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Engineering Bidentate Macromolecular Inhibitors for Trypsin and Urokinase-type Plasminogen Activator

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Ecotin, a dimeric serine protease inhibitor from *Escherichia coli*, is a novel platform for inhibitor design. An approach using the three-dimensional structure of the ecotin–trypsin complex to guide combinatiorial design efforts was taken to create potent bidentate ecotin inhibitors for trypsin and human urokinase-type plasminogen activator (uPA). The ecotin surface loop that was redesigned is composed of residues 67 to 70 (60 s loop), and binds to the target protease at a region 25 Å from the enzyme active site. Two ecotin phage display libraries were constructed to exploit the binding interactions at the 60 s loop. The ecotin $60X_4$ library, in which residues 67 to 70 of ecotin were randomized, was panned against rat and bovine trypsin in parallel for four rounds. Panning against bovine trypsin resulted in enrichment of ecotin phage but did not yield a consensus sequence. Panning against rat trypsin resulted in enrichment as well as the ecotin consensus sequence WGFP at positions 67 to 70. The variant ecotin encoded by this sequence inhibited rat trypsin at 80 pM, a 12-fold improvement over ecotin wild-type (WT). A second generation library, ecotin M84R + $60X_4$ including an additional methionine to arginine substitution at position 84 in the primary binding site of ecotin, was generated for panning against uPA and rat trypsin. Panning against rat trypsin resulted in enrichment but no consensus sequence. Panning against uPA resulted in enrichment as well as the different ecotin consensus sequence WGYR at positions 67 to 70. Ecotin M84R + D70R bound to uPA at 50 pM, a 56,000-fold increase in binding compared to ecotin WT. Furthermore, ecotin M84R + D70R achieved a 13,680-fold preference of specificity towards uPA *versus* rat trypsin. The fact that the 60 s loop of ecotin plays different roles in binding to trypsin and uPA suggests this site can be used to introduce specificity and potency for other members of the serine proteases with a chymotrypsin fold.

Keywords: protease inhibitor; ecotin; uPA; phage display; protein design

Introduction

The serine proteases are a large family of enzymes involved in a wide variety of vital biological processes. The crucial physiological functions of these enzymes in blood coagulation, fibrinolysis, complement pathways, viral maturation, apoptosis and cancer make them important targets for efforts to design and engineer potent and specific inhibitors. A highly selective protease inhibitor can serve as a powerful tool to block key proteolytic activities for dissecting proteolytic pathways and cascades, and elucidating the *in vivo* roles of particular proteases in complex biological processes. Ultimately, this may lead to the development of innovative therapies for life-threatening diseases.

Previous studies have established that, for serine proteases with a chymotrypsin fold, the electrostatic and hydrophobic characteristics of the substrate binding pocket are critical factors in determining substrate specificity (Perona & Craik, 1995). This region has been the major focus of structural, functional and mechanistic studies, as well as inhibitor design efforts. Since many mammalian serine proteases have the same primary specificity, a small molecule inhibitor that only exploits the interactions at the primary binding pocket may not have sufficient discrimination

Abbreviations used: uPA, urokinase-type plasminogen inhibitor; WT, wild-type; HPLC, high performance liquid chromatography; cfu, colony-forming units; LB, Luria broth.

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towards similar enzymes. Macromolecular substrate-like serine protease inhibitors, such as bovine pancreatic trypsin inhibitor (BPTI), bind to the target serine protease through a single loop that indicates the critical P1 residue. This residue fits into the binding pocket of the target protease in a substrate-like conformation to lock the enzyme in a complex formed between the protease and the inhibitor. The common contact between the protease and the inhibitor is small and clustered around the active site of the enzyme. The highly homologous structures of the different enzyme active sites make the design of a highly selective and potent inhibitor for a particular protease a challenging task.

Ecotin, a macromolecular serine protease inhibitor found in the periplasm of *Escherichia coli* (Chung *et al*., 1983), offers a unique platform from which to explore the protease–inhibitor interactions that are distal from the active site. Four surface loops of ecotin form two interface regions between ecotin and the protease resulting in a combined surface area of approximately 2800 Å^2. The enormous buried interface area between ecotin and its target protease is far greater than that of most other protease–inhibitor complexes. In addition, ecotin's unique secondary binding site plays a major role in determining the strength of interaction between ecotin and the bound protease. Through systematic mutagenesis at the 60 s and 100 s loops, we have demonstrated the significance of this secondary binding site for selective inhibition against several proteases, such as rat trypsin and human urokinase-type plasminogen activator (uPA) (Yang *et al*., 1998).

Phage display has been successfully applied to a wide range of peptides and proteins, including antibodies (McCafferty *et al*., 1990), growth hormone (Bass *et al*., 1990), DNA binding proteins (Jamieson *et al*., 1994; Rebar & Pabo, 1994), enzymes (Corey *et al*., 1993; McCafferty *et al*., 1991; Soumillion *et al*., 1994), and macromolecular protease inhibitors (Markland *et al*., 1996a,b; Pannekoek *et al*., 1993; Roberts *et al*., 1992; Wang *et al*., 1995). We have developed an ecotin phage display system that presents fully functional ecotin on the surface of filamentous phage (Wang *et al*., 1995) and used the system to investigate the macromolecular recognition between uPA and ecotin. uPA is a serine protease that plays an active role in extracellular proteolysis, cell migration, and tissue remodeling processes (Fazioli & Blasi, 1994). Because of its implication in cancer metastasis and tumor invasion, uPA has become an important target for drug design and inhibitor development efforts. We have shown previously that a high-affinity inhibitor of uPA could be isolated from a library of phage-displayed ecotin variants at the P1 and P1′ positions (Wang *et al*., 1995). The current study is focused on ecotin's secondary binding site to determine if potency and specificity against target proteases could be obtained through this unique surface region. To achieve this goal, a structure-based approach coupled to phage display has been developed to design uPA inhibitors through stepwise optimization.

Results

Identifying key residues at the 60s loop of the secondary binding site

Taking advantage of its large interface and the unique bidentate mode of interaction, ecotin effectively recognizes a broad range of serine proteases with different substrate specificities. The roles of the four surface loops (the 50 s, 60 s, 80 s and 100 s loops) have been systematically dissected and the key elements responsible for the binding energy of the complex formation have been identified (Yang *et al*., 1998). The results of this region-specific mutagenesis study firmly established the significance of the secondary binding site and showed that this site played different roles with different target enzymes. The complete experimental results and detailed analysis are described in the preceding paper (Yang *et al*., 1998). Eight residues at ecotin's secondary binding site within 4.2 Å of the protease were picked as potential binding determinants for proteases recognition. The residues Trp67, Gly68, Tyr69 and Asp70 in the 60 s loop and residues Arg108, Asn110, Lys112 and Leu113 in the 100 s loop were substituted with alanine. The sharp contrasts of the K_i values of the two ecotin alanine substitution variants at residues 67 to 70 ($60A_4$ and $M84R + 60A_4$) towards bovine trypsin, rat trypsin and human uPA illustrated the sensitivity of the ecotin variants to the differences among the corresponding C-terminal surface regions of the proteases, where the 60 s loop bound to the target enzyme. These experiments confirmed the potential of residues 67 to 70 to serve as novel determinants of specificity and potency of protease inhibition.

