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Modifications outside the proteinase binding loop in *Cucurbita maxima* trypsin inhibitor III (CMTI-III) analogues change the binding energy with bovine β-trypsin

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Abstract Five 26-peptide analogues of the trypsin inhibitor [Pro^{18}]CMTI-III containing Leu or Tyr in position 7 and Val or Tyr in position 27: 1 (Leu^7, Tyr^{27}), 2 (Tyr^7, Val^{27}), 3 (Tyr^7, Tyr^{27}), 4 (Leu^7, Val^{27}) and 5 (Leu^7, Ala^{18}, Tyr^{27}) were synthesized by the solid-phase method. Analogues 1–4 displayed K_a with bovine β-trypsin of the same order of magnitude as the wild CMTI-III inhibitor, whereas for analogue 5, this value was lower by about 3 orders of magnitude. This indicated that for the analogues with Pro (but not with Ala) in position 18, the side-chain interactions between positions 7 and 27 did not play a critical role for the stabilization of the active structure. In addition, these results also suggest that Tyr^7 is involved in an additional aromatic interaction with position 41 of the enzyme.

Key words: Trypsin inhibitor; Synthesis; Activity; Structure stabilization

1. Introduction

Trypsin inhibitors CMTI-I and CMTI-III [1] isolated from squash (*Cucurbita maxima*) seeds (Fig. 1) were the first members of a new family of serine proteinase inhibitors isolated from Cucurbitaceae [2]. Because of the small size (about 30 amino acid residues), compact structure (stabilized by three disulfide bridges) and strong inhibitory activity ($K_a = 10^{11}$–10^{12} M^{-1}) these inhibitors turned out to be very attractive templates for design of new serine proteinase inhibitors. In the last decade we synthesized both CMTI-I and CMTI-III and more than 30 selective trypsin, chymotrypsin and elastase inhibitors [3–9].

The main problem in the synthesis of CMTI inhibitors was a poor yield in the oxidation (refolding) process, which was often not higher than 5%. By the introduction in regions 17–19 and 23–25 of amino acids which induce β-turn formation (these secondary structure elements were found in the three-dimensional structure of CMTI-I [10–12]) we were able to improve the yield in this process significantly [5,13]. In our previous work which aimed at simplifying the structure of CMTI inhibitors we have also proved that the N-terminal dipeptide and C-terminal glycine are not essential for trypsin inhibitory activity [14]. On the other hand, the substitution of certain amino acid residues by glycine considerably lowered the association equilibrium constants (K_a) with bovine β-trypsin by over five orders of magnitude [15]. When we compared the amino acid sequences of the two sets of CMTI analogues designed in these two different approaches, we noticed that in spite of a high sequence homology, two analogues displayed dramatically different trypsin inhibitory activity, measured as K_a: [desR^1,desV^2,G^9,G^{17},G^{19},G^{23},P^{24},N^{25},V^{27}]-CMTI-III (**I′**) $K_a = 8.1 \times 10^5$ M^{-1} [15]; [desR^1,desV^2,desG^{29},G^9,G^{17},P^{18},G^{19}, G^{23},P^{24},N^{25},Y^{27}]-CMTI-III (**1**) $K_a = 1.7 \times 10^{11}$ M^{-1} [13].

The two analogues differ in three positions only. As we have proved in our previous work [14], the deletion of C-terminal Gly in the second analogue, does not affect the inhibitory activity. We can then assume that for such significant differences in the K_a values only two positions, 18 and/or 27, are responsible. The careful analysis of amino acid sequences of other inhibitors of the Cucurbitaceae family [5] have shown a remarkable relation (regularity) between amino acid residues in positions 7 and 27. In all cases, an aliphatic-aromatic pair was present. The only exception were the inhibitors isolated from *Momordica charantia* where in both positions aromatic amino acid residues are present but because they also exhibit the deletion of the dipeptide in the fragment 25–26, their backbones might fold differently from other members of this family. The assumption that side chains of amino acid residues in positions 7 and 27 interact, seemed also to be supported by the 3D structure of CMTI-I [11] where Leu^7 and Tyr^{27} side chains are in a close contact (Fig. 2). In view of the observation discussed we suspected that the dramatically lower trypsin inhibitory activity of the first analogue was caused by the presence of two aliphatic residues in these positions. In order to investigate this we decided to synthesize four CMTI analogues containing all combinations of leucine and tyrosine in positions 7 and 27. These modifications were introduced into the previously designed, fully active analogue [desR^1,desV^2,desG^{29},G^9,G^{17},P^{18},G^{19},G^{23},P^{24},N^{25}]-CMTI-III (Leu^7, Tyr^{27}) (**1**) [13].

