Retardation by the soybean Bowman–Birk inhibitor of elastin hydrolysis catalyzed by leukocyte proteinases

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Abstract The classical Bowman–Birk inhibitor from soya retards strongly the hydrolysis of elastin catalyzed by leukocyte elastase, cathepsin G and a mixture of both. The inhibitory effect is practically unaffected by both the adsorption of the enzymes on elastin and prolongation of the enzymatic reaction.

Key words: Bowman–Birk soybean inhibitor; Human leukocyte elastase; Human leukocyte cathepsin G; Elastin

1. Introduction

Serine proteinases from granule fraction of human polymorphonuclear leukocytes, viz. elastase (HLE) and cathepsin G (HLCG), hydrolyze several important structural proteins of connective tissue including elastin, collagen (types I–V, IX, X, XI), fibronectin, proteoglycans [1,2]. It is commonly believed that the hydrolysis of elastin by HLE plays a role in the destruction of connective tissue in some inflammatory diseases, such as lung emphysema, arthritis, etc. [3,4]. Modulation of the elastinolytic activity of HLE has been a focus of many investigations on therapeutically useful inhibitors [3,4].

HLCG has a low elastinolytic activity [5,6] but is able to stimulate the HLE-catalyzed hydrolysis of elastin from different tissues [6–8]. The stimulation factor (F_{st}) reaches 2.0–2.6 in hydrolysis of bovine neck ligament elastin [6–7] and 1.4–2.9 [7] or 7.2 [6] in hydrolysis of elastin from human lung (the data are shown for [HLE]/[HLCG] = 1:2; $F_{st} = (V_{HLE+HLCG} - V_{HLCG})/V_{HLE}$, where V_{HLE}, V_{HLCG} and $V_{HLE+HLCG}$ are the initial rates of elastinolysis catalyzed by HLE, HLCG and the mixture of the both). The synergistic action of two enzymes on elastin stimulates search for inhibitors which suppress the elastinolytic activity of the both proteinases.

BBI is a 8 kDa protein with two reactive sites. One site binds trypsin, while the other shows affinity to chymotrypsin-like enzymes [9] (including HLCG) and HLE [10]. Earlier we showed that BBI is an effective reversible competitive inhibitor of HLE and HLCG in the reactions with synthetic substrates of low molecular weight [10–12]. According to Bieth [13], two parameters, the association rate constant, k_{on}, and the inhibition constant, K_i, affect the efficiency of the inhibitors in vivo. BBI interacts with HLE with $k_{on} = 3.5 \times 10^4$ $M^{-1} \cdot s^{-1}$ and $K_i = 2.0$ nM [10,12] and with HLCG with $k_{on} = 6.4 \times 10^6$ $M^{-1} \cdot s^{-1}$ and $K_i = 1.2$ nM [10,11]. These values suggest that BBI might be an effective inhibitor of elastin hydrolysis catalyzed by HLE and HLCG. This work was aimed at finding experimental evidence for this hypothesis.

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Abbreviations: BBI, classical soybean Bowman–Birk protease inhibitor; BPTI, basic pancreatic trypsin inhibitor; DMSO, dimethyl sulfoxide; HLE, human leukocyte elastase; HLCG, human leukocyte cathepsin G; MeOSuc-Ala-Ala-Pro-Val-pNA, methoxysuccinyl-Ala-Ala-Pro-Val-*p*-nitroanilide; α_1-PI, α_1-proteinase inhibitor; Suc-Ala-Ala-Pro-Phe-pNA, succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide; F_{st}, stimulation factor.

2. Materials and methods

2.1. Materials

Bovine neck ligament elastin, Brij-35, DMSO, HEPES, MeOSuc-Ala-Ala-Pro-Val-pNA, α_1-PI, Suc-Ala-Ala-Pro-Phe-pNA and Tris-HCl (Sigma, USA); CM-Sephadex C-50 and BrCN-activated Sepharose 4B (Pharmacia, Sweden); Triton X-100 and Dextran T-500 (Loba Chemie, Austria), Bovine trypsin with 49% content of active sites (titrated with *p*-nitrophenyl *p*-guanidinobenzoate [14]) (Spofa, Czechia); BPTI preparation 'Gordox' (Gedeon Richter, Hungary).

