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Stability of protease inhibitors based on the Bowman–Birk reactive site loop to hydrolysis by proteases

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Bowman–Birk proteinase inhibitor proteins contain two inhibitory regions, each of which is encapsulated within nine-residue disulfide-linked loops. It is known that short cyclic peptides that retain the nine-residue disulfide-bridged motif have inhibitory activity, and can be used as models of the natural inhibitor protein. Two factors are important in determining the effectiveness of such inhibitor peptides: the value of the inhibition constant, and rate at which the inhibitor peptide is hydrolyzed by the proteinase. In this paper we report a study of the inhibitory properties and stability towards proteolytic hydrolysis of a family of synthetic peptides derived from the trypsin reactive site loop of the Bowman–Birk inhibitors. The addition of a single amino acid residue to each end of the nine-residue disulfide-linked loop is found to reduce the rate at which the peptide is hydrolyzed. In addition, changing the P2′ residue from Asn→Ile gives inhibitors with considerably enhanced stability to proteolysis, as well as reduced values of K_i. The implications of these factors for the design of inhibitors based on this loop motif is discussed. © Munksgaard 1997.

Key words: Bowman–Birk inhibitor; cyclic peptide; peptide synthesis; protease inhibitor; trypsin

The Bowman–Birk inhibitors (BBI) are bi-headed serine protease inhibitors found within plants of the *Fabaceae* family (1). They are notably present in *Leguminosae* plants (e.g. soybean), in which they play various roles including defense mechanisms against insect attacks (2) and protection of the seed from endogenous protease activity (3). These proteins have a low molecular weight (6–9 kDa) and are characterized by the presence of seven disulfide bridges, which allow the formation of a symmetrical structure consisting of two independent heads located at opposite ends of the molecule. Each head is made of a tricyclic domain in which a binding site is located and enclosed within a nine-residue disulfide linked loop. Crystal structures 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 the nomenclature of Schecter and Berger (8), spans the P3 to the P6′ sequence from one cysteine to the other. Although the sequences of the loops are highly conserved (9), BBIs can be divided into four groups (I–IV) (10) depending on variation at the P1 and P2′ residues. Naturally occurring inhibitors for α-chymotrypsin, elastase and trypsin were identified with the following residues at the P1 site: leucine and phenylalanine for α-chymotrypsin, alanine for elastase, arginine and lysine for trypsin. It is found that the isolated disulfide-linked reactive loop retains inhibitor activity, and there have been extensive studies of various synthetic peptide loops (9, 11–18).

We have recently examined the effect of varying

Abbreviations: Abbreviations used for amino acids follow the IUPAC–IUB Commission of Biochemical Nomenclature in *Eur. J. Biochem.* (1984) **138**, 937; *J. Biol. Chem.* (1989) **264**, 663–673. All amino acids used are of the L-configuration. Other abbreviations are: Acm, acetamidomethyl; Boc, *tert*-butyloxycarbonyl; DCC, *N,N′*-dicyclohexyl carbodiimide; DCM, dichloromethane; DIEA, diisopropylethylamine; DL-BApNA, N^{α}-benzoyl-DL-arginine *p*-nitroanilide; DMAP, 4-dimethylaminopyridine; DMSO, dimethyl sulfoxide; EDT, 1,2-ethanedithiol; FAB-MS, fast atom bombardment mass spectrometry; Fmoc, 9-fluorenylmethoxycarbonyl; FPLC, fast protein liquid chromatography; HBTU, 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HOBt, *N*-hydroxybenzotriazole; HPLC, high-performance liquid chromatography; MTBE, *tert*-methylbutyl ether; NMP, 1-methyl-2-pyrrolidinone; Pmc, 2,2,5,7,8-pentamethylchroman-6-sulfonyl; tBu, *tert*-butyl; TFA, trifluoroacetic acid; TLC, thin-layer chromatography; Tris, 2-amino-2-hydromethylpropane-1,3-diol; Trt, triphenylmethyl.

the P1 residue on inhibition specificity (18). Before starting this work we synthesized various of the loop peptides that have been described in the literature in order to assess which of the different sequences were optimal for inhibition. It is known that these peptide-based inhibitors are slowly hydrolyzed by the enzymes they inhibit (12), although the dominant effect is to act as inhibitors. Hydrolysis is not exclusive to peptides, but is also a feature of protein-based inhibitors (19). To our surprise we found that similar peptide sequences displayed marked differences in the rates at which they are hydrolyzed. In this publication we describe these differences, and examine the factors responsible.

