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SIMULATION OF THE INHIBITORY CYSTATIN SURFACE BY A SYNTHETIC PEPTIDE

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SUMMARY : An inhibitory dodecameric peptide was designed which tentatively mimics the inhibitory site of cystatin C-like structures. Succinylated and mansylated derivatives were also synthesised and assayed for their inhibiting properties towards papain and rat cathepsins B, H and L. All peptides preferentially inhibit cathepsin L and papain as their naturally occurring inhibitor model. A significant increase in inhibition was obtained after mansylation of the crude peptide with Ki values in the micromolar or 0.1 micromolar range. The use and interest of such peptide inhibitors are discussed.

Most of cysteine proteinases structurally related to papain may be inhibited by naturally occurring proteinase inhibitors of the cystatin superfamily (1). Though the mechanism of inhibition remains unclear, comparisons between members of the three main cystatin families suggest that highly conserved amino acid residues are involved in the inhibitory mechanism (2-4). One of these conserved sequences, QVVAG, or its closely related derivatives (see 1 for a review) found in all members of the superfamily was first reported as being part of the inhibitory site. It appeared rapidly however, that other regions in cystatin-like molecules were necessary to obtain a potent inhibition of cysteine proteinases. The peptide bond of the glycyl residue located in the N-terminal region of all members of cystatin-like inhibitory sequences has been thought to be involved in a substrate-like inhibitory site of chicken cystatin and human cystatin C(3). More recent data on the inhibition mechanism of papain by chicken cystatin have demonstrated the importance of one or two residues preceding the conserved Gly residue for binding (6). A third region of chicken cystatin, located close to the C-terminal end, contains a trytophan residue flanked by a conserved proline residue and has been proposed to be part of the papain binding site(4). The recent determination of the X-ray crystal structure of chicken cystatin gives additional support to these data. It shows that all three suspected regions

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ABBREVIATIONS:mansyl,6-(N-methylanilino)naphtalene-2-sulfonyl;
Z,benzyloxycarbonyl; NMec, 7-amino-4-methylcoumarylamide;
AFC, 7-amino-4-trifluoromethylcoumarylamide;
E64, L-trans-epoxysuccinylleucylamino(4-guanido)butane.

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in chicken cystatin are in close proximity and computer modeling suggests that these regions are complementary to the active site of papain (5).

In this study we designed a peptide mimicking the postulated inhibitory site of chicken cystatin on the basis of the available information described above. This peptide corresponds to the sequence KGAGQVVAGPWK and was designated K12K for the sake of brevity. Its inhibitory properties as well as those of some modified analogues have been studied towards papain (EC 3.4.22.2) and rat liver cathepsin B (EC 3.4.22.1), H (EC 3.4.22.16) and L (EC 3.4.22.15).

MATERIAL AND METHODS

PEPTIDES : The peptide corresponding to the sequence KGAGQVVAGPWK was synthesised by the solid phase method of Merrifield (7) using an automated Applied Biosystems 431A peptide synthesiser. To deblock the formyl tryptophan trifluoromethylsulfonic acid was used at 4°C before cleaving the peptide from the resin at room temperature with the same agent. The cleaved peptide was purified on an Aquapore RP 300 CB column by high pressure liquid chromatography in a 0 to 50% acetonitrile gradient starting from a 0.1% trifluoroacetic acid solution in water. Amino-acid analysis of the purified peptide showed a purity higher than 99%. The succinylated peptide was prepared by adding to 8.5 µmoles of peptide dissolved in 1 ml of a 0.1 M phosphate buffer at pH 7.0, 132 µmoles of succinic anhydride. During the reaction the pH was held constant by adding aliquots of a NaOH 1 N solution until complete dissolution of the succinic anhydride. After reaction, the succinylated peptide was separated from the reagents by molecular sieving on a Sephadex G25 column in 1% acetic acid and the eluent was lyophilised. Mansylation (6-(N-methylanilino)naphtalene-2-sulfonylation) of the peptide was performed by incubating 6.3 µmoles of peptide dissolved in 0.5 ml triethylamine buffer pH 10 with 7.5 µmoles mansylchloride (Molecular Probes, Oregon) dissolved in 1ml acetonitrile. After mixing the pH was restored to pH 10 by adding triethylamine and the mixture allowed to react overnight. The mixture was lyophilised and redissolved in 0.1% acetic acid. This solution was subjected to high pressure liquid chromatography using a 10 to 100% acetonitrile gradient. All peptides were lyophilised after high pressure chromatography and redissolved before use. The dimansylated peptide was dissolved in $NaHCO_3$ 0.1M while the other peptides were dissolved in water. Solutions were kept at -20°C until further use. N-trifluoromethylcoumaryl substrate derivatives were a generous gift of Dr J.BIETH Inserm U237 Strasbourg, France). N-methylcoumaryl derivatives were purchased from Bachem (Bubendorf, Switzerland).

