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The role of the P_2' position of Bowman-Birk proteinase inhibitor in the inhibition of trypsin
Studies on P_2' variation in cyclic peptides encompassing the reactive site loop

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Abstract

The role of the P_2' residue in proteinase inhibitors of the Bowman-Birk family was investigated using synthetic cyclic peptides based on the reactive site loop of the inhibitor. A series of 21 variants having different P_2' residues was tested for inhibition of trypsin, and the rate at which they were hydrolysed by this enzyme was also measured. Variation at P_2' was found to result in marked differences in inhibitory potency, with the best sequence (Ile) having a K_i value of 9 nM. Peptides with P_2' Gly, Pro or Glu failed to demonstrate any measurable inhibition ($K_i > 1$ mM). The peptides also displayed significant differences in the rates at which they were hydrolysed, which varied by over three orders of magnitude between the difference sequences. There was found to be overall correlation between the K_i value and the rate of hydrolysis, with peptides that inhibited best also being hydrolysed more slowly. The results are discussed in light of the sequence information for Bowman-Birk inhibitor proteins.

Keywords: Bowman-Birk inhibitor; Proteinase inhibitor; Trypsin; Peptide synthesis

1. Introduction

The Bowman-Birk inhibitors (BBI) are bi-headed serine proteinase inhibitors found within plants of the Fabaceae family [1]. They are notably present in Leguminosa plants (e.g. soybean) in which they are considered to play various roles including protein storage and defence [2,3]. These proteins have a low molecular weight (6–9 kDa) and are characterised by the presence of seven disulphide bridges, which allow the formation of an almost symmetrical structure comprising two independent heads located at opposite sides of the molecule. Each head is made of a tricyclic domain in which a binding site is located

Abbreviations: Fmoc, *N*-α-9-fluorenylmethoxycarbonyl; Pmc, 2,2,5,7,8-pentamethylchroman-6-sulphonyl; Trt, trityl; tBu, *t*-butyl; Boc, butoxycarbonyl; HBTU, 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HOBt, *N*-hydroxybenzotriazole; TFA, trifluoroacetic acid; EDT, 1,2-dithiolethane; MTBE, *tert*-methyl butyl ether; DMSO, dimethylsulphoxide; FAB-MS, fast atom bombardment mass spectroscopy; HPLC, high performance liquid chromatography; DL-BApNA, DL-benzoyl-arginine-*p*-nitroanilide; Cha, cyclohexylalanine; Nle, norleucine

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and enclosed within a nine-residue disulphide linked loop. Crystal and NMR structures of these inhibitors have been reported [4,5]. In addition, the crystal structure of the trypsin domain binding site of BBI from azuki bean complexed with trypsin [6] and the complete BBI from mung bean in ternary complex with trypsin [7] have also been described.

The reactive loop, following Schecter and Berger's nomenclature [8], spans sub-sites P_3 to P_6' from one cysteine to the other. Although the sequences of the loops are highly conserved [9,10], BBIs can be divided into four groups (I–IV) depending on variation at the P_1 and P_2' residues [11]. Naturally occurring inhibitors typically have the following residues at the P_1 site: leucine and phenylalanine (α-chymotrypsin); alanine (pancreatic elastase); arginine or lysine (trypsin). It is found that the isolated disulphide-linked reactive loop retains activity [10–12], which is because such peptides have the same reactive site loop structure as the full-length protein [13,14]. The structural features of the BBI added to their interesting properties have attracted interest in synthetic studies to elucidate specificity [9,11–13,15–20].

It is known that peptide inhibitors based on the BBI reactive site loop motif are slowly hydrolysed by the enzymes they inhibit [15], although the dominant effect is to act as inhibitors. In this respect, their behaviour is similar to the behaviour of full-length protein inhibitors, which form a stoichiometric enzyme-inhibitor complex. Unlike a typical enzyme-substrate complex, however, the energy barrier for hydrolysis is large and unfavourable, resulting in low rates of hydrolysis [3,21,22]. The value of the inhibition constant (K_i) is usually used to rank different inhibitors, however, for the inhibitors that are slowly hydrolysed, their stability is also an important factor [20]. Unfortunately, this aspect of behaviour seems difficult to predict and does not always correlate with the K_i values. Synthetic work on the BBI peptide-based inhibitors has focused mainly on the P_1 site, which confers the primary specificity [18,23]. However, members of the BBI family show wide variation at the P_2' site, and we have recently found marked differences in stability to hydrolysis for peptides having Ile or Asn at the P_2' site [20]. This paper describes a complete characterisation of the effects of variation at P_2' by comparison of 21 trypsin inhibitor peptides that differ at their P_2' residue. It is found that variation of this residue leads to dramatic changes in both K_i values and hydrolysis rate constants of the inhibitor peptides.

