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Peptide Mimics of the Bowman–Birk Inhibitor Reactive Site Loop

Abstract: *Bowman–Birk Inhibitors (BBIs) are small highly cross-linked proteins that typically display an almost symmetrical "double-headed" structure. Each "head" contains an independent proteinase binding domain. The realization that one BBI molecule could form a 1:1:1 complex with two enzymes led early workers to dissect this activity. Now, after three decades of research, it has been possible to isolate the antiproteinase activity as small (~11 residues), cyclic, synthetic peptides, which display most of the functional aspects of the protein. More recently, it has been found that these peptide fragments are not just a synthetic curiosity—a natural 14-residue cyclic peptide (SFTI-1), which too encapsulates the BBI inhibitory motif, is found to occur in sunflowers. This article reviews the properties of BBI-based peptides (including SFTI-1) and discusses the features that are important for inhibitory activity.* Biopolymers (Pept Sci) 66: 79–92, 2002

Keywords: *Bowman–Birk inhibitors; cross-linked proteins; proteinase binding domain; antiproteinase; synthetic peptides; SFTI-1*

BACKGROUND

The Canonical Loop Motif

Naturally occurring inhibitors of proteinase enzymes are generally proteins that act to regulate enzyme activity by forming a tight stoichiometric complex with their target.[1,2] For one of the main families, the serine proteinases, many inhibitor proteins have evolved an inhibitory region that consists of an exposed loop that has a conserved so-called canonical conformation.[2,3] This loop, which is frequently constrained by the presence of disulfide bridges and/or extensive hydrogen-bonding networks, is thought to

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Table I The Three-Dimensional Structures of Bowman–Birk Inhibitors Currently Available[a]

Source	Complexed?	Resolution (Å)	PDB Code	Reference
Adzuki bean (Paseolus angularis)	Trypsin	2.3	1tab	44
Barley (Hordeum vulgare)		1.9	1c2a	26
Mung bean (*Vigna radiata*)	Dengue Virus Ns3-Protease	2.1	1df9	53
Pea (*Pisum sativum*)		2.7	1pbi	54
Soybean PI–II (*Glycine max*)		2.5	1pi2	55
Soybean (*Glycine max*)		2.8	1k9b	56
Soybean (*Glycine max*)		NMR structure	1bbi and 2bbi	33
Soybean (*Glycine max*)	Trypsin	2.3	1d6r	57

[a] Where the coordinates have been deposited in the Brookhaven protein database, the access code is indicated.

adopt the same conformation as that of a productively bound peptide substrate.[1,2] The primary contact region contains the scissile peptide bond P_1—P_1' (nomenclature of Schechter and Berger[4]). The specificity of the inhibition is dictated by the sequence of the reactive site with the P_1 residue dominating the specificity, which typically reflects the substrate preference of the target proteinase.[5,6] One aim of inhibitor research has been to define the minimal requirements for inhibitory activity, in terms both of structural and recognition elements.[6,7] In this regard, the Bowman–Birk family of inhibitors has proved particularly useful; this article reviews those peptides based on the Bowman–Birk inhibitory loop that have thus far been described in the literature.

The Bowman–Birk Inhibitor Family

Background and Structure. The Bowman–Birk inhibitor (BBI) family is a typical canonical serine proteinase inhibitor, which is found in various plant sources. [8,9] The family is named after the workers who first isolated (Bowman, 1946[10]) and subsequently characterized (Birk, 1963[11]) a member of this family from soybean. Plant proteinase inhibitors, though ubiquitous, often do not have a well-defined physiological/functional role. Suggested functions include that of a storage protein, involvement in protection of the seed or whole plant from pathogens, and also regulatory roles in plant development.[12–14]

BBI proteins are small cysteine-rich proteins (typically 60–90 amino acids) with a high degree of sequence homology and a network of highly conserved disulfide bridges[15] (novel members may also exist). About 40 different members of this family have been identified[8,16,17] (SWISSPROT/TrEMBL databank release dates April 3, and March 27,2002),[12] and extensive structural data are available for BBI, both alone and complexed to trypsin (Table I). Most BBI proteins have a symmetrical "double-headed" structure consisting of two tricyclic domains. Each separate domain contains an independent canonical proteinase binding site, and one inhibitor molecule can form a 1:1:1 stoichiometric complex with two different proteinases.[9]

In BBI protein isolated from dicots, the molecular weight is ~8000; monocots have two further classes of BBI, one with a single reactive site and of size ~8000 and the other with two reactive sites and a size of ~16,000.[16,18] A repetitive sequence, of 4000 and 8000, respectively, suggests gene duplication.[16,18] A novel cyclic peptide from sunflowers, SFTI-1, is also structurally a member of this family and represents the smallest known inhibitor, with a single binding site and a molecular weight of 1500.[19]

In addition to the conserved disulfide bridge network in BBI proteins, the majority of BBI inhibitors (including SFTI-1) have a core disulfide-constrained reactive site loop of 9 residues forming a short type VIb β-turn (an 11-residue loop is found at one reactive site of the peanut inhibitors A-II, B-II, and B-III[20]). The structure of this loop is shown in Figure 1. In addition to the cysteine residues, the loop regions have a conserved proline (which incorporates a *cis* peptide bond) at P_3' and serine at P_1'.[20] SFTI-1 retains the core 9 residue loop, but in addition, this peptide is enclosed by backbone cyclization with a distal loop (Figure 2).

Activity and Specificity of BBI Proteins. For BBIs that have two reactive sites, activity is typically either antichymotrypsin/trypsin or trypsin/trypsin. With the trypsin binding domains, the P_1 residue is lysine or arginine; for pancreatic elastase, alanine; and for chymotrypsin, the P_1 residue is leucine or tyrosine. In addition to these well-studied serine proteinases, activity against an increasing number of trypsin-like and chymotrypsin-like proteinases has been described. [9]

