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Acylation of Bowman–Birk Soybean Proteinase Inhibitor by Unsaturated Fatty Acid Derivatives

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Abstract—A procedure was developed for acylation of Bowman–Birk soybean proteinase inhibitor (BBI) by N-hydroxysuccinimide esters of oleic, linoleic, and α-linolenic acids in a dimethyl sulfoxide–dioxane–pyridine mixture. BBI derivatives containing two acylated amino groups were prepared with high yield. The use of the reversible modifier citraconic anhydride in the first stage of synthesis permitted the synthesis of hydrophobized BBI derivatives retaining high antitrypsin and antichymotrypsin activities. It was found that the insertion of two long-chain moieties in the BBI molecule decreases its thermostability.

Key words: Bowman–Birk inhibitor, hydrophobization, organic solvents

Bowman–Birk soybean proteinase inhibitor (BBI), a protein with M_r 8,000, has two active centers: a trypsin-active center (Lys-16–Ser-17) and a chymotrypsin-active center (Leu-43–Ser-44) [1], as demonstrated by X-ray structure analysis [2]. As we previously reported, BBI can inhibit human granulocyte elastase, an important mediator of inflammation [3, 4]. Furthermore, BBI actively inhibits cellular transformation *in vitro* and carcinogenesis *in vivo* [5-8]. Clinical tests of nutrient additives based on BBI as antitumor medications are now being conducted. When taken orally, BBI is present in the colon in active form, but its concentration in blood is low [9]. Thus, it does not reach organs beyond the digestive tract.

Thus, it would be useful to enhance the bioaccessibility of this proteinase inhibitor by increasing its absorption in the intestine; this might be accomplished by making the molecule more hydrophobic. Such methods for *in vitro* modification known from the literature are based on the interaction of proteins or polypeptides with membrane components or their derivatives. One of the most commonly used methods of protein hydrophobization is acylation with fatty acid derivatives [10-12]. Joining three palmitic acid residues to a BBI molecule extends threefold the persistence of the conjugate in blood as compared to the native BBI [13]. Also, palmitoyl–BBI accumulates in the liver, whereas native BBI is quickly eliminated from the body. Recent studies have shown that some unsaturated fatty acids, such as oleic, linoleic, or α-,γ-linolenic, possess antitumor properties [14].

The anticancer properties of BBI combined with the ability of the above-mentioned fatty acids to inhibit tumor cell growth, along with inferred enhancement of membranotropic activity of the inhibitor by insertion of hydrophobic moieties into the protein molecule, may be of great interest for the use of such BBI derivatives as potential prophylactics.

This study is devoted to both the development of methods for modification of BBI with N-hydroxysuccinimide esters of oleic (ol), linoleic (lin), and α-linolenic (α-lin) acids in organic solvent mixtures and determination of the properties of the acylated BBI preparations.

MATERIALS AND METHODS

Materials. A BBI preparation was isolated from soybeans by the method developed in our laboratory [15, 16]. The content of active molecules of the inhibitor determined by titration with both trypsin and chymotrypsin of known active center content was 88% (w/w). N-Hydroxysuccinimide esters of oleic, linoleic, and α-linolenic acids were purchased from Sigma (USA). The trypsin used was a preparation from Merck (Germany). The active enzyme molecule content determined in a weighed sample by titration with N′-guanidine benzoate

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p-nitrophenyl ester [17] was 64%. α-Chymotrypsin was purchased from Merck. The active enzyme molecule content determined in a weighed sample by titration with N-*trans*-cinnamoylimidazole (Sigma) [18] was 57%. N-Benzoyl-L-arginine hydrochloride ethyl ester (BAEE) and N-benzoyl-L-tyrosine ethyl ester (BTEE) were purchased from Merck. Other chemicals used were of high purity and chemical purity grade manufactured in Russia.

Protein concentration was determined by Lowry et al. [19]. Amino group contents in both the native and modified inhibitor preparations were measured spectrophotometrically using 2,4,6-trinitrobenzenesulfonic acid [20]. Antitryptic and antichymotryptic activities of different BBI-containing preparations were estimated using standard methods [21, 22]. Electrophoresis of BBI preparations was carried out in 7% polyacrylamide gels in an acidic buffer system (Reisfeld, pH 4.5).

Synthesis of citraconylated BBI. BBI (40 mg) was dissolved in 1.5 ml of 0.05 M NH_4HCO_3, pH 8.0. Then citraconic anhydride (6 μl, Merck) was added, and the mixture was stirred intensively for 1 h at room temperature. The citraconylated BBI was lyophilized. The preparation thus obtained had two free NH_2 groups per protein molecule. The residual antitryptic activity was 25%.

