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Isolation, properties, and complete amino acid sequence of human secretory leukocyte protease inhibitor, a potent inhibitor of leukocyte elastase

(cathepsin G/trypsin/emphysema/septic shock)

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ABSTRACT A potent inhibitor of human leukocyte elastase (EC 3.4.21.37) and cathepsin G (EC 3.4.21.20) and of human trypsin (EC 3.4.21.4) has been purified from human parotid secretions. The complete amino acid sequence of this protein has been determined. The sequence suggests that the protein has two domains of about 54 amino acids, each of which contains four disulfide bonds. On the basis of a limited homology to other protease inhibitors, the antielastase and antitrypsin activities are thought to be properties of the C-terminal and N-terminal domains, respectively. The affinity of the inhibitor for leukocyte elastase is very high, suggesting a functional role for the protein in preventing elastase-mediated damage to oral and possibly other mucosal tissues.

Human polymorphonuclear leukocytes contain proteolytic enzymes that are essential to their function in phagocytosing necrotic tissue and microorganisms. At the same time, these enzymes pose a significant challenge to the body because their uncontrolled release can lead to the destruction of healthy tissue and circulating proteins. For this reason, there has been significant interest in the distribution and properties of leukocyte protease inhibitors. These proteins are thought to be important in regulating the local activity of the proteases through rapidly forming strong complexes with the enzymes. They might also have clinical value in the treatment of a number of diseases involving excessive proteolytic activity, including emphysema and septic shock.

Thus far, most attention has focused on the therapeutic potential of α_1-antitrypsin (1), the major serum inhibitor of leukocyte proteases (2). However, there are other human protease inhibitors with significant potential for preventing excessive leukocyte-mediated proteolysis *in vivo*. Prominent among these are the acid-stable inhibitors from mucus secretions (3–7), from seminal plasma (8), and from the salivary glands (9, 10). The relationship between these inhibitors is not yet clear. They show a large degree of immunological cross-reactivity, but partial amino acid sequences of the seminal plasma (8) and bronchial mucus inhibitors (4) appear quite different.

Parotid secretions are freer of contaminating proteases than mucus secretions and should therefore be a good source of native material for structural studies of a leukocyte protease inhibitor. We have earlier identified a protein immunologically related to the bronchial mucus leukocyte protease inhibitor in parotid secretions (10). In this communication, we report the purification, properties, and amino acid sequence of this protein. We were surprised to find that its amino acid sequence is different to the partial sequence of both the seminal plasma (8) and bronchial mucus (4) inhibitors published earlier. For this reason, we have given the parotid-derived protein the name secretory leukocyte protease inhibitor (SLPI) to reflect both the type of cell involved in its synthesis and its postulated function.

MATERIALS AND METHODS

Human parotid secretions were collected from volunteers with a suction device fitted over the exit of the parotid ducts (10). Secretion was stimulated by sucking a sour candy. The secretions were stored at −20°C until used.

Peptide *p*-nitroanilides were obtained from Sigma or from Calbiochem. *Staphylococcus aureus* V8 and submaxillary proteases were obtained from Pierce, and endoproteinase Lys-C was obtained from Boehringer Mannheim. Sephadex products were obtained from AB Pharmacia, Uppsala, Sweden. Human leukocyte elastase (EC 3.4.21.37) was prepared as described previously (11) or was purchased from Elastin Products, St. Louis, MO. Human cathepsin G (EC 3.4.21.20) was isolated by the method of Travis *et al.* (12).

SLPI was assayed either enzymatically, by virtue of its ability to inhibit elastase-catalyzed hydrolysis of succinyl-Ala-Ala-Ala-*p*-nitroanilide or methoxysuccinyl-Ala-Ala-Pro-Val-*p*-nitroanilide and chymotrypsin-catalyzed hydrolysis of succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide, or by radioimmunoassay (13) or single radial immunodiffusion (14) using rabbit antibodies against human bronchial mucus inhibitor (15).

