Eur. J. Biochem. **266**, 403–412 (1999) © FEBS 1999

Selection of human elastase inhibitors from a conformationally constrained combinatorial peptide library

Jeffrey D. McBride[1], Hamzah N. M. Freeman[2] and Robin J. Leatherbarrow[1]

[1]*Department of Chemistry, Imperial College of Science, Technology, and Medicine, London, UK;* [2]*Protein Science Unit, Glaxo Wellcome Research and Development Ltd, Stevenage, Herts, UK*

A resin-bound cyclic peptide library was constructed based on the sequence of the reactive-site loop of Bowman–Birk inhibitor, a proteinase inhibitor protein. The constrained loop sequence, which incorporates the minimal proteinase-binding motif, was retained throughout the library, but selected residues known to be important for inhibitor specificity were randomised. The approach was used to create a 'one bead, one peptide' library with 8000 variants resulting from randomization at three target locations in the sequence (P_4, P_1 and P_2'). This library allows us to examine the degree to which variations in this proteinase-binding motif can redirect activity, as well as providing information about the binding specificity of a proteinase target. Screening this library for binding to human leucocyte elastase identified sequences with a strong consensus, and on resynthesis all were found to act as inhibitors, with K_i values as low as 65 nM. Human leucocyte elastase is known to have a substrate preference for small alkyl chains at the P_1 locus, with valine being preferred. However, alanine and not the expected valine was found in 21 out of 23 identified sequences. The remaining two sequences had threonine at P_1, a finding that would be hard to predict based on substrate specificity alone. Further analysis of resynthesized peptides demonstrated that valine substitution results in an analogue that is hydrolysed far more rapidly than ones having library-selected P_1 residues. Testing of the human leucocyte elastase-selected sequences as inhibitors of porcine pancreatic elastase demonstrates a significant difference in the specificity of the P_4 locus between these two proteinases.

Keywords: Bowman–Birk inhibitor; canonical loop; combinatorial chemistry; elastase; peptide library.

Elastases, which are serine proteinases capable of cleaving the connective tissue elastin, are considered to play important roles in the tissue destruction associated with pulmonary emphysema, rheumatoid arthritis, cystic fibrosis, adult respiratory distress syndrome, chronic bronchitis, and pancreatitis [1,2]. For some of these pathological conditions an imbalance between the amount of elastase and endogenous proteinaceous inhibitor(s) has been implicated. As a result, there has been considerable interest in the design of inhibitors that may restore this balance and/or clarify the role of these enzymes in the above diseases. In addition to natural or engineered inhibitor proteins, which have high molecular masses, researchers have also pursued both peptide-based and nonpeptide small inhibitor compounds [2].

In many natural inhibitor proteins, the portion of the inhibitor protein that interacts with the serine proteinase is an extended or 'canonical' loop. This is thought to have a structure similar to that of a productively bound substrate [3,4]. Inhibitors behave as limited proteolysis substrates; residues interacting with proteinase at the reactive site of the inhibitor determine specificity and typically reflect the substrate specificity of the target proteinase. The identity of the P_1 residue (nomenclature of Schechter & Berger [5]) is considered to be the main determinant of specificity [3,6]. Qasim *et al*. [7] have described canonical loops as molecular vices, delivering the P_1 side chain to the S_1 cavity whether the interaction is favourable, or even when it is neutral or locally deleterious. However, the identity of a given P_1 residue alone does not always succeed in defining inhibitory activity. For example, chicken ovomucoid first domain with a P_1 Lys residue is ineffective as an inhibitor of bovine β-trypsin even though this is the preferred P_1 of trypsin substrates [8]. Although many natural inhibitors are selective for the proteinases they target, some inhibitors are more promiscuous and demonstrate activity against a range of enzymes. For example, mucus proteinase inhibitor (P_1 Leu, P_1' Met) is active against human leucocyte esterase (HLE), cathepsin G, chymotrypsin and trypsin [9]; turkey ovomucoid (TOM) (OMTKY3; P_1 Leu, P_1' Glu) is active against chymotrypsin, porcine pancreatic elastase (PPE), *Streptomyces griseus* proteinases A and B, and subtilisins BPN′ and Carlsberg [10].

Redirection and/or enhancement of elastase inhibitory activity resulting from alterations at the P_1 locus have been described following site-directed mutagenesis [11,12] and semisynthesis or peptide synthesis [13,14]. In general, alteration of the P_1 residue is based upon the known substrate preference of the target proteinase. For example, replacement of the P_1 Met in α_1-protease inhibitor by Val has been used to

Correspondence to R. Leatherbarrow, Department of Chemistry, Imperial College of Science, Technology, and Medicine, South Kensington, London SW7 2AY, UK.

Abbreviations: BBI, Bowman–Birk inhibitor; BPTI, bovine pancreatic trypsin inhibitor; CMTI-III, *Curcubita maxima* trypsin inhibitor-III; FAB, fast-atom bombardment; Fmoc, fluoren-9-ylmethoxycarbonyl; HLE, human leucocyte elastase; OMGPP3, peacock pheasant ovomucoid third domain; OMTKY3, turkey ovomucoid third domain; PPE, porcine pancreatic elastase; TOM, turkey ovomucoid.

Enzymes: human leucocyte elastase (HLE; EC 3.4.21.37); porcine pancreatic elastase (PPE; EC 3.4.21.35).

(Received 28 July 1999, accepted 14 September 1999)

overcome oxidation sensitivity, yet retain specificity [15]. On occasion, P_1 alterations have resulted in unexpected activities. P_1 point mutations to the squash seed proteinase inhibitor, *Curcubita maxima* trypsin inhibitor-III (CMTI-III) have been described by McWherter *et al*. [16], who found that of the limited range of variants tested, Phe and Gly were active against HLE. The activity given by a P_1 Gly is striking as this residue provides no side chain for interaction. Inhibitory activity of this variant must therefore rely on more extensive interactions distant to the S_1 site. More recently, Rozycki *et al*. [17] have further reported reduction in activity when the P_4–P_5 residues were deleted at the N-terminus of CMTI-III (which also had the P_1 residue substituted for valine in order to redirect activity towards HLE). In contrast, removal of these two N-terminal residues from the native inhibitor (which displays anti-tryptic activity) did not result in such decreased activity. Not only does this result further emphasize the importance of subsites distant to P_1 in contributing to binding energy with HLE, it suggests that these are correspondingly less important in trypsin binding.

It seems reasonable that efficient redirection of proteinase inhibitory activity would benefit from a more extensive range of modifications that is not limited to the P_1 locus alone. Combinatorial peptide libraries have provided an attractive approach to screening for potential ligands to a given receptor, and have been used to screen for proteinase inhibitors. Phage display libraries have been successfully used to select redirected inhibitor activity, principally involving randomization of the active sites from the Kunitz (or bovine pancreatic trypsin inhibitor, BPTI) family of proteinase inhibitors [18–20]. We have recently used a synthetic combinatorial protocol to create a library of potential inhibitor structures [21], which has the potential advantage of allowing incorporation of nonprotein amino acids. This work is based on the ability of short disulfide-linked peptide sequences that reproduce the extended loop region of Bowman–Birk inhibitor (BBI) proteins to retain inhibitory activity [13,22,23]. Our library contained 8000 cyclic peptide variants obtained by randomization at the P_2, P_1 and P_2' residues [21], and used the 'one bead, one peptide' approach [24,25] to combinatorial peptide synthesis. Screening this library for redirected activity against chymotrypsin identified a consensus-binding motif and allowed the selection of competitive inhibitors with K_i values in the nanomolar range. Using this same approach, we now describe use of a slightly different cyclic peptide library to screen for inhibitors with activity redirected towards HLE. The active sequences that were identified were also tested as inhibitors of PPE to allow a comparison of the specificity of these elastases. These two enzymes have similar activity, share 40% primary sequence identity and a number of HLE and PPE complexes have demonstrated particular topological similarity in their active-site regions [26].

