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Synthesis of a mixture of cyclic peptides based on the Bowman–Birk reactive site loop to screen for serine protease inhibitors

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A peptide mixture containing 21 peptide sequences has been constructed to test the Bowman–Birk inhibitor reactive-site loop motif as the basis of inhibition for a range of serine proteases. The 21 peptides are all based on an 11 amino acid sequence designed from a Bowman–Birk like inhibitor reactive-site loop. Variation has been introduced at the P1 site of the loop, which has been randomised to include all the natural L-amino acids (except for cysteine), plus the non-natural L-amino acids ornithine and norleucine. The mixture of peptides was screened for specific binding to immobilised porcine pancreatic elastase, subtilisin BPN′, α-chymotrypsin, trypsin, anhydro-α-chymotrypsin and anhydrotrypsin. Five peptides from the mixture bind to α-chymotrypsin, two of which also bind to anhydro-α-chymotrypsin, and two peptides bind trypsin, neither of which binds to anhydro-trypsin. The competitive inhibition constants (K_i) and the rates of proteolytic hydrolysis of the individual peptides with their respective enzymes were determined. The rates of hydrolysis were found to vary widely and show little correlation with the K_i values. In the case of the α-chymotrypsin inhibitors, the peptides with the lowest K_i (0.1–0.05 mM) were the only peptides that bound to anhydro-α-chymotrypsin. However, no peptides bound to anhydrotrypsin, suggesting a fundamental difference in the way that α-chymotrypsin and trypsin are inhibited by these cyclic peptides. © Munksgaard 1995.

Key words: anhydro-α-chymotrypsin; anhydrotrypsin; Bowman–Birk inhibitor; serine protease; peptide synthesis

Bowman–Birk inhibitors are a family of serine protease inhibitors with similar structural features (see ref. 1 for a review). They are small proteins (6–9 kD) containing seven disulfide bridges. The disulfide bridges help to form a symmetrical structure consisting of two tricyclic domains, each containing an independent serine protease binding site. Each binding site is enclosed within a nine residue disulfide-bridged loop. Crystal structures and NMR structures of these inhibitors have been resolved (2–4). The crystal structures of the trypsin binding domain of a Bowman–Birk-type inhibitor complexed with trypsin (5), and the complete Bowman–Birk-type inhibitor from mung bean in ternary complex with trypsin (6) have also been reported.

The binding sites of these inhibitors have a very highly conserved amino acid sequence (7). Based on the notation of Schechter and Berger (8), the binding loop spans the P3 to the P6′ sequence from one cysteine to the other. The most variable residues in the binding sites of the natural inhibitors occur at the P1 and P2′ residues, with the P1 defining the specificity of these binding sites for the different serine proteases. Natural inhibitors of trypsin, α-chymotrypsin and elastase have been identified with the following residues at the P1 site: arginine and lysine for trypsin; leucine and phenylalanine for α-chymotrypsin; and alanine for elastase (7).

The disulfide-bridge-enclosed binding loop of the Bowman–Birk inhibitor renders itself an obvious target for the design and synthesis of peptide-based serine protease inhibitors. Several cyclic peptides designed to mimic the binding site of this family of serine protease inhibitors have been synthesised (9–13). In this process the role of the P1 residue has been tested by synthesising various cyclic peptides with different P1 residues

Boc, *tert*-butoxylcarbonyl; DCC, *N,N′*-dicyclohexylcarbodiimide; DCM, dichloromethane; DIEA, diisopropylethylamine; DMAP,dimethylaminopyridine; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; EDT, 1,2-ethanedithiol; Fmoc, 9-fluoromethyloxycarbonyl; HBTU, [2-(*1H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosohate]; HOBT, 1-hydroxybenzotriazole; NMP, *N*-methylpyrrolidone; Pmc, 2,2,5,7,8-pentamethylchroman-6-sulfonyl; PMSF, phenylmethanesulfonyl fluoride; tBu, *tert*-butyl; TFA, trifluoroacetic acid; Trt, trityl.

(12). Five amino acids have been inserted into the P1 site: lysine, D-lysine, arginine, leucine and tyrosine. The first three amino acids are found to confer specificity towards trypsin, and the latter two towards α-chymotrypsin and subtilisin BPN′. These synthetic serine protease inhibitors provide good *in vitro* systems to study protein–protein interactions. They are relatively easy to synthesise and allow the possibility of introducing non-natural amino acids into the system.

An efficient approach to study protein–peptide ligand interactions is through the synthesis and screening of multiple peptide libraries (see refs 14 & 15 for reviews). Several techniques have been developed for this purpose, ranging from an iterative selection and enhancement process with the use of synthetic peptide combinatorial libraries (16) to the synthesis of large-scale random libraries with the concept "one bead, one peptide" (17). More recently this latter technique has been extended to include large-scale screening in solution (18). In this paper we present an easy and cost-effective process to test the Bowman–Birk reactive-site loop against a range of serine proteases using a simple solution mini-library of inhibitors with different specificity-determining P1 amino acids.

By incorporating a single randomised position, it is possible to use Edman sequencing to identify sequences present in a solution mixture of peptides. While this is more limiting than techniques involving libraries comprising many thousands of sequences, it is technically less demanding, and allows screening in solution. It is also highly appropriate for screening protease inhibitors, as their interactions are often dominated by the identity of a single residue position (19).

