by more than one residue. These positions are encoded by mixed nucleotides and were selected from a sequence alignment of eleven different trypsin sequences including human Try1, Try2, Try3, and Try4, bovine Try1 and Try2, rat Try3, pig Tryp, and chicken Try1, Try2, and Try3 (Swiss Prot Database entry). Residue 122 is naturally serine in trypsin but is specifically mutated from cysteine to serine in FX_a. Identical residues are shown in boxes. The segment where most of the analysed sequences from the library have a crossover, is shown in the magenta box, whereas segments with less frequent crossovers are shown in yellow boxes. The numbering follows chymotrypsinogen numbering.

and designed sequences encoding the *C*-terminal subdomain of trypsin (Fig. 2), as described in the Experimental section. The boundary between the two β-barrel subdomains of the serine protease domain was chosen to be located at residue 122 as described by Hopfner *et al.*[5] In positions where the eleven examined trypsin sequences differed, as many of the represented residues as possible were included by using mixed nucleotides in the synthetic oligonucleotides encoding trypsin (*e.g.* N = A, G, C, and T, K= G and T *etc.*). To increase the homology, the codons used in the trypsin sequence were chosen to be as identical to the codons in the FX_a sequence as possible. The DNA fragment obtained after shuffling was ligated into the FX_a phagemid vector (pPhFXCD), which expresses the FX_a–trypsin hybrids as a pIII-fusion protein, resulting in display of a peptide with an *N*-terminal corresponding to activated FX_a (Ile16).

After transformation into *Escherichia coli* TG1 cells, a phage library of 5×10^9 clones was obtained. 50 randomly picked clones were sequenced and all clones were found to be different, which confirmed diversity of the library. Most of the sequences contained the same crossover between FX_a and trypsin, resulting in clones with predominantly the sequence of FX_a and only the *C*-terminal part changed to the sequence of trypsin (Figs. 2 and 3A). Diversity was only incorporated into the trypsin sequence, but a substantial number of point mutations in the FX_a sequence of the shuffled subdomain (generated as a result of the assembly in the DNA shuffling step) contributed to the sequence diversity of the library.

Affinity selection

The library of phage-displayed FX_a–trypsin hybrids was initially subjected to affinity selection by use of biotinylated soybean trypsin inhibitor (SBTI) immobilised on streptavidin-coated magnetic beads. After two rounds of selection and amplification, a population enriched 300 times for SBTI binding was obtained, measured as increase in phage titer after selection. The enzymatic activity of a sample of the total population of phage after the second round of selection was determined and found to be below the activity of a sample from the library (Fig. 4). The SBTI binding capability was found to be significantly higher after the second round of selection (Fig. 4). Seven clones from the second round were randomly picked for sequence analysis. All seven clones (D3–D8 + D10) had different trypsin sequences in the shuffled subdomain and all contained a specific mutation of the active serine at position 195 to glycine (b in Fig. 3A). Clones with these characteristics (trypsin sequence and mutation of serine 195) were not observed in the sequence analysis of the library and must have been specifically selected as a subpopulation from the library. The seven clones were expressed and all were found to bind SBTI by phage ELISA (Fig. 5A), but none of them were enzymatically active (not shown). The obliteration of catalytic activity by the mutation of active site serine 195 reveals a significant selection pressure against active clones, probably because the protease activity represent a disadvantage to the *Escherichia coli* host.[6] The selection enriches the inactive clones because not only do they satisfy the requirement for binding to SBTI but in addition they must have a survival advantage over any active clones that also bind SBTI.

For one clone (D3) the glycine was back-mutated to serine and this new clone was found to be able to process the chromogenic FX_a substrate (S2222) (Fig. 6) proving that the mutation of serine 195 may account for inactivation of the selected clones. These results are a clear demonstration of the principle, "You get what you select for",[7] which is useful to remember when selections do not yield the desired results.