2.2. BBI isolaton

BBI was isolated from soya variety VNIIS-2 by using a modified method of Odani and Ikenaka [15] described elsewhere [10]. The inhibitor was quantitated by direct titration with active site-titrated trypsin and was ca. 100% active with respect to total protein.

2.3. HLE and HLCG isolation

HLE and HLCG were purified from healthy donor blood using a slightly modified method of Baugh and Travis [16]. The procedure included affinity chromatography on BPTI-Sepharose 4B and ion-exchange chromatography on CM-Sephadex C-50. BPTI-Sepharose 4B was prepared by immobilization of desalted and lyophilized 'Gordox' on BrCN-activated Sepharose 4B [16].

Titration of HLE and HLCG with standartized BBI and α_1-PI revealed 93–95% and 75–80% activity of the enzymes, respectively. Specific activity was 11.2 and 4.2 μmol/min·mg for HLE and HLCG, respectively (see section 2.5). HLCG preparation contained no admixture of HLE.

2.4. Protein determination

Protein was determined by the Lowry routine [17].

2.5. Enzyme assays

HLE was determined spectrophotometrically by measuring the rate of generation of *p*-nitroaniline ($\varepsilon_{410} = 8{,}800$ $M^{-1} \cdot cm^{-1}$ [18]) during the hydrolysis of the specific substrate MeOSuc-Ala-Ala-Pro-Val-pNA at 25°C. The reaction mixture contained the substrate (0.32 mM) and the enzyme in the concentration range 10^{-8}–10^{-7}M in 0.1 M HEPES buffer, pH 7.5, 0.5 M NaCl, 0.005% Triton X-100 and 10% DMSO.

HLCG was assayed spectrophotometrically in a similar way using SucAla-Ala-Pro-Phe-pNA as substrate. The reaction mixture contained 0.1 mM substrate and the enzyme in the concentration range 10^{-8}–5×10^{-7} M in 0.1 M HEPES, pH 7.5, 0.5 M NaCl, 0.005% Triton X-100 and 10% DMSO.

All spectrophotometric measurements were performed on a Shimadzu UV-265 FM spectrophotometer.

2.6. Elastin hydrolysis

The hydrolysis was carried out at 37°C in 0.05 M Tris buffer, pH 7.4, contaning 0.15 M NaCl with permanent agitation as described elsewhere [6]. The absorbance of supernatant at 280 nm was used to evaluate the concentration of soluble elastin fragments released in the

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course of enzymatic hydrolysis [6]. Slope $V = A_{280}/t$ was taken as a rate of elastin hydrolysis (solubilization). The rate depends linearly on the enzyme concentration at 0.5–2.4 μM for HLE and HLCG and for the mixture of the both at concentration ratio [HLE]/[HLCG] from 1:1 to 1:2.

In experiments without preadsorption of the enzymes on elastin, the reagents were mixed in the following order: elastin, buffer, inhibitor, enzymes. Preadsorption of the enzymes was achived by incubating elastin and enzymes in a buffer for 5 min at 37°C with agitation prior to addition of the inhibitor. As it was noticed earlier for HLE [19,20] and shown by us for HLCG, the absorption is complete in a matter of 5 min.

3. Results and discussion

3.1. Inhibition of elastinolysis catalyzed by HLE and HLCG without preadsorption of the enzymes on elastin

When HLE and BBI are simultaneously added into a suspension of elastin, the elastinolytic activity of HLE decreases continuously with increasing BBI concentration (Fig. 1, curve 1). Complete inhibition is observed at the $[I]_o/[E]_o$ ratio as low as 5. With eglin C, i.e. one of the best exogenic inhibitors of HLE, the elastinolysis is completely inhibited at $[I]_o/[E]_o = 12.5$ [21].