EXPERIMENTAL PROCEDURES

Materials

Fluoren-9-ylmethoxycarbonyl (Fmoc)-protected amino acids and derivatives were purchased from Novabiochem or Advanced ChemTech with the following side-chain-protecting groups: Ala, Arg (2,2,5,7,8-pentamethylchroman-6-sulfonyl), Asn (trityl), Asp (*tert*Bu), Cys (trityl), Gln (trityl), Glu (anti-tryptic), Gly, His (trityl), Ile, Leu, Lys (*tert*-butyloxycarbonyl; *tert*Boc), Met, Nle, Phe, Pro, Ser (anti-tryptic), Thr (anti-tryptic), Trp (*tert*Boc), Tyr (anti-tryptic), Val. TentaGel-S-NH_2 resin was from Rapp Polymere (Tubingen, Germany). Dimethylformamide and *N*-methylpyrrolidone were peptide synthesis grade from Rathburn Chemicals (Walkerburn, UK) and 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate and 1-hydroxybenzotriazole monohydrate were from SNPE (Croydon, Surrey. UK). 5-Bromo-4-chloro-3-indolyl phosphate, nitroblue tetrazolium, phenol, ethanedithiol, thioanisole, dimethyl sulfoxide, monoclonal anti-rabbit IgG (γ-chain specific) conjugated to alkaline phosphatase, and trifluoroacetic acid were purchased from Sigma. Elastase from human polymorphonuclear leucocytes (HLE, EC 3.4.21.37) was from Elastin Products Co. Inc., Owensville, MO, USA, PPE (EC 3.4.21.35) was from Sigma, and rabbit anti-HLE was from Calbiochem.

Preparation of tethered cyclic peptide library

Synthesis of the library peptide, disulfide-cyclized NH_2-XCTXSXPPQCYGGGGG-Resin, was performed on an ABI 431A peptide synthesizer at 0.25 mmol scale using Fmoc chemistry. The solid-phase resin was TentaGel-S-NH_2, which has a noncleavable linker. 2-(1*H*-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate/1-hydroxybenzotriazole monohydrate [27] activation was used for amino acid couplings at fivefold amino acid excess except for the positions marked X. At these positions, randomization was achieved using preactivated pentafluorophenyl ester or 3-hydroxy-4-oxo-3,4-dihydro-1,2,3-benzotriazine ester amino acid esters at 10-fold excess as described previously [21]. After side-chain deprotection, cyclization was achieved using dimethyl sulfoxide [28]. We have previously demonstrated that this method provides an efficient means of on-resin cyclization, with the generation of active resin-immobilized peptide [29]. Resin was then thoroughly washed and resuspended in NaCl/P_i with 0.05% Tween 20 (NaCl/P_i/Tween) before use.

Preparation of defined sequence cyclic peptides

Peptide sequences identified from the screening procedure were resynthesized by standard solid-phase Fmoc chemistry as described previously using a Shimadzu PSSM-8 synthesizer. The crude peptides were cyclized using dimethylsulfoxide also as described previously [21].

The cyclic peptides were purified by HPLC using a Gilson instrument equipped with a Waters Radial Pak C_{18} column (25 mm × 10 cm, 6-μm particle size) reverse-phase column. Separations were carried out with 0.1% trifluoroacetic acid and acetonitrile as solvents. Peptides were recovered after semi-preparative chromatography by lyophilization. The homogeneity of the synthesized peptides was assessed by TLC, analytical HPLC and MS. Analytical HPLC was performed using a Vydac C_{18} column (150 × 4.6 mm, 5 μm particle size) employing a gradient between water and acetonitrile, each containing 0.1% trifluoroacetic acid (5% acetonitrile for 2.5 min, 5–80% acetonitrile over 22.5 min, 80% acetonitrile for a further 2.5 min before returning to 5% for 2.5 min). Flow rate was 1 mL·min^{-1} with detection at 226 nm. TLC was conducted on prelayered silica-gel 60 F_{254} plates with fluorescent indicator (Merck), and two different solvent systems were used for analysis. Solvent system A was methanol/chloroform/acetic acid (4 : 15 : 1, v/v), and system B was methanol/water/acetonitrile (5 : 5 : 3, v/v); detection was performed using UV (254 nm) and ninhydrin [0.2% (w/v) in acetone]. Mass spectra were recorded on a VG Auto-spec Q instrument operating in the fast-atom bombardment (FAB) mode. In all cases, peptides

were more than 85% pure by HPLC, displayed a single spot on TLC, and the correct mass was confirmed by FAB-MS.

Screening the BBI library against HLE

Screening was performed using a modification of the method described previously [21]. Briefly, HLE was incubated in NaCl/P_i/Tween with sufficient resin to provide ≈ 46 000 beads. The resin was then washed thoroughly before incubating with rabbit anti-HLE in NaCl/P_i/Tween. The washing step was repeated and beads were then incubated with monoclonal anti-rabbit IgG (γ-chain specific) conjugated to alkaline phosphatase. The washing step was again repeated with a final wash of 100 mM Tris (pH 9.5)/0.5 mM $MgCl_2$. Beads were then incubated in the same buffer with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium as alkaline phosphatase substrate. Positively stained beads had a deep purple coloration, and were transferred manually to glass-fibre filters, washed with 40 μL 50 mM HCl, and air-dried. N-Terminal sequencing was performed on either an ABI 476A (PE Biosystems) or an HP G1005A protein sequencer (Hewlett-Packard) using standard sequencing protocols. The beads were prewashed with trifluoroacetic acid and ethyl acetate before sequence analysis, as this was found to reduce the background signal in the first cycle. On the ABI sequencer, the bead was sequenced on the trifluoroacetic acid-washed glass-fibre filter, but on the HP sequencer the bead was trapped at the interface of the hydrophobic and hydrophilic columns of an N-terminal sequencing cartridge.

Inhibition kinetics

Inhibition kinetics for the synthetic peptides were determined using competitive binding studies with MeO-succinyl-Ala-Ala-Pro-Val-*p*-nitroanilide (Sigma) as HLE substrate in 100 mM Tris/500 mM NaCl, pH 7.5. For PPE, succinyl-Ala-Ala-Ala-*p*-nitroanilide (Sigma) was used as the substrate, in 0.1 M Tris pH 8.0, both at 298 K. Substrate hydrolysis was monitored at 410 nm, and substrate concentration calculated from the final absorbance (ε = 8800 $M^{-1}\cdot cm^{-1}$ [30]). Enzyme concentrations were determined from the initial rate of substrate hydrolysis in the absence of inhibitor using values for K_m and k_{cat} of 0.14 mM and 17 s^{-1}, respectively, for HLE [31] and 1.15 mM and 16.6 s^{-1}, respectively, for PPE [32]. Initial velocity data were fitted using the GRAFIT software package [33,34]. K_i values were corrected for substrate competition using the formula $K_i = K_{i(app)}(1 + [S]/K_m)$, where $K_{i(app)}$ is the apparent K_i for a given substrate concentration.

Hydrolysis rates

Hydrolysis of peptides was performed using a 20-fold excess to HLE (4 μM HLE) in 100 mM Tris/500 mM NaCl, pH 7.5 at 298 K. Degradation of the peak corresponding to the cyclic peptide was followed by integration of the peak areas (monitored at 214 nm) after separation by a Micra NPS RP18 reverse-phase column (4.6 × 33 mm, 1.5 μm particle size; Micra Scientific Ltd, Darien, IL, USA) using a Hewlett-Packard HP1100 binary pump system. Separation was performed using a linear gradient between 0.3% trifluoroacetic acid and 0.3% trifluoroacetic acid/90% acetonitrile as solvents.

RESULTS AND DISCUSSION

Design of the library

In this study we have used a cyclic peptide library based on the anti-tryptic loop of BBI protein as the starting point for generation of inhibitors redirected against HLE. Variation was introduced into the library at selected positions in order to generate diversity likely to modify specificity. Interactions with the proteinase are expected to be exclusively via the 'upper-face' of the loop, which spans the region P_4–P_2' [35]. Ser is highly conserved at the P_1' position in BBI proteins [36], and for this reason this residue was not randomised. Similarly, Thr at P_2 shows a high degree of conservation [36]. In our previous

Table 1. Sequences identified from the library after screening for HLE binding. The P_4, P_1 and P_2' positions were randomised to incorporate 20 amino acid variations, and the sequences at these positions are listed for the 23 beads identified from the screening procedure. The table also records the inhibition constant (K_i) found for resynthesized peptide tested against HLE.

