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Biochemistry

Directed evolution of a protein: Selection of potent neutrophil elastase inhibitors displayed on M13 fusion phage

(display phage/library fractionation/protease inhibitor/Kunitz domain)

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ABSTRACT **Inhibitors of human neutrophil elastase were engineered by designing and producing a library of phage-displayed protease inhibitory domains derived from wild-type bovine pancreatic trypsin inhibitor and fractionating the library for binding to the target protease. The affinity of one of the engineered variants for human neutrophil elastase (K_d = 1.0 pM) is 3.6 × 10^6-fold higher than that of the parental protein and exceeds the highest affinity reported for any reversible human neutrophil elastase inhibitor by 50-fold. Thus the display phage method has allowed us to obtain protein derivatives that exhibit greatly increased affinity for a predetermined target. The technology can be applied to design high-affinity proteins for a wide variety of target molecules.**

Filamentous phage displaying small nonfunctional peptides can be selected by virtue of their ability to bind to specific antibodies (1–4) or streptavidin (5). Such fusion phage can also be used to raise antibodies to specific peptide antigens (6). A single-chain antibody (7), a Fab fragment (8), and human growth hormone (9) have been displayed on the surface of filamentous phage. Each phage particle links a particular gene carried within the particle to the gene-encoded protein displayed on the package surface. Directed evolution of binding proteins can be accelerated greatly by constructing a library of phage displaying a multitude of variants of a parental binding protein and fractionating the library to select for fusion phage that display variants with the highest affinity for a target (10). Phage titers of 1 × 10^{13} plaque-forming units (pfu)/ml can readily be obtained; hence, a vast number of display phage can be examined. Further, fusion phage can be amplified and subjected to additional rounds of fractionation. Finally, filamentous phage are resistant to agents that can be used to disrupt protein–ligand interactions. Rapid simultaneous examination of the binding capabilities of a very large number (up to 10^8 with present technology) of mutated proteins can be achieved and applied to improve the affinity of a wide variety of proteins for almost any ligand.

We chose human neutrophil elastase (HNE) as the target for the selection of binding proteins. HNE is an abundant serine protease involved in the elimination of pathogens and in connective tissue restructuring (11). In cases of hereditary reduction of the circulating α_1 antiproteinase inhibitor, the principal physiological inhibitor of HNE (12), or inactivation of α_1 antiproteinase inhibitor by oxidation (smoker's emphysema), extensive destruction of lung tissue results from the uncontrolled elastolytic activity of HNE (13). Hence, a small nontoxic highly efficacious inhibitor of HNE could be of great therapeutic value.

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We describe a library of gene III fusion phage displaying 1000 customized protease inhibitor variants derived from wild-type bovine pancreatic trypsin inhibitor (BPTI). The library was fractionated and fusion phage displaying engineered protease inhibitors with high affinity for HNE were selected. One engineered protease inhibitor is 50-fold more potent than the most potent anti-HNE Kunitz derivative described (14); this protein has 3.6 × 10^6-fold higher affinity for HNE than does BPTI. Thus we demonstrate that the display phage method has allowed us to select protein derivatives exhibiting significantly improved affinity for a predetermined target.

MATERIALS AND METHODS

Reagents and Strains. Restriction endonucleases, T4 DNA ligase, and calf intestinal phosphatase were obtained from New England Biolabs, Sequenase Version 2.0 was from United States Biochemical, and oligonucleotides were from Genosys (The Woodlands, TX). HNE and goat anti-rabbit antibody (alkaline phosphatase conjugate) were obtained from Calbiochem, Reacti-Gel (6×) CDI-activated agarose was from Pierce, and bovine pancreatic trypsin and BPTI were from Sigma. *Escherichia coli* extract was from Promega. Rabbit anti-BPTI serum was a kind gift of T. E. Creighton (MRC Laboratory of Molecular Biology, Cambridge, UK). *E. coli* XL1-Blue was obtained from Stratagene. Suc(OMe)-Ala-Ala-Pro-Val-MCA and Boc-Gln-Ala-Arg-MCA (where Suc is succinyl, MCA is 4-methyl-coumaryl-7-amide, and Boc is *t*-butoxycarbonyl) were from Peninsula Laboratories. Immobilon membrane was from Millipore.