For the HLCG-catalyzed hydrolysis of elastin, complete inhibition is reached at a higher BBI concentration. More than 95% of the elastinolytic activity is inhibited at $[I]_o/[E]_o = 20$ (Table 1).

Apparently, as in the case of other reversible protein inhibitors of HLE [19,21], BBI, on administration, immediately forms a complex with HLE or HLCG, which is stable in the presence of elastin. Preferable complexation of HLE or HLCG with BBI, rather than with elastin, is suggested by analysis of the rates of these three processes. Half-association times, $t_{1/2}$, calculated as $\ln 2/[I]_o \cdot k_{on}$ at $[I]_o >> [E]_o$, equal 1.9 and 0.01 s for the BBI binding to HLE and HLCG, respectively, at $[I]_o = 10$ μM. The complex between HLE and elastin is formed slower, $t_{1/2} = 72.0$ s [21]. There is no data available on kinetics of interaction between HLCG and elastin.

Practically irreversible inhibition of HLE and HLCG by BBI under these conditions ($[E]_o = 0.6$–1.6 μM, $[E]_o/K_i = 500$–$1{,}000$) is in good agreement with the theory of Bieth [13]. The stability of the EI complex in the presence of elastin was additionally confirmed by the following experiment. Elastin (10 mg) was administered to an equimolar mixture of HLE and BBI (1 μM) and incubated for 30 min. There was no elastin hydrolysis after 4 h.

As mentioned earlier [6–8], HLCG was able to stimulate the hydrolysis of elastin by HLE. According to our data F_{st} increases linearly with [HLCG] in the range 0.3–2.0 μM ([HLE] = 1.0 μM). At ratios [HLE]/[HLCG] = 1:1 and 1:2 F_{st} equals 1.35 and 2.0, respectively. These values of F_{st} are in accord with the data reported by other authors for hydrolysis of bovine neck ligament elastin [6–8].

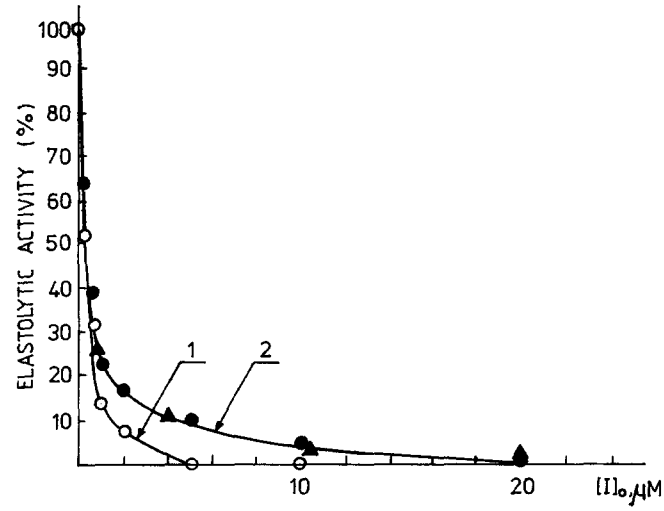

Fig. 1. Inhibition of HLE – catalyzed hydrolysis of elastin by BBI. Curve 1, without preadsorption. Curve 2, with preadsorption of HLE on elastin: the inhibitor was added 5 min (●) and 30 min (▲) after the start of elastinolysis. $[HLE]_o = 1$ μM, 100% = elastinolytic activity of HLE without the inhibitor.

BBI supresses effectively the bi-enzyme elastinolysis under the stimulating conditions (Table 1). An increase in the enzyme ratio [HLE]/[HLCG] from 1:1 to 1:1.5 does not practically affect the degree of inhibition at [BBI] = 100 μM.

