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Selection of catalytically active biotin ligase and trypsin mutants by phage display

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Phage display has been shown to facilitate greatly the selection of polypeptides with desired properties by establishing a direct link between the polypeptide and the gene that encodes it. However, selection for catalytic activities displayed on phage remains a challenge, since reaction products diffuse away from the enzyme and make it difficult to recover catalytically active phage–enzymes. We have recently described a selection methodology in which the reaction substrate (and eventually the reaction product) is anchored on calmodulin-tagged phage–enzymes by means of a calmodulin binding peptide. Phage displaying a catalytic activity are physically isolated by means of affinity reagents specific for the product of reaction. In this study, we investigated the efficiency of selection for catalysis by phage display, using a ligase (the *Escherichia coli* biotin ligase BirA) and an endopeptidase (the rat trypsin His57→Ala mutant) as model enzymes. These enzymes could be displayed on phage as fusion proteins with calmodulin and the minor coat protein pIII. Both the display of functional enzyme and the efficiency of selection for catalysis were significantly improved by using phage vectors, rather than phagemid vectors. In model selection experiments, phage displaying BirA were consistently enriched (between 4-fold and 800-fold) per round of panning, relative to negative controls. Phage displaying the trypsin His57→Ala mutant, a relatively inefficient endopeptidase which cleaves a specific dipeptide sequence, were enriched (between 15-fold and 2000-fold), relative to negative controls. In order to improve the catalytic properties of the trypsin His57→Ala mutant, we constructed a combinatorial phage display library of trypsin mutants. Selection of catalytically active phage–enzymes was evidentiated by increasing phage titres at the different rounds of panning relative to negative control selections, but mutants with catalytic properties superior to those of trypsin His57→Ala mutant could not be isolated. The results obtained provide evidence that catalytic activities can be recovered using phage display technology, but stress the importance of both library design and stringent biopanning conditions for the recovery of novel enzymes.

Keywords: biotin ligase/Bir A/calmodulin/phage display/trypsin

Introduction

The development of enzymes with improved catalytic properties or novel enzymatic functions is a major challenge in biotechnology (Fessner, 1999). Many attempts have been made to endow enzymes with new catalytic activities. One general strategy involves the creation of random combinatorial libraries of mutants associated with an efficient screening or selection scheme. Phage display has been shown to facilitate greatly the selection of polypeptides with desired properties by establishing a direct link between the polypeptide and the gene that encodes (Smith, 1985; Wells *et al.*, 1987; Winter *et al.*, 1994; Greisman and Pabo, 1997). However, selection of phage-displayed enzymes for novel (or improved) catalytic activities remains a challenge, as reaction products (which represent the biochemical memory of the reaction catalysed by the phage–enzyme particle) diffuse away after the reaction is complete, hindering the physical isolation of catalytically active phage. Several enzymes have been expressed on the surface of filamentous phage (Demartis *et al.*, 1999; Legendre *et al.*, 2000). In principle, one could challenge enzyme phage libraries with suicide inhibitors (Soumillion *et al.*, 1994; Legendre *et al.*, 2000; Vanwetswinkel *et al.*, 2000) or transition state analogues (Widersten and Mannervik, 1995). A more general methodology, however, would feature the physical isolation of the phage–enzymes, which convert the substrate of the reaction into the desired product. Such a methodology would directly select for catalysis and would not require knowledge of the structure of the transition state and of the catalytic mechanism.

A number of selection strategies have been proposed, which aim at anchoring the reaction product on the surface of catalytically active phage–enzyme, followed by the physical isolation of the desired phage–enzymes by means of anti-product affinity reagents (Pedersen *et al.*, 1998; Atwell and Wells, 1999; Demartis *et al.*, 1999; Jestin *et al.*, 1999). One of these strategies, recently described by our laboratory, features the stable (but non-covalent) anchoring of reaction substrates on calmodulin-tagged phage–enzymes by means of calmodulin-binding peptide derivatives (Demartis *et al.*, 1999). In our selection scheme, the phage–enzyme catalyses the intramolecular conversion of substrate into product, which cannot dissociate from the phage, since it is anchored to the minor coat protein pIII by means of the stable calmodulin/peptide complex (K_d = 2 pM). Phage displaying the product of reaction can be physically isolated from non-catalytically active phage using anti-product affinity reagents. The calmodulin–peptide complex can be dissociated in a number of ways, e.g. by addition of calcium chelators, liberating the phage–enzymes which can be amplified by bacterial infection.

The selection scheme of Demartis *et al.* (Demartis *et al.*, 1999) was tested in a number of model selection experiments, including those with a calmodulin-tagged subtilisin mutant displayed on phage by means of a phagemid vector, which showed enrichment factors up to 54-fold, relative to negative controls. The paper demonstrated that, among other parameters,

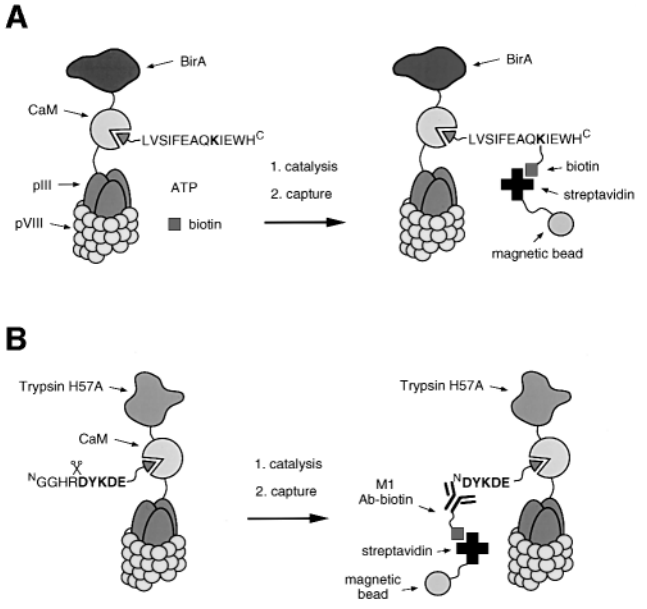

Fig. 1. Scheme for phage selection of enzymatic activity. Calmodulin-binding peptides are used to anchor reaction substrates and products on calmodulin-tagged phage–enzymes. Active phage–enzymes are isolated by specific anti-product reagents. (**A**) Biotin ligase BirA catalyses the biotinylation of lysine in the substrate peptide, containing the LVSIFEAQKIEWH moiety. The reaction product is captured by streptavidin. (**B**) The mutant trypsin H57A cleaves the anchored peptide C-terminally to the amino acid sequence His–Arg. The resulting peptide with an N-terminal DYKDE sequence is captured with the biotinylated monoclonal antibody M1.

good levels of functional enzyme display on phage were essential in order to achieve selection for catalysis. In this work, we aimed at characterizing and improving the parameters which determine the efficiency of selection for catalysis by phage display. We chose a ligase (the *Escherichia coli* biotin ligase BirA) and an endopeptidase (the rat trypsin His57→Ala mutant) as representative model enzymes. BirA is a biotin ligase which catalyses the formation of biotinyl-5′-adenylate from biotin and ATP followed by a covalent attachment of biotin on to a specific lysine residue of the biotin carboxyl carrier protein (BCCP). The trypsin His57→Ala mutant (H57A) is an endopeptidase, which cleaves polypeptides containing the His–Lys or His–Arg substrate motif (Corey *et al.*, 1995) by a mechanism of substrate-assisted catalysis (Carter and Wells, 1987). While the trypsin H57A mutant bears an exquisite substrate specificity, its turnover number k_{cat} is reduced more than 10 000-fold compared with wild-type trypsin. The two enzymes were displayed on phage as fusion protein with calmodulin and the minor coat protein pIII, using novel phage vectors.

