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Mass Spectrometry Reveals Elastase Inhibitors from the Reactive Centre Loop of α_1-Antitrypsin

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Abstract—Peptides derived from the reactive centre loop of α_1-antitrypsin, a serpin, were screened as potential elastase inhibitors by mass spectrometry. An octapeptide, MFLEAIPM, formed a 'stable' ternary complex with porcine elastase: one MFLEAIPM molecule reacted covalently with loss of water, whilst an additional peptide was bound non-covalently. Kinetic analyses suggested that MFLEAIPM may act as an uncompetitive inhibitor and that the activity was associated with the four N-terminal residues.

Uncompetitive enzyme inhibitors[1] have the potential to distinguish between the same enzyme in the presence of different substrates, an attribute that might be exploited to effect tissue specific inhibition. Standard kinetic analyses to identify uncompetitive inhibitors are time consuming and not readily amenable to rapid screening.

Electrospray ionisation (ESI) MS[2,3] has been used to screen for peptides capable of forming specific complexes with porcine pancreatic elastase (PPE). A simple heptapeptide, YPFVEPI (β-casomorphin7 (BCM7)) was shown to form an unusually stable acyl–enzyme complex with PPE. X-ray analysis revealed the peptide was bound in the S_1–S_4 subsites of PPE and was linked covalently to Ser-195.[4] We reasoned that MS has the potential to screen for uncompetitive enzyme inhibitors by identifying compounds which only form adducts with enzyme: substrate or enzyme:substrate analogue complexes.

Sequence comparisons with BCM7 (YP**FVEPI**) identified a similar sequence (M**FLEAI**PMSI, P_8–P_2') in the reactive centre loop (RCL) of a serpin, α_1-antitrypsin (α_1-AT), which served as the starting point for this investigation. Serpins are serine protease inhibitors which comprise at least 10% of plasma proteins.[5–7] They are involved in the regulation of blood coagulation, fibrinolysis, complement activation and inflammation.[5–7] The current mechanism for serpin mediated protease inhibition involves cleavage of a scissile peptide bond on the exposed RCL (P_{15} to P_5') of the serpin forming an initial ester complex and a C-terminal serpin fragment.[7] The initially formed serpin–protease ester complex then undergoes rearrangement to give a more stable complex. Despite the availability of many separate serpin and protease structures, that of a serpin–enzyme complex remains elusive.

ESI MS was used to screen mixtures of PPE and peptides from the RCL of α_1-AT for formation of ternary complexes.[8–13] Since it was considered that uncompetitive inhibitors were more likely to be non-covalently bound, mild conditions were used: samples contained a ca. 1:25 ratio of protein (20 μM) peptide (500 μM), in 10% (maximum) MeOH/H_2O and were introduced to the spectrometer via a nanoflow ESI interface. Mass spectra were an average of 10 scans and were minimally smoothed. Studies have indicated that at higher cone voltages the relative intensities of non-specific adducts (i.e., those not accurately reflecting solution interactions) are reduced.[14,15] In an attempt to select for ternary complexes reflecting solution interactions, analyses were performed using at least two cone voltages (typically 65 and 115 V).

An initial screen led to the discovery that a peptide (MFLEAIPM), identical to residues P_8–P_1 of the RCL of α_1-AT, formed complexes with PPE (Fig. 1). The mass of the major species (b) was 26854.9±2.9 Da, consistent with dehydration and formation of an acyl–enzyme complex between the peptide (951 Da) and PPE (25919 Da). A significant peak at 27804.2±3.6 Da was also observed. This corresponds to addition of a second molecule of MFLEAIPM to the acyl–enzyme complex,

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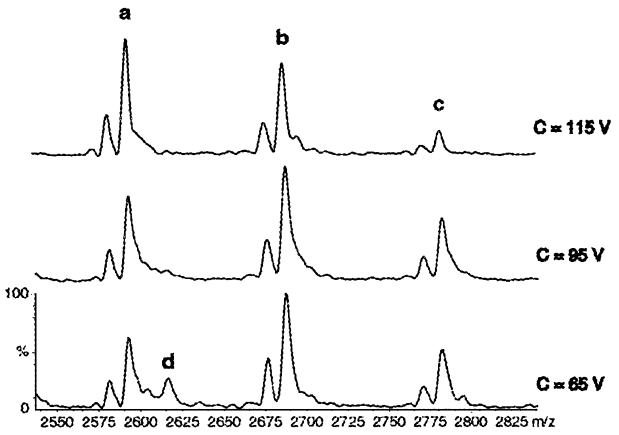