Preparation of Immobilized HNE. HNE was cross-linked to Reacti-Gel (6×) CDI-activated agarose beads as recommended by the manufacturer (Pierce). Prior to use, beads were washed three times with Tris-buffered saline/bovine serum albumin (TBS/BSA) (2) and resuspended as a 50% (wt/vol) slurry in TBS/BSA. A 25% slurry of beads in 50% (vol/vol) glycerol/50% (vol/vol) TBS/BSA was prepared and stored at −20°C.

Construction of a Library of Fusion Phage Displaying 1000 Kunitz Domains. A single-stranded 76-mer oligonucleotide (5′-CCTGCTTTAGCATTGTAGAAATAGCGTKDGR-WSAHASCSAHGCAAGGCCCAGTGTATGGTGGCTC-GAGACAGAAAT-3′) was converted to double-stranded form after annealing to a 20-base-pair primer (5′-GATTTC-TGTCTCGAGCCACC-3′) (15). The double-stranded oligonucleotide was digested with *Xho* I, purified by agarose gel electrophoresis, dephosphorylated, and ligated to the 8.0-kilobase *Xho* I–*Stu* I fragment derived from the M13-derived

Abbreviations: HNE, human neutrophil elastase; pfu, plaque-forming unit(s); BPTI, bovine pancreatic trypsin inhibitor; BSA, bovine serum albumin; Rf, replicative form; MCA, 7-amino-4-methylcoumarin.

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gene III display vector SHO-KUN2 replicative form (Rf) (B.L.R., W.M., K. Siranosian, M. J. Saxena, S.K.G. and R.C.L., unpublished data). The fusion phage encoded by this vector displays a BPTI variant in which residues 39–42 of wild-type BPTI (RAKR) have been converted to MGNG. Ligation samples were used to electroporate XL1-Blue cells, which were plated onto LB/agar plates containing ampicillin at 100 μg/ml, ampicillin-resistant colonies were harvested, and fusion phage were recovered by PEG precipitation. Since the display vector contains a single gene III (encoding the BPTI–III fusion protein), each fusion phage has the potential to display five copies of the fusion protein.

Fractionation of the Fusion Phage Library. Fusion phage (4×10^{11} pfu in 40 μl of TBS/BSA) were added to 10 μl of a 50% slurry of immobilized HNE in TBS/BSA at room temperature. The sample was mixed for 1.5 h, 500 μl of TBS/BSA was added to the sample, and, after an additional 5 min of mixing, the HNE beads were collected by centrifugation for 30 sec in a microcentrifuge at 2000 rpm. Supernatant was removed and the beads were resuspended in 0.5 ml of TBS/0.5% Tween 20, washed for 5 min, and recovered by centrifugation as above. The beads were washed four additional times with TBS/0.5% Tween 20 to reduce nonspecific binding of fusion phage. Beads were washed twice as above with 0.5 ml of 50 mM sodium citrate, pH 7.0/150 mM NaCl containing BSA (1.0 mg/ml), and supernatants from these washes were pooled and neutralized with 1 M Tris (pH 8.0). The beads were washed in this manner with two 0.5-ml samples of each of a series of 50 mM sodium citrate buffers at pH 6.0, 5.0, 4.5, 4.0, 3.5, 3.0, 2.5, and 2.0, and samples were neutralized and titered in duplicate. Fusion phage from the fraction at pH 2.0 were amplified from transductants by incubating 2000 pfu with 100 μl of cells for 15 min at 37°C in 200 μl of 1× minimal A salts followed by addition of 200 μl of 2× LB and incubation for 15 min at 37°C. Portions (100 μl) of the transduction sample were plated onto LB/agar plates containing ampicillin at 100 μg/ml, such that five samples yielded 7000 transduced colonies. Cells were harvested and fusion phage were collected. Amplified fusion phage recovered from the transductants (40 μl, 5.8×10^{10} pfu) were added to 10 μl of HNE beads and subjected to a second enrichment cycle as described above. Fusion phage present in the fraction at pH 2.0 of the second cycle were amplified as above and subjected to a third enrichment cycle for which the input was 1.1×10^{11} pfu. Rf DNA corresponding to selected fusion phage was sequenced using specific primers by the dideoxynucleotide method (16).

