INHIBITORY PROPERTIES OF NONAPEPTIDE LOOP STRUCTURES RELATED TO REACTIVE SITES OF SOYBEAN BOWMAN-BIRK INHIBITOR

Shigeyuki TERADA, Kazuki SATO, Tetsuo KATO and Nobuo IZUMIYA
Laboratory of Biochemistry, Faculty of Science 33, Kyushu University, Higashi-ku, Fukuoka 812, Japan

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1. Introduction

Bowman-Birk inhibitor (BBI) of soybean is a double-headed inhibitor with independent activity against both trypsin (EC 3.4.21.4) and chymotrypsin (EC 3.4.21.1) [1]. The Lys^{16}–Ser^{17} and Leu^{43}–Ser^{44} bonds were assigned to be the anti-tryptic and anti-chymotryptic sites, respectively. Each reactive site is located within a disulfide loop of 9 residues [2]. Cyclic nonapeptides, X–Cys–Thr–Lys–Ser–Asn–Pro–Pro–Gln–Cys–Y (I_a: X=Ac, Y=NH_2; I_b: X=H, Y=OH), corresponding to residues 14–22 in the sequence of BBI by solid-phase method and showed their anti-tryptic activities.

We report how the synthesis by conventional method and the inhibitory activity of cyclic nonapeptides, X–Cys–Ala–Leu–Ser–Tyr–Pro–Ala–Gln–Cys–Y (II_a: X=Ac, Y=NH_2; II_b: X=H, Y=OH), which corresponds to the sequence of 41–49 of BBI involving anti-chymotryptic site. We also examined the effect of replacement at P_2, P_1 and P'_1 residues of I_a and II_a on the inhibitory properties toward trypsin, chymotrypsin and subtilisin BPN′ (EC 3.4.21.14). The anti-chymotryptic site peptides inhibited both chymotrypsin and subtilisin, whereas the natural inhibitor was ineffective to subtilisin.

Residues in peptides and the corresponding subsites in enzyme are numbered according to Schechter and Berger [5]. Abbreviations according to IUPAC-IUB commission (1972), J. Biol. Chem. 247, 977–983, are used throughout. pNA, *p*-nitroanilide; Cys(MeOBzl), *S*-4-methoxybenzylcysteine; Cys(Cam), *S*-carboxamidomethylcysteine. Amino acids other than Gly and D-Lys are of L-configuration.

2. Materials and methods

α-Chymotrypsin (3 X crystallized), trypsin (2 X crystallized) and subtilisin BPN′ (Nagarse) were supplied by Worthington Biochemical Corp. (NJ), by Nutritional Biochemical Corp. (OH) and by Nagase Co. (Osaka), respectively. Natural BBI was prepared from soybeans following [6].

2.1. *Synthesis of II_a and II_b*

The protected nonapeptide (177 mg), Ac–Cys(MeOBzl)–Ala–Leu–Ser(Bzl)–Tyr–Pro–Ala–Gln–Cys(MeOBzl)–NH_2, prepared by condensing Ac–Cys(MeOBzl)–Ala–Leu–N_3 with H–Ser(Bzl)–Tyr–Pro–Ala–Gln–Cys(MeOBzl)–NH_2 was de-blocked by HF in the presence of anisole, and the peptide obtained was oxidized with potassium ferricyanide to form disulfide linkage. The crude product desalted by Sephadex G-15 (50% acetic acid) was purified by Sephadex G-25 (2.8 X 166 cm) using 10% acetic acid as eluent; yield of II_a, 73 mg (54%). Dimer of II_a (28 mg, 21%) was also gained by this procedure.

II_b was obtained from Boc–Cys(MeOBzl)–Ala–Leu–Ser(Bzl)–Tyr–Pro–Ala–Gln–Cys(MeOBzl)–OBzl (90 mg) in the same manner as above; yield of II_b, 20 mg (34%). Details of the synthesis will be reported elsewhere.

2.2. *Synthesis of other peptides*

All the peptides including I_a, I_b and several analogs of I_a and II_a were synthesized by conventional methods, to be reported elsewhere, and are summarized in table 1. Reduced and carboxamidomethylated analog of II_a, an open chain peptide (II_d), was prepared from II_a as for the preparation of I_h [4].

Table 1
Synthetic peptides related to the nonapeptide sequences (14–22 and 41–49) of BBI

Peptide	Structure P_4 P_3 P_2 P_1 P_1' P_2' P_3' P_4' P_5' P_6'
Sequence (14-22) of BBI	-Cys-Thr-Lys-Ser-Asn-Pro-Pro-Gln-Cys-
I_a	Ac-Cys-Thr-Lys-Ser-Asn-Pro-Pro-Gln-Cys-NH_2
I_b	H-Cys-Thr-Lys-Ser-Asn-Pro-Pro-Gln-Cys-OH
I_c	Ac-Cys-Thr-D-Lys-Ser-Asn-Pro-Pro-Gln-Cys-NH_2
I_d	Ac-Cys-Thr-Arg-Ser-Asn-Pro-Pro-Gln-Cys-NH_2
I_e	Ac-Cys-Ala-Lys-Ser-Asn-Pro-Pro-Gln-Cys-NH_2
I_f	Ac-Cys-Thr-Lys-Ala-Asn-Pro-Pro-Gln-Cys-NH_2
I_g	Ac-Cys-Thr-Leu-Ser-Asn-Pro-Pro-Gln-Cys-NH_2
I_h	Ac-Cys(Cam)-Thr-Lys-Ser-Asn-Pro-Pro-Gln-Cys(Cam)-NH_2
Sequence (41-49) of BBI	-Cys-Ala-Leu-Ser-Tyr-Pro-Ala-Gln-Cys-
II_a	Ac-Cys-Ala-Leu-Ser-Tyr-Pro-Ala-Gln-Cys-NH_2
II_b	H-Cys-Ala-Leu-Ser-Tyr-Pro-Ala-Gln-Cys-OH
II_c	Ac-Cys-Ala-Tyr-Ser-Tyr-Pro-Ala-Gln-Cys-NH_2
II_d	Ac-Cys(Cam)-Ala-Leu-Ser-Tyr-Pro-Ala-Gln-Cys(Cam)-NH_2

