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Low Molecular Mass Inhibitors of Bovine β-Trypsin Containing Binding Loop Fragment of CMTI-III*. Synthesis and Evaluation of Inhibitory Activity

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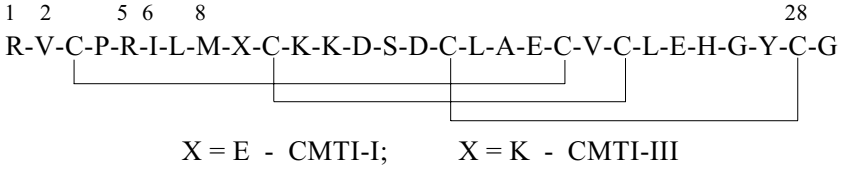
Three peptides with sequences related to the binding loop of *Cucurbita maxima* trypsin inhibitor III were obtained: [G^3,G^{10}]CMTI-III (1-10) (**1**), [A^1,G^3,A^9,G^{10}]CMTI-III (1-10) (**2**) and c(K-A-A-P-R-I-L-M-K-Y-A-E) (**3**). All peptides were synthesized by the solid--phase method using the Fmoc/Bu^t procedure. Peptide **1** revealed a relatively high inhibitory activity (association equilibrium constant with bovine β-trypsin $K_a = 4\times10^9$ [M^{-1}]). Significantly lower activity ($K_a = 3.6\times10^4$ [M^{-1}]) was obtained for peptide **3**. Peptide **2** appeared to be inactive.

Key words: peptidic trypsin inhibitors, solid-phase peptide synthesis

In the seeds of plants from the squash family (*Cucurbitaceae*) polypeptide serine proteinase inhibitors occur [1,2]. Molecules of these inhibitors consist of 27–30 amino acid residues, each including six L-cysteine residues forming three disulfide bridges. The first isolated members of this inhibitors family are CMTI-I and CMTI-III (*Cucurbita maxima* trypsin inhibitor I and III). The reactive site peptide bond in both inhibitors is located between R^5 and I^6 [3].



The X-ray structural examinations of the complex of CMTI-III with bovine β-trypsin [4] have demonstrated that this inhibitor interacts with the enzyme through the exposed binding loop (segment V^2….M^8). The side chain of R^5 (position P1 of the reactive site) penetrates the enzyme substrate pocket, playing an important role in the recognition of the enzyme.

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* Abbreviations: The symbols of amino acids, peptides and their derivatives are in accordance with the 1983 Recommendations of the IUPAC-IUB Joint Commission on Biochemical Nomenclature [*Eur. J. Biochem*., **138**, 9 (1984)] and European Peptide Society [*J. Peptide Sci*., **5**, 465 (1999)]

In the middle of the 1980s we reported the chemical synthesis of both CMTI inhibitors [5–8]. Since then we obtained more than 40 of their analogues. Some of them inhibited the activity of chymotrypsin or elastase [1,2]. Having in mind the potential use of such low-molecular mass proteinase inhibitors in medicine, we decided to obtain CMTI analogues with a simplified structure. We have shown that the N-terminal dipeptides and/or C-terminal Gly from CMTI-III and its analogues can be removed without a significant loss of the trypsin inhibitory activity [1,2]. Continuing this type of investigation, we report here the chemical synthesis of relatively small peptides, which contain a fragment of CMTI-III binding loop. Recently this approach was successfully applied by Leatherbarrow's group to design efficient trypsin [9], chymotrypsin [10] and elastase [11] low molecular inhibitors using the binding loop of the Bowman-Birk inhibitor. The amino acid sequences of peptides synthesized are given below:

(**1**) R-V-G-P-R-I-L-M-K-G [G^3,G^{10}] CMTI-III (1-10)

(**2**) A-V-G-P-R-I-L-M-A-G [A^1,G^3,A^9,G^{10}] CMTI-III (1-10)

(**3**) ┌K-A-A-P-R-I-L-M-K-Y-E┐

Peptide **1** is a 1–10 fragment of CMTI-III, in which two Cys residues involved in the disulfide bridges formation were replaced by Gly residues. Additionally, in peptide **2** two basic amino acid residues, Arg1 and Lys9, were replaced with Ala and Gly residues, respectively. Considering that trypsin hydrolyzed peptide bonds formed by carboxyl groups of these amino acids, their elimination from the molecule might increase the resistance of such a peptide against the enzyme. Homodetic cyclic peptide **3** contains a 4–9 fragment of CMTI-III. Its cyclic structure should preserve the geometry of the binding loop characteristic of the canonical proteinase inhibitor [12], and also increase the proteolytic stability.

We expected that peptides containing reactive site and most of the binding loop's amino acid residues should easily interact with the enzyme and exhibit inhibitory activity to bovine β-trypsin.