The 11-amino-acid sequence designed by Meader *et al.* (9) was chosen as the "scaffold" sequence of our peptide cocktail, being the most appropriate reported inhibitor of this type in terms of its length (only 11 amino acids) and K_i (0.1 mM) values. This design is based on a natural Bowman–Birk inhibitor, D4, from *Macrotyloma axillare*, which inhibits trypsin and α-chymotrypsin (20, 21). A single 0.25-mmol scale peptide synthesis produced a 21-component mixture in which each member has the same 11-amino-acid sequence, except for differing amino acids at the P1 site. The peptide mixture, with the sequence SCTXSIPPQCY from *N*- to *C*- terminus, where **X** is the randomised amino acid, was screened for inhibitors against porcine pancreatic elastase, subtilisin BPN′, trypsin and α-chymotrypsin.

The active site serine (Ser 195) of the serine proteases trypsin and α-chymotrypsin can be chemically modified to dehydroalanine, rendering the enzymes proteolytically inactive (22, 23). Competitive binding studies of natural serine protease inhibitors with the anhydroenzyme and the unmodified protease have shown that in most cases the binding affinity of the inhibitor with the anhydroenzyme is equal to that of the inhibitor with the unmodified enzyme (24). The crystal structure (25) of the complex between anhydrotrypsin and bovine pancreatic trypsin inhibitor (PTI) shows few significant structural changes from that of the native bovine trypsin and PTI (26). In particular, the pyramidalisation of the P1 amide carbonyl in the inhibitor observed in the native complex is maintained in the anhydro-species. These data suggest that the formation of the acyl intermediate is not essential for the binding process of the natural inhibitors with trypsin and α-chymotrypsin. In this paper we also compare the synthetic peptide binding mechanism to that of the natural inhibitors by testing our mixture for peptides binding to anhydro-trypsin and anhydro-α-chymotrypsin.

EXPERIMENTAL PROCEDURES

Materials. The *N*-α-Fmoc protected amino acids, and *N*-α-Fmoc protected amino acid pentafluorophenyl esters were purchased from Calbiochem-Novabiochem (UK) Ltd. or from Bachem (UK) Ltd. The *N*-α-protected Fmoc L-amino acids and the *N*-α-Fmoc-protected L-amino acid pentafluorophenyl esters used in the synthesis were purchased with the following side-chain protecting groups; Ala, Arg(Pmc), Asn(Trt), Asp(tBu), Cys(Trt), Gln(Trt), Glu(tBu), Gly, His(Trt), Ile, Leu, Lys(Boc), Met, Nle, Orn(Boc), Phe, Pro, Ser(tBu), Thr(tBu), Trp, Tyr(tBu), Val. All reagents for the peptide synthesiser, DCC, DMAP, DIEA, HBTU, HOBT, NMP, DMF and DCM, were supplied by Applied Biosystems Ltd. All other reagents and solvents were supplied from Sigma Chemical Co. Ltd. or Aldrich Ltd. unless otherwise stated.

Solid-phase peptide synthesis. All the peptides were synthesised by solid-phase peptide synthesis on an Applied Biosystems (ABI) 431 A peptide synthesiser. The peptides were constructed on *p*-alkoxybenzyl alcohol resin [Bachem (UK) Ltd.]. A four-fold molar excess of amino acid to scale of synthesis was used. The first amino acid was coupled on to the resin by symmetric anhydride activation using 0.5 eq. of DCC in the presence of 0.1 eq. of DMAP. Fmoc protecting groups were removed with 20% piperidine in NMP. The standard ABI "Fast-Moc™" chemistry was used for activation and coupling of the subsequent amino acids. This involves activation of the Fmoc-protected amino acid by equimolar quantities of HBTU/HOBT in DMF (27). Coupling of the activated amino acid to the previous amino acid is performed in the presence of DIEA (1.7 eq.) and NMP. The complete cycle includes up to five NMP washes between coupling and deprotection steps and between deprotection and coupling steps.

Individual peptide synthesis and deprotection. The peptides K11, R11, O11, Y11, F11, M11, L11, NL11, and P11 (Table 1) were synthesised as described above on a 0.025 mmol scale. Cleavage of the peptides from the resin and deprotection of the side chains was performed

TABLE 1

The peptides synthesised individually and the abbreviations used for this paper. All peptides are cyclic through an intramolecular disulfide bridge between the two cysteines. The top row indicates the notation of Schechter and Berger (8)

Individual peptides synthesized											Abbreviation
P4	P3	P2	P1	P1′	P2′	P3′	P4′	P5′	P6′	P7′	
S	C	T	**K**	S	I	P	P	Q	C	Y	K11
S	C	T	**R**	S	I	P	P	Q	C	Y	R11
S	C	T	**O**	S	I	P	P	Q	C	Y	O11
S	C	T	**F**	S	I	P	P	Q	C	Y	F11
S	C	T	**Y**	S	I	P	P	Q	C	Y	Y11
S	C	T	**M**	S	I	P	P	Q	C	Y	M11
S	C	T	**L**	S	I	P	P	Q	C	Y	L11
S	C	T	**NL**	S	I	P	P	Q	C	Y	NL11
S	C	T	**P**	S	I	P	P	Q	C	Y	P11

with a mixture of 0.75 g phenol, 0.25 mL EDT, 0.5 ml thioanisole, 0.5 mL H_2O and 10 mL TFA for 2 h. Each peptide was precipitated in cold *tert*-methyl butyl ether and washed five times with *tert*-methyl butyl ether. The peptide was then dried under vacuum before disulfide bridge formation.

