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SHORT COMMUNICATIONS

Inhibition of free and elastin-bound human pancreatic elastase by human bronchial inhibitor

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Bronchial secretions contain a low molecular mass inhibitor, brI,* capable of inhibiting a variety of serine proteinases including human neutrophil elastase and cathepsin G [1]. The reaction of brI with the former enzyme is extremely fast ($k_{ass} = 10^7\,M^{-1}\,sec^{-1}$) and the elastase–inhibitor complex is very stable ($K_i = 12$ pM) [2]. Theory predicts that with such constants, brI plays an efficient antielastase function at the lung level [3, 4]. The bronchial inhibitor is probably identical to human seminal plasma inhibitor I [5]. The amino acid sequence of the latter has recently been determined by sequence analysis of cDNA reverse-transcribed from an mRNA preparation [6]. This suggests that the preparation of this inhibitor in large quantities by genetic engineering is in active progress. This protein may therefore be used in the mean future as a drug against diseases involving the participation of neutrophil elastase such as lung emphysema or inflammation.

Human pancreas contains one major form of elastase, HPE, named human pancreatic elastase [7] or human pancreatic elastase 2 [8]. This enzyme is thought to induce or to exacerbate acute hemorrhagic pancreatitis [9, 10], a disease for which no efficient medical treatment is available. It was therefore found worth investigating the inhibition of HPE by brI in order to decide whether brI may also be a potential drug for the treatment of the above disease.

Materials and methods

Human leucocyte elastase was isolated from purulent sputum using the method of Martodam *et al.* [11] which involves salt extraction, affinity chromatography on Trasylol-Sepharose and CM-Sephadex chromatography. Published procedures were also used to purify HPE [8], brI [12], α_1-proteinase inhibitor [2] and fibrous human lung elastin [13]. The purity of the soluble proteins was assessed by SDS-polyacrylamide gel electrophoresis at pH 7.4 in 10% gel slabs [14]. The four soluble proteins and the electrophoresis calibration kit (Pharmacia) were treated before electrophoresis as described by Laemmli [15]. Figure 1 shows that HPE, α_1-proteinase inhibitor and leucocyte elastase migrate as single bands. The smearing of the leucocyte elastase band was also observed and discussed by others [11]. The brI migrates as two major bands, in agreement with previous reports [1, 12]. This preparation yielded, however, a single protein band on polyacrylamide gel electrophoresis at pH 4.3 in non-denaturing conditions [16], again in agreement with previous reports [1, 12]. The purity of insoluble elastin was assessed by amino acid composition which was found to be similar to that reported previously [13]. Molarities of enzymes and inhibitors were calculated using molecular masses of 25 kDa, 30 kDa, 14 kDa and 53 kDa for HPE, human leucocyte elastase, brI and α_1-proteinase inhibitor, respectively. The molar concentrations of active enzymes and inhibitors were determined by active-site titration. Human leucocyte elastase was titrated with the active-site titrant Acetyl-$(Ala)_2$-azaAla-*p*-nitrophenylester [17] purchased from Enzyme Systems Products (Livermore, CA), which showed that our preparation was 80% active. This enzyme was used to titrate brI and α_1-proteinase inhibitor as described previously [2]. The former inhibitor was found to be 65% active while the latter was 80% active. The HPE preparation was titrated with α_1-proteinase inhibitor [18] and was found to contain 70% active enzyme. The molarities of HPE and brI indicated in the results section correspond to concentrations of active proteins.

The inhibition of free HPE by brI was assessed using the synthetic substrate Suc-$(Ala)_2$-Pro-Phe-pNA [19] from Bachem. In the experiments reported in Fig. 2, increasing amounts of brI were reacted with constant amounts of HPE (final concentration: 0.1 μM) in a total volume of 495 μl of 0.2 M Tris–HCl pH 8.0. After 20 min at 25°, 5 μl of 90 mM Suc.$(Ala)_2$-Pro-Phe-pNA in N-methylpyrrolidone was added to the mixtures and the absorbance at 410 nm was recorded for a few minutes to measure the reaction rates. The inhibition of elastin-bound HPE by brI was monitored as follows. Insoluble human lung elastin was suspended at a concentration of 7 mg.ml^{-1} in 20 mM Tris–HCl, pH 8.0, 37°. Constant amounts of HPE (0.91 μM) were added to the continuously stirred elastin suspensions. After 15 min, variable amounts of brI were added to these mixtures. The rate of elastin solubilization was then measured as described previously [13]: at selected time intervals aliquots were removed from the suspensions and subsequently acidified, centrifuged and read at 280 nm.

Results

The inhibition of HPE by brI was first assessed using Suc-$(Ala)_2$-Pro-Phe-pNA as an elastase substrate. With [brI] = [HPE] = 13 nM, no appreciable inhibition was observed, whatever the preincubation time of the enzyme + inhibitor mixture. By contrast, reaction of 13 nM HPE with 130 nM brI yielded significant inhibition. When such mixtures were incubated for variable periods of time before addition of substrate, maximum inhibition was obtained within the time required to mix the reagents. On the other hand, the extent of inhibition was found to decrease with increasing substrate concentrations. From the above observations it may be concluded [20] that brI inhibits HPE through formation of a reversible complex which equilibrates rapidly and undergoes fast substrate-induced dissociation.

