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Cathepsin B, L, and S Cleave and Inactivate Secretory Leucoprotease Inhibitor*

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A number of serine proteases, matrix metalloproteases, and cysteine proteases were evaluated for their ability to cleave and inactivate the antiprotease, secretory leucoprotease inhibitor (SLPI). None of the serine proteases or the matrix metalloproteases examined cleaved the SLPI protein. However, incubation with cathepsins B, L, and S resulted in the cleavage and inactivation of SLPI. All three cathepsins initially cleaved SLPI between residues Thr[67] and Tyr[68]. The proteolytic cleavage of SLPI by all three cathepsins resulted in the loss of the active site of SLPI and the inactivation of SLPI anti-neutrophil elastase capacity. Cleavage and inactivation were catalytic with respect to the cathepsins, so that the majority of a 400-fold excess of SLPI was inactivated within 15 min by cathepsins L and S. Analysis of epithelial lining fluid samples from individuals with emphysema indicated the presence of cleaved SLPI in these samples whereas only intact SLPI was observed in control epithelial lining fluid samples. Active cathepsin L was shown to be present in emphysema epithelial lining fluid and inhibition of this protease prevented the cleavage of recombinant SLPI added to emphysema epithelial lining fluid. Taken together with previous data that demonstrates that cathepsin L inactivates α_1-antitrypsin, these findings indicate the involvement of cathepsins in the diminution of the lung antiprotease screen possibly leading to lung destruction in emphysema.

Secretory leucoprotease inhibitor (SLPI)[1] is an 11.7-kDa protein produced by the mucosal surfaces of epithelial cells and also by macrophages and neutrophils (1–4). It is found predominantly in the upper airways of the lungs and in salivary secretions (5, 6). SLPI was initially identified as a potent inhibitor of neutrophil elastase (NE) and is thought to provide significant protection for the lung epithelial surfaces against NE released from activated or disintegrating neutrophils (7). In recent years SLPI has been recognized to have anti-bacterial, anti-viral, and anti-inflammatory properties. These include the prevention of human immunodeficiency virus replication in monocytic cells (8), the down-regulation of lipopolysaccharide-induced production of tumor necrosis factor-α (3), and matrix metalloprotease synthesis (9) by monocytic cells, and inhibition of inflammatory lung injury caused by deposition of IgG-immune complexes (10).

Cathepsins B, L, and S are cysteine proteases produced by macrophages, fibroblasts, and epithelial cells in the lung and have been demonstrated to possess elastolytic activity (11). Cathepsins B, L, and S all work at acidic pH and their proteolytic activity is neutralized at neutral pH with the exception of cathepsin S (12). Cathepsin L activity has been shown to be elevated in bronchoalveolar lavage (BAL) fluid from emphysema (13) and the release of cathepsin B and S from macrophages has been induced by cigarette smoking (14, 15). Indeed, cathepsin L has been shown to cleave and inactivate α_1-antitrypsin (16), the major serine protease inhibitor present in the lung (17).

A large percentage of the elastolytic activity of cultured macrophages has been attributed to cathepsins (18) and it is believed that protease-antiprotease imbalance such as occurs in emphysema may result in destruction of local tissue (19). A variety of other proteases are produced by activated macrophages and neutrophils in the emphysematous lung including the matrix metalloproteases, MMP-1, -8, -9, and -12 as well as a number of serine proteases (20, 21). However, SLPI is believed to be proteolytically stable due to the large number of disulfide bridges present in its structure (22). To date, only NE has been demonstrated to cleave oxidized SLPI, at a 2:1 molar ratio, and has no action on the native molecule (5). Here we report that the cathepsins cleave and inactivate SLPI, both *in vitro* and *in vivo*.

EXPERIMENTAL PROCEDURES

Materials—Recombinant human SLPI, human SLPI quantikine enzyme-linked immunosorbent assay, goat anti-human SLPI IgG, normal mouse IgG$_1$ isotype control, and purified human MMP-1, MMP-8, and MMP-9 were obtained from R&D Sytems (Abingdon, Oxon, United Kingdom). Cathepsins B and L, purified from human liver, purified bovine cathepsin S were from CN-Biosciences (Nottingham, UK). Z-Arg-Arg-*p*-nitroanilide, Z-Phe-Arg-*p*-nitroanilide, and Z-Phe-Phe-CHN$_2$ were obtained from Bachem (Saffron Walden, England) and CA-074 was obtained from SERVA (Heidelberg, Germany). Neutrophil elastase, proteinase 3, and cathepsin G purified from sputum were obtained from Elastin Products Company, Inc. (Owensville, MO). *N*-Methoxy-succinyl-Pro-Ala-Ala-Val-*p*-nitroanilide was

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[1] The abbreviations used are: SLPI, secretory leucoprotease inhibitor; NE, neutrophil elastase; MMP, matrix metalloprotease; PAGE, polyacrylamide gel electrophoresis: ELF, epithelial lining fluid; BAL, bronchoalveolar lavage; HPLC, high performance liquid chromatography.

purchased from Sigma (Poole, Dorset, UK). E-64 was obtained from Roche Molecular Biochemicals (Lewes, East Sussex, UK). TCEP was obtained from Pierce (Rockford, IL). Microcon-3 columns were from

Millipore (Watford, England). Western blotting reagents were obtained from Tropix (Bedford, MA).

Enzyme Assays—SLPI was assayed by measuring its inhibition of human neutrophil elastase activity on *N*-methoxy-succinyl-Pro-Ala-Ala-Val-*p*-nitroanilide as previously described (23).

