Chymotrypsin Inhibitors Identified from a Second-Generation Template Assisted Combinatorial Peptide Library

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Introduction

Proteinases are essential components of a wide variety of biological processes [1] 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 [2]. Specific endogenous 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. Natural proteinaceous proteinase inhibitors have been reviewed by Laskowski and Kato [3] and more recently by Bode and Huber [4]. In almost all inhibitors of the serine proteinases isolated and characterised thus far, the portion of the inhibitor that interacts with the proteinase is an extended or 'canonical' loop, often stabilised by intramolecular disulphide bonds. This loop is usually thought to have a structure similar to that of a productively bound substrate [3, 4], although this assumption has recently been questioned [5]. 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 (notation of Schechter and Berger [6]) is considered to be the main determinant of specificity [3].

It is possible to reproduce the loop region of one of these proteins, Bowman-Birk inhibitor (BBI) by short peptide sequences incorporating a minimal disulphide-linked nonapeptide [7, 8]. We have previously demonstrated the utility of this model peptide to generate and screen a template-assisted combinatorial library for chymotrypsin inhibitors [9]. This study employed randomisation at the P_2, P_1 and P_2' residues using the "one bead, one peptide" approach [10] and was able to identify active sequences against chymotrypsin with K_i values ~20nM (Table 1).

Table 1. *Active sequences identified from the initial BBI library for binding to chymotrypsin. Randomised positions corresponding to the P_2, P_1 and P_2' positions are highlighted in bold. Peptide-resin was sequenced only to the P_2' position. Data from [9].*

Sequence of isolated peptide-resin bead H_2N-SC-**P_2**-**P_1**-S-**P_2'**-PPQCY-(G_5-spacer)-Resin			Frequency selected from screen	K_i for re-synthesised 11mer peptide, nM
P_2	**P_1**	**P_2'**		
T	F	I	5	19
T	F	L	1	20
T	F	Nle	2	19
T	Y	I	2	17

Whilst the identity of the P_1 residue of this first-generation library was in line with the known specificity of this enzyme, it was striking that all active sequences had Thr as the P_2 residue. Subsequently, a kinetic and structural analysis [11; JDM, RJL & Brauer, A.B.E., in preparation] have revealed that this residue is optimal and serves a dual role in enzyme recognition and loop maintenance (via internal hydrogen bonding). As a consequence, it seems likely that further enhancement of activity might benefit from randomisation at another position, P_4, which is located on the upper face or contact region of the peptide. To do this, we elected to screen a second-generation library in which this position plus the P_1 and P_2' positions have been randomised (the P_2 is fixed as Thr). Synthesis and screening of this second-generation library was performed essentially as described previously [9]. Briefly, biotinylated chymotrypsin was incubated in PBS with sufficient resin to provide approximately 40,000 beads. The resin was then washed thoroughly before incubating with Extravadin-alkaline phosphatase (Sigma) in PBS. The washing step was repeated and then beads were incubated with 5-Bromo-4-Chloro-3-Indolyl Phosphate and Nitroblue tetrazolium as alkaline phosphatase substrate in 100mM Tris pH 9.5, 0.5 mM $MgCl_2$. Positively stained beads were visualised using a Zeiss Stemi-2000-C microscope, 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 Hewlett Packard HP G1005A sequencer.

Inhibition kinetics for peptides identified from the screen were determined using competitive binding studies with succinyl-Ala-Ala-Pro-Phe-7-amido-4-methyl coumarin as chymotrypsin substrate.

Results and Discussion

The native Bowman-Birk inhibitor has a symmetrical structure consisting of two tri-cyclic domains, each with an independent proteinase binding site [12] forming a "bow-tie" like structure. For trypsin binding domains, the P_1 residue is lysine or arginine; for elastase, alanine, and for chymotrypsin, the P_1 residue is leucine or tyrosine [13]. 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 good 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 determine the randomised sequences.

Table 2. *Sequences identified from the second generation BBI library screened for binding to chymotrypsin. Randomised positions corresponding to the P_4, P_1 and P_2' positions are highlighted in bold. Peptide-resin was sequenced only to the P_2' position.*

Sequence of isolated peptide-resin bead H_2N-**P_4**-CT-**P_1**-S-**P_2'**-PPQCY-(G_5-spacer)-Resin			Frequency selected from screen	K_i for re-synthesised 11mer peptide, nM
P_4	**P_1**	**P_2'**		
Nle	F	I	3	3.5
V	F	F	2	31.5
F	F	I	1	3.5
Y	F	F	1	5.8
Q	F	L	1	3.4
Q	F	Nle	1	5.3
Q	L	Nle	1	114
Nle	L	I	1	224
Nle	L	F	1	310

Our screening results (Table 2) reveal a preference for phenylalanine at the P_1 site, which is in accordance with the well-reported substrate specificity. However, it is interesting to note that in both this and our original study [9], a tryptophan residue was not detected. Leucine was also found at this locus, and is consistent with the presence of this residue in a number of BBI loops [11] including soybean BBI which has well documented anti-chymotryptic activity [14, 15]. Like the first library, the P_2' position shows a preference for large apolar aliphatic side chains, however in addition, phenylalanine is also now identified here.

The main difference between this new library and our earlier report is that we have now varied the P_4 position. Overall, there is a general preference for both hydrophobic residues and glutamine at this site, with norleucine being found most frequently and also providing one of the lowest K_i values. In our earlier studies, all members of the library had serine at P_4. After varying this locus we have now identified sequences that are over 5-fold more potent than in our earlier studies. Indeed, it is interesting that several of the sequences display K_i values lower than the best studied proteinaceous BBI (Literature values for Soybean BBI activity against chymotrypsin are 6.4 nM and 13 nM [14, 15]). This indicates that the peptide loop is more than capable of mimicking the larger protein and illustrates the benefits of library screening procedures.

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