Peptides were sequenced by using a Beckman 890 spinning-cup sequencer (at the University of California, Davis) and an Applied Biosystems (Foster City, CA) 401A gas phase sequencer (at Synergen). Phenythiohydantoin derivatives of amino acids were identified by HPLC and by gas chromatography or TLC.

Reverse-phase HPLC was performed on Synchrom RP8 columns (Synchrome, Linden, IN) in 0.1% trifluoroacetic acid, using acetonitrile gradients of either 1% or 0.5%/min.

The amino acid compositions of the peptides in Table 2 were determined as described by Tarr and coworkers (16). Amounts were calculated relative to the methylthiohydantoin of tyrosine. The results are reported as moles of amino acid per mole of peptide.

RESULTS

The purification of SLPI from human parotid secretions is summarized in Table 1. Six liters of secretions were mixed with 300 ml of pH 6.0, 0.5 M sodium acetate, brought to pH 6.0 by addition of acetic acid, and centrifuged. The supernatant was applied directly to a 2.5 × 40 cm SP-Sephadex C-50 column equilibrated with 5 mM NaCl/0.05 M acetate buffer, pH 6.0, and eluted with a linear gradient of 5 mM to

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Abbreviations: SLPI, secretory leukocyte protease inhibitor; CMCys, carboxymethylcysteine.

Table 1. Purification of SLPI

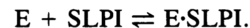
Step	Total vol, ml	Total protein, mg	Total SLPI, mg	Recovery, %
Parotid secretion	6000	5280	5.7	100
SP-Sephadex C-50	75	736	4.6	81
Sephadex G-50	18	4.9	4.0	70

The amount of SLPI was determined by radioimmunoassay (13). Total protein was determined according to Lowry *et al.* (17), using bovine serum albumin as a standard. Titration of granulocyte elastase with the isolated SLPI gave a final amount of purified inhibitor of 4.2 mg.

1 M NaCl in pH 6.0 acetate buffer. Material reacting with antibodies to the protease inhibitor from bronchial mucus eluted at about 0.5 M NaCl. These fractions were pooled and concentrated to 3 ml by ultrafiltration through an Amicon UM2 membrane.

The concentrate was applied to a 1.6 × 100 cm column of Sephadex G-50 equilibrated with 0.6 M NaCl/0.05 M Tris·HCl buffer, pH 7.4, and eluted with the same solution. Material reacting with the antibody appeared in fractions corresponding to an apparent molecular weight of 12,000. The protein in these fractions is essentially homogeneous by $NaDodSO_4$/polyacrylamide gel electrophoresis (Fig. 1*A*) and by reverse-phase HPLC (data not shown). An immunoblot of the $NaDodSO_4$/polyacrylamide gel of the protein and of parotid secretions showed that the purified SLPI has the same size as the single immunoreactive component of the secretions (Fig. 1*B*).

Initial measurements indicated that SLPI inhibits leukocyte elastase and cathepsin G through forming a 1:1 complex with these enzymes. To determine whether the protein could be an important inhibitor of leukocyte proteases *in vivo*, we measured its affinity for these enzymes *in vitro*. A standard amount of each enzyme was mixed with various amounts of the inhibitor and allowed to come to equilibrium. The residual free enzyme was determined by assay with a standard substrate, and the ratio of free to total enzyme (E) was plotted against inhibitor concentration and compared to that predicted on the basis of the mechanism



$$E + SLPI \rightleftharpoons E{\cdot}SLPI.$$

The best fit between the theoretical line and the data is provided when the K_d of the SLPI·elastase complex and the SLPI·cathepsin G complexes are set at $2 \pm 1 \times 10^{-10}$ M and $5 \pm 2 \times 10^{-9}$ M, respectively (Fig. 2). In a similar experiment (data not shown), the K_d of the complex of SLPI with human anionic trypsin was found to be 4×10^{-9} M.

For sequencing, SLPI was chromatographed on a C_8 reverse-phase column. The protein was then reduced and carboxymethylated with iodo[^{3}H]acetic acid (21) and repurified by reverse-phase HPLC before sequencing or proteolytic digestion. The complete sequence of the protein is presented in Fig. 3. The sequence was determined as follows.