Although the 60 s loop was located as the starting point for engineering ecotin, limited understanding of the interactions at this region made it difficult for the *de novo* design of a specific ecotin variant for a particular target protease. On the other hand, it was equally difficult to test all the amino acid combinations on this surface loop individually, since the total number of variants is 160,000 (20^4), beyond the limit of our assay methodology. A combinatorial method was developed to approach this problem by taking advantage of the *in vitro* selection power of phage display, to directly isolate strong binding ecotin variants from a comprehensive 60 s loop library.

Designing and constructing ecotin phage display libraries at the 60 s loop

The ecotin phage display vector pBSeco-gIII expresses the fusion proteins of full-length ecotin connected to the C-terminal domain of filamentous phage minor coat proteins pIII *via* a GlyGlyGly lin-

ker. With the addition of VCSM13 helper phage, the ecotin–pIII fusion protein is assembled onto phage particles. Phage carrying this fusion has ecotin activity and can bind to the immobilized protease on the solid surface. The three enzymes, bovine trypsin, rat trypsin and uPA, were coated onto polystyrene Petri dishes and remained active as monitored by *p*-nitroanilide release of N^{α}-benzyloxy-carbonyl-L-Gly-Pro-Arg-*p*-nitroanilide substrate after 30 minutes of incubation at room temperature (25°C).

Taking advantage of three-dimensional structure information, two different ecotin libraries were designed to randomize key residues, thereby permitting isolation of the optimal cognate inhibitor for a target protease. The first library ecotin, $60X_4$, had four residues randomized at positions 67 to 70 of the 60 s loop. This library was used to pan against bovine trypsin and rat trypsin separately. The results of the multiple alanine substitution experiments showed that the 60 s loop was not determinant of binding for bovine trypsin (Yang *et al.*, 1998). This suggests that bovine trypsin would bind to many 60 s loop ecotin variants equally well, regardless of the identities of the residues at positions 67 to 70. In contrast, only a small subset of ecotin variants within the 60 s loop library should inhibit rat trypsins with high affinity. For rat trypsin inhibition, the side-chain contribution from the 60 s loop was important in the absence of strong favorable electrostatic interactions at the P1 position. In this case, selective binding towards rat trypsin would certainly put greater constraints upon the nature of amino acid moieties at positions 67 to 70. Panning the ecotin $60X_4$ library with rat trypsin would likely produce one or a few strong binders from the millions of library members.

The second library, ecotin M84R + $60X_4$, combined a favorable P1 Arg residue with the randomized 60 s loop. This library was designed to encode ecotin variants that inhibit uPA with high potency. The K_i of ecotin M84R + $60A_4$ against uPA is 1470 nM, several hundred-fold higher than the K_i of ecotin M84R, suggesting that four amino acid substitutions 25 Å away from the active site were sufficient to cause a dramatic change in the strength of the interaction. Since the inhibition of uPA was so sensitive to the residue substitutions at the 60 s loop even in the presence of M84R, we reasoned that the uPA panning experiment with the ecotin M84R + $60X_4$ library had a high probability of generating a subset of ecotin variants that recognize uPA with increased potency and specificity.

The two ecotin libraries were constructed through Kunkel (1985) mutagenesis. The NNS (N = A,C,G,T,S = C,G) encoding scheme allowed a maximum of 1.0×10^6 possible DNA sequences that represent 1.6×10^5 possible amino acid residue combinations. The ecotin $60X_4$ library contained approximately 5×10^6 individual clones; the ecotin M84R + $60X_4$ library contained approximately 50×10^6 individual clones. The completeness of the library was calculated using the equation:

$$N = \ln(1 - p)/\ln[1 - (1/n)] \quad (1)$$

where N is the number of total individual clones in the library, n is the number of possible combinations, and p is the probability that any clone can be found in the library given a library size N. In both cases, the sizes of the final libraries indicated that these libraries were well over 99% complete in representing all the possible four amino acid sequence combinations at positions 67 to 70. Both libraries were characterized for completeness using the same procedure. Random individual clones were isolated from the libraries. Their plasmid DNA and phage were purified using standard procedures. A *Bam*HI/*Hin*dIII restriction digest of the sample plasmid DNA was used to monitor the total size of the ecotin phage display vector and the size of the DNA fragment containing the ecotin–gIII fusion. All the individual clones characterized contained a full-length insert. Double-strand nucleotide sequencing of the ecotin M84R + $60X_4$ library members revealed that 60% of the library members were variants. The heterologous nucleotide sequences within the correct reading frame at the designed positions for 67 to 70 encoded random amino acid residues without significant deviation from the expected frequency distributions. An immuno-blot assay of the library phage sample using rabbit anti-ecotin antibodies detected that ecotin was expressed on phage. Bovine trypsin activity inhibition assays of the phage library mixture further ensured that the ecotin variants displayed on phage were still active. Selected ecotin variants were over-expressed, purified and characterized kinetically (see below).

Panning of the ecotin $60X_4$ library with bovine and rat trypsin

Throughout this study, two criteria have been adopted to assess the effectiveness of a panning experiment. One was the total recovery of phage eluted from the plates. A rigorous panning protocol should allow the enrichment of strong binding clones over background in the course of iterative panning and amplification. The enrichment could be indirectly measured by comparing the phage recovery in the library panning experiment to the recovery in a control experiment where no protease was coated onto the polystyrene plate solid support. The number of phage recovered from nonspecific background binding with the plate and the blocking agent was less than 10^5 under our normal experimental conditions. A typical round of ecotin phage library panning with significant enrichment would yield 10^6 to 10^8 phage from the elution, given that the input phage was in the range of 10^{10} to 10^{11}. Although an elevated recovery usually suggested an increase of positive clones on the

pool of panning intermediates, other factors that were not directly related to the *in vitro* binding between ecotin and the immobilized protease might also lead to an artificially high recovery. These factors included the emergence of certain deletion phage variants that possessed a significant growth advantage in the liquid amplification procedure, or a sub-population of the phage library that bound to the solid support to the blocking agent. To ensure that the *in vitro* selection was based solely on the strength of the interactions between ecotin phage and immobilized protease, another more stringent criterion was also applied to evaluate the outcome of a panning experiment: the emergence of one or a few consensus sequences in the final population of the ecotin phage library. Ecotin variants encoded by these consensus sequences were than made and characterized kinetically for their affinities towards the immobilized protease used in the panning experiment.