To measure K_i, we have reacted constant concentrations of HPE with increasing concentrations of brI and have measured the elastase activities of the mixtures with constant concentrations of Suc-$(Ala)_2$-Pro-Phe-pNA. The data shown in Fig. 2 were used to estimate K_i(app) by an iterative least-squares fit to the following equation [20]:

* Abbreviations used: brI, bronchial inhibitor; HPE, human pancreatic elastase; Suc-$(Ala)_2$-Pro-Phe-pNA, N-succinyl-dialanyl-prolyl-phenylalanine-*p*-nitroanilide.

$$a = 1 - \frac{[E^\circ] + [I^\circ] + K_i(\text{app}) - \{([E^\circ] + [I^\circ] + K_i(\text{app}) - 4\,[E^\circ]\,[I^\circ]\}^{1/2}}{2\,[E^\circ]}$$

where a is the fractional activity defined in the legend to Fig. 2, $[E^\circ]$ and $[I^\circ]$ are the total HPE and brI concentrations, respectively and K_i(app) is the apparent equilibrium dissociation constant of the enzyme–inhibitor complex. K_i(app) was found to be 92 ± 4.5 nM. Figure 2 shows that the theoretical curve generated using this value fits well the experimental points. The substrate-independent equilibrium constant, K_i, was derived from $K_i = K_i(\text{app})/(1 + [S^\circ]/K_m)$ [17] using $K_m = 2.5$ mM [19] and $[S^\circ] = 0.9$ mM (Fig. 1). K_i was found to be 67 ± 3.3 nM.

The rate constants k_{ass} and k_{diss} ($K_i = k_{diss}/k_{ass}$) could not be measured. However, the limits of these constants may roughly be estimated. We have shown that reacting 13 nM HPE with 130 nM brI yields "instantaneous" inhibition, indicating that the half-life of the association process is less than 1 sec. Hence, we get $k_{ass} \geq 10^7\,M^{-1}\,sec^{-1}$ and $k_{diss} \geq 0.6\,sec^{-1}$.

HPE was found to be a potent elastinolytic enzyme on human lung elastin (Fig. 3). Under the present experimental conditions, HPE had a specific activity of 8.3 μg elastin solubilized per ml per min per 1 μM enzyme, i.e. more than twice that of human leukocyte elastase on the same substrate [13]. The action of brI on elastin-bound elastase was investigated by adding inhibitor to pre-incubated elastin-elastase suspensions. Figure 3 clearly demonstrates the ability of brI to inhibit matrix-bound elastase. In a separate experiment, HPE was reacted with elastin for 15 min at 37° in the conditions given in Fig. 3, after which, the mixture was centrifuged and the elastase activity of the supernatant was measured with Suc-$(Ala)_2$-Pro-Phe-pNA. This showed that 75% of HPE was bound to elastin. brI is thus able to displace HPE from its complex with elastin. The data shown in the insert of Fig. 3 were used to estimate a K_i(app) of 180 ± 50 nM. This value is 2.7-times higher than the substrate-independent equilibrium dissociation constant of the complex, indicating that elastin efficiently competes with brI for the binding of HPE.

Discussion

Our data are the first to demonstrate that brI inhibits free and elastin-bound HPE. Schiessler *et al.* [21] showed, however, that human seminal plasma inhibitor I, which is probably identical to brI [5], is able to inhibit HPE with a K_i of about 100 nM. These authors did not investigate the action of the seminal inhibitor on elastin-bound elastase.

Acute hemorrhagic pancreatitis is a severe disease characterized by intensive peritoneal bleeding and shock. Pancreatic elastase is thought to be responsible for the vascular injury accompanying this pathological state [9, 10]. There is no specific drug available for the treatment of this injury. The bovine basic pancreatic trypsin inhibitor, frequently used in acute pancreatitis, does not inhibit human pancreatic elastase [7]. By contrast, brI forms an enzymatically inactive complex with this enzyme and may thus be considered as a potential drug for treating the onset of acute hemorrhagic pancreatitis. Moreover, brI also inhibits ela-

