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

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Abstract: In an earlier study (McBride JD, Freeman N, Domingo GJ, Leatherbarrow RJ. Selection of chymotrypsin inhibitors from a conformationally-constrained combinatorial peptide library. *J. Mol. Biol.* 1996; **259**: 819–827) we described a resin-bound cyclic peptide library, constructed based on the sequence of the anti-tryptic reactive site loop of Bowman–Birk Inhibitor (BBI), a proteinase inhibitor protein. This library was used to identify re-directed chymotrypsin inhibitors with K_i values as low as 17 nM. We have now extended this work by constructing an enhanced library in which a further position, at the P_4 site of the inhibitor, has been randomized. This new library has variation at three target locations (P_4, P_1 and P'_2) within the inhibitory loop region, producing 8000 variants. Screening this library allowed selection of new inhibitor sequences with K_i values as low as 3.4 nM. The success of this approach is reflected by the fact that the inhibition constant given by the selected peptide sequence is slightly lower than that reported against chymotrypsin for the most studied full length BBI protein, Soybean BBI 2-IV. Copyright © 2000 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: chymotrypsin; combinatorial library; peptide library; peptide synthesis; proteinase inhibitor

INTRODUCTION

Proteinases play roles in a range of diverse biological processes including the digestion of food, the cascade systems of blood clotting and complement, activation of hormones, and the degradation of endogenous proteins [1]. Natural inhibitors of proteinases have important roles in regulation; in addition many different synthetic inhibitors have been described, which are often of interest for reason of therapeutic intervention [2]. Specific endogenous protein-based inhibitors are commonly found, and represent the highest level at which proteolytic activity can be regulated. Alterations in the balance between proteinase and inhibitor are implicated in a number of pathological conditions affecting normal physiology. In almost all protein-based inhibitors of the serine proteinases isolated and characterized to

Abbreviations: AMC, 7-amino-4-methylcoumarin; BBI, Bowman–Birk Inhibitor; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; DMF, dimethylformamide; HBTU, 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HOBt, 1-hydroxybenzotriazole monohydrate; HPLC, high performance liquid chromatography; NBT, nitroblue tetrazolium; OdhBt, 3-hydroxy-4-oxo-3,4-dihydro-1,2,3-benzotriazine ester; OPfp, pentafluorophenyl ester; PBS, phosphate-buffered saline; P_n, the nth residue of a substrate or inhibitor on the carbonyl side of the bond hydrolysed by a proteinase; P'_n, the nth residue of a substrate or inhibitor on the amino side of the bond hydrolysed by a proteinase; TFA, trifluoroacetic acid; TLCK, $N\varepsilon$-*p*-tosyl-L-lysine chloromethyl ketone; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol.

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date, the portion of the inhibitor that interacts with the proteinase is an external loop that has an extended or 'canonical' conformation [3,4]. This structure is thought to be similar to that of a productively bound substrate [3] and in the majority of inhibitor families the loop organization is primarily stabilized by intramolecular disulphide bonds. Inhibitors behave as limited proteolysis substrates; residues interacting with proteinase at the reactive site of the inhibitor determine specificity and typically reflect the substrate preference of the target proteinase. The identity of the P_1 residue (notation of Schechter and Berger [5]) is considered the main determinant of specificity for most proteinases [3,6,7]. However, interactions with subsites outside P_1 are also important for specificity [8,9].

Synthetic peptides that incorporate the loop region from one of these serine proteinase inhibitor proteins, Bowman–Birk inhibitor (BBI), retain inhibitory activity provided they retain the disulfide that links the ends of the loop [10–14]. We have previously demonstrated that peptides based on this region are useful starting points for production of template-assisted combinatorial libraries of potential proteinase inhibitors [15]. In this first study, the library incorporated randomization at the P_2, P_1 and P'_2 residues using a 'one bead, one peptide' approach [16] and was able to identify active sequences against chymotrypsin with K_i values $\sim$20 nM. The overall strategy is summarized as follows. The active loop region from the full length BBI protein is synthesized so that it remains tethered to synthesis resin after deprotection and disulphide bond formation. Beads that represent a one bead, one peptide library are then screened for proteinase binding activity by adding chymotrypsin that is tagged with biotin such that beads that bind chymotrypsin can be detected by an enzyme linked assay using avidin-alkaline phosphatase. Such beads are identified under a low power optical microscope and the peptide sequence that they contain is read by automated Edman degradation. Once identified, active sequences can then be resynthesized and tested for inhibition activity in solution.

