Human leukocyte elastase inhibition by Bowman-Birk soybean inhibitor

Discrimination of the inhibition mechanisms

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Abstract The reaction between human leukocyte elastase and soybean Bowman-Birk inhibitor has been studied. The inhibition was found to be due to slow tight binding of the inhibitor. The interaction of BBI with HLE was shown to involve two steps: the rapid formation of an initial EI complex, with a K_i of 28 nM, followed by a slow equilibrium conversion to a tighter-binding EI* complex with a final K_i^* of 2.3 nM. At pH 7.5 and 25°C, k_{on} was 3.5×10^4 M^{-1} s^{-1} and k_{off} was 1.0×10^{-4} s^{-1}.

Key words: Bowman-Birk soybean inhibitor; Human leukocyte elastase; Kinetic constant; Inhibition constant

1. Introduction

The classical soybean Bowman-Birk proteinase inhibitor (BBI) is well known as an ancestor of the Bowman-Birk inhibitor family [1]. BBI is a protein of low molecular mass (8000 Da). It has 71 amino acid residues, and contains 7 disulphide bridges. Classical BBI, called a double-headed inhibitor, simultaneously inhibits trypsin via one reactive site, Lys_{16}-Ser_{17}, and α-chymotrypsin via another, Leu_{43}-Ser_{44} [1–3]. The ability of classical BBI to inhibit human granulocyte elastase (HLE) and cathepsin G reactions with synthetic and natural substrates has also been demonstrated [4–8]. HLE and human granulocyte cathepsin G are believed to be involved in the pathogenesis of a variety of diseases [9]. The high-efficiency inhibitors of neutral leukocyte proteinases have been extensively studied because of their physiological significance, and potential therapeutic implications [10]. It should be noted, however, that the inhibition kinetics of HLE by the Bowman-Birk inhibitors have not yet been studied. In this paper, the formal inhibition mechanisms were discriminated and the kinetic constants of interaction between BBI and HLE and dissociation constants were determined.

2. Materials and methods

2.1. Materials

α_1-PI, MeOSuc-Ala-Ala-Pro-Val-pNA, HEPES, DMSO, *p*-nitrophenyl *p'*-guanidinobenzoate, Triton X-100 (Sigma, USA), bovine trypsin (Olaine Chemical Plant, Latvia) with 49% content of active sites (titrated with *p*-nitrophenyl *p'*-guanidinobenzoate) [11]. HLE was purified as reported previously [5]. The classical BBI was isolated from soybean cultivar VNIIS-2 according to a modified method of Odani and Ikenaka [2] described elsewhere [4].

2.2. Active site titration

Titration of HLE with standardized BBI and α_1-PI revealed 93–95% activity of enzyme. α_1-PI and BBI were quantified by direct titration with the active site-titrated trypsin, and were found to be 47% (α_1-PI) and 100% (BBI) active with respect to the total protein.

2.3. Protein determination

Protein was assayed according to Lowry et al. [12].

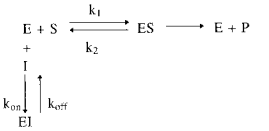
2.4. Kinetic procedure

HLE activity measurements were made with MeO-Suc-Ala-Ala-Pro-Val-pNA as a substrate, and its hydrolysis was followed by monitoring the release of *p*-nitroaniline at 410 nm ($\varepsilon_{410}=8800$ M^{-1} cm^{-1}) (Shimadzu UV-265 FM recording spectrophotometer). A 1 ml cuvette (1 cm path length) containing 0.1 ml substrate (10^{-3} M in Me_2SO) and 0.3–0.8 ml BBI in 0.1 M HEPES buffer, pH 7.5, containing 0.005% Triton X-100 and 0.5 M NaCl, was incubated at 25°C for 5–10 min in a jacketed holder in the spectrophotometer cell compartment. Injection of 0.1 ml HLE solution initiated the reaction. The final concentrations of HLE, substrate and BBI were 4.3 nM, 0.1 mM and 30–80 nM, respectively.

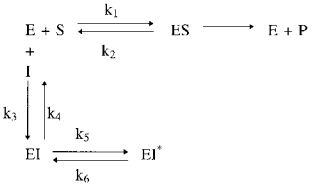
2.5. Data analysis

To assess the equilibrium and kinetic parameters of HLE-BBI interaction, the method, offered for slow-binding inhibitors was used [13–15]. There are two basic mechanisms [14] (A and B) for slow inhibition of enzymes.

Mechanism A:



Mechanism B:



The integrated equation (Eq. 1) describes product concentration in the presence of competitive slow-binding inhibitor which interacts with enzyme according to mechanism A or B, if $[I]_o \gg [E]_o$ and $[P] \ll [S]_o$ [15].

$$P = \upsilon_S t + (\upsilon_o - \upsilon_S)[1-\exp(-kt)]/k \quad (1)$$

where P is the product concentration at any time t, υ_o is the initial and υ_S is the final steady-state velocities, k is the apparent first-order rate constant for the establishment of the equilibrium between EI and EI*.