ENZYMES : Crystallized papain (Boehringer-Mannheim) was prepared and titrated as described before (8) using the irreversible epoxide inhibitor E64 as an active site titrant, and was used at a final concentration of 2.5 nM. Rat liver cathepsins B, H and L were purified and titrated as previously described (9) and used at final concentrations of 3 nM, 10 nM and 3.75 nM respectively.

KINETICS MEASUREMENTS : Inhibition constants (Ki) were calculated according to the method of Dixon (10), plotting 1/V versus I, at three different substrate concentrations. Alternatively, true Ki values were determined from Ki(app) values when Michaelis constants were known (11) and using the following equation which refers to a competitive mechanism:

$$Ki = Ki(app) / 1+S/Km.$$

Z-Arg-Arg-NMec, Arg-NMec were first used as substrates for cathepsins B, H respectively whereas Z-Phe-Arg-NMec was chosen for cathepsin L and papain. However, using N-methyl-coumarin as a fluorophore resulted in a fluorescence background when mansylated peptides were reacted. AFC derivatives (AFC), the excitation spectrum of which does not overlap with that of mansyl groups, were therefore chosen as substrates to react with fluorescent inhibitors. All substrates were used at final concentrations ranging from 1 to 75 µM. Assays were carried out in 0,1 M phosphate buffer pH 6.0, 1mM EDTA, 2 mM dithiothreitol, 0,1% Brij 35 for cathepsins B and L and in 0,1 M phosphate buffer pH 6.8, 1mM EDTA, 2 mM dithiothreitol, 0,1% Brij 35 for cathepsin H and papain. Enzyme solutions were incubated for 5 minutes at 30°C with increasing amounts (0 to 500 µM final) of inhibitory peptides before substrate was added to start the reaction. Recordings were made on a Jobin-Yvon JY3D spectrofluorimeter setting excitation and emission wavelenghts at 350nm and 460nm respectively for N-methylcoumaryl derivatives and at 400nm and 480nm respectively for N-trifluoromethylcoumaryl derivatives (12).

RESULTS AND DISCUSSION

Numerous attempts have been made to design synthetic inhibitory peptides which mimic reactive sites of protein inhibitors of serine proteinases (reviewed in 13). The design of synthetic inhibitors of cysteine proteinases has not been developped to the same extent . First attempts in this field were carried out by Teno et al (14-15) who synthesised QVVAG derivatives as well as a tridecapeptide corresponding to a sequence in T-kininogen which also includes the QVVAG sequence. However the former appeared to be poor inhibitors of papain and cathepsin B whereas the latter lacked any inhibitory activity. This is not surprising since the recent elucidation of the 3D architecture of chicken cystatin gave an additionnal proof that at least three regions in chicken cystatin were involved in the binding and inhibition of cysteine proteinases (5). The intimate mechanism of inhibition by cystatin-like inhibitors is not yet clarified however: parallel studies of the interaction between other members of the cystatin superfamily and cysteine proteinases have shown that the mechanism of binding and inhibition may be somewhat different from that described for chicken cystatin. In particular several cystatin like molecules including kininogen fragments which lack the N-terminal part containing the critical glycyl residue have been shown to retain potent inhibitory activity towards cysteine proteinases (16-18). It is therefore uncertain that all members of the cystatin superfamily obey the same mechanism to bind and inhibit their target enzymes. Nevertheless we designed in this study a water soluble dodecameric peptide which includes amino acids from the three critical regions of chicken cystatin thought to play a role in the inhibitory activity. Several derivatives were also constructed to better understand the mechanism of interaction.

Succinylation of the side chains of lysyl residues was first carried out to investigate the possible effects of positive charges on inhibition. Fig 1 shows the high pressure reverse phase chromatographic profile of K12K after succinylation which demonstrates that over 80% of the material is disubstituted whereas about 5 to 10% is monosubstituted and 5% remains in the native state. Therefore no further purification was performed to assay inhibitory properties.

