Human bronchial leucocyte proteinase inhibitor

Rapid isolation and kinetic analysis with human leucocyte proteinases

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Bronchial leucocyte proteinase inhibitor (BLPI) is an 11000 M_r protein found in human mucous secretions. This inhibitor apparently controls the serine proteinases elastase and cathepsin G, released from extravascular polymorphonuclear leucocytes. A simple, single-step chromatographic procedure for the isolation of BLPI based on its affinity for chymotrypsin was developed. The purified inhibitor was homogeneous by electrophoresis and gel filtration. Amino acid analyses were in close agreement with previous reports, and showed BLPI to be rich in proline and cystine, but lacking histidine. We have further characterized the role of BLPI with respect to human leucocyte elastase and cathepsin G by close examination of the kinetic parameters. Additionally, we have determined the kinetics of association (k_{on}) and dissociation (k_{off}) for BLPI with bovine trypsin and chymotrypsin. Equilibrium dissociation constants (K_i) of 1.87×10^{-10} M, 4.18×10^{-9} M, 8.28×10^{-9} M and 2.63×10^{-8} M were obtained for human leucocyte elastase, cathepsin G, bovine trypsin and chymotrypsin, respectively. These results are discussed with respect to BLPI's possible function *in vivo* and its role relative to other inhibitors in bronchial secretions.

Human bronchial mucous secretions contain inhibitors of neutral serine proteinases, and the majority of this inhibitory activity was shown by Hochstrasser *et al.* (1972) to be due to a low- M_r , acid-stable protein. This protein, which will be called bronchial leucocyte proteinase inhibitor (BLPI), is particularly interesting because it inhibits both the major serine proteinases produced by human polymorphonuclear leucocytes, elastase and cathepsin G. Ohlsson *et al.* (1977) subsequently isolated BLPI by using affinity chromatography on trypsin bound to Affi-Gel 10, followed by gel filtration and ion-exchange chromatography. BLPI has been shown to be identical with the human seminal plasma inhibitor (HUSI-1) by amino acid analysis (Ohlsson *et al.*, 1977) and immunological cross-reactivity (Schiessler *et al.*,

1978). The apparent function of BLPI is the control of leucocyte proteinases released from extravascular leucocytes in the upper airways and various other mucous secretions. If proteolysis and its control in the lung are to be understood, the role of BLPI must be more clearly defined.

As an aid to the study of this inhibitor we report a simple method for the purification of BLPI based on its affinity for bovine chymotrypsin. Extensive amino acid analyses have been performed and are compared with previous data.

Equilibrium dissociation constants for BLPI and several proteinases have been reported by Hochstrasser *et al.* (1981), but association and dissociation rates have been examined for HLE only (Gauthier *et al.*, 1982). In an effort to obtain more definitive data we have measured the association and dissociation rates of BLPI with cathepsin G, chymotrypsin and trypsin, and have re-examined the reaction of BLPI with HLE to clarify discrepancies in the previous reports. These data are compared with existing information to delineate more clearly the function of BLPI in bronchial secretions.

Abbreviations used: BLPI, bronchial leucocyte proteinase inhibitor; α_1 -PI, α_1 -proteinase inhibitor (α_1 -antitrypsin); HLE, human leucocyte elastase; pNA, *p*-nitroanilide; -OEt, ethyl ester; Suc-, succinoyl.

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Experimental

Materials

The substrates Suc-(Ala)₂-Pro-Val-pNA, Suc-(Ala)₃-pNA, Suc-(Ala)₂-Pro-Phe-pNA, *N*-benzoyl-L-Tyr-OEt, *N*-benzoyl-DL-Arg-pNA, *N*-benzoyl-L-Tyr-pNA and *p*-nitrophenylguanidine benzoate were purchased from Sigma. Chromatographically pure porcine pancreatic elastase (type III), bovine trypsin, porcine trypsin, chymotrypsin, hen ovalbumin, soya bean trypsin inhibitor, horse heart myoglobin, horse heart cytochrome *c*, and 5,5'-dithiobis-(2-nitrobenzoic acid) were also products of Sigma. Human α_1 -PI was prepared as described by Travis & Johnson (1981). The leucocyte proteinases HLE and cathepsin G were purified from purulent sputum according to Martodam *et al.* (1979). Sephadex G-50 and Sepharose 4B gels were obtained from Pharmacia.