Inactivation of SLPI—SLPI inactivation was determined by measuring NE inhibitory activity following incubation with cathepsin B, L, or S over a 60-min time period. SLPI (8 nmol) was incubated alone or
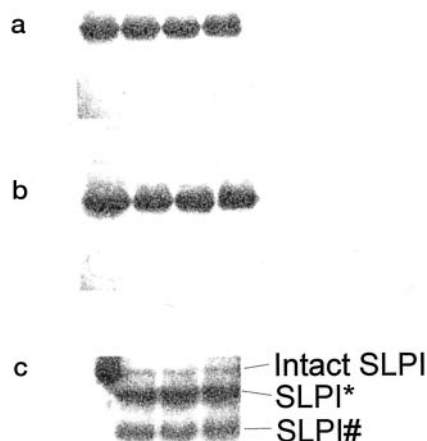
FIG. 1. **SDS-PAGE analysis of SLPI incubated with various proteases.** *a*, SLPI was incubated with a number of serine proteases, MMPs, and cysteine proteases and electrophoresed on a 17.5% SDS-PAGE. *First lane*, SLPI standard; *second lane*, SLPI + NE; *third lane*, SLPI + cathepsin G; *fourth lane*, SLPI + proteinase 3. *b*, *first lane*, SLPI standard; *lane 2*, SLPI + MMP-1; *third lane*, SLPI + MMP-8; *fourth lane*, SLPI + MMP-9. *c*, *first lane*, SLPI standard; *second lane*, SLPI + cathepsin B; *third lane*, SLPI + cathepsin L; *fourth lane*, SLPI + cathepsin S.
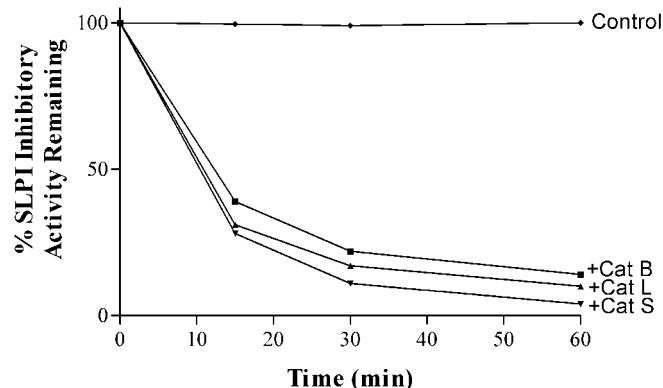
FIG. 2. **Effect of cathepsin B, L, and S on SLPI inhibitory activity.** SLPI was incubated at pH 5.5 alone (*Control*) or with cathepsin B (*Cat B*), cathepsin L (*Cat L*) and cathepsin S (*Cat S*) at a SLPI: cathepsin ratio of 15:1, 400:1, and 400:1, respectively. Residual neutrophil elastase inhibitory activity is plotted relative to the zero time control.
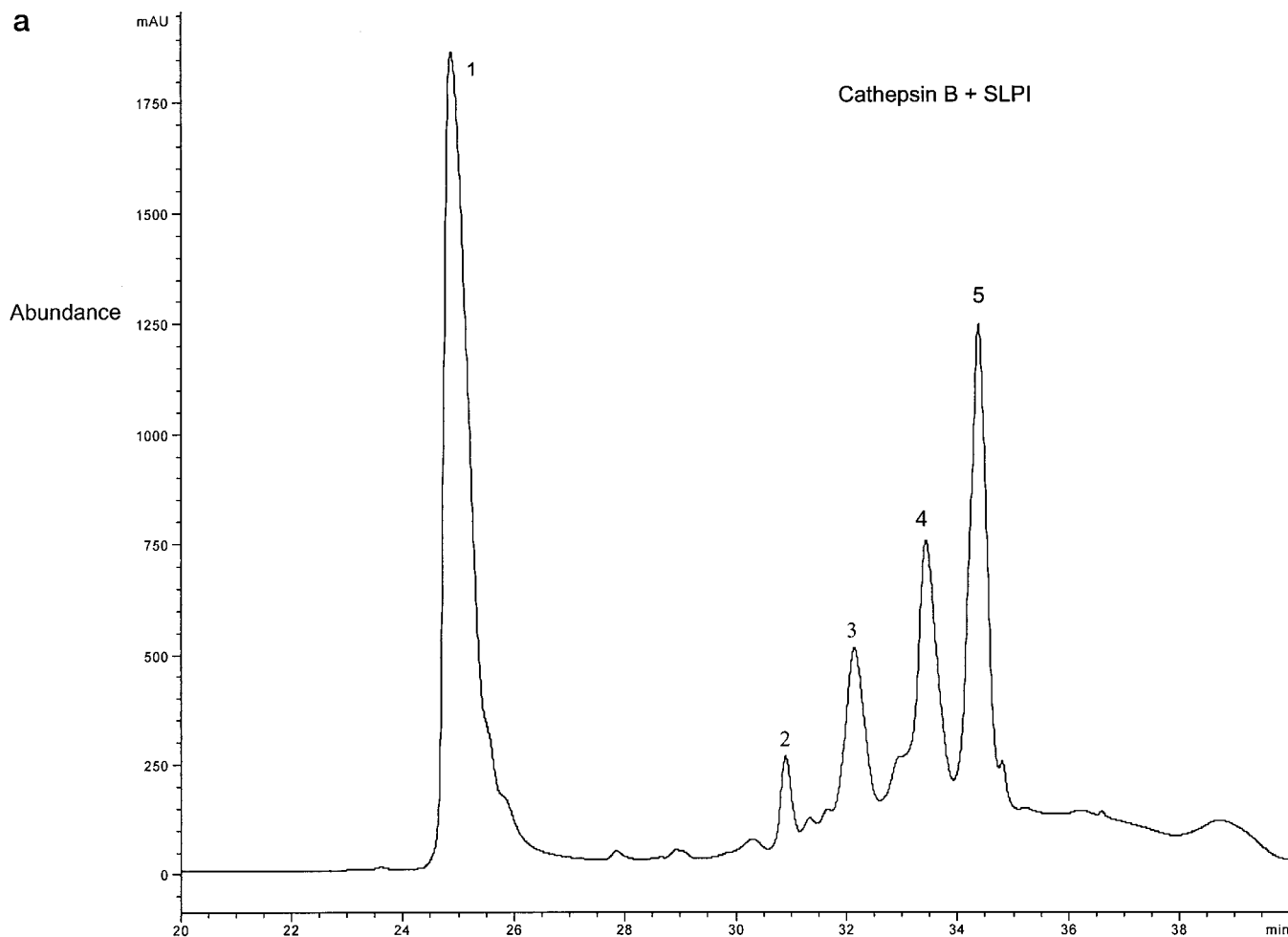
FIG. 3. **HPLC analysis of SLPI incubated with cathepsins B, L, and S.** Cathepsin B (*a*), L (*b*), and S (*c*) were incubated with SLPI in the absence of dithiothreitol for 48, 8, and 1 h, respectively. The samples were neutralized with 0.2 M Tris, pH 8.0, and 1 μM E-64, dried and reconstituted in 6 M guanidine HCl with TCEP (1 mM). The samples were then separated by HPLC. Five peaks were obtained for each incubation, corresponding to the various SLPI products obtained from each incubation.
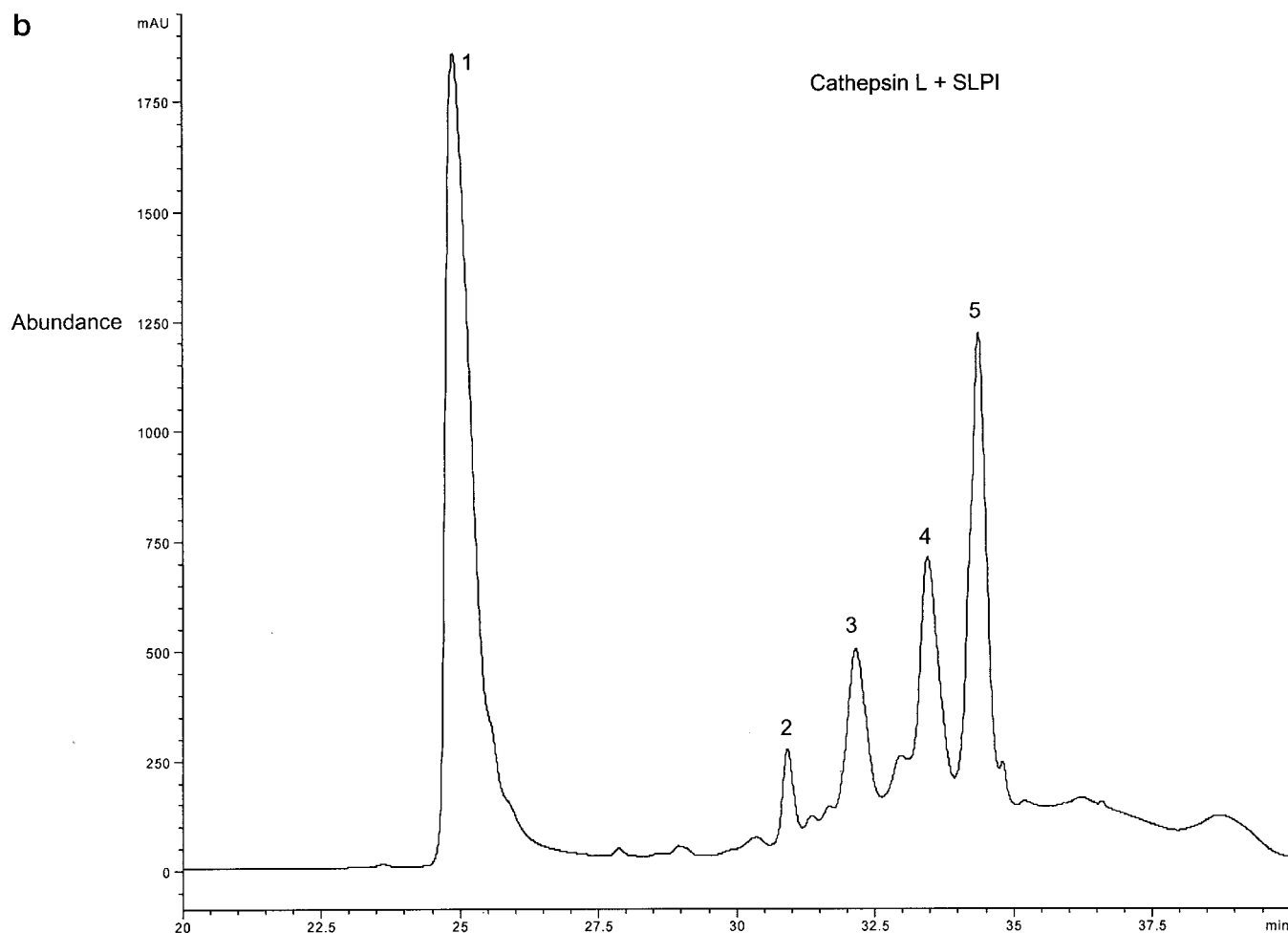
b