The identity of the P_1 residue found from screening our first-generation library was broadly in line with the known specificity of chymotrypsin, with P_1 Phe and Tyr residues being selected. However, chymotrypsin is known to have greatest preference for Trp at P_1 [7,17]. The lack of P_1 Trp in the sequences found is investigated further by examining the inhibition properties of loop peptides having a P_1 Trp residue.

Another striking result from our earlier study was that all active inhibitor sequences had Thr as the P_2 residue. Subsequently, kinetic and structural analyses ([8]; Brauer ABE, McBride JD, Leatherbarrow RJ, in preparation) have revealed that this residue is optimal and serves a dual role in enzyme recognition and maintenance of structural integrity (via internal hydrogen bonding). As a consequence, it seems likely, therefore, that further enhancement of activity might benefit from randomization at another position, P_4, which is located on the upper face or contact region of the peptide. To do this, we have chosen to screen a second-generation library where the P'_2 position is fixed as Thr, but randomization is incorporated at P_4, P_1 and P'_2.

MATERIALS AND METHODS

Synthesis of Tethered Cyclic Peptide Library

Synthesis of the template peptide, NH_2-XCTXSXP-PQCYGGGGG-Resin (Figure 1), was performed on an ABI 431A peptide synthesizer at 0.25 mmol scale using 9-fluorenylmethoxycarbonyl (Fmoc) chemistry essentially as described previously [15]. The first glycine was coupled to TentaGel-S-NH_2 resin (Rapp Polymere, Tubingen, Germany; 0.23 meq g^{-1}) as the symmetric anhydride using 0.5 equivalents of DCC. 2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate/

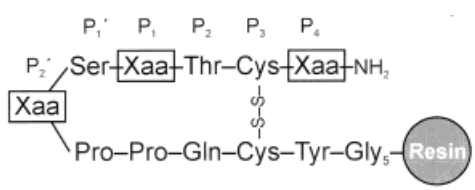

Figure 1 Sequence of the synthetic library. Peptides were attached to TentaGel-S-NH_2 resin. Five Gly residues were introduced to act as a spacer between the inhibitor sequence and the resin bead. The inhibitory sequence of 11 residues was then added with the positions marked Xaa representing points of randomization. The disulfide was formed after deprotection. Randomization used all natural amino acids with the exception of cysteine but the inclusion of norleucine, which results in a library size of 8000 variants. The figure also shows the location of the subsites (P_1, P_2 etc.) using the nomenclature of Schechter and Berger [5].

1-hydroxybenzotriazole monohydrate (HBTU/HOBt) [18] activation was used for subsequent amino acid couplings at a four fold excess except for the positions marked X. At these locations, randomization was achieved by splitting the resin into 20 polypropylene tubes, each containing a ten fold excess of separate amino acid (comprizing the common amino acids minus cysteine but with norleucine) as their pentafluorophenyl ester (Opfp) or 3-hydroxy-4-oxo-3,4-dihydro-1,2,3-benzotriazine ester (ODhBt) (serine and threonine) with one equivalent of HOBt in 2 ml DMF. Resin was re-pooled into the reaction vessel of the ABI 431A and excess reagent removed by filtration before performing *N*-terminal deprotection by standard protocols on the synthesizer. All *N*-terminal deprotections were monitored using an ultra violet (UV) detector, attached to the synthesizer as recommended by the manufacturer. Side chain protecting groups were removed using a scavenger mixture of 15 ml trifluoroacetic acid (TFA), 0.75 ml water, 0.75 ml thioanisole, 0.375 ml ethanedithiol and 1.125 g phenol, washed thoroughly with methanol and dried. Cyclization was achieved by modification of the method described by Tam *et. al.* [19] for solution peptides. The resin was shaken for 48 h at room temperature in 1 l 20% dimethylsulphoxide (DMSO), 5% acetic acid (adjusted to pH 6 with aqueous ammonia). Resin was then thoroughly washed and resuspended in phosphate-buffered saline (PBS) prior to use. We have previously shown that this procedure results in efficient on-resin cyclization for the BBI peptides [20].