Selection including deselection with peptide substrate

The selection steps were repeated, with a FX_a specific peptide substrate (Fig. 7) added to the phage solution before and during capture on biotinylated SBTI. Catalytically active clones, able to cleave the peptide substrate and release the product rapidly,

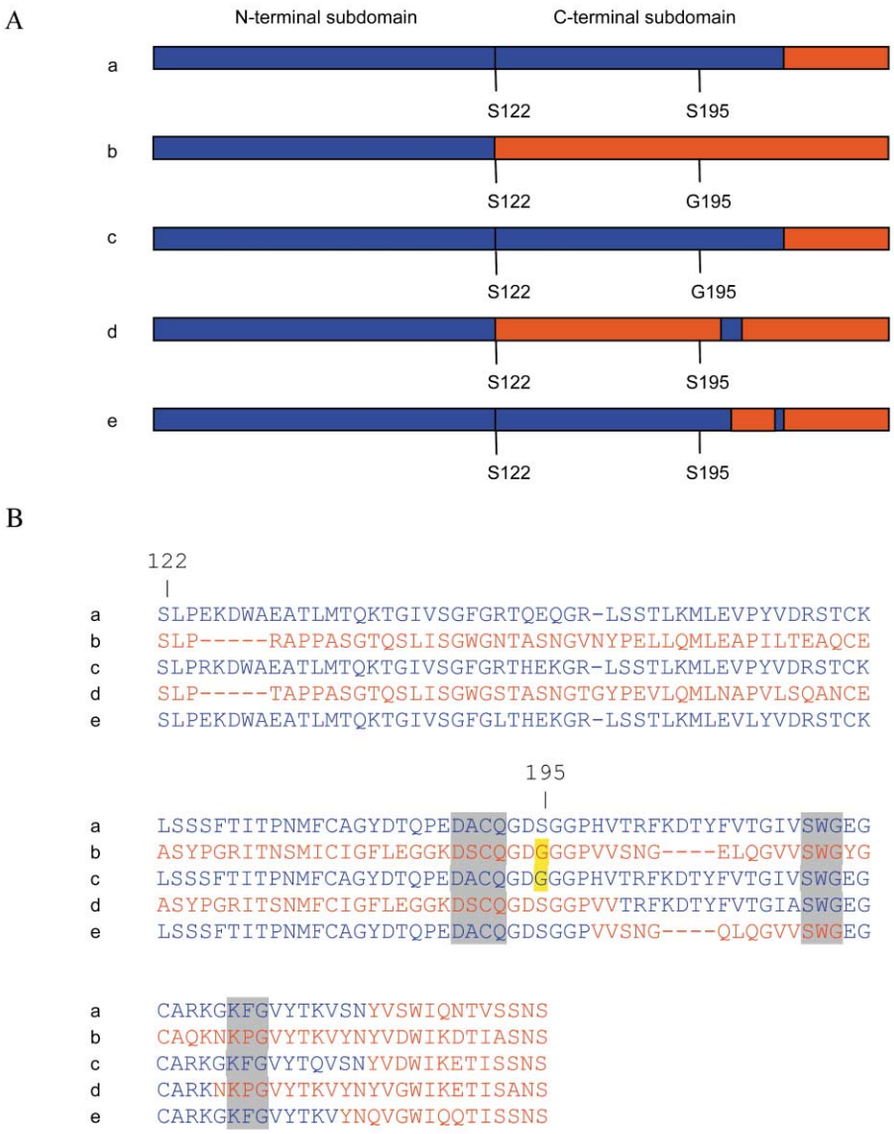

Fig. 3 Examples of clones. Blue represents the sequence of FX_a and red represents the sequence of trypsin. A: Schematic representation. S195 or G195 defines if the residue in position 195 is serine or glycine, respectively. The *C*-terminal subdomain is the subdomain that has been subjected to shuffling. a) represents a clone with predominantly the sequence of FX_a and only the most *C*-terminal part changed to the sequence of trypsin, b) represents a clone with trypsin sequence in the *C*-terminal subdomain and serine 195 mutated to glycine, c) represents the same clone as a), although serine 195 is mutated to glycine, d) represents a clone containing more than one crossover in the *C*-terminal subdomain and with the sequence of trypsin in the region around S195 that defines the S1 site specificity, and e) represents a clone containing more than one crossover in the *C*-terminal subdomain and with the sequence of FX_a in the region around S195 that defines the S1 site specificity. B: Sequences of the *C*-terminal subdomain of clones representing the examples in A. Amino acid residues comprising the S1 subsite are shown in grey boxes. a) is represented by the R5 clone, b) is represented by the D3 clone, c) is represented by the R7 clone, d) is the P1 clone, and e) is the P4 clone.

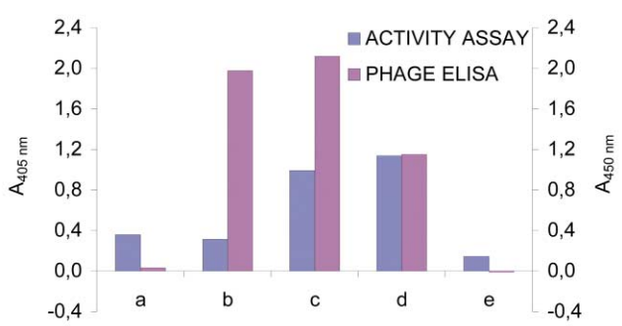