Peptide	Sequence of isolated resin bead P_4-C-T-P_1-S-P_2'-P-P-Q-C-Y			Number of times this sequence was found	K_i for resynthesized peptide (μM)
	P_4	P_1	P_2		
1	Nle	A	I	5	0.065
2	Nle	A	L	2	0.092
3	M	A	I	1	0.090
4	I	A	L	1	0.55
5	F	A	I	1	0.26
6	F	A	L	1	0.31
7	Y	A	I	1	0.13
8	Y	A	L	1	0.32
9	W	A	F	1	0.97
10	F	A	W	1	0.65
11	W	A	V	1	2.77
12	V	A	W	1	1.01
13	R	A	I	4	0.26
14	Nle	T	I	1	0.41
15	W	T	I	1	1.7

BBI-based library, screening against chymotrypsin resulted in the identification of only Thr at this locus for all active inhibitors, although 20 variants were present at this position within the library [21]. Further analysis of all included amino acids at this locus has confirmed Thr as the optimal residue [36]. In the crystal structures of both BBI–trypsin [35] and OMTKY3–HLE complex [26] (where a Thr residue is also found at P_2), the P_2 Thr is found to make close contact with His57. Further, in the structure of BBI complexed with trypsin [37], the hydroxyl function of this Thr is involved in hydrogen bonds within the loop structure. As the S_2 subsite of HLE is considered to be similar to that of other mammalian Ser proteinases including PPE [26], this locus was therefore not varied. In the current communication, we have therefore elected to randomize at the remaining positions of the upper face, namely P_1, P_2' and P_4. Each site had one of 20 different amino acids, giving a library with 8000 different components. The library was synthesized on TentaGel resin, and included a five-residue polyGly spacer between the 11-residue inhibitor template and the resin. This spacer, which was also present in our previous library [21], was included to minimize possible steric hindrance between the proteinase and the resin.

Sequences discovered by screening the inhibitor library with HLE

Screening of the library was performed using an indirect enzyme-linked immunoassay. This was done using a relatively low concentration of HLE with the aim of selecting only the most tightly binding species. Detection of bound HLE then employed further incubation with rabbit anti-HLE followed by monoclonal anti-rabbit IgG (γ-chain specific) conjugated to alkaline phosphatase. Visualization of positive beads used the enzyme activity of alkaline phosphatase to generate deeply stained beads [21,24]. The screening procedure identified a total of 23 highly positive beads from a sample of ≈ 46 000 beads that were analysed. This means that ≈ 1 in 2000 peptide sequences were active in the screening assay, a similar proportion to that found for chymotrypsin binding in our earlier study [21]. The active beads were isolated using a pair of dissecting tweezers and their peptide sequences determined by the Edman method using an automated peptide sequencer. Unambiguous identification of the randomized positions was possible in each case, and the sequences found are listed in Table 1. Each of the different 11-residue core sequences was resynthesized, purified and analysed for inhibition of HLE in solution. In our earlier study we found that 10 of 13 identified sequences were active as inhibitors of chymotrypsin, and the remaining three presumably represented nonspecific binding or the binding was an artefact [21]. However, in the present study we found that all 23 peptide sequences inhibited HLE after resynthesis, and their K_i values are also listed in Table 1. The K_i values range from 0.065 to 2.77 μM, with 15 different sequences being identified.

The peptide sequence found most frequently by the screening procedure (P_4 Nle, P_1 Ala, P_2' Ile) was also found to have the lowest K_i value of those peptides tested, indicating that the screening process is selective for tight binding. This sequence also represents the overall consensus for all the positive beads that were analysed. It seems likely that the contributions to binding from each of the three loci varied are additive, so that their combination gives the best available inhibitor, as has been described in other proteinase inhibitor interactions [7]. Sufficient resin was screened so that, on average, 5.75 copies of each sequence would be present. The consensus sequence itself was found on five separate beads, which means it is possible that all of the beads having this sequence were identified by the screening protocol. At least one sequence (peptide 4) returned residues at the randomised positions that correspond to a natural elastase inhibitor, elafin (P_4 Ile, P_1 Ala, P_2' Leu).

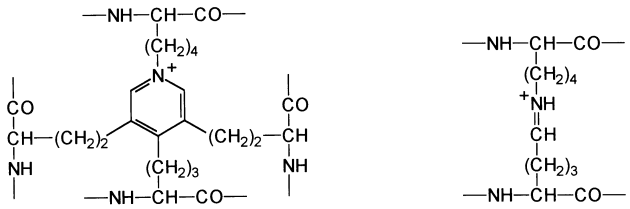

Fig. 1. Structure of two common elastin cross-linking residues, desmosine and dehydrolysinonorleucine [50].

The P_1 position. The screening results against HLE indicate a high preference for alanine in the P_1 position, with 21 out of 23 of the sequences having Ala at this locus. This result is broadly consistent with the known substrate specificity of elastases for small alkyl side chains, although it is generally considered that the preferred P_1 for HLE is valine [38–40], and this has been the most widely utilized residue at this locus in the design of inhibitors [2]. It is therefore quite striking that no P_1 sequences containing Val were recovered from the screening process. Natural macromolecular inhibitors with activity against HLE are known that have at the P_1 position Met (α_1-proteinase inhibitor [41], guamerin [42]), Leu (eglin c [43], mucus proteinase inhibitor [9]) and Ala (elafin [44]). In addition, semisynthetic BPTI-derived inhibitors have been shown to display a preference for Val at P_1 [45], and the most active OMTKY3-derived inhibitors have P_1 Ile [46]. Two of the sequences listed in Table 1 have a Thr residue at P_1. Although these sequences proved to be less potent inhibitors than the corresponding Ala variants, they were still found to give K_i values around the micromolar level. It is interesting that neither the available substrate specificity data nor the sequence information for natural inhibitors with activity against HLE would have led us to predict finding Thr at P_1, which illustrates the benefits of library-screening procedures. It should be noted that Thr has been identified as the P_1 residue by sequence alignment in peacock pheasant ovomucoid third domain (OMGPP3; TCTTEHRP, P_4-P_4') but to our knowledge, no activity has been reported for this protein [47]. Although a P_1 Thr is not normally considered to be a usual HLE substrate, hydrolysis by HLE of the Thr87-Thr88 sequence in a polypeptide linking the inhibitory Kunitz-1 and Kunitz-2 domains of tissue factor pathway inhibitor has been described [48]. This confirms that HLE is able to interact with a P_1 Thr residue. Replacement of Thr226 in PPE by Asp226 in HLE has suggested the design of inhibitors with positively charged P_1 side chains [49]. However, no positively charged P_1 side chains were detected by the library screening. This is in agreement with results obtained by Lu *et al.* [12] for P_1 variations to OMTKY3 whereby charged residues and side chains with increased steric bulk were found to decrease the K_a when compared with the parent inhibitor (Leu at P_1).

The P_4 position. A preference for large apolar or aromatic groups is observed in the P_4 position consistent with the structure of the S_4 pocket [26], and NLe is found here with the greatest frequency. Substrates with Lys at P_4 have been found to

Table 2. Effect of P_1 variation alone on HLE inhibition constants. The effect of P_1 variation alone was tested within the original nonoptimized cyclic peptide template (S-C-T-P_1-S-I-P-P-Q-C-Y). The inhibition constants against HLE are tabulated. NI, no inhibition at the concentrations tested; $K_i > 1000$ μM.