The ecotin $60X_4$ library was panned against two serine proteases, bovine and rat trypsin, which were coated onto separate polystyrene plates. After the first round of panning, the elution fraction from the two plates were pooled and amplified separately on multiple LB/ampicillin plates to generate two intermediate libraries for the next round of panning. The two panning experiments were then carried out in parallel for the subsequent amplification rounds. Careful precautions were taken to avoid cross-contamination between the bovine trypsin and rat trypsin panning. Four rounds of panning were completed before the final sequencing of the individual clones. For both proteases, there was a significant increase in both total recovery of phage eluted and the percentage of phage recovered after each round (data not shown), suggesting the enrichment of phage carrying ecotin variants that bind to the two immobilized proteases. The individual clones from the final round of bovine trypsin and rat trypsin panning were sequenced. The DNA sequencing results showed that panning the ecotin $60X_4$ library against bovine trypsin resulted in a heterologous population of sequences and no consensus (see Table 6, below). It confirmed that the impact of 60 s loop side-chains on binding was minimal under such conditions. On the other hand, panning with the ecotin $60X_4$ library against rat trypsin generated a clear consensus sequence Trp-Gly-Phe/Leu-Pro for positions 67 to 70 (Table 1 and Table 6). The strong selection for Trp and Gly at positions 67 and 68 were highly significant. At position 69, all 18 clones contained large, hydrophobic residues such as Phe, Leu, Ile, Tyr, Trp and Met. Phe and Leu were the most predominant residues of the consensus, with five and four occurrences, respectively. Pro emerged from the library as a predominant residue at position 70. Not surprisingly, the wild-type sequence Trp-Gly-Tyr-Asp appeared three times, confirming that this sequence was also a good solution for rat trypsin binding. Another result that further substantiated the consensus sequence was that the selection operated at the amino acid level of the nucleotide level. Since the library was encoded by a mixture of NNS codons, amino acids, such as Gly, Leu and Pro, could be encoded by two or three codons. Indeed, among the 18 sequences, different codons were found with comparable frequencies for Gly (12 GGG and 5 GGC), Leu (2 CTG, 2 CTC) and Pro (4 CCC and 4 CCG).

Table 1. The consensus sequence from library $60X_4$ panning against rat trypsin

Position:	67	68	69	70
	Y	G	F	I
	W	G	I	Q
	W	G	F	T
	W	G	L	P
	W	Q	L	P
	W	G	L	P
	W	G	W	G
	W	G	F	N
	R	G	Y	P
	W	G	F	S
	W	G	Y	D
	W	G	Y	D
	W	G	F	P
	W	G	Y	D
	W	G	L	W
	W	G	M	P
	W	G	M	P
	W	G	I	P
Consensus	W	G	F	P
Occurrence	16	17	5	8
$(P_o - P_e)/\sigma$[a]	20.9	15.5	6.0	6.7
Consensus			L	
Occurrence			4	
$(P_o - P_e)/\sigma$			1.9	
WT	W	G	Y	D

[a] P_e, the expected frequency of possible NNS (N = A,C,G,T,S = C,G) codons; P_o, the observed frequency of codons in the clones sequenced; n, number of clones sequenced; $\sigma = [P_e(1 - P_e)/n]^{1/2}$.

Inhibition kinetics of ecotin variants Y69F + D70P and Y69L + D70P

To show that the panning experiments selected for strong binders, the variant ecotins encoding the consensus sequences Trp-Gly-Phe-Pro and Trp-Gly-Leu-Pro at positions 67 to 70 were cloned into the expression vector pTacTacEcotin (McGrath *et al.*, 1991). Ecotin Y69F + D70P and Y69L + D70P were expressed and purified. Their K_i values against bovine trypsin, rat trypsin and human uPA are listed in Table 2. The kinetic data show that for both trypsins, ecotin Y69F + D70P and ecotin Y69L + D70P bound tighter than WT ecotin. Ecotin Y69F + D70P was the stronger binder with a lower K_i (bovine trypsin, 30 pM; rat trypsin, 80 pM). The similar increase in affinity by ecotin Y69F + D70P for both trypsins suggested a general optimization of the hydrophobic packing at the secondary binding site. For uPA, ecotin Y69F + D70P bound better (670 pM) than ecotin WT (2800 pM) and ecotin Y69L + D70P bound worse (20,200 pM), implying

Table 2. K_i of ecotin variants from library $60X_4$

Variant	Bovine trypsin (pM)	Rat trypsin (pM)	uPA (μM)
WT	310 ± 60	930 ± 160	2.8 ± 0.2
Y69F + D70P	30 ± 10	80 ± 10	0.67 ± 0.08
Y69L + D70P	190 ± 30	460 ± 50	20.2 ± 1.8

that the inhibition against uPA was extremely sensitive to changes at the 60 s loop and that the uPA-ecotin interface differed from the trypsin–ecotin interface at the secondary site. Clearly, high-affinity ecotin variants modified at the secondary site can be selected from a phage display library.

Liquid *versus* plate amplification of library M84R + $60X_4$

The isolation of an inhibitor with improved binding towards rat trypsin suggested that this approach could be used to identify a high-affinity inhibitor for uPA. To reduce the panning cycle time and simplify the experimental procedure, the phage amplification process was changed from plate amplification to liquid amplification. Although panning of the ecotin $60X_4$ library was conducted with plate amplification, no serious consequences were expected in switching the amplification protocols. The ecotin M84R + $60X_4$ library was panned with uPA through liquid amplification for four rounds. However, there was no significant increase of phage recovery in each round of the experiment. When samples from the final round were characterized, a significant fraction of the clones were deletions of the full-length ecotin phage clones. In fact, the outcome of the panning process was very sensitive to both the fraction of initial positive clones in the library and the panning protocol. Due to their selective growth advantage, the spontaneously occurring deletion phage grew faster in liquid culture, thereby gaining a significant advantage in competition with the full-length ecotin phage. By iterative enrichment, the clones that carry the deletion sequences could be amplified at the early stage of library propagation and dominated the final population.

Since the amplification procedure was a critical factor for the success of the panning experiments, the plate amplification protocol was further refined to eliminate the nutrient selection pressure for phage growth in liquid culture. A ''nursing protocol'' was developed to ensure that the small fraction of positive clones in the initial input library were not lost during amplification (see Materials and Methods). This protocol used plate amplification to minimize the selective pressure for nutrients and short incubation times to limit growth, preventing certain clones from dominating the pool of selected variants. The low density growth on solid media and limited propagation times were essential in the first round of library amplification for subsequent selection of desired variant clones.