In this paper we consequently describe the synthesis and inhibitory activity of the following analogues: [desR^1,desV^2, desG^{29},Y^7,G^9,G^{17},P^{18},G^{19},G^{23},P^{24},N^{25},V^{27}]-CMTI-III (Tyr^7, Val^{27}) (**2**); [desR^1,desV^2,desG^{29},Y^7,G^9,G^{17},P^{18},G^{19},G^{23},P^{24}, N^{25},Y^{27}]-CMTI-III (Tyr^7, Tyr^{27}) (**3**); [desR^1,desV^2,desG^{29},G^9, G^{17},P^{18},G^{19},G^{23},P^{24},N^{25},V^{27}]-CMTI-III (Leu^7, Val^{27}) (**4**);

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Abbreviations: CMTI, *Cucurbita maxima* trypsin inhibitor; Acm, acetamidomethyl; Boc, tert-butyloxycarbonyl; des, removal of amino acid residue; E, residual enzyme concentration; HPLC, high performance liquid chromatography; I_0, initial inhibitor concentration; K_a, association equilibrium constant; K_m, substrate Michaelis constant; NPGB, 4-nitrophenyl 4′-guanidinobenzoate; RP, reversed phase; RT, retention time

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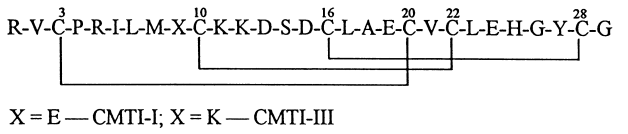

Fig. 1. The amino acid sequence and the disulfide bridge connectivities for CMTI-I and CMTI-III.

[desR^1,desV^2,desG^{29},G^9,G^{17},G^{19},G^{23},P^{24},N^{25}]-CMTI-III (Leu^7, Ala^{18}, Tyr^{27}) (**5**).

The last analogue (numbered as **5**) differs from analogue **1** in one position only. Pro^{18} present in the first one is replaced with the naturally occurring Ala^{18} residue. It should be stressed that in both analogues also positions 7 and 27 are occupied by the amino acid residues (Leu and Tyr, respectively) found in wild CMTI inhibitors. By the synthesis of analogue **5** and the comparison of its inhibitory activity with the relevant data obtained for analogue **1** we intended to investigate the influence of position 18 on the binding energy with bovine β-trypsin.

2. Materials and methods

Amino acid derivatives were supplied by Protein Research Foundation (Osaka, Japan), Bachem Feinchemikalien (CH-4416 Bubendorf) and Fluka AG (CH-9470 Buchs), and except for Gly were of the L-configuration. Chloromethyl resin with capacity of 0.7 meq/g was purchased from Fluka AG. Sephadex G-10 and Sephadex G-25 were purchased from Pharmacia Fine Chemicals (S-75104 Uppsala). Tris was obtained from Serva Feinbiochemica (W-6900 Heidelberg). Other chemicals used for peptide synthesis and HPLC-grade acetonitrile were obtained from Fluka AG. Bz-D,L-Arg-4-nitroanilide, Bz-Val-Gly-Arg-4-nitroanilide, NPGB and Triton X-100 were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Bovine β-trypsin was prepared according to Liepnicks and Light [16].

3. Experimental

3.1. Peptide synthesis

All peptides were synthesized by the solid-phase method using Boc chemistry. Cleavage of peptides from the resin, removal of Acm groups, oxidation (refolding) of the linear peptides, purification of synthetic inhibitors and amino acid analysis were performed as described previously [14,17]. Yields of the synthetic and purification steps, HPLC analyses and association equilibrium constants of the synthesized inhibitors with bovine β-trypsin are summarized in Table 1.