General assay conditions

Inhibitory activities for all experiments were measured by assaying residual enzyme activity after incubating enzyme and inhibitor. Enzyme and inhibitor incubation times were based on either known or determined half times of association ($t_{1/2, on}$) and the enzyme concentration in the reaction (Bieth, 1980). HLE activity was assayed with either Suc-(Ala)₂-Pro-Val-pNA (Castillo *et al.*, 1979) or Suc-(Ala)₃-pNA (Bieth *et al.*, 1974), and the latter substrate was used to assay porcine pancreatic elastase also. Chymotrypsin activity was assayed with either Bz-Tyr-OEt (Kang & Fuchs, 1973), or Bz-Tyr-pNA (Bundy, 1963). Suc-(Ala)₂-Pro-Phe-pNA and Bz-Tyr-OEt (Kang & Fuchs, 1973) were used to assay cathepsin G activity. The trypsin were assayed by the method of Erlanger *et al.* (1961) using Bz-Arg-pNA as the substrate. One inhibitory unit is defined as the amount of inhibitor that decreases the rate at which chymotrypsin catalyses the hydrolysis of Bz-Tyr-OEt by 1.0 A_{256}/min . Assays were performed in a total volume of 1.0 ml either as a stopped assay, by allowing the hydrolysis of substrate to proceed for a fixed period of time, followed by the addition of 30 μl of conc. acetic acid to stop the reaction, or by measuring the rate of hydrolysis directly with a Cary 219 spectrophotometer equipped with a strip chart recorder. Representative stopped assays were checked by continuous monitoring for linearity. All assays were linear and there was no indication of substrate-induced complex dissociation. The purification factor was based on A_{280} . The various enzyme concentrations employed in different experiments necessitated the use of substrates with either increased or decreased sensitivity. The molar concentrations of proteins were based on published $A_{280}^{1\%}$ and M_r values as follows: HLE,

9.85 and 30000 (Baugh & Travis, 1976); cathepsin G, 6.64 and 23500 (Travis *et al.*, 1978); bovine trypsin, 14.3 and 23800 (Cunningham, 1954); bovine chymotrypsin, 20.5 and 25600 (Wilcox, 1970); porcine pancreatic elastase, 22.0 and 25900 (Shotton, 1970); porcine trypsin, 13.5 and 23400 (Travis & Liener, 1965); α_1 -PI, 5.3 and 52000 (Travis & Johnson, 1981); and BLPI, 7.8 and 11200 (the present paper). Molarities were corrected for active site concentrations as described below.

Amino acid analysis

Samples were hydrolysed *in vacuo* for 12, 24, 48, and 72 h with 6M-HCl at 110°C, followed by duplicate analyses. The values for serine and threonine were obtained by extrapolation to time 0. The 72 h values were taken for valine and isoleucine, and the 24 and 48 h data were averaged for the values of the other amino acids. The sample for tryptophan analysis was subjected to alkaline hydrolysis (Hugli & Moore, 1972), and the sample for amino sugar content was hydrolysed for 5 h with 4M-HCl at 100°C. Samples were analysed on a Beckman 119 CL amino acid analyser. A newly developed chromatographic method was used in the analysis of tryptophan and glucosamine (Johnson, 1983). The $A_{280}^{1\%}$ was calculated from the recovery of amino acids relative to the A_{280} of the sample. The protein was examined for free thiol groups by reaction with 5,5'-dithiobis-(2-nitrobenzoic acid) (Habeeb, 1972).

Determination of active site concentrations

The active site concentrations of porcine pancreatic trypsin and chymotrypsin were determined by the method of Chase & Shaw (1967) to be 79% and 85% of total sites, respectively. Porcine trypsin of known active site concentration was used to titrate a stock solution of α_1 -PI, which was used as a secondary standard for the active site determinations of HLE, cathepsin G, bovine trypsin, and porcine pancreatic elastase, and as a check on the active site titrations of bovine chymotrypsin.