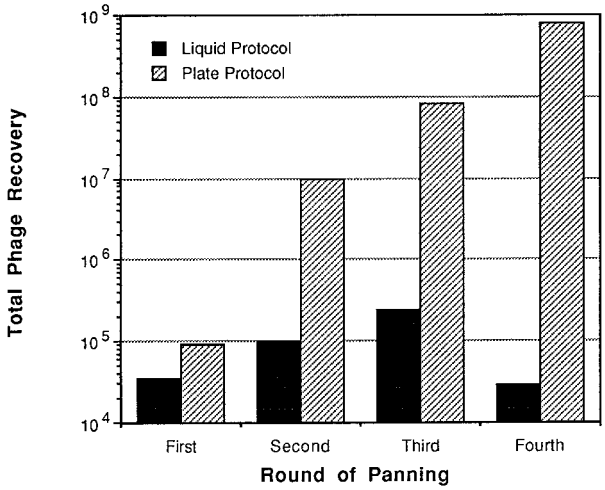

Figure 1. Comparison of liquid and plate amplification. The total phage yields *versus* the round of panning from the liquid and plate amplification procedures are compared. The plate amplification protocol (e.g. nursing protocol, see Results) was clearly superior to the liquid amplification protocol. The panning experiment was conducted by panning the ecotin M84R + $60A_4$ library against uPA for four rounds of amplification. The phage yield from liquid amplification round one to four was 3.5×10^4, 1.0×10^5, 2.4×10^5 and 2.9×10^4, respectively. The phage yield from plate amplification rounds one to four was: 9.2×10^4, 9.8×10^6, 8.2×10^7 and 8.0×10^8, respectively.

Specifically, *E. coli* colonies infected with phage were grown on multiple, large LB/ampicillin plates with appropriate density (2.5×10^4 to 6.0×10^4) to ensure the separation of individual colonies. The same ecotin M84R + $60X_4$ library was panned with uPA again using the new method. The plate amplification significantly increased the number of phage recovered from the elution, indicating the enrichment of clones that bind to uPA. The comparison of the phage yields from the two methods is shown in Figure 1. By adopting this nursing protocol, we have not only obtained a final phage sample that was highly enriched over the background (10^7 to 10^8 in total *versus* less than 10^5), but also isolated a clear consensus sequence (see the following section). These results show that the conditions of the amplification protocol had a direct impact on the outcome of the *in vitro* selection process.

Panning of the ecotin M84R + $60X_4$ library with rat trypsin and uPA

The ecotin M84R + $60X_4$ library was panned against rat trypsin and uPA in parallel with intermediate plate amplification steps. Similar to the results from panning the ecotin $60A_4$ library, a significant increase of phage recovery was observed in the eluates in each of the four rounds of panning for both ligands. Again, the panning results con-

Table 3. The consensus sequence from library M84R + 60X$_4$ panning against uPA

Position:	67	68	69	70	K_i (pM)
	W	G	Y	D	
	W	G	H	R	
	M	G	Y	P	
	W	G	Y	R	
	Y	G	Y	R	
	W	G	H	R	
	W	E	F	P	
	W	G	N	R	
	W	G	Y	R	
	W	G	Y	Q	
	N	G	Y	R	
	W	G	Y	P	
	W	G	F	G	
	W	G	Y	P	
	W	G	Y	P	
	F	G	Y	K	
	W	G	Y	G	
	W	G	Y	W	
Consensus	W	G	Y	R	50 ± 40
Occurrence	14	17	13	8	
$(P_o - P_e)/\sigma$	18.2	15.5	16.8	5.1	
Consensus				P	80 ± 20
Occurrence				4	
$(P_o - P_e)/\sigma$				2.8	
M84R	W	G	Y	D	3600 ± 630

firmed our observations for prior mutagenesis experiments. In rat trypsin binding, the dominant role of the electrostatic interaction with Arg84 completely masked the impact of the 60 s loop. Thus panning against rat trypsin did not generate a consensus sequence (data not shown), even though in the final round of panning, the phage recovery from the acid elution exceeded 10^8. As anticipated, a consensus sequence was found in panning against uPA (Table 3). Furthermore, this consensus sequence Trp-Gly-Tyr-(Arg/Pro) was different from the previous consensus Trp-Gly-(Phe/Leu)-Pro from panning the ecotin 60X$_4$ library against rat trypsin, suggesting that the *in vitro* selection process was indeed dependent on the nature of the immobilized protease. At positions 67 to 68, the same residues Trp and Gly were selected from the random sequence pool with high affinity. At position 69, the wild-type residue Tyr occurred in nearly 75% of the sequences. The Asp at position 70 was replaced with Arg (eight times) and Pro (four times). This was the only residue that differed from the wild-type sequence. Again, the nucleotide sequences of the 18 samples from uPA panning showed a mixture of codons encoding the selected amino acids such as Gly (14 GGG and 3 GGC), Pro (3 CCG and 1 CCC) and Arg (5 AGG and 3 CGG), strengthening the conclusion that these residues were selected on the basis of their contributions to increase the affinity towards uPA.

Inhibition kinetics of ecotin M84R + D70R and ecotin M84R + D70P

Ecotin M84R + D70R and ecotin M84R + D70P were constructed by cloning the specific variant sequences from the phage clone into the expression vector pTacTacEcotin, taking advantage of a pair of common restriction sites *Bam*HI/*AatII* that flanked 90% of the ecotin gene. The two variants were purified to homogeneity *via* reverse phase HPLC. Their K_i values against uPA were determined and are listed in Table 4. The K_i of ecotin M84R + D70R was lower (50 pM) than that of ecotin M84R + D70P (80 pM), mirroring their relative occurrence in the consensus sequences. The D70R substitution alone reduced the K_i 72-fold, resulting in a 50 pM inhibitor of uPA, the tightest binding competitive inhibitor of uPA reported to date. The one amino acid substitution at position 70 generated a significant impact on the affinity of these two ecotin variants.

Table 4. Designing specificity and potency of ecotin

	WT	M84R	M84R + D70R
K_i (rat trypsin) (nM)	0.93 ± 0.16	0.38 ± 0.10	0.22 ± 0.04
K_i (uPA) (nM)	2800 ± 160	3.6 ± 0.6	0.05 ± 0.04
K_i(uPA)/K_i (rat trypsin)	3010	10	0.22
Preference value *versus* ecotin WT	1	300	13680

Preference value is calculated by dividing the ratio of K_i (uPA)/K_i (rat trypsin) of ecotin WT by that of ecotin M84R or M84R + D70R. This value reflects the fold change in preference of the variant ecotin compared to ecotin WT for a given protease.

The increased potency and specificity of ecotin M84R + D70R validates the strategy to optimize the affinity of ecotin towards uPA through a stepwise approach. The inhibition of rat trypsin can be used as a benchmark to assess the effectiveness of this methodology. Ecotin M84R + D70R, with K_i against rat trypsin of 220 pM, was a better inhibitor of uPA than rat trypsin. This result was in sharp contrast with the preference of WT ecotin for rat trypsin over uPA by over 3000-fold. Table 4 summarizes the ecotin K_i values towards rat trypsin and uPA generated through the combination of region-specific mutagenesis and phage display. The specificity of ecotin has been successfully converted from one serine protease to the other with a significant increase in potency at the same time. The overall specificity preference was 13,680-fold.