3.2. Determination of trypsin-inhibitor equilibrium association constants

The association constants were measured by the method developed in the laboratory of M. Laskowski, Jr. [18,19]. Bovine β-trypsin-inhibitor interaction was determined in 0.1 M Tris-HCl, pH 8.3 buffer containing 20 mM $CaCl_2$ and 0.005% Triton X-100 at 22°C. The measurement was carried out at an enzyme concentration of 3.01×10^{-10} M. The inhibitor concentrations varied from 0 to 6.5×10^{-10} M. The residual enzyme activity was measured with Bz-Val-Gly-Arg-4-nitroanilide as a substrate. Different conditions, due to significantly lower K_a value, were applied for the analogue **5**; enzyme concentration 3.40×10^{-8} M, inhibitor concentrations varied from 0 to 7.40×10^{-8} M. Bz-D,L-Arg-4-nitroanilide was used as a substrate. In all cases the initial substrate concentration was below 0.1 K_m. The experiment data were analyzed based on the residual enzyme concentration, [E], versus the initial inhibitor concentration, [I]. The details of the non-linear least square procedure are described elsewhere [19,20]. The inhibition of bovine β-trypsin by the five new analogues is shown in Fig. 3.

4. Results

As indicated in Table 1, with the exception of peptide **5**, the other four analogues showed a very strong trypsin inhibitory activity. Their K_a values measured with bovine β-trypsin were of the same order of magnitude as those obtained for the wild CMTI-I and -III molecules. The last analogue **5**, with Ala residue in position 18, showed significantly lower inhibitory activity (the K_a value was about three orders of magnitude lower than that obtained for the other analogues). These results suggest that the side-chain interactions between Leu^7 and Tyr^{27} are less important for the inhibitor stabilization, but

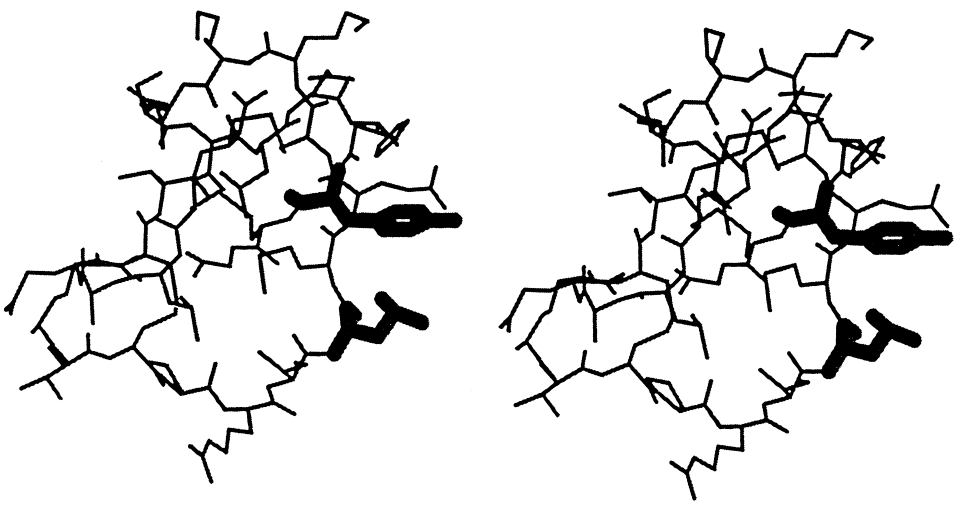

Fig. 2. Stereo view of CMTI-I adopted from file 2cti retrieved from Brookhaven Protein Data Bank [21]. Residues Val^7, Tyr^{27} are marked by bold lines.