Phage displaying the calmodulin-tagged active enzyme were captured with anti-product reagents according to the selection scheme depicted in Figure 1. For BirA selections, we used the peptide GAAARWKKAFIAVSAANRFKKISTSGGGP-GG**LVSIFEAQKIEWH** as reaction substrate, which contains a sequence capable of high-affinity binding to calmodulin (underlined) fused to a biotinylatable peptidic tag (bold) (Schatz, 1993). The lysine residue of the peptide moiety LVSIFEAQKIEWH is biotinylated. Selections with trypsin mutants were performed with peptide GG**HR***DYKDE*GGG-AAARWKKAFIAVSAANRFKKIS, which contains a calmodulin binding sequence (underlined), fused to a peptidic appendix (bold), which can be cleaved by the trypsin H57A mutant. Upon cleavage, the resulting calmodulin-binding peptide displays an N-terminal DYKDE sequence (italics), which is bound specifically by the M1 antibody (Demartis *et al.*, 1999). The DYKDE sequence is not bound by the M1 antibody when it is located in internal peptide positions (Hopp *et al.*, 1988; Huber *et al.*, 1999). We demonstrated in model selection experiments that phage–enzymes could be enriched on the basis of their catalytic activity and we investigated the parameters which dictate the efficiency of selection. Finally, a phage library of trypsin mutants was constructed, characterized and selected, with the aim of isolating improved trypsin mutants, which were able to cleave the His–Lys or His–Arg substrate motifs.

Materials and methods

Materials

The *E.coli* strain TG1 (K12, Δ(*lac-pro*), *sup*E, *thi*, *hsdD5*/F′*traD*36, *proA*$^+$B$^+$, *lacI*^{q}, *lacZ*ΔM15) was used as a host for phage production and gene expression. Peptides were synthesized and purified by HPLC as described (Montigiani *et al.*, 1996). Peptide identity was confirmed by amino acid analysis and by MALDI-TOF mass spectrometry (Protein Service Labor, ETH Zürich).

The fluorogenic substrates benzyloxycarbonyl-Gly-Gly-Arg-4-methyl-7-aminocoumarin and benzyloxycarbonyl-Gly-His-Arg-4-methyl-7-aminocoumarin were purchased from Bachem Bioscience (Bubendorf, Switzerland).

Ampicillin was purchased from Applichem (Darmstadt, Germany) and tetracycline from Sigma (Buchs, Switzerland). All the restriction enzymes and polymerases were purchased from Appligene Oncor (Basel, Switzerland), Axon Lab (Baden, Switzerland) and Boehringer Mannheim (Rotkreuz, Switzerland). T4 DNA ligase was obtained from Eurogentec (Seraing, Belgium). DNA primers were purchased from Eurogentec (Seraing, Belgium) and Microsynth (Balgach, Switzerland).

Construction of vectors

DNA manipulations were performed according to standard protocols (Sambrook *et al.*, 1989).

pCHH1 is a derivative of fd-tet-DOG1 (Clackson *et al.*, 1991) in which the multiple cloning site was altered. An insert containing a unique restriction site *Sfi*I was prepared by polymerase chain reaction (PCR) using the primers Bsrfdba and LeaderSfifo and fd-tet-DOG1 as template. The PCR product was digested with the restriction enzymes *Bsr*GI and *Sfi*I and ligated into fd-tet-DOG1.

pCHH2 was constructed by cloning the genes of rat anionic trypsin II, a 13-amino acid linker and calmodulin sequentially into the vector pCHH1. The cDNA of rat anionic trypsin II was obtained from rat pancreas using a reverse transcriptase (Pharmacia, Dübendorf, Switzerland), followed by PCR amplification using the primers Tyba and Tyfo. The gene coding for the 13-amino acid linker and calmodulin was PCR amplified from the vector pSD4 (Demartis *et al.*, 1999) with the primers Linkba and CaMfoNot. An *Sfi*I restriction site and an overlapping sequence were appended to the trypsin gene in a PCR reaction using the primers SfincobaTsn and Linkfo. The two PCR products were assembled in a PCR reaction with the primers SfincobaTsn and CaMfoNot and ligated into the restriction sites *Sfi*I and *Not*I of the vector pCHH1.

pCHH3 is a derivative of pCHH1 which harbors the genes of trypsin H57A, a 13-amino acid linker and calmodulin. The

vector was cloned with the same strategy as for pCHH2. The mutation H57A was inserted into the trypsin gene as follows. Two gene fragments were PCR amplified with the primer pairs SfincobaTsn/Ty57Afo and Ty57Aba/Linkfo, respectively. The two PCR fragments were assembled and amplified with the primers SfincobaTsn and Linkfo.

pCHH4 is a derivative of pCHH1 in which a dummy DNA fragment of 930 base pairs was inserted into the restriction sites *Sfi*I and *Not*I.

pSD17 is a phagemid vector based on pSD4 (Demartis *et al.*, 1999). The biotin ligase gene of *E.coli* was PCR amplified from vector pGEX-BirA (a kind gift from Prof. Petri Saviranta, University of Turku, Finland) (Saviranta *et al.*, 1998) with the primers BirbaNco1 and BirfoNot and cloned into the *Nco*I and *Not*I sites of pSD4.

pSD22 is an fd-tet-DOG1 (Clackson *et al.*, 1991) based vector in which the genes of BirA, a 13-amino acid linker and calmodulin were inserted. The fused genes of BirA, the 13-amino acid linker and calmodulin were PCR amplified from pSD17 with the primers BirbaApa1 and PhVecCamfoXho and subcloned into the *Apal*I and *Xho*I sites of fd-tet-DOG1.

pCHH5 is a vector for the expression of hexahistidine-tagged wild-type trypsin. The gene of rat anionic trypsin II was inserted into the *Sfi*I and *Not*I sites of the expression vector pDN321 which is a derivative of pUC119SNpolymyc (Neri *et al.*, 1996). The gene was PCR amplified from pCHH2 with the primers SfincobaTsn containing the *Sfi*I restriction site and TsnHisGlyfo containing the *Not*I restriction site and a hexahistidine tag.

pCHH6 was constructed following the same strategy as for the vector pCHH5. It harbors the trypsin mutant H57A instead of the wild-type trypsin. The gene for the mutant trypsin was PCR amplified from pCHH3 with the primers SfincobaTsn and TsnHisGlyfo.

pCHH7 is a vector for the expression of the hexahistidine-tagged trypsin mutant H57K. The mutation H57K was introduced into wild-type trypsin as follows. Two gene fragments were PCR amplified with the primer pairs Sfincobatsn/H57Kfo and TsnHisGlyfo/tsnlib-mut55-57ba, respectively. These two PCR fragments were assembled and amplified with the primers Sfincobatsn and TsnHisGlyfo. The resulting gene was cloned with *Sfi*I/*Not*I sites into pDN321, a derivative of pUC119SN polymyc (Neri *et al.*, 1996).