Peptide	P_1	K_i (μM)
16	A	3.4
17	V	4.6
18	M	NI
19	T	15.9

be less reactive than those with P_4 Ala while those with aromatic or large hydrophobic groups at this subsite are more reactive [50]. An unexpected finding is Arg in this position for 4/23 of the identified inhibitors as, in addition to hydrophobic residues, Arg is also found in the S_4–S_5 region of both HLE (Arg217) and PPE. In the TOM complex with HLE, the side chain of Ala (P_4) runs parallel with Arg217 [51], and it has been suggested that a negatively charged residue at the P_4 (or P_5) locus would facilitate a favourable electrostatic interaction [51]. Indeed, negatively charged groups have been used in this region of synthetic inhibitors in order to exploit electrostatic interactions at this locus [52]. However, the library results do not reflect this. In contrast, the crystal structure of methoxy-succinyl-Ala-Ala-Pro-Ala chloromethyl ketone in complex with HLE shows that the Arg217 side chain is directed away from the P_4 residue [53]. The results are consistent with the observation that ovomucoid third domains derived from chestnut bellied scaled quail and Gambel's quail, which differ only at P_4 (Asp and Ala, respectively), show a 160-fold reduction in K_a against PPE when an acidic group is present [54]. Arg is found as the P_3 residue in both elafin [55] and guamerin [42], and the PPE–elafin structure [56] shows that the P_3 Arg guanidino group forms intramolecular hydrogen bonds with carbonyl of P_1' Met. In the BBI template, P_3 is occupied by one of the two cysteines constraining the peptide and so is not available for interaction. Interestingly, for the reduction in activity against HLE reported by Rozycki *et al*. [17] when the P_4–P_5 residues of CMTI-III were deleted, Val-Arg are the respective residues (which also had the P_1 residue substituted for Val in order to redirect activity towards HLE).

Elastin is a highly cross-linked protein rich in Gly, Ala, Val and Pro residues [2,57]. Two common cross-linking residues are desmosine and dehydrolysinonorleucine (Fig. 1). Both are characterized by multiple methylene groups and an imino group. The two most frequently found residues from our screening (Nle and Arg) have features in common with dehydrolysinonorleucine, which could explain their selection. This finding is consistent with the work of Yasutake & Powers [50], which studied the effect of protected Lys derivatives [Lys(Z), Lys(Bz), Lys(benzimidoyl) and Lys(picolinoyl)] at P_2, P_3 and P_4 as surrogates for desmosine within short peptide substrates. In general, it was found that increased reactivity was obtained at P_4 and P_3 by these analogues [optimal Lys(picolinoyl) at P_4]. It was also found that a positive charge at P_4 (Lys) or both a positive charge and aromatic ring [Lys(benzimidoyl)] were detrimental. Taken together, these findings suggested that HLE hydrolysis of elastin occurs near these cross-links rather than the Lys-rich regions of tropoelastin or those regions of elastin containing noncross-linked Lys residues. In contrast, PPE was found to be much less reactive and, in particular, would not accept the Lys and derivative residues at P_4, which may indicate a more restricted S_4 pocket.

The P_2' position. The P_2' position also shows a preference for large apolar side chains with Ile being found at this locus with greatest frequency, and Ile or Leu together accounting for 83% of the sequences identified. Natural endogenous elastase inhibitors have Ile (α_1-proteinase inhibitor) or Leu (mucus proteinase inhibitor, elafin) at P_2'. On the leaving side of the scissile bond, Tyr at P_2' in the TOM–HLE complex makes the most contact with HLE, principally interacting with Ile151 in the S_2' pocket [26]. Crystal structures of the complex with OMTKY3 or with substrate show that in PPE and HLE the S_2' pocket is lined by Leu, Leu and Leu, Ile, respectively [26]. Thus the P_2' specificity is likely to be similar between these enzymes.

Phe, Trp and Val were also found at this position (Table 1). Phe is present at P_2' in guamerin [42]. The ovomucoid third domains from Indian peafowl and OMTKY3 differ only at P_2' (His and Tyr, respectively). Comparison of their K_a values for PPE shows that the value for OMTKY3 is 44-fold higher [54], which may indicate some preference for hydrophobic residues over basic residues at this locus.

Effect of P_1 variation on activity

In order to compare the results of the library-selected sequences with the outcome of P_1 alteration alone, the dissociation constants of selected analogues were recorded, based on the original template sequence (Table 2). The variants synthesized were selected on the basis of substrate specificity, and incorporated Ala, Thr, Val or Met at P_1. All variants were found to display considerably lower activity than the library-selected sequences, with P_1 Ala having marginally better activity than the corresponding Val analogue. The Met variant displayed no measurable activity against HLE (or PPE). In order of K_i, we find Ala < Val < Thr < Met.

As each of these peptides had a P_4 Ser residue (present in the original trypsin inhibitor template) which was not identified in any library-selected sequences, we performed a further comparison using an optimal library-selected P_4 residue. Table 1 shows that Nle was the most frequent P_4 residue, and so we substituted the same P_1 residues in the sequence NleCTXSIPPQCY (Table 3). All variants now show measurable activity. As Ile is present at P_2' of both peptide series, a significant contribution to binding must arise from the P_4 Nle. In particular, it is noteworthy that the P_1 Met variant only shows measurable activity in combination with P_4 Nle. The K_i values remain in the order Ala < Val < Thr < Met, but are around

Table 3. Effect of P_1 variation within an optimized sequence on HLE inhibition constants. The effect of P_1 variation was tested within a sequence that contained an optimized residue at P_4 (Nle) (Nle-C-T-**P_1**-S-I-P-P-Q-C-Y). The inhibition constants against HLE are tabulated.

		K_i (μM)	
Peptide	P_1	HLE	PPE
20	V	0.13	6.7
21	M	11.4	5.8
1	A	0.065	0.7
14	T	0.41	1.5

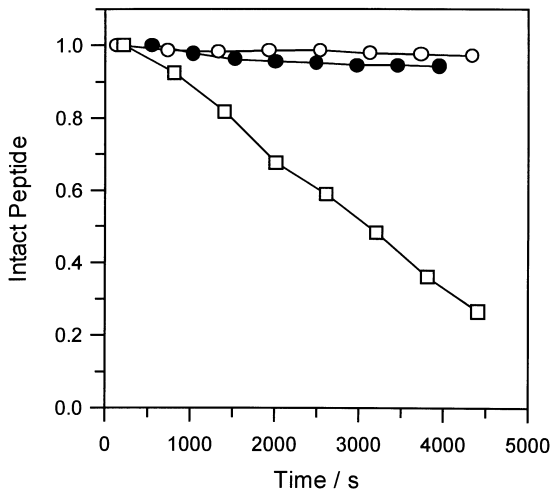

Fig. 2. Comparative hydrolysis rates for the consensus library-selected sequences [cyclic NleCTASIPPQCY (○) and NleCTTSIPPQCY (●) and a non-library-selected variant [cyclic NleCTVSIPPQCY (□)]. Hydrolysis was followed by HPLC as described in Experimental procedures.

50-fold lower than with a P_4 Ser residue. It should be noted that the Val variant has a K_i lower than most of the library-selected sequences, even though no P_1 Val sequences were detected by screening. The reasons for this are considered below.

Although in our system we find that P_1 Ala gives a lower K_i for HLE than does P_1 Val, this is not the case for all systems that have been studied. As Val is generally considered to be the preferred residue at the P_1 locus, it is common for researchers to replace this residue with Val when re-directing the specificity of natural inhibitors [58,59]. The Kazal inhibitor BPTI has often been studied because of its small size (58 residues) and potent inhibitory properties. A number of P_1 variants of this protein have been prepared by semisynthesis [45]. Of the variants tested against HLE, Val was found to give the lowest K_i (0.11 nM) and this was 22-fold lower than the Ala variant. The order of potency was Val > Nva (also referred to as Ape) > aminobutyric acid > allo-isoleucine > Ile Ala Leu (the native inhibitor with P_1 Lys has a K_i for HLE of 3.5 μM). Similarly, Lu *et al.* [12] have recently screened a wide range of natural and engineered P_1 variants of OMTKY3. In this study, the order of potency in terms of K_a was Ile > aminobutyric acid > Val > Nva > Cys > Leu > Thr > Ala > Nle for HLE, and aminobutyric acid > Nva > Nle > Ala > Thr > Leu > Met > Val > Ile for PPE. McRae *et al.* [38] have used the reactive-site region of α_1-proteinase inhibitor to evaluate substrates of HLE and found that the sequence derived from α_1-proteinase inhibitor was not optimal. Subtle differences, for example between Ser and Thr at P_1', were observed primarily in terms of k_{cat}. This result is consistent with our data, and once more demonstrates that optimal inhibitor and substrate sequences are not necessarily the same.