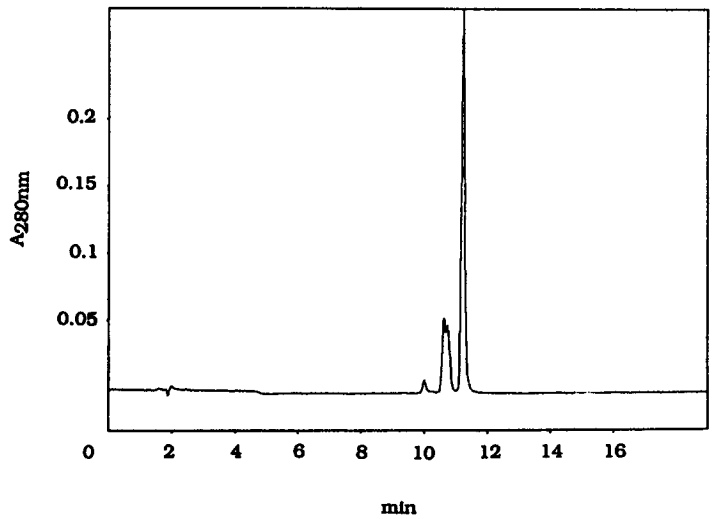

Figure 1 : HPLC profile of the succinylated peptide. A 10 to 70% acetonitrile gradient was used and the optical density measured at 280 nm. The first peak corresponds to unmodified K12K peptide, the second double peak to the two forms of monosuccinylated K12K and the third peak to the disuccinylated K12K.

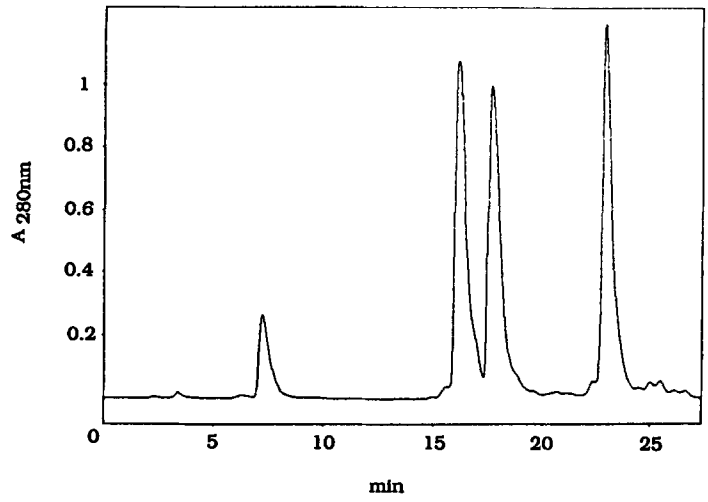

Figure 2 : HPLC profile of the mansylated peptide. A 10 to 100% acetonitrile gradient was used and the optical density measured at 280 nm. The first peak corresponds to the unmodified K12K peptide, the second to the N-terminal lysine monomansylated peptide MnsK12K, the third one to the C-terminal lysine monomansylated peptide K12KMns and the fourth one to the dimansylated peptide (MnsK12KMns).

Mansylation of the K12K peptide was also carried out to possibly enhance its interaction with proteases: mansyl groups, though they have no intrinsic affinity towards papain, have been reported to combine with this enzyme in the region of its active site when attached to a substrate peptide of suitable length and structure. As a result, a significant decrease of the Km value was obtained(19). Another interesting feature resulting from the introduction of such a fluorescent probe into the K12K peptide is to allow direct interaction studies through modifications of fluorescence properties. Mansylation of the K12K peptide was made as described under Methods. As shown on fig 2, four peaks were separated after high pressure liquid chromatography using a 10 to 100% acetonitrile gradient. Those corresponded to the unreacted peptide, the mono-substituted peptides and the di-substituted peptide respectively as assessed from their absorption spectra (fig 3). Measurements of energy transfer between the tryptophan residue of the peptide and the mansyl groups (data not shown) suggested that the first mansylated peptide was substituted at the N-terminal lysine while the second mansylated peptide was substituted at the C-terminal lysine, nearest to the tryptophan residue.