DNA primers: BirbaApa1, 5′-GGCGGCCACAGTGCACAGGTCCAAGATAACACCGTGCCACTGAAATTG-3′; BirbaNco1, 5′-AATCGACCCATGGCCCAGGTCCAGGATAACACCGTGCCACTGAAATTG-3′; BirfoNot, 5′-GAGTCATTCTGCGCCGCGTTTTCTGCACTACGCAGGGATATTTC-3′; Bspbafd, 5′-CCGTTTAATGGAAACTTCCTCATG3′; Bsrfdba, 5′-TACGTTGATTTGGGTAATGAATATC-3′; CaMfonot, 5′-CCGATTGCGGCCGCCTTTGCTGTCATCATTTGTACA-3′; H57Kfo, 5′-CTTGGATGCGGGACTTATAGCATTTAGCTGCAGACACCACCCA-3′; LeaderSfifo, 5′-TGATGCGGCCGCGGCTATGGCCGGCTGGGCCGCATAGAAAGGAACAACTAAAGGAAT-3′ Linkba, 5′-ACCGCCACTTCCACCGCCACCAGAACCACCGTTGGCAG-3′; Linkfo, 5′-CCACCAGAACCACCGTTGGCAGCAA TTGTGTCCTGA-3′; PhVecCamfoXho, 5′-GGCCCGCTCGAGCTTTGCTGTCATCATTTGTACAAAC-3′; SFINCOBATSN, 5′-ATGCGGCCCAGCCGGCCATGGCCATCGTTGGAGGATACACCTGC-3′; TsnHisGlyfo, 5′-CCATTGCGGCCGCTATCAGTGATGGTGATGGTGATGGCCACCGTTGGCAGCAATTGTGTCCTG-3′; Ty57Aba, 5′-GTGTCTGCAGCTGCTTGCTATAAGTCCCGCATCCAAG3′; Ty57Afo, 5′-CTTATAGCAAGCAGCTGCAGACACCACCCACTGG-3′

Phage preparation and characterization

Phages were produced in *E.coli* TG1 cells at 20°C for 20 h in 2YT medium supplied with tetracycline (10 μg/ml final concentration), isolated and purified using polyethylene glycol (PEG) according to standard procedures (Nissim *et al.*, 1994).

The number of infective phage particles was determined by incubating phage with an excess of exponentially growing *E.coli* TG1 cells for 30 min at 37°C and plating them immediately. The concentration of physical phage particles was measured by absorption spectroscopy (Day *et al.*, 1978).

The display of protein on phage was analysed by Western blotting. A total of 5×10^8 infective phage were denatured by heating at 90°C for 5 min in the presence of 20 μl of reducing SDS-sample buffer. The samples were loaded on a 10% (w/v) polyacrylamide gel (Novex, Frankfurt, Germany), separated by electrophoresis and transferred on to a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany). The membrane was blocked with PBS buffer [phosphate-buffered saline; 50 mM phosphate (pH 7.4), 100 mM NaCl] containing 1% (w/v) milk powder and proteins were detected with an anti-pIII murine monoclonal antibody (Tesar *et al.*, 1995) followed by a secondary anti-mouse HRP-goat IgG antibody (Sigma, St. Louis, MO). The peroxidase activity was detected by electrochemiluminescence using an ECL kit from Amersham (Dübendorf, Switzerland).

Calmodulin binding activity was assayed by phage ELISA. Phages were incubated with the biotinylated peptide CAAARWKKAFIAVSAANRFKKIS, washed, blocked and immobilized on streptavidin-coated plates (Boehringer Mannheim, Rotkreuz, Switzerland) as described (Demartis *et al.*, 1999).

Fluorogenic activity assays of wild-type trypsin displayed on phage (pCHH2) were performed in 50 mM Tris–HCl pH 8.0, 20 mM $CaCl_2$, 100 mM NaCl at 25°C using Z-Gly-Gly-Arg-AMC (AMC = aminomethylcoumarin) as substrate. Fluorescence measurements were carried out with an excitation wavelength of 381 nm and an emission wavelength of 455 nm. Typically, 50 μl of phage [5×10^{11} transducing units (t.u.)/ml] were added to 950 μl of buffer containing 50 mM Z-Gly-Gly-Arg-AMC substrate.

Model selection experiments with trypsin H57A

Trypsin H57A–calmodulin–phage were blocked in TBSC (50 mM Tris–HCl pH 7.4, 100 mM NaCl, 1 mM $CaCl_2$) containing 3% (w/v) BSA for 30 min at room temperature. These phages (10^{10} t.u.) were incubated with the peptides DYKDEGGGAAARWKKAFIAVSAANRFKKIS, GGHRDYKDEGGGAAARWKKAFIAVSAANRFKKIS, GAAHYDYKDEGGGAAARWKKAFIAVSAANRFKKIS and GDDDDKDYKDEGGGAAARWKKAFIAVSAANRFKKIS in individual reactions. The final concentration of peptide was 1 μM in a 400 μl volume (5 mM $CaCl_2$, 20 mM Tris–HCl pH 8, 100 mM NaCl). Reactions were carried out for 30 min at 37°C, then the unbound peptide was removed by precipitating the phage with 100 μl of ice-cold PEG solution [20% (w/v) polyethylene glycol, 2.5 M NaCl) followed by centrifugation at 13 000 r.p.m. for 1 min. The phage pellet was dissolved in 100 μl of TBSC containing 1% (w/v) milk powder and incubated with 1 μg/ml biotinylated M1 anti-FLAG antibody (Kodak, New Haven, CT) for 20 min at room temperature. A 50 μl volume of pre-blocked streptavidin-coated M280

Dynabeads (10 mg beads/ml; Dynal, Oslo, Norway) were added and mixed for 20 min. The magnetic beads were captured on a magnet (Dynal) and washed seven times with TBSC–Tween-20 (0.1%) and four times with TBSC. Phages were eluted in 300 µl of TBS (50 mM Tris–HCl pH 7.4, 100 mM NaCl) containing EDTA (10 mM), saturated with calcium chloride and used to infect exponentially growing TG1 cells (OD = 0.4). The cells were plated on 2YT agar plates containing tetracycline (10 µg/ml).

Model selection experiments with BirA

BirA–calmodulin–phage (pSD22, 5×10^8 t.u.) pre-blocked in TBSC–BSA (3%, w/v) were incubated with the peptide GAAARWKKAFIAVSAANRFKKISTSGGGPGGLVSIFE-AQKIEWH (10^{-7} M), 0.1 M biotin, 10 mM ATP, 12 mM $MgCl_2$, 1 mg/ml inorganic pyrophosphatase (Sigma) and 0.06% Tween-20 in a total volume of 200 µl of TBSC. In negative control reactions either biotin or peptide was omitted, and in positive control reactions the phage were incubated with the biotinylated CAAARWKKAFIAVSAANRFKKIS peptide (Demartis *et al.*, 1999). All individual reactions were incubated for 20 min at room temperature. Phage were then PEG precipitated twice (as described above) and resuspended in 200 µl of TBSC containing milk powder (3%, w/v) and BSA (1 mg/ml). Phage were captured, washed and eluted from streptavidin-coated beads as described (Demartis *et al.*, 1999).

In competition experiments, phage of two different species were mixed and captured as described above. After selective elution, phage was used to infect *E.coli* bacteria. The primers LMB3 (5′-CAGGAAACAGCTATGAC-3′) and fdseq (5′-GAATTTTCTGTATGAGG-3′) were used for PCR screening of individual colonies.

Construction of the trypsin library

The trypsin library was constructed using partially degenerate primers in a PCR-based method, which introduced random mutations at positions 55, 57, 102 and 214 of rat anionic trypsin II. Primers containing the codon 5′-NNK-3′ were used. They allow the integration of all 20 amino acids and only one stop codon. The stop codon UAG is suppressed in E-suppressor strains, such as TG1. For residue 57, the codon 5′-DNK-3′ was used to knock out histidine in this position. This codon also prevented the amino acids Pro and Gln at this position. Four overlapping PCR fragments were produced with the primer pairs Bspbafd/Tsnlin-mut55–57fo, Tsnlib-mut55–57ba/Tsnlib-mut102fo, Tsnlib-mut102ba/Tsnlib-mut214fo and Tsnlib-mut214ba/fdseq1 (15 s at 94°C, 45 s at 56°C, 1 min at 72°C for 10 cycles and 15 s at 94°C, 1 min at 58°C, 2 min at 72°C for 15 cycles). Gel-purified PCR fragments were assembled in a single reaction with the primers Bspbafd and fdseq1, double-digested with *Sfi*I/*Not*I and ligated into 5 µg of *Sfi*I/*Not*I-digested pCHH4 phage vector. A 930 bp dummy fragment between the restriction sites *Sfi*I and *Not*I of pCHH4 allowed a good separation of doubly digested vector from an agarose gel (0.8%, w/v), from which the DNA was recovered with QIAEX II gel extraction kit (Qiagen, Hilden, Germany). The ligation product was purified in a phenol extraction followed by ethanol–sodium acetate precipitation and washed twice with 70% ethanol. The vector was transformed into electrocompetent TG1 *E.coli* cells, which were then plated on agar plates supplemented with 10 µg/ml tetracycline at 30°C overnight. The colonies were pooled by washing the large plates (20 cm diameter) with 7.5 ml of 2YT + 2.5 ml of glycerol. Cells were frozen in liquid nitrogen and stored at –80°C. Phage were produced and purified as described above.

