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Study of Antiproteinase Activity of Acylated Derivatives of Bowman–Birk Soybean Proteinase Inhibitor

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Abstract—The effect of acylation of Bowman–Birk soybean proteinase inhibitor (BBI) by derivatives of various unsaturated fatty acids on inhibition of trypsin, α-chymotrypsin, and human leukocyte elastase was investigated. Inhibition (K_i) and kinetic (k_{ass}, k_{diss}) constants of interaction between proteases and acylated BBI derivatives were determined. For mono-, di-, and triacylated BBI derivatives, insertion of two oleic residues into the BBI molecule was demonstrated to be more potent for exhibiting antiproteinase activity.

Key words: Bowman–Birk inhibitor, acylation, unsaturated fatty acids, inhibition constant, trypsin, α-chymotrypsin, human leukocyte elastase

Studies of covalent hydrophobization of proteins demonstrate that such modifications mainly result in disturbance of their native conformation, giving rise to changes in such important physicochemical properties as biological activity, kinetic characteristics of the enzyme–substrate interaction, thermal stability, etc. [1, 2]. Thus, it was shown that acylation of insulin by palmitic acid derivatives results in significant decrease in its activity [3]. However, modification of subtilisin by chloranhydrides of caprylic and palmitic acid results in 15- and 2-3-times increase in its thermal stability at 45 and 65°C, respectively [4]; this seems to be caused by retardation of autolysis due to steric hindrance arising from the presence of fatty residues on the enzyme surface.

Earlier we described acylation of classical soybean Bowman–Birk inhibitor (BBI) [5]. BBI efficiently inhibits cell transformation *in vitro* and carcinogenesis *in vivo* [6-9]; BBI-based food dopants are now being clinically tested as anti-carcinogenic preparations.

If modified BBI is to be a potent drug, the inhibitory action of the native protein should be retained. The native BBI efficiently inhibits trypsin (Tp) (K_i ~ 0.14 nM) [10] and less efficiently inhibits α-chymotrypsin (CTp) (K_i ~ 6.4 nM) [11] and human leukocyte elastase (HLE) (K_i ~ 2.0 nM) [12]. It should be noted that hydrophobic forces play an important role in interaction between CTp and HLE with protein inhibitors because the binding site of both enzymes mainly consists of nonpolar amino acids [13, 14]. We suggested that BBI hydrophobization via insertion of various residues of unsaturated fatty acids such as oleic (ol), linoleic (lin), and linolenic (α-lin) may result in more efficient inhibition of HLE and CTp due to additional hydrophobic contacts.

The antiproteinase action of BBI preparations containing from one to three amino groups acylated by derivatives of various unsaturated fatty acids is the subject of the present work.

MATERIALS AND METHODS

Materials. The BBI preparation was isolated from soybean by the method developed by us earlier [12, 15]. The active molecule content determined by titration of the inhibitor with trypsin and α-chymotrypsin with known active site content was 88 wt. %.

N-Hydroxysuccinimide esters of oleic, linoleic, and α-linolenic acids were from Sigma (USA).

Tp was from Merck (Germany). The active molecule content determined by titration with *p*-nitrophenyl ester of *p′*-guanidine benzoic acid according to Chase and Show [16] was 64%.

CTp was from Merck. The active molecule content determined by titration with N-*trans*-cinnamoylimidazole from Sigma according to Shonbaum et al. [17] was 57%.

HLE preparation was isolated by Larionova et al. [12]. The active molecule content determined by titration with BBI was 60%.

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Ethyl ester of N-benzoyl-L-arginine hydrochloride (BAEE) and ethyl ester of N-benzoyl-L-tyrosine (BTEE) were from Merck. MeOSuc(L-Ala)$_2$ProValpNa was from Sigma. Other reagents were chemically pure and extra pure grade products produced in Russia.