Production and Purification of Protease Inhibitors in *E. coli*. An expression vector was constructed from an M13 derivative MB48 (17) that encodes a *phoA* signal peptide–mature BPTI–mature M13 gene VIII fusion protein. A stop codon was introduced between the DNA sequences encoding BPTI and encoding mature gene VIII protein in MB48 by site-specific mutagenesis using a 24-mer oligonucleotide (5′-CGCCACCGCGGATCCTTCCACTAC-3′) to create the expression vector BS1. The 125-base-pair *Xho* I–*Eag* I fragments derived from Rf DNAs encoding the Epi1, -3, and -7 inhibitors were ligated to the 8.0-kilobase *Xho* I–*Eag* I fragment derived from BS1 Rf. Positive clones were identified by dot-blot analysis of fusion phage derived from plaques using radiolabeled oligonucleotides (Epi1, 5′-GCGTGGGAAGAAAGCGATGCA-3′; Epi3, 5′-TGAGAAGAAACCGACGCA-3′; Epi7, 5′-GCGTGGGAACATAGCGACGCA-3′). Phage stocks were prepared, and 500-ml cultures of XL1-Blue cells ($OD_{600} = 0.5$) were infected with the above M13 expression vectors at a multiplicity of infection of 10. Thirty minutes after infection, isopropyl β-D-thiogalactoside was added to a final concentration of 0.5 mM and cultures were incubated overnight at 37°C. Cells were removed from cultures by two centrifugations at 5000 × *g* for 20 min and supernatant was filtered through a 0.45-μm (pore size) membrane and adjusted to pH 7.5 with 1 M Tris·HCl (pH 7.5). The filtrates were loaded onto a column (100-μl packed bed volume) of immobilized HNE, which was subsequently washed with 50 mM Tris·HCl (pH 7.5). Bound engineered protease inhibitor was eluted with 250 mM KCl/20 mM HCl, and samples were neutralized with 1 M Tris·HCl (pH 8.0). Affinity-purified engineered protease inhibitor was subjected to electrophoresis on SDS/urea/15% polyacrylamide gels (18) and protein was quantitated either by silver staining or by Western blot analysis after electrotransfer onto an Immobilon membrane. Western blots were developed using anti-BPTI rabbit serum (previously incubated with an *E. coli* extract), followed by goat anti-rabbit antibody conjugated to alkaline phosphatase.

Kinetic Analysis. Inhibitor was incubated with protease in 50 mM Tris·HCl, pH 8.0/150 mM NaCl/1 mM $CaCl_2$/0.05% Triton X-100 for 30 min at room temperature. Residual enzyme activity was assayed at room temperature using a Perkin–Elmer model 650-15 fluorescence spectrophotometer. Reaction rates were linear for at least the first 10 min. The following assay conditions were employed: for HNE, 1.0 nM enzyme/0.1 mM Suc(OMe)-Ala-Ala-Pro-Val-MCA substrate (where Suc is succinyl); for bovine pancreatic trypsin, 0.022 nM enzyme/0.1 mM Boc-Gln-Ala-Arg-MCA substrate (where Boc is *t*-butoxycarbonyl). By the method used, the largest and smallest K_d values that could be measured for inhibitors of HNE are 2 nM and 1 pM, respectively. Inhibition plots were determined at least twice and K_d values were calculated based on all data obtained from inhibition plots.

RESULTS

Construction of a Library of Gene III Fusion Phage Displaying Engineered Protease Inhibitors. Fusion phage dissociate from immobilized HNE at a specific pH characteristic of the particular BPTI variant displayed (B.L.R., W.M., K. Siranosian, M. J. Saxena, S.K.G. and R.C.L., unpublished data). Each characteristic pH is a direct function of the known K_d values of a set of BPTI mutants. Therefore, pH-dependent elution can be exploited to fractionate a library of fusion phage displaying BPTI variants exhibiting a range of affinities for HNE.