Fig. 4 Enzymatic activity and SBTI binding capability of samples of a) total phage preparations of the library and of the second rounds of b) affinity selection, c) selection including deselection with peptide substrate, and d) selection including deselection with peptide substrate and BPTI, respectively. e) M13 KO7 helper phage were used to determine the background contribution. The enzymatic activity was determined by an activity assay ($A_{405\ nm}$) and the SBTI binding capability was determined by phage ELISA ($A_{450\ nm}$).

will be able to eventually be captured by SBTI, whereas the enzymatically inactive, although SBTI binding, derivatives will be trapped on the substrate. Furthermore the strategy will deselect clones that suffer from product inhibition or only have low enzymatic activity.

After two rounds of selection and amplification, the population was enriched by a factor of 100 for SBTI binding phage. The enzymatic activity of a sample of the total population of phage after the second round of selection was found to be significantly higher than the enzymatic activity of a sample from the library (Fig. 4), which confirms that this selection protocol has enriched the population in active enzymes. Eight colonies from the second round were picked for sequence analysis and all clones were found to be different. Two clones (P2, P3) were homologous to the clones described in the previous selection with the sequence of trypsin in the shuffled subdomain and mutation of serine 195 to glycine (b in Fig. 3A). Four clones (P5–P8) had the same crossover as most of the clones identified in the library with primarily the sequence of FX_a in the shuffled