Discussion

The consensus sequences generated from rat trypsin and human uPA panning revealed that residues 67 and 68 of ecotin were essential in high-affinity binding against the target serine protease. In the tetrameric complex of ecotin–trypsin, Trp67 of ecotin provided the majority of the hydrophobic interactions at the binding interface. In fact, this tryptophan side-chain was within direct van der Waals distance of residues 91, 233, 234, 236, 237 and 240 (chymotrypsin numbering system) of the protease. In all three proteases, amino acid residues 234 and 237 were aromatic side-chains. Residue

234 is either Tyr (rat and bovine trypsin) or Phe (uPA) and residue 237 is a conserved Trp. The stacking of aromatic rings between ecotin and the protease provides a tightly packed hydrophobic interface. Due to tryptophan's large side-chain volume and buried surface area, substituting Trp67 of ecotin with any other amino acid might create an unfilled cavity that destabilizes the ecotin–protease complex. Thus Trp67 appears to be an integral part of the hydrophobic ''core'' of the secondary binding site. On the other hand, Gly68 was also selected from both panning experiments. This residue probably plays a more structural role to maintain the proper flexibility and main-chain conformation of the 60 s loop. Since Trp67 and Gly68 were conserved at the 60 s loop, the other two residues, Tyr69 and Asp70, were the only candidates to provide differential recognition towards target proteases.

The ecotin consensus sequence Trp-Gly-(Phe/Leu)-Pro at positions 67 to 70 that resulted when the ecotin library was panned against rat trypsin represented an overall improvement of the hydrophobicity at positions 69 to 70. Both Phe and Leu are more polar than Tyr, which has a hydroxyl group. In addition to its hydrophobicity, proline was commonly found at various types of turns to lock the surface loops into stable conformations. Pro70 was presumably selected for this reason. The tenfold increase in affinity of ecotin Y69F + D90P towards both bovine and rat trypsin illustrated the modest improvement of side-chain packing in the vicinity of residues 60 and 70. In the case of uPA inhibition, the charge reversion substitution of D70R suggests that an electrostatic interaction could also be important for this position. In the crystal structure of the trypsin–ecotin complex, Asp70 of ecotin is very close to Asn93 of rat trypsin. In fact, the O^{δ} of Asp70 is within hydrogen bonding distance (2.94 Å) of the N^{δ} of Asn93. Although the crystal structure of the ecotin–uPA complex is not available, it is possible to build a complex model based on the high resolution structures of the rat trypsin–ecotin complex and the catalytic domain of human uPA. The recently published 2.5 Å structure of the uPA protease domain has a similar topology to trypsin (Spraggon *et al*., 1995). By superimposing the catalytic triad of uPA and rat trypsin, a model of the ecotin–uPA complex was constructed. The polypeptide backbone conformation of uPA overlaid with that of rat trypsin very well with an r.m.s. of 0.66 Å. Most of the differences occurred in several surface loops. However, the region near the secondary binding site and close to position 93 of the protease were highly conserved (Figure 2). Residue 70 of ecotin is close to residue 93 of uPA in the model of the ecotin–uPA complex. In this context, the results of the uPA panning are easily interpreted. In uPA, residue 93 was an Asp instead of an Asn. The consensus sequence Arg70 from the ecotin M84R + $60X_4$ library panning against uPA was indeed an ideal choice to provide a counter charge to stabilize Asp93 by forming a salt bridge between these two side-chains. Thus, we have shown improvement in affinity through the creation of alternative intermolecular electrostatic interaction and also refined the structural model of the ecotin–uPA complex. Since the D70R substitution decreased the K_i 72-fold from 3.6 nM to 50 pM, this favorable charge substitution alone was responsible for about 2.5 kcal/mol of binding energy improvement.

The agreement between the results from the multiple alanine substitution experiments and the phage display experiments suggests that mutagenesis data of the 60 s loop can be used to design ecotin phage libraries that will lead to a consensus sequence in a panning experiment. To extend the scope of these efforts to other parts of the secondary site (e.g. 100 s loop), the impact of a particular surface loop under different contexts was analyzed. The free energy differences of binding for the different ecotin variants were calculated and compared. The K_i values of all ecotin variants were converted to free energy terms with the equation $\Delta G = -RT\ln K$. The impact of the 60 s loop when the P1 residue is an Arg can be calculated as the $\Delta\Delta G = \Delta G_{(M84R + 60A4)} - \Delta G_{(M84R)}$. From our data in the previous study, the $\Delta\Delta G$ values of the 60 s and/or 100 s loop in the presence of the M84R substitution were calculated and are listed in Table 5. A high absolute value of $\Delta\Delta G$ will be a good indication that the specific surface loop is a key contributor to protease recognition. The outcome of the ecotin phage display experiment is summarized in Table 6 for the comparison between the free energy difference and the *in vitro* selection process. For ecotin $60A_4$ binding to rat trypsin, the $\Delta\Delta G$ was −5.02 kcal/mol and a consensus sequence was observed when the ecotin $60X_4$ library was panned against rat trypsin. Similarly, for ecotin M84R + $60A_4$ against uPA, the $\Delta\Delta G$ was −3.56 kcal/mol and a consensus sequence was observed when the ecotin M84R + $60X_4$ library was panned against uPA. Since the high $\Delta\Delta G$ values of the ecotin mutated in the binding determinants of the 60 s loop (Table 5) correlate with the emergence of a consensus sequence (Table 6), we think that this analysis can be used to identify areas that are important for high-affinity binding. Since the $\Delta\Delta G$ values of the ecotins mutated in the binding determinants of the 100 s loop (Table 5) are comparable to that of the 60 s loop for uPA binding, the 100 s loop should have a major impact on the strength of ecotin–uPA complex formation. Panning a phage display library at this region will likely generate novel consensus sequences encoding ecotin variants with high affinity towards uPA. The multiple alanine substitution experiments establish a rigorous strategy from which future combinatorial selection or screening approaches might be rationally designed and developed. Combining region-specific mutagenesis with the comprehensive scope of phage display, this affinity optimization procedure has