General methods. Hydrophobized BBI derivatives were obtained by the method developed by us earlier [5]. Protein concentration was determined according to Lowry [18]. The amino group content in the native and modified inhibitor preparations was monitored spectrophotometrically using 2,4,6-trinitrobenzenesulfonic acid [19]. Anti-tryptic and anti-chymotryptic activities of various BBI-containing preparations were evaluated according to [20, 21]. Electrophoresis of BBI preparations was performed in 7% polyacrylamide gel in acidic buffer (Reisfeld, pH 4.5) [22].

The kinetic and equilibrium constants of interaction between enzymes and acylated BBI derivatives were determined from kinetics of steady-state establishment in the enzyme–inhibitor–substrate triple system according to the method developed by Morrison [23]. A set of kinetic curves of inhibition at constant concentrations of the enzyme and substrate and various concentrations of the inhibitor was obtained. Equation (1) relating concentration of the product (P) with reaction time t for competitive, slowly reacting, and tightly binding inhibitors was used as the model:

$$[P] = V_s t + (V_0 - V_s)[1 - \exp(-k_{app} t)]/k_{app} + d, \quad (1)$$

where V_0 is the initial and V_s is the steady-state velocities, k_{app} is the rate constant describing the steady-state establishment, d is deviation of [P] from zero at $t = 0$;

$$k_{ass}[I]_0 = k_{app}(1 - V_s/V_0)(1 + [S]_0/K_m),$$

$$k_{diss} = k_{app} V_s/V_0,$$

$$K_i = k_{diss}/k_{ass},$$

where k_{ass} and k_{diss} are the rate constants of formation and decomposition of the enzyme–inhibitor complex EI and K_i is the equilibrium inhibition constant. To use Eq. (1), the following conditions must be met: $[I]_0 >> [E]_0$ and $[P] << [S]_0$, where $[I]_0$, $[E]_0$, and $[S]_0$ are the initial inhibitor, enzyme, and substrate concentrations in the system, respectively.

The values of V_0, V_s, and k_{app} were calculated using the program Origin-6 for Windows (Microcalc Software, USA).

Determination of kinetic and equilibrium constants of interaction between Tp and acylated BBI derivatives. The standard measurement was performed as follows: 0.05-0.1 ml of BBI preparation of proper dilution was placed in a 1-ml cuvette; the volume of solution in the cuvette was brought to 0.8 ml with 0.05 M Tris-HCl buffer, pH 8.0, containing 0.02 M $CaCl_2$. After careful stirring, 0.1 ml of BAEE solution was added (the substrate concentration in the cuvette was $1.5 \cdot 10^{-4}$ M). The reaction was initiated by addition of 0.1 ml of Tp aqueous solution acidified with HCl to pH 3.0 (the active Tp concentration in the cuvette was 33 nM) and the optical density was monitored at 253 nm using a Shimadzu UV-265 FW spectrophotometer (Japan).

Determination of kinetic and equilibrium constants of interaction between CTp and acylated BBI derivatives. BBI preparation (0.05-0.1 ml) of proper dilution was placed in a 1-ml cuvette; the volume of solution in the cuvette was brought to 0.8 ml with 0.05 M Tris-HCl buffer, pH 8.0. After careful stirring, 0.1 ml of 1.27 mM BTEE was added. The reaction was initiated by addition of $4.0 \cdot 10^{-7}$ M CTp in 1.0 mM HCl and the optical density was monitored at 256 nm using the Shimadzu UV-256 FW spectrophotometer.

Determination of kinetic and equilibrium constants of interaction between HLE and acylated BBI derivatives. BBI preparation (0.05-0.1 ml) of proper dilution was placed in a 1-ml cuvette; the volume of solution in the cuvette was brought to 0.8 ml with 0.1 M HEPES, pH 7.5, 0.5 M NaCl, and 0.005% Triton X-100. After careful stirring, 0.1 ml of 1.0 mM MeOSuc(L-Ala)$_2$ProValpNa solution in DMSO was added. The reaction was initiated by addition of 0.1 ml of $1.6 \cdot 10^{-8}$ M HLE and the optical density was monitored at 410 nm using the Shimadzu UV-256 FW spectrophotometer.