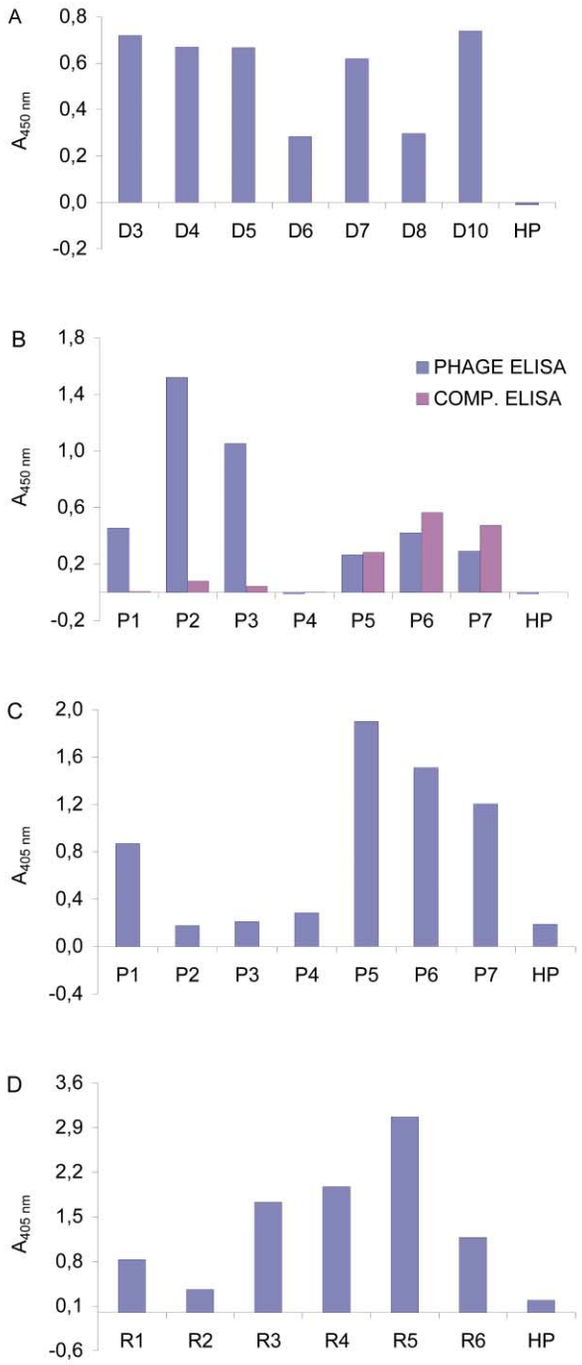

Fig. 5 SBTI binding capability and/or enzymatic activity of different selected clones. M13 KO7 helper phage (HP) were used to determine the background contribution. A: SBTI binding capability of clones obtained after the second round of affinity selection determined by phage ELISA. B: SBTI binding capability and BPTI inhibition determined by phage ELISA and competition ELISA, respectively. Both performed with clones obtained after the second round of selection including deselection with peptide substrate. C: Enzymatic activity of the same clones as in B determined by activity assay. D: Enzymatic activity of clones obtained after the second round of selection including deselection with peptide substrate and BPTI, determined by activity assay.

subdomain and only the most *C*-terminal part changed to the sequence of trypsin (a in Fig. 3A). The remaining two clones contained more than one crossover. From the sequence in the region that defines the S1 site specificity, we anticipate that one (P1) is predominantly trypsin specific (d in Figs. 3A and B) and the other (P4) is predominantly FX_a specific (e in Figs. 3A and B).[8,9] Most importantly, six clones (P1 and P4–P8) had retained serine at position 195. Except P1, these clones contain either a

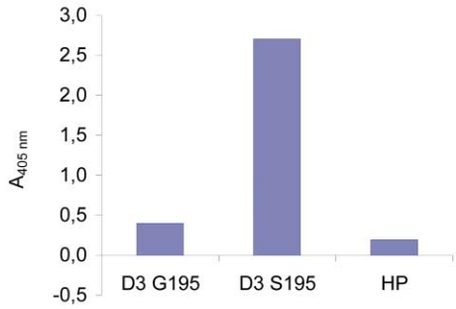

Fig. 6 Enzymatic activity of the D3 clone obtained after the second round of affinity selection (D3 G195) and the clone in which the glycine in position 195 of D3 is backmutated to serine (D3 S195), respectively. M13 KO7 helper phage (HP) were used to determine the background contribution. The enzymatic activity was determined by an activity assay.

GGIQGR↓GSGK

Fig. 7 The FX_a-specific peptide substrate. The underlined residues in the peptide sequence, comprise a recognition sequence for cleavage by FX_a and the arrow highlights the scissile bond.