Acylation of citraconylated BBI in organic solvent medium. Citraconylated BBI (10 mg) was dissolved for 10 min in 5 ml dimethyl sulfoxide (DMSO). N-Hydroxysuccinimide ester of a corresponding acid in dioxane (0.5 ml) was then added to the solution. Then pyridine (0.2 ml) was added, and the mixture was agitated for 20 h at room temperature. The organic solvents were distilled away under vacuum.

Removal of protective moiety and separation of native and modified BBI. The samples were decitraconylated as we reported previously [23].

The $(ol)_2$BBI and $(lin)_2$BBI preparations were separated from the native protein by precipitation under acidic conditions. To do this, hydrochloric acid (1 mM) was added to the mixture of the native amd modified BBI until a pellet formed. After centrifugation (10 min, 1,600 rpm), the pellet was washed several times with water acidified to the corresponding pH value and then lyophilized.

The $(\alpha\text{-lin})_2$BBI preparation was separated from the native protein by ion-exchange chromatography on DEAE-Sephadex A-25 (Pharmacia, Sweden). The sample was sorbed in 0.05 M CH_3COONH_4 solution, pH 6.2. The material was eluted with a linear gradient of CH_3COONH_4 (0.05-0.3 M), pH 6.2-4.6. Fractions containing the acylated preparation were pooled and lyophilized.

Study of thermal inactivation of BBI preparations. The resulting preparations were dissolved in 0.1 M NaCl, pH 7.0, and placed into a water bath at 75 or 95°C. Aliquots were cooled in an ice bath, and antitryptic activity was measured. The protein concentration in the samples was 0.1 mg/ml.

RESULTS AND DISCUSSION

The BBI molecule has six NH_2 groups (five ε-NH_2-Lys and one α-NH_2 of the N-terminal Asp). By titration with 2,4,6-trinitrobenzenesulfonic acid, we found only five free NH_2 groups. The active center of the BBI molecule that interacts with trypsin is known to contain Lys-16, and its modification may lead to complete loss of antitryptic activity because of the disruption of an ionic bond between the amino group of Lys-16 in BBI and the carboxyl group of Asp-177 involved in the trypsin-binding center [24]. Actually, the experiments showed that modification of BBI with fatty acid derivatives without previous protection of the trypsin-active center retains high antichymotryptic activity (80%), whereas antitryptic activity falls to 10%.

One of the most common ways to reversibly protect protein ε-NH_2 groups is the use of citraconic anhydride [25]. Hence, we used previously citraconylated BBI preparation in our further work on hydrophobization of BBI.

It is known from the literature [26, 27] that organic solvents or their mixtures used as modification media may have a denaturing effect on proteins. The native conformation of a protein can be affected under the influence of organic solvents, resulting in decrease of its biological activity. Thus, we first studied the influence of organic solvent mixture (DMSO, dioxane, pyridine) on the activity of citraconylated and then decitraconylated BBI preparation, BBI(org). To eliminate the possibility that the citraconyl groups were not completely removed, we titrated the free amino groups with 2,4,6-trinitrobenzenesulfonic acid (TNBS) and demonstrated that BBI(org), as well as native BBI, contains five free NH_2 groups. Moreover, we demonstrated the absence of citraconylated BBI in the preparation of BBI(org) by PAGE (Fig. 1). As seen, the citraconylated BBI preparation con-

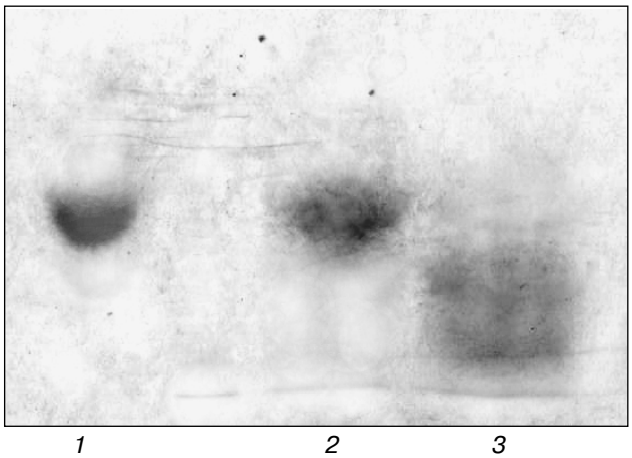

Fig. 1. Electrophoresis of BBI preparations in polyacrylamide gel (Reisfeld, pH 4.5): *1*) BBI; *2*) BBI(org); *3*) citraconylated BBI.