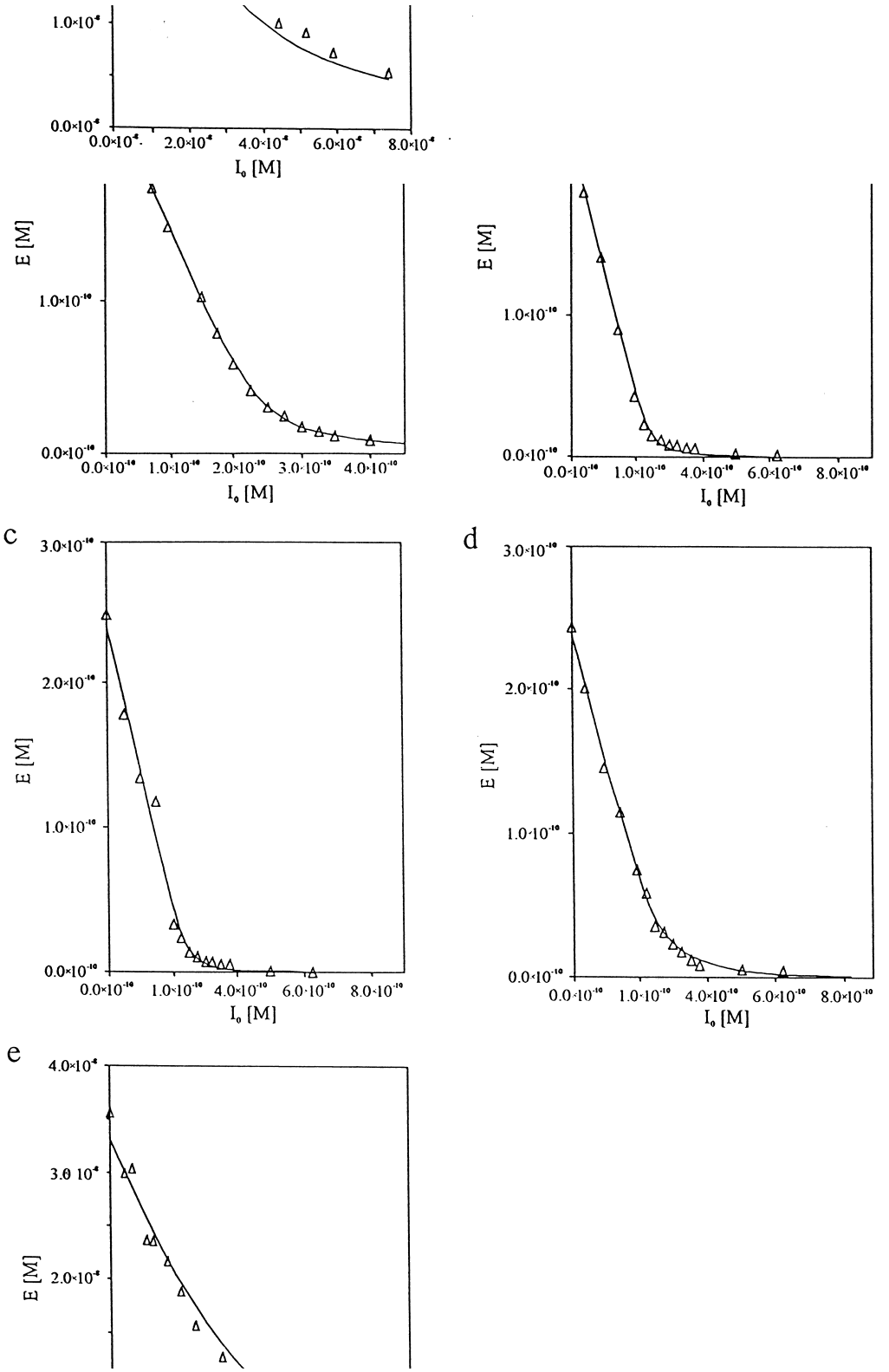

Fig. 3. Inhibition curves of bovine β-trypsin by; (a) analogue **1**, (b) analogue **2**, (c) analogue **3**, (d) analogue **4**, (e) analogue **5**.

Table 1
Details of the synthetic steps, retention time of the HPLC analysis and association equilibrium constants for CMTI-III analogues with bovine β-trypsin

Analogue[a]	Amount[b] (mg) (yield %)					RP C_{18} HPLC[c] RT	K_a (M^{-1})
	Acm peptide		Reduced peptide	Oxidized peptide			
	crude	purified		crude	purified		
1	392	274 (70%)	147 (54%)	92 (63%)	10.1 (11.0%)	13.0 (a)	1.7×10^{11}
2	383	201 (52%)	150 (74%)	102 (68%)	12.0 (11.8%)	8.1 (b)	6.0×10^{11}
3	610	252 (41%)	190 (75%)	122 (64%)	13.0 (10.6)	8.2 (b)	5.7×10^{11}
4	323	195 (60%)	110 (56%)	66 (60%)	8.2 (12.4%)	7.8 (b)	1.5×10^{11}
5	410	208 (51%)	160 (77%)	105 (66%)	14.0 (13%)	12.0 (a)	1.4×10^{8}

