

Selection of Human Elastase Inhibitors from a Conformationally-Constrained Combinatorial Peptide Library

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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 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 randomisation at three target locations in the sequence [1, 2]. Screening this library for binding to human leukocyte 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.

Introduction

Proteinases are essential components of a wide variety of biological processes [3] including the digestion of food, the cascade systems of blood clotting and complement, activation of hormones, and the degradation of endogenous proteins. The regulation of proteinase activity is therefore of great importance both *in vivo*, and for reason of therapeutic intervention [4]. Specific endogenous proteinaceous inhibitors represent the final level at which proteolytic activity can be regulated and alterations in the balance between proteinase and inhibitor are implicated in a number of pathological conditions affecting the normal physiology of the organism. For example, genetic deficiency of α_1 -proteinase inhibitor has been implicated in a number of diseases. In particular, deficiency is believed to lead to an excess of active leukocyte elastase resulting in pulmonary emphysema due to destruction of lung elastin [5]. Natural proteinaceous proteinase inhibitors have been reviewed by Laskowski and Kato [6] and more recently by Bode and Huber [7]. In almost all serine proteinases inhibitors isolated and characterised thus far, the portion of the inhibitor that interacts with the proteinase is an extended loop, which in the majority of inhibitor families is stabilised by intramolecular disulphide bonds. Inhibitors behave as limited proteolysis substrates, and the scissile bond is referred to as the reactive site. Residues interacting with proteinase at the

reactive site of the inhibitor determine the specificity, and are labelled according to the notation of Schechter and Berger [8].

It is possible to retain inhibitory activity by reproducing the loop region of one of these inhibitor proteins, Bowman-Birk inhibitor (BBI), by short peptide sequences incorporating a minimal disulphide-linked nonapeptide [9, 10]. We have recently demonstrated the utility of this model peptide to screen a defined peptide mixture of P₁ variants [10]. We have further generated a cyclic peptide library by randomisation at the P₂, P₁ and P₂' residues using the "one bead, one peptide" approach [2] to combinatorial peptide synthesis. Screening this library against chymotrypsin identified a consensus binding motif and allowed the selection of competitive inhibitors with K_i values in the nanomolar range [1].

Materials and Methods

Synthesis of tethered cyclic peptide library

Synthesis of the library peptide, NH₂-XCTXSXPPQCYGGGGG-Resin, was performed on an ABI431A peptide synthesiser at 0.25 mmole scale using Fmoc chemistry. HBTU/HOBT [11] activation was used for amino acid couplings at 4 fold excess except for the positions marked X. At these positions, randomisation was achieved as described previously [1]. Following side chain deprotection, cyclisation was achieved using DMSO [12]. We have previously demonstrated that this method provides an efficient means of on-resin cyclisation, with the generation of active resin-immobilised peptide [13]. Resin was then thoroughly washed and resuspended in PBS-T (PBS with 0.05% Tween-20) prior to use.

Synthesis of untethered cyclic peptides

Peptide sequences identified from the screen were re-synthesised as previously [1]. The cyclic peptides were purified by HPLC using a Waters Radial Pak C₁₈ column (25 mm x 10 cm) with 0.1% TFA and acetonitrile as solvents. Peptides were characterised by FAB-MS and analysed by TLC and analytical HPLC using a Vydac C₁₈ column (15 cm x 4.6 mm), again with 0.1% TFA and acetonitrile as solvents.

Screening the BBI library against Human Leukocyte Elastase (HLE)

Screening was performed essentially as described previously [1]. Briefly, HLE (Elastin Products Co. Inc., Missouri) was incubated in PBS-T with sufficient resin to provide approximately 46,000 beads. The resin was then washed thoroughly before incubating with rabbit anti-HLE (Calbiochem) in PBS-T. The washing step was repeated and then beads were incubated with monoclonal anti-rabbit IgG (γ chain specific) conjugated to alkaline phosphatase (Sigma Chemical Co.). The washing step was again repeated with a final wash of 100 mM Tris pH 9.5, 0.5 mM MgCl₂. Beads were then incubated with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium as alkaline phosphatase substrate in the same buffer. Positively stained beads were transferred to glass fibre filters, washed with 40 μl 50 mM HCl, and air dried. N-terminal sequencing was performed by Edman degradation on an ABI 477A peptide sequencer.

