Biochemical and Biophysical Research Communications **269,** 81–84 (2000)
doi:10.1006/bbrc.2000.2257, available online at http://www.idealibrary.com on IDEAL®

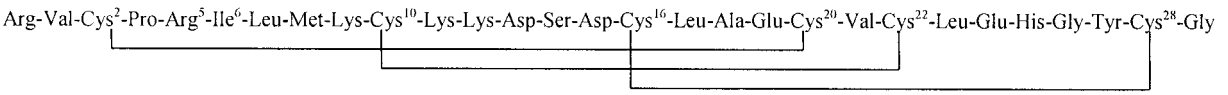
Design, Chemical Synthesis and Kinetic Studies of Trypsin Chromogenic Substrates Based on the Proteinase Binding Loop of *Cucurbita maxima* Trypsin Inhibitor (CMTI-III)

Adam Lesner, Krzysztof Brzozowski, Gotfryd Kupryszewski, and Krzysztof Rolka[1]
Faculty of Chemistry, University of Gdańsk, PL-80-502 Gdańsk, Poland

Received January 14, 2000

A series of trypsin chromogenic substrates with formula: Y-Ala-X-Abu-Pro-Lys-pNA, where X = Gly, Ala, Abu, Val, Leu, Phe, Ser, Glu and Y = Ac, H; pNA = *p*-nitroanilide was synthesized. The *Cucurbita maxima* trypsin inhibitor CMTI-III molecule was used as a vehicle to design the trypsin substrates. To evaluate the influence of position P_4 on the substrate–enzyme interaction, kinetic parameters of newly synthesized substrates with bovine β-trypsin were determined. The increasing hydrophobicity of the amino acid residue (Gly, Ala, Abu, Val) introduced in position P_4 significantly enhanced the substrate specificity (k_{cat}/K_m) which was over 8 times higher for the last residue than that for the first one. The introduction of residues with more hydrophilic side chain (Glu, Ser) in this position reduced the value of this parameter. These results correspond well with those obtained using molecular dynamics of bovine β-trypsin with monosubstituted CMTI-I analogues, indicating that in both trypsin substrate and inhibitor position 4 plays an important role in the interaction with the enzyme.

The interactions between serine proteases and their inhibitors have been extensively studied by several groups and were already described in excellent review articles (1, 2). In enzyme–inhibitor complexes, about 10–15 residues, the so-called proteinase binding loop of the inhibitor, are in contact with enzyme. Their specific nature strongly affects both strength and specificity of enzyme–inhibitor interactions (3). In the last decade we focused our interest on one of the smallest and strongest ($K_a = 6.8 \times 10^{11}$ M^{-1}) inhibitor of bovine β-trypsin CMTI-III (*Cucurbita maxima* trypsin inhibitor) (4–6) isolated from squash seeds. The amino acid sequence and disulfide bridge connectivities of this inhibitor are given below:



The Arg^5-Ile^6 peptide bond is the reactive site of the inhibitor (named P_1—P_1' according to Shechter and Berger (7)). The fragment Val^2-Lys^{11} directly interacting with the enzyme, is the binding loop of this inhibitor (3).

Using the native sequence as a vehicle we were able to obtain synthetic CMTI-III analogues which inhibited human leukocyte elastase (8, 9) and chymotrypsin (9). By chemical synthesis of a series of CMTI-III analogues we have also shown the influence of the amino acid residues from outside of the proteinase binding loop on the trypsin inhibitory activity (10).

Generally, substrates of serine proteases interact with the enzyme by the same mechanism as do the inhibitors. In this case, the specificity is usually lower (as measured by K_m), and obviously the hydrolysis of P_1—P_1' peptide bond (measured by k_{cat}) is much faster as compared with that of effective inhibitors (11). Several attempts were made to design chromogenic proteinase substrates using the binding loop of proteinase inhibitors or natural occurring substrates. Such efforts were successful in the case of human cathepsin G,

[1] To whom correspondence should be addressed. Fax: 0048-58-3412681. E-mail: krzys@chemik.chem.univ.gda.pl.