DNA primers: Bspbafd, 5′-CCGTTTAATGGAAAC-TTCCTCATG-3′; fdseq1, 5′-GAATTTTCTGTATGAGG-3′; Tsnlib-mut102ba, 5′-ATCATGCTGATCAAGCTCTCTTC-3′; Tsnlib-mut102fo, 5′-GAAGAGAGCTTGATCAGCATGATM-NNATTGTTCAGGGTCTTCCTATCG-3′; Tsnlib-mut214ba, 5′-TGGGGCTATGGCTGTGCCCTGC-3′; Tsnlib-mut214fo, 5′-GCAGGGCACAGCCATAGCCCCAMNNGACAATGCCC-TGCAGCTCTCC-3′; Tsnlib-mut55-57ba, 5′-TGCTATAAG-TCCCGCATCCAAG-3′; Tsnlib-mut55-57fo, 5′-TTGGATGC-GGGACTTATAGCAMNHAGCMNNAGACACCACCCA-CTGGTCAT-3′.

Trypsin library selection

Biopanning experiments on the trypsin library were performed essentially as described in the section Model selection experiments with trypsin H57A. Between 10^9 and 10^{11} phage were subjected to selection in each round of panning. The washing conditions were less stringent in the first round of selection [three times with TBSC–Tween-20 (0.1%) and three times with TBSC]. The reaction time was reduced to 1 min in the third and fourth rounds of panning; however, the reaction could proceed for some time during the PEG precipitation step. Eluted phages were used to infect an excess of exponentially growing TG1 cells, which were then plated on 2YT agar plates containing tetracycline (10 µg/ml). The colonies were pooled and used to produce new phages.

Expression and purification of trypsin

Trypsin mutants were PCR-amplified from single colonies using the primers Sfincobatsn and TsnHisGlyfo. The primer TsnHisGlyfo adds a hexahistidine tag C-terminally to the trypsin mutant. PCR products were *Sfi*I/*Not*I digested and ligated into the expression vector pDN321, a derivative of pUC119SNpolymyc (Neri *et al.*, 1996).

The vector contains a PelB leader sequence, which directs the recombinant proteins into the periplasm. A 5 ml overnight culture of *E.coli* strain TG1 containing the mutant plasmids was used to inoculate 400 ml of 2YT medium containing 100 µg/ml ampicillin and 0.1% glucose. Cells were grown at 37°C with vigorous shaking until an OD_{600} of 0.6 was reached. Protein expression was induced by adding IPTG to a final concentration of 1 mM. Cells were incubated for an additional 12 h at 20°C and then harvested. The periplasmic fraction was obtained as described (Hennecke *et al.*, 1997). A modified extraction buffer (50 mM Tris–HCl pH 8, 300 mM NaCl, 1 mM EDTA and 1 mg/ml polymyxin) was used which is compatible with nickel affinity chromatography purification.

The native fraction of the expressed protein was purified by nickel affinity chromatography. An 8 ml volume of periplasmic extract (from a 400 ml culture) was supplied with imidazole (15 mM final concentration) and $MgCl_2$ (final concentration 1 mM), incubated with 200 µl of Ni-NTA agarose (Qiagen) and mixed for 60 min on a rotary shaker (200 r.p.m.) at 4°C. The lysate–Ni-NTA mixture was loaded into a column (Bio-Rad, Glattbrugg, Switzerland) and washed twice with 6 ml of a buffer containing 50 mM Tris–HCl pH 8, 300 mM NaCl and 15 mM imidazole by gravity flow. The enzyme was eluted in 200 µl of buffer containing 50 mM Tris–HCl pH 8, 300 mM NaCl and 250 mM imidazole. Alternatively, the column was first washed with 6 ml of a denaturing buffer (50 mM Tris–HCl pH 8, 300 mM NaCl, 4 M urea and 15 mM

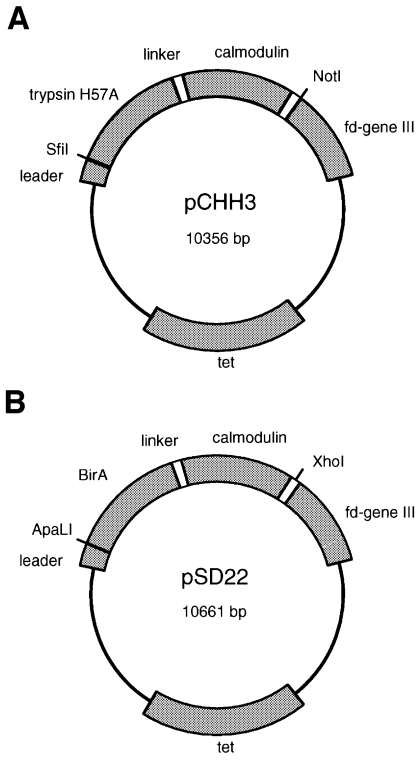

Fig. 2. Schematic representation of the phage vectors for the display of calmodulin-tagged enzyme on filamentous phage. Leader, fd-gene III leader sequence; trypsin H57A, gene of rat anionic trypsin II with the mutation H57A; linker, 13-amino acid linker GGSGGGGSGGGGS; tet, tetracycline resistance gene; BirA, biotin ligase gene. (**A**) Vector pCHH3 harbors the genes for the fusion protein of fd-gene III leader sequence, trypsin H57A, calmodulin and fd-protein III. (**B**) pSD22 contains the genes for the fusion protein of fd-gene III leader sequence, BirA, calmodulin and fd-protein III.

imidazole) in order to remove ecotin, which is bound to some of the trypsin mutants.

Activity measurement of mutant trypsin

Fluorescence assays were performed by following the release of free aminomethylcoumarin (AMC) from the substrates Z-Gly-Gly-Arg-AMC and Z-Gly-His-Arg-AMC. Time-dependent fluorescence intensity was measured with a VersaMAX Gemini plate spectrofluorimeter (Molecular Devices, Sunnyvale, CA). A typical assay consisted of a 100 µl volume of 50 mM Tris–HCl pH 8.0, containing 10 mM $CaCl_2$, 100 mM NaCl, 5 µM–2 mM Z-Gly-Gly-Arg-AMC or Z-Gly-His-Arg-AMC and 0.1–1 µM of a trypsin mutant at 25°C for 2 h. Fluorescence measurements were carried out with an excitation wavelength of 381 nm and an emission wavelength of 455 nm.

Results

Display of calmodulin-tagged enzymes on phage

The efficient display of functional enzyme–calmodulin fusion on phage is an absolute prerequisite for the selection of enzymatic activities according to the strategy outlined in Figure 1 and described in the Introduction. In this study, we displayed the two enzymes trypsin H57A and BirA as calmodulin–pIII fusion on filamentous phage.