A library of gene III fusion phage comprising up to 1000 displayed engineered protease inhibitors derived from wild-type BPTI was constructed. A variegated oligonucleotide (MUT001) was designed to mutate the DNA encoding residues 15–19 of wild-type BPTI (Fig. 1). Prior to construction of the library, the mutations R39M, A40G, K41N, and R42G were introduced into wild-type BPTI (B.L.R. *et al.*, unpublished data). We have shown that these alterations enhance the affinity of a BPTI variant for HNE (B.L.R. *et al.*, unpublished data). The P1 residue of Kunitz domains (Lys-15

Structural Position		P1	P1′	P2′	P3′	P4′
BPTI position		15	16	17	18	19
Wild type Sequence		K	A	R	I	I
Allowed amino acids		V F M / L I	G A	V F M / L I	F I / S T	K Q T / S P stop
DNA	5′-	A T C / G G / T	G C T / G	A T C / G G / T	A C C / T T	A A A / C C / T

FIG. 1. Sequence of the mutagenic region of the oligonucleotide used to mutate residues 15–19 of the (R39M, A40G, K41N, R42G)BPTI mutant and the encoded amino acids are indicated. The oligonucleotide contains 1746 DNA sequences that encode 1000 protein sequences.

in BPTI) (19) is considered to be the principal determinant of the inhibitory specificity (20). The MUT001 oligonucleotide encodes any one of five hydrophobic amino acids (phenylalanine, leucine, valine, methionine, and isoleucine) at position 15. This is consistent with the known substrate specificity of HNE (21). The replacement of Lys-15 with aliphatic hydrophobic residues yields proteins with higher affinity for HNE than BPTI (20). At position 16, either glycine or alanine is encoded. These amino acids prevail at the corresponding positions in a variety of BPTI homologues (22). The variegation scheme at position 17 is identical to that at position 15. We anticipated that a hydrophobic residue at this position might enhance the binding of BPTI variants to HNE. Finally, at positions 18 and 19, four (phenylalanine, serine, threonine, and isoleucine) and five (serine, proline, threonine, lysine, and glutamine) amino acids, respectively, are encoded. These amino acids are found in the corresponding positions of BPTI homologues that bind HNE (22). Although the amino acids included in the variegation scheme were chosen because they might facilitate binding to HNE, it is not possible to predict which combination is optimal for high-affinity binding.

The MUT001 library of fusion phage was constructed by cassette mutagenesis. Dot-blot analysis indicated that of 4900 transformants harvested, 81% (≈3970) contained substantial alterations within the P1 region from the mutagenesis (data not shown). Since the total number of potentially different DNA sequences in the MUT001 library is 1728, we calculated that the phage library displays $1 - \exp(-3970/1728) = 0.90$ of the potential engineered protease inhibitors, assuming equal representation of the 1728 DNA sequences.

Selection of Engineered Protease Inhibitors with High Affinity for HNE. Fusion phage obtained from ampicillin-resistant transformants were pooled and incubated with HNE-agarose beads. Bound phage were recovered by washing the beads sequentially with buffers of decreasing pH. Fig. 2 illustrates that the largest percentage of input phage that bound to the HNE matrix was recovered in the pH 5.0 wash in the first enrichment cycle. The elution peak exhibited a trailing edge at low pH, suggesting that a small proportion of the total bound fusion phage might elute from HNE beads at a pH of <5.0. Further, phage SHO-KUN1, displaying a BPTI variant, (K15L)BPTI, with moderate affinity (K_d = 2.9 nM) for HNE (20), were eluted from HNE beads as a peak centered on pH 4.75 (B.L.R. *et al.*, unpublished data). Since the highest peak in the first passage of the MUT001 library was centered on pH 5.0, we infer that some members of the library display variants having moderate-to-high affinity for HNE.

