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Active site chemical mutagenesis of *Ecballium elaterium Trypsin Inhibitor II*: New microproteins inhibiting elastase and chymotrypsin

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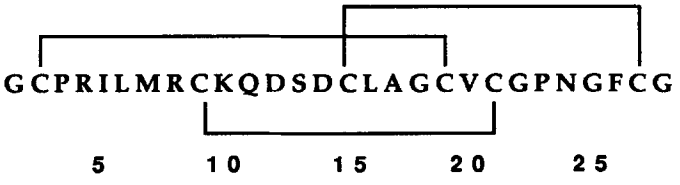
Seven microproteins analogous to *Ecballium elaterium Trypsin Inhibitor II.* were synthesized. The study of their inhibiting power showed a change in selectivity from trypsin to elastase for 5 of the compounds and to α-chymotrypsin for another one. A striking characteristic that appeared during this synthetic approach was the ability of the 28 amino acid peptides to refold and close correctly the 3 disulfide bridges, giving in each case an active compound.

Seeds of *Ecballium Elaterium*(jumping cucumber) are a rich source of protein inhibitors of proteinases : we recently isolated, sequenced, and characterized the main trypsin inhibitor [1], which, following the international nomenclature, is designed as EETI II [2].

Its primary structure indicates that it belongs to the squash family [3], a recently discovered family of serine protease inhibitors [4] of small peptides rich in disulfide bridges. The chemically synthesized EETI II [5] is the shortest microprotein inhibiting a serine protease so far known, with an association constant of 8.10^{11} M^{-1}. It contains 28 amino acid residues and three disulfide bridges ; the primary structure is outlined below.



The primary structure of EETI II

Though the primary structure is closely related to other microproteins extracted from various cucurbitacaea, its chemical synthesis was facilitated by an unique

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feature of the C-terminal sequence, where a -Gly-21-Pro-22-Asn-23-Gly-24- β-turn forming section behaves as a strong template to drive the correct folding in high yield and selectivity. Large quantities of EETI II could be prepared, enough to make a complete 2D NMR study that, followed by a DISGEO modelisation of all 15 possible disulfide bridge combinations made possible the assignment of the disulfide bridges [6]. A preliminary radiocrystallographic study of the crystals obtained for the 1/1 complex with porcine trypsin has confirmed this attribution [7].

EETI II behaves as a "no-turnover" substrate, the cleaved product retaining full association towards the enzyme ; by analogy with the other members of the squash family, we located the scissile bond at -Arg-4-Ile-5- . These residues occupy the P1 and P'1 subsites of the enzyme respectively. On the basis of other examples reported in the literature [8], it can be anticipated that the modification of these residues could deeply alter the specificity of EETI II and direct it to other serine proteinases.

Experimental

Synthesis : The products were synthesized according to the solid phase procedure described previously [9]. t-Boc-Nα-protected amino acids are sequentially introduced using BOP coupling reagent [10]. After complete deprotection with HF, ring closure is performed by air-oxidation in the following conditions : The crude peptides were dissolved in water (1mg/ml), pH was adjusted to ≈ 8 and kept at this value throughout the reaction with DIEA ; the solution was vigorously stirred at room temperature until no thiol was detected by DTNB [11] tests (≈ 50 hours). The cyclization was monitored by reverse-phase HPLC using a Merck Lichrosorb RP-18 column (5μm, 12.5cm x 0.4cm I.D.) and a buffer system consisting of solvent A = 0.1%TFA in H_2O and solvent B = 60% CH_3CN in H_2O + 0.1% TFA.

After cyclisation, the solutions were filtered, loaded onto a semi preparative HPLC column (Whatman ODS3 10μm, 50cm x 2.2cm I.D.) and eluted in a gradient mode with the buffer system described above. In each case, one single fraction corresponding to the expected compound was collected and lyophilized (yield ≈ 10%). The peptides composition was determined by amino acid analysis ; the structure of the parent compound EETI II was further ascertained by full sequence determination.

Enzyme assays : Bovine trypsin from Cooper Biomedical, bovine chymotrypsin from Sigma CC., porcine elastase from Sigma CC. were used for the present study. The substrates Nα-benzoyl-arginyl-ethyl ester , Nα-benzoyl-arginyl-p-nitro-anilide and Nα-acetyl-L-tyrosine ethyl ester were obtained from Fluka; Nα-benzoyl-L-arginyl-7-amido-4-methyl-coumarin, Nα-succinyl-L-alanyl-L-alanyl-L-phenylalanyl-7-amido-4-methyl-coumarin and Nα-succinyl-L-alanyl-L-alanyl-L-alanyl-p-nitroanilide from Bachem. Initial rates of enzyme activity were measured as a function of time on a Kontron spectrophotometer or a Perkin-Elmer spectrofluorimeter. When necessary, controls were achieved on chosen samples to ascertain quantitatively the presence of both substrate and product by HPLC. Ka values were determined at high dilution by the method of Green & Work [12] as modified by Laskowski [13]. In the high dilution assays, glassware were avoided and quartz used routinely : the time to reach equilibrium for the enzyme-inhibitor associations was optimized for each peptide. The buffer in all

cases was 0.2M Tris (pH 8.3), 0.01% Triton, and the substrates were about 10-fold lower than Km concentration. Kd values were evaluated by a computerized fitting of the experimental values of Vrel = f (inhibitor) with the theoretical given by the equation :

$$Vrel = \frac{1+\frac{Km}{S}}{1+\frac{Km}{S}+\left(\sqrt{\left(1+\frac{Et-It}{Kd}+\frac{S}{Km}\right)^2+4.\frac{It}{Kd}\left(1+\frac{S}{Km}\right)}-\left\{1+\frac{Et-It}{Kd}+\frac{S}{Km}\right\}\right)\frac{Km}{2S}}$$

describing the system :

$$E + S \rightleftharpoons ES \longrightarrow \text{products}$$

$$E + I \rightleftharpoons EI$$

$[EI] = 1/Kd\,[E].[M]$ $\qquad$ $Et = [E] + [EI] + [ES]$

$[ES] = 1/Km\,[E].S$ $\qquad$ $It = [I] + [EI]$

where Kd is the enzyme-inhibitor dissociation complex, Km the Michaelis constant, Et the total concentration of the enzyme, It the total concentration of the inhibitor, S the initial concentration of the substrate.
Scheme 1 gives an example of these determinations.