RESULTS AND DISCUSSION

The BBI molecule consists of 71 amino acid residues and has two active sites, chymotrypsin- and elastase-reactive (Leu43-Ser44) and trypsin-reactive (Lys16-Ser17) [24]. The distance between these sites is 2.8 nm, and the diameter of the molecule is 3.9 nm. In spite of its small size, the BBI molecule contains seven disulfide bridges; that is why it has rigid conformation and high thermal stability [25].

Five ε-amino groups of lysine residues (Lys6, 16, 37, 63, and 69) and one amino group of N-terminal aspartic acid are constituents of the BBI molecule; the contact areas between the latter and proteases include Lys16, 37, and 63 (Fig. 1), Lys16 being in the trypsin-reactive site of the inhibitor [26].

Earlier we demonstrated that BBI modification without preliminary protection of the trypsin-reactive site results in complete loss in anti-tryptic activity [5]. That is why the hydrophobized preparations were prepared with preliminary reversible protection of the Lys16 amino group.

The method of acylation of soybean inhibitor with derivatives of unsaturated fatty acids we suggested earlier allows obtaining not only active but homogenous BBI preparations with various contents of the modified NH_2

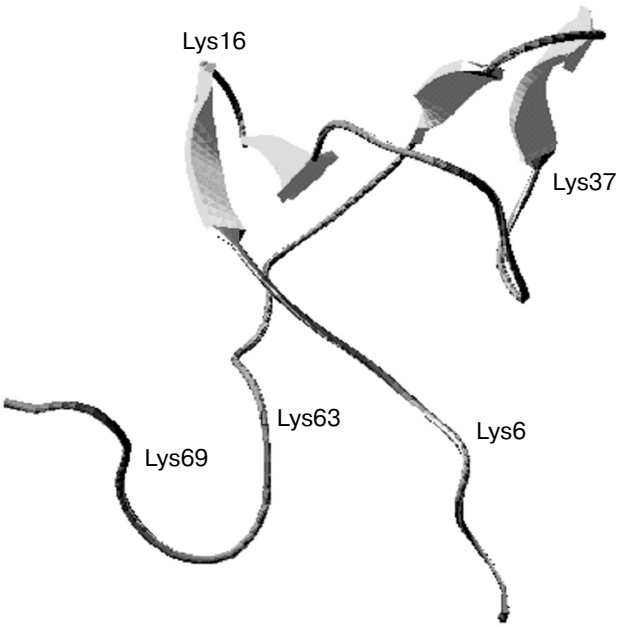

Fig. 1. Tertiary structure of BBI molecule.

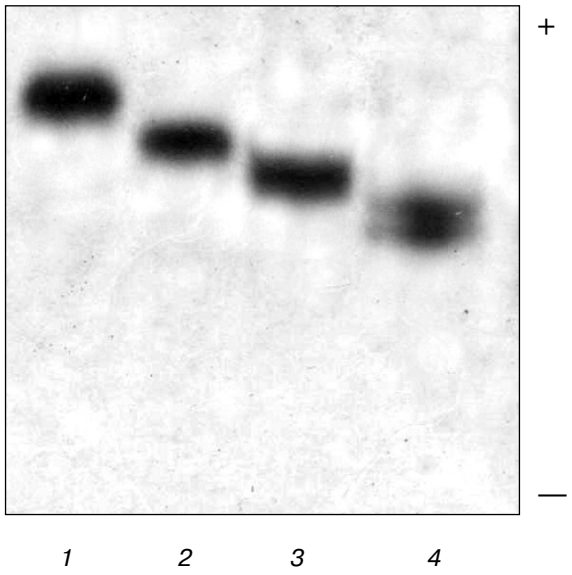

Fig. 2. Electrophoresis in 7% polyacrylamide gel at pH 4.5 of various preparations containing: *1*) native BBI; *2*) $(ol)_1$BBI; *3*) $(ol)_2$BBI; *4*) $(ol)_3$BBI.

groups as well. Homogeneity of acylated BBI preparations was demonstrated by electrophoresis in polyacrylamide gel at pH 4.5 (Fig. 2).