Our results clearly show that although the P_1 site is important for activity, other P and P′ sites must be optimized in order to obtain good inhibition. Similar conclusions have been drawn by other workers. In an attempt to improve further antielastase activity, additional alteration of K15V BPTI at the P_1' and P_2' positions (A16S, R17I) to match those of the α_1-proteinase inhibitor was found to result in a 40-fold decrease in K_d [59]. The importance of the P_4–S_4 interaction in PPE has been described by Thompson & Blout [60]. Using a limited range of peptide amides, these authors were able to demonstrate P_4 interactions that could either strengthen enzyme–substrate binding or those that could increase the rate of hydrolysis.

Effects on hydrolysis rates

We and others have previously noted that BBI peptides often show marked differences in the rates at which they are hydrolysed by proteinase [36,61,62]. Differences in hydrolysis rates are a potential factor likely to affect the sequences selected from the library, as sequences that are rapidly hydrolysed would not be detected. During analysis of peptides with Val at P_1, it became apparent that this peptide seemed more susceptible to hydrolysis. To examine this, a time course of peptide hydrolysis was followed for selected analogues. Figure 2 records a hydrolysis time course, monitored using HPLC, for P_1 Thr, Ala and Val peptides (P_4 and P_2' are Nle and Ile in each case). Sequences with Thr and Ala at P_1 were identified from the library screening; P_1 Val sequences might have been expected, and result in a low K_i value, but were not found during screening. The results show that the library-selected variants are significantly more stable to hydrolysis than the non-library-selected sequence (P_1 Val), which is rapidly turned over at a rate some 20-fold greater than the corresponding library-selected sequences. This effect, which is consistent with the substrate specificity of HLE, means that any sequences with a P_1 Val would almost certainly have hydrolysed during the incubation period of the screening. It is therefore not surprising that no such sequences were discovered.

Effect of synthesized variants against PPE

To examine any significant differences in specificity, we therefore tested all the variants synthesized against PPE. The K_i values are listed in Table 4.

Table 4. PPE inhibition constants of peptides identified by HLE screening. Sequences identified from the library after screening for HLE binding were further tested for inhibition of PPE. The K_i ratio shows the selectivity of the inhibitor for HLE, with larger values representing greater selectivity.

	Sequence of isolated resin bead P_4-C-T-P_1-S-P_2'-P-P-Q-C-Y				
Peptide	P_4	P_1	P_2'	K_i (μM)	K_i PPE/K_i HLE
1	Nle	A	I	0.7	10.8
2	Nle	A	L	0.85	9.2
3	M	A	I	1.2	12.9
4	I	A	L	0.75	1.4
5	F	A	I	1.5	5.8
6	F	A	L	1.4	4.4
7	Y	A	I	0.07	0.55
8	Y	A	L	0.27	0.83
9	W	A	F	3.3	3.4
10	F	A	W	1.1	1.7
11	W	A	V	13	4.7
12	V	A	W	0.23	0.23
13	R	A	I	14.2	55
14	Nle	T	I	1.5	3.6
15	W	T	I	3.8	2.2

Library-selected sequences. In general, the HLE-selected sequences display higher K_i values against PPE (Table 4). This is not unexpected, as the sequences were selected on the basis of HLE binding activity. The ratio K_i PPE/K_i HLE gives some measure of selectivity. Peptide 13, with Arg at P_4, results in the largest ratio, while three peptides, 7, 8 and 12, show slightly lower K_i values for PPE. It is worth noting that a significant difference at the S_4–S_5 pocket between HLE and PPE complexed with methoxysuccinyl-Ala-Ala-Pro-Ala chloromethyl ketone has been observed by Navia *et al*. [53]. The difference can largely be attributed to the deletion of Ser217 in PPE, changing the overall conformation of the corresponding loop and, in particular, altering the orientation of Arg217A in the pocket. In HLE, this residue is directed away from the P_4 residue.

Other P_1 variants. In general, the various peptides have lower activity against PPE than HLE. The P_1 Val substitution results in particularly poor PPE inhibition, consistent with the known specificity of these enzymes [39,63]. The presence of Nle at P_4 is found to enhance activity, as was the case with HLE, suggesting that they share similarities in P_4 specificity. The order of the K_i values is Ala < Thr < Met < Val, which is quite different from the HLE findings. Like HLE, Ala provides the best K_i of the variants tested, but the Met variant results in a lower K_i for PPE than HLE. The order of preference is slightly different from that found for a range of OMTKY3 P_1 variants [12], which could be due to the presence in our studies of optimized P_4 and P_2'.

Overall we find significant differences between PPE and HLE in terms of their specificities for the various inhibitor peptides used in the present study.

How well do our peptides mimic the BBI canonical loop?

Our approach to the design of potent peptide-based competitive inhibitors has been to incorporate the canonical loop feature BBI protein within a constrained peptide template. When a functional domain or scaffold is excised in this manner and used to generate a tethered library there are various structural consequences.

(a) A terminal amino group is introduced in the molecule, which will be positively charged unless the peptide is N-acetylated (acetylation is not possible if Edman sequencing is to be used for identification of active components).

(b) The C-terminus is anchored to the solid phase, which could restrict binding.

(c) The N-terminal residue(s) is likely to be more flexible than in the parent protein. The peptide library described in the present study was truncated at P_4. As noted by Thompson & Blout [60] in a study of PPE-catalysed hydrolysis of peptide amides, the lack of a potential P_5–S_5 interaction may influence P_4 specificity compared with full-length inhibitors.

(d) Many long-range interactions potentially present in the protein structure are missing.

A switch from the parent protein to a peptide mimetic may therefore lead to a great loss of preorganization. It is therefore reassuring that the inhibitors selected from the library not only possess low hydrolysis rates against the target proteinase HLE, but also display K_i values approaching that of the native 'classical' soybean BBI [64]. This suggests that the peptides do adopt the same conformation as the original protein. In addition, we have recently determined an NMR-derived solution structure for an 11-mer BBI loop peptide, which shows that the peptide in solution has almost exactly the same structure as the corresponding region within the parent protein (A. B. E. Brauer & R. J. Leatherbarrow, unpublished work).

Comparison of these K_i values with those of other elastase inhibitors

In general, the peptides identified in the present study (lowest K_i value 65 nM) have inhibition constants that are higher (i.e. they are less potent) than those of elastase inhibitor proteins. Chemically synthesized elafin has a K_i of 2 nM for HLE and 6 nM for PPE [65]. Eglin c, which is an extremely potent elastase inhibitor, has a K_i of 75 pM for HLE [66]. For soybean BBI, Larionova *et al*. [64] report a K_i for HLE of 28 nM (initial EI complex), K_i^* = 2.3 nM (further tighter EI* complex); a considerably lower value of 11 μM has previously been reported by Bieth *et al*. [67] for PPE. However, it must be stressed that the peptides in the current study are considerably smaller than the full-length inhibitor proteins.

Comparison with other peptide inhibitors of HLE

Linear peptides based on the reactive site of α_1-proteinase inhibitor are substrates rather than inhibitors [63]. In contrast, cyclic versions displayed some activity, albeit poor, with K_i values ranging from 0.39 to 17 mM [63]. Very recently, Yavin & Fridkin [68] have described a set of overlapping 15-mer peptides from C-reactive protein. Unlike the parent protein, some of these peptides were found to demonstrate both anti-HLE and cathepsin G activity. The best peptide (EILIFWSKDIGYSFT) gave K_i values of 0.18 μM and 0.25 μM, respectively. The bicyclic peptide Ac-CKSAICALSYPAQCFC based on the antichymotryptic domain of classical BBI has been tested with K_i = 43 μM [61], which is approximately fourfold higher than reported for the parent BBI against PPE [67].