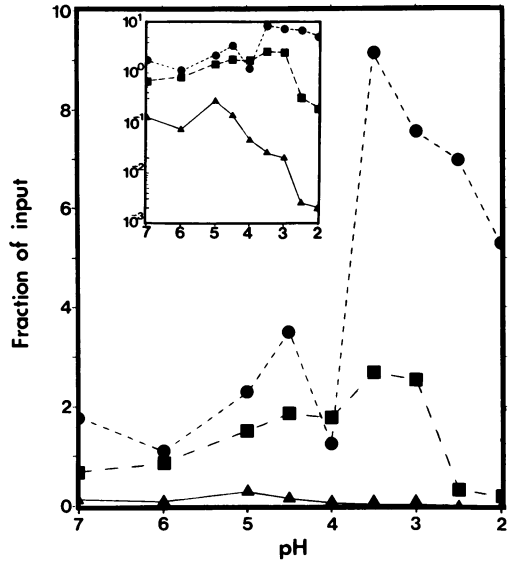

FIG. 2. Fractionation of the fusion phage library. Variegated fusion phage (4×10^{11} pfu) were added to immobilized HNE and bound phage were recovered by washing with buffers of decreasing pH. The total pfu recovered in each pH fraction as a percent of the input ($\times 10^1$) is plotted as a function of pH (▲). Fusion phage from the fraction at pH 2.0 of the first cycle were amplified and subjected to a second enrichment cycle (■); input was 5.8×10^{10} pfu. Fusion phage from the fraction at pH 2.0 of the second cycle were amplified and subjected to a third enrichment cycle (●); input was 1.1×10^{11} pfu. The insert illustrates the same data plotted on a semilogarithmic scale.

Phage from the fraction at pH 2.0 of the first enrichment cycle were amplified and subjected to a second round of enrichment. The largest percentage of input phage that bound to HNE beads was recovered in the fraction at pH 3.5 (Fig. 2). A smaller peak centered on pH 4.5 was apparent and may represent residual fusion phage from the first passage of the library that eluted at pH 5.0. The percentage of total input phage that eluted at pH 3.5 in the second cycle exceeded that which eluted at pH 5.0 in the first cycle. This is indicative of more avid binding of some members of the fusion phage library to the HNE matrix, suggesting that selection for fusion phage displaying BPTI variants with higher affinity for HNE had occurred.

Phage from the fraction at pH 2.0 of the second enrichment cycle were amplified and subjected to a third round of fractionation. The largest percentage of input phage was recovered in the fraction at pH 3.5 (Fig. 2). The percentage of input phage that eluted at pH 3.5 was greater in the third enrichment cycle than in the second. By comparison, SHO-KUN4 phage, which display a BPTI variant, (K15V, R17L)BPTI with high affinity for HNE (K_d = 60 pM) (14), were eluted from HNE beads as a peak centered on pH 4.0 (B.L.R. *et al.*, unpublished data). A significant selection for fusion phage displaying high affinity for the HNE matrix has occurred. Furthermore, that these phage were eluted from HNE beads at a pH of <4.0 suggests that engineered protease inhibitors displayed by these phage possess higher affinities for HNE than (K15V, R17L)BPTI (i.e., a K_d < 60 pM).

Characterization of Selected Clones. Twenty plaques from the fraction at pH 2.0 of the third enrichment cycle were picked at random and plaque-purified three times. Each phage strain was amplified and characterized with respect to affinity for immobilized HNE and the Rf DNA was sequenced. Five of the eight unique clones examined (Epi1, -3, -5, -6, and -7) exhibit elution peaks centered on pH 3.5 (data not shown), with no minor peak centered on pH 4.5. The remaining three clones (Epi2, -4, and -8) have elution profiles that peak in the range of pH 4.0–3.5.

The DNA sequences that encode the engineered protease inhibitors were determined and the amino acid sequences predicted from the DNA sequences of the P1 region are shown in Table 1. The sequences of Epi1, -3, and -7 appeared four, six, and five times, respectively. By assuming that the 1728 DNA sequences encoded by the MUT001 oligonucleotide were present at equal frequencies in the library, the prevalence of Epi1, -3, and -7 suggests that these mutant proteins have the highest affinity for HNE of the BPTI variants obtained from the library.

Only valine or isoleucine appear at the P1 position. No example of leucine, phenylalanine, or methionine at the P1 position was observed, consistent with the observation that BPTI variants having one of these amino acids at the P1 position exhibit significantly lower affinities for HNE than their counterparts containing valine or isoleucine (20).

Phenylalanine is favored at position 17, appearing in 12 of 20 clones. Methionine appears at position 17 when valine is present at position 15. At position 18, phenylalanine is observed in all 20 clones. This result is surprising and could

Table 1. Amino acid sequences in the region of variegation (residues 15–19) for the selected engineered protease inhibitor (Epi) clones predicted from the Rf DNA sequence

Clone	Sequence					Frequency
	15	16	17	18	19	
Epi3	V	G	F	F	S	6
Epi7	V	A	M	F	P	5
Epi1	I	A	F	F	P	4
Epi5	I	A	F	F	Q	1
Epi6	V	G	F	F	Q	1
Epi2	I	A	L	F	K	1
Epi4	V	A	I	F	P	1
Epi8*	V	A	I	F	K	1

Frequency of occurrence of each sequence among 20 random clones derived from the pH 2.0 fraction of the third enrichment cycle of the MUT001 library is shown.