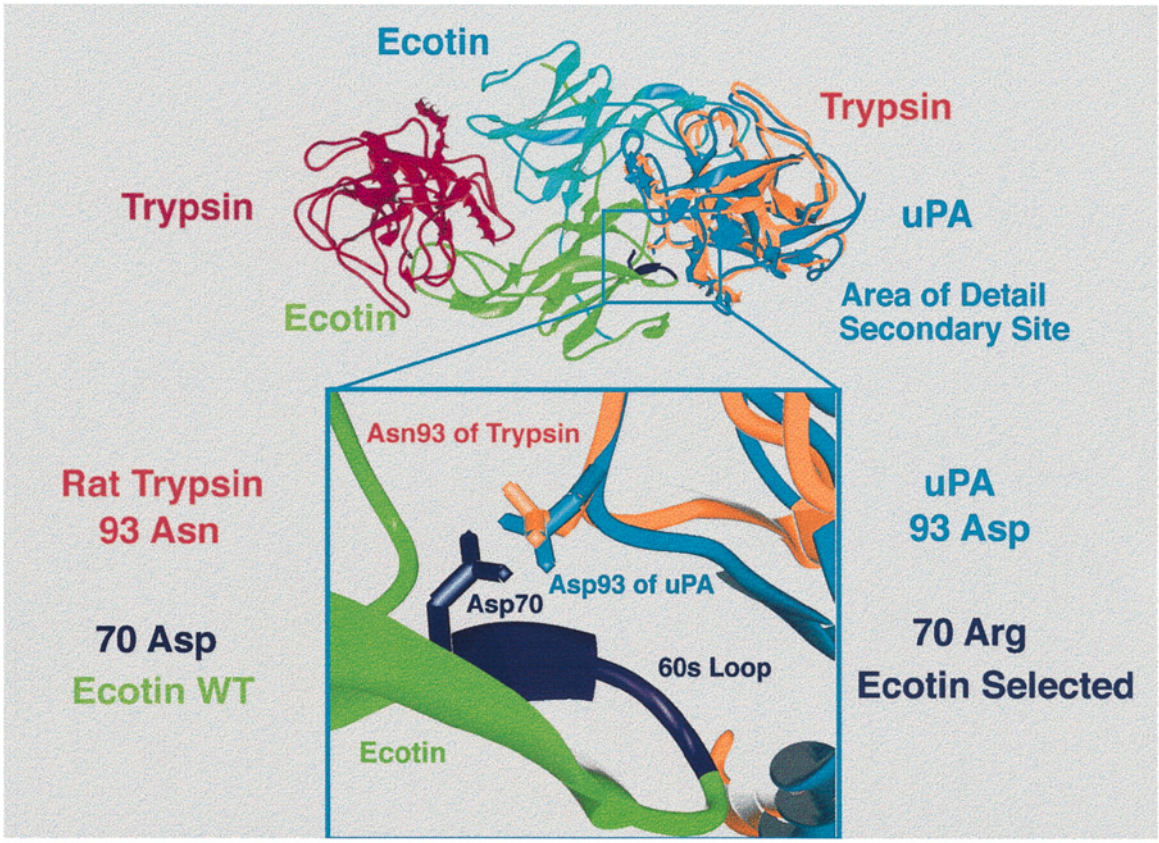

Figure 2. Modeled electrostatic interaction at the 60 s loop of ecotin. The three-dimensional structure of uPA is superimposed with one trypsin molecule in the ecotin–trypsin tetrameric complex by matching the residues Ser195, His57, Asp102, and Asp189 of each enzyme using the program MidasPlus (Computer Graphics Laboratory, UCSF). The RMS deviation of these amino acid residues is 0.66 Å. The two ecotin molecules are colored green and cyan, the two rat trypsin molecules are colored red and blue. One uPA molecule is colored orange. The 60 s loop of one ecotin molecule is colored purple. The area of detail illustrates the relative positions of residue 70 of one ecotin molecule (Asp in ecotin WT; Arg in selected variant) and residue 93 (Asn in trypsin; Asn in uPA) of the bound protease, suggesting potential electrostatic interactions between these two side-chains.

the potential to be applied to other systems of macromolecular recognition as well.

The highly differentiated and specialized roles of the mammalian serine proteases demand inhibitors that interact with their target enzymes with strong selectivity. Both conventional small molecule and macromolecular inhibitor design approaches are constrained by the distinctive structural features at the proximity of the active site and binding pocket of the target enzyme. This limitation is partially due to our current knowledge, which has been based on serine proteases on the reactive mechanism and substrate specificity of model enzymes such as trypsin and chymotrypsin. However, under physiological conditions, many mammalian serine proteases take advantage of a large array of cofactors to develop sophisticated mechanisms to control and regulate the potency and specificity of precise proteolytic events. For example, thrombin, a key enzyme that cleaves fibrinogen and forms fibrin clots in the blood coagulation pathway, is regulated by heparin, α_2-macroglobulin, antithrombin III, thrombomodulin (Stubbs & Bode, 1995) and monovalent ions such as Na^+ (Dang & Di Cera, 1996). Through several cofactor interactions that are distal to the active site, thrombin achieves a high level of fine tuning and balance between its coagulation and anticoagulation activities in an intertwined web of biological pathways in haemostasis, platelet aggregation, tissue remodeling, mitosis and chemotaxis. In the case of uPA, the serine protease domain can form a high-affinity complex with several key partners such as PAI-1, PAI-2, proteases nexin-1 and α_2-macroglobulin receptor (Andreasen *et al.*, 1994; Fazioli & Blasi, 1994). These examples illustrate the variety of structural features that are involved in the macromolecular recognition between serine proteases and other macromolecules *in vivo* that takes advantage of binding sites other than the primary recognition pocket. It also suggests unexploited opportunities of using other protease surface regions as the basis of inhibitor design and engineering.

Ecotin offers a unique platform from which to investigate and utilize the contribution from a binding region distal to the primary binding site for protease inhibition. The dimeric macromolecular inhibition has special structural features for

Table 5. Ecotin variants $\Delta\Delta G$ (kcal/mol)

$\Delta\Delta G$	Bovine trypsin	Rat trypsin	uPA
$\Delta G(60A_4) - \Delta G(WT)$	0.10	−5.02	−3.16
$\Delta G(M84R + 60A_4) - \Delta G(M84R)$	−0.26	0.44	−3.56
$\Delta G(M84R + 100A_4) - \Delta G(M84R)$	0.65	1.33	−4.16
$\Delta G(M84R + 60A_4 + 100A_4) - \Delta G(M84R)$	−0.34	−0.16	−5.31

ΔG was calculated by converting K_i to $RT\ln(K_i)$, where $T = 298$ K (25°C). The relative free energy was the ΔG difference between corresponding variants. According to this scale, a 100-fold difference in K_i will be equal to −2.73 kcal/mol.

innovative methods of inhibitor design and engineering. By modulating the amino acid residues at the 60 s loop, another level of control has been achieved in designing the specificity and potency of ecotin variants. The secondary binding site of ecotin not only facilitated the fine-tuning of the molecular recognition towards many known homologous enzymes, but also provided additional side-chain conformational flexibility to accommodate other serine proteases with similar scaffolds. These experimental results demonstrated that ecotin has several distinct advantages as a generic starting point for inhibitor design. The first advantage is the electrostatic and hydrophobic surface diversity available in the contact regions between the ecotin dimer and two protease molecules. Secondly, the combinatorial approach of phage display makes it feasible and highly efficient to search and sort the large repertoires of ecotin surface loop variants. Finally, the crystal structures of ecotin–protease complexes can served as a framework for designing inhibitors against enzymes with unknown structures. By taking advantage of the unique secondary binding site of ecotin, it is possible to improve the affinity against a target proteases through stepwise optimization of various surface loops involved in the binding interaction. This approach will be especially powerful for enzymes that share the same primary substrate specificity.