Progressive curves for HLE inhibition were fitted by non-linear regression to Eq. 1 using a Sigma Plot program for DOS (USA). Using the Marquardt method, the program performs simultaneous optimization of several parameters. As initial approaches for the three

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Abbreviations: BBI, classical soybean Bowman-Birk proteinase inhibitor; HLE, human leukocyte elastase; α_1-PI, α_1-proteinase inhibitor; MeO-Suc-Ala-Ala-Pro-Val-pNA, methoxysuccinyl-Ala-Ala-Pro-Val-*p*-nitroanilide; DMSO, dimethyl sulfoxide

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empirical parameters υ_o, υ_S and k, values calculated using the graphic method [16] were used. To verify the data obtained, the calculation was repeated with several other values of initial approximations.

For mechanism A the following equations were used to calculate the association rate constant (k_{on}) and dissociation rate constant (k_{off}) [15]:

$$k = k_{off} + k_{on}[I]_o/(1 + [S]_o/K_m) \quad (2)$$

$$k_{off} = k\upsilon_S/\upsilon_o \quad (3)$$

The inhibition constant K_i^* was calculated according to Eq. 4 from the final steady-state velocity data [15].

$$K_i^*(1 + [S]_o/K_m) = [I]_o/(\upsilon_c/\upsilon_S - 1) - \upsilon_S[E]_o/\upsilon_c \quad (4)$$

where υ_c is the control velocity in the absence of BBI.

For mechanism B, the apparent first-order rate constant can be expressed as:

$$k = k_6 + k_5\left[\frac{[I]_o/K_i}{1 + [S]_o/K_m + [I]_o/K_i}\right] \quad (5)$$

where K_i is the constant of the first equilibrium ($K_i = k_4/k_3$ [15]).

The kinetic rate constants (k_5, k_6) and overall inhibition constant, K_i^*, are defined in Eqs. 6–9 [15].

$$k_6 = k\upsilon_s/\upsilon_o \quad (6)$$

$$\upsilon_o = \frac{k_{cat}[E]_o[S]_o}{K_m(1 + [I]_o/K_i) + [S]_o} \quad (7)$$

$$\upsilon_S = \frac{k_{cat}[E]_o[S]_o}{K_m(1 + [I]_o/K_i^*) + [S]_o} \quad (8)$$

$$k_6/(k_5 + k_6) = K_i^*/K_i \quad (9)$$

3. Results and discussion

3.1. The inhibition parameters of HLE by BBI

Fig. 1 shows the progressive inhibition of HLE by BBI in the presence of the substrate MeO-Suc-Ala-Ala-Pro-Val-pNA. The rate of substrate hydrolysis decreased from an initial rate (υ_o) to a much slower steady-state rate (υ_S) according to the first-order rate constant (k) Eq. 1.

k is related to the k_{on} and k_{off} by Eqs. 2 and 3. Thus, a replot of the k vs. $[I]_o$ (Fig. 2) afforded these constants. Linear regression analysis provided accurate estimation of $k_{on}/(1+[S]_o/K_m)$. The k_{on} was calculated using $[S]_o = 0.1$ mM, $K_m = 0.055$ mM and was equal to $(3.5 \pm 0.5) \times 10^4$ M^{-1} s^{-1}. Linear regression analysis also provided the error on k_{on}. As can be seen, the line intercepts the ordinate giving an accurate determination of k_{off} (see Eq. 2). In addition, the dissociation rate constant was calculated for each inhibitor concentration from Eq. 3. The k_{off} and its error were then computed from the individual values and were equal to $(1.0 \pm 0.2) \times 10^{-4}$ s^{-1}. The values of K_i^* calculated from to Eq. 4 was equal to (2.0 ± 0.5) nM. Taking into account the values of kinetic rate constants we may conclude that BBI is a slow-binding inhibitor of HLE.

3.2. Mechanistic consideration of HLE inhibition by BBI

An important problem in contemporary enzymology is the determination of the mechanism responsible for slow-binding inhibition. Slow-binding inhibitors are distinguished from their classical counterparts by diminished values of k_{on} and k_{off} which are typically several orders of magnitude smaller than those of classical inhibitors.

For mechanism A, it is assumed that the interaction of a competitive inhibitor with enzyme is slow because its concentration is low and/or it encounters barriers for its binding at the site of substrate interaction. For mechanism B it is assumed that there is an initial rapid interaction between the enzyme and competitive inhibitor to form EI which then undergoes a slow conversion to a tighter-binding EI* complex.