FIG. 3—*continued*

with cathepsin B (0.5 nmol), cathepsin L (20 pmol), or cathepsin S (20 pmol) in 0.1 M sodium acetate buffer, pH 5.5, containing 1 mM EDTA, 1 mM dithiothreitol in a 100-μl volume at 37 °C. Samples (5 μl) were removed from each incubation over a 60-min time period and mixed with 15 μl of 0.2 M Tris, pH 8.5, containing 1 μM E-64. The samples and controls were then assayed for anti-NE activity.

SDS-PAGE—SLPI (4 nmol) was incubated with neutrophil elastase (0.5 nmol), proteinase 3 (0.5 nmol), and cathepsin G (0.5 nmol) in 0.1 M HEPES, 0.5 M NaCl, pH 7.5, for 24 h at 37 °C. SLPI (4 nmol) was also incubated with MMP-1 (0.5 nmol), MMP-8 (0.5 nmol), and MMP-9 (0.5 nmol) in 50 mM Tris, 0.15 M NaCl, 10 mM CaCl₂, 1 mM ZnCl₂, pH 7.5, for 24 h at 37 °C. Finally, SLPI (4 nmol) was incubated with cathepsin B, L, and S in 0.1 M sodium acetate buffer, pH 5.5, containing 1 mM EDTA, 1 mM dithiothreitol in a 50-μl volume at 37 °C for 1 h. 5-μl samples were removed after each incubation and serine protease activity was neutralized with 1 mM phenylmethylsulfonyl fluoride, MMP activity with 1 mM EDTA and cathepsin activity with 0.2 M Tris, pH 8.5, containing 1 μM E-64. The samples were boiled for 5 min in an equal volume of SDS sample treatment buffer, containing 1 mM β-mercaptoethanol, prior to electrophoresis in a 17.5% polyacrylamide gel. Gels were then stained in Coomassie Brilliant Blue R-250 and destained as previously described (38).

HPLC Mass Spectrometry—SLPI (4 nmol) was incubated with cathepsin B, L, and S for 48, 8, and 1 h, respectively, in 0.1 M sodium acetate buffer, pH 5.5, containing 1 mM EDTA (without dithiothreitol) in a 50 μl volume at 37 °C. Cathepsin activity was neutralized with 0.2 M Tris, pH 8.5, containing 1 μM E-64 and the samples were lyophilized and reconstituted in 6 M guanidine HCl at a concentration of 1 μg/ml. Tris[2-carboxyethyl]phosphine (final concentration 1 mM, added to assure unambiguous identification of each peptide) was added to each sample and incubated at room temperature for 15 min. 20 μl (20 μg) of SLPI was loaded onto the HPLC, separated, and analyzed by electrospray mass spectrometry as previously described (24). Mass spectra were deconvoluted with the software provided by the instrument manufacturer (Agilent Technologies, Palo Alto, CA, Chemstation version 8).

Expected masses were calculated from the SLPI sequence by the GP-MAW program (Lighthouse Data, Odense, Denmark).