Increasing amounts of α_1 -PI were incubated for 30 min with 0.52 nmol of porcine trypsin in 0.05M-Tris/HCl/0.05M-NaCl, pH 8.0, in a total volume of 980 μl . The residual enzyme activity was monitored at 410 nm after adding 20 μl of 20 mM-Bz-Arg-pNA in dimethyl sulphoxide to the reaction mixture. The reactions were run for 10 min and then stopped by the addition of 30 μl of conc. acetic acid. α_1 -PI was determined to be 100% active based on linear regression analyses (r 0.99) of these data. Similar experiments were performed with α_1 -PI as a secondary standard to determine the active site concentrations of all other proteinases. Inhibitor and enzyme mixtures were incubated for a

period of not less than $5 \times t_{i, on}$, based on the association-rate data of Beatty *et al.* (1980), and the enzyme concentration in the reaction.

Other enzymes were subsequently titrated with BLPI to determine the molar binding ratios of BLPI and enzyme required for complete inhibition of enzyme. Enzyme concentrations chosen were of the order of 10^{-7} M for HLE and cathepsin G and 10^{-6} M for trypsin and chymotrypsin. These concentrations were high enough to give linear titrations (Bieth, 1974).

Association rate determinations (k_{on})

The association rates for BLPI with HLE, cathepsin G, bovine trypsin, and chymotrypsin, were determined by the method of Vincent & Lazdunski (1972), under second-order rate conditions. Equimolar mixtures of enzyme and inhibitor, based on the above titrations, were incubated for increasing time periods in a total volume of 980 μ l. Remaining enzyme activity was measured as described earlier by monitoring the change in A_{410} after the addition of substrate.

These experiments were performed in 0.1M-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethane sulphonic acid], pH 7.5 at 25°C. The reaction buffer for HLE was 1M in NaCl, and the buffers for cathepsin G, chymotrypsin and bovine trypsin were 0.05M in NaCl. In addition, the buffer for chymotrypsin reactions contained 8% dimethyl sulphoxide to maintain Bz-Tyr-pNA solubility, and 10mM-CaCl₂. The final enzyme concentrations used in each determination were as follows: 0.033 μ M for HLE, 0.55 μ M for cathepsin G, 1.04 μ M for trypsin and 2.14 μ M for chymotrypsin. Substrate concentrations ranged from 0.3 to 0.8mM.

Dissociation rate determination (k_{off})

Dissociation rates were determined by adding α_1 -PI to complexes of BLPI and enzyme, and measuring the amount of enzyme transferred from the BLPI-proteinase complex to α_1 -PI in a time-course reaction (Vincent & Lazdunski, 1972; Gauthier *et al.*, 1982). Initially, equimolar mixtures of BLPI and enzyme, based on the aforementioned titrations, were incubated until zero enzyme activity was recorded ($7 \times t_{i, on}$ for association). The dissociation of the BLPI-proteinase complex was measured by monitoring the α_1 -PI inhibitory activity of 50 μ l aliquots taken from the reaction mixture (500 μ l) and added to 930 μ l of 0.1M-Hepes/1M-NaCl, pH 7.5, containing an equal amount of porcine pancreatic elastase. Following a 15min incubation, the residual elastase activity, which equalled the amount of BLPI-proteinase complex, was measured after the addition of 20 μ l of 20mM-Suc-(Ala)₃-pNA in dimethyl sulphoxide.

The enzyme concentrations used in the initial incubations were: 2.15 μ M for HLE, 5.15 μ M for cathepsin G, 10.4 μ M for trypsin and 10.1 μ M for chymotrypsin. The ratio of k_{off}/k_{on} was used to calculate the equilibrium dissociation (K_i) for the interaction of each proteinase with BLPI.