The gene of trypsin H57A was fused to the calmodulin gene and cloned into the phage vector pCHH1, a derivative of fd-tet-DOG1 (Clackson *et al.*, 1991). The resulting vector pCHH3 is shown in Figure 2a. Bioactive rat anionic trypsin II was

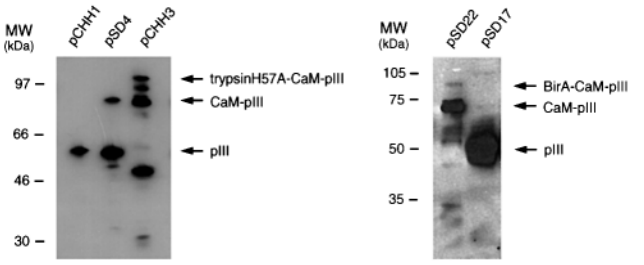

Fig. 3. Western blot analysis of enzyme–calmodulin–pIII fusion protein. Denatured proteins from phages were loaded on a 10% (w/v) polyacrylamide gel, separated by electrophoresis and transferred on to a nitrocellulose membrane. The pIII and pIII fusion proteins were immunostained with mouse monoclonal antibodies against pIII and anti-mouse HRP. Left panel: pCHH1 is a phage vector which displays wild-type pIII on phage; pSD4 is a phagemid vector which displays calmodulin fused to pIII (Demartis *et al.*, 1999); pCHH3 is a phage vector which displays trypsin H57A–calmodulin fusion protein. Right panel: pSD22 (phage vector) and pSD17 (phagemid vector) display BirA–calmodulin fusion protein.

displayed on phage without the pro-peptide, in analogy to wild-type trypsin which had previously been functionally displayed on phage as a fusion to the C-terminal portion (residues 198–406) of pIII (Corey *et al.*, 1993). Here we linked the calmodulin-tagged enzyme to the entire pIII protein (residues 1–406). Fluorogenic activity assays, monitoring the release of free AMC from the substrate Z-Gly-Gly-Arg-AMC, performed on phage displaying wild-type trypsin–calmodulin fusion (pCHH2), showed that the phage–enzyme is catalytically active (data not shown). The activity of trypsin H57A on phage (pCHH3) could not be measured, as the turnover number of this mutant is too low (Corey *et al.*, 1995).

The fusion of BirA and calmodulin genes was cloned into the vector fd-tet-DOG1. The resulting construct (pSD22) comprises the DNA coding for the signal sequence, BirA, a 13-amino acid linker, calmodulin and the gIII protein (Figure 2b). From these vectors, phage were produced using standard procedures (Clackson *et al.*, 1991; Griffiths *et al.*, 1994). For all phage constructs, high titres were obtained ($>10^9$ t.u.) per ml in bacterial supernatants). The functionality of calmodulin displayed on phage was confirmed by phage ELISA, using a biotinylated calmodulin-binding peptide and streptavidin-coated microtitre plates as described (Demartis *et al.*, 1999).

The display of calmodulin-tagged enzyme on phage was further analysed by Western blotting using an anti-pIII monoclonal antibody (Tesar *et al.*, 1995). The immunoblot confirmed that trypsin H57A–calmodulin fusion is efficiently displayed on phage (Figure 3). Additionally, some degradation products of the fusion protein can be observed. The same pattern of bands is observed in the immunoblot of phage displaying the wild-type trypsin–calmodulin fusion (pCHH2). By comparison, trypsin–calmodulin–pIII fusions can hardly be detected in immunoblot experiments, whenever phage particles are produced using phagemid vectors (Demartis *et al.*, 1999). The fusion protein BirA–calmodulin–pIII (pSD22) could be detected by immunoblot experiments, which were dominated by a band corresponding to calmodulin–pIII fusion, indicating extensive proteolysis at the level of the linker between BirA and calmodulin (Figure 3). This low level of functional display of BirA on phage has consequences on the results of the selection experiments (see below).

Model selection experiments with BirA

We systematically investigated the performance of selection for catalysis using BirA–calmodulin phage, according to the

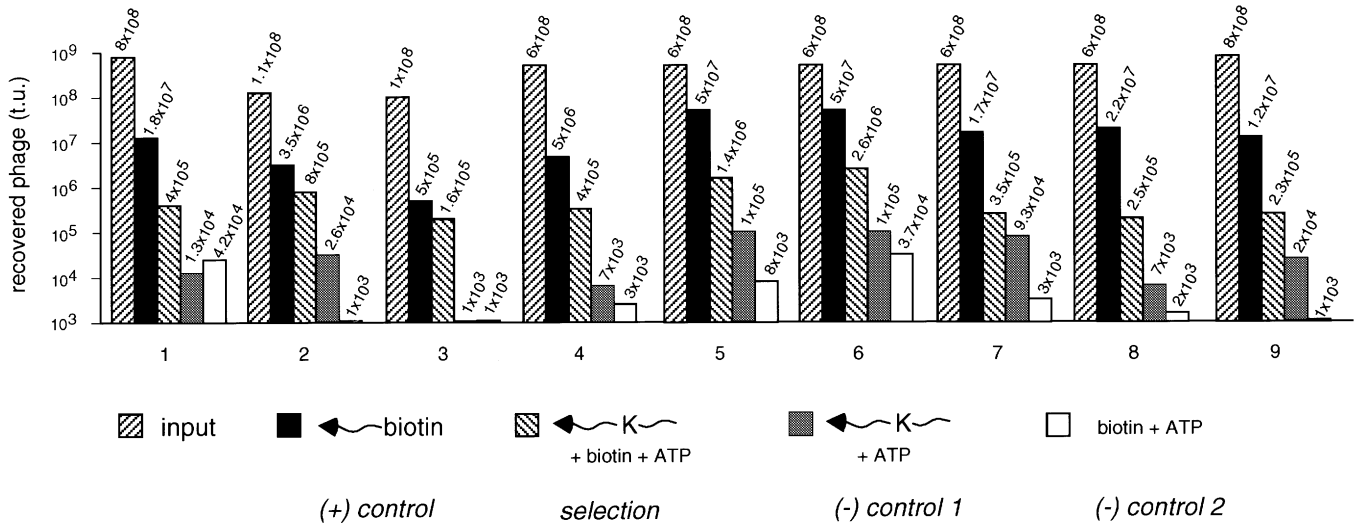

Fig. 4. Model experiments of selection for biotin ligase activity. Phage titres (input titre and titre of rescued phage) are plotted for different selection experiments. The legend indicates the relevant reagents which are included in or omitted from the selection experiments and positive or negative control experiments. Phage titres rescued at the end of the selection experiment are consistently higher than titres in negative control experiments, in which some reaction components are omitted. See also Materials and methods.

strategy described in the Introduction (Figure 1). The selection scheme relies on the efficient capture of catalytically active phage displaying biotinylated peptides, by means of streptavidin-coated magnetic beads and on the selective separation of phage particles from unreacted biotin, by means of PEG precipitation.

In order to monitor biopanning efficiency, control reactions were performed as follows. A biotinylated calmodulin-binding peptide was used as positive control, simulating the reaction product of the biotinylation reaction of Figure 1a. In negative controls, either biotin or the substrate peptide was not added to the reaction mixture. The phage–enzyme (~5×10^8 phage particles in each of the four separate reactions) was allowed to catalyse the reaction for 20 min at room temperature, before PEG precipitation and streptavidin capture. Nine independent sets of experiments were performed, in order to assess the reproducibility of the biopanning results. The phage titres rescued after each individual selection experiment are presented in Figure 4. On average, ~1–10% of the input phage was recovered in positive control selections, performed with the biotinylated peptide, indicating a good level of functional display of calmodulin on phage and an efficient phage capture scheme. Selection experiments performed with peptide GAAARWKKAFIAVSAANRFKKISTSGGGPGGLVSIFEAQKIEWH, biotin and ATP gave consistently higher titres (range: between 4- and 800-fold) compared with negative control experiments, in which either the peptide or biotin had been omitted (Figure 4). Notably, the phage titres rescued in the selection experiments were consistently lower (range: between 3- and 100-fold) than positive control selections, indicating a lower level of BirA display on phage, compared with the level of calmodulin display (Figure 3), with a variability among different batches of phage preparation.