EXPERIMENTAL PROCEDURES

Materials

The *p*-alkoxybenzyl alcohol resin (Wang resin) and (4-(2′,4′-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxyacetamidonorleucyl)-4-methyl benzhydrylamine resin (Rink amide MBHA resin) were from Novabiochem Ltd. (UK). N^{α}-Fmoc amino acids were purchased from Novabiochem Ltd. or from Bachem Ltd. (UK). These amino acids had the following side-chain protecting groups: Ala, Arg (Pmc), Asn (Trt), Cys (Acm), Cys (Trt), Gln (Trt), Ile, Lys (Boc), Pro, Ser (tBu), Thr (tBu), Tyr (tBu). All reagents for the peptide synthesizer: DCC, DMAP, DIEA, HBTU, HOBT, NMP, DMF, and DCM were supplied by Applied Biosystems Ltd. (Warrington, UK) or Rathburn Chemicals Ltd. (Walkerburn, Scotland). All other reagents and solvents were supplied from Sigma or Aldrich unless otherwise stated.

Peptide synthesis

The peptides used in this study are shown in Table 1. All the peptides were synthesized using an Applied Biosystems 431A peptide synthesizer at a 0.1 mmol synthesis scale using previously described protocols (20–22). Peptides **1** and **2**, which had a *C*-terminal amide, were synthesized using Rink amide MBHA resin (0.4–0.6 mmol/g) (23). Peptides **3–11** were constructed on Wang resin (0.6–0.8 mmol/g) to generate peptides with a *C*-terminal carboxyl group (24).When using Wang resin, a four-fold molar excess of the first amino acid was coupled by symmetric anhydride activation (70 min) using 0.5 equiv. of DCC in the presence of 0.1 equiv. of DMAP with NMP as solvent. The resin was then capped with a four-fold molar excess of benzoic anhydride (70 min) to avoid any parallel synthesis.

Fmoc protecting groups were removed by addition of 20% piperidine in NMP. This procedure was performed twice (6 min each cycle), and the extent of deprotection after each cycle was monitored using a UV flow-through detector (Pharmacia UV-1) to observe the Fmoc-piperidine adduct generated after deprotection (25). Such reaction monitoring confirmed the addition of each amino acid during the syntheses (coupling efficiency estimated from peak heights, >97% at each step). For all the sequences **1–11** it was found that complete (>97%) deprotection was achieved in the first deprotection cycle.

Activation of the Fmoc-protected amino acid used 0.9 equiv. HBTU/HOBT in DMF (22, 26), and coupling of the activated amino acid to the previous amino acid was performed in NMP with DIEA (1.7 equiv.). The activated Fmoc-amino acid was coupled at four-fold molar excess for 15 min.

Peptides **1**, **2**, **4**, **7** and **9** were acetylated after completion of the synthesis by the addition of a four-fold molar excess of acetic anhydride in NMP (15 min) followed by five washes with DCM.

Cleavage of the peptides from the resin and deprotection of the side chains was performed using a multicomponent scavenger cleavage mixture: 84% TFA–6% phenol–4% thioanisole–4% H_2O–2% EDT for 3 h at room temperature. The cleaved deprotected peptide was filtered in order to remove the resin, precipitated in cold MTBE and collected by centrifugation. The peptides were then lyophilized.

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). Analytical HPLC was performed using a C_{18} Waters Radial Pack column (8 mm × 10 cm, 4 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. Samples were eluted with a linear gradient, with detection at 223 nm. The gradient was typically run from 0–50% B over 20 min, then from 50–100% B over 5 min. For the analytical column flow rates of 2 mL min^{-1} were used; for preparative purposes the flow rate was 10 mL min^{-1}. The peptides in this study elute typically at ca. 30% B when linear and ca. 25% B when cyclized.

Peptides were also analysed by TLC using two different solvent systems (A, $CH_3OH:CH_2Cl_2:CH_3COOH$ 4:15:1; B, $CH_3OH:H_2O:CH_3CN$ 5:5:3) on silica plates (Merck KGaA, Germany). All peptides ran as a single spot that was visualised using Mary's spray reagent [0.4% 4,4′-bis(dimethylamino) benzyhydrol in acetone].

FAB-MS was performed using a VG Autospec mass spectrometer with Cs ion bombardment. Peptides were analyzed in a matrix of 3-nitrobenzyl alcohol.