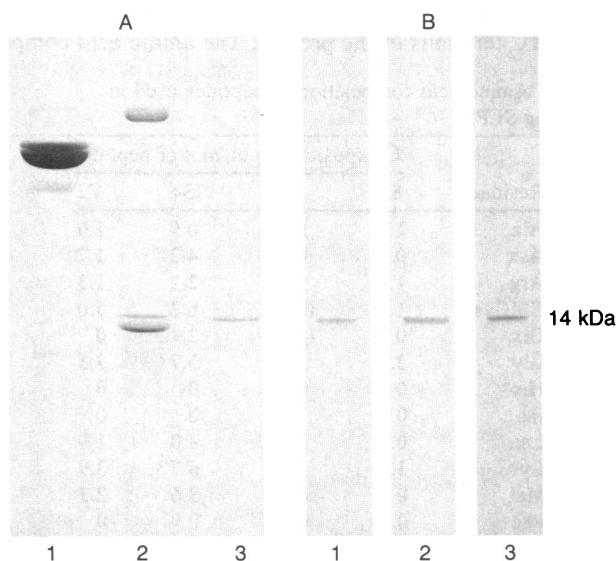


FIG. 1. $NaDodSO_4$/polyacrylamide gel electrophoresis (according to ref. 18) of parotid secretion (lanes 1), SLPI after SP-Sephadex C-50 chromatography (lanes 2), and after gel filtration on Sephadex G-50 (lanes 3). All samples were reduced with 10 mM dithiothreitol before electrophoresis. (*A*) The proteins were stained with Coomassie R 250 after electrophoresis. (*B*) Immunoblotting (19) of the gel, using rabbit antiserum to bronchial mucus inhibitor (15) and peroxidase-coupled goat anti-rabbit IgG (Dako Immunoglobulin, Copenhagen) and 3-amino-9-ethylcarbazole (20).