Figure 1. ESI mass spectra (m/z) of PPE (at charge state +10) after addition of MFLEAIPM peptide: (a) intact PPE; (b) MFLEAIPM covalently bound to PPE with loss of H_2O; (c) one molecule of MFLEAIPM covalently bound to PPE with loss of H_2O, with a second molecule of MFLEAIPM non-covalently bound; (d) (at charge state +11) MFLEAIPM non-covalently bound to PPE. Each peak appears as a doublet due to use of a heterogeneous mixture of intact and truncated PPE lacking its C-terminal Asn.

but without dehydration (c). Increasing the cone voltage to 115 V did not completely remove this peak indicating selective binding of the second peptide. An adduct was also observed at 65 V corresponding to MFLEAIPM bound to PPE but without loss of water (d). This adduct was removed upon increasing the cone voltage to 115 V suggesting that it was a non-covalent interaction. When enantiomeric-MFLEAIPM, prepared as a control, was analysed, some formation of a complex of PPE plus one molecule of *ent*-MFLEAIPM was observed.[14] The lack of evidence both for dehydration of this complex and for the formation of a ternary complex, indicated that it reflects a relatively non-specific association.

Kinetic analysis of the inhibition by MFLEAIPM indicated apparent uncompetitive inhibition (Table 1, entry 4).[9–13] *Ent*-MFLEAIPM showed no inhibitory activity (Table 1, entry 5). MFLEAIPM was not a trypsin inhibitor (Table 2, entry 4), possibly due to the presence of a Met at the P_1 position. Kinetic analysis with trypsin of MFLEAIPK, in which the P_1 Met is substituted with Lys, showed competitive inhibition kinetics (Table 2, entry 5), consistent with this peptide binding at the trypsin S subsites. Analysis of MFLEAIPK with PPE revealed uncompetitive inhibition (Table 1, entry 6), suggesting the uncompetitive activity was more likely to be associated with the N-terminal rather than the C-terminal end of the peptide. Addition of residues to the C-terminus of MFLEAIPM resulted in mixed inhibition (Table 1, entries 1–3). Thus, the presence of additional C-terminal residues may favour competitive binding.

Removal of the P_8 Met (Table 1, entry 12) had no discernible effect on inhibition activity. Substitution of the P_7 Phe with an acetyl group (Table 1, entry 13) caused a drop in inhibitory activity. Further truncation, with or without acetyl substitution, from the N-terminus led to loss of inhibition (Table 1, entries 14–18). In contrast, truncation of up to four residues from the C-terminus had little effect on activity; a tetrapeptide, MFLE, maintained the uncompetitive activity (Table 1, entry 10). The inhibitory activity was therefore ascribed to the N-terminal residues of the original octapeptide.

Table 1. Kinetic analysis of synthetic peptides using Suc-AAPA-*p*Na and PPE[a]

	Peptide sequence	K_i (mM)	K_i' (mM)
1	Met-Phe-Leu-Glu-Ala-Ile-Pro-Met-Ser-Ile-Pro-Pro-Glu	0.19±0.02	2.0±1.0
2	MFLEAIPMSI	0.10±0.07	0.34±0.13
3	MFLEAIPMS	5.8±3.8	1.2±0.2
4	MFLEAIPM	—	0.44±0.07
5	*ent*-MFLEAIPM	x	x
6	MFLEAIPK	—	0.64±0.07
7	MFLEAIP	—	0.22±0.02
8	MFLEAI	Not determined	
9	MFLEA	Not determined	
10	MFLE	—	0.37±0.07
11	Ac-MFLEAIPM	Not determined	
12	FLEAIPM	—	0.53±0.11
13	Ac-LEAIPM	—	0.89±0.28
14	LEAIPM	x	x
15	Ac-EAIPM	x	x
16	EAIPM	x	x
17	Ac-AIPM	x	x
18	AIPM	x	x

[a]N-Terminally truncated peptides were synthesised with and without a protecting acetyl group as a control for the positively charged amino group. MFLEAI, MFLEA and Ac-FLEAIPM were insoluble in the assay conditions. K_i = inhibition constant for competitive inhibition, K_i' = inhibition constant for uncompetitive inhibition. x = no inhibition observed up to the solubility limit of the peptide.