Patient Characteristics—Sixteen individuals with emphysema (all smokers) and 15 healthy volunteers (all non-smokers) were studied. Emphysema patients attended the outpatient clinic at Beaumont Hospital, Dublin. All subjects had normal AAT levels and phenotypes as determined by nephelometry and isoelectric focussing. Diagnosis of emphysema was based on previous guidelines (25) including medical history, chest roentgenography, pulmonary function, and CT scan. Symptoms of a respiratory tract infection were absent for the 6 weeks preceding lavage. Arterial blood gas analysis performed on all subjects while they breathed room air showed no clinically significant hypoxemia or hypercapnea. Informed consent was obtained from all subjects and the study was approved by the Beaumont Hospital Ethics Committee.

Bronchoalveolar Lavage (BAL)—BAL was performed with 120 ml of sterile saline as described by Klech and Pohl (26). Recovered fluid was filtered through sterile gauze to remove debris and mucus and was then centrifuged at 300 × g and the supernatants were stored at −70 °C until analyzed. Cell number was determined by a hemocytometer and cell differentiation was carried out by May-Grunwald Giemsa staining of cytospin preparations.

Determination of Epithelial Lining Fluid (ELF)—This was carried out by measuring urea nitrogen in serum and BAL as previously described (23). This gave an accurate determination for the actual amount of epithelial surface liquid present in each BAL sample.

Determination of SLPI Levels and Activity in Emphysema and Normal ELF—SLPI levels were determined by sandwich enzyme-linked immunosorbent assay, as previously described (23). SLPI activity was determined as previously described (23). Briefly, increasing amounts of BAL are titrated against a fixed concentration of NE (2 nM) and porcine pancreatic elastase (2 nM). AAT, SLPI, and elafin, the other significant antiprotease present in the lung (23), can all inhibit NE. However, AAT and elafin both inhibit porcine pancreatic elastase whereas SLPI does not (23). Therefore, the difference in the
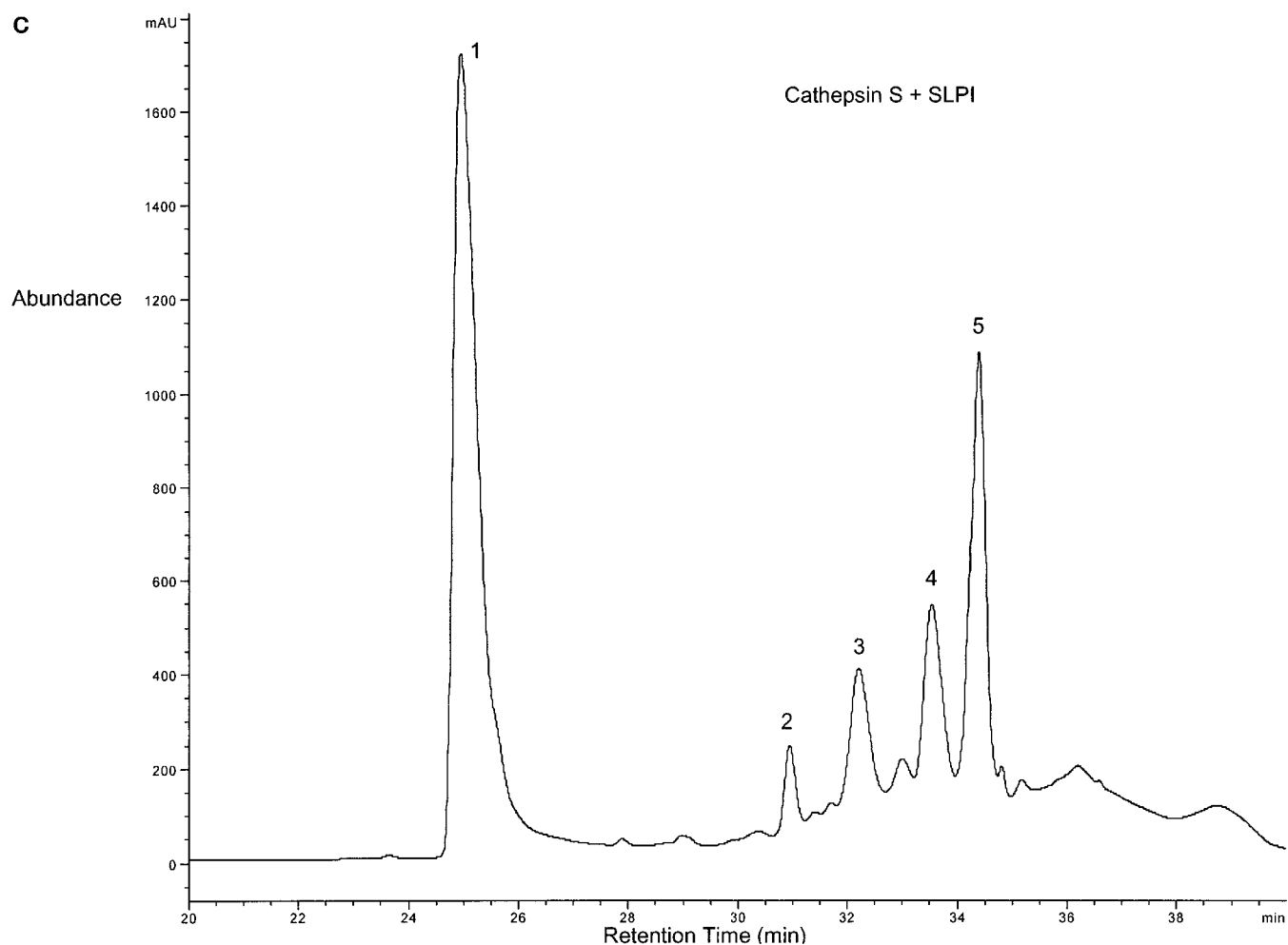
FIG. 3—*continued*

amount of BAL required to inhibit NE compared with amount needed to inhibit porcine pancreatic elastase is taken to be equivalent to the SLPI activity present in BAL. Values were corrected for ELF and expressed as micromolar/ml ELF.

Western Blotting of SLPI in ELF—BAL samples were concentrated 20-fold using Microcon-3 columns. Equal amounts of ELF were electrophoresed on 15% SDS-PAGE and blotted onto nitrocellulose. After blocking in I-Block (Tropix), SLPI was detected using affinity purified rabbit anti-SLPI IgG (1:1000 in I-block) for 1 h followed by incubation with alkaline phosphatase-labeled goat anti-rabbit IgG (1:7500) for 1 h. Development was carried out using the Tropix Western Kit.

Measurement of Cathepsin Activity in COPD and Control ELF—Cathepsin B and L activities in BAL were measured using Z-Arg-Arg-p-nitroanilide (0.1 mM) for the estimation of cathepsin B activity and Z-Phe-Arg-p-nitroanilide (0.1 mM), in the presence of the cathepsin B inhibitor CA-074 (10 μg/ml), for the estimation of cathepsin L activity. The incubation buffer was 0.2 M sodium acetate, pH 5.5, containing 1 mM EDTA, 1 mM dithiothreitol. The buffer was made 10% acetonitrile for the cathepsin L activity measurement. Values were corrected for ELF and expressed as micromolar p-nitroaniline released per min/ml ELF.