Inhibition kinetics and analysis of hydrolysis rate

Inhibition kinetics for peptides identified from the screen were determined using competitive binding studies with MeO-Suc-Ala-Ala-Pro-Val-*p*-nitroanilide (Sigma) as HLE substrate and 100 mM Tris, 500 mM NaCl pH 7.5 as buffer. Calculation of K_i values from rate versus inhibitor concentration data was as described previously for chymotrypsin inhibition [1].

Hydrolysis of peptides was performed using a 20 fold excess to HLE in 100 mM Tris, 500 mM NaCl pH 7.5. Degradation of the peak corresponding to the cyclic peptide was monitored using a Hewlett Packard HP1100 binary pump system equipped with a Micra NPS RP18 reverse phase column (4.6 x 33 mm, 1.5 μ m particle size) with 0.1% TFA and 90 % acetonitrile as solvents.

Results

The screening procedure identified a total of 23 highly positive beads from a sample of *ca.* 46,000 beads that were analysed. Peptide sequencing allowed in each unambiguous identification of the randomised positions, and the sequences found are listed in Table 1. Each of the different sequences found were resynthesised, purified to homogeneity and analysed for inhibition of HLE in solution. All of these peptides inhibited HLE, and the K_i values are also listed in Table 1.

Table 1. Sequences identified from the library. The P_4 , P_1 and P_2' positions were randomised, and the sequences at these positions are listed for the 23 beads identified from the screening procedure

Sequence of isolated resin bead			Frequency selected from HLE screen	K_i for re-synthesised peptide
<div><div></div><div>P₄-C-T-P₁-S-P₂'-P-P-Q-C-Y</div></div>				
P ₄	P ₁	P ₂ '		
Nle	A	I	5	65 nM
Nle	A	L	2	92 nM
M	A	I	1	90 nM
I	A	L	1	0.55 μM
F	A	I	1	0.26 μM
F	A	L	1	0.31 μM
Y	A	I	1	0.13 μM
Y	A	L	1	0.32 μM
W	A	F	1	0.97 μM
F	A	W	1	0.65 μM
W	A	V	1	2.77 μM
V	A	W	1	1.01 μM
R	A	I	4	0.26 μM
Nle	T	I	1	0.41 μM
W	T	I	1	1.7 μM

Discussion

In this work we demonstrate the applicability of the “one bead, one peptide” [2] approach to generate both cyclic conformationally-constrained peptides in general and to screen proteinase inhibitors in particular. The native Bowman-Birk inhibitor has a symmetrical structure consisting of two tri-cyclic domains, each with an independent proteinase binding site [14] forming a “bow-tie” like structure. Inhibitory activity for members of this protein family has been reported for trypsin, pancreatic elastase, and for chymotrypsin [15]. Serine is highly conserved at the P_1' position, and for this reason this residue was not randomised in the template peptide. This ‘template’ peptide was based on the anti-tryptic loop of the Bowman-Birk Inhibitor, D4, from *Macrotyloma axillare* [9]. This loop serves as a good template since a single disulphide bond maintains loop stability; this and the relatively short sequence ensures the possibility of both maximal coupling efficiency and high yield of the correct conformation. In addition, the reactive site is sufficiently near the N-terminus to minimise the number of sequencing steps required to identify the randomised residues in the vicinity of this bond.

The P_1 Position

The screening results against HLE indicate a high preference for alanine in the P_1 position. This is broadly consistent with the known substrate specificity of elastases for small alkyl side chains, although it is generally accepted that the preferred P_1 for HLE in terms of substrate specificity is valine [16]. It is therefore quite striking that no P_1 sequences containing valine were recovered from the screening process. Natural macromolecular inhibitors with activity against HLE are known which have at P_1 methionine (α_1 -proteinase inhibitor, guamerin), leucine (eglin c) and alanine (elafin). Two of the sequences listed in Table 1 had a threonine residue at P_1 . Although these sequences proved to be less potent inhibitors than the corresponding alanine 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 known sequence information for natural HLE inhibitors would have lead us to predict finding threonine at P_1 , which illustrates the benefits of library screening procedures.