Synthesis of Untethered Cyclic Peptides

Peptide sequences identified from the screen were re-synthesized as described previously [21] using a Shimadzu PSSM-8 synthesizer at 0.025 mmol scale with HBTU/HOBt activation (using tyrosine precoupled Wang resin, and five fold amino acid excess). Following side chain deprotection and cleavage from resin using 1 ml of the previously described mixture of scavengers, peptides were precipitated in cold *tert*-methyl butyl ether and dried. Cyclization was performed at a peptide concentration of 0.05 mg ml^{-1} overnight in the cyclization mixture described for the library. The cyclic peptides were then purified by high performance liquid chromatography (HPLC) using a Waters Radial Pak C_{18} column (25 mm $\times$ 10 cm) with 0.1% TFA and acetonitrile as solvents. Peptides were characterized by fast atom bombardment-mass spectrometry (FAB-MS) and analysed by analytical HPLC using a Vydac C_{18} column (4.6mm $\times$ 10cm, 5 μm particle size), again with 0.1% TFA and acetonitrile as solvents on a Beckman System Gold system. In all cases, the synthetic peptides had the expected mass and eluted as a single component on HPLC, with estimated purities of $>90\%$ based on integration of peak area.

Preparation of Biotin-Chymotrypsin

Bovine α-chymotrypsin (*N*ε-*p*-tosyl-L-lysine chloromethyl ketone (TLCK) treated, Sigma–Aldrich, Poole, UK) was labelled with 20 fold excess biotin-*N*-hydroxy succinimide ester in 50 mM $NaHCO_3$ pH 9 as described previously [15]. Labelled enzyme was purified by affinity chromatography using soybean trypsin inhibitor-Sepharose (prepared as described by Feinstein *et al.* [22]). The column was run in 50 mM Tris, 500 mM NaCl pH 8 at a flow rate of 2 ml min^{-1}. Bound chymotrypsin was eluted with 10 mM HCl, concentrated using a Centricon 10 (Millipore (UK) Ltd, Watford, UK), then stored at $-20\,^{\circ}C$ prior to use.

Screening of the BBI Library

Screening was performed essentially as described previously [15]. Briefly, biotin-chymotrypsin was incubated in PBS with sufficient resin to provide approximately 40000 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 (BCIP) and nitroblue tetrazolium (NBT) as alkaline phosphatase substrate in 100 mM Tris pH 9.5, 0.5 mM $MgCl_2$. Positively stained beads were visualized 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 a Hewlett Packard G1000A protein sequencer, using protocols recommended by the manufacturer.

Inhibition Kinetics

Inhibition kinetics for peptides identified from the screen were determined using competitive binding studies with succinyl-Ala-Ala-Pro-Phe-7-amino-4-methylcoumarin (AMC) as chymotrypsin substrate. Assays were performed at pH 7.8 in 144 mM Tris buffer at 25 °C using a CytoFluor series 4000 microplate reader (PE Biosystems, Warrington, UK) with excitation and emission set at 360 and

460 nm, respectively. Substrate concentration was determined by calibrating instrument response with 0–20 μM AMC and then by determining the release of AMC in the presence of excess chymotrypsin. The active chymotrypsin concentration was determined by titration with *p*-nitrophenol acetate (Bender *et al.*, 1966 [29]), and peptide concentrations by their absorbance at 280 nm [23]. Typical assay conditions were 0.25 nM enzyme and 56 μM substrate. Initial velocity data were fitted using the GraFit software package to determine K_i. These values were corrected to account for competition by substrate ($K_i = K_{i\ (observed)}/(1 + [S]/K_m)$); the K_m for this substrate was found to be 45 μM.