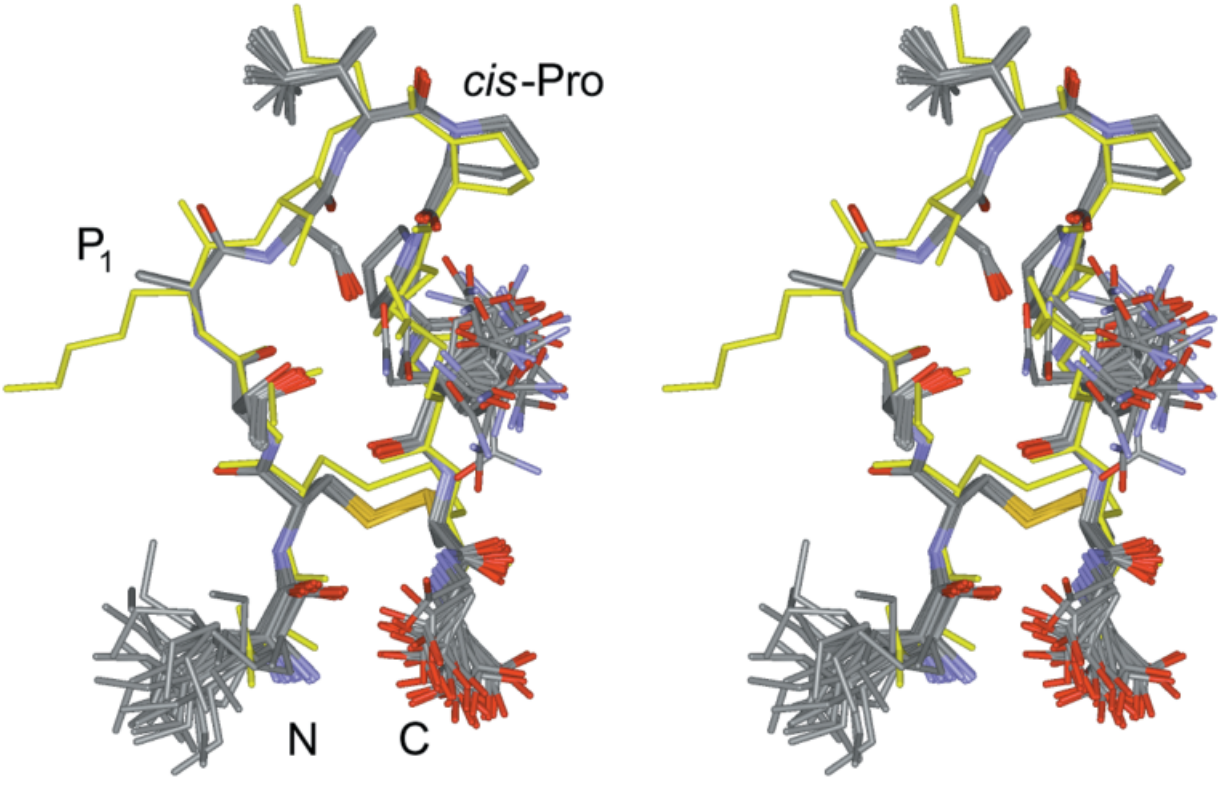

FIGURE 1 Stereo view of the family of 30 best simulated annealing structures calculated from the NMR data (gray) of peptide 38[29] superimposed onto the x-ray structure of the reactive site loop (yellow) from mung bean BBI protein.[51] The positions of the P_1 residue and the *cis*-Pro at P_3' are indicated, as are the locations of the N- and C-termini. The disordered side chain of the C-terminal residue Tyr_{11} is not shown for clarity.

Variants of BBI proteins generated by semisynthesis suggest that Phe is the optimal P_1 residue for chymotrypsin and that inhibition follows the series F > W > L > M > V > A > DW > G > desL.[21] Deimination of Arg to citrulline at both reactive sites destroyed trypsin activity in peanut B-III, but altered chymotrypsin activity only marginally.[22] At P_1' inhibition of chymotrypsin by soybean BBI was found to follow the series S > A > T > V > L >G.[23]

Although dual-headed BBI proteins are able to inhibit two proteinases at the separate reactive sites, there are exceptions and there have been documented alterations in the relative affinity when one site is already occupied. Soybean BBI inhibits duodenase with a K_i of 4 n*M*, but when precomplexed with trypsin the K_i is 400 n*M*.[24] Peanut inhibitor was found to be devoid of antichymotryptic activity when precomplexed with trypsin, and vice versa.[25] These results suggest that though the two reactive sites are approximately 40 Å apart,[26] it is possible that in some instances there is steric hindrance when two independent proteinases attempt to bind.

INHIBITION BY SYNTHETIC BBI LOOPS

Historical Perspective and the Progressive Reduction in Size

Peptidic derivatives of the inhibitory loop regions from the BBI family have been shown to retain both significant inhibitory activity and structural features of the parent proteins.[27–29] This observation was recently confirmed by the discovery and isolation of a related inhibitor from sunflower seeds (SFTI-1).[19,30] This low M_r inhibitor comprising only 14 residues appears to be derived from the antitryptic loop of BBI proteins, but is unique in having a cyclic peptide backbone and therefore no free termini. It is possible that its cyclic structure results from the action of a transpeptidase.[19] Small peptidic inhibitors may be more widespread than previously thought,[12] and other inhibitors having cyclic backbones related to existing inhibitor families are known.[31,32]

The realization that soybean BBI could form a 1:1:1 complex with two separate proteinases led early

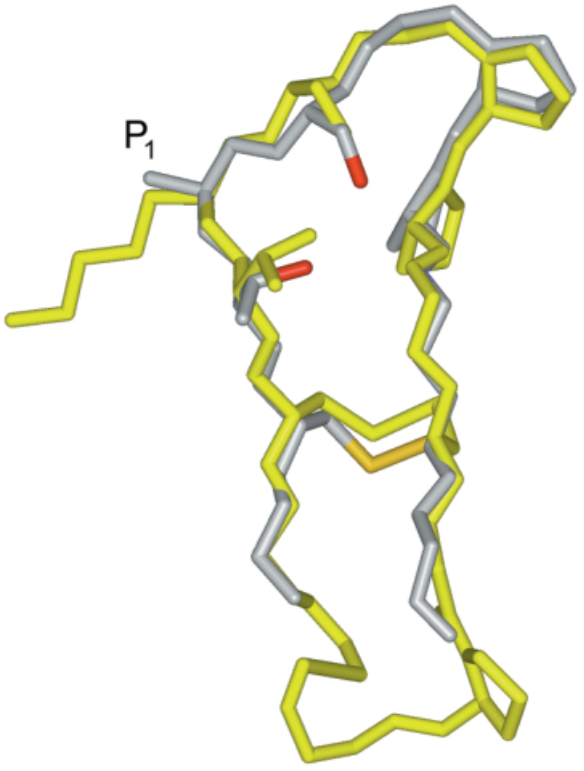

FIGURE 2 Superimposition of the averaged minimized simulated annealing structure of the entire peptide backbone of the anti-HNE peptide 38 (gray)[29] onto the corresponding atoms of the bicyclic Sunflower trypsin inhibitor 1, peptide 47 (yellow).[29] The RMS deviation between the two structures is 0.72 Å. Most side chains are omitted for clarity.

workers to determine if they could dissect these two independent activities. By a combination of cyanogen bromide and pepsin treatment, it was found possible to separate activity of the antitryptic loop.[15] While antitryptic activity was retained, the antichymotryptic activity was greatly reduced. This implies that there are fewer stabilizing tertiary interactions in that domain, a feature confirmed by the NMR structure of the BBI protein.[33] The retention of inhibitory activity that followed fragmentation of BBI protein stimulated several workers to investigate the minimal size requirements. Nishino et al.[34] first synthesised a cyclic peptide with activity based on the core nine residues of the antitryptic loop (residues 14–22). Since then many publications have described mono-, bi-, and tricyclic peptides designed to mimic the activity of the BBI. Those BBI-based peptides reported in the literature prior to 1995 are listed in Table II. The peptides described by Nishino with Lys at P_1 were found to inhibit trypsin with micromolar K_i values.[35] Similar results were also reported by Terada et al.[36]; in addition, they found that changing the P_1 residue allowed alteration of specificity with Lys or Arg inhibiting trypsin, while Leu or Tyr allowed inhibition of chymotrypsin and subtilisin (Table III lists variants that differ mainly at position P_1). More extended sequences that include a second disulfide show some improvements in terms of lowered K_i and/or improved stability toward hydrolysis. [37–39]Activity is dependent on disulfide cyclization and noncyclic peptides have little or no inhibitory activity[40] (for example, peptide 12).