Cathepsin L Inhibition and Neutralization Experiments—Emphysema BAL samples were preincubated with inhibitors of cathepsin B (CA-074, 50 μM) and cathepsin L (Z-Phe-Phe-CHN$_2$, 50 μM) for 30 min at 37 °C followed by incubation with recombinant SLPI (2 μg) for 24 h at 37 °C. Samples were analyzed for SLPI cleavage by Western blot. Similarly, emphysema BAL samples were incubated with monoclonal anti-cathepsin L IgG (10 μg/ml) or with isotype control IgG (10 μg/ml) for 1 h at 37 °C. The samples were then incubated with recombinant SLPI (2 μg) for 4 h at 37 °C and analyzed for SLPI cleavage by Western analysis. The effect on the anti-NE activity of each sample was also measured.

RESULTS

Proteolytic Susceptibility of SLPI—SDS-PAGE analysis demonstrated that incubation of SLPI with serine proteases (Fig. 1a) or MMPs (Fig. 1b) did not lead to cleavage of SLPI. However, cathepsin incubation with SLPI revealed a very similar cleavage pattern for all three cathepsins resulting in the generation of two SLPI cleavage fragments with approximate molecular masses of 7.5 kDa (SLPI*) and 4.5 kDa (SLPI#) (Fig. 1c). This is not entirely surprising considering that cathepsin B, L, and S have a very similar substrate profile and probably cleave at very similar sites.

Effect of Cathepsin Incubation on SLPI Anti-NE Activity—SLPI did not lose anti-NE activity over the duration of the experiment when incubated in 0.1 M sodium acetate buffer (Fig. 2) indicating that acidic pH does not affect anti-NE activity. Incubation of SLPI:cathepsin B at a molar ratio of 15:1 resulted in a decrease in anti-NE activity to 14% of control (Fig. 2). However, inactivation of SLPI anti-NE activity by cathepsin L and S (to 10 and 4% of control, respectively, Fig. 2) was achieved at molar ratios of SLPI:cathepsin of 400:1 indicating that SLPI is a much better substrate for cathepsins L and S than cathepsin B.

Analysis of Cathepsin-SLPI Cleavage Sites—HPLC separation of the products obtained following incubation of cathepsins B, L, and S with SLPI resulted in the initial formation of three species (Fig. 3, peaks 1, 2, and 5). The mass spectrum of peak 1 obtained from the HPLC is shown in Fig. 4a. The deconvoluted mass of this mass was 7,353.4 Da, identifying it
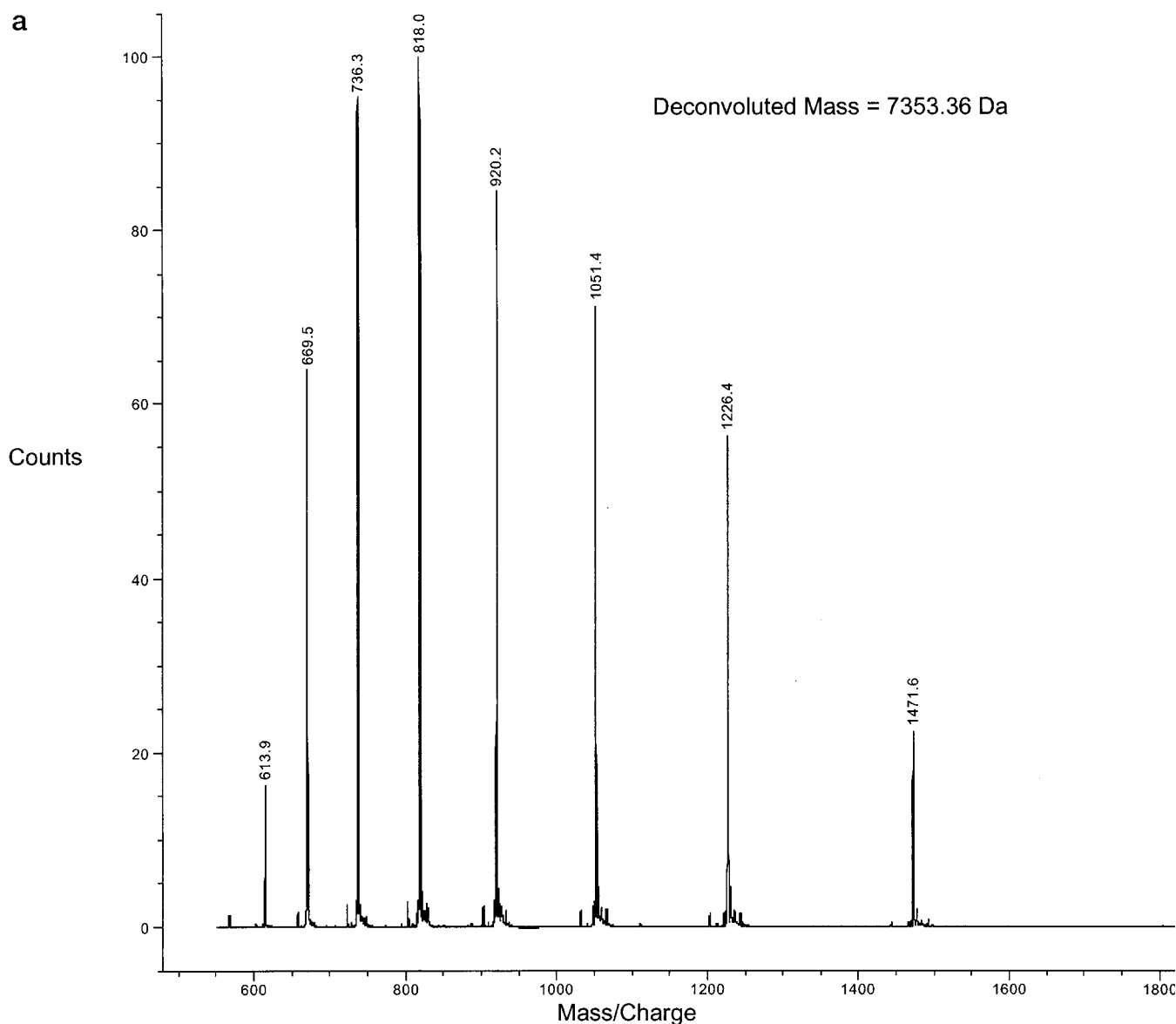
a

