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Conjugation of the Bowman-Birk Soybean Proteinase Inhibitor with Hydroxyethylstarch

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ABSTRACT

The classical Bowman-Birk soybean proteinase inhibitor was modified by hydroxyethylstarch. The modified inhibitor retained the capacity for simultaneous binding of trypsin and human leukocyte elastase. The inhibition constants, K_i, of bovine trypsin, α-chymotrypsin and human leukocyte elastase (HLE) increased not more than 10-, 1.5-, and 20-fold, respectively, on modification of the inhibitor. The less effective inhibition is presumably due to the steric hindrance brought about by the conjugation with polysaccharide molecules. The results obtained indicate the pronounced structure differences of the binding regions for trypsin and chymotrypsin/leukocyte elastase in the modified preparation.

Index Entries: Bowman-Birk soybean inhibitor; hydroxyethylstarch; conjugate; modification; leukocyte elastase.

Abbreviations: BBI, the classical Bowman-Birk soybean proteinase inhibitor; HLE, human leukocyte elastase; HES, hydroxyethylstarch.

INTRODUCTION

The inhibitors of neutral leukocyte proteinases have attracted increasing attention over the past ten years because human leukocyte elastase (HLE) and human leukocyte cathepsin G are the main mediators of inflammation *(1,2)*. One of the most accessible sources of HLE inhibitors is *Fabaceae*. The Bowman-Birk soybean proteinase inhibitor (BBI) is the ancestor of a family of Bowman-Birk inhibitors *(3,4)*. BBI binds

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trypsin-, chymotrypsin like enzymes and leukocyte proteinases rapidly and with high affinity *(5–8)*. According its kinetic parameters BBI may be considered as a promising antiproteolytic drug *(9)*.

BBI is a protein of low Mr (8000 Da) *(3)*. In fact the in vivo delivery of BBI to target organs had many limitations, including epithelial and endothelial barriers. Therefore, when BBI is fed very little is taken up into bloodstream and delivered to the organs *(10)*. After intravenous injection, BBI is rapidly eliminated by the reticuloendothelial cells and from the kidneys *(11,12)*, with a half-life of 3.9 min *(12,13)*. Chemical modification of proteins with biocompatible soluble polymers is one of the most promising ways to overcome these problems *(14)*. Previously, a number of macromolecular derivatives of BBI have been synthesized *(11,13,15–17)*. This paper describes the modification of BBI with hydroxyethylstarch (HES), which is widely used in the United States and Japan as a plasma expander *(18)*. HES has a half-life of 24 h and a complete removal from the human body occurs in 30 d *(18)*. BBI was successfully used for the treatment of experimental endotoxemia *(19)* and HES is useful in septicemia and shock *(18)*. Combining the best properties of its two parts, HES-BBI could show a superior therapeutic effect.

MATERIALS AND METHODS

Materials

Bovine trypsin (E.C. 3.4.21.4) and α-chymotrypsin (E.C. 3.4.21.1) were from the Olaine chemical plant (Olaine, Latvia).

HLE (E.C. 3.4.21.11) was purified using the method of Baugh and Travis *(20)*. BBI was isolated from strains of soybean "Krapinka" and "VNIIS-2" as described previously *(5)*. HES (weight-average Mr 100–160 kDa, substitution degree 0.6–0.7) was synthesized by alkylation of partially hydrolyzed amylopectin starch with ethylene oxide *(21)*. Glutaraldehyde, benzoyl-L-arginine ethyl ester (BAEE), benzoyl-L-tyrosine ethyl ester (BTEE), and MeO-Suc-Ala-Ala-Pro-Val-pNA were from Sigma, St. Louis, MO. TSK-Gel Toyopearl HW-60 was from Toyo Soda MFG, Shizuoka, Japan. Triton X-100 was from Serva, Heidelberg, Germany.

Modification of BBI with Hydroxyethylstarch

Activation of Hydroxyethylstarch

HES (100 mg) was added to 10 mL 5% cyanuric chloride in a mixture of dioxane-o-xylene (1:1 v/v). The mixture was stirred for 30 min at room temperature. The activated polysaccharide was then thoroughly washed with dioxane and dried.

Coupling of BBI to 4,6-Dichloro-s-Triazinehydroxyethylstarch

Activated HES (100 mg) was reacted with a 2 or 20 mL solution of BBI (5 mg/mL) in 0.05 *M* borate buffer, pH 8.5, for 20 h at room temperature.

The unreacted chlorines were blocked by adding ethanolamine into the solution until its concentration became equal to 1 *M* and stirring for 4 h. Then the mixture was applied to a Toyopearl HW-60 column (3.5 × 44 cm) equilibrated with 5% acetic acid containing 1 *M* NaCl. The first protein band was desalted on the same column in 5% acetic acid and then lyophilized. Elution of polysaccharide was followed by monitoring the phenol-sulfuric acid reaction at 485 nm *(22)*. The protein content in modified BBI was quantified by the method of Lowry et al. *(23)* using purified BBI as a standard.

Enzyme and Inhibitor Activity Assays

Trypsin was titrated with the covalently binding substrate 4-nitrophenyl guanidinobenzoate by the method of Shaw et al. *(24)*.