*Epi8 carries an unexpected alteration, Y21S, outside the region of variegation. This additional mutation may have been introduced during DNA synthesis or manipulation.

not have been predicted from previous mutational studies or on any current theoretical grounds. Finally, at position 19, four of the five amino acid possibilities were observed; proline occurred in 10 of 20 isolates. Of the residues targeted for mutagenesis, residue 19 is nearest to the predicted boundary of the interaction surface of an engineered protease inhibitor with HNE (19). Thus a variety of amino acids can be accommodated at position 19 without significantly compromising the HNE binding capacity of the engineered protease inhibitors.

Kinetic Determination of the Affinities of Engineered Protease Inhibitors for HNE. The genes encoding Epi1, -3, and -7 were expressed in *E. coli* and the engineered protease inhibitors were purified by affinity chromatography using immobilized HNE. The purified protein was at least 95% pure as judged by silver staining of polyacrylamide gels (data not shown). To examine inhibitory activity, we incubated purified engineered protease inhibitor with HNE for 30 min and determined residual enzymatic activity using a fluorometric assay. K_d values were determined by the method of Green and Work (23) using a computer-assisted best-fit program. The three engineered protease inhibitors are extremely potent inhibitors of HNE, having K_d values for HNE of 1.0 pM (Epi1, Fig. 3 *Left*), 2.8 pM (Epi3), and 2.7 pM (Epi7). Under the same conditions, we measured the K_d value between HNE and (K15V, R17L)BPTI to be 51 pM (Fig. 3 *Right*), in accord with ref. 14. The dissociation constant of BPTI for HNE is 3.6 μM (24); the dissociation constant of these BPTI derivatives for HNE is more than six orders of magnitude lower. None of the three engineered protease inhibitors inhibited trypsin when present in equimolar concentration. This is consistent with the loss of five basic residues of BPTI that interact with trypsin. Furthermore, Epi1 protein does not show measurable affinities for either immobilized porcine pancreatic elastase or human cathepsin G (data not shown). Thus Epi1 appears to be a specific inhibitor of HNE and is not a promiscuous inhibitor of serine proteases that prefer hydrophobic amino acid substrates.

DISCUSSION

A library of fusion phage that display BPTI proteins with designed amino acid substitutions within the predicted interface with HNE was constructed. We chose wild-type BPTI as the parental protein for this library because it is a small stable protein whose three-dimensional structure has been determined (19). Furthermore, a variety of BPTI homologues are known (22), and BPTI has been expressed in *E. coli* (25, 26) and shown to be safe when administered as a drug (27, 28). We calculate that this fusion phage library displays ≈900 of the potential 1000 BPTI variants. To our knowledge, this is the largest population of mutated proteins derived from a single parental protein that has been examined for binding properties in any one study.

As seen in Fig. 2, pH elution profiles afford a means of monitoring the fractionation process with a high degree of resolution. Only 2.0×10^{-4}% of the input phage (8.1×10^5 pfu) was recovered in the fraction at pH 2.0 from the first enrichment cycle of the library, a proportion small enough to constitute a true selection. Other investigators have employed a single low pH elution buffer to recover bound fusion phage (1–5). In the present study, this would have yielded a more heterogenous population of fusion phage and necessitated the use of additional enrichment cycles, instead of the three cycles employed here, to select for phage displaying engineered protease inhibitors with the highest affinity for HNE.