Disulfide bridge formation is always an efficiency-determining step in cyclic peptide synthesis. We found the DMSO oxidation method described by Tam *et al.* (28) to be a most efficient method for intramolecular disulfide bridge formation, in contrast to air oxidation which gave significant amounts of side products, including multimeric products. After deprotection, the dry peptides were dissolved in 5% aqueous acetic acid to make a 0.3–0.5 mg/mL solution. The pH was raised to 6 by careful addition of aqueous ammonia. DMSO was then slowly added to make up a 20% (v/v) DMSO solution, and the solution was stirred for 24 h at 21 °C. This method was found to give 90% yield of cyclic peptide with low levels of side products, polymers and linear peptides (yields are estimated from analytical HPLC profiles).

Cleavage of the peptides from the resin and cyclisation of the peptides were followed by analytical reversed-phase chromatography on a Gilson HPLC system with a C18 Waters Radial Pak column (8 mm × 10 cm). The aqueous and polar phases used were water and acetonitrile, respectively, each containing 0.1% TFA. Purification of the peptides was performed on a semi-preparative C18 Waters Radial Pak column (25 mm × 10 cm). All the peptides were characterised by mass-spectroscopy to verify that the correct sequence was present.

Peptide mixture synthesis. The *C*-terminal portion of the 11 amino-acid peptide, up to the Ser P1′ residue, was synthesised and the *N*-terminus deprotected on the ABI 431 A peptide synthesiser using a 0.25 mmol synthesis scale as described above. The resin was then separated into different reaction vessels for each amino acid that was to be coupled at the P1 site. All the natural L-amino acids (except for cysteine), L-norleucine and L-ornithine were used to create a 21-component mixture. Ornithine was coupled as an Fmoc-protected amino acid (as described above), since the pentafluorophenyl ester is not commercially available. For the remainder of the amino acids, pentafluorophenyl esters were used to facilitate the manual coupling step. A tenfold excess of one of the *N*-α-Fmoc-protected amino acid pentafluorophenyl esters dissolved in 4 mL of NMP was added to each reaction vessel. One equivalent of HOBT to amino acid was included to catalyse the reaction (29, 30). After 2 h, completion of the reaction was confirmed using the ninhydrin test (31). All 21 resin samples were then pooled together, and washed with NMP and DMF several times. The pooled resin sample was returned to the ABI 431A peptide synthesiser for Fmoc removal from the P1 residue. The remaining three residues ThrP2 to SerP4 were then added on the synthesiser as described earlier.

The peptide mixture was cleaved from the resin and side-chain deprotected as described previously. The cyclisation and purification of the peptide mixture were performed using DMSO oxidation and reverse phase chromatography as for the individual peptides.

Immobilisation of serine proteases for affinity chromatography. Four serine proteases, porcine pancreatic elastase (Sigma Chemical Co. Ltd., UK, type I), Subtilisin BPN′ (Sigma Chemical Co. Ltd., type XXVI), trypsin (Sigma Chemical Co. Ltd., type III) and α-chymotrypsin (Sigma Chemical Co. Ltd., type 1-S) were immobilised separately on to CNBr-activated Sepharose 4B beads (Pharmacia Ltd.). The beads were washed and reswollen in 1 mM HCl. The serine protease was dissolved in 0.5 M NaCl, 0.1 M $NaHCO_3$ (pH 8.3) buffer to give 5 mg protein per mL of Sepharose gel, and mixed for 2 h at room temperature. The gel suspension was then washed and incubated with 1 M ethanolamine (pH 8.0) for a further 2 h to block the

remaining active groups on the gel. Unbound protein was removed by washing the gel alternately with high and low pH buffer solutions five times using 0.5 M NaCl, 0.1 M $NaHCO_3$ (pH 8.3) buffer and 0.5 M NaCl, 0.1 M acetate (pH 4.0) buffer. The enzyme-immobilised beads were tested for proteolytic activity with their respective chromogenic substrates; elastase: succinyl-Ala-Ala-Ala-*p*-nitroanilide (sAAA-pNA); subtilisin and α-chymotrypsin: succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (sAAPF-pNA); and trypsin: benzoyl-arginine-*p*-nitroanilide (L-BApNA). All substrates were supplied by Sigma Chemical Co. Ltd.