In summary, a combination of site-directed mutagenesis and phage display approaches was taken to study the interactions between ecotin and several serine proteases. The secondary binding site of ecotin was shown to play a critical role for certain proteases. Phage display libraries of ecotin variants were then made at these surface loops and used for panning against the target proteases. A protocol was developed that permitted identification of two distinctive consensus sequences from panning the ecotin variant phage libraries with rat trypsin and uPA. In both cases, the consensus sequence encoded ecotin variants with higher affinity for the target protease. This study provided a general strategy for engineering potency of a macromolecular serine protease inhibitor by modulating various components of the network of extended interactions between the inhibitor and the protease.

Materials and Methods

Materials

Enzymes and reagents for the manipulation of DNA were purchased from Promega (Madison, WI) or New England Biolabs (Beverly, MA) and were used following the manufacturer's instructions. The *E. coli* strain JM101, XL-1 Blue F′ and the VCSM13 helper phage were from Stratagene (La Jolla, CA). The *E. coli* ecotin gene deletion strain IMΔecoJ was derived from JM101 and was a generous gift from Dr Iain Murray (I. Murray & C. S. Craik, unpublished results). Low molecular mass uPA (LMuPA) was obtained from American Diagnostica (Greenwich, CT). Rat trypsin was expressed in *E. coli* using the expression vector pZ3 and purified as described (Higaki *et al.*, 1989). Bovine trypsin was from Sigma (St. Louis, MO). [α-^{35}S]dATP was from DuPont NEN (Boston, MA).

Sequenase Version 2.0 sequencing kit was from U.S. Biochemical Corp (Cleveland, OH). GeneClean® was from Bio101, Inc (La Jolla, CA). Oligonucleotides were synthesized with an Applied Biosystems 391 DNA synthesizer (Foster City, CA). Falcon polystyrene Petri dishes were from Becton Labware (Lincoln Park, NJ). All other chemicals were of reagent grade or better and were used without further purification.

Plasmid and library constructions

Mutagenesis was performed by the method of Kunkel (1985). All mutations have been confirmed at the DNA level by sequencing. The vector pBS eco-gIII was used to construct phage libraries of ecotin $60X_4$ and ecotin $M84R + 60X_4$. A deletion and frameshift mutation was introduced at residues 67 to 70 of ecotin by primer 5′-C AAA ACG CTG GAA GG TAT TAT GTC TTT GAT-3′ to make pBS eco-gIIIΔ60. This construct was used as template to make the ecotin $60X_4$ library by primer 5′-AAC AAA ACG CTG GAA GGC *NNS NNS NNS NNS* TAT TAT GTC TTT GAT AAA GTC AG-3′ ($N = A/C/G/T$, $S = C/G$). Primer 5′-GT TCC CCG GTT AGT ACT AGG ATG GCC TGC C-3′ was used to introduce an

Table 6. Summary of ecotin phage library panning results

Library	Bovine trypsin	Rat trypsin	uPA
Ecotin $60A_4$	High recovery, no consensus	High recovery, consensus	–
Ecotin $M84R + 60A_4$	–	High recovery, no consensus	High recovery, consensus

M84R mutation in pBSeco-gIIIΔ60 to generate pBSeco-gIIIM84RΔ60. This vector was then used to generate the ecotin M84R + $60X_4$ library by the 60 s loop library primer 5′-AAC AAA ACG CTG GAA GGC *NNS NNS NNS NNS* TAT TAT GTC TTT GAT AAA GTC AG-3′ ($N = A/C/G/T$, $S = C/G$). Both libraries of ecotin phage had four positions 67 to 70 randomized. The ecotin phage display vector pBSeco-gIII and expression vector pTacTacEcotin were mutated to carry an *Aat*II site by primer 5′-CA GAC AAT GTA GAC GTC AAG TAC CGC GTC-3′ at amino acid 125 of ecotin to facilitate the cloning between the two vectors. All ecotin variants obtained from panning experiments could be directly cloned into the expression vector pTacTacEcotin. For library phage preparation, the mutagenesis reaction mixture was purified by Geneclean, redissolved in water, electroporated into F′ XL-1 Blue cells, and grown in 100 ml of 2YT/ampicillin for one to two hours. The culture was then divided into two portions. One portion was transferred into fresh 2YT/ampicillin medium and grown for 8 to 12 hours. The cells were harvested by centrifugation and the double-stranded plasmid was prepared using a Promega Midiprep Kit. This DNA sample was the ecotin phage library stock. The other portion of the culture was subsequently diluted to an A_{600} of 0.25, and then infected with VCSM13 helper phage. This infected culture was grown for six hours at 37°C with rigorous shaking, and the phage were harvested as described in the section of ecotin phage preparation below.

Ecotin mutagenesis, expression and purification

Ecotin and ecotin variants were expressed and purified as described (Yang *et al.*, 1998).

Determination of ecotin K_i values against target proteases

Ecotin and ecotin variant K_i values against bovine, rat trypsin and uPA were determined as described (Yang *et al.*, 1998).

Ecotin phage preparation

For the preparation of pBSeco-gIII ecotin bacteriophage, plasmid DNAs were transformed into a male strain (F′) of IMΔecoJ. A single colony selected on ampicillin plates was grown in 3 ml of 2YT medium containing 60 μg/ml ampicillin at 37°C for eight hours. The culture was diluted into 100 ml of 2YT/ampicillin, grown to $A_{600} = 0.25$, and infected with the helper phage VCSM13 at a multiplicity of infection of approximately 100 helper phage per cell. The infected culture was allowed to grow at 37°C with shaking for approximately six hours. Phage particles were harvested by precipitation with one fifth volume of 20% polyethylene glycol 8000, 2.5 M NaCl at 4°C overnight, centrifugation at 6000 *g* for 40 minutes, and resuspension in 1 ml TE buffer. Phage stocks were stored at 4°C for up to six months. Phage titers typically ranged from 10^{10} to 10^{11} cfu/μl culture and were stable within six months.

Ecotin library panning

Polystyrene Petri dishes (35 mm, Falcon 1008) were coated with 1 ml of 10 μg/ml bovine trypsin, rat trypsin or LMuPA in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM K_2HPO_4, pH 7.5) overnight at 4°C, and excess binding sites were blocked with 5% non-fat dry milk PBS solution for two hours. Phage were added to the dishes in buffer containing 1 ml PBS/0.5% Tween 20 and were incubated for two to 24 hours with gentle agitation at 4°C. Solutions containing the phage were then removed and the dishes were washed nine times with 5 ml PBS/0.5% Tween 20. Bound phage were serially eluted by incubation with 900 μl of 0.1 M HCl/glycine solution (pH 2.2) with gentle shaking for 15 minutes at room temperature. Three elutions were performed and the eluates were neutralized with 167 μl of 1 M Tris-HCl (pH 8.8).