Circular dichroism (CD) is used for studying the secondary structure of proteins. The secondary structure of the BBI molecule does not contain α-helix, but every domain consists of three β-sheets [27-29]. CD spectra of acylated BBI conjugates proved the absence of significant changes in the secondary structure of the protein compared with the native BBI (Fig. 3).

Kinetic and equilibrium inhibition constants of Tp by the native and hydrophobized BBI preparations are presented in Table 1.

Tp specificity is defined by peculiarities of its binding pocket (residues 183-194 and 214-228), namely the presence of the negatively charged Asp189 residue. Thus, in BBI binding to Tp, Lys16 of the inhibitor plays the main role, interacting electrostatically with Asp189 of the enzyme molecule.

As shown, preparations with one acylated amino group retain high affinity to Tp. However, with increase in the number of modified amino groups, a tendency for some decrease in the value of k_{ass} in observed (Table 1). This can be rationalized by disruption of high complementarity of interacting surfaces of the enzyme and inhibitor due to the presence of long-chain fatty acid residues in the BBI molecule (Fig. 4).

The average length of C_{18} fatty acid residue is 26 Å. As shown, the shortest distances between Lys37 and Lys63 of BBI molecule incorporated into the contact area of interaction with proteases and the surface of enzyme molecule are 12 and 20 Å, respectively. That is why partial screening of the binding site of hydrophobized BBI preparations by fatty acid residues is possible; this results in decreased efficiency of Tp inhibition. The higher the degree of BBI modification, the higher is the probability of acylation of both Lys37 and Lys63. But in spite of this, all the BBI derivatives obtained remain rather potent Tp inhibitors.

Analogous data for interaction between BBI derivatives and CTp are given in Table 2.

It should be noted that insertion of one hydrophobic residue into the protein molecule results only in significant decrease in the k_{diss} value; it means that the com-

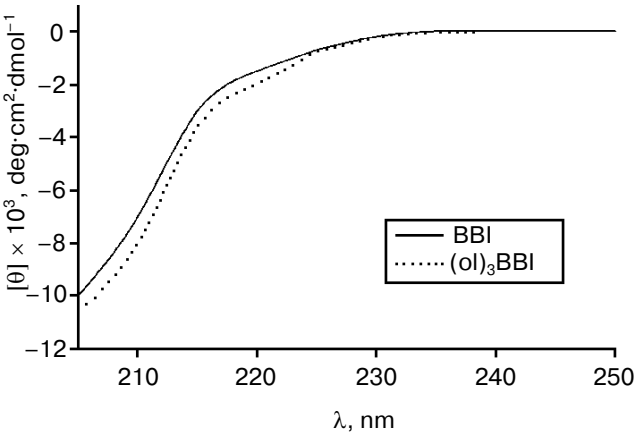

Fig. 3. CD spectra of BBI preparations.

Table 1. Kinetic and equilibrium inhibition constants of Tp by BBI derivatives

Preparation	$k_{ass} \times 10^{-6}$, $M^{-1} \cdot sec^{-1}$	$k_{diss} \times 10^{4}$, sec^{-1}	K_i, nM
BBI	1.1 ± 0.2	4.2 ± 1.1	0.14 ± 0.02
$(ol)_1$BBI	2.6 ± 0.6	5.7 ± 1.4	0.22 ± 0.06
$(lin)_1$BBI	2.4 ± 0.5	4.3 ± 1.0	0.18 ± 0.05
$(\alpha\text{-lin})_1$BBI	1.7 ± 0.4	2.2 ± 0.5	0.13 ± 0.03
$(ol)_2$ BBI	6.7 ± 1.6	5.9 ± 1.5	0.89 ± 0.25
$(lin)_2$BBI	0.41 ± 0.1	3.4 ± 0.8	0.83 ± 0.23
$(\alpha\text{-lin})_2$BBI	0.38 ± 0.1	2.7 ± 0.7	0.64 ± 0.21
$(ol)_3$ BBI	0.75 ± 0.2	6.9 ± 1.7	0.93 ± 0.26
$(lin)_3$BBI	0.67 ± 0.2	6.1 ± 1.5	0.91 ± 0.26
$(\alpha\text{-lin})_3$BBI	0.62 ± 0.1	5.3 ± 1.3	0.85 ± 0.23