3.2. BBI inhibition of elastin-bound HLE and HLCG

All the above data refer to the systems in which the enzymes are added simultaneously or after the inhibitor. The results might be different, if the enzymes preadsorbed on elastin are used. It is known, for example, that adsorption of HLE on elastin protects the enzyme from the action of physiological α_1-PI. The residual enzymatic activity equals 25% and does not decrease further on addition the inhibitor [19]. Apparently, the steric hindrance disfavors the association between the inhibitor and the enzyme adsorbed on insoluble substrate. In contrast, low molecular weight protein inhibitors, secretory leukocyte

Table 1
Suppression by BBI of elastinolysis catalyzed by leukocyte proteinases

$[HLE]_o$, μM	$[HLCG]_o$, μM	$[I]_o$, μM	Residual elastolytic activity (%)	
			Without preadsorption of enzymes of elastin	Without preadsorption of enzymes of elastin
0.0	0.9	0.0	100	100
0.0	0.9	20.0	5	11
0.0	0.9	100.0	0	8
0.6	0.6	0.0	100	100
0.6	0.6	20.0	1	
0.6	0.6	50.0	0	
0.6	0.6	100.0	0	7
0.6	0.9	0.0	100	100
0.6	0.9	100.0	0	10

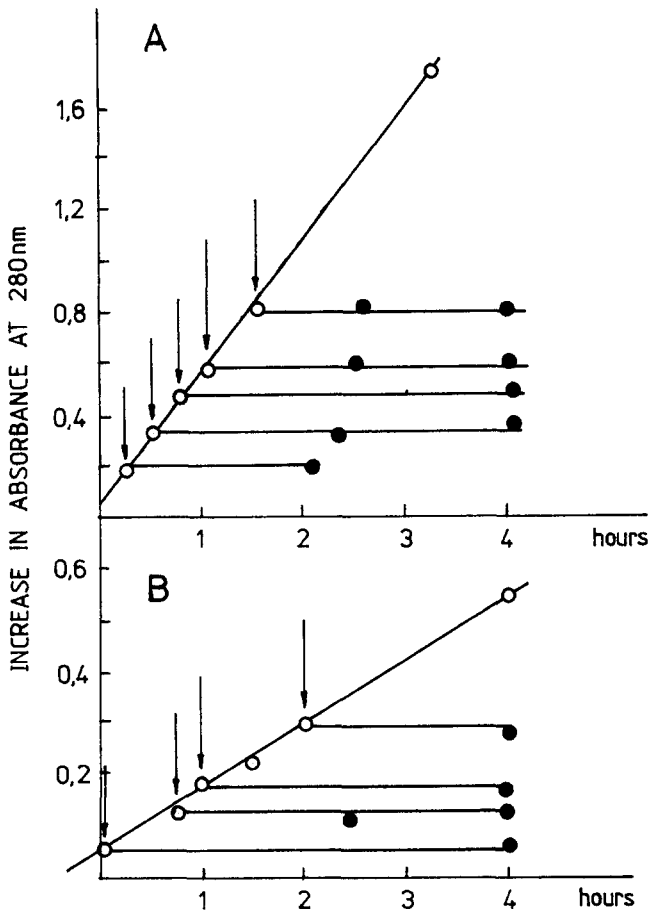

Fig. 2. Inhibition of HLE (panel A) and (HLE+HLCG) (panel B) mediated hydrolysis of elastin by BBI. The points of inhibitor administration are shown by arrows. Hydrolysis of elastin in the absence of the inhibitor (○); hydrolysis of elastin after addition of inhibitor (●). A: $[HLE]_o$ = 1.2 μM; B: $[HLE]_o$ = 0.6 μM, $[HLCG]_o$ = 0.6 μM.

proteinase inhibitor and eglin C, inhibit HLE in solution and on the support in a similar way [19,21].