Synthesis of peptide 1. This synthesis followed essentially the protocol of Nishino *et al.* (15). The bicyclic 16-mer peptide was synthesized as CH_3CO-Cys(Acm)-Gln(Trt)-Asp(tBu)-Ala-Ala-Cys(Trt)-Thr(tBu)-Lys(Boc)-Ser(tBu)-Asn(Trt)-Pro-Pro-

TABLE 1

*The sequences of the synthesized peptides with their respective abbreviations as used in this paper. Peptides **1–5** have an Asn residue at the P2′ position; peptides **6–11** have an Ile residue. All peptides are cyclic through an intramolecular disulfide bridge represented by the lines joining the cysteine residues. The location of the P2–P2′ sites are indicated*

	P2 P1 P1′ P2′
1	CH_3CO-Cys-Gln-Asp-Ala-Ala-Cys-Thr-Lys-Ser-Asn-Pro-Pro-Gln-Cys-Arg-Cys-NH_2
2	CH_3CO-Cys-Thr-Lys-Ser-Asn-Pro-Pro-Gln-Cys-NH_2
3	H-Cys-Thr-Lys-Ser-Asn-Pro-Pro-Gln-Cys-Tyr-OH
4	CH_3CO-Cys-Thr-Lys-Ser-Asn-Pro-Pro-Gln-Cys-OH
5	H-Ser-Cys-Thr-Lys-Ser-Asn-Pro-Pro-Gln-Cys-Tyr-OH
6	H-Cys-Thr-Lys-Ser-Ile-Pro-Pro-Gln-Cys-OH
7	CH_3CO-Cys-Thr-Lys-Ser-Ile-Pro-Pro-Gln-Cys-OH
8	H-Cys-Thr-Lys-Ser-Ile-Pro-Pro-Gln-Cys-Tyr-OH
9	CH_3CO-Cys-Thr-Lys-Ser-Ile-Pro-Pro-Gln-Cys-Tyr-OH
10	H-Ser-Cys-Thr-Lys-Ser-Ile-Pro-Pro-Gln-Cys-OH
11	H-Ser-Cys-Thr-Lys-Ser-Ile-Pro-Pro-Gln-Cys-Tyr-OH

Gln(Trt)-Cys(Trt)-Arg(Pmc)-Cys(Acm)-Rink amide MBHA resin. This gives Acm protecting groups on the outer cysteine residues, and Trt protecting groups on the inner cysteines. After deprotection, the CH_3CO-Cys(Acm)-Gln-Asp-Ala-Ala-Cys-Thr-Lys-Ser-Asn-Pro-Pro-Gln-Cys-Arg-Cys(Acm)-NH_2 peptide was isolated by FPLC (Pharmacia) gel filtration on a column of Sephadex G-25 (Pharmacia, bead size 20–80 μm, 2 cm × 40 cm) with water as the eluant. Formation of the inner disulfide was by air oxidation. The peptide (13.5 mg), deprotected except for the Acm groups, was dissolved in distilled water at a concentration of 0.1 mg/mL. The pH was carefully raised to 8.5 by addition of aqueous ammonia, and the peptide solution was then left to stir for 72 h. The remaining free thiols were monitored by reaction with Ellman's reagent (27). After 72 h the residual free thiol content was 2%. Deprotection of the Acm protecting groups on the two outer cysteines was performed directly on this crude peptide mixture using mercury(II) acetate. Mercury(II) acetate (2 equiv. to cysteine) was added to the solution, and left to stir for 1 h. 2-Mercaptoethanol (2 equiv.) was then added to the reaction mixture, which was left to stir for a further hour. Precipitated mercuric sulfate was removed by filtering the reaction mixture through a pad of Celite® 521 filter agent (Fisons). The outer disulfide was then introduced by a further air oxidation step as described above. The bicyclic product was isolated by preparative HPLC, where the major peak was the desired product (40% yield after isolation). The peptide was characterized by FAB-MS.