Comparison with other peptide/protein library screening results

A phage library of the Kazal inhibitor BPTI has previously been used to screen for binding to HLE [18]. Although this report employed randomization at a larger number of sites than the present study and was able to identify potent inhibitors, the number of amino acids coded at each locus was much lower than in the present communication. At the P_1 site, coding for Val, Leu, Phe, Ile and Met was included on the basis of known substrate specificity of HLE; both Val and Ile were identified from the selection procedure. However, neither Ala nor Thr was coded for in this library, making direct comparison with our results difficult. The remaining subsites were P_1' (G/A), P_2' (V/L/I/F/M), P_3' (F/I/T/S) and P_4' (K/Q/T/S/P). It was expected that hydrophobic residues at P_2' might enhance binding. Of those amino acids allowed, Ile (5%), Leu (10%), Met (25%) and Phe (60%) were identified by the screening procedure. In general agreement, we found Ile, Leu, Phe and Val but with Ile and Leu most frequent. None of our sequences had Met at P_2', and, although we also identified Trp, this was not present in the phage library.

Influence of extended interactions and elastic or flexible S_1 in HLE

Although both HLE and PPE are known to have an extended substrate-binding site [60,69], several lines of evidence suggest that HLE possesses a less rigid, more flexible S_1 pocket than do PPE, chymotrypsin or trypsin. For extended peptide

substrates, the P_1 specificity may be more strict and modulated by the remote subsites accommodating the extended substrate residues.

Substrates. Increasing substrate length is known to improve the catalytic efficiency of amide bond hydrolysis by Ser proteinases, and this is typically reflected in increased k_{cat} [70], which is equal to the rate-limiting acylation step. For HLE, this has been attributed to the significant effect of subsites on acylation rate. In the absence of an extended chain and therefore remote interactions, HLE, unlike PPE, displays a broad specificity at P_1 [71]; for example, Tyr can be tolerated reasonably well. During hydrolysis of an extended chain, subtle changes in the structure of HLE result in a narrowing of the S_1 pocket [69,71]. Extended interactions appear to fulfil several functions: enhancing catalytic efficiency [72]; enlisting operation of the charge-relay system [69]; regulating P_1 specificity [71].

Inhibitors. Adaptation of the P_1 binding is also demonstrated by the structure of HLE in complex with methoxysuccinyl-Ala-Ala-Pro-Val-CH_2Cl [73]. The β-branched side chain is accommodated in P_1 with slight tilting of the main chain. Simultaneously, the S_1 pocket shrinks and adapts to the smaller side chain. Recent results of P_1 variation of TOM also suggest that in HLE the S_1 site is more flexible than in trypsin, *S. griseus* proteinase A, *S. griseus* proteinase B, subtilisin Carlsberg or PPE [12]. In this study, β-branched P_1 side chains were found to be highly deleterious to all but HLE. Further analysis of the K_a for the isomeric pair Hse/Thr, revealed that Thr was found to be deleterious for all other proteinases studied but advantageous for HLE and PPE. Other evidence suggesting an important role for subsites other than P_1 comes from studies of P_1 variation in CMTI-III [16]. Inhibition was found with P_1 Val, Ile, Gly, Leu, Ala, Phe and Met; inhibition by P_1 Gly means that there must be contribution from other subsites as this residue has no side chain.

Conclusions

We have successfully used a tethered peptide library to discover new HLE inhibitors. Using a library allows more efficient screening than could be obtained by testing individual inhibitors, which is exemplified by the library-selected sequences giving lower K_i values than could be obtained by simple P_1 substitutions alone. Our results show that the screening procedure used identifies those sequences that offer the best compromise between low K_i and low hydrolysis rate. In the context of finding the 'best' inhibitor sequence, this represents an obvious benefit. One surprising finding is that, contrary to expectations, sequences that correspond to the optimal substrate specificity do not necessarily make the best inhibitors (at least in the system studied). This is exemplified by comparison of sequences with P_1 Val or Ala. The Val-containing sequences are better substrates, but Ala variants are better inhibitors in this particular system. Clearly, this has significant implications for proteinase inhibitor design, which often relies on substrate specificity data.

ACKNOWLEDGEMENTS

This work was supported by the BBSRC and Glaxo-Wellcome Research. We would like to thank the following people: John Barton (Department of Chemistry, Imperial College) for performing the mass spectroscopy; Norman Gray (Glaxo-Wellcome) for his assistance with the hydrolysis assays; Robin Smith (Glaxo-Wellcome) for the gift of HLE. We are indebted to Malcolm Weir of Glaxo-Wellcome for his continued support and encouragement. Presented in part at the Fifth International Symposium on Solid Phase Synthesis & Combinatorial Chemical Libraries, Imperial College, University of London, UK, 2–6 September 1997.

REFERENCES

1. Geokas, M.C., Rinderknecht, H., Swanson, V. & Haverback, B.J. (1968) The role of elastase in acute hemorrhagic pancreatitis in man. *Lab. Invest.* **19**, 235–239.
2. Edwards, P.D. & Bernstein, P.R. (1994) Synthetic inhibitors of elastase. *Med. Res. Rev.* **14**, 127–194.
3. Laskowski, M. Jr & Kato, I. (1980) Protein inhibitors of proteinases. *Annu. Rev. Biochem.* **49**, 593–626.
4. Bode, W. & Huber, R. (1992) Natural protein proteinase-inhibitors and their interaction with proteinases. *Eur. J. Biochem.* **204**, 433–451.
5. Schechter, I. & Berger, A. (1967) On the size of the active site in proteases. I. Papain. *Biochem. Biophys. Res. Commun.* **27**, 157–162.
6. Bode, W. & Huber, R. (1991) Ligand binding: proteinase–protein inhibitor interactions. *Curr. Opin. Struct. Biol.* **1**, 45–52.
7. Qasim, M.A., Ganz, P.J., Saunders, C.W., Bateman, K.S., James, M.N.G. & Laskowski, M. (1997) Interscaffolding additivity. Association of P-1 variants of eglin C and of turkey ovomucoid third domain with serine proteinases. *Biochemistry* **36**, 1598–1607.
8. Kato, I., Schrode, J., Kohr, W.J. & Laskowski, M. (1987) Chicken ovomucoid: determination of its amino acid sequence, determination of the trypsin reactive site, and preparation of all 3 of its domains. *Biochemistry* **26**, 193–201.
9. Boudier, C. & Bieth, J.G. (1994) Oxidized mucus proteinase inhibitor: a fairly potent neutrophil elastase inhibitor. *Biochem. J.* **303**, 61–68.
10. Ardelt, W. & Laskowski, M. (1985) Turkey ovomucoid 3rd domain inhibits 8 different serine proteinases of varied specificity on the same …Leu^{18}-Glu^{19}… reactive site. *Biochemistry* **24**, 5313–5320.
11. Longstaff, C., Campbell, A.F. & Fersht, A.R. (1990) Recombinant chymotrypsin inhibitor-2: expression, kinetic analysis of inhibition with alpha-chymotrypsin and wild-type and mutant subtilisin Bpn′, and protein engineering to investigate inhibitory specificity and mechanism. *Biochemistry* **29**, 7339–7347.
12. Lu, W.Y., Apostol, I., Qasim, M.A., Warne, N., Wynn, R., Zhang, W.L., Anderson, S., Chiang, Y.W., Ogin, E., Rothberg, I., Ryan, K. & Laskowski, M. (1997) Binding of amino acid side-chains to S-1 cavities of serine proteinases. *J. Mol. Biol.* **266**, 441–461.
13. Nishino, N., Aoyagi, H., Kato, T. & Izumiya, N. (1977) Studies on the synthesis of proteinase inhibitors. I. Synthesis and activity of nonapeptide fragments of soybean Bowman-Birk inhibitor. *J. Biochem. (Tokyo)* **82**, 901–909.
14. Favel, A., Lenguyen, D., Colettipreviero, M.A. & Castro, B. (1989) Active-site chemical mutagenesis of ecballium-elaterium trypsin inhibitor-II: new microproteins inhibiting elastase and chymotrypsin. *Biochem. Biophys. Res. Commun.* **162**, 79–82.
15. Carrell, R. (1984) Therapy by instant evolution. *Nature (London)* **312**, 14.
16. McWherter, C.A., Walkenhorst, W.F., Campbell, E.J. & Glover, G.I. (1989) Novel inhibitors of human-leukocyte elastase and cathepsin G: sequence variants of squash seed protease inhibitor with altered protease selectivity. *Biochemistry* **28**, 5708–5714.
17. Rozycki, J., Kupryszewski, G., Rolka, K., Ragnarsson, U., Zbyryt, T., Watorek, W. & Wilusz, T. (1997) The smallest peptide inhibitors of human leukocyte elastase (HLE): structural requirements for inhibition of HLE based on *Cucurbita maxima* trypsin inhibitor III (CMTI-III) analogues. *Pol. J. Chem.* **71**, 176–180.
18. 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 elastase inhibitors displayed on M13 fusion phage. *Proc. Natl Acad. Sci. USA* **89**, 2429–2433.
19. Markland, W., Ley, A.C., Lee, S.W. & Ladner, R.C. (1996) Iterative optimization of high-affinity protease inhibitors using phage display. 1. Plasmin. *Biochemistry* **35**, 8045–8057.