2. Materials and methods

2.1. Materials

Synthesis was carried out using *p*-alkoxybenzyl alcohol resin (Wang resin), which was purchased from Novabiochem (UK). *N*-α-Fmoc amino acids were purchased from Novabiochem (UK) or from Bachem (UK). These amino acids were used having the following side-chain protecting groups: Ala, Arg (Pmc), Asn (Trt), Asp (tBu), Cha, Cys (Trt), Glu (tBu), Gln (Trt), Gly, His (Trt), Ile, Leu, Lys (Boc), Met, Nle, Phe, Pro, Ser (tBu), Thr (tBu), Trp (Boc), Tyr (tBu) and val. All reagents for the peptide synthesiser were supplied by Rathburn Chemicals (Walkerburn, UK). All other reagents and solvents were from Sigma or Aldrich unless otherwise stated.

2.2. Peptide synthesis

The peptides used in this study are listed in Table 1. All peptides were synthesised by standard solid-phase techniques using Fmoc chemistry [24] and HBTU/HOBt activation [25] as described previously [18,20] using a PSSM-8 multiple peptide synthesiser (Shimadzu). Reactions were performed at a 0.05 mmol synthesis scale with a five-fold excess of amino acid at each cycle.

Cleavage of peptides from the resin and deprotection of the side chain was performed using a multi-component scavenger cleavage mixture: 90% TFA, 5% phenol, 2% thioanisole, 2% H_2O, 1% EDT stirring overnight at room temperature. The cleaved deprotected peptide was filtered in order to remove the resin, precipitated in cold MTBE and collected by centrifugation. The precipitation step was repeated four times and the peptide then dried in a desiccator.

Disulphide bridge formation was performed using DMSO oxidation [26]. The dried peptides were dissolved in 20% (v/v) DMSO, 5% acetic acid to give a 0.02 mg/ml solution. The pH was adjusted to 6 with aqueous ammonia, and stirring was performed at

room temperature for 24 h. All peptides were purified by reversed-phase HPLC using a Gilson HPLC 720 system with a semi-preparative C18 Waters Radial Pack column (25 mm × 10 cm, 6 μm particle size, 6 nm pore size). For both preparative and analytical use, the aqueous and polar phases used were water (A) and acetonitrile (B) respectively, each containing 0.1% TFA (v/v). All peptides were characterised by FAB-MS and analytical reverse phase HPLC. In each case the peptides had the expected mass, and were >90% pure by HPLC.

2.3. Inhibition kinetics

The inhibition kinetics of the various peptides measured against bovine pancreatic trypsin (Sigma) were determined by competitive binding studies [18,27] using the chromogenic substrate DL-BApNA (Sigma). All experiments were performed at 25°C in Tris-HCl (50 mM, pH 7.8) containing $CaCl_2$ (10 mM), and monitored through the change of absorbance at 412 nm upon hydrolysis of the substrate by the enzyme. Under these conditions the values for k_{cat} and K_m are 2.4 s^{-1} and 2.16 mM, respectively. The enzyme concentration was calculated from the initial rate of substrate hydrolysis [18]. Substrate concentration was obtained from the final absorbance at 412 nm after complete hydrolysis ($\varepsilon = 8480$ M^{-1} cm^{-1}), The peptide concentrations were determined by optical density at 280 nm [28]. All data were processed using non-linear regression [29] using the GraFit software package [30] to find K_i.

2.4. Hydrolysis rate constants

The rate of hydrolysis of the inhibitory peptides by trypsin was determined by analysing the rate at which inhibited enzyme regained activity. Experiments were performed at 25°C in Tris-HCl (50 mM, pH 7.8) containing $CaCl_2$ (10 mM), using the chromogenic substrate DL-BApNA. Sufficient inhibitor was added to trypsin so that the enzyme was ~90% inhibited. Aliquots were taken at increasing times and the rate of substrate hydrolysis monitored by the change of absorbance upon the addition of substrate (final concentrations: trypsin $\sim 10^{-7}$ M; substrate 5×10^{-5} M). Inhibitor hydrolysis is accompanied by a regain of enzyme activity, the rate of which was calculated from these data assuming a first order process as described by Eq. 1:

$$\text{Activity} = (100 - \text{initial})(1 - e^{-kt}) + \text{initial}. \quad (1)$$

In this equation the activity varies from the *initial* value to 100% with k being the first order rate constant for the process.