Table 2. Kinetic and equilibrium inhibition constants of CTp by BBI derivatives

Preparation	$k_{ass} \times 10^{-5}$, $M^{-1} \cdot sec^{-1}$	$k_{diss} \times 10^{4}$, sec^{-1}	K_i, nM
BBI	2.0 ± 0.4	23.0 ± 5.7	11.5 ± 2.8
$(ol)_1$BBI	2.0 ± 0.4	2.4 ± 0.5	1.2 ± 0.3
$(lin)_1$BBI	1.7 ± 0.4	2.5 ± 0.5	1.4 ± 0.3
$(\alpha\text{-lin})_1$BBI	1.4 ± 0.3	2.8 ± 0.7	2.0 ± 0.4
$(ol)_2$ BBI	88.0 ± 21.0	2.4 ± 0.5	0.02 ± 0.001
$(lin)_2$BBI	65.0 ± 14.0	3.2 ± 0.8	0.05 ± 0.002
$(\alpha\text{-lin})_2$BBI	38.0 ± 9.0	4.2 ± 1.0	0.11 ± 0.02
$(ol)_3$ BBI	1.2 ± 0.3	4.7 ± 1.1	3.9 ± 1.1
$(lin)_3$BBI	1.1 ± 0.2	5.2 ± 1.2	4.6 ± 1.2
$(\alpha\text{-lin})_3$BBI	1.1 ± 0.2	6.5 ± 1.5	5.9 ± 1.5

Table 3. Kinetic and equilibrium inhibition constants of HLE by BBI derivatives

Preparation	$k_{ass} \times 10^{-5}$, $M^{-1} \cdot sec^{-1}$	$k_{diss} \times 10^{4}$, sec^{-1}	K_i, nM
BBI	0.37 ± 0.09	0.10 ± 0.02	2.7 ± 0.06
$(ol)_1$BBI	5.2 ± 1.3	3.5 ± 1.0	0.67 ± 0.09
$(lin)_1$BBI	3.7 ± 0.8	4.1 ± 1.1	1.1 ± 0.2
$(\alpha\text{-lin})_1$BBI	1.4 ± 0.35	8.7 ± 2.2	6.2 ± 1.8
$(ol)_2$ BBI	76.0 ± 20.0	2.3 ± 0.6	0.03 ± 0.001
$(lin)_2$BBI	8.6 ± 2.4	2.6 ± 0.7	0.30 ± 0.06
$(\alpha\text{-lin})_2$BBI	6.1 ± 1.5	3.2 ± 0.9	0.52 ± 0.08
$(ol)_3$ BBI	1.5 ± 0.3	1.1 ± 0.3	6.9 ± 1.7
$(lin)_3$BBI	0.19 ± 0.05	1.4 ± 0.4	7.4 ± 1.9
$(\alpha\text{-lin})_3$BBI	0.22 ± 0.05	1.7 ± 0.5	7.8 ± 2.1

plexes of the enzyme with acylated BBI derivatives are more tightly bound than the native BBI–CTp complex. However, diacylated BBI derivatives form more tightly bound complexes with CTp with the rate ~20-40 times higher than that for the native inhibitor.