*Synthesis of peptides **2–11**.* The remaining peptides, which contain a single disulfide and are analogues of each other, were constructed in linear form using the protocols described above using the on-line monitoring to confirm the addition of each residue. Analytical HPLC of the crude peptides shows that the purity at this stage is typically ca. 80–90%, and so DMSO oxidation to generate the disulfide (28) was performed without prior purification. The lyophilized peptides (typically 10 mg) were dissolved in a 5% aqueous acetic acid solution to make a 0.2–0.4 mM solution. The pH was raised to 6 by addition of aqueous ammonia. DMSO was then slowly added to 20% (v/v), and the solution was left to stir for 24 h at 25 °C. This method was found to give ca. 50% isolated yield of cyclic peptides **2–11**. Each cyclic peptide was purified by preparative HPLC, and purity confirmed by analytical HPLC of the isolated product. All peptides **2–11** gave essentially one peak after purification (purity by integration >90% in each case). The purified peptides were analyzed by FAB-MS, which confirmed that the peptides had the expected masses, and by TLC using two different solvent systems. Table 2 summarizes the analytical information for each of the peptides used in this study. Representative analytical data are shown in Fig. 1 for peptide **11**. This records the

TABLE 2
Analytical information about the peptides used in this study

Peptide	HPLC retention time (min)	R_f value on TLC		Mass	
		A	B	Expected	Found
1	10.0	0.48	0.05	1763.04	1763
2	9.6	0.43	0	1016.17	1017
3	13.6	0.45	0.30	974.11	974
4	6.5	0.40	0.01	1017.16	1017
5	12.4	0.51	0.08	1224.37	1225
6	8.6	0.42	0	973.17	974
7	9.0	0.45	0.06	1016.21	1016
8	12.0	0.53	0.18	1136.34	1137
9	12.4	0.40	0.27	1179.39	1179
10	8.3	0.44	0.01	1060.24	1061
11	11.2	0.55	0.14	1223.42	1224

Analytical HLPC was performed on a C_{18} column using a linear gradient between water and acetonitrile, each containing 0.1% TFA. The gradient was run from 0 to 50% acetonitrile over a period of 20 min. The R_f values from TLC are recorded using two different solvent systems, A and B, was outlined in the methods section. Masses are from FAB-MS.

coupling efficiencies found during synthesis, the analytical HPLC trace of the final product by HPLC and the FAB-MS characterization.

Inhibition kinetics

The inhibition constants of the different inhibitors against trypsin were determined by competitive binding studies (18, 29) using the chromogenic substrate DL-BApNA. 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. The enzyme concentrations were calculated from the initial rate of substrate hydrolysis (18). Substrate concentration was obtained from the final absorbance at 412 nm after complete hydrolysis ($\varepsilon = 8480\ \mathrm{M}^{-1}\ \mathrm{cm}^{-1}$). The peptide concentrations were determined by optical density at 280 nm for peptides having a *C*-terminal tyrosine residue (using $\varepsilon = 1400\ \mathrm{M}^{-1}\ \mathrm{cm}^{-1}$), and by dry weight of purified peptide for the remainder. All data were processed using non-linear regression using the program GraFit (30) as described previously (18).

For tight-binding inhibitors it is possible to measure directly the enyzme : inhibitor ratio at which complete inhibition occurs (29). Any deviation from a 1 : 1 ratio implies that the enzyme or the inhibitor is not fully active. As described previously (18), we consistently find that BBI peptides appear less than 100% active, and typically show only 20–40% activity in such titrations, despite being homogeneous by chemical criteria. At least two factors contribute to this: partial hydrolysis of the peptides during the incubation time prior to the initial measurement, and the presence of a mix of *cis/trans* isomers of the proline residue at the P3′ position within the sequence giving active and inactive conformers, respectively (unpublished data, ref. 17). For the K_i values calculated here we have elected to quote the K_i for the active fraction, rather than for the entire mixture. This approach compensates for any loss of active inhibitor due to hydrolysis during the incubation period, which is advantageous for the inhibitors that are more rapidly hydrolyzed.

Hydrolysis rates

The rate of hydrolysis of the inhibitory peptides by trypsin was determined by analyzing the rate at which inhibited enzyme regains activity. Experiments were performed at 25 °C 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 ca. 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 ca. 10^{-7} M; substrate ca. 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 eqn. (1):

$$v = (v_\infty - v_0) \times \{1 - \exp(-kt)\} + v_0 \qquad (1)$$

In this equation the rate, v, increases with time t, from the initial rate, v_0, to the final uninhibited rate, v_∞, with a rate constant for hydrolysis, k.

RESULTS AND DISCUSSION

There are two important factors determining the effectiveness of protease inhibitors that are based on peptide sequences. The first and most widely reported is the value of the inhibition constant, K_i. The second is the rate at which an inhibitor is hydrolyzed by the protease. In common with full-length inhibitor proteins (19), the peptide-based protease inhibitors described here are substrates as well as inhibitors, and so for best effect the hydrolysis rate should be low. An ideal inhibitor would have a combination of a low K_i value together with a low rate of hydrolysis.