EXPERIMENTAL

Starting materials: All amino acid derivatives were obtained from Bachem AG (Bubendorf, Switzerland) and except for glycine were of L-configuration. Tenta Gel S AC FmocGly resin with capacity 0.22 mmol/g (peptide **1** and **2**) and FmocGlu (OBu^t)-Trt(2-Cl)Cl resin with capacity 0.83 mmol/g (peptide **3**) were purchased from Rapp Polymere (Tübingen, Germany). Sephadex G-10 and Sepharose 4B were obtained from Pharmacia (Uppsala, Sweden). Tris was purchased from Serva Fine Chemicals (Heidelberg, Germany). Acetic acid (AcOH), (benzotriazol-1-yloxy) tripyrrolidinophosphnium hexafluorophosphate (PyBOP), dichloromethane (DCM), diisopropylcarbodiimide (DIPCDI), diethyl ether (Et_2O), diisopropylethylamine (DIPEA), dimethylformamide (DMF), dimethylsulfoxide (DMSO), N-hydroxybenzotriazole (HOBt), methanol (MeOH), N-methylpyrrolidone (NMP), piperidine, trifluoroacetic acid (TFA), trifluoroethanol (TFE) and triisopropylsilane were obtained from Flucka AG (Buchs, Switzerland). Acetonitrile HPLC-grade and bovine β-trypsin (EC3.4.21.4) were purchased from Merck AG (Darmstadt, Germany). Bz-D,L-Arg-4-nitroanilide (BAPNA), Bz-Val-Gly-Arg-4-nitroanilide, triton X-100 and anhydrotrypsin immobilized on agarose gel were obtained from Sigma Chemicals Co. (St. Louis, U.S.A.).

Solid-phase peptide synthesis: Fmoc-Gly resin (peptide **1** and **2**) or FmocGlu(OBu^t) resin (peptide **3**) were used for automated solid-phase synthesis on an Applied Biosystem Model 430A synthesizer. A standard machine programme was used. Reversible protection of the α-amino groups was accomplished throughout the synthesis with Fmoc and, in addition, the amino acid side chains were protected as follows: Arg(Pbf), Lys(Boc), Tyr(Bzl) and Glu(OBu^t). Deblockings were performed with 20% piperidine in a mixture of DMF-NMP (1:1 v/v) with 1% Triton X-100. After each stage, the resin was washed with a mixture of DMF-DCM-NMP (1:1:1 v/v) with 1% Triton X-100. Following the completed synthesis and removal of Fmoc protection from the N-terminal α-amino group, peptidyl-resins were washed with DMF, MeOH, Et_2O and dried carefully *in vacuo*.

Cleavage from the resin and side chain protecting groups deblocking in peptides 1 and 2: Cleavage from 1 g of each of both peptidyl-resins and removal of side chain protecting groups were performed with a deoxidated mixture of 88% TFA, 5% phenol, 2% triisopropylsilane and 5% H_2O for 2 hours at room temperature. Then the resins were filtered and washed with cooled TFA. The peptides were precipitated with cooled Et_2O, filtered and dissolved in 1 M AcOH. Then the solvents were removed *in vacuo* and the peptide was dissolved in water and lyophilized. The peptides were purified on a Sephadex G-10 column eluted with 5% AcOH with a flow rate 1 ml/min. The elutes were fractioned and monitored for absorbance at 254 nm. The fractions comprising the major peak were collected and lyophilized. Purity of the analogues was checked by HPLC.

Cleavage from the resin of peptide 3: After deblocking of the N-terminal Fmoc group, the cleavage of the linear protected peptide from the resin was performed with the mixture AcOH/TFE/DCM (2:2:6) for 2 hours. Protected peptide was precipitated with cooled Et_2O, filtered and dried.

Cyclization of peptide 3 and side chain protecting groups deblocking: Cyclization of linear protected peptide **3** was performed in DMF using a mixture of peptide/PyBOP/DIPEA (1:6:12) for 24 hours. High dilution of substrates was used. After removal of solvent, side chain protecting groups were deblocked with 88% TFA in DCM solution. The product was precipitated with cooled Et_2O, filtered and dissolved in 1M AcOH. The solvents were removed *in vacuo* and the peptide was dissolved in water and lyophilized. The product was purified in the same manner as peptides **1**and **2**.

Determination of trypsin inhibitory activity: The trypsin inhibitory activity was determined, according to Erlanger *et al*. [13], with BAPNA as a substrate. Peptides **1** and **3** revealed the inhibitory activity, peptide 2 was inactive.

Affinity chromatography of peptides 1 and 3: The column (1×5 cm) with immobilized anhydrotrypsin on Sepharose 4B gel was stabilized with 0.025 Tris-HCl buffer (pH-8.3) containing 20 mM $CaCl_2$ and 1% Triton X-100. The peptide dissolved in 15 ml of the buffer was applied to the column and washed with the buffer for 20 min with a flow rate 1 ml/min. Then the column was washed with water and the pure product was eluted with 1 mM HCl. Fractions were monitored for absorbance at 254 nm. The fractions collected were evaporated *in vacuo*, dissolved in water and lyophilized, to give peptides **1** and **3**. The purity of peptides synthesized was 97% (peptide **1**) and 98% (peptide **3**), as determined by HPLC.