Results and discussion

Purification and characterization

Sputum collected daily from patients with chronic bronchitis was stored at -15°C until used. The thawed sputum was made 0.5M in NaCl and 20mM in HCl in order to dissociate BLPI-proteinase complexes. Trichloroacetic acid was added to a final concentration of 3% (w/v), which denatured humoral inhibitors and several other proteins. The viscosity of this mixture was reduced by homogenizing in a blender for 15s at low speed prior to centrifugation at 30000g for 20min. The supernatant fraction was made 0.05M in Tris base, adjusted to pH 8.0 with 10M-NaOH and added to chymotrypsin-Sepharose (25ml of gel/l of sputum) which had been prepared by coupling the enzyme to CNBr-activated Sepharose at pH 6.5 (Marsh *et al.*, 1974). After stirring for 1h at room temperature the BLPI-chymotrypsin-Sepharose was collected on a glass fritted Buchner funnel and washed with 0.05M-Tris/HCl/0.5M-NaCl, pH 8.0. The gel was poured into a chromatography column and washed with the pH 8.0 buffer until the A_{280} was 0. The BLPI was eluted, in a trailing peak (Fig. 1),

Fig. 1. Chymotrypsin-Sepharose affinity chromatography. The trichloroacetic acid supernatant fraction from 1 litre of sputum was added to 25 ml of chymotrypsin-Sepharose at pH 8.0. The affinity gel was washed with 2000 ml of 0.05M-Tris/HCl/0.5M-NaCl, pH 8.0 and poured into a column. The column (1.5cm $\times$ 14cm) was washed with the pH 8.0 buffer until the A_{280} was 0, and BLPI was eluted with 0.025M-NaCl, pH 1.75. Fractions (2.8ml each) were collected every 3 min at room temperature.

Fig. 2. *Gel filtration chromatography* BLPI (1.16mg) was chromatographed on a Sephadex-G 50 (superfine grade) column (1.6cm $\times$ 85cm) in 0.05M-Tris/HCl/0.1M-NaCl, pH 8.0, at room temperature. Fractions (1.0ml) were collected at 5min intervals and their A_{225} was measured. The elution positions of the standard proteins used to calibrate the column are indicated. The correlation coefficient for the log M_r versus K_{av} plot of standards was 0.99; the M_r of BLPI was calculated to be 12900.

with 0.025M-HCl/0.5M-NaCl, pH 1.75. Active fractions were pooled and concentrated on an Amicon UM-2 (2000 M_r cut-off) membrane. The yield from six isolations averaged 5mg/l of sputum, for a 350-fold purification (based on A_{280}) and a 25% recovery of inhibitory activity. Chromatography of the preparation on Sephadex G-50 (superfine grade) yielded a single symmetrical peak corresponding to an M_r of 12900 (Fig. 2). This column was monitored at 225nm in order to detect other components such as peptides, but none were found. Chymotrypsin inhibitory activity coincided with the A_{225} peak, and the specific activity of the fractions varied from 70.9 (inhibitory units/mg) on the front side of the peak to 57 on the back side. The preparation was also homogeneous by electrophoresis (Fig. 3) at pH 6.8 in a high resolution system for cationic proteins (Thomas & Hodes, 1981). Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of reduced BLPI in 18% gels yielded two major and two minor bands. This finding was indicative of internal peptide bond cleavages possibly resulting from proteolysis by enzymes in the sputum and/or by the chymotrypsin of the affinity matrix during purification. Titration of chymotrypsin (0.12 μ M) and Cathepsin G (0.26 μ M) with BLPI, using Bz-Tyr-OEt as substrate, yielded linear plots intersecting at 1.0mol of BLPI/mol of proteinase, indicative of 1:1 binding.

Fig. 3. *Polyacrylamide-gel electrophoresis* The trichloroacetic acid supernatant fraction (0.5ml) (a) and purified BLPI (40 μ g) (b) were electrophoresed in 10% polyacrylamide gels. The pH of the 3% stacking gel was 8.0 and the separating gel had a pH of 6.8. The gels were buffered with Mops (4-morpholine propanesulphonic acid) only while the reservoir contained Mops/histidine at pH 6.8. The anode was at the top and cathode at the bottom. After electrophoresis for 4h at 12mA/gel, the gels were stained with Coomassie Brilliant Blue R-250 containing formaldehyde and acetic acid as fixatives.