Anhydro-trypsin and anhydro-α-chymotrypsin were prepared from trypsin and α-chymotrypsin, respectively, using the method described by Ako *et al.* (22, 23). The reactions were performed on Sepharose-immobilised enzyme prepared as above. Quenching of enzyme activity of the two enzymes was verified by exposing the enzyme-bound beads to their chromogenic substrates. Binding activity of the anhydro-enzymes immobilised on the beads was confirmed with affinity chromatography using the natural inhibitors, Soybean trypsin inhibitor (Sigma Chemical Co. Ltd.), and Barley chymotrypsin inhibitor-2 produced in this laboratory (32), for anhydro-trypsin and anhydro-α-chymotrypsin, respectively, and the Bowman–Birk inhibitor (Sigma Chemical Co. Ltd.) for both anhydro-enzymes.

Affinity chromatography. Selection of the peptide serine protease inhibitors was performed by passing a sample of the peptide mixture through a 3 mL column of immobilised serine protease. Sample (0.5 mL, 1 mg/mL) was loaded on to the columns at a flow rate of 0.2 mL/min in 0.1 M Tris–HCl, pH 8.0 (with 0.1 M NaCl, 0.12 M $CaCl_2$). Specifically bound peptide samples were desalted by analytical reversed-phase chromatography as described above. After lyophilisation, the samples were processed by *N*-terminal peptide sequencing on an Applied Biosystems 467A protein sequencer.

The following samples were loaded on to the different affinity columns: cyclic peptide mixture; linear peptide mixture (free -SH groups were maintained during the assay by use of oxygen-free buffers); Bowman–Birk inhibitor; Soybean trypsin inhibitor; and Barley chymotrypsin inhibitor 2.

Inhibition kinetics. The inhibition constants were measured for various active peptides identified from the peptide screening. These were determined by competitive binding studies (33) using the chromogenic substrates sAAPF-pNA, L-BApNA and sAAA-pNA, for α-chymotrypsin, trypsin and porcine pancreatic elastase, respectively. All assays were performed at pH 7.8 in 50 mM Tris–HCl containing 10 mM $CaCl_2$. The peptide concentrations were determined by the optical density (OD) at 280 nm. The enzyme concentrations were calculated from the initial rate of substrate hydrolysis monitored at 412 nm. The substrate concentration was determined from the final OD at 412 nm after complete hydrolysis. All data were processed using non-linear regression using the GraFit software package (34).

Proteolytic hydrolysis rates. The rates of hydrolysis of the individual peptides with their respective serine proteases were determined using capillary electrophoresis and/or reversed-phase HPLC to monitor the formation of hydrolysis products. In both cases, excess peptide to enzyme concentrations were used, typically a reaction mixture of 8×10^{-4} M peptide with 1×10^{-7} M enzyme. All reaction mixtures were incubated in 50 mM Tris–HCl, 10 mM $CaCl_2$, pH 7.8. The peptide concentrations were determined by the OD at 280 nm, and the enzyme concentrations by their hydrolytic activity in the presence of their chromogenic substrates (see above).

For the capillary electrophoresis a Beckman P/ACE™ 2000 series system was used with a bare fused silica 47 cm long, 50 mm bore capillary. The samples were loaded on to the capillary tube by high-pressure loading over 5 s, and the separation was carried out over 15 min with a potential difference of 18.80 kV at 25°C using Tris–HCl pH 8 as running buffer. Samples were taken from the same reaction mixture at increasing time intervals for up to 1800 min. Peak heights of intact peptide and the cleaved products were plotted against time to obtain the rates of hydrolysis.

Data from the capillary electrophoresis (CE) experiments was confirmed and complimented by similar hydrolysis experiments performed on reversed-phase HPLC. The peptide was incubated with the enzyme in the same ratios as for the CE experiments. Aliquots were removed from the reaction mixture at increasing time intervals over 1800 min. The reaction was stopped by immediate pH lowering and freezing of the sample. The samples were then analysed by analytical reversed-phase chromatography in a 0–60% acetonitrile–water (both 0.1% TFA) over 9 min gradient at 2 mL/min.

RESULTS

Affinity chromatography

The following serine proteases were immobilised to CNBr-activated Sepharose beads: subtilisin BPN′, pancreatic elastase, trypsin, anhydro-trypsin, α-chymotrypsin, anhydro-α-chymotrypsin. Soybean trypsin inhibitor was found to bind to trypsin and anhydrotrypsin, whereas Barley chymotrypsin inhibitor-2 bound to α-chymotrypsin, anhydro α-chymotrypsin and subtilisin BPN′. The natural Bowman–Birk inhibitor bound to trypsin, anhydrotrypsin, α-chymotrypsin and anhydro-α-chymotrypsin. These results confirm that the various affinity columns displayed the expected binding specificities.