To study the inhibition of elastin-bound HLE and HLCG we investigated the adsorption of enzymes as it was described by Bruch and Bieth [19]. The adsorption of HLE is a function of the ratio $[E]_o/[S]_o$ in the range of HLE concentrations used. HLE is adsorbed on elastin almost completely (more than 90%) only at $[E]_o/[S]_o$ lower 0.04 nmol HLE per mg elastin. This differs from the data reported elsewhere [19,22] where the HLE activity was completely adsorbed at 0.2–0.25 nmol HLE per mg elastin. The discrepancy may be due to variable properties of elastin from different sources or due to using different isolation procedures [19, 22]. Sorption capacity of elastin for HLCG is higher than that for HLE and equals 0.2 nmol HLCG per mg elastin.

Further studies are performed with the proteinases completely adsorbed on elastin. Administration of BBI into these systems allows to reach almost complete inhibition of elastinolysis but higher concentrations of BBI than in the case of simultaneous addition of enzymes and inhibitor are required. A complete suppression of elastin-bound HLE is reached only at the ratio $[I]_o/[E]_o$ = 15–20 (Fig. 1, curve 2). For HLCG adsorbed on elastin, 90% of elastinolytic activity is inhibited at $[I]_o/[E]_o$ = 20 (Table 1). Five-fold enhancement of the BBI concentration decreases the residual HLCG activity by 3% only.

On addition of BBI to a mixture of both proteinases preadsorbed on elastin (Table 1) residual activity increases with enhancement of HLCG concentration.

Likewise other low molecular weight protein inhibitors [19] BBI is, apparently, able to induce the dissociation of HLE- and HLCG-elastin complexes to form the EI complex. However, a small fraction of HLCG bound to elastin is not available to the inhibitor.

One of the possible factors affecting the efficiency of the inhibition is the duration of preincubation of proteinases with elastin. Hydrolysis of elastin by HLE could be described by the following kinetic scheme [21]:

$$E + S \overset{(1)}{\rightleftharpoons} ES \overset{(2)}{\rightleftharpoons} ES'' \overset{(3)}{\longrightarrow} E + P$$

The rapid and reversible complex formation **(1)** is followed by slow reversible 'isomerization' **(2)** of the encounter complex. Steady-state elastinolytic reaction **(3)** can then occur. Steps **(1)** and **(2)** do not involve elastin degradation [21]. In our experiments HLE is usually preadsorbed on elastin for 3–5 min as recommended elsewhere [19–22]. The incubation time is shorter than the time ($t_{99\%}$) required to complete the formation of ES'' ($t_{99\%}$ = 8.4 min) [21]. Addition of an inhibitor during the formation of complex ES induces its rapid dissociation and thereby suppression of elastinolysis is effective.

Longer interaction between HLE and elastin prior to administrating the inhibitor can reduce the inhibitory effect. This phenomenon was observed with eglin C and human pancreatic elastase [23]. The inhibiting efficiency decreased consequently from 95 to 75% on addition of the inhibitor after 10, 20, 60, 90 min from the begining of the elastinolysis. The effect was accounted for by the fact that a fraction of the enzyme became more strongly bound to elastin as the reaction proceeded and thus was not available to the inhibitor [23]. Similar experiments were performed with BBI and HLE as well as with BBI and the mixture of both proteinases. The inhibition degree is more than 95% in the case of HLE-catalyzed elastinolysis when the inhibitor is added 5 or 90 min after the start (Fig. 2, panel A). The dependency of the degree of inhibition on the BBI concentration was obtained for two periods of time 30 and 60 min. This is practically the same as in the case where BBI was added 5 min after initiation (Fig. 1, curve 2). When elastinolysis is catalyzed by the HLE and HLCG mixture (Fig. 2, panel B) the inhibition is equal to 90% on addition of BBI both immediately after adsorption and after 30–120 min from the start of the reaction. These results suggest that BBI is effective in suppressing elastinolysis catalyzed by leukocyte proteinases regardless a step of elastin solubilization.

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