For those peptides listed in Table III, the 11-mer peptide described by Maeder et al. [41] (peptide no. 22) displayed a lower K_i value against trypsin than was found in the studies using 9-mer sequences. It appears that having an additional residue each side of the 9-mer core improves the inhibition properties and the stability of the peptide.[39] The residue to the N-terminal side of the 9-mer core provides the P_4 subsite, which has been found to contribute significantly to interactions with chymotrypsin.[42] The disulfide itself appears only necessary to organize the structure; in the whole protein it is possible to remove this disulfide without impairing activity,[43] although here the remaining structure is presumably sufficient to organise the reactive site loop. For SFTI-1, this is also confirmed by the cyclic variants, (Table IV lists SFTI-1 based inhibitors; Table V compares both BBI and SFTI-1 inhibitors of decreasing size) 49 and 50 lacking the inner disulfide. Modest alteration in the K_a is described for peptide 49, and although an increase in K_i is seen for peptide 50, it remains active in contrast to the inactive linear variant (peptide 51).

Like BBI peptides, reduction of SFTI-1 to the core 9 residues also results in activity loss against trypsin (peptides 58 and 54). It is possible that this loss will be more significant for other proteinases, as has been found with other BBI peptides. In general, BBI proteins (including SFTI-1) have the nine amino acid core shown in Figure 3.

Reduction to the core 9–11 residues also typically involves the introduction of new termini. Acetylation of the N-terminal and generation of a C-terminal amide provides one approach to capping these new charged termini (Table I, peptides 1–20 and 24); however, some loss in activity results (peptide 11, cf. peptide 10). Another approach is the replacement of the disulfide and enclosure of the loop with D-Pro and L-Pro (peptides 43–46 and 52–59). However, although this provides a native-like structure, loss of potential contacts with the target enzyme gives reduced activity. For example, peptide 22 displays a K_i of ca. 10 n*M* against trypsin compared with 103 n*M* for peptide 43. Comparing peptides 44–46 with 32–34, there appears much greater antichymotrypsin ac-

Table II Peptides Based on the Reactive Site Loop of BBI and First Reported in the Literature Prior to 1995[a]

No.	Amino Acid Sequence																						K_i Values (μM)				
							P_4	P_3	P_2	P_1	P'_1	P'_2	P'_3	P'_4	P'_5	P'_6								TR	CH	SUB	PPE	Ref.
1							*Ac*—	C	T	K	S	N	P	P	Q	C	—NH_2							0.75, 0.17				35,39
2								C	T	K	S	N	P	P	Q	C								3.8,				35
3							*Ac*—	C	T	D-K	S	N	P	P	Q	C	—NH_2							850				36
4							*Ac*—	C	T	R	S	N	P	P	Q	C	—NH_2							1.3				36
5							*Ac*—	C	A	K	S	N	P	P	Q	C	—NH_2							16				36
6							*Ac*—	C	T	K	A	N	P	P	Q	C	—NH_2							3				36
7							*Ac*—	C	T	L	S	N	P	P	Q	C	—NH_2							NI	5.7	2900		36
8							*Ac*—	(C)	T	K	S	N	P	P	Q	(C)	—NH_2							100				36
9							*Ac*—	C	A	L	S	Y	P	A	Q	C	—NH_2								52	11		36
10							*Ac*—	C	A	L	S	Y	P	A	Q	C	—NH_2								180	170		36
11							*Ac*—	C	A	Y	S	Y	P	A	Q	C	—NH_2								13	55		36
12							*Ac*—	(C)	A	L	S	Y	P	A	Q	(C)	—NH_2								290, 3450	NI		36, 58
13			*Ac*—	C	D	Q	A	A	C	T	K	S	N	P	P	Q	C	R	C	—NH_2				0.15, 0.039				37, 39
14			*Ac*—	C	D	Q	A	A	(C)	T	K	S	N	P	P	Q	(C)	R	C	—NH_2				24				37
15			*Ac*—	(C)	D	Q	A	A	C	T	K	S	N	P	P	Q	C	R	(C)	—NH_2				0.57				37
16			*Ac*—	(C)	D	Q	A	A	(C)	T	K	S	N	P	P	Q	(C)	R	(C)	—NH_2				NI				37
17			*Ac*—	C	K	S	A	I	C	A	L	S	Y	P	A	Q	C	F	C	—NH_2					0.71		43	38
18			*Ac*—	C	K	S	A	I	(C)	A	L	S	Y	P	A	Q	(C)	F	C	—NH_2					36			38
19			*Ac*—	(C)	K	S	A	I	C	A	L	S	Y	P	A	Q	C	F	(C)	—NH_2					1.4			38
20			*Ac*—	(C)	K	S	A	I	(C)	A	L	S	Y	P	A	Q	(C)	F	(C)	—NH_2					75			38
21							S	T	C	T	K	S	I	P	P	Q	C	H	S					1				41
22								S	C	T	K	S	I	P	P	Q	C	Y						0.1, 0.01, 0.009, 0.045				39–41, 59
23									C	A	L	S	Y	P	A	Q	C							NI	NI			52
24								*Ac*—	C	T	L	S	N	P	P	Q	C	—NH_2						NI	25			52
25	Q	P	C	C	D	S	C	R	C	T	K	S	I	P	P	Q	C	H	C	A	N			0.12				27
26	Q	P	C	C	D	S	C	R	C	T	K	S	I	P	P	Q	C	H	C	A	N			0.04				28

[a] Unless indicated otherwise, peptides have free amino and carboxylic acid termini (*Ac*, N-acetylated; NH_2, carboxyl amide). The peptides are cyclized via a disulfide bridge between P_3 and P'_6 unless otherwise indicated. (C) indicates a protected cysteine residue that is not involved in a disulfide bridge. Head-to-tail cyclized peptides are represented by enclosing the sequence in square brackets. For peptides 25 and 26, the disulfide bridge configuration was not elucidated. TR, CI, and SUB represent the enzymes trypsin, α-chymotrypsin, and subtilisin BPN′, respectively. Where differing K_i values have been reported, the table records the respective values for the references cited. NI, no inhibition.