Table 1. Properties of BBI preparations

Preparation	Antitryptic activity, %	Antichymotryptic activity, %	Separation of native and modified BBI	λ_{max}, nm	Proportion of hidden Tyr residues, %
BBI	100	100		283.3	44
BBI(org)*	84	83		284.3	49
$(\alpha\text{-lin})_2$BBI	70	79	ion-exchange chromatography	284.5	50
$(lin)_2$BBI	73	80	sedimentation (pH 2.9)	284.8	51
$(ol)_2$BBI	71	83	sedimentation (pH 3.2)]	285.3	58

* BBI(org) is citraconylated BBI preparation incubated in the acylating medium without the acylating agent and then decitraconylated.

taining two NH_2 groups according TNBS titration gives a blurring spot with higher electrophoretic mobility than either the native BBI or BBI(org) and has virtually no native protein.

The data in Table 1 show that the organic solvents chosen for acylation of the inhibitor with fatty acid derivatives have practically no effect on either its tryptic or chymotryptic activity.

Table 1 shows some methods for separation of the native protein when purifying hydrophobized BBI specimens. A method based on the ability of protein globules to aggregate at lowered pH due to the interaction of hydrophobic residues on their surfaces was applied for both $(ol)_2$BBI and $(lin)_2$BBI.

In contrast, no aggregation was observed in pH range 2.0-12.0 for the $(\alpha\text{-lin})_2$BBI preparation: α-linolenic acid has three double-bonds, so, unlike both oleic and linoleic acids, it is more hydrophilic and has higher solubility in polar solvents. As seen from Fig. 2, native BBI and $(\alpha\text{-lin})_2$BBI can be separated by ion-exchange chromatography.

The absence of the native protein in hydrophobized BBI preparations purified by the methods used was established with by electrophoresis in polyacrylamide gel (data not shown). All BBI preparations modified with fatty acid derivatives were purified with high yield (~75%).

The study of the influence of hydrophobization on the activity of purified BBI preparations showed that all the preparations retain high inhibitory activity against both chymotrypsin (95% of that for BBI(org)) and trypsin (85% of that for BBI(org)).

To demonstrate the higher hydrophobicity of the acylated derivatives of BBI, the second derivatives of the UV absorption spectra were investigated. Aromatic amino acid residues are known to be distributed between the interior areas and the surface of protein globules, so that only some are exposed to the solvent. The BBI molecule contains Tyr-45 (close to the chymotrypsin-active center) and Tyr-59 (exposed to polar solvent, solvated with five H_2O molecules, and located close to both Lys-37 and Lys-63 that might undergo modification) [28]. The data

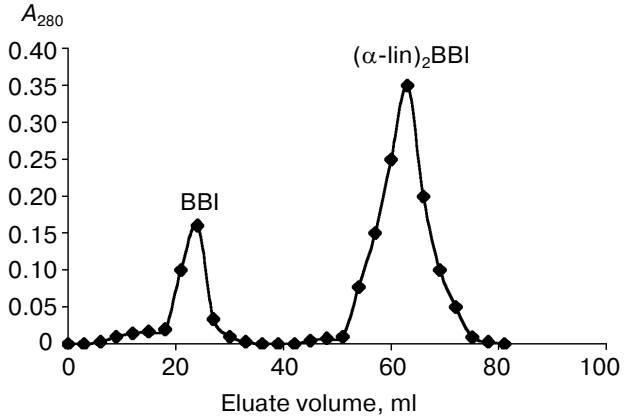

Fig. 2. Separation of acylated $(\alpha\text{-lin})_2$BBI from native BBI by ion-exchange chromatography on DEAE-Sephadex A-25 in the presence of 0.05-0.3 M CH_3COONH_4, pH 6.2-4.6.