Amino acid analysis of this preparation (Table 1) was in very close agreement with that of Ohlsson *et al.* (1977) for BLPI and Schiessler *et al.* (1978) for HUSI-1. Examination of the protein for free thiol groups with Ellman's reagent after denaturation in sodium dodecyl sulphate (Habeeb, 1972) indicated that all 14 of the cysteine residues were disulphide linked. The combined cystine and proline content equals 25%, and these residues probably make a significant contribution to the stability of this protein. The major difference between our data and those of others is that we found one residue/mol each of tryptophan and glucosamine. These are difficult residues to quantify, and they may have

Table 1. *Amino acid analysis of human BLPI*

Residue	Analysis (residues/molecule)			
	Present work		Data of Ohlsson <i>et al.</i> (1977)	Data of Schiessler <i>et al.</i> (1978)
	Found	Integer		
Asp	8.51	9	9	9
Thr	3.90	4	4	4
Ser	5.88	6	6	6
Glu	6.69	7	5	8
Pro	13.23	13	11	12
Gly	9.13	9	9	9
Ala	3.03	3	4	3
Cys	14.14	14	14	12
Val	5.02	5	7	5
Met	3.26	3	3	3
Ile	0.76	1	1	2
Leu	4.99	5	5	6
Tyr	1.77	2	2	2
Phe	1.96	2	2	2
His	0	0	0	0
Lys	14.20	14	13	15
Arg	4.44	4	4	5
Trp	0.62	1	0	0
Glucosamine	0.44	1	0	0
Total		103	99	103
Calculated M_r		11 243	10 332	10 970

been missed by previous investigators. From amino acid analysis data a minimum M_r of 11243 was calculated, which was close to the value obtained by gel filtration. An $A_{280}^{1\%}$ of 7.8 was determined from amino acid analysis data, corresponding to a molar absorption coefficient of $8770\text{M}^{-1}\cdot\text{cm}^{-1}$ for M_r 11200. These values, which have not been reported previously, are consistent with the presence of one tryptophan and two or three tyrosine residues, in good agreement with the amino acid analysis given in Table 1. The $A_{280}^{1\%}$ and M_r of 11243 were used to calculate the inhibitor's molar concentration in subsequent studies.

Whereas other methods for BLPI purification have employed ion exchange and gel-filtration chromatography in addition to affinity chromatography on trypsin-bound agarose, our method, using chymotrypsin-Sepharose, yielded a homogeneous preparation in a single chromatographic step. As our subsequent data show, BLPI-chymotrypsin complexes are more stable than trypsin complexes and may explain why chymotrypsin proved to be a superior affinity ligand. Both electrophoresis and gel-filtration data demonstrate the homogeneity of the preparation. The purity of our preparation is consistent with that of Ohlsson *et al.* (1977) as evidenced by amino acid analysis and immunological cross-reactivity (K. Ohlsson, personal communication). Schiessler *et al.* (1978)

found that BLPI inactivated HLE, cathepsin G, bovine trypsin, and bovine chymotrypsin, but failed to inhibit porcine elastase or porcine trypsin. The inhibition spectrum of our preparation is in total agreement with this report. Thus, there seems to be little doubt that this inhibitor is the major leucocyte proteinase inhibitor of sputum described by Hochtrasser *et al.* (1972) and Ohlsson *et al.* (1977).

Inhibition kinetics

The rates of association for BLPI with HLE, cathepsin G, bovine trypsin and chymotrypsin were determined according to Vincent & Lazdunski (1972) as described in the Experimental section. In its simplest form, the interaction of enzyme and inhibitor, for reversible inhibition, may be written as:

where k_{on} is the rate of association and k_{off} is the rate of dissociation for EI complexes. We have determined the association rate of BLPI with the aforementioned proteinases under second-order rate conditions where the rate was dependent on the concentration of both enzyme and inhibitor

Fig. 4. Association rates for BLPI with various proteinases

Equimolar mixtures of enzyme and inhibitor were incubated for increasing time intervals, and the percentage of enzyme activity remaining is plotted versus time. A second-order replot of these data (inset), in accordance with eqn. (3) (see the text), gives a slope equal to k_{on} , and the y -intercept equals $[E_0]$. The enzymes studied were: (a), HLE; (b), cathepsin G; (c), trypsin; (d), chymotrypsin.