Prolonged incubation of these peptide inhibitors with trypsin results in proteolytic hydrolysis of the peptide P1–P1′ reactive bond (18). This leads to a regain of enzyme activity with time, which can be used to determine the hydrolysis rate of the inhibitor. A typical hydrolysis profile is shown in Fig. 2, which records the regain of activity when a peptide based on the BBI inhibitory loop is incubated with trypsin. Analysis of such profiles affords a simple means of measuring hydrolysis rate constants. Table 3 presents the hydrolysis rate constants determined for three

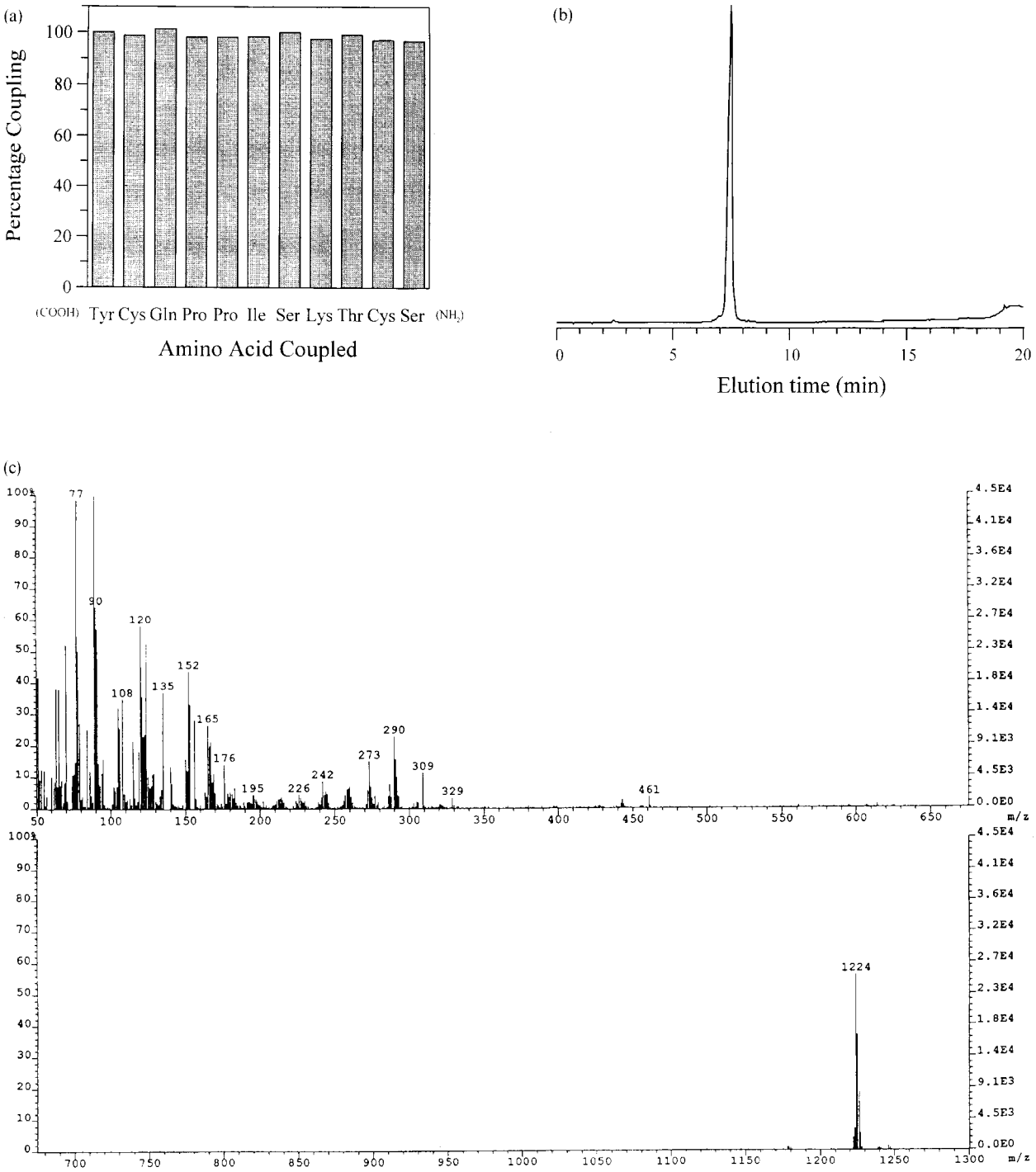

FIGURE 1

Characterization of peptide **11**. (a) Coupling efficiencies for each step of the synthesis. The quantity of Fmoc-piperidine adduct released after each deprotection step was measured with the aid of a UV flow monitor. The peak heights are plotted relative to the initial deprotection cycle. (b) Analytical HPLC trace of the purified cyclic peptide. The aqueous and polar phases used were water and acetonitrile, each containing 0.1% TFA. Samples were eluted with a linear gradient at 2 mL min^{-1}, with detection at 223 nm. The gradient was from 0–70% acetonitrile over 17 min, then from 70–100% acetonitrile over 1 min followed by 100% acetonitrile for 1 min. (c) FAB-MS spectrum. Expected mass 1223.42, found 1224.

peptide sequences that have been described in the literature (**1**, **2** and **11**), and also lists K_i values for these peptides. Peptide **2** is a nine-residue cyclic peptide (11), peptide **1** is an extended 16-residue version of this with two disulfides (12), and peptide **11** is an 11-mer cyclic peptide (16). Based on their inhibition constants, all three are effective inhibitors of trypsin. However, we find that these sequences display very different susceptibility to proteolytic hydrolysis. The enhanced stability of peptide **1** over peptide **2** has been noted previously (12), and attributed to the presence of the additional disulfide. However, the difference between peptides **2** and **11** is most striking, with **11** being hydrolyzed 244 times more slowly.