0006-291X/00 $35.00

TABLE 1

Physiochemical Properties of Substrates Studied and Their Kinetic Parameters with Bovine β-Trypsin

Substrate: Y-Ala-X-Abu-Pro-Lys-pNA	MW Calc. (found)	Rt (HPLC) min	K_m μM	k_{cat} s^{-1}	k_{cat}/K_m M^{-1} s^{-1}	$\Delta\Delta G$(X/Gly) kJ/mol
Ac-Ala-Gly-Abu-Pro-Lys-pNA	618.7 (620)	13.6[b]	338.0	43.0	127219.0	—
Ac-Ala-Ala-Abu-Pro-Lys-pNA	632.8 (634)	14.0[a]	66.5	10.3	154887.3	−0.48
H-Ala-Ala-Abu-Pro-Lys-pNA	590.7 (592)	12.6[a]	238.8	13.8	57983.3	−0.19
Ac-Ala-Abu-Abu-Pro-Lys-pNA	646.7 (648)	15.5[a]	28.9	10.5	363321.8	−2.57
Ac-Ala-Val-Abu-Pro-Lys-pNA	660.4 (662)	15.7[a]	17.6	19.0	1079545.5	−5.24
H-Ala-Val-Abu-Pro-Lys-pNA	618 (619.3)	9.1[b]	28.7	9.6	334494.7	−2.37
Ac-Ala-Ile-Abu-Pro-Lys-pNA	674.8 (676)	16.1[a]	32.3	17.3	535604.8	−3.53
H-Ala-Ile-Abu-Pro-Lys-pNA	632.7 (634)	15.2[a]	16.9	32.0	189349.1	−0.97
Ac-Ala-Phe-Abu-Pro-Lys-pNA	708 (709.4)	14.4[b]	41.1	15.1	367397.2	−2.59
H-Ala-Phe-Abu-Pro-Lys-pNA	666 (668.1)	11.8[b]	157.1	23.0	156278.0	−2.86
Ac-Ala-Glu-Abu-Pro-Lys-pNA	689.7 (691)	14.4[a]	21.3	299.0	140675.3	−2.41
Ac-Ala-Ser-Abu-Pro-Lys-pNA	648.7 (651)	14.3[a]	29.4	377.3	128231.1	−1.94

[a,b] HPLC analysis was performed on a Gold System (Beckman, USA) using an RP C_{18} 10 μm Ultrasphere (Beckman, USA) column (4.6 × 250 mm). Solvent system: (A): 0.1% TFA, (B) 80% acetonitrile in A, linear gradients (a) 20–80% B for 20 min and (b) 30–80% B for 20 min (flow rate 1 ml/min) were applied. Fraction were monitored at λ = 226 nm. Molecular weights of the peptides obtained were determined on a Trio-3 FAB (VG-Masslab, Great Britain) mass spectrometer.

when the sequence of the inhibitor binding loop (serpins) was chosen to obtain new sensitive fluorogenic substrates (12). Other attempts were made to design plasmin substrates based on the sequence of binding loop of the plasmin inhibitor (13) or in the case of tissue kalikrein, when the sequence of kininogen were used to determine the substrate specificity (14). In this paper we describe chemical synthesis and kinetic studies of a series of chromogenic substrates with 4-nitroanilide moiety (pNA) at the C-terminus, designed on the amino acid sequence of the binding loop of CMTI-III.

Based on the sequence of fragment P_1—P_4 the following substrates were obtained: Y-Ala-X-Abu-Pro-Lys-pNA (where Y = Ac, H and X = Gly, Ala, Abu, Val, Ile, Phe, Ser, Glu). Cys Residue was replaced with the residue of L-aminobutyric acid (isosteric replacement). Another modification was introduced in position P_1; Arg was substituted with Lys. As shown earlier (15), such modification did not affect the inhibitory activity of CMTI-III toward bovine β-trypsin. In position P_4 (X) various amino acid residues were introduced in order to investigate their influence on the interaction between the substrate and bovine β-trypsin.

MATERIALS AND METHODS

Synthesis of substrates. Fmoc-Lys (Boc)-pNA was prepared in the reaction of Fmoc-Lys(Boc) with *p*-nitroaniline applying the mixed anhydride method (16). After removal of Boc protection from the ε-amino group of L-lysine, Fmoc-Lys-pNA was purified on a chromatographic column (100 cm × 5 cm) packed with Silica Gel 60, using DCM:MeOH (9:1) as an eluent (flow rate 1 ml/min). The purity of the final product was checked by TLC (solvent system: DCM: MeOH, 10:2). In the next step, Fmoc-Lys-pNA was attached to 2-chlorotritylchloride resin (substitution 1.46 meq/mol) using a method described in the literature (17). The derivative thus obtained (Fmoc-Lys(resin)-pNA) was used in the solid-phase method applying the Fmoc chemistry to obtain chromogenic substrates. After completing the synthesis, peptides were cleaved from the resin using solution of 5% TFA in DCM and purified on a Sephadex LH-20 column