Table III Families of Variants Differing Mainly at the P_1 Position[a]

	Amino Acid Sequence											K_i Values (μM)					
No.	P_4	P_3	P_2	P_1	P_1'	P_2'	P_3'	P_4'	P_5'	P_6'		Tase	HNE	PPE	TR	CH	Ref.
22	S	C	T	K	S	I	P	P	Q	C	Y	30			0.1, 0.01, 0.009, 0.045	NI	39–41,59
27	S	C	T	R	S	I	P	P	Q	C	Y	78			.02, .09	NI	40,59
28	S	C	T	Orn	S	I	P	P	Q	C	Y	>10,000			NI, 62	NI	40,59
29	S	C	T	M	S	I	P	P	Q	C	Y		>1,000			2	40,49
30	S	C	T	L	S	I	P	P	Q	C	Y					9	40
31	S	C	T	Nle	S	I	P	P	Q	C	Y					4	40
32	S	C	T	F	S	I	P	P	Q	C	Y	>2,000			NI, 26	0.07, 0.019	17,40,46,59
33	S	C	T	Y	S	I	P	P	Q	C	Y					0.05, 0.017	40,46
34	S	C	T	W	S	I	P	P	Q	C	Y					0.043	42
35	S	C	T	V	S	I	P	P	Q	C	Y		4.6				49
36	S	C	T	A	S	I	P	P	Q	C	Y		3.4				49
37	S	C	T	T	S	I	P	P	Q	C	Y		15.9				49
38	Nle	C	T	A	S	I	P	P	Q	C	Y		0.065	0.7			49
39	Nle	C	T	L	S	I	P	P	Q	C	Y					0.224	42
40	Nle	C	T	V	S	I	P	P	Q	C	Y		0.13	6.7			49
41	Nle	C	T	T	S	I	P	P	Q	C	Y		0.41	1.5			49
42	Nle	C	T	M	S	I	P	P	Q	C	Y		11.4	5.8			49
43		[DP	T	K	S	I	P	P	I	P]					0.103	>100	45
44		[DP	T	Y	S	I	P	P	I	P]		>25				4.5	45
45		[DP	T	F	S	I	P	P	I	P]		>20				4.8	45
46		[DP	T	W	S	I	P	P	I	P]		>20				1.4	45

[a] Details as in Table II. Tase, human β-tryptase; HNE, human neutrophil elastase.

tivity for a disulfide-constrained loop with additional exocyclic residues than without (Table III).

Systematic Variation at Single Position's Within the Inhibitor

Residues important to the structure–function aspects of potential BBI-based peptides can be inferred from the reactive loop sequences of known proteins (Figure 2 previously). Most of those conserved residues are in the "front side"[44] or contact region of the loop/peptide. An alanine scan of a minimized peptide has been presented by Descours et al., peptides 53–59.[45] This reconfirms the importance of those conserved residues. Significant activity losses against trypsin are seen at positions P_2, P_1, P_2', and P_3'.

The P_1 Position. A number of studies have analyzed P_1 variation in BBI peptides[35–38,40] (Table III). Changes at P_1 frequently result in large changes in specificity, which are broadly in line with the substrate specificity of the proteinase in question. This correlation of the P_1 identity for inhibitors and substrates is commonly found in proteinase inhibitors in general.[1,5,6] For example, trypsin inhibitors tend to have at P_1 Arg or Lys, as would be found for trypsin substrates. Using a peptide mixture of 20 variants based on peptide 22, Domingo et al.[40] screened P_1 variants against trypsin, chymotrypsin, and elastase (the identified residues are also given in Table III).

The P_2 Position. The role of the P_2 residue within the core loop has been investigated within the sequence SC**X**FSIPPQCY (cyclized via the cysteines) where **X** was replaced by 26 different amino acids[17] (Table VI). It was found that Thr was the optimal residue at this position when inhibiting of chymotrypsin. The Thr side chain has a dual role: intraloop hydrogen bonding (via the —OH group) and formation of hydrophobic interactions with the enzyme (via the $—CH_3$ group).[26] This result explains why in studies selecting chymotrypsin inhibitors from a partially randomized BBI peptide combinatorial library, only sequences having a P_2 Thr residue were returned.[46]

The P_2' Position. The role of this residue in trypsin binding has been analyzed within the sequence SCTKS**X**PPQCY[47] (Table VII). A total of 21 peptides were compared; the optimal residue was found to be Ile, which resulted in a K_i value of 9.5 nM.

Table IV Peptides Based on the Sunflower Inhibitor (SFTI-1) Sequence and Derivatives[a]

	Amino Acid Sequence													K_i Values (μM)								
No.		P_4	P_3	P_2	P_1	P'_1	P'_2	P'_3	P'_4	P'_5	P'_6				TR	CHY	CatG	THR	FXa	MaT	uPA	EL	Ref.
47	[G	R	C	T	K	S	I	P	P	I	C	F	P	D]	0.0001, (K_a 1.1×10^{10}), 0.013, 0.0005, 0.00106, 0.0003	7.4, 2.3	0.0001 5	136, 5.05	NI	0.0092	500	105	19,30,45, 50,60,6
48	G	R	C	T	K	S	I	P	P	I	C	F	P	D	0.0121, (K_a 9.9×10^9), 0.0012								30,50,6
49	[G	R	Abu	T	K	S	I	P	P	I	Abu	F	P	D]	(K_a 4.6×10^9)								50
50	[G	R	G	T	K	S	I	P	P	I	G	F	P	D]	0.027								61
51	G	R	G	T	K	S	I	P	P	I	G	F	P	D	NI								61
52	[DP	R	C	T	K	S	I	P	P	I	C	F	P]		0.011	6.6							45
43			[DP	T	K	S	I	P	P	I	P]				0.103	>100							45
53			[DP	A	K	S	I	P	P	I	P]				1								45
54			[DP	T	A	S	I	P	P	I	P]				>10								45
55			[DP	T	K	A	I	P	P	I	P]				0.73								45
56			[DP	T	K	S	A	P	P	I	P]				1.92								45
57			[DP	T	K	S	I	A	P	I	P]				>25								45
58			[DP	T	K	S	I	P	A	I	P]				0.114								45
59			[DP	T	K	S	I	P	P	A	P]				0.74								45
44			[DP	T	Y	S	I	P	P	I	P]				>25	4.5							45
45			[DP	T	F	S	I	P	P	I	P]				>20	4.8							45
46			[DP	T	W	S	I	P	P	I	P]				>20	1.4							45

[a] Details as in Table II. CatG, cathepsin G; THR, thrombin; FXa, factor Xa; MaT, Matryptase; uPA, Elastase (EL).