Increased stability to proteolysis is a highly desirable feature for any protease inhibitor, and therefore it is important to discover why peptide **11** should have such enhanced stability over peptide **2**. In order to investigate its causes, a series of peptides have been constructed which allow an assessment of the relative contributions of the various differences between peptides **2** and **11**. Although the sequences of these two peptides are largely common, there are several differences: they have different residues at the P2′ position within the loop, they differ in length, peptide **2** has a *C*-terminal amide whereas peptide **11** possesses a *C*-terminal carboxyl, peptide **2** is *N*-acetylated while peptide **11** is not. In order to investigate the reasons for the enhanced stability of peptide **11**, a series of analogues (**3–10**) were synthesized. The hydrolysis by trypsin of each peptide analogue was measured, and their respective hydrolysis rates were calculated. These values, together with the inhibition constants for these peptides, are listed in Table 3.

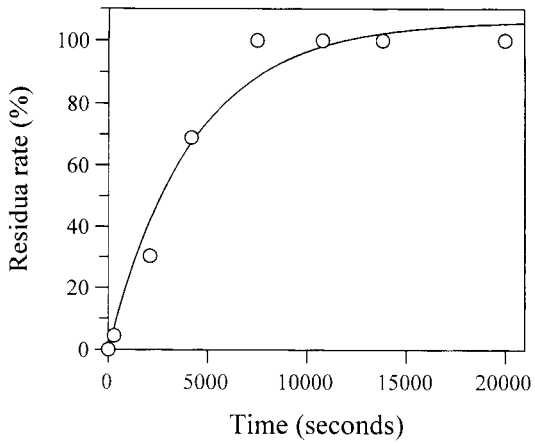

FIGURE 2

Hydrolysis of inhibitor peptide **10** by trypsin. Enzyme inhibitor complex was formed, and then the rate of substrate hydrolysis following addition of substrate to an aliquot of the complex was recorded at various time intervals. Initially the enzyme was almost totally inhibited, but with time the inhibitor peptide was hydrolyzed and so the rate of substrate hydrolysis increased. All rates are referenced to rate of hydrolysis of the substrate by uninhibited enzyme. The substrate concentration for this experiment was 5.36×10^{-5} M and the enzyme concentration 2.46×10^{-7} M. The solid line represents the best fit first-order rate curve [eqn. (1)], from which the inhibitor hydrolysis rate constant was derived.

TABLE 3

Comparison of the stability of the various peptide inhibitors used in this study to hydrolysis by trypsin. The table also shows the K_i *values of the different peptides with the same enzyme*

Peptide	Length	K_i value (μM)	Hydrolysis rate ($mol_{peptide}\ mol_{enzyme}^{-1}\ s^{-1} \times 10^{-3}$)
1	16	0.039[a]	29
2	9	0.17[b]	2370
3	10	0.46	522
4	9	0.267	9050
5	11	0.223	416
6	9	0.068	111
7	9	0.105	510
8	10	0.024	81
9	10	0.108	195
10	10	0.045	78
11	11	0.0095[c]	9.7[d]

[a] Lit. 0.15 μM (11).
[b] Lit. 0.75 μM (11) and 0.76 μM (12).
[c] Lit. 0.01 μM (18).
[d] Lit. $9 \times 10^{-3}\ mol_{peptide}^{-1}\ mol_{enzyme}^{-1}\ s^{-1}$ (18).