Application of the cyclic peptide mixture to the im-

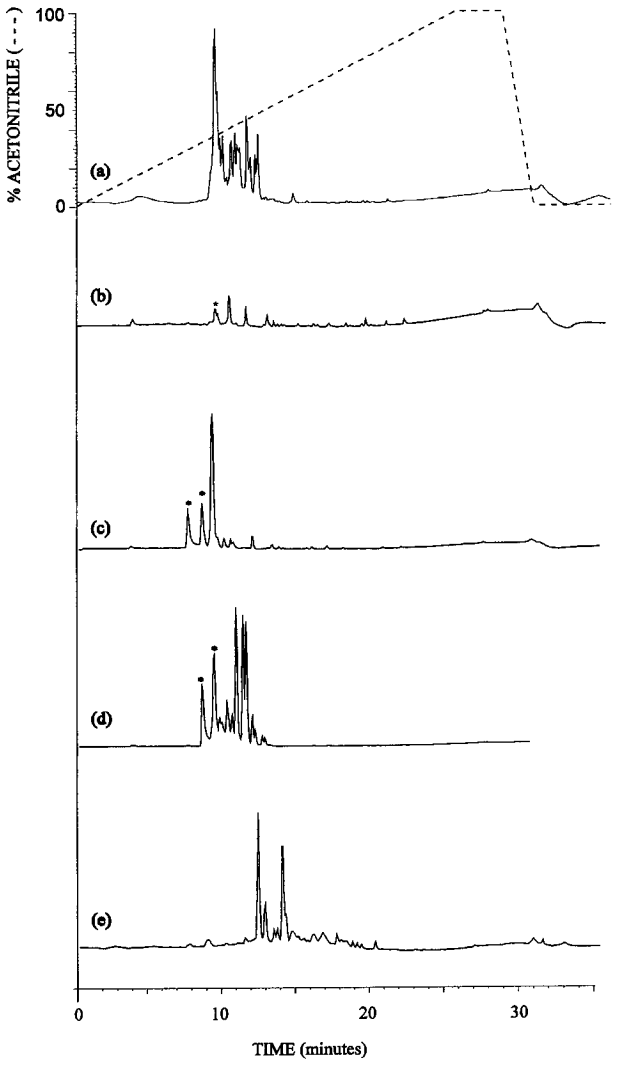

FIGURE 1

Analytical reversed-phase traces of (a) the intact cyclic peptide mixture and the fractions of the mixture which were bound to the different affinity columns, (b) porcine pancreatic elastase, (c) trypsin, (d) α-chymotrypsin and (e) anhydro-α-chymotrypsin. The retention times of the components of the peptide mixture bound to the different affinity columns vary, illustrating how different fractions of the mixture are binding to each serine protease. New peaks with shorter retention times to those in the peptide mixture also arise (marked with an asterisk) from the affinity columns with active enzyme. These are not found with anhydro-α-chymotrypsin. Sequencing results identified them as hydrolysis products (see Results). The number of peptides bound to anhydro-α-chymotrypsin is less than the number of peptides bound to α-chymotrypsin.

mobilised enzyme columns resulted in a subfraction binding to the trypsin, α-chymotrypsin and anhydro-α-chymotrypsin, and a small amount of binding to pancreatic elastase. Weak binding of the mixture to elastase was manifested by a relatively small elution peak at low pH compared to the other columns. There was no detectable binding to anhydro-trypsin or subtilisin BPN′. The linear peptide mixture did not bind to any of the affinity chromatography columns.

Figure 1 shows typical reversed-phase traces of the different fractions of the peptide mixture which bound to each immobilised enzyme. The raw mixture is revealed as a complex batch of peaks on the HPLC trace. The material binding to the various affinity columns is clearly a subset of the parent peptide mixture, as the traces show less diversity. Peaks marked with an asterisk, and only present in the active protease samples, were identified as hydrolysis products by peptide sequencing.

Peptide sequencing

N-Terminal sequencing was used to identify which components of the mixture were selected by the various affinity columns. Sequencing of the peptide samples from the affinity chromatography with active serine proteases shows the presence of two *N*-termini, resulting in the parallel sequencing of two peptide sequences. The two sequences indicate that proteolysis occurs specifically and exclusively at the P1–P1′ site (Table 2). As a consequence of this, some proline was detected at cycle 4 of the sequence irrespective of whether proline was present at the P1 site. In order to resolve this ambiguity, a peptide with a proline at the P1 residue was individually synthesised and kinetically tested for its activity. No proteolysis was found with the anhydro-enzymes and only intact peptides eluted form the anhydro-α-chymotrypsin column.

Peptide sequencing of the intact peptide mixture confirmed the presence of all intended components in our mixture. The sequencing data of the mixture fractions that bound to the affinity columns confirmed that each serine protease exhibited specific affinity towards different peptides of the mixture (Table 3), as suggested by

TABLE 2

The amino acids identified at each sequencing reaction for two separate samples; α-chymotrypsin and anhydro-α-chymotrypsin. In α-chymotrypsin two sequences run in parallel as a result of hydrolysis. No hydrolysis products were identified for anhydro-α-chymotrypsin. The results are shown for eight consecutive reactions. The fourth residue is the random residue (X), the components of which are shown in Table 3

Residue	α-Chymotrypsin		Anhydro-α-chymotrypsin
	N-Terminus of peptide	Hydrolysis product	Full sequence
1	S	S	S
2	C	I	C
3	T	P	T
4	X	P	X
5	S	Q	S
6	I	C	I
7	P	Y	P
8	P	–	P

TABLE 3

The residues detected at the fourth residue in N- *to* C- *terminal sequencing of the cyclic peptides which bound to the different affinity columns. The proline detected from the hydrolysis product has been omitted. The residues are listed from left to right in order of decreasing recovery. Residues recovered at less than 25% of the highest recovered residue for each respective enzyme are in parentheses*

Enzyme affinity	Amino acids detected at residue 4 (the P1 site of the inhibitor)				
Trypsin	Lys	Arg	(Phe)	(Tyr)	
α-Chymotrypsin	Phe	Tyr	Met	Leu	Nle
Anhydro-α-chymotrypsin	Phe	Tyr	(Met)		
Pancreatic elastase[a]	Leu	Met			

[a] The elastase affinity column retained much less peptide material than the other columns (less than 5% of the amount bound to the trypsin column, using the same conditions), indicating only weak binding.