Table V What Is the Minimum Size to Maintain Inhibitory Activity?[a]

No.	Amino Acid Sequence																							K_i Values (μM)		
								P_4	P_3	P_2	P_1	P'_1	P'_2	P'_3	P'_4	P'_5	P'_6								TR	CHY	Ref.
47							[G	R	C	T	K	S	I	P	P	I	C	F	P	D]				0.0001, (K_a 1.1×10^{10}), 0.013, 0.0005, 0.00106, 0.0003	7.4, 2.3	19,30,45, 50,60,61
48							G	R	C	T	K	S	I	P	P	I	C	F	P	D				0.0121, (k_a 9.9×10^9), 0.0012		30,50,61
52							[DP	R	C	T	K	S	I	P	P	I	C	F	P]					0.11	6.6	45
43									[DP	T	K	S	I	P	P	I	P]							0.103	>100	45
26	Q	P	C	C	D	*Q*	A	A	C	T	K	S	I	P	P	Q	C	R	C	H	C	A	N	0.04		28
13			*Ac*—	C	D	*Q*	A	A	C	T	K	S	N	P	P	Q	C	R	C	—NH_2				0.039		39
22								S	C	T	K	S	I	P	P	Q	C	Y						0.001		39,40
60								*Ac*—	C	T	K	S	I	P	P	Q	C	Y						0.108		39
61									C	T	K	S	I	P	P	Q	C	Y						0.024		39
62								S	C	T	K	S	I	P	P	Q	C							0.045		39
63								*Ac*—	C	T	K	S	I	P	P	Q	C							0.105		39
64									C	T	K	S	I	P	P	Q	C							0.068		39
65								S	(C)	T	K	S	I	P	P	Q	(C)	Y						NI		39

[a] Details as in Table II.

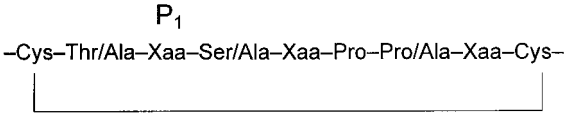

FIGURE 3 The amino acid sequence of the core disulfide-linked reactive site sequence from BBI. Xaa represents any amino acid. The location of the P_1 residue is indicated.

Combinatorial Libraries of BBI Peptides

Advances in combinatorial and multiple peptide synthesis have allowed the creation and analysis of synthetic libraries of BBI-based peptides where, using a tethered (or solution phase) sequence, strategic positions likely to be important for interaction with proteinases are be varied.

$P_2/P_1/P_2'$ Library. In the first study of this kind, McBride et al.[46] constructed a library having 8000 variations by targeting P_2, P_1, and P_2' for randomization with all DNA-encoded amino acids except cysteine but including norleucine (Table VIII). Chymotrypsin-binding sequences were visualized by an enzymatic color reaction. Binding to roughly 1 in 1000 beads was observed and the sequence of 13 beads was determined by the Edman method. Following resynthesis, ten sequences showed activity and had K_i values of ~20 n*M* for chymotrypsin. These sequences showed a strong consensus: at P_2 all sequences had Thr; P_1 was Tyr or Phe; P2′ was Ile, Leu, or Nle. The preference for aromatic residues at P_1 is consistent with the substrate preference of chymotrypsin.[48] These results are also in accord with previous work where the P_1 residue alone was varied.[40]

$P_4/P_1/P_2'$ Library. In an extension of this above work, a similar 8000- component library was analyzed having randomization at P_4, P_1, and P_2'[42] (Table IX). In this library the optimal Thr residue was fixed at P_2 and analysis of chymotrypsin binding identified twelve active sequences. The consensus inhibitor se-

Table VI Influence on the P_2 Position (Shaded) on Inhibitory Activity[a]

	Amino Acid Sequence										
No.	P_4	P_3	P_2	P_1	P_1'	P_2'	P_3'	P_4'	P_5'	P_6'	K_i Values (μM) for CHY
32	S	C	T	F	S	I	P	P	Q	C	Y	0.019
66	S	C	S	F	S	I	P	P	Q	C	Y	0.4
67	S	C	Hse	F	S	I	P	P	Q	C	Y	2.6
68	S	C	allo-Thr	F	S	I	P	P	Q	C	Y	130
69	S	C	D-Thr	F	S	I	P	P	Q	C	Y	NI
70	S	C	Q	F	S	I	P	P	Q	C	Y	7.1
71	S	C	N	F	S	I	P	P	Q	C	Y	17
72	S	C	A	F	S	I	P	P	Q	C	Y	0.57
73	S	C	G	F	S	I	P	P	Q	C	Y	4.4
74	S	C	M	F	S	I	P	P	Q	C	Y	2.2
75	S	C	Abu	F	S	I	P	P	Q	C	Y	0.13
76	S	C	V	F	S	I	P	P	Q	C	Y	1.3
77	S	C	Nva	F	S	I	P	P	Q	C	Y	0.28
78	S	C	Nle	F	S	I	P	P	Q	C	Y	1.3
79	S	C	L	F	S	I	P	P	Q	C	Y	2.4
80	S	C	I	F	S	I	P	P	Q	C	Y	13.4
81	S	C	tBu-Gly	F	S	I	P	P	Q	C	Y	560
82	S	C	F	F	S	I	P	P	Q	C	Y	8.7
83	S	C	W	F	S	I	P	P	Q	C	Y	9
84	S	C	Y	F	S	I	P	P	Q	C	Y	17.1
85	S	C	E	F	S	I	P	P	Q	C	Y	84
86	S	C	D	F	S	I	P	P	Q	C	Y	98
87	S	C	H	F	S	I	P	P	Q	C	Y	1.4
88	S	C	R	F	S	I	P	P	Q	C	Y	1.8
89	S	C	K	F	S	I	P	P	Q	C	Y	2.8
90	S	C	P	F	S	I	P	P	Q	C	Y	5.2

[a] Data from McBride et al.[17] Details as in Table II.

Table VII Influence on the P_2' Position (Shaded) on Inhibitory Activity[a]

	Amino Acid Sequence											
No.	P_4	P_3	P_2	P_1	P_1'	P_2'	P_3'	P_4'	P_5'	P_6'		K_i Values (μM) for TR
91	S	C	T	K	S	T	P	P	Q	C	Y	3.4
92	S	C	T	K	S	S	P	P	Q	C	Y	6.2
93	S	C	T	K	S	Q	P	P	Q	C	Y	17
94	S	C	T	K	S	N	P	P	Q	C	Y	2.5
95	S	C	T	K	S	A	P	P	Q	C	Y	23
96	S	C	T	K	S	G	P	P	Q	C	Y	>1000
97	S	C	T	K	S	V	P	P	Q	C	Y	2.6
98	S	C	T	K	S	M	P	P	Q	C	Y	3.1
99	S	C	T	K	S	Nle	P	P	Q	C	Y	0.5
100	S	C	T	K	S	L	P	P	Q	C	Y	1
22	S	C	T	K	S	I	P	P	Q	C	Y	0.009
101	S	C	T	K	S	Cha	P	P	Q	C	Y	5.5
102	S	C	T	K	S	F	P	P	Q	C	Y	1.2
103	S	C	T	K	S	W	P	P	Q	C	Y	20
104	S	C	T	K	S	Y	P	P	Q	C	Y	2
105	S	C	T	K	S	E	P	P	Q	C	Y	>1000
106	S	C	T	K	S	D	P	P	Q	C	Y	26
107	S	C	T	K	S	H	P	P	Q	C	Y	3.5
108	S	C	T	K	S	R	P	P	Q	C	Y	1
109	S	C	T	K	S	K	P	P	Q	C	Y	4.2
110	S	C	T	K	S	P	P	P	Q	C	Y	>1000

[a] Data from Gariani et al.[47] Details as in Table II.

quence had at P_4 Nle, P_1 Phe, and P_2' Ile, and displayed a K_i value of 3.5 n*M*, which gives this sequence a better inhibition constant than is found for any full-length BBI protein inhibiting chymotrypsin. Screening of this P_4, P_1, and P_2' bead library against human leukocyte elastase (HLE) has also been described[49] (Table IX). A total of 23 active binding beads were analyzed in this study, with 21 of these being found to have Ala at P_1 and the remaining two Thr. The consensus sequence had at P_4 Nle, P_1 Ala, and P_2' Ile; the K_i for this sequence against HLE was 65 n*M*.