A comparison of the pH elution profiles shown in Fig. 2 indicates that the greatest enrichment was achieved in proceeding from the first to the second enrichment cycle. The pH elution profile for a fourth enrichment cycle was highly similar to that of the third cycle (data not shown), suggesting that little or no additional enrichment had been achieved. Hence, only three enrichment cycles were required to select

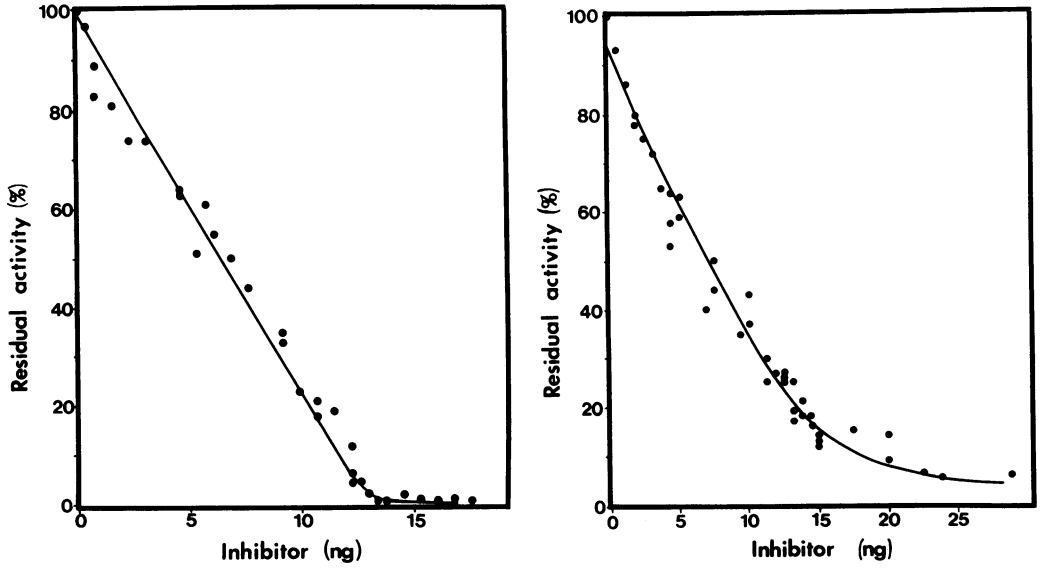

FIG. 3. Inhibition of HNE by affinity-purified Epi1 protein. (*Left*) Effect of increasing amounts of Epi1 protein on the percent residual activity of HNE. (*Right*) Effect of the (K15V, R17L)BPTI variant. A computer-assisted best-fit program was used to fit the data to the theoretical curve shown.

fusion phage displaying BPTI variants with high affinity for HNE.

For other applications, the number of enrichment cycles required to select a high-affinity mutant will depend on the diversity within the library and the relative affinities of mutants for the target. If one particular mutant exhibits a substantially higher affinity for a target than others, a single enrichment cycle may suffice.

The engineered protease inhibitors selected here are, to our knowledge, the most potent anti-HNE Kunitz-type inhibitors described to date. The affinity of Epi1 for HNE was 50-fold higher than that of the (K15V, R17L)BPTI variant and 200-fold higher than that of another well-characterized proteinase inhibitor of HNE, anti-leukoproteinase (29). Furthermore, the affinities of these engineered proteins for trypsin are substantially less than that of the parental protein BPTI. Due to their potent anti-HNE activity, small size, and stability, these proteins may be of value in controlling the destructive elastolytic activity of HNE symptomatic of emphysema and cystic fibrosis.

Cwirla *et al.* (4) selected phage from a library of phage that display random hexapeptides for binding to a monoclonal antibody raised against the peptide YGGFL. None having affinity for the target antibody as high as the original peptide antigen were found, nor was the parental peptide YGGFL found. It has been suggested that multicopy display of peptides on the five gene III proteins per phage virion results in tenacious multivalent binding of fusion phage to immobilized targets. Multicopy display of BPTI as a gene III fusion protein did not affect our ability to select variants exhibiting extremely high affinities for HNE.

The technology described here enables the selection of fusion phage on the basis of their affinity for a specific ligand. This technology is highly versatile and can be employed in an iterative manner to improve the affinity of proteins for a variety of ligands. A single-chain antibody (≈250 amino acids) (7) and human growth hormone (191 amino acids) (9) have been displayed on the surface of phage as gene III fusion proteins. Thus, there does not seem to be a serious limitation on the size of displayed proteins. More important than size is structural integrity; hence, one should introduce complete domains of proteins (10). Although display of a single-chain antibody is of interest, we believe that much smaller stable proteins can exhibit equivalent levels of diversity, show extremely high affinity and specificity, and have a number of advantages over antibodies as therapeutic or diagnostic agents.