TABLE 4

The hydrolysis rates of the different peptides when they are incubated in excess with α-chymotrypsin for the M11, NL11, L11, F11, Y11 peptides and trypsin for the R11, K11 and O11. These results are compared to the K_i *values of the different peptides with the same enzyme. No inhibition or hydrolysis was detected for the Orn at P1 site peptide with trypsin*

Cyclic peptide	Hydrolysis rate (mol (peptide) s^{-1} mol enzyme)$^{-1}$ $\times 10^{-3}$	Relative rates to the hydrolysis rate of the M11 peptide	K_i values (μM)
M11	0.6	1	2
L11	1	2	9
NL11	4	6	4
F11	2	3.5	0.07
Y11	7	12	0.05
R11	200	344	0.02
K11	9	15	0.01
O11	–	–	–

reversed-phase chromatography. Trypsin bound to peptides with lysine (K11) or arginine (R11) at the P1 site (the fourth residue of the peptide) and to a lesser extent phenylalanine (F11) and tyrosine (Y11). Pancreatic elastase bound to peptides with leucine or methionine at the P1 site. However, the sequencing recoveries for pancreatic elastase were extremely low as a result of weak binding to the affinity column. α-Chymotrypsin bound to the peptides with phenylalanine (F11), tyrosine (Y11), methionine (M11), leucine (L11) or norleucine (NL11) at the P1 site. Anhydro-α-chymotrypsin only bound significantly to F11 and Y11, together with low recoveries of M11. Affinity chromatography performed on the individual peptides K11, R11, O11, Y11, F11, M11, L11 and NL11, on the different enzyme columns confirmed the results obtained with the peptide mixture. The peptide P11 was found not to bind to any of the affinity columns.

Inhibition kinetics

Based on the sequencing results, individual peptides with different P1 sites were synthesised to test their inhibition properties. As described earlier, a peptide with a proline at the P1 site was also synthesised as a control. Competitive inhibition constants (K_i) were measured for the different peptides with trypsin, α-chymotrypsin and elastase. For the tighter-binding inhibitors the inhibition data provided the equivalence point for complex formation, and so measures of the active peptide concentration. In all cases the peptide material was found to be considerably less than 100% active, for example the K11 peptide showed a 1/6 portion of active material. We attribute this to differing *cis*/*trans* isomers of the Pro residues (unpublished data) in the P3′ and P4′ sites resulting in a mix of active and inactive conformers. Accordingly all our data have been corrected to use the concentration of active peptide for the K_i calculations. Table 4 shows the K_i values for those peptides which exhibited inhibition. The K_i of the parent natural inhibitor, D4, with trypsin is approximately 10^{-9} M (21). This is still an order of magnitude lower than our tightest binding inhibitor.

The peptide with Pro at the P1 site (P11) was found not to inhibit any of the serine proteases. The peptides with Lys and Arg at P1 site are inhibitors of trypsin but not of α-chymotrypsin. Peptides with Phe, Tyr, Met, Leu or Nle at the P1 site are inhibitors of α-chymotrypsin but not of trypsin. F11 and Y11 exhibit the tightest binding with α-chymotrypsin. The lowest K_i values are submicromolar and are found for F11 and Y11 with α-chymotrypsin and K11 and R11 with trypsin. L11 and M11 exhibit no measurable inhibition properties ($K_i > 1$ mM) towards pancreatic elastase, although the presence of hydrolysis products in the sequencing results suggests they may act as substrates. None of the α-chymotrypsin inhibitors were found to show measurable trypsin inhibition, and *vice versa*. The weak binding of two of the chymotrypsin inhibitors (F11 and Y11) to immobilised trypsin is not matched by measurable inhibition in the solution assays. This could indicate that the binding of these species was non-specific, or that the conditions of the enzyme assay (relatively high substrate levels) masked any weak interactions. The peptide with ornithine at P1 was inactive against all proteases tested.

Rates of proteolytic hydrolysis

The rates of proteolytic hydrolysis for several of the synthesised peptides were measured using capillary electrophoresis and/or reversed phase chromatography. Figure 2 illustrates four capillary electrophoresis traces taken during tryptic hydrolysis of the K11 cyclic peptide. The peak corresponding to the intact peptide decreases with time as a new peak arises at the same rate corresponding to the hydrolysis product.