Hydrolytic Stability. BBI peptides bind in the same manner as substrates and are competitive enzyme inhibitors; though hydrolysis rates are generally low, it is found that some sequences hydrolyze more readily than others. Once the peptides have hydrolyzed, they are no longer able to act as inhibitors.[35] The susceptibility of peptides to hydrolysis is sequence dependent and does not necessarily correlate with inhibitory potency.[17,39,40,47,49] Inhibitory sequences identified using the above combinatorial libraries have been shown to demonstrate favorable hydrolysis rates when compared to single variation

Table VIII Peptides Identified from a $P_2/P_1/P_2'$ Randomized Library (Positions Shaded) Screened Against Chymotrypsin[46]

	Amino Acid Sequence											
No.	P_4	P_3	P_2	P_1	P_1'	P_2'	P_3'	P_4'	P_5'	P_6'		K_i Values (μM) for CHY
32	S	C	T	F	S	I	P	P	Q	C	Y	0.019
111	S	C	T	F	S	L	P	P	Q	C	Y	0.02
112	S	C	T	F	S	Nle	P	P	Q	C	Y	0.019
33	S	C	T	Y	S	I	P	P	Q	C	Y	0.017

[a] Details as in Table II.

Table IX Peptides Identified from a $P_4/P_1/P'_2$ Randomized Library (Positions Shaded) Screened Against Chymotrypsin or Elastase[42,49]

	Amino Acid Sequence											K_i Values (μM)		
No.	P_4	P_3	P_2	P_1	P'_1	P'_2	P'_3	P'_4	P'_5	P'_6		CHY	HNE	PPE
32	Nle	C	T	F	S	I	P	P	Q	C	Y	0.0035		
113	V	C	T	F	S	F	P	P	Q	C	Y	0.0315		
114	F	C	T	F	S	I	P	P	Q	C	Y	0.0035		
115	Y	C	T	F	S	F	P	P	Q	C	Y	0.0058		
116	Q	C	T	F	S	L	P	P	Q	C	Y	0.0034		
117	Q	C	T	F	S	Nle	P	P	Q	C	Y	0.0053		
118	Q	C	T	L	S	Nle	P	P	Q	C	Y	0.114		
119	Nle	C	T	L	S	I	P	P	Q	C	Y	0.224		
120	Nle	C	T	L	S	F	P	P	Q	C	Y	0.310		
121	Nle	C	T	A	S	I	P	P	Q	C	Y		0.065	0.7
122	Nle	C	T	A	S	L	P	P	Q	C	Y		0.092	0.85
123	M	C	T	A	S	I	P	P	Q	C	Y		0.09	1.2
124	I	C	T	A	S	L	P	P	Q	C	Y		0.55	0.75
125	F	C	T	A	S	I	P	P	Q	C	Y		0.26	1.5
126	F	C	T	A	S	L	P	P	Q	C	Y		0.31	1.4
127	Y	C	T	A	S	I	P	P	Q	C	Y		0.13	0.07
128	Y	C	T	A	S	L	P	P	Q	C	Y		0.32	0.27
129	W	C	T	A	S	F	P	P	Q	C	Y		0.97	3.3
130	F	C	T	A	S	W	P	P	Q	C	Y		0.65	1.1
131	W	C	T	A	S	V	P	P	Q	C	Y		2.77	13
132	V	C	T	A	S	W	P	P	Q	C	Y		1.01	0.23
133	R	C	T	A	S	I	P	P	Q	C	Y		0.26	14.2
37	Nle	C	T	T	S	I	P	P	Q	C	Y		0.41	1.5
134	W	C	T	T	S	I	P	P	Q	C	Y		1.7	3.8

[a] Details as in Table II.

alone.[17,42,49] SFTI-1 is also susceptible to hydrolysis,[12] and hydrolysis rates have been determined against trypsin for these peptides.[47,50]

Structural Analysis

Several x-ray and NMR structures of BBI/SFTI peptides have recently been published (Table X) and in part shed light on those previous empirical findings from synthetic variants. The x-ray structure of the complex between a 22 amino acid BBI peptide and trypsin demonstrates these peptides adopt the same overall conformation when bound to the active site as the corresponding region of the parent protein.[27,28] Likewise, the x-ray structure of SFTI-1 in complex also reveals an almost identical conformation.[19] The solution structure of synthetic BBI and SFTI-1 peptides is also remarkably close to that found in the BBI protein.[29,30] It appears that the disulfide-linked 9-residue motif, having a *cis*-Pro and internal hydrogen bonding, represents a stable folding unit (Figures 1 and 2). The retention of structure in the synthetic peptides accounts for their continued activity as inhibitors.

The families of 30 lowest energy structures calculated from the NMR data of peptide 38 (pdb 1hd9, Figure 1) show an exceptionally high degree of convergence with average pairwise RMS deviations over the backbone of the disulfide-linked nonamer binding loop of 0.11 Å, indicating high structural rigidity.[29] Deviations between this structure and those of BBI protein binding loops lie in the range 0.62–0.8 Å. This peptide structure meets the requirements of the canonical conformation from P_3 to P_2', the front side of the inhibitors, which interacts with the enzyme surface.[44,51] The peptide structures (and BBI proteins) deviate in P_3' from the canonical conformation, due to the *cis* peptide bond in that position, which is the center of a type VIb β-turn. This arrangement is characteristically found in all BBI protein structures as the essential part of the back side or "secondary contact region" from P_3' to P_8.[44,51] This region acts principally to restrain the active site region in an inhibitory conformation but does not primarily inter-

Table X Current Three-Dimensional Structures for BBI and SFTI-1 Based Peptides and NMR Studies[a]

Source	Peptide No.	Complexed?	Resolution (Å)	PDB Code	Reference/Notes
Mung bean (*Vigna radiata*) (fragment)	28	Trypsin	2.1	1smf	22 amino acid fragment, only one disulfide resolved from 6 cysteines[27]
	29	Trypsin	2.2	1g9i	In cyclohexane. Synthetic 22aa peptide, three disulfides resolved[28]
BBI	38		NMR	1hd9	Monocyclic 11mer, HNE inhibitor, smallest peptide-structure[29]
	22		NMR	1gm2	Synthetic peptide[62]
	23, 24		NMR		[52]
Sunflower (*Helianthus annuus*)	47	Trypsin	1.65	1sfi	Isolated peptide[19]
	47		NMR	1jb1	Synthetic peptide[30]
	48		NMR	1jbn	Acyclic synthetic peptide SFTI-1[30,45]
	43, 47, 52		NMR		Synthetic peptides[45]