Table 2. Kinetic analysis of synthetic peptides with trypsin, using the reporter substrate Ac-R-*p*Na; chymotrypsin (chy-T), using Suc-AAPF-*p*Na and HNE using MeO-AAPV-*p*Na

	Peptide sequence	K_i (mM) (trypsin)	K_i (mM) (chy-T)	K_i (mM) (HNE)
1	MFLEAIPMSIPPE	x	0.24±0.02	0.019± 0.001
2	MFLEAIPMSI	x	0.16±0.01	0.027± 0.002
3	MFLEAIPMS	x	3.1±0.7	0.19± 0.02
4	MFLEAIPM	x	2.3±0.4	0.27± 0.03
5	MFLEAIPK	0.54±0.05	x	x
6	MFLE	x	x	x

Further substitutions/truncations were used to probe the importance of the MFLE residues. Replacement of N-terminal Met with an acetyl group, to give Ac-FLE, led to loss of inhibition (Table 3, entry 1). The tripeptide, FLE, was a weak apparent uncompetitive inhibitor (Table 3, entry 2). These results suggest that the 'full' uncompetitive activity is a function of all four residues within the MFLE peptide. Replacement of P_6 Leu with Ala, gave a peptide, MFAE, which was too insoluble to analyse. Replacement of P_5 Glu with Ala in MFLE changed the inhibition activity from uncompetitive to mixed (Table 3, entry 3).

In vivo α_1-AT also inhibits human neutrophil elastase (HNE) and chymotrypsin. Analysis of MFLEAIPM with chymotrypsin and HNE demonstrated competitive

Table 3. Kinetic analysis of synthetic peptides using Suc-AAPA-*p*Na and PPE[a]

	Peptide sequence	K_i (mM)	K_i' (mM)
1	Ac-Phe-Leu-Glu	x	x
2	Phe-Leu-Glu	—	1.4±0.3
3	Met-Phe-Leu-Ala	1.1±0.4	0.89±0.35
4	Met-Phe-Ala-Glu	Not determined	

[a]The N-terminally truncated peptide was synthesised with and without a protecting acetyl group as a control for the positively charged amino group. MFAE was insoluble in the assay conditions.

inhibition ($K_i = 2.3 \pm 0.4$ mM and 0.27 ± 0.03 mM, respectively) (Table 2, entry 4). MFLEAIPK was not found to be an inhibitor of either enzyme, probably due to the presence of a lysine residue at the P_1 position. There was no observed inhibition of chymotrypsin or HNE by MFLE (Table 2, entry 6). Together these results suggest that the uncompetitive activity of the MFLEAIPM/MFLE peptides is specific to PPE. It is interesting to note that addition of residues to the C-terminal end of MFLEAIPM significantly increased the degree of inhibition observed for both enzymes.

The mode of action and 'proof' of the uncompetitive nature of the inhibition will require further studies. However, the apparent uncompetitive inhibition may involve binding interactions previously unexploited and be of interest in the design of inhibitors. Since, it is probable that the covalently bound MFLEAIPM occupies the S subsites, the non-covalently bound, 'uncompetitive', MFLEAIPM must bind elsewhere. This proposal is supported by the observation that both MFLEAIPM and MFLEAIPK are uncompetitive PPE inhibitors, but only MFLEAIPM forms an acyl–enzyme complex with PPE. Further, the ESI MS studies suggest that the uncompetitive inhibitors bind to acylated PPE and it is reasonable to propose that they operate via stabilisation of this complex. If the uncompetitive inhibitors lie in the PPE S′ subsites, it is possible they displace the 'hydrolytic' water.[4] thereby preventing cleavage of the acyl–enzyme complex and leading to concomitant stabilisation.

It is possible the 'uncompetitive' inhibition observed for MFLEAIPM and related peptides may reflect stabilising interactions during the rearrangement of an initially formed acyl–enzyme complex en route to the final 'stable' complex.[16–18] However, given that the uncompetitive inhibition by the MFLE and related sequences can only be observed after serpin cleavage, the interaction is probably not related directly to formation of the stable serpin–enzyme complex.

A C-terminal fragment resulting from cleavage of the α_1-AT RCL has been shown to be present in human tissue.[19,20] The fragment, named short peptide from α_1-antitrypsin (SPAAT), contains the MFLEAIPM sequence at its N-terminus and the normal scissile bond of the RCL remains uncleaved. Cleavage of SPAAT at the P_1–P_1' scissile bond by HNE or chymotrypsin would subsequently release MFLEAIPM to act as an inhibitor. It is possible that SPAAT and MFLEAIPM constitute part of a 'cascade of mechanisms' derived from α_1-AT by which serine proteases are inhibited.

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