Ecotin library amplification

Equal volumes of three eluates were pooled for subsequent amplification and characterization. Two amplification protocols were used in this experiment. In the liquid amplification procedure, 900 μl of phage elution pool was incubated with 9 ml of fresh grown IMΔecoJ lawn cells for 30 minutes at 37°C with gentle shaking. The infected lawn cells were transferred to 200 ml of 2YT/ampicillin liquid medium and continued to grow to an A_{600} of 0.25, and infected with VCSM13 helper phage. The infected culture was grown for six to ten hours at 37°C with shaking, then harvested and precipitated as described above. In the plate amplification procedure, 9 ml of fresh grown IMΔecoJ lawn cells was incubated wit 900 μl of phage elution pool at 37°C for 30 minutes with gentle shaking. The cells were then plated on multiple LB/ampicillin plates (polystyrene Petri dishes, 150 mm, Falcon 3025) and grown for six to ten hours. Cells from these plates were recovered by soaking each plate with 5 ml of 2YT and scraping the cells into the medium. The 2YT medium containing cells from plates was collected in 100 ml of 2YT/ampicillin and grown for one to two hours. This cell culture was then diluted in 100 ml 2YT/ampicillin to an $A_{600} = 0.25$, and infected with VCSM13 helper phage. The infected culture was grown for six hours at 37°C with shaking, and the phage were harvested as described above.

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References

Andreasen, P. A., Sottrup-Jensen, L., Kjoller, L., Nykjaer, A., Moestrup, S. K., Petersen, C. M. & Gliemann, J. (1994). Receptor-mediated endocytosis of plasminogen activators and activator/inhibitor complexes. *FEBS Letters*, **338(3)**, 239–245.

Bass, S., Greene, R. & Wells, J. A. (1990). Hormone phage: an enrichment method for variant proteins with altered binding properties. *Proteins: Struct. Funct. Genet.* **8(4)**, 309–314.

Chung, C. H., Ives, H. E., Almeda, S. & Goldberg, A. L. (1983). Purification from *Escherichia coli* of a peri-

plasmic protein that is a potent inhibitor of pancreatic proteases. *J. Biol. Chem.* **258(18)**, 11032–11038.
Corey, D. R., Shiau, A. K., Yang, Q., Janowski, B. A. & Craik, C. S. (1993). Trypsin display on the surface of bacteriophage. *Gene,* **128(1)**, 129–134.
Dang, Q. D. & Di Cera, E. (1996). Residue 225 determines the Na(+)-induced allosteric regulation of catalytic activity in serine proteases. *Proc. Natl Acad. Sci. USA,* **93(20)**, 10653–10656.
Fazioli, F. & Blasi, F. (1994). Urokinase-type plasminogen activator and its receptor: new targets for antimestastic therapy? *Trends Pharmacol. Sci.* **15(1)**, 25–29.
Higaki, J. N., Evnin, L. B. & Craik, C. S. (1989). Introduction of a cysteine protease active site into trypsin. *Biochemistry,* **28(24)**, 9256–9263.
Jamieson, A. C., Kim, S. H. & Wells, J. A. (1994). In vitro selection of zinc fingers with altered DNA-binding specificity. *Biochemistry,* **33(19)**, 5689–5695.
Kunkel, T. A. (1985). Rapid and efficient site-specific mutagenesis without phenotypic selection. *Proc. Natl Acad. Sci. USA,* **82(2)**, 488–492.
Markland, W., Ley, A. C. & Ladner, R. C. (1996a). Iterative optimization of high-affinity protease inhibitors using phage display. 2. Plasma kallikrein and thrombin. *Biochemistry,* **35(24)**, 8058–8067.
Markland, W., Ley, A. C., Lee, S. W. & Ladner, R. C. (1996b). Iterative optimization of high-affinity proteases inhibitors using phage display. 1. Plasmin. *Biochemistry*, **35(24)**, 8045–8057.
McCafferty, J., Griffiths, A. D., Winter, G. & Chiswell, D. J. (1990). Phage antibodies: filamentous phage displaying antibody variable domains. *Nature,* **348(6301)**, 552–554.
McCafferty, J., Jackson, R. H. & Chiswell, D. J. (1991). Phage-enzymes: expression and affinity chromatography of functional alkaline phosphatase on the surface of bacteriophage. *Protein Eng.* **4(8)**, 955–961.
McGrath, M. E., Erpel, T., Browner, M. F. & Fletterick, R. J. (1991). Expression of the protease inhibitor ecotin and its co-crystallization with trypsin. *J. Mol. Biol.* **222(2)**, 139–142.
Pannekoek, H., van Meijer, M., Schleef, R. R., Loskutoff, D. J. & Barbas, C. d. (1993). Functional display of human plasminogen-activator inhibitor 1 (PAI-1) on phages: novel perspectives for structure-function analysis by error-prone DNA synthesis. *Gene,* **128(1)**, 135–140.
Perona, J. J. & Craik, C. S. (1995). Structural basis of substrate specificity in the serine proteases. *Protein Sci.* **4(3)**, 337–360.
Rebar, E. J. & Pabo, C. O. (1994). Zinc finger phage: affinity selection of fingers with new DNA-binding specificities. *Science,* **263(5147)**, 671–673.
Roberts, B. L., Markland, W., Ley, A. C., Kent, R. B., White, D. W., Guterman, S. K. & Ladner, R. C. (1992). Directed evolution of a protein: selection of potent neutrophil elasatase inhibitors displayed on M13 fusion phage. *Proc. Natl Acad. Sci. USA,* **89(6)**, 2429–2433.
Soumillion, P., Jespers, L., Bouchet, M., Marchand-Brynaert, J., Winter, G. & Fastrez, J. (1994). Selection of beta-lactamase on filamentous bacteriophage by catalytic activity. *J. Mol. Biol.* **237(4)**, 415–422.
Spraggon, G., Phillips, C., Nowak, U. K., Ponting, C. P., Saunders, D., Dobson, C. M., Stuart, D. I. & Jones, E. Y. (1995). The crystal structure of the catalytic domain of human urokinase-type plasminogen activator. *Structure,* **3(7)**, 681–691.
Stubbs, M. T. & Bode, W. (1995). The clot thickens: clues provided by thrombin structure (published erratum appears in *Trends Biochem. Sci.* (1995) March **20(3)**, 131). *Trends Biochem. Sci.* **20(1)**, 23–28.
Wang, C. I., Yang, Q. & Craik, C. S. (1995). Isolation of a high affinity inhibitor of urokinase-type plasminogen activator by phage of ecotin. *J. Biol. Chem.* **270(20)**, 12250–12256.
Yang, S. Q., Wang, C. I., Gillmor, S. A., Fletterick, R. J. & Craik, C. S. (1998). Ecotin: a serine protease inhibitor with two distinct and interacting binding sites. *J. Mol. Biol.* **279**, 945–957.

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