TGA or TAG substitution in different positions and are probably selected because it reduces the expression of displayed proteases, which are forced by the selection pressure to be active. Although the *E. coli* TG1, that were used for phage expression, are an UAG but not an UGA suppressor strain, phages that display the clones containing the TGA or TAG substitution, respectively, appeared to be expressed at the same level (not shown). Selection of clones containing stop codons has previously been observed by Atwell *et al.* on display of subtiligase.[10] Expression of the clones containing the TGA substitution must be explained by codon read-through, probably by $tRNA^{Trp}$.[11]

Seven of the eight clones (P1–P7) were expressed individually and analysed for binding to SBTI by phage ELISA (Fig. 5B). Only P4 did not bind SBTI. To distinguish between trypsin and FX_a specificity at the S1 site, a competitive ELISA assay with bovine pancreatic trypsin inhibitor (BPTI) was performed. BPTI is a substrate-like trypsin inhibitor that binds FX_a with a very low K_a value,[12] partly because of Lys in the P1 position of the binding loop sequence, compared to Arg in the P1 position of SBTI, which binds both trypsin and FX_a.[13,14] The clones (P1–P3) with the sequence of trypsin in the region that defines the S1 site specificity were almost completely blocked for SBTI binding after addition of BPTI (Fig. 5B), whereas clones (P5–P7) with the sequence of FX_a in the same region were not affected by addition of BPTI. The enzymatic activity was also determined (Fig. 5C). Clones with Ser at position 195 were active (P1, P5–P7), although the P4 clone that also failed to bind SBTI was not active. The two clones with Gly at position 195 (P2, P3) were also not active as expected.

Selection including deselection with peptide substrate and BPTI

To avoid selection of unwanted derivatives with predominantly trypsin specificity, the selection steps were repeated once more and this time the phage were deselected with BPTI in addition to the peptide substrate. After two rounds of selection and amplification, the population was enriched 200 times for SBTI binding phage. The enzymatic activity of a sample of the total phage population after the second round was found to be significantly higher than the enzymatic activity of the sample from the library (Fig. 4). Again this confirms that the selection protocol has enriched the population in active enzymes. Eight colonies from the second round were picked for sequence analysis. They were all found to be different but homologous to the four clones from the previous selection with primarily the

sequence of FX_a in the shuffled subdomain and only the most *C*-terminal part changed to the sequence of trypsin (a and c in Fig. 3A). Two clones (R7, R8) were inactivated by the glycine mutation at position 195 (c in Fig. 3A), while six clones (R1–R6) had retained serine at this position (a in Fig. 3A). All the clones contained either a TGA or a TAG substitution in different positions and one clone even contained two TAG substitutions. The six clones with serine at position 195 (R1–R6) were expressed individually, and the enzymatic activity was determined (Fig. 5D). All clones were active, although one (R2) only shows low activity.

To demonstrate that deselection with the peptide substrate accounts for the selection of active variants, a competition ELISA with the peptide substrate was performed (Fig. 8). This shows that the binding of the active R5 clone to SBTI was not affected by the added peptide substrate whereas the inactive D3 clone showed a reduced binding when the peptide substrate was included, demonstrating that some of the inactive enzyme displaying phage were trapped on the substrate.

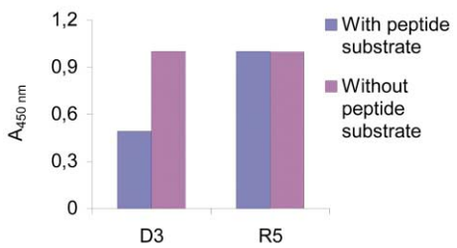

Fig. 8 SBTI binding capability and peptide substrate inhibition determined by phage ELISA and competition ELISA, respectively, performed on the D3 and R5 clone.

In summary, we have shown that a combination of affinity selection and appropriate deselection can select a population of phage-enzymes with increased proteolytic activity and maintain FX_a specificity for most of the clones, determined by their ability to discriminate between BPTI and SBTI. A screening step, in which clones are examined separately on different substrates, may be included to ascertain the specificity of each clone.