α-Chymotrypsin was titrated by *N*-trans-cinnamoyl-imidazole *(25)*.

BBI was titrated with the active site-titrated bovine trypsin. The antitryptic activity was assayed spectrophotometrically by measuring the inhibition of the tryptic hydrolysis of BAEE *(26)*. The antichymotrypsin activity was assayed according to the modified method of Hummel *(27)*, as described by Kress, et al., *(28)* using BTEE as a substrate. The antielastase activity was assayed using MeO-Suc-Ala-Ala-Pro-Val-pNA as substrate *(29)*. The concentration of elastase in solution was determined by the rate of hydrolysis of the substrate in the absence of the inhibitor with $k_{cat} = 17\ s^{-1}$ and $K_m = 1.4 \times 10^{-4}\ M$ *(29)*. It was shown that 15 min preincubation sufficed to form proteinase-inhibitor complexes under the conditions of the assay.

Determination of Dissociation Constants of Proteinase-Inhibitor Complexes (K_i)

The values of inhibition constants (K_i) were determined from the titration curves by measuring the enzymatic activity of equilibrium mixtures formed of a constant amount of proteinases and increasing amounts of inhibition using the approach reported elsewhere *(30)*. K_i^{app} was calculated using eq. (1). K_i^{app} and $[E^o]$ are optimizing parameters of eq. (1). Selection of the optimal values of K_i^{app} and $[E^o]$ was carried out by the nonlinear regression method using the Sigma Plot for Windows. Using the Marquardt method, the program perform simultaneous optimization of several parameters. The value of K_i^{app} calculated by the graphic method *(31)* and the value of $[E^o]$ determined by the titration of the active sites or the values of specific preparation activity of the corresponding proteinases were used as initial approximations.

To verify certainty of data obtained, the calculation was repeated with several other values of initial approximations.

$$a = 1 - \frac{([E^0] + [I^0] + K_1^{app}) - \{([E^0] + [I^0] + K_1^{app})^2 - 4[E^0][I^0]\}^{1/2}}{2[E^0]} \quad (1)$$

where **a** is the fractional activity (rate in the presence of inhibitor/rate in its absence, $([E^o] - [EI])/[E^o]$); $[E^o]$ is the initial active enzyme concentration; $[I^0]$ is the initial inhibitor concentration, the active inhibitor for native BBI and the total protein for the modified BBI preparations; K_i^{app} is the apparent constant of dissociation of *EI* complex.

The true values of the dissociation constants of enzyme-inhibitor complexes (K_i) were calculated by eq. (2):

$$K_1^{app} = K_i(1+[S^0]/K_m \quad (2)$$

where $[S^0]$ is the initial substrate concentration which is used for the enzyme assay; K_m is the Michaelis constant. The K_m values employed: 3×10^{-6} *M* for trypsin-catalysed BAEE hydrolysis *(32)*; 2.2×10^{-5} *M* for α-chymotrypsin-catalysed BTEE hydrolysis *(27)*; 1.4×10^{-4} *M* for HLE-catalysed MeO-Suc-Ala-Ala-Pro-Val-pNA hydrolysis *(29)*.

RESULTS AND DISCUSSION

Hydroxyethylstarch was conjugated through the free amino and hydroxy groups of the tyrosine residues of the BBI molecule, which contains five ε-amino groups of lysine residues, one N-terminal aspartic acid amino group and two tyrosine hydroxy groups *(33)*. Cross-linking HES with cyanuric chloride and then via BBI is also possible. Earlier, we showed the occurrence of these effects upon modification of basic pancreatic trypsin inhibitor by polysaccharides activated by cyanuric chloride *(34)*. To increase the yield of HES-BBI during separation from the native inhibitor, we carried out gel-filtration on Toyopearl which is a hydrophilic polymer completely different from dextran or agarose gels. We checked Toyopearl HW-50, 55, and 60 and demonstrated that the best results were obtained in the latter case. The content of protein in the modified BBI was 3 and 6 mg per g of the preparation when the initial ratio BBI/HES was 1:10 and 1:1, respectively.

We measured the antiproteinase activity of the synthesized conjugates. Figs. 1–3 show the titration curves of trypsin, α-chymotrypsin and HLE by some preparations of HES-BBI. The true inhibition constants were found using the dependence of the K_i^{app} on $(1 + [S^0]/K_m)$ according to Eq. (2) and were summarized in Table 1.