We have demonstrated the utility of this technology by selecting highly potent inhibitors of a clinically important enzyme, HNE. Given that this process lends itself to automation, one could rapidly select for many binding proteins of value.

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1. Smith, G. P. (1985) *Science* **228,** 1315–1317.
2. Parmley, S. F. & Smith, G. P. (1988) *Gene* **73,** 305–318.
3. Scott, J. K. & Smith, G. P. (1990) *Science* **249,** 386–390.
4. Cwirla, S. E., Peters, E. A., Barrett, R. W. & Dower, W. J. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 6378–6382.
5. Devlin, J. J., Panganiban, L. C. & Devlin, P. E. (1990) *Science* **249,** 404–406.
6. Greenwood, J., Willis, A. E. & Perham, R. N. (1991) *J. Mol. Biol.* **220,** 821–827.
7. McCafferty, J., Griffiths, A. D., Winter, G. & Chiswell, D. J. (1990) *Nature (London)* **348,** 552–554.
8. Kang, A. S., Barbas, C. F., Janda, K. D., Benkovic, S. J. & Lerner, R. A. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 4363–4366.
9. Bass, S., Greene, R. & Wells, J. A. (1990) *Proteins* **8,** 309–314.
10. Ladner, R. C. & Guterman, S. K. (1990) Int. Patent Appl. W090/02809.
11. Travis, J. (1988) *Am. J. Med.* **84,** Suppl. 6A, 37–42.
12. Heidtmann, H. & Travis, J. (1986) in *Proteinase Inhibitors*, eds. Barrett, A. J. & Salvesen, G. (Elsevier, New York), pp. 441–456.
13. Cantor, J. O. & Turino, G. M. (1989) in *Elastin and Elastases, Vol. II*, eds. Robert, L. & Hornebeck, W. (CRC, Boca Raton, FL), pp. 159–168.
14. Auerswald, E. A., Bruns, W., Horlein, D., Reinhardt, G., Schnabel, E. & Schroder, W. (1989) U.K. Patent Appl. GB 2,208,511A.
15. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. & Struhl, K. (1987) *Current Protocols in Molecular Biology* (Wiley, New York), Vol. 1, Sect. 8.2.8.
16. Sanger, F., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 5463–5467.
17. Markland, W., Roberts, B. L., Saxena, M. J., Guterman, S. K. & Ladner, R. C. (1991) *Gene*, **109,** 13–19.
18. Laemmli, U. K. (1970) *Nature (London)* **227,** 680–685.
19. Gebhard, W., Tschesche, H. & Fritz, H. (1986) in *Proteinase Inhibitors*, eds. Barrett, A. J. & Salvesen, G. (Elsevier, New York), pp. 375–388.
20. Beckmann, J., Mehlich, A., Schroder, W., Wenzel, H. R. & Tschesche, H. (1988) *Eur. J. Biochem.* **176,** 675–682.
21. Nakajima, K., Powers, J. C., Ashe, B. M. & Zimmerman, M. (1979) *J. Biol. Chem.* **254,** 4027–4031.
22. Creighton, T. E. & Charles, I. G. (1987) *Cold Spring Harbor Symp. Quant. Biol.* **52,** 511–519.
23. Green, N. M. & Work, E. (1953) *Biochem. J.* **54,** 347–352.
24. Vincent, J.-P. & Lazdunski, M. (1972) *Biochemistry* **11,** 2967–2977.
25. Marks, C. B., Vasser, M., Ng, P., Henzel, W. & Anderson, S. (1986) *J. Biol. Chem.* **261,** 7115–7118.
26. Marks, C. B., Naderi, H., Kosen, P. A., Kuntz, I. D. & Anderson, S. (1987) *Science* **235,** 1370–1373.
27. Bidstrup, B., Royston, D., Sapsford, R. & Taylor, K. (1989) *J. Thorac. Cardiovasc. Surg.* **97,** 364–372.
28. Carrel, T., Bauer, E., Laske, A., von Segesser, L. & Turina, M. (1991) *Lancet* **337,** 673.
29. Smith, C. E. & Johnson, D. A. (1985) *Biochem. J.* **225,** 463–472.