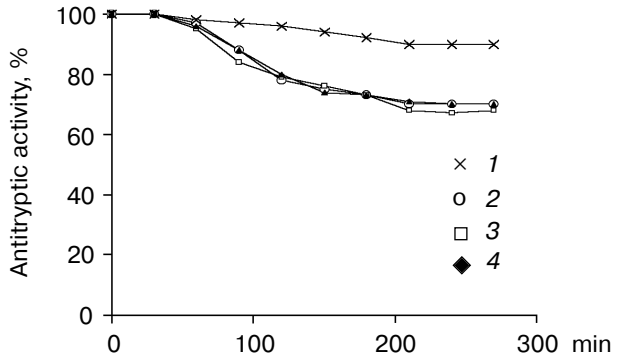

Fig. 3. Thermal inactivation of BBI preparations (75°C, 0.1 M NaCl, pH 7.0). *1*) Native BBI; *2*) $(\alpha\text{-lin})_2$BBI; *3*) $(lin)_2$BBI; *4*) $(ol)_2$BBI.

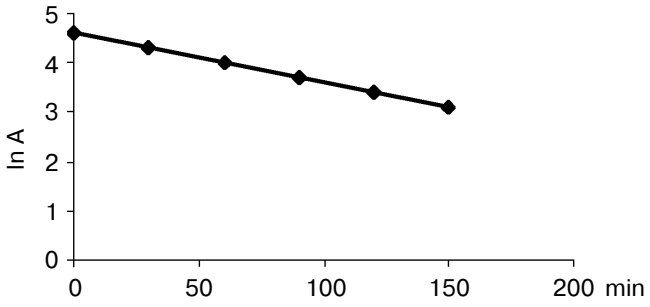

Fig. 4. Semi-logarithmic plot of kinetic curve of thermal inactivation of $(lin)_2$BBI (95°C, 0.1 M NaCl, pH 7.0).

(Table 1) show that the wavelength of maximum absorbance (λ_{max}) is shifted to higher values for (α-lin)$_2$BBI compared to BBI(org). This shows that Tyr residues are less accessible to the polar solvent, i.e., water. Preparations of $(lin)_2$BBI and $(ol)_2$BBI show larger long-wavelength shifts of their spectra. Increased shielding of Tyr in the series (α-lin)$_2$BBI, $(lin)_2$BBI, and $(ol)_2$BBI may result from the increase in rotational freedom of the hydrophobic groups relative to the protein molecule due to reduction in the number of double bonds in their chains.

The number of buried Tyr residues in the protein molecule was calculated by the method of Shevchenko et al. [29]. As seen from Table 1, the fraction of buried Tyr residues increases by 5-14% on the insertion of hydrophobic groups. Organic solvents had a significant effect on BBI conformation. The long-wavelength shift of the absorption peak (λ_{max}) for the BBI(org) spectrum compared to BBI indicates decreased polarity of the tyrosine microenvironment in BBI(org). The protein becomes more hydrophobic than native BBI but remains active.

The incorporation of hydrophobic moieties into a protein can alter not only its catalytic activity, but also its thermal stability [30]. Thus, we studied the influence of incubation of BBI preparations at elevated temperatures on antitryptic activity. Under nearly physiological conditions (0.1 M NaCl, pH 7.0), the activities of both native and modified BBI remained unchanged after 5 h incubation at 37°C. At 75°C, native BBI activity decreased by 10%, whereas the activities of modified preparations decreased by 30% (Fig. 3) after 4 h.

Table 2. Rate constants of thermal inactivation of BBI preparations (95°C, 0.1 M NaCl, pH 7.0)

Preparation	$k_{in} \times 10^4$, sec^{-1}
BBI	0.55 ± 0.13
$(ol)_2$BBI	1.8 ± 0.4
$(lin)_2$BBI	1.7 ± 0.4
(α-lin)$_2$BBI	1.9 ± 0.5

Residual activities of hydrophobized and native BBI were 30 and 70%, respectively, after 2 h at 95°C. The thermal inactivation rate constants of acylated BBI derivatives at 95°C were approximately equal for different fatty acid moieties and were three times higher than for the native BBI (Table 2, Fig. 4).

As seen, the modified BBI preparations are more susceptible than BBI to irreversible damage caused by external factors, e.g., increased temperature.

The BBI molecule has seven disulfide bonds [28]. Therefore, its conformation is rather rigid and its thermal stability is high. The decrease in thermal stability of acylated preparations at high temperatures may result from changes in the native conformation of the protein due both to the influence of the organic medium used for the modification and the incorporation of the long-chain moieties into the protein molecule, as supported by spectrophotometry (Table 1). Such alteration in the native conformation of the protein does not prevent the manifestation of high anti-proteinase activity by the hydrophobized BBI preparations.

Thus, a method has been described for the synthesis of hydrophobized derivatives of BBI. It provides preparations which, in spite of lower thermal stability than the native BBI, retain high antitryptic and antichymotryptic activities.

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