In spite of the fact that the canonical mechanism of association of proteinases with their protein inhibitors was proposed by Laskowski et al. [17] rather long ago, it was recently demonstrated [18–20] that every proteinase-inhibitor pair has

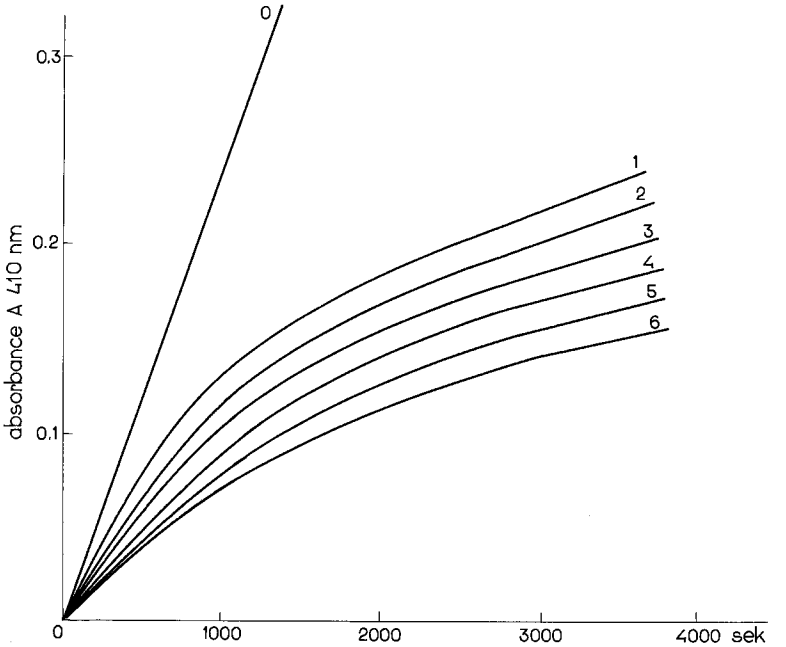

Fig. 1. Hydrolysis of MeOSucAlaAlaProValpNA by HLE in the presence of BBI. BBI concentrations in (nM): 0.0 (0), 30.0 (1), 40.0 (2), 50.0 (3), 60.0 (4), 70.0 (5), 80.0 (6). $[S]_o = 0.1$ mM, $[E]_o = 4.3$ nM, 0.1 M HEPES, pH 7.5, 0.5 M NaCl, 0.005% Triton X-100.

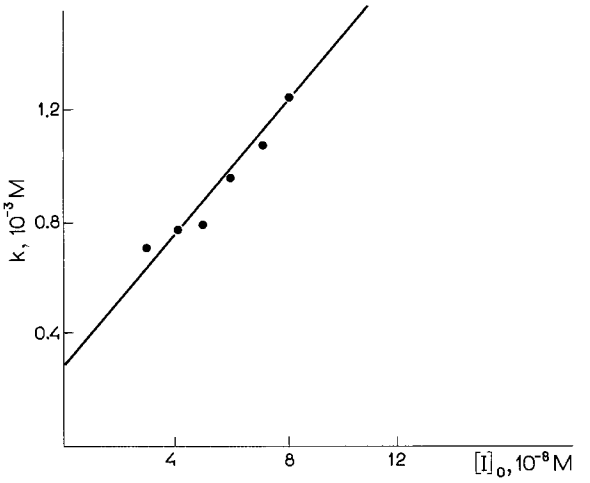

Fig. 2. Dependence of first-order rate constant for the approach to steady state on concentration of BBI.

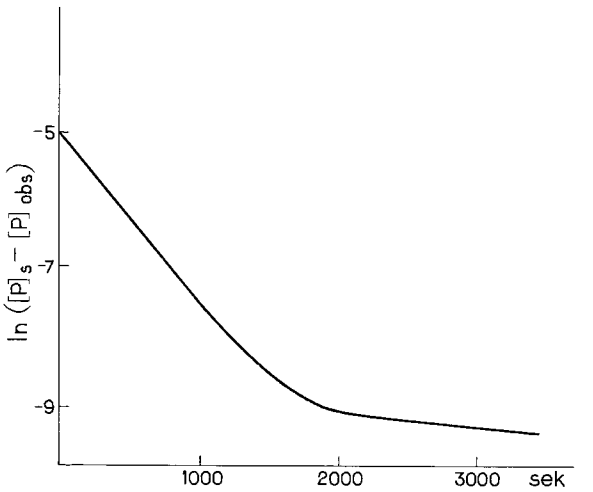

Fig. 3. Plot of $\ln([P]_s-[P]_{obs})$ against t for the inhibition of HLE by BBI. Data taken from curve 6 in Fig. 1.

its own interaction peculiarities including various number of stages in this scheme.