[a] Where the coordinates have been deposited in the Brookhaven protein database, the access code is indicated.

act with the enzyme.[51] The existence of a *cis* peptide bond in peptides was first shown by Pavone et al.[52] The stability and consequently the biological activity of these loops is supported by an extensive intramolecular hydrogen-bond network. The side-chain hydroxyl group of the P_2 Thr is the center of a bifurcated hydrogen bond. The main-chain to main-chain hydrogen bond between P_2 and P_1' appears to be instrumental in projecting the P_1 side chain outward for the primary interaction with the enzyme. This helps explain the improved activity of many peptides (Thr provides the lowest K_i of P_2 variants listed in Table VI) derived from the antitryptic loops of BBI. The other branch interacts with the side-chain hydroxyl of P_1' Ser, a residue also well conserved in natural sequences (Ref. 9, Figure 3). In addition to P_2 Thr and P_1' Ser, P_2' Ile, P_3' Pro, and the cysteine residues at P_3 and P_6' are well defined in the NMR structure of peptide 38. The side chain of the exocyclic P_4 residue, Nle, is pointed toward an interaction with enzyme surface thereby contributing to binding.[29]

Opening of the SFTI-1 structure (peptide no. 48, pdb code 1jbl [30]) generates two new N- and C-termini, like most BBI peptides, including peptide 38 (pdb code 1hd9).[29] For SFTI-1, this appears to result in moderate weakening of the internal H-bonds. The NMR structure of synthetic SFTI-1 also demonstrates that an H-bond between Gly H^N and Asp14 $O^{\delta 1}$ predicted by the x-ray structure is unfavorable in solution.[30] Overlay of the crystal structure of SFTI-1 (peptide 47) and peptide 38 shows extremely similar conformations (Figure 2). It seems likely, therefore, that the exocyclic loop in SFTI-1 is not essential to maintain structure, although it may contribute to the stability of the molecule.

Summary of Structural and Sequential Requirements of Active BBI-Based Peptides

Cyclic BBI/SFTI-1 peptides have a solution structure that incorporates the canonical serine proteinase inhibitor motif. Three principal restraining features are particularly important for the structural integrity and inhibitory activity of these peptides.

1. Covalent cyclization. Reducing the disulfide bond to form linear peptides results in the loss of inhibition.[35,36,40]
2. A *cis*-Pro in P_3' as part of the type VIb β-turn, which is absolutely conserved in all BBI proteins.[17] NMR and x-ray structures of SFTI-1 and BBI-based peptides show that the *cis* conformation is retained in the isolated peptide.
3. A transannular hydrogen bond network in which the side chain hydroxyl groups of the P_2 Thr and P_1' Ser of the front side interact with each other and main chain atoms on the back side of the loop.[17]

Together, these allow small peptide sequences independently to form a biologically active canonical loop, to the extent that naturally occurring small pep-

tide inhibitor sequences can be found (SFTI-1). This suggests that this disulfide-linked β-hairpin peptide represents an independent structural motif .[29]

On the contact or front side of the core BBI loop, principal residues that appear to be available for synthetic variation allowing redirection of activity include P_4, P_1, and P_2'. Those other residues that are conserved BBI sequences appear to have roles in maintaining the conformation of the peptide loop.

CONCLUSION

Small cyclic peptides based on the reactive site of BBI protein retain the structure and activity of the canonical loop region from the parent protein. This almost unique circumstance allows the construction of highly potent proteinase inhibitors that build on the design principles found in natural proteinase inhibitors but that are easily synthesized. The potency of these short peptide loops made it surprising, perhaps, that nature bothers to produce full-length proteins to act as inhibitors. However, the more recent discovery of SFTI-1, a 14-residue fully cyclic peptide found naturally in sunflowers, has reconfirmed the biological relevance of the work on peptide fragments initiated in the 1970s by Nishino[34,35] and Terada.[36]

REFERENCES

1. Laskowski, M., Jr.; Kato, I. Ann Rev Biochem 1980, 49, 593–626.
2. Bode, W.; Huber, R. Eur J Biochem 1992, 204, 433–451.
3. Bode, W.; Fernandez-Catalan, C.; Nagase, H.; Maskos, K. APMIS 1999, 107, 3–10.
4. Schechter, I.; Berger, A. Biochem Biophys Res Commun 1967, 27, 157–162.
5. 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. J Mol Biol 1997, 266, 441–461.
6. Qasim, M. A.; Ganz, P. J.; Saunders, C. W.; Bateman, K. S.; James, M. N.; Laskowski, M., Jr. Biochemistry 1997, 36, 1598–1607.
7. Wenzel, H. R.; Tschesche, H. In Peptides Synthesis, Structures, and Applications; Gutte, B., Ed.; Academic Press: San Diego, CA, 1995; pp 321–362.
8. Birk, Y. Int J Peptide Protein Res 1985, 25, 113–131.
9. McBride, J. D.; Leatherbarrow, R. J. Curr Med Chem 2001, 8, 909–917.
10. Bowman, D. E. Proc Soc Exp Biol Med 1946, 63, 547–550.
11. Birk, Y.; Gertler, A.; Khalef, S. Biochem J 1963, 87, 281–284.
12. Konarev, A. V.; Anisimova, I. N.; Gavrilova, V. A.; Vachrusheva, T. E.; Konechnaya, G. Y.; Lewis, M.; Shewry, P. R. Phytochemistry 2002, 59, 279–291.
13. Green, T. R.; Ryan, A. C. Science 1972, 175, 776–777.
14. Richardson, M. In Methods in Plant Biochemistry; Rogers, L., Ed.; Academic Press: New York, 1991; Vol 5, pp 259–305.
15. Odani, S.; Ikenaka, T. J Biochem (Tokyo) 1973, 74, 697–715.
16. Prakash, B.; Selvaraj, S.; Murthy, M. R.; Sreerama, Y. N.; Rao, D. R.; Gowda, L. R. J Mol Evol 1996, 42, 560–569.
17. McBride, J. D.; Brauer, A. B.; Nievo, M.; Leatherbarrow, R. J. J Mol Biol 1998, 282, 447–458.
18. Odani, S.; Koide, T.; Ono, T. J Biochem (Tokyo) 1986, 100, 975–983.
19. Luckett, S.; Garcia, R. S.; Barker, J. J.; Konarev, A. V.; Shewry, P. R.; Clarke, A. R.; Brady, R. L. J Mol Biol 1999, 290, 525–533.
20. Ikenaka, T.; Norioka, S., In Proteinase Inhibitors; Barrett, A. J., Salvesen, G. S., Eds.; Elsevier Science Publishers, Amsterdam, 1986, 2.
21. Odani, S.; Ono, T. J. Biochemistry 1980, 88, 1555–1558.
22. Kurokawa, T.; Hara, S.; Norioka, S.; Teshima, T.; Ikenaka, T. J Biochem (Tokyo) 1987, 101, 723–728.
23. Odani, S.; Ikenaka, T. J. Biochemistry 1978, 84, 1–9.
24. Gladysheva, I. P.; Zamolodchikova, T. S.; Sokolova, E. A.; Larionova, N. I. Biochemistry (Mosc) 1999, 64, 1244–1249.
25. Tur Sinal, A.; Birk, Y.; Gertler, A.; Rigbi, M. Biochim Biophys Acta 1972, 263, 666–672.
26. Song, H. K.; Kim, Y. S.; Yang, J. K.; Moon, J.; Lee, J. Y.; Suh, S. W. J Mol Biol 1999, 293, 1133–1144.
27. Li, Y. L.; Huang, Q. C.; Zhang, S. W.; Liu, S. P.; Chi, C. W.; Tang, Y. Q. J. Biochemistry 1994, 116, 18–25.
28. Zhu, G.; Huang, Q.; Zhu, Y.; Li, Y.; Chi, C.; Tang, Y. Biochim Biophys Acta 2001, 1546, 98–106.
29. Brauer, A. B. E.; Kelly, G.; McBride, J. D.; Cooke, R. M.; Matthews, S. J.; Leatherbarrow, R. J. J Mol Biol 2001, 306, 799–807.
30. Korsinczky, M. L.; Schirra, H. J.; Rosengren, K. J.; West, J.; Condie, B. A.; Otvos, L.; Anderson, M. A.; Craik, D. J. J Mol Biol 2001, 311, 579–591.
31. Hernandez, J. F.; Gagnon, J.; Chiche, L.; Nguyen, T. M.; Andrieu, J. P.; Heitz, A.; Trinh Hong, T.; Pham, T. T.; Le Nguyen, D. Biochemistry 2000, 39, 5722–5730.
32. Heitz, A.; Hernandez, J. F.; Gagnon, J.; Hong, T. T.; Pham, T. T.; Nguyen, T. M.; Le-Nguyen, D.; Chiche, L. Biochemistry 2001, 40, 7973–7983.
33. Werner, M. H.; Wemmer, D. E. Biochemistry 1992, 31, 999–1010.
34. Nishino, N.; Aoyagi, H.; Kato, T.; Izumiya, N. Experientia 1975, 31, 410–412.
35. Nishino, N.; Aoyagi, H.; Kato, T.; Izumiya, N. J. Biochemistry 1977, 82, 901–909.