The P_4 Position

Preference for large apolar or aromatic groups is observed in the P_4 position consistent with the structure of the S_4 pocket [17], and norleucine is found here with the greatest frequency. In addition, arginine is also observed at this position. Arginine is found as the P_3 residue in both elafin [18] and guamerin [19]. In the BBI template, P_3 is occupied by one of the two cysteines constraining the peptide.

The P_2' Position

The P_2' position also shows a preference for large apolar side chains with isoleucine being found at this locus with greatest frequency, and isoleucine or leucine together accounting for 83% of the sequences identified.

The peptide sequence that was 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 procedure 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 these three loci are additive, so that their combination gives the best available inhibitor. 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 likely that all of the beads having this sequence were identified by the screening protocol.

Effects of Hydrolysis

In order to evaluate further the omission of valine and methionine as the P_1 residue in the sequences discovered, we have substituted these amino acids within the consensus sequence (the P_4 and P_2' remain Nle and Ile respectively). The Met variant was found to have a significantly higher K_i ($11.4 \mu\text{M}$) than those sequences found by the library approach, which explains why this sequence was not amongst those identified. The Val variant, however, was found to have a K_i that is within the range of values for the sequences in Table 1 ($0.13 \mu\text{M}$), although this still makes the Val variant a poorer inhibitor than an Ala substituant. During analysis of the Val variant, it became apparent that this peptide was being turned over far more rapidly than the corresponding P_1 Ala sequence, and further analysis reveals a significant difference in their respective hydrolysis rates (Fig. 1). The valine variant has a turnover some 20 fold greater than the corresponding library selected sequence. This effect, which is consistent with the substrate specificity of HLE [16], means that any sequences with a P_1 valine would almost certainly have hydrolysed during the incubation period of the screening. It is therefore not surprising that no such sequences were discovered.

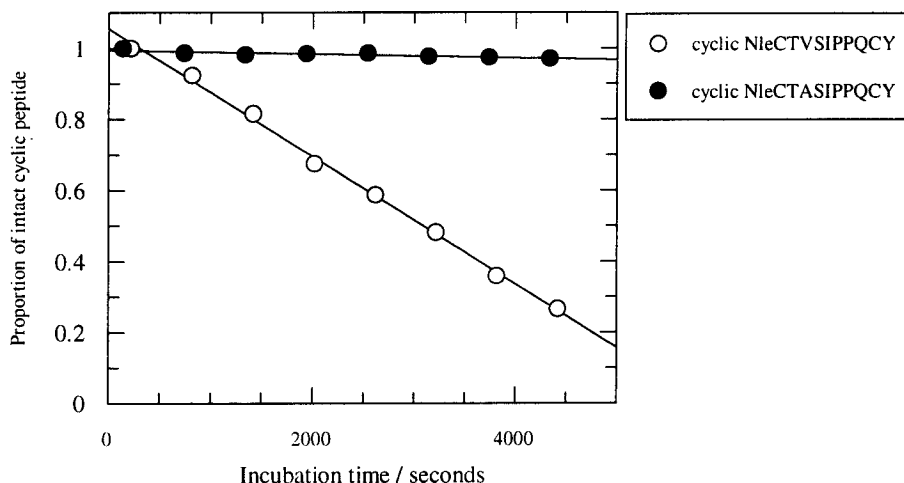


Figure 1. Comparative hydrolysis rates for the consensus library selected sequence (cyclic NleCTASIPPQCY) and a non-library selected variant (cyclic NleCTVSIPPQCY). Hydrolysis was followed by HPLC as described in the methods.

Conclusions

It seems that the screening procedure 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, as is shown by comparison of sequences with P₁ Val or Ala. Clearly, this has significant implications for proteinase inhibitor design, which often relies on substrate specificity data.

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