Determination of trypsin-inhibitor association equilibrium constant (K_a) of peptide 1 and peptide 3: Association equilibrium constant was measured by the method developed by M. Laskowski [14,15]. Bovine β-trypsin-inhibitor interaction was determined in 0.1 M Tris-HCl, 20 mM $CaCl_2$, 0.005% Triton X-100, pH 8.3 at 22°C. The measurement was carried out at an enzyme concentration 7.06×10^{-10} M. The inhibitor concentration varied from 0 to 19.8×10^{-10} M. The concentration of the active form of the inhibitor was determined by titration with the enzyme using BAPNA as a substrate. The residual enzyme concentration was developed with Bz-Val-Gly-Arg-4-nitroanilide as a substrate. The initial substrate concentration never exceeded 0.1 of the value of the substrate Michaelis constant Km. The experimental points were analyzed based on the plot of the residual enzyme concentration, [E], *versus* the initial inhibitor concentration [I_0] (Fig. 1). The Ka value is given in Table 1. The details of the non linear least square procedure were described elsewhere [14,15].

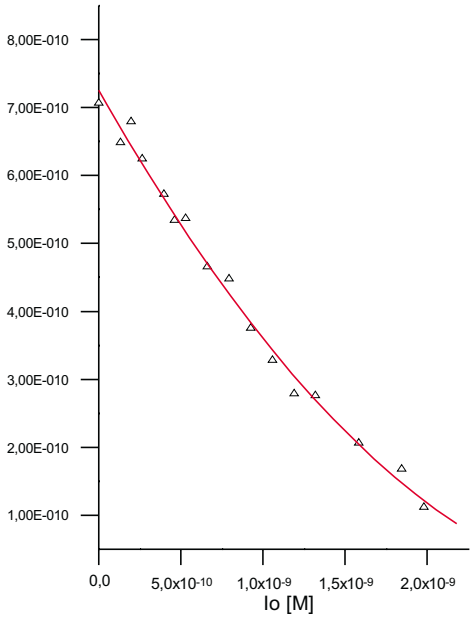

Figure 1. Inhibition of bovine β-trypsin with peptide **1**.

Table 1. Retention time of HPLC, association equilibrium constant (K_a) with bovine β-trypsin and molecular ion of peptides **1**, **2** and **3**.

Inhibitor	C8 RP HPLC[a]		K_a [M^{-1}]	Molecular ion[c]	
	R_T	k'		calc.	found
CMTI-III [16]	–	–	6.8×10^{11}	–	–
Peptide 1[b]	9.60	2.75	4×10^{9}	1126.3	1126.5
Peptide 2[b]	11.03	4.15	non-active	965.9	966
Peptide 3[b]	20.43	2.85	3.6×10^{4}	1372.8	1373.9

[a]HPLC was performed on a Beckman Gold System chromatograph with a C8 RP Knauer column (5 μm particle size, 4.6×250 mm). Solvent system: 0.1% TFA, (B) 80% acetonitrile in A, linear gradient from 20 to 80 for 20 min, flow rate 1 ml/min, A_{226}.
[b]Amino acid composition of the peptides is consistent with expectation.
[c]Molecular spectra (MS FAB) were measured with a VG Masslab TRIO-3. Ion source was equipped with an argon gun. Energy of the argon ion was 8 keV.

RESULTS AND DISCUSSION

Three peptides with sequences based on that of the binding loop of CMTI-III were synthesized by the solid-phase method. Fmoc/Bu^t procedure was used for the synthesis. Products were purified on a Sephadex G-10 column and affinity chromatography (peptides **1** and **3**) and characterized by MS-FAB. Purity of all peptides was checked with RP HPLC (Table 1). The association equilibrium constant (K_a) with bovine β-trypsin was determined for both active peptides.

The K_a value obtained for peptide **1** is by about two orders of magnitude lower than that determined for the parent CMTI-III. It is worth noticing the prolongation of the time of incubation of peptide with the enzyme to 24 hours did not change its inhibitory activity, which means that the peptide discussed did not behave as a substrate. Such a dual character is very often observed for peptidic proteinase inhibitors [9]. The replacement of basic amino acids residues, except the one present in position P1 (peptide **2**), caused the loss of trypsin inhibitory activity. In relation to the activity of the first analogue this is a very interesting observation. A question arises not only why peptide **1** possesses inhibitory activity, but also how to explain its stability. We postulate that basic amino acid residues present at the N- and C- termini interact with the appropriate subsites of the enzyme in such a way that Arg5 (position P1) cannot deeply penetrate the substrate pocket of trypsin, and therefore the peptide bond Arg^5-Ile^6 is not hydrolyzed. This hypothesis has to be verified by structural studies of the complex of peptide **1** with bovine β-trypsin (in progress). Unfortunately, cyclic peptide **3** appeared to be a rather weak inhibitor. The determined association equilibrium constant was by seven orders of magnitude lower than that measured for CMTI-III. As already emphasized, despite many families of inhibitors established, the geometry of their binding loops is very similar. Therefore, one can assume that the conformational constraint introduced into peptide **3** weakens its interaction with trypsin as compared with CMTI-III. This is also supported by the fact that this peptide contains two basic amino acid residues situated in a similar position as in peptide **1**, but its conformational rigidity does not allow their optimum interaction with the enzyme.

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