3. Results and discussion

3.1. Effect of P_2' variation on K_i values

It is found that variation of the P_2' residue results in a large effect on the measured K_i values, which range from 9 nM to >1 mM for the peptides studied (Table 1). These results confirm a significant influence of interactions involving the P_2' residue on binding to the enzyme. The lowest K_i value is given by peptide P_2'-Ile. Large aliphatic side chains are generally found to give the best inhibitors, with P_2'-Nle and P_2'-Leu providing the next lowest K_i values. Positively charged residues (Arg, Lys) are tolerated at P_2', and have relatively low K_i values. However, negatively charge side chains give poor inhibitors, with the peptide P_2'-Glu not providing any measurable inhibition. From the concentrations tested in our assays, this indicates that the K_i value for this variant must be >1 mM.

Two other peptides showed no measurable inhibition in our assays: P_2'-Gly and P_2'-Pro. These residues are the ones most likely to cause structural alterations, as a Gly has much greater conformational flexibility than other amino acids, while a Pro has restricted conformational freedom [31]. It is, therefore, possible that introduction of these residues at P_2' results in peptides that do not have the appropriate conformation for binding. This result is consistent with site-directed mutagenesis experiments on whole BBI protein, which have shown that introduction of a Pro at P_2' results in an inactive inhibitor [32].

The results in Table 1 are consistent with the work of McBride et al. [19], where a library of BBI loop peptides having 8000 combinations of P_2, P_1 and P_2' (20 possibilities at each position) was screened for chymotrypsin binding. The active chymotrypsin-binding peptides (total 10 sequences) were found to

Table 1
Inhibition of trypsin by various peptides that differ at the P_2' position

P_2' residue[a]	K_i value (μM)	Hydrolysis rate constant ($\times 10^{-4}$)(mol peptide/mol enzyme/s)
Ala	23 (±3.4)	260 (±30)
Arg	1.0 (±0.2)	6.7 (±0.8)
Asn	2.5 (±0.4)	42 (±5)
Asp	26 (±3)	200 (±30)
Cha	5.5 (±0.9)	36 (±7)
Gln	17 (±1.3)	141 (±22)
Glu	–[b]	2000[c]
Gly	–[b]	400[c]
His	3.5 (±0.4)	34 (±2)
Ile	0.009 (±0.001)	0.9 (±0.09)
Leu	1.0 (±0.2)	4.9 (±1)
Lys	4.2 (±1.5)	14 (±1)
Met	3.1 (±0.34)	66 (±8)
Nle	0.5 (±0.04)	3.0 (±0.6)
Phe	1.2 (±0.1)	15 (±2)
Pro	–[b]	–[d]
Ser	6.2 (±1.0)	105 (±8)
Thr	3.4 (±0.8)	63 (±9)
Val	2.6 (±0.2)	17 (±0.4)
Trp	20 (±3)	2500 (±280)
Tyr	2 (±0.4)	26 (±2)

The values in parentheses are the standard errors obtained from the data fitting.
[a]All peptides had the sequence NH_2-S-C-T-K-S-**Xaa**-P-P-Q-C-Y-OH, where the P_2' residue is denoted Xaa. An intramolecular disulphide joins the two cysteine residues.
[b]No inhibition is observed ($K_i > 1$ mM).
[c]Estimated from HPLC traces showing hydrolysis of peptide at various time intervals.
[d]No hydrolysis was detected.

have at P_2' Ile (seven sequences), Nle (two sequences) and Leu (one sequence). Although the present study records the effect of P_2' variation on K_i values for trypsin, there is a striking parallel with the K_i values recorded in Table 1, which suggests that chymotrypsin and trypsin have similar requirements at P_2'.

3.2. *Effect of P_2' variation on hydrolysis rate constants*

The hydrolysis rate constants of these peptides by trypsin are given in Table 1. Hydrolysis was determined by adding inhibitor to a solution of trypsin, then monitoring the recovery of trypsin activity with time. For this reason, it was only possible to record hydrolysis rate constants for those peptides that were functional as inhibitors. Regain of trypsin activity is assumed to be due to inhibitor hydrolysis, and provides a simple way of monitoring turnover of the inhibitor peptides. Control experiments using HPLC to monitor peptide integrity (not shown) demonstrated that hydrolysis correlated with regain of activity, allowing simpler kinetic assays to be used to monitor inhibitor cleavage.

The peptides were found to show differences of over three orders of magnitude in their rate of hydrolysis. Peptide P_2'-Ile was hydrolysed most slowly, while peptide P_2'-Trp was hydrolysed most rapidly. For the peptides that did not inhibit trypsin, hydrolysis was monitored by HPLC. The peptides with P_2' Gly and Glu were found to hydrolyse very rapidly (Table 1). For the peptide with Pro at P_2', no apparent hydrolysis was found. This suggests that this peptide does not interact with the enzyme, which could result from it adopting a different structure to the other peptides studied.