To demonstrate further that the enrichment of enzyme-tagged phage in selection experiments was due to its catalytic activity, BirA–CaM–phage (pSD17) was incubated with phage displaying calmodulin and an irrelevant enzyme (GST–CaM–phage; pSD8) (Demartis *et al.*, 1999) in a 1.5:1 ratio in the presence of substrate peptide and biotin. After reaction, washing of the inactive phage–enzyme and elution with EDTA, phages were used to infect *E.coli* bacteria. After this single round of selection, PCR screening of individual colonies showed that the ratio of BirA to GST phages was 11:1 (enrichment factor = 7). As a negative control, BirA–CaM–phage and GST–CaM–phage (1.5:1 ratio) were incubated in a reaction mixture omitting either biotin or the substrate peptide. In both experiments there was a slight enrichment of the negative control GST phage over BirA phage (enrichment factor = 0.7).

It is tempting to speculate that BirA mutants, with improved folding properties and higher levels of display on phage, should allow an even more efficient recovery of catalytically active phage–enzymes. Under the experimental conditions used, the k_{cat} of BirA towards the biotin carboxyl carrier protein is 0.6 s^{-1} (Xu and Beckett, 1994). Furthermore, it can be excluded that the substrate peptide is biotinylated in an uncatalysed reaction, as k_{uncat} is negligible for this reaction.

Model selection experiments with the trypsin mutant H57A

In model selection experiments, we tested whether phage displaying trypsin H57A can be enriched for catalysis over appropriate negative controls. Selection experiments were performed as depicted schematically in Figure 1b, using the substrate peptide GGHRDYKDEGGGAAARWKKAFIAVSAANRFKKIS and allowing for a 30 min. reaction time (k_{cat} = 0.002 s^{-1} for peptides containing the His–Arg substrate) (Corey *et al.*, 1995). The trypsin mutant H57A displayed on phage recognizes the His–Arg sequence and cleaves the peptide C-terminally to this site. The monoclonal antibody M1 is used as capture reagent (Hopp *et al.*, 1988), binding to peptides carrying the sequence DYKDE (underlined) at the N-terminal extremity, but not peptides with the DYKDE sequence at internal positions (Huber *et al.*, 1999). A PEG precipitation step was performed before phage capture by means of streptavidin-coated magnetic beads, pre-incubated with the biotinylated M1 antibody. In positive control experiments, the peptide DYKDEGGGAAARWKKAFIAVSAANRFKKIS was used, which represents the product of the cleavage reaction. As a negative control, the peptides GAAHYDYKDEGGGAAARWKKAFIAVSAANRFKKIS and GDDDDKDYKDEGGG-

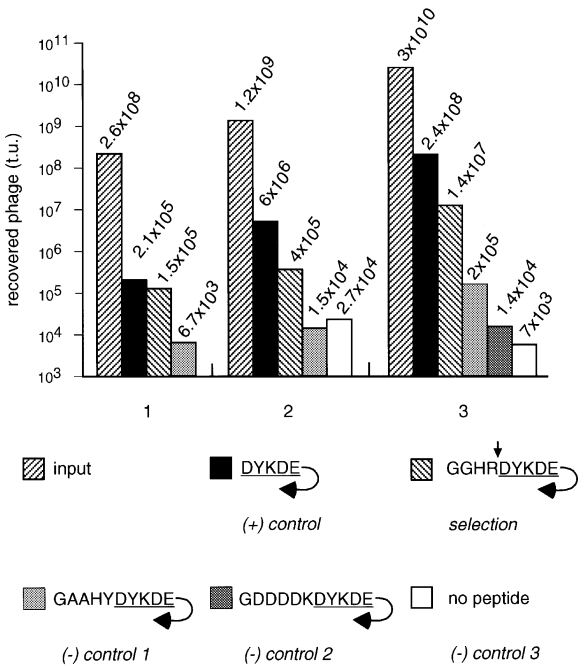

Fig. 5. Model experiments of selection for endoproteolytic activity. Phage titres (input titre and titre of rescued phage) are plotted for different selection experiments. The legend indicates the relevant reagents which are included in or omitted from the selection experiments and positive or negative control experiments. Phage titres rescued at the end of the selection experiment are consistently higher than phage titres in negative control experiments, in which some reaction components are omitted. See also Materials and methods.

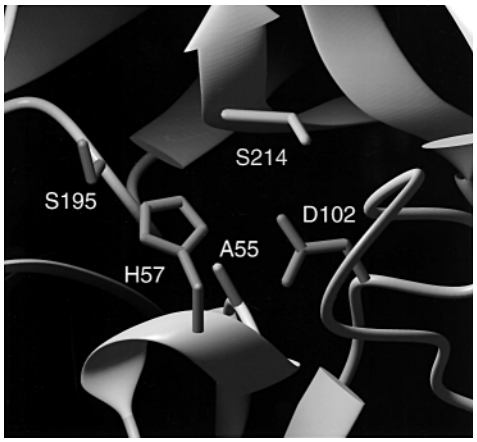

Fig. 6. Ribbon drawing of the S1 pocket of rat anionic trypsin II. Residues S195, H57 and D102 form the catalytic triad. Amino acids H57 and D102 as well as the two amino acids A55 and S214 in the proximity of the active site are randomly mutated in the trypsin library. Histidine is not allowed in amino acid position 57. Atomic coordinates were obtained from the Brookhaven Protein Data Bank (entry 1ANE).

AAARWKKAFIAVSAANRFKKIS were used, which contain only either the His or the Lys residue located N-terminally relative to the DYKDE sequence and are therefore not cleaved by trypsin H57A. In another negative control experiment, no peptide was added to the phage.

Three independent sets of experiments were performed in which phage were incubated with different peptides in five individual reactions. Elution titres of phage–enzyme anchoring the substrate peptide were reproducibly higher (range: between 15- and 2000-fold) compared with the titres of negative control selections (Figure 5). While these results demonstrate that phage displaying trypsin H57A can be enriched for catalysis, phage captured with the peptide DYKDEGGGAAARWKKAFIAVSAANRFKKIS (positive control) gave consistently higher titres (range: between 1.4- and 17-fold) compared with selection for catalysis experiments (Figure 5). The more efficient phage rescue in the positive control experiments can be explained by the fact that not all phage display an enzyme (Figure 3), stressing once again the importance of efficient functional enzyme display on phage. Additionally, the endogenous *E.coli* protein ecotin, a nanomolar inhibitor of trypsin, might partially inhibit the enzymatic reaction on phage (Chung *et al.*, 1983). However, enrichment factors did not improve when phage–enzymes were dialysed prior to selections (data not shown).

Construction of a library of trypsin mutants

In substrate-assisted catalysis (Carter and Wells, 1987), the His residue in the catalytic triad of a serine protease is mutated into a different residue. At the same time, a His residue is inserted in the P2 position of the peptidic substrate. To a certain extent, the His residue in the substrate complements the missing histidine in the catalytic triad, leading to an active protease with a two-amino acid substrate specificity (e.g. His–Lys or His–Arg for the trypsin H57A mutant (Corey *et al.*, 1995). Structural studies of substrate-assisted catalysis of the trypsin mutant H57A revealed that the catalytic histidine residue provided by the substrate does not optimally restore the stereoelectronic arrangement of the catalytic triad (Corey *et al.*, 1995). In particular, it is expected that Asp102 of the catalytic triad may not assist catalysis. We therefore aimed at creating a new trypsin mutant, which maintains the substrate specificity of trypsin H57A and which has an improved catalytic activity.