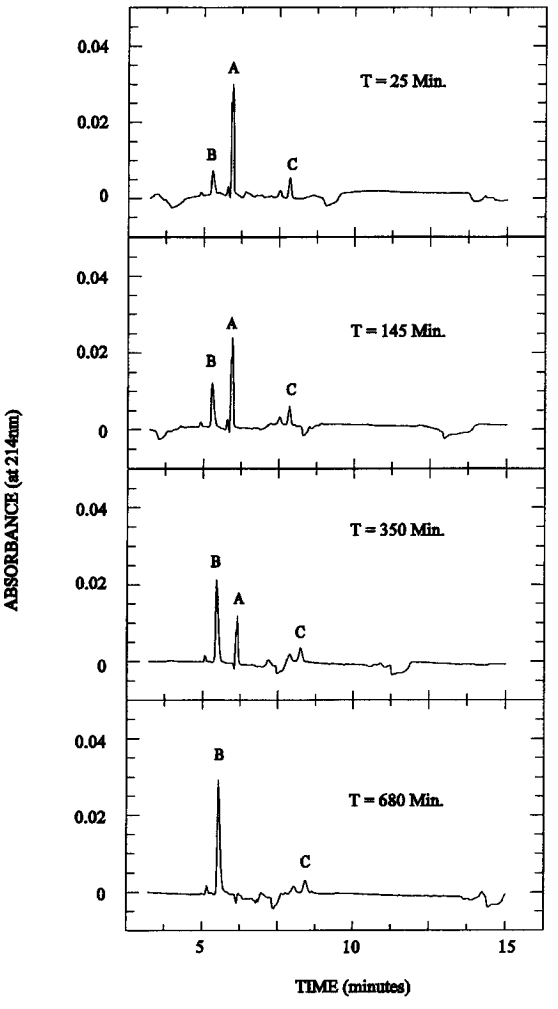

FIGURE 2

Hydrolysis of the inhibitor peptide K11 by the protease was followed over time by capillary electrophoresis (CE). The figure shows four CE traces taken at four different times for a reaction mixture of the K11 peptide incubated with trypsin. The peaks labelled A, B and C are the intact cyclic peptide, the hydrolysis product and trypsin, respectively. Only one hydrolysis product peak is detected with CE, which increases as the intact peptide decreases. The trypsin peak remains constant.

From the CE data the rates of decrease of the intact peptide peak and the rates of increase of the hydrolysis product peak were found to match within experimental error and give the rates of hydrolysis of the cyclic peptides with their serine proteases. Figure 3 shows the rates of increase of the hydrolysis products and the rates of decrease of the cyclic peptide for the K11 cyclic peptide in a CE hydrolysis experiment.

Table 4 compares the measured rates of hydrolysis of the different peptides with trypsin and α-chymotrypsin with their K_i values. To facilitate comparison, all hydrolysis rates have been presented relative to that of M11 with α-chymotrypsin, which exhibited the slowest

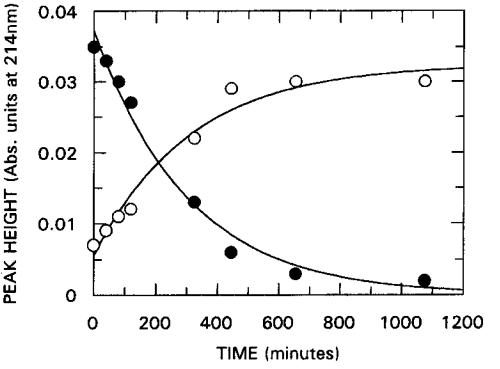

FIGURE 3

Hydrolysis of excess cyclic peptide K11 with trypsin was followed by capillary electrophoresis (CE). Peak heights corresponding to the intact peptide and hydrolysis product after increasing times of incubation were determined from the CE traces (Fig. 2). This graph compares the rate of increase in the hydrolysis product peak (empty circles) height with the rate of decrease in intact peptide peak (filled circles) height. Solid lines show best-fit first-order rate curves.

rate. The R11 peptide is hydrolysed at a significantly faster rate than all the other peptides followed by K11 and Y11, all of which exhibit the tightest binding and lowest K_i values. O11 exhibits no detectable binding to trypsin and no hydrolysis. For M11, L11 and NL11, which exhibit relatively weak binding properties to α-chymotrypsin, the rates of hydrolysis are slow. We distinguish F11 from these last three inhibitors because it binds relatively tightly to α-chymotrypsin and anhydro-α-chymotrypsin, as does Y11, but it is significantly more resistant to hydrolysis than Y11.

DISCUSSION

In this paper we have presented a cost-effective technique for testing a peptide mimic of the Bowman–Birk reactive site loop for a series of serine proteases. The peptide mixture is now available to test against serine proteases other than those presented in this paper. This method, although limited by the quantity of variants (only one randomisation site is possible), is simple to synthesise, and the screening procedure is also technically trivial. The procedure should be applicable to other systems. The suitability to our system hinges on several factors; the peptide synthesis and cyclisation could be achieved at high yields; the randomised site is only four residues into the sequence from the *N*-terminus, where *N*-terminus sequencing is still quite efficient, and the hydrolysis product occurs exclusively at the P1–P1′ amide bond after the randomised site.