20. Markland, W., Ley, A.C. & Ladner, R.C. (1996) Iterative optimization of high-affinity protease inhibitors using phage display. 2. Plasma kallikrein and thrombin. *Biochemistry* **35**, 8058–8067.
21. McBride, J.D., Freeman, N., Domingo, G.J. & Leatherbarrow, R.J. (1996) Selection of chymotrypsin inhibitors from a conformationally-constrained combinatorial peptide library. *J. Mol. Biol.* **259**, 819–827.
22. Odani, S. & Ikenaka, T. (1973) Scission of soybean Bowman-Birk proteinase inhibitor into two small fragments having either trypsin or chymotrypsin inhibitory activity. *J. Biochem. (Tokyo)* **74**, 857–860.
23. Terada, S., Sato, K., Kato, T. & Izumiya, N. (1978) Inhibitory properties of nonapeptide loop structures related to reactive sites of soybean Bowman-Birk inhibitor. *FEBS Lett.* **90**, 89–92.
24. Lam, K.S., Salmon, S.E., Hersh, E.M., Hruby, V.J., Kazmierski, W.M. & Knapp, R.J. (1991) A new type of synthetic peptide library for identifying ligand-binding activity. *Nature (London)* **354**, 82–84.
25. Furka, A., Sebestyen, F., Asgedom, M. & Dibo, G. (1991) General method for rapid synthesis of multicomponent peptide mixtures. *Int. J. Pept. Protein Res.* **37**, 487–493.
26. Bode, W., Meyer, E. & Powers, J.C. (1989) Human leukocyte and porcine pancreatic elastase: X-ray crystal structures, mechanism, substrate-specificity, and mechanism-based inhibitors. *Biochemistry* **28**, 1951–1963.
27. Knorr, R., Trzeciak, A., Bannwarth, W. & Gillessen, D. (1989) New coupling reagents in peptide chemistry. *Tetrahedron Lett.* **30**, 1927–1930.
28. Tam, J.P., Wu, C.R., Liu, W. & Zhang, J.W. (1991) Disulfide bond formation in peptides by dimethyl sulfoxide: scope and applications. *J. Am. Chem. Soc.* **113**, 6657–6662.
29. McBride, J.D., Harbeck, B. & Leatherbarrow, R.J. (1996) Resin-coupled cyclic peptides as proteinase inhibitors. *Protein Peptide Lett.* **3**, 193–198.
30. Erlanger, B., Kokowski, N. & Cohen, W. (1961) The preparation and properties of two new chromogenic substrates of trypsin. *Arch. Biochem. Biophys.* **95**, 271–278.
31. Nakajima, K., Powers, J.C., Ashe, B.M. & Zimmerman, M. (1979) Mapping the extended substrate binding site of cathepsin G and human leukocyte elastase. Studies with peptide substrates related to the alpha 1-protease inhibitor reactive site. *J. Biol. Chem.* **254**, 4027–4032.
32. Bieth, J., Spiess, B. & Wermuth, C.G. (1974) The synthesis and analytical use of a highly sensitive and convenient substrate of elastase. *Biochem. Med.* **11**, 350–357.
33. Leatherbarrow, R.J. (1992) *GraFit 3.0*. Erithacus Software Ltd, Staines, UK.
34. Leatherbarrow, R.J. (1990) Using linear and nonlinear-regression to fit biochemical data. *Trends Biochem. Sci.* **15**, 455–458.
35. Lin, G.D., Bode, W., Huber, R., Chi, C.W. & Engh, R.A. (1993) The 0.25-nm X-ray structure of the Bowman–Birk-type inhibitor from mung bean in ternary complex with porcine trypsin. *Eur. J. Biochem.* **212**, 549–555.
36. McBride, J.D., Brauer, A.B.E., Nievo, M. & Leatherbarrow, R.J. (1998) The role of threonine in the P-2 position of Bowman–Birk proteinase inhibitors: studies on P-2 variation in cyclic peptides encompassing the reactive site loop. *J. Mol. Biol.* **282**, 447–457.
37. Voss, R.H., Ermler, U., Essen, L.O., Wenzl, G., Kim, Y.M. & Flecker, P. (1996) Crystal structure of the bifunctional soybean Bowman–Birk inhibitor at 0.28-nm resolution: structural peculiarities in a folded protein conformation. *Eur. J. Biochem.* **242**, 122–131.
38. McRae, B., Nakajima, K., Travis, J. & Powers, J.C. (1980) Studies on reactivity of human leukocyte elastase, cathepsin G, and porcine pancreatic elastase toward peptides including sequences related to the reactive site of alpha 1-protease inhibitor (alpha 1-antitrypsin). *Biochemistry* **19**, 3973–3978.
39. Harper, J.W., Cook, R.R., Roberts, C.J., McLaughlin, B.J. & Powers, J.C. (1984) Active-site mapping of the serine proteases human-leukocyte elastase, cathepsin g, porcine pancreatic elastase, rat mast-cell protease-I and protease-Ii, bovine chymotrypsin-α and *Staphylococcus aureus* protease V-8 using tripeptide thiobenzyl ester substrates. *Biochemistry* **23**, 2995–3002.
40. Stein, R.L., Trainer, D.A. & Wildonger, R.A. (1985) Neutrophil elastase. *Annu. Rep. Med. Chem.* **20**, 237–246.
41. Carrell, R., Jeppsson, J.O., Laurell, C.B., Brennan, S.O., Owen, M.C., Vaughan, L. & Boswell, D.R. (1982) Structure and variation of human α1-antitrypsin. *Nature (London)* **298**, 329–334.
42. Jung, H.I., Kim, S.I., Ha, K.S., Joe, C.O. & Kang, K.W. (1995) Isolation and characterization of guamerin, a new human-leukocyte elastase inhibitor from Hirudo–Nipponia. *J. Biol. Chem.* **270**, 13879–13884.
43. Heinz, D.W., Hyberts, S.G., Peng, J.W., Priestle, J.P., Wagner, G. & Grutter, M.G. (1992) Changing the inhibitory specificity and function of the proteinase inhibitor eglin-c by site-directed mutagenesis: functional and structural investigation. *Biochemistry* **31**, 8755–8766.
44. Ying, Q.L. & Simon, S.R. (1993) Kinetics of the inhibition of human-leukocyte elastase by elafin, a 6-kilodalton elastase-specific inhibitor from human skin. *Biochemistry* **32**, 1866–1874.
45. Beckmann, J., Mehlich, A., Schroder, W., Wenzel, H.R. & Tschesche, H. (1988) Preparation of chemically mutated aprotinin homologs by semisynthesis: P1 substitutions change inhibitory specificity. *Eur. J. Biochem.* **176**, 675–682.
46. Bigler, T.L., Lu, W.Y., Park, S.J., Tashiro, M., Wieczorek, M., Wynn, R. & Laskowski, M. (1993) Binding of amino-acid side-chains to preformed cavities: interaction of serine proteinases with turkey ovomucoid 3rd domains with coded and noncoded P_1 residues. *Protein Sci.* **2**, 786–799.
47. Laskowski, M., Kato, I., Ardelt, W., Cook, J., Denton, A., Empie, M.W., Kohr, W.J., Park, S.J., Parks, K., Schatzley, B.L., Schoenberger, O.L., Tashiro, M., Vichot, G., Whatley, H.E., Wieczorek, A. & Wieczorek, M. (1987) Ovomucoid 3rd domains from 100 avian species: isolation, sequences, and hypervariability of enzyme-inhibitor contact residues. *Biochemistry* **26**, 202–221.
48. Petersen, L.C., Bjorn, S.E., Olsen, O.H., Nordfang, O., Norris, F. & Norris, K. (1996) Inhibitory properties of separate recombinant Kunitz-type-protease-inhibitor domains from tissue-factor-pathway inhibitor. *Eur. J. Biochem.* **235**, 310–316.
49. Sinha, S., Watorek, W., Karr, S., Giles, J., Bode, W. & Travis, J. (1987) Primary structure of human neutrophil elastase. *Proc. Natl Acad. Sci. USA* **84**, 2228–2232.
50. Yasutake, A. & Powers, J.C. (1981) Reactivity of human-leukocyte elastase and porcine pancreatic elastase toward peptide 4-nitroanilides containing model desmosine residues: evidence that human-leukocyte elastase is selective for cross-linked regions of elastin. *Biochemistry* **20**, 3675–3679.
51. Bode, W., Wei, A.Z., Huber, R., Meyer, E., Travis, J. & Neumann, S. (1986) X-ray crystal structure of the complex of human-leukocyte elastase (PMN elastase) and the 3rd domain of the turkey ovomucoid inhibitor. *EMBO J.* **5**, 2453–2458.
52. Regan, J., McGarry, D., Bruno, J., Green, D., Newman, J., Hsu, C.Y., Kline, J., Barton, J., Travis, J., Choi, Y.M., Volz, F., Pauls, H., Harrison, R., Zilberstein, A., BenSasson, S.A. & Chang, M. (1997) Anionic- and lipophilic-mediated surface binding inhibitors of human leukocyte elastase. *J. Med. Chem.* **40**, 3408–3422.
53. Navia, M.A., McKeever, B.M., Springer, J.P., Lin, T.Y., Williams, H.R., Fluder, E.M., Dorn, C.P. & Hoogsteen, K. (1989) Structure of human neutrophil elastase in complex with a peptide chloromethyl ketone inhibitor at 1.84-Å resolution. *Proc. Natl Acad. Sci. USA* **86**, 7–11.
54. Empie, M.W. & Laskowski, M. (1982) Thermodynamics and kinetics of single residue replacements in avian ovomucoid 3rd domains: effect on inhibitor interactions with serine proteinases. *Biochemistry* **21**, 2274–2284.
55. Wiedow, O., Schroder, J.M., Gregory, H., Young, J.A. & Christophers, E. (1990) Elafin – an elastase-specific inhibitor of human skin: purification, characterization, and complete amino acid sequence. *J. Biol. Chem.* **265**, 14791–14795.
56. Tsunemi, M., Matsuura, Y., Sakakibara, S. & Katsube, Y. (1996) Crystal structure of an elastase-specific inhibitor elafin complexed