The selection scheme is based on the same principle as the counter-selection protocol described for selection of β-lactamase activity from a library of phage displayed TEM-1 β-lactamase mutants.[15] They used a suicide inhibitor for trapping of active enzymes, but as demonstrated here the efficiency of the technique is not dependent on the availability of a suitable suicide inhibitor.

Advanced selection strategies based on catalytic function have been proposed.[16–19] These are very elaborate techniques based on intraphage conversion of substrate into product, whereby the phage is released from or attached to a solid support. Furthermore, these strategies have the potential problem that only a single conversion of substrate into product is required for selection of a certain clone. It is therefore impossible to distinguish between clones with a high or low turnover number. The selection strategy presented in this paper is based on a simple affinity selection coupled to catalytic function by providing a selective advantage to clones with the ability to undergo multiple turnovers and the strategy of adding a suitable substrate to the selection mixture could be useful in most selections for enzymatic activity.

Experimental

Phagemid construction

The fragment encoding the amino acid residues 82–484 (full coding reading frame numbering) of bovine FX was cloned from a cDNA obtained from total bovine liver. The fragment encoding the activated serine protease domain of bovine FX (amino acid residues 234–492, full coding reading frame numbering or 16–270, chymotrypsinogen numbering, used if nothing else is mentioned) was amplified by PCR using the primers 5′-GCGTGGAGGCCCAGCCGGCCATGGCCATAGTGGGCGGCAGGGACTG and 5′-CGACCTGCGGCCGCCAGCGGC AGTGGCGGCGGCACGGT CCAGGTGGCAGGGGCTTC. The PCR product was digested with *SfiI* and *NotI* and ligated into the corresponding sites of pCANTAB 5 E phagemid (Amersham Biosciences, Denmark). A *NheI* restriction site was introduced along with substitution of cysteine 122 with serine using the QuikChange™ Site-directed Mutagenesis Kit (Stratagene, USA) and the primers 5′-GTGGCGCCCGCTAGCCTGCCCGAGAAGG and 5′-CTTCTCGGGCAGGCTAGCGGGCGCCACG to create pPhFXCD phagemid. All primers and oligonucleotides were from DNA Technology A/S (Denmark).

Preparation of substrates for the DNA shuffling reaction

The 514 bp fragment encoding the *C*-terminal subdomain of bovine FX_a was amplified by PCR from pPhFXCD using the primers 5′-CGAGCGTGCACGGCGCCCGCTAGCCTGC (PH-vec-fw) and 5′-GGTCCTGGACCGGATGGTGATGGTGATGTGC (PH-vec-rev). The 421 bp fragment encoding the *C*-terminal subdomain of trypsin was prepared by PCR assembly of the seven oligonucleotides 5′-GCGCCCGCTAGCCTGCCCASMGCCCCTCCAGCCDCCGGCACCVAGAGCCTCATCAGCGGCT GGGGGAACACGSYCTCGARTGGCDYGRRCTACCCGGASSWGCTCCAAATGCTGRASGCGCC, 5′-CACTGTCGCCCTGGCAGGAGTCCTTGCCCCCTTCGAGGAAGCCGABGCAGAWCATGYTGYTCGTAATCYKGCCGGGGTAGGMCGMCTCACACTBGGCSTSSSTCAGGABGGG CGCSTYCAGCATTTGG, 5′-CACTGTCGCCCTGGCAGGAGTCCTTGCCCCCTTCGAGGAAGCCGABGCAGAWCATGYTGYTCGTAATCYKGCCGGGGTAGGMCGMCTCACAGYYGGCSTSSSTCAGGABGGGCGCSTYCAGCATTTGG, 5′-CTGCCAGGGCGACAGTGGCGGCCCCGTCGTCAGCAACGGCVAGCTCCAAGGCGTCGTCAGCTGGGGAAKCGGGTGCGCGCWGAAGAACARGCCCGGCGTCTAC ACCAAGGTCTAC, 5′-CTFCCAGGGCGACAGTGGCGGCCCCGTCGTCAGCAACGGCVAGCTCCAAGGCGTCGTCAGCTGGGGABACGGGTGCGCGCWGAAGAACARGCCCGGCGTCTACACCAAGGTCTAC, 5′-GATGGTGATGGTGATGTGCGGCCGCTGAGTTGGMCGMAATGGTKGTCTKGATCCAGYYTGTGACGTAGTAGACCTTGGTGTAGACG, and 5′-GATGGTGATGGTGATGTGCGGCCGCTGAGTTGGMCGMAATGGTSTSCTKGATCCAGYYGACGTAGTTGTAGACCTTGGTGTAGACG followed by PCR amplification using the primers PH-vec-fw and PH-vec-rev. Free primers were removed from the DNA fragments by agarose gel electrophoresis followed by electrophoresis onto NA45 DEAE membrane (Schleicher and Schuell, Germany). The isolated DNA fragments were eluted with 1.5 M LiCl, and ethanol precipitated.