For BBI preparations containing three acylated amino groups, the rate of CTp binding is shown to decrease (the values of k_{ass} are two times lower than that for the native BBI), but along with this the forming complexes are tightly bound (the values of k_{diss} are 3-5 times lower than that for the native BBI). As a whole, all hydrophobized BBI derivatives inhibit CTp more efficiently than the native BBI does, diacylated preparations being more efficient (K_i(BBI)/K_i($(ol)_2$BBI) = 575) than monoacylated derivatives (K_i(BBI)/K_i($(ol)_1$BBI) = 9.5) and triacylated BBI conjugates (K_i(BBI)/K_i($(ol)_3$BBI) = 2.9).

Catalytic sites of Tp and CTp are known to coincide, however, the main distinction is in the structure of their binding sites (for CTp, 183-194 and 215-228 residues, the binding pocket size is 10 × 5.5 × 4 Å) [30]. For CTp, this area is hydrophobic and formed mainly by nonpolar side chains (Asp189 residue of the Tp binding site in CTp is changed for Ser189). Thus, we suggest that it is increase in BBI hydrophobicity due to insertion of fatty acid residues into the molecule that results in increased affinity to CTp.

The data on interaction of modified BBI preparations with HLE are given in Table 3.

The tertiary structure of the native elastase is unknown because its crystals are too small for X-ray structure analysis [31]. However, there are many data on the structure of HLE complexes with various synthetic substrates and proteins in the literature [32-34]. Electrostatic and hydrophobic forces are shown to prevail in interaction between the enzyme and various ligands. The binding pocket of elastase contains a hydrophobic cavity, and not only synthetic inhibitors and inhibitors of protein origin but also *cis*-unsaturated fatty acids (K_i(oleic acid) ~$9.0 \cdot 10^{-6}$ M, K_i(vaccenic acid) ~$1.5 \cdot 10^{-5}$ M, K_i(linoleic acid) ~$2.4 \cdot 10^{-5}$ M) are able to suppress its esterase activity. Ashe and Zimmerman [35] suggest that a certain chain conformation which is attained due to the presence of *cis*-unsaturated bond is necessary for inhibition of elastase by fatty acids.

As shown in Table 3, mono- and triacylated conjugates of soybean inhibitor retain high affinity to HLE compared with the native BBI. Anti-elastase activity of BBI containing two fatty acid residues is significantly higher than that of the native BBI. Thus, k_{ass} for $(ol)_2$BBI is 200 times larger than that for BBI and affinity increases approximately two orders of magnitude.

It is interesting that as a result of BBI modification by derivatives of unsaturated fatty acids we first of all obtained increased affinity of these preparations to CTp and to the lesser extent, to HLE. It is known that the hydrophobic cavity of the binding site of CTp is significantly larger than that of HLE. Thus, on interaction with

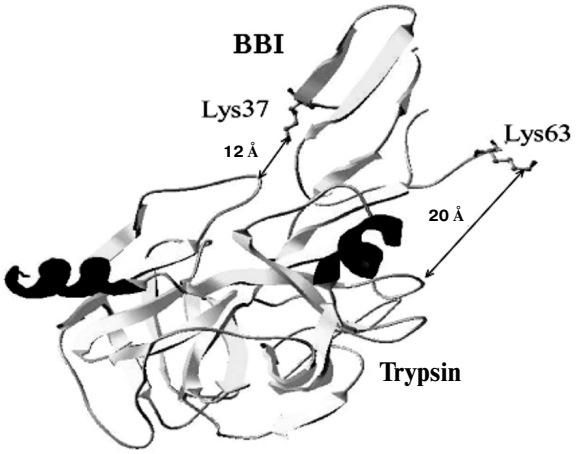

Fig. 4. Tertiary structure of the BBI–Tp complex.

triacylated BBI derivatives, steric hindrance manifests itself more clearly in the case of HLE.

Thus, in our study homogeneous BBI preparations with various numbers of amino groups acylated by unsaturated fatty acids were synthesized. It was demonstrated that of mono-, di-, and triacylated BBI derivatives, insertion of two acyl groups into the protein molecule is optimal for exhibiting anti-proteinase activity by hydrophobized preparations.

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