2.3. *Enzyme assays and kinetic measurements*

Esterase activities of chymotrypsin and trypsin were determined spectrophotometrically at 25°C with Ac–Tyr–OEt [7] and Tos–Arg–OMe [8] as substrates, respectively. *p*-Nitroanilides were employed to examine amidase activities at 410 nm; Bz-Arg-pNA for trypsin [9] and Boc–Gly–Ala–Leu–pNA (prepared in our laboratory; k_{cat}/K_m=970 $M^{-1} \cdot s^{-1}$, K_m=0.62 mM, at pH 8.0) for subtilisin BPN'. The inhibition constants (K_i) were calculated from the initial rate of hydrolysis at various concentrations of the substrate by the methods in [10,11].

3. Results and discussion

II_a and II_b inhibited chymotrypsin competitively, though the activities were about 10^4-times weaker than natural inhibitor (table 2). In contrast to the anti-tryptic site nonapeptides [4], dimer of II_a showed similar inhibitory activity of that of II_a. Replacement of P_1 residue (Leu) by Tyr enhanced the potency of inhibition in accord with primary specificity of substrate. Interestingly the most potent inhibitor of chymotrypsin is I_g, an analog of anti-tryptic site peptide. Relatively high activity (K_i=290 μM) of the

Table 2
Competitive inhibition of chymotrypsin and subtilisin BPN' by several peptides and natural BBI

Peptide	K_i (μM) for chymotrypsin	K_i (μM) for subtilisin
	Ac-Tyr-OEt[a]	Boc–Gly–Ala–Leu–pNA[b]
I_a	no inhibition	no inhibition
I_g	5.7	2900
II_a	52	11
II_b	180	170
II_c	13	55
II_d	290	–
Dimer of II_a	45	170
BBI	0.008	no inhibition

[a] 0.05 M phosphate buffer (pH 7.0) containing 3% methanol at 25°C
[b] 0.05 M phosphate buffer (pH 8.0) containing 16% *N,N*-dimethylformamide at 25°C

open chain analog (II_d) indicates that the loop structure with a disulfide bond is not indispensable for the inhibitory property to chymotrypsin, but it may contribute to the conformational specificity [12] by positioning P_4–P'_2 residues of the inhibitor to the rigid optimum conformation for binding to the enzyme.

Natural BBI has been known not to inhibit bacterial proteinase [13]. We confirmed it with subtilisin BPN' (table 2). All the peptides related to anti-tryptic site were ineffective for this enzyme except I_g, which showed very weak inhibition. However, the anti-chymotryptic site fragment, II_a, strongly inhibited subtilisin BPN' (K_i=11 μM, table 2), indicating the broad specificity of this loop itself. This observation is interesting from both the evolutional and the structural points of view on BBI molecule. The lack of strict specificity of these model inhibitors compared to natural BBI may be the results of either slightly different conformation or the lack of other contact regions such as P_4 amino acid residue in these peptides, or both.

To gain further understanding the role of amino acid residues near anti-tryptic site of BBI, we tested the inhibitory activity of several analogs of I_a on esterase and amidase activities of trypsin (table 3).

Table 3
Competitive inhibition of trypsin by several peptides and natural BBI

Peptide	K_i (μM) for trypsin	
	Tos–Arg–OMe	Bz–Arg–pNA
I_a	0.75	0.76
I_b	3.6	3.9
I_c	860	850
I_d	0.90	1.3
I_e	16	16
I_f	3.5	3.0
I_g	no inhibition	no inhibition
I_h	–	100
BBI	0.003	–

0.05 M Tris buffer (pH 8.0 for Tos–Arg–OMe and pH 8.2 for Bz–Arg–pNA) containing 0.01 M $CaCl_2$ at 25°C

Introduction of D-Lys into P_1 residue practically abolished the inhibitory activity. Replacement of Lys (P_1) residue by Arg did not affect the anti-tryptic potency. Replacements of two amino acid residues (Thr and Ser; adjacent to the reactive site) afforded different effects: alteration in P_2 caused a higher increase in K_i value than P'_1 (table 3). This can be explained by the insufficient contact of P_2 side chain (methyl group) of I_e with S_2 subsite (Leu^{99}) of the enzyme. Replacing of the P_1 residue (Lys) of I_a by Leu abolished the anti-tryptic activity (I_g in table 3), but it converted the peptide into chymotrypsin inhibitor (table 2). This will provide a good support for the hypothesis of evolution of double-headed inhibitor [14]. Enzymatic mutation of P_1 residue has been achieved for Kunitz soybean trypsin inhibitor [15] and basic pancreatic trypsin inhibitor [16].

All of the peptides tested here was stable during the incubation period. Somatostatin having similar molecular size, possible reactive site (Lys–X), and cyclic structure with a disulfide bond did not inhibit trypsin but was hydrolyzed rapidly by the enzyme. C-Terminal undecapeptide of ovine prolactin, H–Leu–Asn–Cys–Arg–Ile–Ile–Tyr–Asn–Asn–Asn–Cys–OH, did not inhibit trypsin and chymotrypsin [17].

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