DNA shuffling

Shuffling was carried out essentially as described by Stemmer.[20] 10–50 bp fragments, generated by DNaseI (Stratagene, USA) treatment of a 1 : 1 mixture of the PCR fragments encoding the *C*-terminal subdomain of FX_a and trypsin, respectively, were reassembled by PCR without added primers using the following PCR program: 94 °C, 1 min; 40 cycles of 94 °C, 30 s; 55 °C, 30 s; 72 °C, 30 s; and 72 °C, 5 min. After 40-fold dilution of the reaction mixture into PCR mix supplemented with 0.8 μM of primer 5′-CGAGCGTGCACGGCGC and 5′-GGTCCTGGACCGGATGG, respectively, and 30 additional cycles of PCR (94 °C, 1 min; 30 cycles of 94 °C, 30 s; 55 °C, 30 s; 72 °C, 1 min; and 72 °C, 5 min) the reassembled fragments of 400–500 bp were obtained.

Library construction

After digestion with *NheI* and *NotI*, the reassembled fragments were ligated into the corresponding sites of pPHFXCD. The ligation mixture was transformed into freshly prepared *Escherichia coli* TG1 cells by electroporation, plated on TYE agar containing 100 mg L^{-1} ampicillin and 2% (w/v) glucose and incubated at 30 °C overnight. Colonies were pooled and after addition of glycerol to final 16% (v/v) the cells were stored at −80 °C as the glycerol library stock. A serial dilution of a sample of the transformed cells was used to estimate the number of clones in the library by colony counting.

Phage preparation

Phage were produced by infecting exponentially growing cells from the glycerol library stock at 37 °C (at $OD_{600\ nm}$= 0.5) with M13 KO7 helper phage (5×10^9 cfu mL^{-1} of culture). After infection the culture medium was changed to 2xTY containing 100 mg L^{-1} ampicillin and 50 mg L^{-1} kanamycin. After overnight incubation at 25 °C, the phage particles from the supernatant were isolated by polyethylene glycol precipitation ($\frac{1}{3}$ supernatant volume of 20% (w/v) PEG 6000; 2.5 M NaCl) and resuspended in TE buffer (10 mM Tris-HCl, pH 8.0; 0.1 mM EDTA) in 1/10 of the supernatant volume.

Biotinylation

SBTI (Type I-S, Sigma-Aldrich Denmark A/S) was biotinylated by using EZ-Link™ NHS-SS-Biotin (Pierce, USA) following the manufactures instructions. Finally the biotinylated SBTI was gelfiltrated into 10 mM Tris-HCl, pH 7.4; 150 mM NaCl and stored at 4 °C. The concentration was estimated from the light absorption at 280 nm and the assumption that the $A(1\%)_{280}$ value for biotin-SBTI is 1 g L^{-1} cm^{-1}.