3.3. *Correlation of hydrolysis rate constants and K_i*

Fig. 1 shoes a logarithmic plot of hydrolysis rate constant versus K_i. It is found that there is overall correlation between these two factors. Peptides that have the lowest K_i values also tend to be hydrolysed more slowly, whereas a high K_i is associated with rapid hydrolysis. It is, therefore, the case that tight binding via the P_2' location is associated with slow hydrolysis. It may be possible to interpret these results by considering how binding energy would be

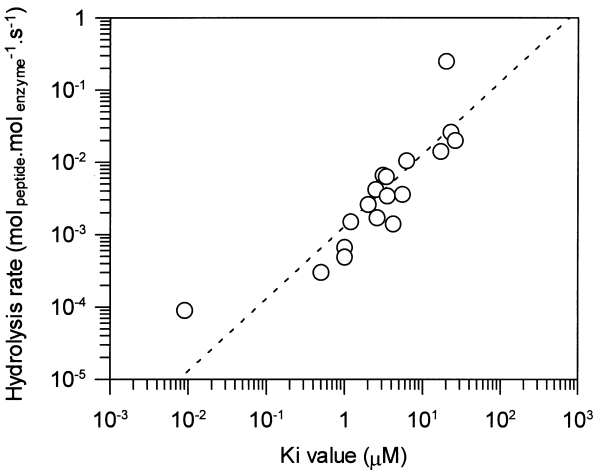

Fig. 1. Correlation of hydrolysis rate constant and inhibition constant (K_i) for the peptides used in the current study. Peptides that did not display measurable inhibition (P_2'-Glu, P_2'-Gly and P_2'-Pro) are not included. The dotted line is arbitrary, and is included to facilitate comparison.

utilised in an 'ideal' enzyme-substrate interaction. As pointed out by Fersht [33,34], it is catalytically advantageous for an enzyme to bind substrates *weakly*, and maximisation of rate requires *high* values of K_M. In this respect, these proteinase inhibitors function by being non-ideal substrates in that they have K_M values (assumed to be equivalent to K_i) that are very low. Indeed, the plot in Fig. 1 is an excellent illustration of the deleterious influence on rate that results from strong substrate binding (low K_M or K_i).

It should be noted that the correlation between K_i and hydrolysis rate constant found in this study does not always apply for substitutions at other locations with BBI reactive site loops. We have previously reported that sequence alterations at P_1 [18] and at P_2 [9] show little or no correlation between K_i values and hydrolysis rate constants. We can only speculate on the reason for this apparent discrepancy. One possibility is that these results reflect differences between P and P′ site interactions, with the P′ site binding, which will only be involved in interactions prior to acyl enzyme formation, showing the simpler correlation between binding energy and rate.

3.4. P_2' variation in BBI proteins

The P_2' position is one of the most variable residues within the nine-residue reactive site loop of BBI proteins. Of 47 sequences listed in McBride et al. [9], the following residues are found at the P_2' position: Ile (26), Met (7), Asn (7), Gln (2), Tyr (2), Glu (1), Phe (1), Arg (1). The residue that occurs most frequently, Ile, is that found in the present study to result in the lowest K_i and smallest hydrolysis rate constant. The indicates either that a P_2' Ile satisfies the specificity requirements for many of the proteinase targets inhibited by BBIs, and/or that Ile at P_2' is optimal for the structural organisation of the BBI reactive site loop.

3.5. Structural details of the P_2'-S_2' interaction

Structures are available for the complexes with trypsin of a BBI sub-domain [35], a BBI-derived tricyclic peptide [14], and intact BBI protein [7]. These reveal that the P_2' residue fits into a well-defined apolar S_2 pocket. By inspection of these structures it might be anticipated that large aliphatic side chains would provide optimal packing within the pocket, in line with the experimentally observed K_i values (Table 1).

3.6. Overall conclusions

The introduction of various amino acids in the anti-trypsin BBI synthetic peptide sequence resulted in inhibitors that changed K_i values and hydrolysis rate constants by more than three orders of magnitude. Thus, although the P_1 residue is usually considered the prime determinant of specificity, the nature of the P_2' residue is also important in directing the specificity of the inhibitor towards its cognate enzyme. The P_2' substitution that produced the most potent trypsin inhibitor (P_2'-Ile) is also the residue most frequently found in natural BBI sequences. The conservation implies that this residue is likely to function well against a range of different proteinases.

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