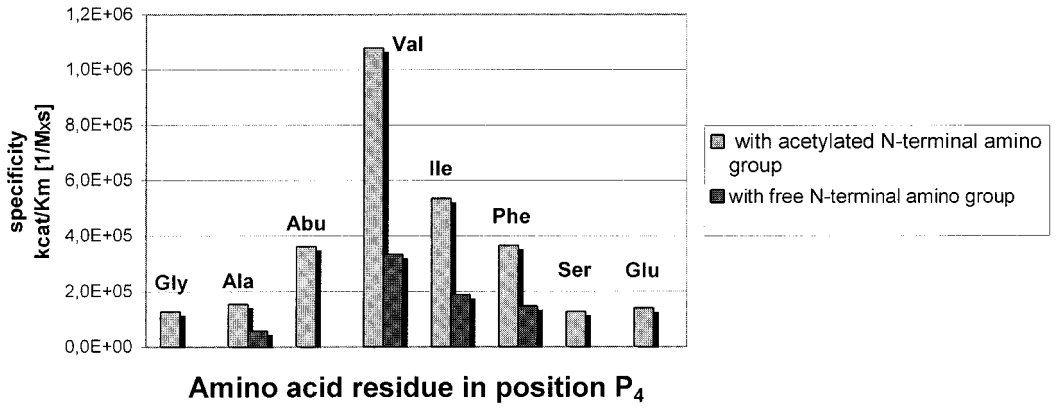


FIG. 1. The influence of amino acid in position 4 of substrates studied on specificity constants with bovine β-trypsin.

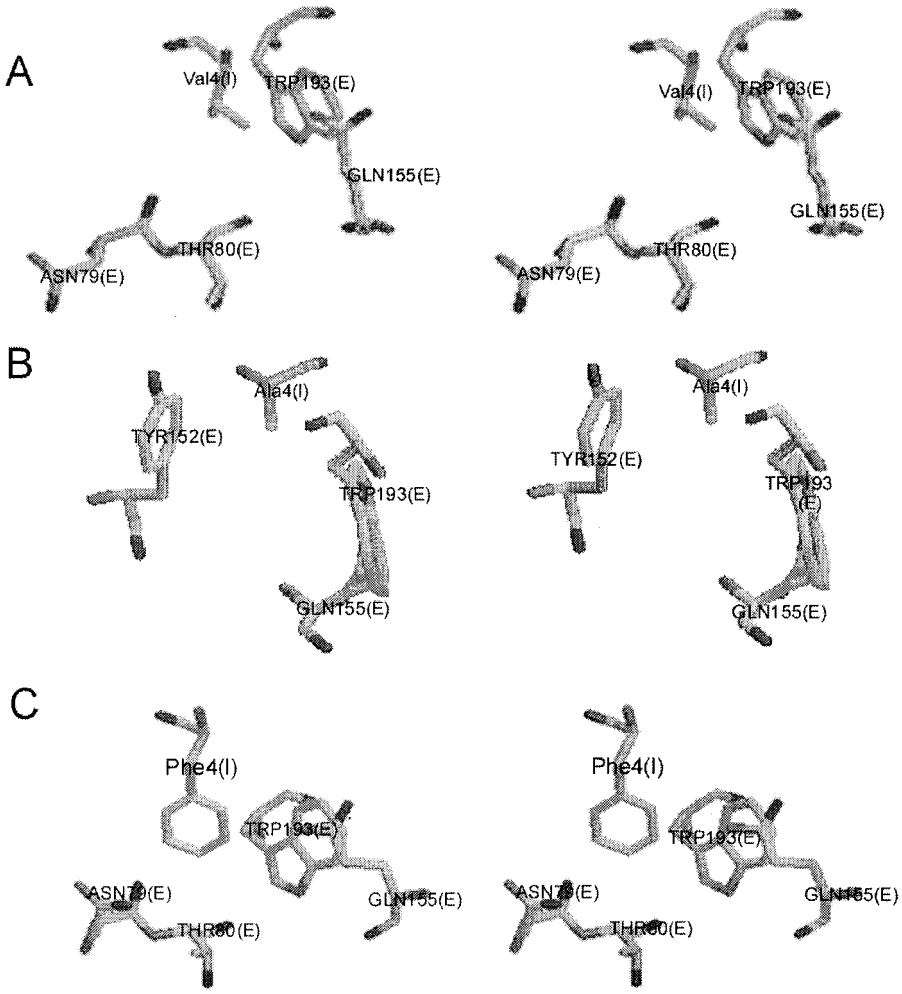


FIG. 2. Stereo view of the complex between bovine β-trypsin and CMTI-I (22) (A), [Phe^5] CMTI-I (B), and [Ala^5] CMTI-I (C). Only position P_4 of the inhibitor and amino acid residues with the closest contact of the enzyme are shown.