Crude K12K peptide as well as its derivatives were assayed for their inhibitory properties towards papain and rat liver cathepsins B, H and L. Ki values were determined according to Dixon (10) and are reported in Table 1. All peptides retain significant inhibitory properties towards these cysteine proteases though to varying extents. This depends both on the nature of the protease used and on the mansylation of the K12K peptide. As expected, a far better inhibition was obtained using mansylated peptides with Ki values 100 to 500 times lower than those obtained with the crude peptide. The best inhibition was obtained in any case with the dimansylated peptide which could be indicative of a more favourable conformation to interact with cysteine proteinases.

Positive charges brought by lysyl residues have no effect on the inhibitory properties since no significant change of Ki values were obtained using succinylated K12K.

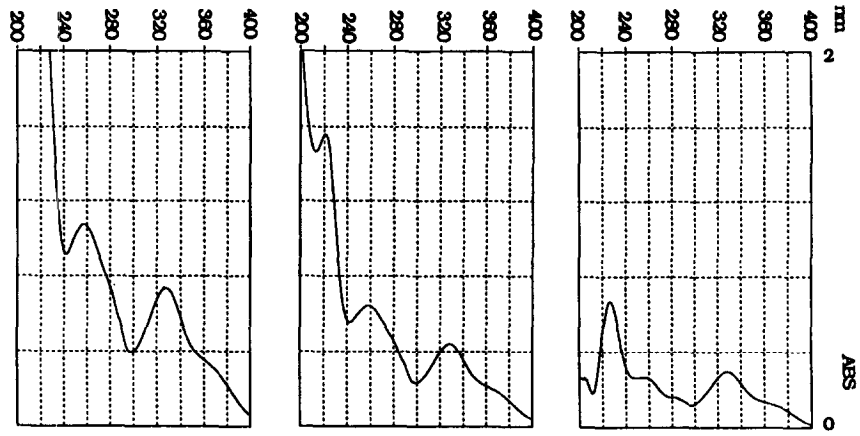

Figure 3 : Absorption spectra of the three mansyl-substituted peptides. From left to right, spectra corresponding to respectively MnsK12K, K12KMns and MnsK12KMns i.e the second, the third and the fourth peak in the HPLC profile of Figure 2.

On the other hand, it is worth noticing that lower Ki values were observed using papain and cathepsin L whatever the peptide used, a result also obtained with naturally occurring inhibitors of the cystatin C family which served as models to design the K12K peptide (20).

A first attempt to simulate by synthetic peptides discontinuous protein surfaces which interact with antibodies has been reported by Atassi et al.(21). An extension of that approach by Geysen et al.(22) resulted in the definition of "mimotope" which tags a molecule able to bind to the antigen combining site of an antibody but not necessarily identical with the epitope inducing the antibody. To generalize this concept to all protein-protein interactions, inhibitory peptides which were synthesized using the same strategy may be called cystatin mimotopes. Of prime interest is the fact that these peptide mimotopes may bind to several subsites on the target protease (13) which is of considerable interest to understand the mechanism of interaction with naturally occurring inhibitors. However, no synthetic structure has been

Table 1 : Ki values (μM) for the interaction of the K12K peptide and its modified analogues with papain and rat liver cathepsins

Enzyme	Ki for				
	K12K	Succinylated K12K	MnsK12K	K12KMns	MnsK12KMns
	μM				
Papain	140	143	3.8	3.2	0.5
Cathepsin L	105	n.d	5.2	3.5	0.4
Cathepsin B	1370	n.d	25	6.4	1.8
Cathepsin H	1000	n.d	30	10.0	5.0

n.d : not determined.

designed so far which retains as potent inhibitory properties as its protein model. This could be due to secondary events in the protein structure increasing the binding affinity after recognition of the protease or to conformational restrictions of the sequence within the cystatin structure. As a result, the Ki values for the peptide inhibitors are generally several order of magnitude higher than those of natural inhibitors. However the Ki values reported here for the interaction between cathepsin L or papain and the mansylated mimotope account among the lowest ever reported for peptide inhibitors (13, 23), thus encouraging further studies in this way. In particular, transition state analogue inhibitors which retain more potent inhibitory activity could be generated from this peptide structure (23). On the other hand, the design of peptide mimotopes as protease inhibitors could also be used to raise antibodies which may react with their naturally occuring proteinase inhibitor model.

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