We screened for inhibitors for four common serine proteases: porcine pancreatic elastase, subtilisin BPN′, α-chymotrypsin and trypsin. No inhibitors were found for subtilisin BPN′, and only low recoveries of peptides with leucine (L11) and methionine (M11) at the P1 for

porcine pancreatic elastase. Kinetic assays of these peptides with elastase showed no inhibition properties, but they were found to be substrates. The five peptides with leucine (L11), norleucine (NL11), methionine (M11), phenylalanine (F11) and tyrosine (Y11) at the P1 site were found to bind α-chymotrypsin and all were found to inhibit this enzyme. Only peptides with leucine and tyrosine have been reported before as inhibitors of α-chymotrypsin (11, 12), illustrating the utility of approaches that use randomised sequences. The two peptides with lysine (K11) and arginine (R11) at the P1 site were found to bind trypsin. The ornithine peptide (O11), which differs from the lysine peptide by a single methylene group, was not found to bind to trypsin. As this was somewhat surprising, we synthesised this peptide individually to test for inhibition. Studies with purified O11 confirmed the previous results, showing neither detectable inhibition of trypsin nor hydrolysis by trypsin.

The linear peptide mixture, whereby all the cysteines were reduced, showed no inhibition towards any of the enzymes. This suggests that the conformational restriction imposed by the disulfide bond in the cyclic peptide mixture is essential for binding to serine proteases.

The cyclic peptides act both as inhibitors and substrates. Our results show that differences in the P1–S1 interactions modulate both hydrolysis, a kinetic property, and inhibition, which reflects binding affinities. These differences in the enzymatic hydrolysis rates between inhibitors of the same enzyme are independent of the K_i values (Table 4). For example, the rate of hydrolysis of Y11 with α-chymotrypsin is almost four times that of F11 with α-chymotrypsin, although their K_i values are similar. This could be due to subtle differences in the way the peptide interacts with the protease, leading to a more or less favourable orientation of the scissile bond for hydrolysis. It is noted that the trypsin inhibitors R11 and K11 both exhibit a relatively high rate of hydrolysis with trypsin, suggesting a favourable orientation of the scissile bond for hydrolysis in their inhibitor–enzyme complexes. These observations are in accord with recent work by Hedstrom *et al.* (35, 36), who show that there is no obvious correlation between the binding affinity and the catalytic activity for various peptidic substrates and active site directed inhibitors of trypsin, trypsin S1 pocket mutants and chymotrypsin.

The cyclic peptide mixture was also screened for binding with anhydro-α-chymotrypsin and anhydrotrypsin. The peptides F11 and Y11 bound to anhydro-α-chymotrypsin, but no peptides bound to anhydrotrypsin even though the K_i values of the R11 and K11 are in the same sub-micromolar range as F11 and Y11. This difference between the two enzyme systems suggests that interactions with the active site Ser195 may be important for complex formation in the case of trypsin but not so for α-chymotrypsin. This is supported by the relative hydrolysis rates of the four peptides with their respective proteases (Table 4). Both trypsin inhibitors, R11 and K11, are hydrolysed faster than any of the chymotrypsin inhibitors. This might reflect a more intimate interaction with the active site Ser195 in the trypsin–peptide complex and explain why the anhydro-trypsin fails to bind to these peptides. Formation of the α-chymotrypsin-peptide complex does not appear to involve these active site interactions, and consequently it is possible to have a tight inhibitor such as F11 of α-chymotrypsin with a relatively slow rate of hydrolysis. We suggest that M11, NL11 and L11 are weak binding inhibitors as a result of non-optimal interactions in the S1 pocket (relatively high K_i values). They are therefore slow to form the acyl intermediate and so are also poor substrates (illustrated by their relatively slow hydrolysis rates).

The X-ray structure of the Bowman–Birk type inhibitor in a ternary complex with porcine trypsin (6) shows the sub-van der Waals contact between the active site serine O^{γ} (195) of the trypsin and the P1 carbonyl carbon of the inhibitor loop to be significantly smaller (210 pm) than previously observed in trypsin-inhibitor complexes, suggesting that the interaction is more important in this type of inhibitor. It would be interesting to see if the distortion of the scissile bond is retained in the interactions of the Bowman–Birk-type inhibitors with α-chymotrypsin. Affinity chromatography of the natural complete Bowman–Birk inhibitor show that it binds to anhydro-trypsin and anhydro-α-chymotrypsin, although precise binding affinities were not measured. Previous studies of natural inhibitors with trypsin, α-chymotrypsin and their anhydro-analogues suggest that there is little difference in binding between the active enzyme and its anhydro-analogue (24, 25, 37). Our results imply that the loss in conformational restriction of the cyclic peptide inhibitors in comparison to their parent inhibitors has imposed new interaction demands for the formation of the complex between the inhibitor and the serine protease. It is also possible that the greater conformational freedom of the peptides relative to the intact protein will lead to rapid hydrolysis of the amide bond once the pre-acyl intermediate is formed.

The studies of a simple mixture of 21 peptides with serine proteases and their anhydro equivalents have provided a mechanistic insight into the nature of serine protease inhibition by cyclic peptides. Optimal inhibition is produced by tight binding coupled with low hydrolysis rates. Our results show that each of these effects is modulated by changes in P1. They also suggest a difference in the association requirements of these Bowman–Birk-type cyclic peptides with trypsin, which involves interactions with the catalytic Ser (195), and α-chymotrypsin in which this seems less important. We are currently performing studies to examine these interactions more specifically.

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