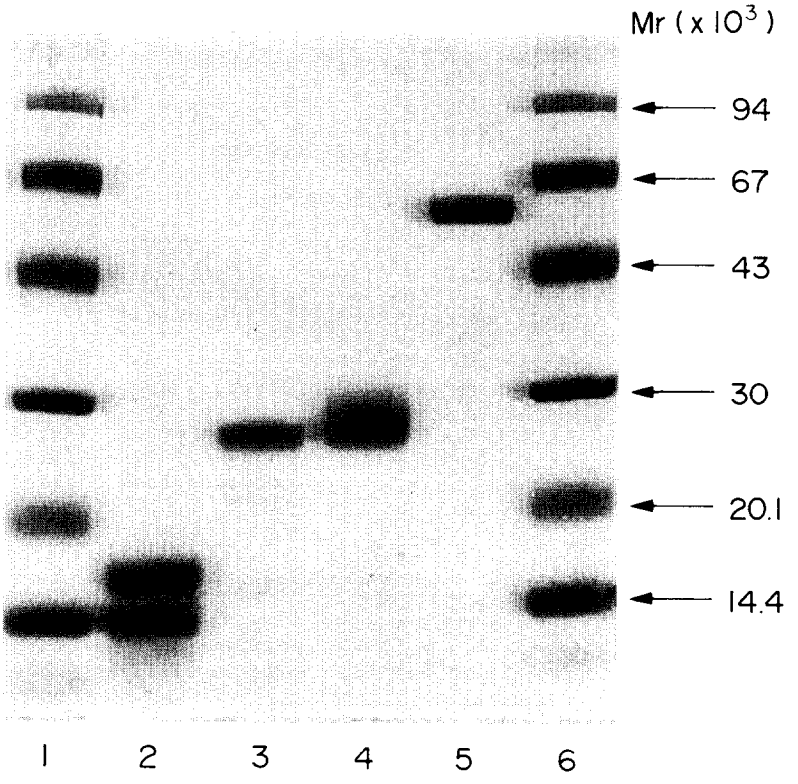

Fig. 1. SDS-polyacrylamide gel electrophoresis of the proteins used in the present investigation. Lanes 1 and 6: molecular mass markers comprised of phosphorylase B, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor and lactalbumin (from top to bottom), lanes 2–5: brI (7.4 μg), HPE (5.9 μg), leucocyte elastase (5.4 μg) and α_1-proteinase inhibitor (4.8 μg), respectively.

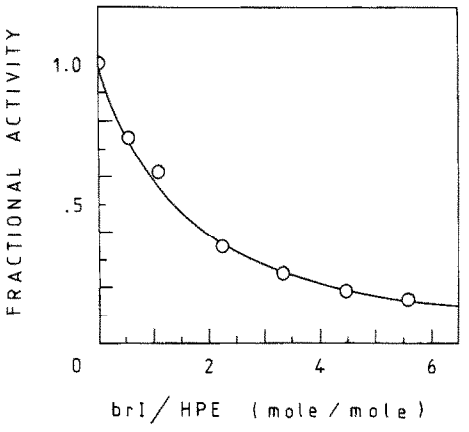

Fig. 2. Inhibition of free HPE by brI. Conditions: HPE = 0.1 μM, Suc-(Ala)$_2$-Pro-Phe-pNA = 0.9 mM, pH 8.0, 25°. The fractional activity is the ratio of the rate of substrate hydrolysis in the presence of brI to that in the absence of inhibitor; (○) = experimental points; (——) = theoretical curve generated using the equation given in the text and a computer—calculated K_i(app) value of 92 nM.

stin-bound elastase, an enzyme–substrate complex that certainly forms *in vivo*. The latter property is of particular interest since α_1-proteinase inhibitor, the endogeneous pancreatic elastase inhibitor, is poorly efficient on elastin-bound elastase (Laurent and Bieth, paper in preparation).

The question is now: how much brI should be administered in order to get efficient inhibition of elastase *in vivo*? Theory predicts that with a reversible inhibitor, 99% inhibition is observed for [I°]/K_i or [I°]/K_i(app) = 100 (refs 3 and 4). The *in vivo* brI concentration, [I°], should therefore be 18 μM (0.25 mg/ml) to get almost full inhibition of elastin-bound elastase. Taking into account this concentration and the rough estimate of k_{ass}, we get a delay time of inhibition [3] of 27 msec. This indicates that with such a concentration of drug, the inhibition will be efficient from both a static and a dynamic point of view. In addition to acting on human pancreatic elastase, brI will also efficiently inhibit human leukocyte elastase, its physiological target [2]. The inhibitor may therefore also decrease the inflammatory reaction that accompanies acute pancreatitis.

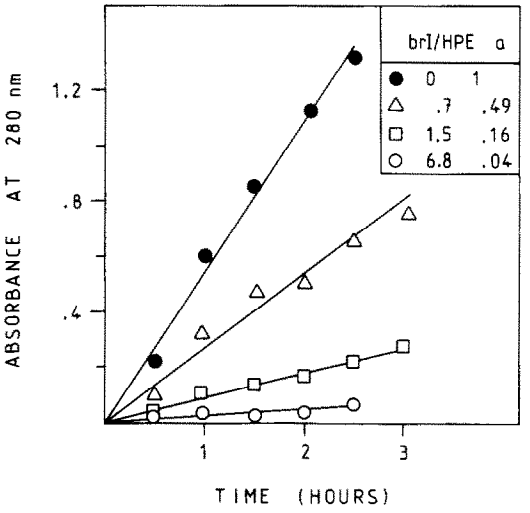

Fig. 3. Kinetics of human lung elastin solubilization by HPE in the absence (●) and in the presence of increasing amounts of brI (△, □, ○). Conditions: HPE = 0.91 μM, elastin = 7 mg.ml^{-1}, pH 8.0, 37°. The insert shows the fractional activity *a* (defined in Fig. 1) as a function of the molar ratio of brI to HPE.

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