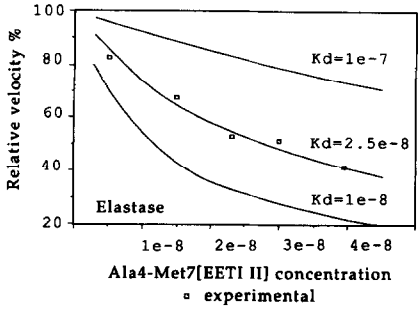
Results and Discussion

Seven EETI II analogues bearing modification in positions 4 and/or 5 have been prepared; Met-7 was also replaced by nor-leucine for a higher chemical stability of the products. In every case, the cyclisation of the disulfide bridges occurred in high yield and selectivity.

Table 1 gives a summary of the results obtained for EETI II analogues, all 28 amino-acids long.

Nor-leucine replacement of methionine-7, which has been successful in other biologically active peptides [14] results in a loss of inhibitory power of EETI II, thus confirming the importance of this residue present in all the squash trypsin inhibitors. The same effect can be observed with the elastase inhibitors.

The changes brought about by subsite S1 mutation are spectacular, since the replacement of Arg-4 by either Ala or Phe exerts strong differential effects, weakening the parent interaction with trypsin and enhancing the new one with elastase or chymotrypsin respectively. Changes in secondary sites may enhance these constants as in Nle-4, Ser-5, Nle-7-[EETI II] as compared with Nle-4, Nle-7-[EETI II] and prompt us towards a rational molecular designing of therapeutically



SCHEME 1

TABLE 1. Dissociation constants of EETI analogues towards three serine proteinases (- means very low if none inhibition)

EETI II derivatives	Trypsin	Elastase	α-Chymotrypsin
EETI II (1-28)	$1\ 10^{-12}$ M	-	-
Nle-7	$4\ 10^{-10}$ M	-	-
Ala-4	-	$2.5\ 10^{-8}$ M	
Ala-4, Nle-7	-	$6\ 10^{-7}$ M	
Nle-4, Nle-7	-	$3\ 10^{-6}$ M	
Nle-4, Ser-5, Nle-7	-	$8\ 10^{-7}$ M	
Val-4, Nle-7	-	$5\ 10^{-6}$ M	
Phe-4, Nle-7	-	-	$2\ 10^{-7}$ M

active molecules able to prevent protease mediated pathologies. The S1 replacements presented here are by no mean exhaustive : the small size of these microproteins, together with the unfailing correct refolding of the peptide chain, probably through correct disulfide bridge forming, yielding each time a product inhibiting the target protease, leads us to believe that less known or more complex serine proteases could be specifically inhibited by controlled replacements in the active site of EETI II.

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References

1. Favel, A., Mattras,H., Coletti-Previero, M-A., Zwilling, R., Robinson, E.A. & B. Castro (1988) Int. J. Peptide Protein Res , in press.
2. Wilusz, T., Wieczorek, M., Polanowski, A., Denton, A., Cook, J. & Laskowski, M. Jr. (1983) Hopppe-Seyler's Z. Physiol. Chem. 364, 93-95.
3. Wieczorek, M., Otlewski, O., Cook, J., Parks, K., Leluk, J., Wilimowska-Pelc, A., Polanowski, A., Wilusz, T. & Laskowski, M. Jr. (1985) Biochem. Biophys. Res. Com. 126, 646-652.
4. Powers, J.C. & Harper, W. (1986) in Proteinase Inhibitors, Barrett, A.J. & Salvesen, G. Ed. Elsevier, 55-152.
5. Le-Nguyen, D., Nalis, D., Heitz, A. & Castro, B. (1988) Proc. 20th European Peptide Symposium, Tubingen , in press.
6. Heitz, A., Chiche, L., Le-Nguyen, D. & Castro. B. (1989) Biochemistry under press.
7. J.P. Mornon personal communication
8. Carrell, R.W., Jeppsson, J.-O., Laurell, C.-B., Brennan, S.O., Owen, M.C., Vaughan, L. & Boswell, R. (1982) Nature 298, 329-334.
9. Le-Nguyen, D., Heitz, A. & B. Castro. (1987) J. Chem. Soc. Perkin I, 1915.
10. Le-Nguyen, D. & Castro, B. (1988) in Peptide Chemistry 1987, Shiba T. & Sakakibara S. Ed. Protein Research Foundation, Osaka , 231-238.
11. Ellman, G.L., (1973) Arch. Biochem. Biophys. 82, 70-77.
12. Green, N.M. & Work, E. (1953) Biochem. J. 54, 347-352.
13. Park, S.J., Tashiro, M., Empie, M.W., Cook, J.A. and Laskowski, M. (1984) Fed. Proc. 43, 1962-1963. .
14. Bodanszky, M., Natarajan, S., Hahne, W. & Gardner, J. (1977) J. Med. Chem. 20, 1047-1050.