(Fig. 4). The rate of complex formation is given by the equation (Bieth, 1980):

$$\frac{d[E]}{dt} = k_{on}[E][I] - k_{off}[EI] \quad (2)$$

Since no complex dissociation was observed over the time course of each assay, the last term in eqn. (2) may be excluded. Under these conditions, k_{on} may be analysed by eqn. (3):

$$\frac{1}{[E]} = \frac{1}{[E_0]} + k_{on}(t) \quad (3)$$

where $[E_0]$ is the initial (or total) enzyme concentration, $[E]$ is unassociated (or free) enzyme, and t is time. Plots of $1/[E]$ versus t (Fig. 4 insets) were linear and intercepted the $1/[E]$ axis at or near $1/[E_0]$, as expected. The slopes of these lines equalled the respective association rates (k_{on}).

The dissociation rates of BLPI–proteinase complexes were determined by adding an equimolar amount of α_1 -PI to previously formed (1:1) BLPI–proteinase complexes. α_1 -PI forms irreversible complexes with dissociated proteinase and serves as an enzyme ‘trap’. Samples of this mixture were assayed to determine the amount of free and enzyme-complexed α_1 -PI. This assay is based on the observation that α_1 -PI inhibits porcine pancreatic elastase on a mol/mol basis, whereas BLPI does not inhibit this enzyme. Plots of $\ln[\text{BLPI–proteinase complex} (\%)]$ versus time (Fig. 5) were linear with slopes equal to the respective dissociation rates (k_{off}). The association and dissociation rates are listed in Table 2 for comparison. Equilibrium dissociation constants (K_i) were calculated from the ratio of k_{off}/k_{on} (Table 2).

Based on these data there is little doubt that BLPI preferentially inhibits HLE relative to

Fig. 5. Dissociation rates for BLPI with various proteinases

Equimolar mixtures of BLPI and proteinase were incubated until zero proteinase activity was recorded, at which time an equal molar amount of α_1 -PI was added. By measuring the inhibitory activity of α_1 -PI against porcine pancreatic elastase from 50 μ l aliquots of the reaction mixture, the percentage of BLPI-proteinase complex remaining over a period of time could be determined. Plots of $\ln[\text{BLPI-proteinase complex (\%)}]$ versus time were linear, with slopes equal to the respective dissociation rates (k_{off}). Enzymes: (a), HLE; (b), cathepsin G; (c), trypsin; (d), chymotrypsin.

cathepsin G. The K_i that we have determined from HLE with BLPI is one order of magnitude higher than the value of $1.2 \times 10^{-11} \text{ M}$ reported by Gauthier *et al.* (1982). In both cases, K_i was determined by calculating the ratio of $k_{\text{off}}/k_{\text{on}}$. The k_{on} determined by Gauthier *et al.* (1982) ($1.1 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$) is two orders of magnitude higher than the value we have obtained, while our k_{off} is one order of magnitude lower than their value ($1.3 \times 10^{-4} \text{ s}^{-1}$). These discrepancies may be explained as follows. The kinetic approach employed by Gauthier *et al.* (1982) involved competition experiments in which HLE was added to mixtures of α_1 -PI and BLPI. This method was an adaptation of Vincent & Lazdunski's (1972) competition experiments employing one inhibitor and two enzymes. When we attempted to apply the approach of Gauthier *et al.* (1982) for the measurement of BLPI-cathepsin G association rates, the method failed because plots of $\log(a/a-x)$ versus $\log(b/b-y)$ (where a = initial concn. of α_1 -PI, x = concn. of α_1 -PI-cathepsin G complex, b = initial concn. of BLPI and y = concn. of BLPI-cathepsin G complex) did not have a positive slope or pass through the origin as required by theory. Re-examination of the data presented by Gauthier *et al.* (1982) revealed a similar problem; i.e., plots of $\log(a/a-x)$ versus $\log(b/b-y)$ using their data yielded a line parallel to the $\log(a/a-x)$ axis. The inconsistency of these data with the theory is indicative of an erroneous result. These findings were suggestive of an association of porcine pancreatic elastase with BLPI, but no association could be detected by us under conditions such that the association rate of BLPI and porcine pancreatic elastase must be less than $10^2 \text{ M}^{-1} \cdot \text{s}^{-1}$. The reason why the approach of Gauthier *et al.* (1982) failed is not immediately clear, but may be related to the competition of a reversible inhibitor (BLPI), with an irreversible one (α_1 -PI), and/or the introduction of a second enzyme to measure the irreversible inhibitor (α_1 -PI).