Effect of C*-terminal amide*

Peptide **4** was synthesized to allow comparison of the effect of having a *C*-terminal amide (peptide **2**) or acid (peptide **4**). It was found that a *C*-terminal amide group had a moderate stabilizing effect on hydrolysis rates, which were reduced four-fold in comparison to the free acid. This difference is not a major factor in the increased stability of peptide **11** (and in any case operates in favor of the less stable peptide **2**). For this reason, all remaining peptides were synthesized with *C*-terminal acidic groups to facilitate better comparison with peptide **11**.

Effect of the P2′ position

Peptides **2** and **11** differ within the disulfide-linked loop at the P2′ position, where peptide **2** has an asparaginyl and peptide **11** an isoleucyl residue. To assess the involvement of this residue, peptide **5** was synthesized, differing from **11** only at the P2′ location. It is found that the presence of Ile at P2′ gives a peptide that is 43 times more resistant to hydrolysis than the same peptide having Asn at P2′. The factor by which the Ile substitution stabilizes the peptide was found to vary slightly depending on the length of the peptide sequence into which the change is introduced. In nine-mer sequences (comparison of **4** with **7**) the stabilization is found to be 18-fold.

However, it is clear that the presence of an Ile residue at P2′ results in a significant decrease in the rate at which the peptide is hydrolyzed by the protease.

Effect of peptide length
The length of the peptide was varied between 9 and 11 residues in order to determine the effect on hydrolysis rates. Variants of the 11-mer sequence (**11**) were created that lacked the *N*-terminal Ser (**8**, **9**), the *C*-terminal Tyr (**10**), or both these residues (**6**, **7**).

C-*Terminal Tyr.* The contribution of the *C*-terminal Tyr residue can be judged by comparing the hydrolysis rates of peptides **6** and **8**, or peptides **10** and **11**. The stabilization going from **6** to **8** is found to be only a factor of 1.4; between **10** and **11** the factor is 8. We also compared the *N*-acetylated forms of peptides **6** and **8**, peptides **7** and **9**; in this case the additional Tyr affords a factor of 2.6 in stability. The magnitude of the stabilization afforded by addition of the *C*-terminal Tyr to the base nine-residue loop sequence is therefore less than the Ile/Asn substitution, and comparable to the effect of replacing the *C*-terminal acidic group of the nine-mer sequence with an amide.

N-*Terminal Ser.* The contribution of the *N*-terminal Ser residue can be assessed by comparison of the hydrolysis rates of peptides **6** and **10**, or **8** and **11**. Peptide **10** is found to be more stable than peptide **6** by a factor of 1.4, and **11** more stable than **8** by a factor of 8. These results are very similar to those found for the addition of the *C*-terminal Tyr: the stabilization of an 11-mer sequence over the corresponding 10-mer is greater than that of a 10-mer over the corresponding nine-mer.

In conclusion, the addition of an extra amino acid residue at each end of the nine-residue loop sequence results in an inhibitor that is more resistant to hydrolysis. The effect of each individual addition is similar, and adds a factor of between 1.4 and 8; the addition of both residues together stabilizes the peptide by a factor of ca. 12.

Effect of N-*acetylation*
Peptides **6** and **8** were compared with *N*-acetylated versions (**7** and **9**) to see the effect on hydrolysis rates. In both cases the *N*-acetylated forms of the peptide were less stable, by factors of 4.6 and 2.4, respectively.

Overall conclusion
The difference in hydrolytic stability between peptides **2** and **11** is marked: peptide **2** has a hydrolysis rate 244 times that of peptide **11**. However, this effect has various contributing factors. The largest single factor is the change at the P2′ position. Comparison of otherwise identical 11-residue sequences shows that a P2′ Asn results in 43 times faster hydrolysis than the P2′ Ile variant. Addition of an extra residue at each end of the nine-mer sequence provides the remaining stabilization, with each additional residue having roughly equal contribution.