(120 cm × 3.5 cm) eluted with methanol (flow rate 1 ml/min). Fractions were analyzed using a UV $\lambda = 254$ nm monitor (LKB, Sweden). Fractions containing the homogenous peptide were combined and lyophilized. The purity of all peptides was higher than 98% as judged by the HPLC analysis.

Kinetic studies. The concentration of bovine β-trypsin stock solution was determined by titration with NPGB (18). The stock of the substrate solution was prepared by dissolving about 24 mg of peptide in 1 ml DMSO. Substrates stock solution was diluted: 2, 4, 5, 6, 8, 10, 12, 15 and 20 times. The enzymatic hydrolysis of substrates was performed in 0.1 M Tris-HCl (pH 8.3) buffer at 25°C. Measurements were performed at enzyme concentrations in the range $5.5–10^{-8}$ to $1–10^{-10}$ M using a Cary 3E spectrophotometer (Varian, Australia). The increase in absorbency at 410 nm resulting from *p*-nitroaniline release was measured as a function of time. For each compound at least five measurements were performed (systematic error expressed as a standard deviation never exceeded 10%). Kinetic parameters Michaelis constant (K_m), turnover number (k_{cat}) and specificity constant k_{cat}/K_m for each substrate were determined based on the equation (19)

$$v/E_o = k_{cat}/(1 + K_m/S),$$

where v is the velocity, E_o is the enzyme concentration, k_{cat} is the turnover number, S is the substrate concentration S and v are variables. Calculations were carried out using the commercial Steady-State Enzyme Kinetics program (20). Based on the kinetic parameters K_m, k_{cat}, k_{cat}/K_m, the relative change in free Gibbs energy was calculated employing the equation $\Delta\Delta G = RT \ln(k_{cat}/K_m)_X/(k_{cat}/K_m)_{Gly}$, where X is the amino acid residue in position P_4 (21) (Table 1).

Molecular dynamics. Molecular dynamics of the CMTI-I inhibitor was performed on its starting structure based on the CMTI-I–bovine β-trypsin complex (22). Two monosubstituted structures with Phe and Ala in position P_4 of CMTI-III in complexes with bovine β-trypsin were analyzed. Constraints imposed on linearity and geometry of peptide bonds were used. Force constants were 10 kcal/mol in both cases. Molecules were surrounded by 9 Cl^- counterions and 6825 TIP3P water molecules (23), filling a 66.73 × 72.06 × 65.80 Å rectangular box. 300 Steps of minimization were applied prior to molecular dynamics experiment. In calculation the Shake procedure (24) was applied to all bonds involving hydrogens. Simulation was run at a constant temperature of 298 K. All calculations were carried out using the SANDER module of the AMBER (25) package, with the AMBER 4.1 force-field parameters. Molecular dynamics simulation was run by 250 ps with a 0.5 fs time step at a constant pressure. All

computations were carried in the Academic Computer Center (TASK) in Gdańsk.

RESULTS AND DISCUSSION

Molecular weights, HPLC analysis and results obtained from the kinetic studies of peptide substrates with bovine β-trypsin are summarized in Table 1. As can be seen in Table 1 and Fig. 1, position P_4 plays an important role in the substrate–trypsin interaction. The differences can be observed for all kinetic parameters (K_m, k_{cat}, k_{cat}/K_m), but they were substantial for the last parameter—the specificity constant. In the case of the substrate with Val in position P_4 (this amino acid residue is present in wild CMTI-III inhibitor) k_{cat}/K_m obtained is above 8 times as high as that measured for the analogue with Gly or Ala and 3 times for Phe in the position discussed, respectively. This is also reflected by the value (5 kJ/mol) of the relative change of the free Gibbs energy for the analogues with Val and Gly residue, respectively. This value for obvious reasons is lower than that calculated for the monosubstituted serine protease inhibitor. Also the acetylation of positively charged N-terminal amino group stabilizes the substrate–enzyme complex. In all four cases (Ala, Val, Ile, Phe in position 4) *N*-acetyl substrates displayed significantly higher values of k_{cat}/K_m. The results described above correspond well with the interaction of bovine β-trypsin with its inhibitors. In the CMTI-I-bovine β-trypsin complex (22, 26) the amino acid residues in position P_4 (originally Val) interacts with three amino acid residues of the enzyme (Gln^{155}, Tyr^{152} and Trp^{193}). The closest contact (about 3–3.5 Å) which is between isobutyl side chain of Val and the indole ring of Trp^{193} (Fig. 2A) seems to be hydrophobic-aromatic interaction. The introduction of residues with shorter and less hydrophobic side chains into discussed position of CMTI-I resulted in the reduction of kinetic parameters due to the lack of these interactions. In the complex of [Ala^5] CMTI-III and bovine β-trypsin, the residue of Ala had no significant contact (all are above 4Å) with the enzyme (Fig. 2B). On the other hand, the Phe residue introduced in the position discussed deeply penetrate the S_4 subsite (Fig. 2C) making few destabilizing contacts (phenyl ring of Phe in close contact with hydroxyl and carbonyl oxygens of Asn^{79} (2.93 Å) and Thr^{80} (3.02 Å)), which probably caused the decrease in the specificity. We believe that in the case of [Ile^5] CMTI-I a similar effect would be observed.