with porcine pancreatic elastase determined at 1.9 angstrom resolution. *Biochemistry* **35**, 11570–11576.

57. Gowda, D.C., Luan, C.H., Furner, R.L., Peng, S.Q., Jing, N., Harris, C.M., Parker, T.M. & Urry, D.W. (1995) Synthesis and characterization of the human elastin W4 sequence. *Int. J. Pept. Protein Res.* **46**, 453–463.
58. Rosenberg, S., Barr, P.J., Najarian, R.C. & Hallewell, R.A. (1984) Synthesis in yeast of a functional oxidation-resistant mutant of human alpha-1-antitrypsin. *Nature (London)* **312**, 77–80.
59. Wenzel, H.R. & Tschesche, H. (1995) Reversible inhibitors of serine proteinases. In Peptides Synthesis, Structures, and Applications (Gutte, B., ed.), pp. 321–362. Academic Press, San Diego, CA.
60. Thompson, R.C. & Blout, E.R. (1973) Dependence of the kinetic parameters for elastase-catalyzed amide hydrolysis on the length of peptide substrates. *Biochemistry* **12**, 57–65.
61. Ando, S., Yasutake, A., Waki, M., Nishino, N., Kato, T. & Izumiya, N. (1987) Anti-chymotrypsin and anti-elastase activities of a synthetic bicyclic fragment containing a chymotrypsin-reactive site of soybean Bowman-Birk inhibitor. *Biochim. Biophys. Acta* **916**, 527–531.
62. Gariani, T. & Leatherbarrow, R.J. (1997) Stability of protease inhibitors based on the Bowman–Birk reactive site loop to hydrolysis by proteases. *J. Pept. Res.* **49**, 467–475.
63. Powers, J.C., Yasutake, A., Nishino, N., Gupton, B.F. & Kam, C.M. (1981) Synthetic elastase inhibitors and their role in the treatment of disease. In *Peptides: Synthesis, Structure, Function* (Rich, D.H. & Gross, E., eds), pp. 391–399. Pierce Chemical Co., Rockford, IL.
64. Larionova, N.I., Gladysheva, I.P. & Gladyshev, D.P. (1997) Human leukocyte elastase inhibition by Bowman-Birk soybean inhibitor; discrimination of the inhibition mechanisms. *FEBS Lett.* **404**, 245–248.
65. Tsunemi, M., Kato, H., Nishiuchi, Y., Kumagaye, S. & Sakakibara, S. (1992) Synthesis and structure-activity relationships of elafin, an elastase-specific inhibitor. *Biochem. Biophys. Res. Commun.* **185**, 967–973.
66. Braun, N.J., Bodmer, J.L., Virca, G.D., Metzvirca, G., Maschler, R., Bieth, J.G. & Schnebli, H.P. (1987) Kinetic studies on the interaction of eglin-c with human-leukocyte elastase and cathepsin-G. *Biol. Chem. Hoppe Seyler* **368**, 299–308.
67. Bieth, J.G., Kandel, M.J., Zreika, M. & Pochon, F. (1983) Interaction of protein substrate and inhibitors with alpha-2-macroglobulin-bound proteinases. *Ann. N.Y. Acad. Sci.* **421**, 209–217.
68. Yavin, E.J. & Fridkin, M. (1998) Peptides derived from human C-reactive protein inhibit the enzymatic activities of human leukocyte elastase and cathepsin G: use of overlapping peptide sequences to identify a unique inhibitor. *J. Pept. Res.* **51**, 282–289.
69. Stein, R.L. (1985) Catalysis by human-leukocyte elastase. 4. Role of secondary-subsite interactions. *J. Am. Chem. Soc.* **107**, 5767–5775.
70. Fersht, A.R. (1974) Catalysis, binding and enzyme-substrate complementarity. *Proc. R. Soc. Lond. B. Biol. Sci.* **187**, 397–407.
71. Stein, R.L. (1985) Catalysis by human-leukocyte elastase. 3. Steady-state kinetics for the hydrolysis of *para*-nitrophenyl esters. *Arch. Biochem. Biophys.* **236**, 677–680.
72. Kraut, J. (1977) Serine proteases: structure and mechanism of catalysis. *Annu. Rev. Biochem.* **46**, 331–358.
73. Wei, A.Z., Mayr, I. & Bode, W. (1988) The refined 2.3 Å crystal-structure of human-leukocyte elastase in a complex with a valine chloromethyl ketone inhibitor. *FEBS Lett.* **234**, 367–373.