[a]Amino acid composition of the analogues is consistent with expectation.
[b]All results refer to 1 g of peptide resin.
[c]HPLC was performed on: (a) an RP C_{18} 10 μm Bondapak column (3.9×250 mm). Solvent system: (A) 0.1% TFA, (B) 80% acetonitrile in A (linear gradient from 30 to 80% of B for 15 min, and then isocratic conditions in 80% B for 5 min, flow rate 1.0 ml/min, monitored at 254 nm), (b) RPC_{18} 10 μm Bondapak column (3.9×250 mm) under isocratic conditions in 35% B, flow rate 1 ml/min, monitored at 254 nm.

probably only when Pro is present in position 18 (analogues **2**–**4**).

In our previous papers [5,13] we demonstrated the role of two β-turns present in the regions 17–20 and 23–26 on the oxidation (refolding) yields. Based on those results we suggested that the second region had stronger influence on the refolding pathway and the stabilization of the three-dimensional structure of CMTI inhibitors. Now, we assume that the role of the first β-turn is more important. It is particularly significant when we consider differences in the inhibitory activity of two pairs of analogues: **4/I′** and **1/5**, which were found to be over five and three orders of magnitudes, respectively. The analogues differ in one position only, Ala^{18} present in **I′** and **5** was replaced by Pro in analogues **4** and **2**. This amino acid residue (Pro) was a part of the tripeptide Gly^{17}-Pro^{18}-Gly^{19} which was introduced into CMTI-III analogues for the promotion of the β-turn formation [13]. In the case of analogues with Pro^{18} (**2**–**4**), the more important factor is the effect that stabilizes the β-turn in fragment 17–20. The role of the hydrophobic interactions between positions discussed in the stabilization of the three-dimensional structure is less pronounced. The K_a values are of the same order of magnitude. The presence of Ala (the amino acid residue with much greater conformational freedom) in position 18 might allow, when the interaction between side chains of amino acid residues in positions 7 and 27 are not strong enough, to adopt by the binding loop a conformation which is not favorable for the interaction with trypsin. In fact, Ala^{18} is present in the second position of the type II′ β-turn in both, the complex of CMTI-I-bovine β-trypsin [10] and in the structure of the free inhibitor as determined by the NMR method [11]. As already stressed in both papers, this position in the type II′ β-turn is very often occupied by Gly but not by Ala. In contrast to what was observed for the analogues with Pro in position 18, the influence on the inhibitory activity of the interaction between side chains of amino acid residues in positions 7 and 27 for the analogues with Ala in position 18 is rather significant. The K_a value for analogue **5** is about three orders of magnitude lower than that obtained for the parent compound **1**. In both analogues a pair Leu^7, Tyr^{27} is present. For the analogue **I′**, which differs from **5** basically in position 27 only, the inhibitory activity was more than two orders of magnitude lower. This reflects the presence of an aliphatic pair Leu^7, Val^{27} in the first analogue. The interaction between side chains of these amino acid residues is not strong enough to form the conformation of the binding loop which would be the most favorable for the interaction with the enzyme.

Another interesting point is that the interaction between the amino acid residue in position 7 of the inhibitor and Phe^{41} of trypsin can occur, giving a detectable energy contribution to the enzyme-inhibitor stabilization. Both analogues with Tyr instead of Leu in position 7 demonstrated about four times higher K_a values. According to the X-ray study of the CMTI-I-bovine β-trypsin complex, the primary contact segment of the inhibitor runs antiparallel to the trypsin segment Ser^{214}-Gly^{216} [10], and the presence of the hydrogen bond between inhibitor Leu^7 and enzyme Phe^{41} has been pointed out. We suppose that an aromatic amino acid residue (such as Tyr) in position 7 of the inhibitor can also induce additional hydrophobic (aromatic-aromatic) interaction with the aromatic ring of the trypsin Phe^{41} residue. According to Burley and Petsko [22], such single aromatic-aromatic interaction contribute about 1 Kcal/mol to the protein-protein complex stabilization. This is in line with our results, which indicate that possible aromatic-aromatic interaction of the inhibitor Tyr^7 and the enzyme Phe^{41} give about 0.8 Kcal/mol to the enzyme-inhibitor complex stabilization, and also with theoretical calculations of the energy interaction of the analogues discussed with bovine β-trypsin (W. Brandt, Halle, Germany, personal communication).

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