The results presented above clearly indicate that position P_4 of trypsin substrates plays an important role in the interaction with enzyme. In addition we would like to stress that the sequence derived from the proteinase binding loop of the CMTI-III inhibitor may be useful for designing substrates of the corresponding target enzyme (in this case bovine β-trypsin). A series of substrates with the acetylated N-terminal amino group and with hydrophobic amino acid residue in position P_4 (Val, Ile) have a low K_m, moderate k_{cat}, and relatively high specificity k_{cat}/K_m that make them suitable for several applications. We used these compounds successfully during determination of the association equilibrium constants (K_a) of inhibitors with bovine β-trypsin.

ACKNOWLEDGMENTS

This work was supported by the University of Gdansk (Grant No. BW-8000-5-0104-8). The authors thank Professor J. Szafranek and his co-workers from the Faculty of Chemistry, Gdansk University for performing mass analyses.

REFERENCES

1. Laskowski, M., *et al.* (1980) *Annu. Rev. Biochem.* **49,** 593.
2. Laskowski M., *et al.* (1989) Protein Recognition of Immobilized Ligands, 149–168, A. R. Liss Inc., New York.
3. Bode, W., *et al.* (1992) *Eur. J. Biochem.* **204,** 433–451.
4. Polanowski, A., *et al.* (1980) *Acta Biochim. Pol.* **27,** 371–382.
5. Wilusz, T., *et al.* (1983) *Hoppe-Seyler's Z. Physiol. Chem.* **364,** 93–95.
6. Holak, T. A., *et al.* (1989) *J. Mol. Biol.* **210,** 649–654.
7. Schechter, J., and Berger, A. (1968) *Biochem. Biophys. Res. Commun.* **27,** 157–162.
8. Różycki, J., *et al.* (1994) *Biol. Chem. Hoppe-Seyler* **375,** 289–291.
9. Różycki, J., *et al.* (1991) *Biol. Chem. Hoppe-Seyler* **372,** 63–68.
10. Jaśkiewicz, A., *et al.* (1998) *FEBS Lett.* **436,** 174–178.
11. Perona, J. J., and Creik, C. S. (1995) *Protein Sci.* **4,** 337–360.
12. Rehault, S., *et al.* (1999) *J. Biol. Chem.* **274,** 13810–13817.
13. Fiedler, F. (1987) *Eur. J. Biochem.* **163,** 303–312.
14. Kiss, J., *et al.* (1985) *Biochem. Biophys. Res. Commun.* **131,** 928–934.
15. Jaśkiewicz, A., *et al.* (1997) *Biochem. Biophys. Res. Commun.* **240,** 869–871.
16. Schutkowski, M., *et al.* (1995) *Int. J. Peptide Protein Res.* **45,** 257–265.
17. Bernhardt, A., *et al.* (1997) *J. Peptide Protein Res.* **50,** 143–148.
18. Chase, T., and Shaw, E. (1970) *Methods Enzymol.* **19,** 20–27.
19. Segel, I. H. (1993) Enzyme Kinetics, Chap. 2. Wiley–Interscience, California.
20. Cornish Bowden, A. (1995) Analysis of Enzyme Kinetics Data, Oxford Univ. Press, New York.
21. Fersht, A. (1985) Enzyme Structure and Mechanism, Freeman and Company, London.
22. Berstein, F. C., *et al.* (1977) *J. Mol. Biol.* **112,** 535–542; Protein Data Bank, Chemistry Department, Brookhaven National Laboratory, Upton, NY 11973, File 1 PPE.
23. Jorgensen, W. L., *et al.* (1982) *J. Chem. Phys.* **79,** 926–935.
24. Hess, B., *et al.* (1997) *J. Comp. Chem.* **18,** 1463–1472.
25. Pearlman, D. A., *et al.* (1995) AMBER 4.1, University of California, Manual, pp. 98–149.
26. Holak, T. A., *et al.* (1989) *J. Mol. Biol.* **210,** 649–654.