A trypsin library was constructed which bears mutations in the proximity of the trypsin's active site. We hoped that the residues selected for mutation would complement the catalytic unit formed by Ser195 and the histidine residue provided by the substrate. We combinatorially randomized Ala55, Asp102 and Ser214 (Figure 6). Additionally, His57 was combinatorially mutated with codon DNK, which does not allow for a histidine residue at this position. Ala55 points into the catalytic cavity and interacts with His57, which together with Asp102 and Ser195 constitutes the catalytic triad of the enzyme. Ser214 points towards the catalytic triad and forms a hydrogen bond with Asp102. It has been shown that single mutations of these residues do not disrupt the overall structure of trypsin (Corey and Craik, 1992; McGrath *et al.*, 1992; Takeda-Shitaka and Umeyama, 1998). Using partially degenerate oligonucleotidic primers in a PCR assembly protocol and the phage vector pCHH4 (see Materials and methods), we constructed a library containing $>5\times10^5$ transformants, which was significantly larger than the calculated theoretical complexity of 1.36×10^5 possible amino acid combinations. Sequencing of randomly picked clones indicated that the library had been properly constructed and that the expected diversity was present at the desired positions (Table I). Phage were produced from cells representing the trypsin library, obtained by electroporation. However, we found that this phage population was less infective (0.01%) than the monoclonal phage used in model selections, as determined by measuring transforming units by bacterial infection and number of phage particles by UV absorption methods (Day and Wisemann, 1978).

Table I. Sequence data of trypsin variants in the library and of trypsin variants selected after three and four rounds of panning

Round of panning	Clone	A55[a]		H57[a]		D102[a]		S214[a]	
0	Lib-1	P	CCT	A	GCT	I	ATT	S	AGT
	Lib-2	N	AAT	S	AGT	Y	TAT	H	CAT
	Lib-3	A	GCG	S	TCG	T	ACT	F	TTT
	Lib-4	V	GTT	N	AAT	S	AGT	H	CAT
	Lib-5	T	ACG	T	ACA	A	GCG	Q	CAG
	Lib-6	V	GTT	S	TCT	H	CAT	Y	TAT
	Lib-7	R	CGT	T	ACT	N	AAC	S	TCT
	Lib-8	R	CGG	S	TCT	H	CAT	L	TTG
	Lib-9	Q	CAG	S	AGT	H	CAT	R	CGA
	Lib-10	L	CTG	Y	TAC	T	ACT	Y	TAT
3	3rd-1	I	ATT	T	ACG	N	AAT	P	CCT
	3rd-2	T	ACT	S	TCG	P	CCT	T	ACT
	3rd-3	T	ACT	S	TCG	P	CCT	T	ACT
	3rd-4	N	AAT	T	ACT	P	CCT	F	TTT
	3rd-5	Q	CAG	I	ATT	T	ACG	L	CTT
	3rd-6	L	CTG	S	TCT	T	ACT	I	ATT
	3rd-7	R	CGT	S	TCT	H	CAT	G	GGA
4	4th-1	I	ATT	S	TCT	A	GCT	H	CAT
	4th-2	M	ATG	I	AAT	A	GCT	S	TCG
	4th-3	Q	CAG	F	TTT	K	AAG	L	CTT
	4th-4	K	AAG	G	GGT	T	ACT	A	GCA
	4th-5	P	CCT	A	GCT	N	AAT	H	CAT
	4th-6	P	CCG	S	TCT	T	ACT	D	GAT
	4th-7	S	TCT	G	GGT	P	CCT	H	CAT
	4th-8	F	TTT	Y	TAT	Y	TAT	H	CAG
	4th-9	P	CCT	G	GGT	Q	CAG	H	CAT

[a]The amino acid residues (and the corresponding codons) are listed which were found in clones of the trypsin library at positions 55, 57, 102 and 214. In wild-type trypsin these residues are Ala, His, Asp and Ser, respectively.

Table II. Biopanning experiments with the trypsin library

Round of panning	Phage titre (t.u.)			
	Input[a]	Output positive control[b]	Output selection[c]	Output negative control[d]
1	10^9	2×10^5	10^5	1.5×10^3
2	8×10^{10}	2.6×10^7	1.4×10^7	3.4×10^4
3	10^{11}	2×10^7	1.5×10^7	1.1×10^5
4	3×10^{10}	10^6	5×10^5	1×10^3

[a]Infective phages subjected to the selection.
[b]The peptide DYKDEGGGAAARWKKAFIAVSAANRFKKIS representing the product of the reaction.
[c]The peptide GGHRDYKDEGGGAAARWKKAFIAVSAANRFKKIS representing the substrate of the reaction.
[d]No peptide.

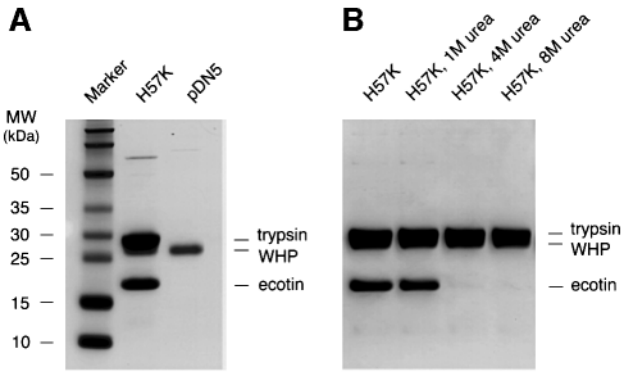

Fig. 7. SDS–PAGE analysis of trypsin mutants expressed in *E.coli* and purified by nickel affinity chromatography. (**A**) Lane 1: trypsin mutant H57K (Corey *et al.*, 1992) purified using plasmid pCHH7. WHP is a contaminant metal binding protein. The endogenous trypsin inhibitor ecotin is co-purified with the trypsin mutant. Lane 2: proteins purified using plasmid pDN5 (Neri *et al.*, 1996) are shown. Plasmid pDN5 is similar to pCHH7, but does not contain the trypsin gene. (**B**) During nickel affinity purification, the column was washed with buffer containing urea in order to remove the ecotin bound to trypsin H57K. This purification procedure was monitored by polyacrylamide gel electrophoresis. Proteins recovered in different washing steps are indicated.

Selection for improved catalysis and characterization of mutant trypsins

Four rounds of panning were performed with the phage library of trypsin mutants described in the previous section, using GGHRDYKDEGGGAAARWKKAFIAVSAANRFKKIS as peptide substrate and following the selection scheme described in Figure 1b. In parallel, positive control selections were performed using the peptide DYKDEGGGAAARWKKAFIAVSAANRFKKIS, and also negative control selections, in which no peptide was added. In the first round of selection, 10^9 infective phage were used, representing each trypsin variant ~10 000 times on average. Enzymes were allowed to react for 20 min at 37°C in the first two rounds of panning, while the reaction time was reduced in the third and fourth rounds of selection. Phage titres rescued at the end of each round of panning are listed in Table II. Surprisingly, elution titres of the first round of selection were enriched more than 60-fold, compared with the negative control. The number of phage captured in this selection was nearly as high as in the positive control. Similar enrichment factors were observed in the three successive rounds of panning.

In order to characterize the selected mutants, their genes were sequenced (Table I) and subcloned into an *E.coli* expression vector, appending a hexahistidine tag at the C-terminus, to facilitate purification of the recombinant protein by nickel-affinity chromatography. On average, 0.1 mg of pure protein was obtained from a 400 ml culture. Purified trypsin variants from the library and variants selected after three rounds of panning were analysed on a Coomassie Brilliant Blue-stained SDS–PAGE gel. Three proteins of different molecular weight were typically visible on the gel (Figure 7). The band with the highest molecular weight represents the trypsin mutant, which was identified by Western blotting using an anti-pentahistidine antibody (data not shown). The protein band immediately below trypsin corresponds to the endogenous WHP protein of *E.coli* (Wolfing *et al.*, 1994). This contaminant protein binds to metal and was therefore purified by nickel affinity chromatography. The protein band migrating at 18 kDa was identified as the trypsin inhibitor ecotin (Chung *et al.*, 1983). This protein was co-purified in the nickel affinity chromatographic procedure, as it binds to trypsin. The fact that ecotin binds to some of the trypsin mutants suggests that at least these mutants are properly folded.