Hydrolysis Rates

Hydrolysis of peptides was performed using a 20 fold excess to chymotrypsin (3.43 μM chymotrypsin) in 25 mM Tris, pH 7.8 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, Illinois, USA) using a Hewlett Packard HP1100 binary pump system. Separation was performed using a linear gradient between 0.3% TFA and 0.3% TFA/90% acetonitrile as solvents. The incubation time is taken to be the time from addition of enzyme until the time sample was applied to the column, where the low pH conditions ensure that enzyme activity is minimal.

RESULTS

The new second-generation library (Figure 1) incorporated randomization at P_4, P_1 and P'_2, and screening a sample of ~40000 beads allowed 12 beads to be identified as chymotrypsin binders. The sequences of the peptides found on these beads, determined via the Edman method, are shown in Table 1. This table also records the inhibition constants against chymotrypsin, which were determined using re-synthesized peptides. The sequence P_4 Nle, P_1 Phe and P'_2 Ile was found on three independent beads; P_4 Val, P_1 Phe and P'_2 Phe was found twice and the remaining sequences once only. Screening our earlier library [15] against chymotrypsin, we obtained Phe (80%) and Tyr (20%) at P_1; for our current library we found no sequences with Tyr at this position but did recover three sequences having P_1 Leu. At P'_2 we had previously obtained inhibitors with Ile, Leu or Nle; each of these were recovered again and, in addition, we found some sequences with Phe at this location. The P_4 position was not randomized in our earlier study; the present results found Nle, Val, Phe, Tyr and Gln returned.

To investigate the inhibition properties of peptides having a P_1 Trp, the peptide SCTWSIPPQCY was synthesized for comparison with the consensus peptide of our previous chymotrypsin library, SCTFSIPPQCY (both peptides were cyclic by disulfide linking their cysteine residues). The K_i values for these peptides against chymotrypsin were

Table 1 Sequences Identified from the Second-generation BBI Library. Randomized Positions Corresponding to the P_2, P_1 and P'_2 Positions are Highlighted. Peptide-resin was Sequenced only to the P'_2 Position. The K_i value for Inhibition of Chymotrypsin by These Sequences, Determined Following Re-synthesis and Purification of Each Peptide Species, is also Shown

Sequence of isolated peptide-resin bead H_2N-**P_4**-CT-**P_1**S-**P'_2**-PPQCY-(G_5-spacer)-Resin			Number of times this sequence was found	K_i for re-synthesized 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

determined to be 43 nM and 19 nM, respectively. However, during these assays it was apparent that the Trp-containing peptide was far more rapidly hydrolysed. A comparison of the relative hydrolysis of these peptides is shown in Figure 2.

DISCUSSION

The native BBI protein has a symmetrical structure consisting of two tri-cyclic domains, each with an independent proteinase binding site [24] forming a 'bow-tie' like structure. For trypsin binding domains, the P_1 residue is lysine or arginine; for pancreatic elastase it is alanine; for chymotrypsin the P_1 residue is leucine or tyrosine [25]. Serine is highly conserved at the P'_1 position [8], and so this residue was not randomized in the present study. The peptide sequence used was based on the antitryptic loop of the BBI, D4, from *Macrotyloma axillare* [13]. This loop serves as a good template since a single disulphide bond constrains the loop, 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 minimize the number of sequencing steps required to determine the randomized sequences in the vicinity of this bond using the Edman method. A schematic structure of this loop, showing the location of the residues that are randomized in the current study, is shown in Figure 1. We have shown that synthetic 11-mer peptides based on the reactive site loop of BBI retain in solution the structure of the reactive site loop found in the complete protein (Brauer ABE, McBride JD, Leatherbarrow RJ. in preparation). This makes such synthetic peptides good models for the original protein inhibitor.