Effects on inhibition constants
The addition of a peptide inhibitor to a solution of trypsin results in loss of protease activity due to the formation of an inhibitor–enzyme complex, as is shown in Fig. 3. Fitting of these data allows calculation of the inhibition constant (K_i). The K_i values against trypsin for all the synthetic peptides used in this study are given in Table 1.

The results in this table show that peptide **11**, as well as being the most resistant of those tested to hydrolysis, displays the lowest K_i value. However, the difference in K_i between the peptides is less marked than the difference in hydrolysis rates.

Effect of the P2′ position
Comparison of all pairs of peptides that are identical apart from the Asn/Ile substitution at the P2′ position (**4** with **7**; **3** with **8**; **5** with **11**) shows that in each case the Ile peptide displays a lower K_i value. On average, the Ile peptides have K_i values that are 15-fold lower than the corresponding Asn peptides.

Effect of peptide length
For peptides with Asn at P2′ (**3**–**5**) the K_i values are similar irrespective of peptide length. However, the Ile series peptides (**6**–**11**) do show a tendency for reduced K_i values as additional *N*- and *C*- terminal residues are added to the inner nine-residue loop.

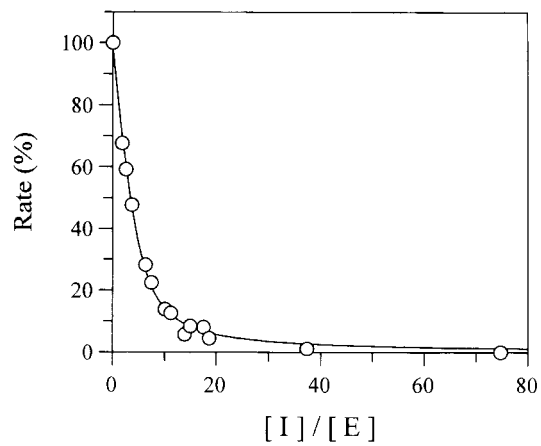

FIGURE 3
Inhibition of trypsin by peptide **10**. The enzyme and the inhibitor were pre-incubated for 5 min prior to measuring the rate of substrate hydrolysis. The substrate concentration was 5.36×10^{-5} M and the enzyme concentration 2.46×10^{-7} M. The solid line shows the best fit non-linear regression curve, from which the K_i value was derived.

Comparison of the group of peptides **6**, **8**, **10** and **11** shows that addition to the inner nine-residue sequence of the *N*-terminal Ser reduces the K_i by around 1.5–2.5-fold, and addition of the *C*-terminal Tyr reduces it by 2.8–4.5-fold. The effect of going from the 9-residue to the 11-residue sequence is to reduce the K_i 6.8 fold, showing that the individual effects are additive.

Correlation between hydrolysis rates and K_i *values*

It is generally the case that peptidic protease inhibitors are substrates as well as inhibitors, and this is certainly true for peptides based on the BBI loop sequence. It is therefore important for inhibitor design that hydrolysis is minimized. The results that are presented in this paper show that there is little correlation between the K_i value of one of these peptides and the rate at which hydrolysis occurs, although there is some tendency for peptides with low K_i values to show low hydrolysis rates. Very large differences in hydrolysis rates can be displayed by sequences that show similar K_i values. These results are in line with previous work from this laboratory, where a series of BBI loop peptides differing in the P1 position were found to show little correlation between K_i values and rates of proteolytic hydrolysis (18).

SUMMARY

The results of this study show that the selection of peptide-based inhibitors needs to consider criteria other than just the value of inhibition constant. The stability of these inhibitors is an equally important factor. Unfortunately, this aspect of behavior seems difficult to predict, although it can easily be measured experimentally. For inhibitors of trypsin based on the Bowman–Birk loop structure, our results show that a marked increase in stability to hydrolysis is gained by having an isoleucyl residue at the P2′ position rather than Asn. The residues found at this position in natural BBI protein sequences are Ile, Met, Asn and Lys, with Ile being the most common residue (10). This suggests that natural selection has favored sequences with low hydrolysis rates. We are currently studying the effects of introducing more extensive variations at this location in an attempt to examine these factors further.

In addition, we find that sequences based on the minimal nine-residue disulfide-linked loop are considerably less stable to hydrolysis than 11-residue sequences that possess an additional amino acid at each end of the loop. The combination of this with an Asn→Ile change at P2′ explains the marked additional stability of the 11-residue sequence of Maeder *et al.* (16) over other nine-residue sequences (11).

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