In contrast, Hochstrasser *et al.* (1981) have reported a K_i of $2.5 \times 10^{-9} \text{ M}$ for BLPI and HLE using the method of Green & Work (1953). They have also reported a value of $2.7 \times 10^{-8} \text{ M}$ for BLPI and cathepsin G, which is one order of magnitude higher than our value, and values of $4.0 \times 10^{-9} \text{ M}$

Table 2. Comparison of kinetic data for various proteinases with BLPI

For an explanation of experimental details see the text. Complex stability is equivalent to the half time of dissociation, as explained in the text. Calculations of delay time and complex stability are based on the molar concentrations of BLPI in sputum as determined by Kramps *et al.* (1984).

Enzyme	$k_{\text{on}} (\text{M}^{-1} \cdot \text{s}^{-1})$	$k_{\text{off}} (\text{s}^{-1})$	$K_i (\text{M})$	Delay time (s)	Complex stability (h)
HLE	1.06×10^5	1.99×10^{-5}	1.87×10^{-10}	8.04	9.67
Cathepsin G	2.48×10^4	1.04×10^{-4}	4.18×10^{-9}	34.35	1.85
Trypsin	2.24×10^4	1.85×10^{-4}	8.28×10^{-9}	38.03	1.04
Chymotrypsin	1.16×10^3	3.05×10^{-5}	2.63×10^{-8}	734.30	6.31

and $7.0 \times 10^{-10} \text{ M}$ were listed for bovine trypsin and chymotrypsin, respectively. This K_i for bovine trypsin is in close agreement with our data, but our K_i for chymotrypsin is two orders of magnitude higher than theirs. These discrepancies seem to be due to the methods employed. We believe our data to be more correct because the approach of Vincent & Lazdunski (1972) is more rigorous than the method of Green & Work (1953), which is subject to considerable error at the equivalence point, and which was employed by Hochstrasser *et al.* (1981).

For an inhibitor to be physiologically efficient two requirements must be met. First, k_{on} must be sufficiently fast to completely remove a potentially damaging proteinase from a susceptible tissue. The delay time of inhibition, as described by Bieth (1980), is the time required for complete inhibition of a proteinase *in vivo* and is given by the equation:

$$\text{delay time} = \frac{5}{k_{\text{on}}[I_0]} \quad (4)$$

The BLPI concentration of sputum has recently been reported to be as high as $5.87 \times 10^{-6} \text{ M}$ (Kramps *et al.*, 1984). Based on this concentration and our association rates for HLE and cathepsin G, delay times of 8 s and 25 s can be calculated respectively for the control of these proteinases in the upper respiratory tract. The second requirement is that the complexes formed must be stable. For inhibitors such as α_1 -PI and α_1 -antichymotrypsin, this requirement is easily fulfilled since these inhibitors bind covalently to their respective enzymes and are non-reversible (Travis & Salvesen, 1983). BLPI, however, exhibits reversible binding and therefore dissociation rates must be slow enough to provide a stable complex.

The half-time of dissociation ($t_{1/2, \text{off}}$) is given by the equation (Bieth, 1980):

$$t_{1/2, \text{off}} = \frac{0.693}{k_{\text{off}}} \quad (5)$$

Using the k_{off} values for HLE and cathepsin G as obtained from our work, the half-times of dissociation for these proteinase complexes are 10 h and 2 h, respectively. Fryksmark *et al.* (1983) have further documented the stability of the BLPI-HLE complex by showing that these complexes are stable for at least 24 h. The proportion of free enzyme at equilibrium is also dependent on the ratio of $[E_0]/K_i$ (Bieth, 1974). Since BLPI concentrations are generally much greater than the amount of HLE and cathepsin G that may be present in the bronchial mucus (Ohlsson *et al.*, 1983), $[E_0]$ may be replaced by $[I_0]$, and the overall reaction is essentially first-order with respect to $[I_0]$ under these conditions. If the concentration of BLPI within the

upper respiratory tract is $5.87 \times 10^{-6} \text{ M}$, as reported by Kramps *et al.* (1984), the ratios of $[I_0]/K_i$ for HLE and cathepsin G are 3.14×10^4 and 1.40×10^3 respectively, which are more than sufficient for complete proteinase inhibition.