The progress curves for the inhibition of HLE by BBI were found to be biphasic, as illustrated in Fig. 1. In the absence of the inhibitor the steady-state velocity for the hydrolysis of MeO-Suc-Ala-Ala-Pro-Val-pNA was reached immediately. However, in the presence of BBI there is a slow decrease in both (initial and steady-state) substrate hydrolysis rates, which vary as a function of the inhibitor concentration. Analysis of these results can be used to derive the mechanism of HLE-BBI interaction.

The characteristics υ_0 and k in Eq. 1 exhibit a different dependence on the inhibitor concentration for these two mechanisms. The initial velocity is independent of $[I]_o$ for mechanism A, but decreases as the concentration of the inhibitor increases for mechanism B [14,15]. Linearity of plots of $1/\upsilon_0$ vs. $[I]_o$ (not shown) suggests that BBI binds to HLE according to mechanism B. Further, k varies as a linear function of $[I]_o$ (Fig. 2) at BBI concentration up to 80 nM. From a formal point of view this fact is consistent with mechanism A. The inhibition of HLE by BBI, and the inhibition of HLE [21] and human pancreatic elastase [22] by eglin C, are examples where it is problematic to distinguish whether the mechanism of slow inhibition belongs to the A or B type. Mechanism B seemed to be transformed into mechanism A because the range of inhibitor concentrations is insufficient to give saturation of binding at the first step in mechanism B [14].

The k_{on} value for association of BBI with HLE is 4 orders of magnitude lower than the rate constant for a bimolecular diffusion-controlled reaction. It may support the suggestion that the interaction between HLE and BBI occurs via a rapidly formed complex EI followed by its slow rearrangement into EI* (mechanism B).

An additional method of analysis of the results was used for discrimination of the investigated mechanisms. For two-step interaction between the inhibitor and the enzyme, the formation of the reaction product as a function of time is:

$$[P]_{obs} = [P]_s - c_1 e^{-r_1 t} - c_2 e^{-r_2 t} \quad (10)$$

where $[P]_{obs}$ is the product concentration actually observed, and $[P]_s$ is the product concentration when t approaches infinity [18].

It can be seen from Eq. 10 that a plot of $\ln([P]_s-[P]_{obs})$ against t should give a curve resolved into two straight lines with slopes of r_1 and r_2 which are a combination of the rate constants. Such a plot for the course of MeOSucAlaAlaProValpNA hydrolysis by HLE in the presence of BBI, as shown in Fig. 3, suggested the formation of an intermediate for the binding of HLE with this inhibitor.

Consequently, the measured rate constants k_{on} and k_{off} are complex values consisting of microscopic terms. We calculated the rate constants of the elementary stages of the BBI and HLE interaction. The results are summarized in Table 1. Contrary to the general mechanism suggested by Laskowski, the K_i was found to be rather small and the stable complex EI* had a dissociation constant that was only 1 order of magnitude lower than that of the initial complex EI. A similar situation has been observed with human pancreatic elastase and eglin C where the initial inhibition constant, K_i was 0.3 μM [22]. The value of the ratio $k_5/K_i = 4.3\times10^4$ M^{-1} s^{-1} was in good correspondence with that of k_{on} for BBI-HLE interaction.

Although the biological and physicochemical properties of the classical soybean BBI have been well documented, crystallographic analyses have not yet yielded its refined structure at high resolution. Nevertheless, Werner and Wemmer have reported the three-dimensional structure of BBI in solution [23] and identified the binding surface of BBI in contact with α-chymotrypsin [24]. It should be noted that the anti-

Table 1
Kinetic and inhibition constants for HLE-BBI interaction (pH 7.5, 25°C)

K_I (nM)	K_I^* (nM)	k_{on} ($\times10^4$ M^{-1} s^{-1})	k_{off} ($\times10^{-4}$ s^{-1})	k_5 ($\times10^{-3}$ s^{-1})	k_6 ($\times10^{-4}$ s^{-1})
	2.0 ± 0.5	3.5 ± 0.5	1.0 ± 0.2		
28 ± 0.5	2.3 ± 0.5	$k_5/K_i = 4.3$		1.2 ± 0.2	1.0 ± 0.2

chymotryptic domain of the inhibitor is responsible for the interaction with HLE and cathepsin G. The surface of contact between BBI and α-chymotrypsin involved residues 39–48 of anti-chymotryptic domain β-hairpin as well as residues 32, 33 and 37 in the anti-chymotryptic domain loop of the inhibitor [24]. The conformation of the binding loop in the BBI-chymotrypsin complex is very similar to the canonical conformation observed in the crystal structure of the bound Bowman-Birk-type trypsin inhibitor from mung bean [25], as well as the Kazal inhibitor family and the potato inhibitor I family [24]. This can be the structural basis confirming that the inhibition of HLE by BBI occurs by the substrate-like standard Laskowski and Kato mechanism [26]. This mechanism was multiply reported to hold for serine proteinase interaction with the above-mentioned families of protein inhibitors [27].

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