Deconvoluted Mass = 7353.36 Da

FIG. 4. **Electrospray mass spectrum of SLPI fragments obtained following HPLC.** *a*, mass spectrum of peak 1 obtained from the HPLC following separation of SLPI incubated with cathepsin B, L, or S. The deconvoluted mass of peak 1 was 7,353.4 Da (*a*), identifying it as SLPI residues 1–67 (calculated mass = 7,353.7 Da). *b*, mass spectrum of peak 5 obtained from the HPLC following separation of SLPI:cathepsin mixtures. The deconvoluted mass was found to be 4,389.8 Da (*b*), identifying it as SLPI residues 68–107 (calculated mass = 4,390.4 Da).

as SLPI residues 1–67 (calculated mass = 7,353.7 Da). Likewise, the mass spectrum for peak 5 is shown in Fig. 4*b*, and the deconvoluted mass for this peak was 4,389.8 Da, identifying it as SLPI residues 68–107 (calculated mass = 4,390.4 Da). Mass spectrometric analysis of peak 2 established that this peak was SLPI residues 75–107 (observed mass = 3,580.9 Da; calculated mass = 3,581.3 Da). The other peaks in the chromatogram were the small amount of residual wild-type SLPI (Peak 4, observed mass = 11,725.5 Da, calculated = 11, 725.5 Da) and the monomethionine sulfoxide SLPI (peak 3, observed mass = 11, 741.5 Da, calculated = 11,741.5 Da). These results indicated that in the case of all three cathepsins the initial site of cleavage in the SLPI molecule occurred after the threonine 67 residue. Cleavage at the Thr[67]-Tyr[68] bond was followed by trimming of the 68–107 peptide to generate the 75–107 peptide. The SLPI active site for NE inhibition occurs at Met[72]-Leu[73] (23) and destruction of this site by cathepsin B, L, and S action clearly resulted in the loss of anti-NE activity observed in Fig. 2. The presence of oxidized SLPI in SLPI in the commercial preparation has

been previously identified by HPLC mass spectrometry analysis of native SLPI (data not shown) and did not occur as a result of SLPI incubation with cathepsins B, L, or S. Interestingly, the oxidized SLPI species appeared to be relatively resistant to cleavage by any of the cathepsins since it was present at approximately the same concentration before and after incubation with cathepsins B, L, and S.

Patient Characteristics—Clinical and lavage characteristics of the patient and control groups are shown in Table I. All emphysema patients had impaired pulmonary function with FEV_1 values of <70%, decreased FEV_1/FVC and DL_{co} values compared with the controls ($p < 0.001$). ELF recovery for emphysema and controls was very similar (0.87 ± 0.3 ml *versus* 0.89 ± 0.18 ml). Emphysema BAL samples contained a greater number of cells compared with controls ($3.5 \times 10^7 \pm 0.8 \times 10^7$ *versus* $2 \times 10^7 \pm 0.4 \times 10^7$) and an increased percentage of macrophages (97 *versus* 89%, $p < 0.001$). Neutrophil percentages were not significantly different between both groups (2 *versus* <1%).
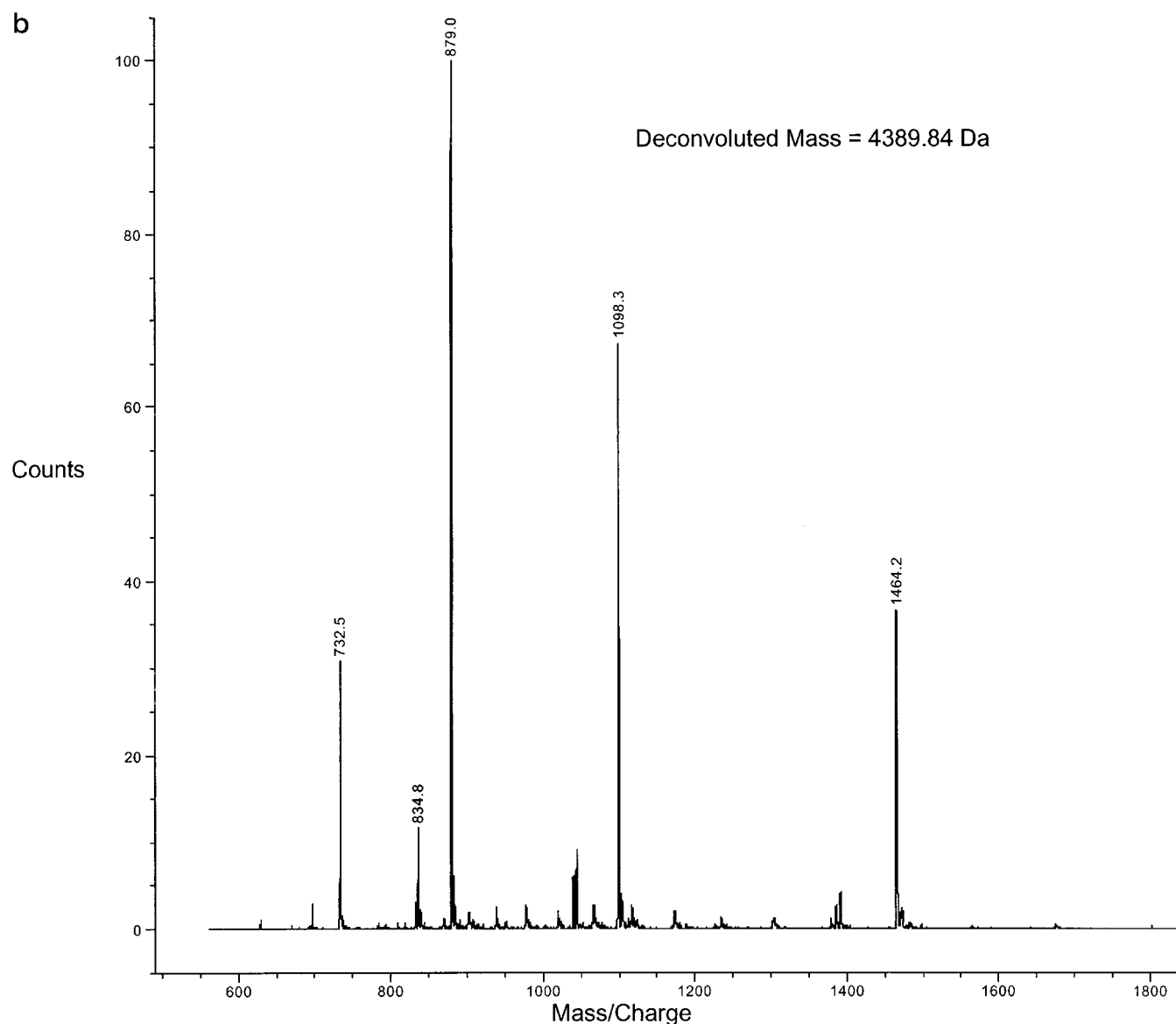
Cathepsin Inactivation of Secretory Leucoprotease Inhibitor

b

Deconvoluted Mass = 4389.84 Da

FIG. 4—*continued*

TABLE I

Patient characteristics

Table describing the pulmonary function test values. ELF values, and cell types and number present for the emphysema and control patient population examined in this study.