Selection on library

For each selection, 5×10^{11}–9×10^{12} cfu phage were blocked in a total volume of 1 mL of PBS (140 mM NaCl; 2.7 mM KCl; 8.1 mM Na_2HPO_4; 1.1 mM KH_2PO_4) containing 3% (w/v) skimmed milk powder for 20 min at room temperature. For the selections including deselection, the GGIQGRGSGK peptide (Clonestar Biotech, Czech Republic) or the peptide and BPTI (Type I-P, Sigma-Aldrich Denmark A/S), respectively, were included in the blocking step. The final concentration of peptide and BPTI, respectively, was 200 μg mL^{-1}. Streptavidin-coated M280 Dynabeads (20 μL, 10 mg beads mL^{-1}, Dynal Biotech, Norway) were coated with 10 μg biotinylated SBTI in 1 mL of PBS containing 3% skimmed milk powder at room temperature for 30 min. The magnetic beads were isolated using a magnet (Dynal Biotech, Norway) and unbound biotinylated SBTI was removed by washing the beads (once with PBST (PBS containing 0.1% (w/v) TWEEN 20) and twice with PBS). The blocked phage were mixed and incubated with the SBTI-coated magnetic beads. After 30 min at room temperature the beads were washed (twice with PBST and PBS, respectively) and bound phage were eluted by incubation in 1 mL of PBS containing 50 mM DTT for 30 min at room temperature. An excess of exponentially growing TG1 cells were infected with the eluted phage, plated on TYE agar containing 100 mg L^{-1} ampicillin and 2% glucose and incubated overnight at 30 °C. Colonies were pooled and used to produce new phage. The phage titer after selection was estimated from a serial dilution of a sample of the infected cells and colony counting.

Phage and competition ELISA assay

Streptavidin-coated wells (NUNC A/S, Denmark) were coated with biotinylated SBTI (10 μg mL^{-1}; 100 μl/well) in 150 mM NaCl; 100 mM Tris-HCl, pH 8.0; 0.1% Tween 20 at 37 °C for one hour. Wells were washed (once with PBST and twice with PBS) and blocked with PBS containing 3% skimmed milk powder for one hour at 37 °C. After washing the wells (once with PBST and twice with PBS), 100 μL pre-blocked phage (5×10^{10}–9×10^{11}cfu) were applied/well in PBS containing 3% skimmed milk powder. For the competition ELISA assay, BPTI or peptide substrate, respectively, to a final concentration of 200 μg mL^{-1} was included in the blocking step. After one hour incubation at room temperature, the wells were washed (five times with PBST and PBS, respectively) and bound phage were detected using the HRP/Anti-M13 Monoclonal Conjugate (Amersham Biosciences, Denmark) at a 1/2000 dilution and the TMB One-Step Substrate System (DAKO, Denmark) following the manufactures instructions. The ELISA signal was determined by reading the plate at 450 nm.

Enzyme activity assay

The enzyme activity was determined by using the chromogenic FX_a substrate S2222 (*N*-benzoyl-L-isoleucyl-L-glutamylglycyl-L-arginine-*p*-nitroaniline, Chromogenix, Italy). 100 μL phage suspension (2.5×10^{11}–4.5×10^{12}cfu) in 100 mM NaCl; 50 mM Tris-HCl, pH 8.0; 1 mM $CaCl_2$ was mixed with 50 μL of 3 mM S2222. Activity was determined by recording the formation of *p*-nitroaniline at 405 nm after 19 hours of incubation at room temperature.

Mutagenesis

The glycine at position 195 of one specific clone was back-mutated to serine by using the QuikChange™ Site-directed Mutagenesis Kit (Stratagene, USA) and the primers 5′-GCC-AGGGCGACAGTG GCGGCCCC and 5′-GGCCGCCACT-GTCGCCCTGGCAGG.

Nucleotide sequence analysis

Nucleotide sequencing was carried out using the BigDye™ Terminator version 3.0 DNA sequencing Kit (Applied Biosystems, Sweden).

Acknowledgments

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