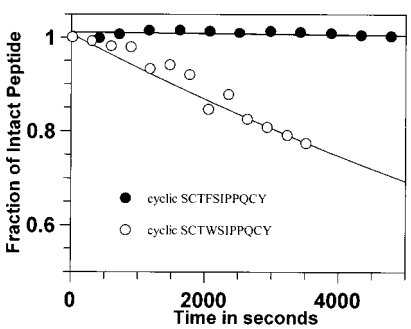

Figure 2 Comparative hydrolysis rates for the consensus sequence selected from the first library (SCTFSIPPQCY) and a non-library selected sequence (SCTWSIPPQCY).

Our screening results reveal a preference for phenylalanine at the P_1 site, which is broadly in accord with the well-reported substrate specificity of chymotrypsin [17]. Leucine was also found at this locus, and is consistent with the presence of this residue in a number of BBI loops [8] including soybean BBI which has well documented anti-chymotryptic activity [26,27]. However, the sequences with leucine at P_1 were found to have poorer binding. In our previous library, we found sequences with Tyr at P_1; no such sequences were discovered on this occasion, although it is possible that this is just a statistical factor and that sequencing of more beads would have revealed P_1 Tyr sequences.

It is interesting to note that in both this and our original study [15], a tryptophan residue was not detected at P_1. On the basis of both substrate specificity [17] and studies with other canonical inhibitors [7] we would have expected Trp to be one of the best residues. However, no Trp residues are found at P_1 in natural BBI proteins [8]. To test this, we synthesized a Trp-containing sequence and compared this to an analogue having Phe at P_1. The results show that the Trp P_1 peptide was a respectable inhibitor at short incubation times, but was hydrolysed many times more rapidly than the Phe P_1 sequence (Figure 2). The result is interesting, as it shows that binding and turnover are not strictly correlated, an observation that we have also made with other BBI-related sequences [14,28]. In this case, the Trp variant clearly has more substrate-like behaviour with a turnover rate considerably greater than the corresponding library selected sequence. This effect means that any sequences with a P_1 Trp would almost certainly have hydrolysed during the incubation period of the screening. It is, therefore, not surprising that no such sequences were discovered during the present study or in our earlier work.

At the P'_2 position, our results show a preference for large apolar side chains. This is consistent with the results found with our earlier chymotrypsin library [15], and is broadly similar to the specificity found for trypsin inhibition at this locus [9].

Comparing the K_i values of the library used in this study and in our previous one [15], it can be seen that a much wider distribution is seen in the newer library, with some values lower and some higher than the original library. It seems highly probable that relative hydrolysis rate is a crucial factor in our library selection process, as by necessity the beads are incubated with enzyme for several minutes in order to select binders. We have

previously shown that P_2 Thr substitutions, which were included in the first library, result in increased hydrolysis rates [8]. As all sequences in the second library had an optimized P_2, it seems likely that they will be, on average, more resilient to hydrolysis.

The main difference between this new library and our earlier report is that we have now varied the P_4 position. Overall, there is greater variation at this position than we have observed at the others varied, suggesting that the contribution of P_4 towards specificity in chymotrypsin binding is correspondingly less. Large apolar residues or glutamine residues were present in the peptides, with norleucine being found most frequently and also providing one of the lowest K_i values. This is a similar result to that found when screening for inhibitors of human elastase [21]. In our earlier screening study for chymotrypsin inhibitors [15], all members of the library had a serine residue at P_4. When this position is allowed to vary, it is interesting that no P_4 Ser sequences are returned, demonstrating that a serine residue is less than optimal for this target enzyme. By optimizing this locus, we have now allowed identification of sequences that are over five fold more potent than in our earlier studies. Upon re-synthesis, several of the sequences discovered also display K_i values slightly lower than the best studied proteinaceous BBI (Literature values for Soybean BBI activity against chymotrypsin are 6.4 nM [26] and 13 nM [27]). This indicates that the peptide loop is more than capable of mimicking the larger protein.

Acknowledgements

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