Though the K_i value for inhibition of trypsin and HLE by HES-BBI increased approximately by an order of magnitude, the affinity of the conjugate to α-chymotrypsin practically did not change. The values of K_i are slighly influenced by the protein content in the preparations. These results are explicable by taking account of the BBI structure. BBI macromolecule consists of two similar domains. One of them has the trypsin-reactive site (Lys^{16}-Ser^{17}), the other the α-chymotrypsin/HLE reactive site (Leu^{43}-Ser^{44}) *(33)*. It should be noted that the amino group of Lys^{16} may be modified during the coupling. Fortunately, the chymotrypsin/HLE

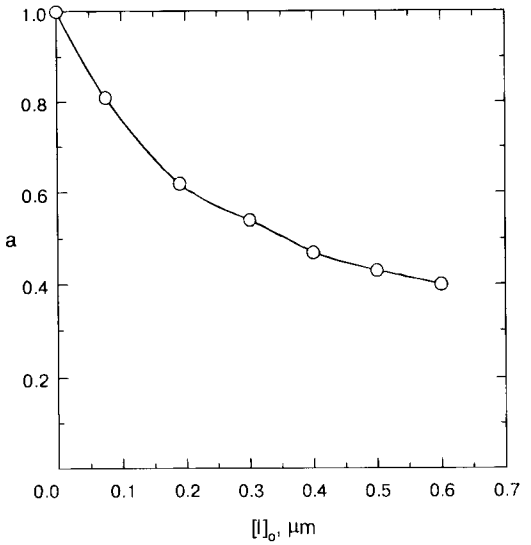

Fig. 1. The residual enzymatic activity of trypsin as a function of protein concentration in HES-BBI conjugate (6 mg protein/g). $[E^0]$ = 6.8 n*M*, [BAEE] = 6×10^{-4} *M*, 0.05*M* Tris HCl, 0.02 *M* $CaCl_2$, pH 8.0, 25°C.

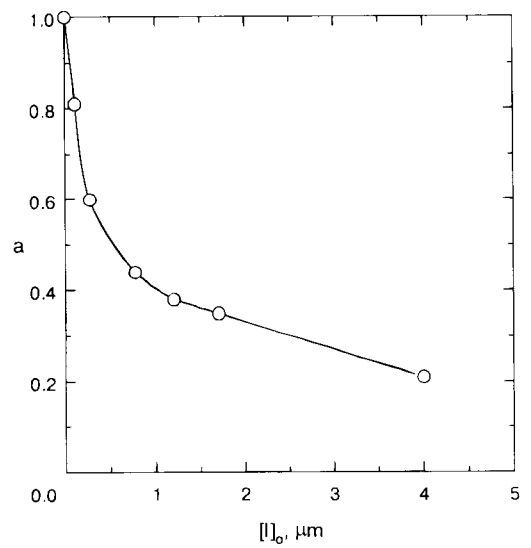

Fig. 2. The residual enzymatic activity of α-chymotrypsin as a function of protein concentration in HES-BBI conjugate. (6 mg protein/g). $[E^0]$ = 50 n*M*, [BTEE] = 4×10^{-4} *M*, 0.05*M* Tris HCl, pH 8.0, 25°C.

reactive site of BBI does not react directly with the activated hydroxy groups of HES.

Nevertheless the steric effects obviating the multiple contacts between HLE and BBI molecules may be the reason for some weakening of HLE inhibition by polysaccharide-modified inhibitor.

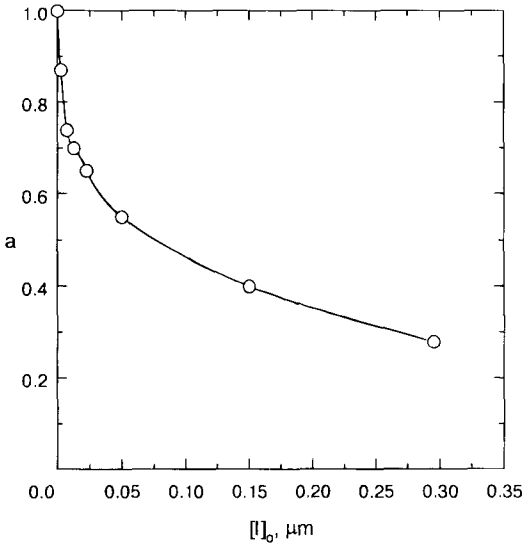

Fig. 3. The residual enzymatic activity of HLE as a function of protein concentration in HES-BBI conjugate (3 mg protein/g). [E^0] = 8.7 n*M*, [MeO-Suc-Ala-Ala-Pro-Val-pNA] = 10^{-3}*M*, 0.05 *M* HEPES, 1*M* NaCl, pH 7.5, 25°C.

Table 1
The K_i for Trypsin, α-Chymotrypsin, and Human Leukocyte Elastase Inhibition by BBI-Containing Preparations

Enzyme	K_i, nM		
	BBI	HES-BBI	
		3 mg BBI/g preparation	6 mg BBI/g preparation
trypsin	0.14 [5]	0.60	1.3
α–chymotrypsin	6.40 [5, 7]	10.0	10.0
HLE	2.0 [5]	15.0	38.0

To verify that HES-BBI is still a "double-headed" inhibitor, we added HLE to a mixture of trypsin and HES-BBI (3 mg protein/g) preincubated for 15 min. A decrease in the elastase activity in 5 min without increasing the trypsin activity is consistent with the retention of two independent proteinase-binding sites in HES-BBI.

So, these results indicate that the modified BBI remained a fairly strong inhibitory agent capable of simultaneous binding to trypsin- and α-chymotrypsin-like proteinases. If the retention time of HES-BBI in vivo is similar to that for HES, the potential of BBI as antiproteolytics could improve.

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