In order to measure the activity of the trypsin mutants, the inhibitor ecotin had to be removed. The purification protocol was therefore modified for trypsin mutants which were shown to co-purify ecotin. During nickel affinity chromatography, ecotin was eliminated by washing the column with 4 M urea (Figure 7). The activity of the trypsin mutant H57K increased by a factor of 4 when ecotin was removed by urea treatment, confirming the validity of the purification procedure used (data not shown). The catalytic properties of the trypsin variants selected after three and four rounds of panning were measured

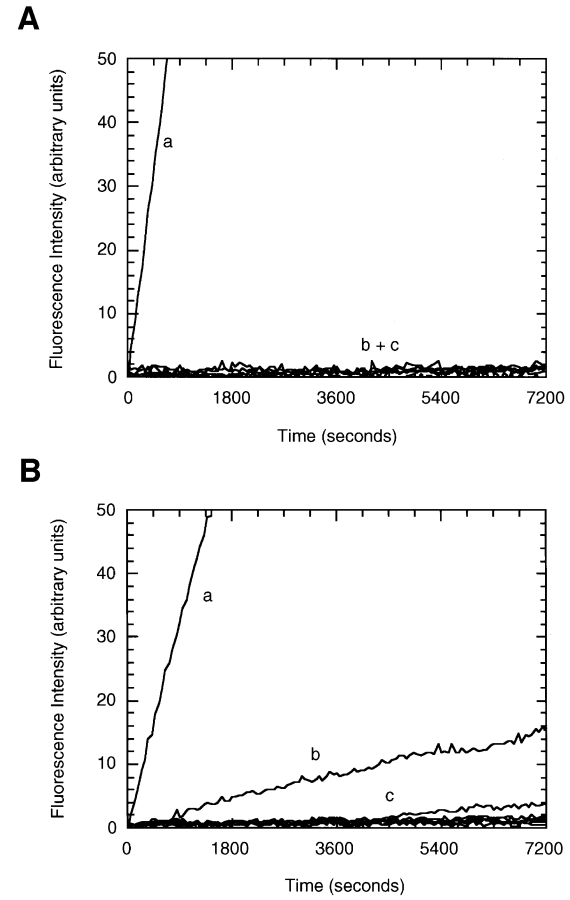

Fig. 8. Fluorogenic activity assay of trypsin mutants. Assays were performed by following the release of free coumarin (AMC) from the tripeptide substrate Z-Gly-Gly-Arg-AMC (**A**) and Z-Gly-His-Arg-AMC (**B**), respectively. (a) Trypsin H57K; (b) trypsin H57A; (c) trypsin mutants selected after three rounds of panning. Protein concentration in the assay was ~0.3 μM.

in fluorogenic assays using the substrates Z-Gly-Gly-Arg-AMC and Z-Gly-His-Arg-AMC. The enzymes selected were expected to cleave preferentially the substrate Z-Gly-His-Arg-AMC, which contains a histidine residue in position P2. The endoproteolytic activity of 30 trypsin mutants was screened. The trypsin mutants H57A and H57K were used as controls (Corey and Craik, 1992). Trypsin mutant H57K cleaved both AMC substrates, whereas trypsin H57A cleaved only the substrate Z-Gly-His-Arg-AMC, as expected (Figure 8).

None of the clones examined, either before or after four rounds of selection, showed an endopeptidase activity comparable to that of trypsin H57A (Figure 8). The results indicate that more work is needed to demonstrate that the selection strategy (Figure 1) can yield desired biocatalysts using large libraries. They also may reflect that the biopanning conditions used were not sufficiently stringent or a wrong choice of the amino acid positions which were combinatorially mutated.

Discussion

Selection strategies, which aim at anchoring the reaction product on the surface of catalytically active phage–enzyme, have been used in model selection experiments with subtiligase (Atwell and Wells, 1999), glutathione-*S*-transferase and subtilisin (Demartis *et al.*, 1999), DNA polymerase (Jestin *et al.*, 1999) and a nuclease (Pedersen *et al.*, 1998). In those experiments, enrichment factors as large as 100-fold (relative to negative controls) could be documented in some cases. In most cases, the performance of phage selection appeared to be limited by the efficiency of enzyme display on phage. Furthermore, the relevance of these promising selection schemes for the isolation of improved enzymes out of large protein repertoires remains to be demonstrated.

In this work, we implemented the selection scheme of Demartis *et al.* (Demartis *et al.*, 1999) for two different classes of enzymes: an endoprotease and a ligase. As expected, the use of phage vectors (rather than phagemids) appears to improve enzyme display on phage dramatically. However, proteolytic degradation (particularly prominent in the case of biotin ligase) may also limit the number of enzyme molecules displayed on each phage particle. It is tempting to speculate that the selection scheme of Figure 1 may yield enzyme mutants with improved folding properties and superior display on phage, from libraries obtained, for example, by DNA shuffling (Stemmer, 1994).

In model selection experiments, phage displaying BirA were consistently enriched (between 4- and 800-fold) per round of panning, relative to negative controls. In competition experiments we showed that the enzymes act on the substrate displayed on the same phage rather than on the substrate of other phage. Phage displaying BirA mixed with phage displaying an irrelevant enzyme were enriched by a factor of 10 per round of selection.

Phage displaying the trypsin His57→Ala mutant, a relatively inefficient endopeptidase which cleaves a specific dipeptide sequence, were enriched (between 15- and 2000-fold), relative to negative controls. These promising results suggest that the selection scheme of Figure 1 may be useful for the isolation of novel and/or improved catalysts. This task remains a significant challenge in the field of protein engineering, particularly when large protein repertoires are selected with phage display methodologies.

In order to improve the catalytic properties of the trypsin His57→Ala mutant, we constructed a combinatorial phage display library of trypsin mutants. Selection of catalytically active phage–enzymes was shown by increasing phage titres at the different rounds of panning relative to negative control selections, but mutants with catalytic properties superior to those of trypsin His57→Ala mutant could not be isolated. These results indicate that further work is needed to demonstrate the usefulness of phage–enzyme selection schemes at the level of large combinatorial libraries. The catalytically active His57→Ala mutant was contained in the library, but was not recovered after four rounds of biopanning (Table I). This result suggests that more stringent selection schemes will have to be implemented, e.g. by blocking the reaction with suitable inhibitors after addition of the substrate to the phage and before the capture step (Figure 1). A potential problem with selections performed with peptidic substrates is represented that proteolytic cleavage by the His57→Ala mutant is extremely slow (k_{cat} = 0.002 s^{-1}) and approaches the level of background proteolysis in mixtures of unpurified biological material. In the future, we will direct our attention to trypsin mutants with an intact catalytic triad in order to start the selection with more active trypsin variants. The substrate specificity could be altered at the P1 position of the peptide by changing the binding pocket of trypsin. A human enzyme with novel

substrate specificity, which maintains its catalytic activity in serum, could be used for effective tumor targeting. The antibody-mediated delivery of such enzymes to the tumor environment (Halin and Neri, 2001) will allow the selective generation of drugs from non-toxic precursors ('pro-drugs') *in situ*, therefore sparing normal tissues and improving the therapeutic index of available chemotherapeutic agents (Niculescu-Duvaz *et al.*, 1999).

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