36. Terada, S.; Sato, K.; Kato, T.; Izumiya, N. FEBS Lett 1978, 90, 89–92.
37. Nishino, N.; Izumiya, N. Biochim Biophys Acta 1982, 708, 233–235.
38. Ando, S.; Yasutake, A.; Waki, M.; Nishino, N.; Kato, T.; Izumiya, N. Biochim Biophys Acta 1987, 916, 527–531.
39. Gariani, T.; Leatherbarrow, R. J. J Pept Res 1997, 49, 467–475.
40. Domingo, G. J.; Leatherbarrow, R. J.; Freeman, N.; Patel, S.; Weir, M. Int J Pept Protein Res 1995, 46, 79–87.
41. Maeder, D. L.; Sunde, M.; Botes, D. P. Int J Pept Protein Res 1992, 40, 97–102.
42. McBride, J. D.; Freeman, H. N.; Leatherbarrow, R. J. J Pept Sci 2000, 6, 446–452.
43. Philipp, S.; Kim, Y. M.; Durr, I.; Wenzl, G.; Vogt, M.; Flecker, P. Eur J Biochem 1998, 251, 854–862.
44. Tsunogae, Y.; Tanaka, I.; Yamane, T.; Kikkawa, J.; Ashida, T.; Ishikawa, C.; Watanabe, K.; Nakamura, S.; Takahashi, K. J. Biochemistry 1986, 100, 1637–1646.
45. Descours, A.; Moehle, K.; Renard, A.; Robinson, J. A. Chembiochem 2002, 3, 318–323.
46. McBride, J. D.; Freeman, N.; Domingo, G. J.; Leatherbarrow, R. J. J Mol Biol 1996, 259, 819–827.
47. Gariani, T.; McBride, J. D.; Leatherbarrow, R. J. Biochim Biophys Acta 1999, 1431, 232–237.
48. Schellenberger, V.; Braune, K.; Hofmann, H. J.; Jakubke, H. D. Eur J Biochem 1991, 199, 623–636.
49. McBride, J. D.; Freeman, H. N. M.; Leatherbarrow, R. J. Eur J Biochem 1999, 266, 403–412.
50. Zablotna, E.; Kazmierczak, K.; Jaskiewicz, A.; Stawikowski, M.; Kupryszewski, G.; Rolka, K. Biochem Biophys Res Commun 2002, 292, 855–859.
51. Lin, G. D.; Bode, W.; Huber, R.; Chi, C. W.; Engh, R. A. Eur J Biochem 1993, 212, 549–555.
52. Pavone, V.; Isernia, C.; Saviano, M.; Falcigno, L.; Lombardi, A.; Paolillo, L.; Pedone, C.; Buoen, S.; Naess, H. M.; Revheim, H.; Eriksen, J. A. J Chem Soc Perkin Trans 2 1994, 1047–1053.
53. Murthy, H. M.; Judge, K.; DeLucas, L.; Padmanabhan, R. J Mol Biol 2000, 301, 759–767.
54. Li de la Sierra, I.; Quillien, L.; Flecker, P.; Gueguen, J.; Brunie, S. J Mol Biol 1999, 285, 1195–1207.
55. Chen, P.; Rose, J.; Love, R.; Wei, C. H.; Wang, B. C. J Biol Chem 1992, 267, 1990–1994.
56. Voss, R. H.; Ermler, U.; Essen, L. O.; Wenzl, G.; Kim, Y. M.; Flecker, P. Eur J Biochem 1996, 242, 122–131.
57. Koepke, J.; Ermler, U.; Warkentin, E.; Wenzl, G.; Flecker, P. J Mol Biol 2000, 298, 477–491.
58. Dittmann, K. H.; Gueven, N.; Mayer, C.; Rodemann, H. P. Protein Eng 2001, 14, 157–160.
59. Scarpi, D.; McBride, J. D.; Leatherbarrow, R. J. J Pept Res 2002, 59, 90–93.
60. Long, Y. Q.; Lee, S. L.; Lin, C. Y.; Enyedy, I. J.; Wang, S.; Li, P.; Dickson, R. B.; Roller, P. P. Bioorg Med Chem Lett 2001, 11, 2515–2519.
61. Jaulent, A. M.; McBride, J. D.; Leatherbarrow, R. J. In Peptides: The Wave of the Future. Proceedings of the Second International and Seventeenth American Peptide Symposium; Lebl, M., Houghten, R. A., Eds.; American Peptide Society, San Diego, California, USA, 2001, 547–548.
62. Brauer, A. B. E.; Kelly, G.; Matthews, S. J.; Leatherbarrow, R. J. J Biomol Struct Dynam 2002, 20, 59–70.