These results indicate that BLPI is primarily an HLE inhibitor, but when HLE is fully inhibited, BLPI will begin to control cathepsin G. The difference in the k_{on} of BLPI with HLE and cathepsin G may not be significant *in vivo* if the concentration of BLPI is sufficiently higher than the sum of HLE and cathepsin G concentrations in bronchial secretions. Present information indicates that this is the case under normal conditions, but the finding of free HLE in secretions from patients with chronic bronchitis (Ohlsson & Tegner, 1975) shows that the system of inhibitors (mainly BLPI) in the upper airways can become saturated. As the concentration of HLE approaches that of BLPI, cathepsin G would be free to attack tissues. We have found that the metalloelastase from *Pseudomonas aeruginosa* can catalytically inactivate BLPI (Johnson *et al.*, 1982), which may also contribute to uncontrolled proteolysis both directly and via inhibitor destruction. Additionally, Carp & Janoff (1980) have found that cigarette smoke partially inactivates BLPI, but the significance of this is difficult to evaluate in the absence of kinetic data. We have recently shown that BLPI is also susceptible to oxidative inactivation by ozone (C. E. Smith & D. A. Johnson, unpublished observations). Thus, a loss of BLPI activity in pathological situations could result in the degradation of host tissue proteins by HLE and cathepsin G.

The role of BLPI with respect to α_1 -PI and α_1 -antichymotrypsin within the upper respiratory tract is not clear. The association rates of HLE and cathepsin G with α_1 -PI and α_1 -antichymotrypsin respectively (Beatty *et al.*, 1980), are faster than with BLPI (the present data). However, the molar concentration of BLPI is approx. 10 times greater than the blood plasma inhibitor concentrations in the upper respiratory tract (Ryley & Brogan, 1973). Based upon the concentration data of Ryley & Brogan (1973) and the association rate data of Beatty *et al.* (1980), the delay times for inhibition of HLE and cathepsin G by α_1 -PI and α_1 -antichymotrypsin in sputum are calculated to be 0.15 s and 0.45 s respectively. Thus, the blood plasma inhibitors should be 55 times more efficient in controlling HLE and cathepsin G than BLPI under normal circumstances. However, during acute infection, both HLE and cathepsin G can increase to high levels (Ohlsson & Tegner, 1975), saturating the plasma inhibitors. Ohlsson *et al.* (1984) have shown that BLPI is an acute phase reactant with serum levels reaching ten times normal. Consistent with this report, Stockley *et al.* (1984) found that

BLPI was responsible for 75% of the HLE inhibitory activity in bronchoalveolar lavage from patients with chronic bronchitis. These findings suggest that BLPI's physiological importance may be to inhibit these enzymes during periods of acute inflammation. Our dissociation rate studies, in agreement with those of Fryksmark *et al.* (1983), indicate that HLE and cathepsin G bound to BLPI would be transferred to α_1 -PI and α_1 -antichymotrypsin, respectively.

Additionally, BLPI may aid the plasma proteinase inhibitors in controlling proteinases after they have bound to their protein substrates. Reilly & Travis (1980) showed that α_1 -PI would not fully inhibit HLE pre-absorbed to elastin. Hornebeck & Schnebli (1982) found that the low- M_r inhibitor from the leech known as eglin C (M_r 8100) would inhibit HLE even after pre-absorption of the proteinase to elastin. By analogy BLPI, which is also of low M_r , may serve to inhibit leucocyte proteinases after they have bound to their substrates.

Our improved isolation method should facilitate additional study of this inhibitor in other secretions and tissues as well as bronchial mucus. The kinetic data obtained in this study are intended to provide a benchmark for future experiments designed to examine the effects of amino acid modification, including oxidation, on the function of BLPI.

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