	Controls	Emphysema	
Age (years)	55 ± 4	62 ± 2	
Sex	11 Male, 4 female	13 Male, 3 female	
Smoking status	15 Non-smokers	16 Smokers	
FEV_1 (%)	90 ± 5	50 ± 6	*$p < 0.001$
FEV_1/FVC	87 ± 5	49 ± 4	*$p < 0.001$
DL_{CO} (%)	76 ± 3	38 ± 7	*$p < 0.001$
ELF (ml)	0.87 ± 0.18	0.89 ± 0.3	
Cells	2×10^7 ($\pm 0.4 \times 10^7$)	3.5×10^7 ($\pm 0.8 \times 10^7$)	
Macrophages (%)	86 ± 3	97 ± 1	*$p < 0.01$
Neutrophils (%)	<1	2	

SLPI Levels, Activity, and Status in Emphysema and Control ELF—SLPI levels were found to be significantly lower in emphysema ELF compared with control ELF (0.37 ± 0.12 μM/ELF *versus* 0.67 ± 0.21 μM/ELF, $p < 0.05$, Fig. 5*a*. Likewise, SLPI activity was found to be lower in emphysema ELF compared with control ELF (0.18 ± 0.07 μM/ELF *versus* 0.42 ± 0.1 μM/ELF, $p < 0.05$, Fig. 5*b*). Thus, SLPI status in emphysema and control BAL samples was investigated by Western blotting.

BAL samples were concentrated using Microcon-3 columns in order to detect SLPI by Western blot. Attempted analysis of SLPI in BAL samples by SDS-PAGE followed by staining with Coomassie Blue R-250 gave a confused picture due to the presence of other small molecular weight species in BAL with a molecular weight similar to SLPI. SLPI was detected as a band at ~12 kDa in all normal BAL samples and a representative blot is shown in Fig. 5*b*, *insert 1* (*lane 2*). However, analysis of
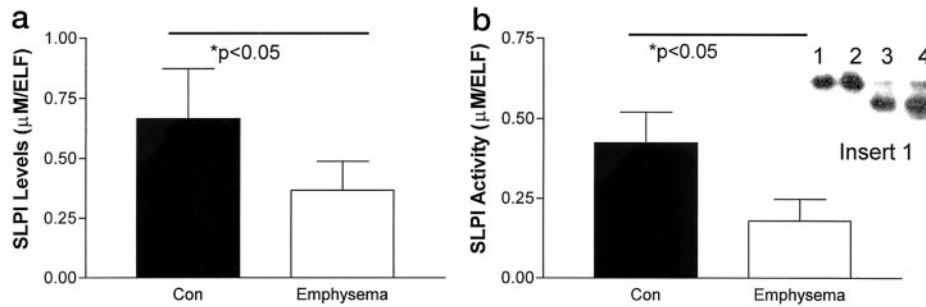
FIG. 5. **SLPI levels, activity, and status in emphysema and control ELF.** SLPI levels in the emphysema group were found to be significantly lower than the corresponding control group (a, $p < 0.05$). Values are presented as micromolar SLPI/ml ELF. Likewise, SLPI activity was also significantly lower in the emphysema group (b, $p < 0.05$). Values are presented as micromolar active SLPI/ml ELF. SLPI in emphysema BAL was examined by Western blot and a representative figure is shown. b, *insert 1*, *lane 1*, SLPI standard; *lane 2*, control BAL; *lane 3*, emphysema BAL; *lane 4*, recombinant human SLPI plus cathepsin L.
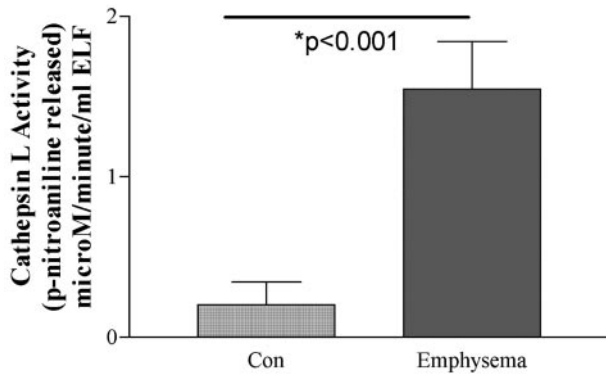

FIG. 6. **Cathepsin activity in emphysema and control ELF.** Emphysema and control BAL samples were examined for cathepsin L activity (using Z-Phe-Arg-*p*-nitroanilide) in the presence of CA-074. Cathepsin L activity was significantly increased in the emphysema group (Fig. 6, $p < 0.001$). Values are presented as micromolar *p*-nitroaniline produced per min/ml of ELF.
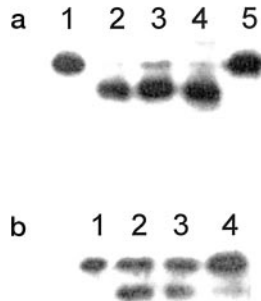

FIG. 7. **Neutralization of cathepsin L activity in emphysema ELF.** Effect of inhibiting cathepsin L cleavage of SLPI, added to emphysema BAL, using a synthetic inhibitor of cathepsin L. a, *first lane*, SLPI standard; *second lane*, SLPI + emphysema BAL; *third lane*, SLPI + emphysema BAL + dimethyl sulfoxide; *fourth lane*, SLPI + emphysema BAL + CA-074 (50 μM); *fifth lane*, SLPI + emphysema BAL + Z-Phe-Phe-CHN$_2$ (50 μM). Effect of inhibiting cathepsin L cleavage of SLPI, added to emphysema BAL, using a neutralizing antibody to cathepsin L. b, *first lane*, SLPI standard; *second lane*, SLPI + emphysema BAL; *third lane*, SLPI + emphysema BAL + isotype control IgG; *fourth lane*, SLPI + emphysema BAL + anti-cathepsin L IgG.

SLPI in emphysema BAL demonstrated that it was present as a cleaved product with some native intact SLPI remaining (Fig. 5b, *insert 1*, *lane 3*). The size of the SLPI cleavage product was equivalent to that obtained when SLPI was incubated with purified cathepsin and analyzed separately by Western blot (Fig. 5b, *insert 1*, *lane 4*). Only the native SLPI and the larger 7.5-kDa SLPI fragment can be detected by Western blot. The smaller 4.5-kDa SLPI fragment cannot be detected by Western blot presumably due to degradation of the epitope specific for

the anti-SLPI IgG by cathepsin activity. In light of these results we investigated the different protease activities present in emphysema ELF that might be cleaving and inactivating SLPI.

Determination of Proteolytic Activities in BAL—The presence of active cathepsin L in ELF samples from individuals with emphysema has previously been demonstrated therefore, we measured the activities of cathepsins B and L in our emphysema and control BAL samples. Cathepsin B activity was present in 4 COPD BAL samples but was not significantly elevated above controls (data not shown). Cathepsin L activity was demonstrated in 15 emphysema BAL and 2 control BAL samples, with cathepsin L activity being significantly increased in emphysema ELF compared with control ELF (Fig. 6, $p < 0.001$). Therefore, this result suggested that cathepsin L was the possible candidate protease responsible for the cleavage and inactivation of SLPI in emphysema ELF.

Cathepsin L Inhibition and Neutralization Experiments—Incubation of emphysema BAL with recombinant SLPI resulted in SLPI cleavage (Fig. 7a, *lane 2*) with a pattern similar to that seen with endogenous SLPI present in emphysema BAL (Fig. 5b, *insert 1*, *lane 3*) and recombinant SLPI incubated with purified cathepsin (Fig. 5b, *insert 1*, *lane 4*). Incubation of emphysema BAL with inhibitors of cathepsin B and L followed by incubation with SLPI in 0.1 M sodium acetate buffer, pH 5.5, containing 1 mM EDTA, resulted in the cleavage of SLPI in the presence of cathepsin B inhibitor (Fig. 7a, *lane 4*). However, no cleavage of SLPI was observed in the presence of cathepsin L inhibitor (Fig. 7a, *lane 5*). Incubation under the same conditions using dimethyl sulfoxide (the solvent that both inhibitors was dissolved in) also resulted in SLPI cleavage (Fig. 7a, *lane 3*) indicating that dimethyl sulfoxide did not inhibit the cathepsin L activity in emphysema BAL. This confirmed that inhibition of cathepsin L in emphysema BAL prevented cleavage of SLPI. The findings in this experiment were confirmed using several emphysema BAL samples incubated with recombinant SLPI (data not shown).

Emphysema BAL was also incubated with IgG to cathepsin L or isotype control IgG followed by incubation with SLPI as above. These experiments demonstrated that isotype IgG did not prevent SLPI cleavage from occurring (Fig. 7b, *lane 3*) but anti-cathepsin L IgG prevented significant SLPI cleavage (Fig. 7b, *lane 4*). The findings in this experiment were also confirmed using several emphysema BAL samples incubated with recombinant SLPI (data not shown).

DISCUSSION

SLPI is cleaved by the elastolytic cysteine proteases cathepsins B, L, and S resulting in the inactivation of SLPI anti-NE activity. Previous studies have shown that SLPI is susceptible to inactivation by bacterial proteases (27, 28).

However, in this study we have demonstrated that SLPI can be cleaved and inactivated by members of the cathepsin family. Investigation of emphysema ELF revealed that SLPI levels and activity were decreased compared with ELF from controls. Analysis of SLPI by Western blot revealed that SLPI was intact in control ELF but SLPI cleavage products were present in emphysema ELF with a banding pattern similar to that obtained by Western blotting when recombinant SLPI was incubated with purified cathepsin. Cathepsin L activity was shown to be increased significantly in emphysema ELF and the use of a synthetic inhibitor for cathepsin L and a neutralizing antibody for human cathepsin L were both capable of inhibiting the cleavage of recombinant SLPI added to emphysema BAL. This result shows that cathepsin L-mediated SLPI cleavage occurs *in vivo* in emphysema and probably decreases the antiprotease screen present in the emphysema lung.

SLPI is composed of two highly homologous domains, an N-terminal domain composed of residues 1–54 and a C-terminal domain consisting of residues 55–107 (29). Four disulfide bridges are present in each domain, a feature that was thought to make the SLPI molecule inaccessible to proteolytic action. Leucine at position 72 has been demonstrated to be the active residue for NE inhibition and the methionine at position 73 is has been shown to be susceptible to oxidation which renders SLPI inactive toward NE (30). It is also thought that the region between residues 67–74 of the SLPI molecule act as a docking region for a number of proteases (3). Therefore, our observation that cathepsins B, L, and S proteolytically cleave SLPI at Thr^{67}-Tyr^{68} and subsequently remove residues 68–74 indicates that the region surrounding this bond is susceptible to cleavage leading to inactivation of SLPI's anti-NE activity.

The other major serine protease inhibitor of the respiratory tract, α_1-antitrypsin, also possesses proteolytically susceptible sites and oxidation sensitive residues in its active site between residues 350 and 358. This region can be cleaved by a variety of proteases including MMP-1, MMP-7, MMP-8, MMP-9, and *Pseudomonas* elastase resulting in a loss in the anti-NE activity of α_1-antitrypsin (31–35). In addition, cathepsin L can also inactivate α_1-antitrypsin by cleavage at bonds Glu^{354}-Ala^{355} and Met^{358}-Ser^{359} (16). Similarly to SLPI, α_1-antitrypsin also possesses oxidation-sensitive methionine residues in its active site (24). Therefore, together with the data presented in this paper, cathepsin L is the only protease identified to date that is capable of inactivating both SLPI and α_1-antitrypsin, the major antiproteases of the upper and lower respiratory tracts. However, it seems likely that the cathepsins play an important role in inflammatory lung diseases. As well as the ability to inactivate antiproteases, the cathepsins L and S are capable of degrading lung connective tissue such as elastin (36, 37). In this study we have shown that active cathepsin L is present at significantly elevated levels in emphysema ELF compared with control ELF fluid. It has also been demonstrated previously that smokers have increased levels of cathepsin L compared with healthy controls (13, 14) and cigarette smoking has been demonstrated to induce cathepsin S activity in alveolar macrophages (15). The evidence we have presented in this study shows that cathepsin activity present in the respiratory tract of smokers may result in SLPI inactivation. This would have consequences for the anti-NE activity of the lung

in disease states in which cathepsins are present in active concentrations. The destruction of the anti-NE activity of both SLPI and α_1-antitrypsin by cathepsin activity may result in a dramatically decreased antiprotease screen in the disease states resulting in subsequent lung damage.

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Cathepsin B, L, and S Cleave and Inactivate Secretory Leucoprotease Inhibitor

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