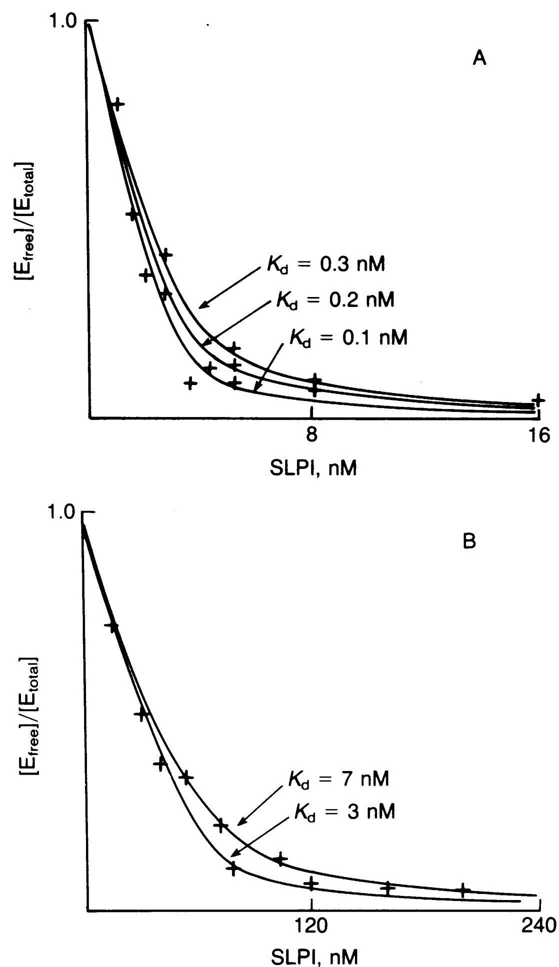


FIG. 2. Titration of leukocyte elastase (*A*) and cathepsin G (*B*) with SLPI. (*A*) Elastase (0.1 μg) in 0.9 ml of Tris·HCl (200 mM, pH 7.5) containing 0.1% human serum albumin was mixed with SLPI and allowed to stand at 37°C for 15 min. Then 100 μl of a 10 mM solution of methoxysuccinyl-Ala-Ala-Pro-Val-*p*-nitroanilide (Sigma) in dimethyl sulfoxide was added to start the reaction. (*B*) Cathepsin G (1 μg) in 0.45 ml of Tris·HCl (200 mM, pH 7.5) containing 0.1% human serum albumin was mixed with SLPI and allowed to stand at 25°C for 15 min. Then 50 μl of a 100 mM solution of succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide in dimethyl sulfoxide was added to start the reaction. The initial velocity of the reaction, determined from the rate of increase in absorbance at 410 nm, was used to calculate the concentration of free enzyme. The lines are calculated by using the known concentrations of enzyme and SLPI and the K_d values shown.

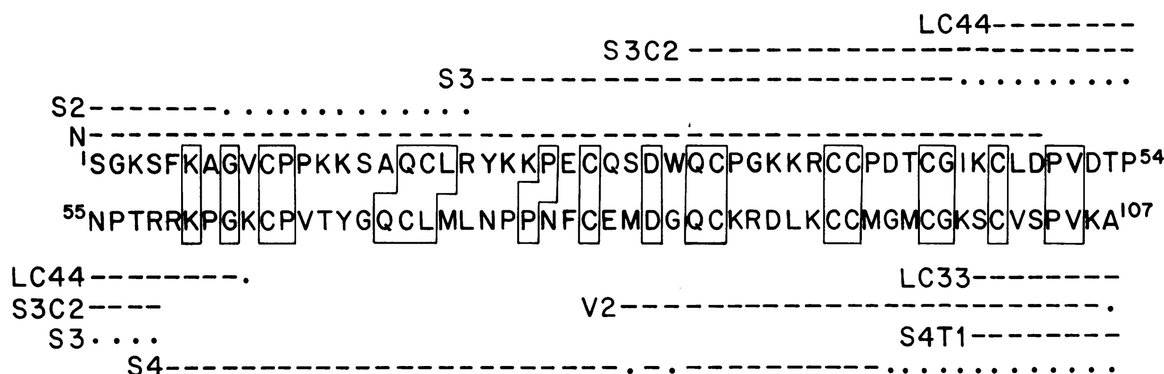


FIG. 3. The complete amino acid sequence of SLPI is presented in the standard one-letter code in a manner that emphasizes the homologies between the first and second halves of the protein. The key peptides used to determine the sequence are shown adjacent to the sequence and are discussed in the text. N refers to sequence determined by Edman degradation of the reduced carboxymethylated protein. A dash indicates that this residue was positively identified; a dot indicates that this residue is presumed to be present in the peptide on the basis of amino acid composition but was not positively identified.

(*i*) Ten nanomoles of the reduced carboxymethylated protein was subjected to automated Edman degradation. This experiment gave good sequence information up to residue Asp-49 and fragmentary information beyond that point.

(*ii*) Key peptides for further sequence determination were isolated by digestion of the reduced carboxymethylated protein with submaxillary protease (23). Reverse-phase HPLC of submaxillary protease digests of SLPI gave two clean peaks corresponding to peptides S2 and S3 and several peaks of material corresponding to peptide S4 (Fig. 4). The sequence (Fig. 3) and amino acid composition (Table 2) of peptide S2 show that it corresponds to the N-terminal 20 amino acids of the protein and resulted from a cleavage after Arg-20, in good accord with the known specificity of this enzyme. The sequence (Fig. 3) and composition (Table 2) of peptide S3 show that it also results from cleavage of the Arg-Tyr bond between residues 20 and 21 and served to confirm the sequence determined from the N terminus through Cys-39. However, this peptide did not further extend our knowledge of the protein sequence because it gave only fragmentary information beyond residue 39. Further information came from sequencing a peptide from a chymotryptic digest of S3, peptide S3C2. This peptide results from cleavage of S3 after Trp-30. It gave good sequence data up to Asp-52 and fragmentary information as far as Arg-58 (Fig. 3). From this result, it appears that peptide S3 results from cleavage after Arg-58 and that the Arg-CMCys bond at residues 37–38 is not a substrate for submaxillary protease.

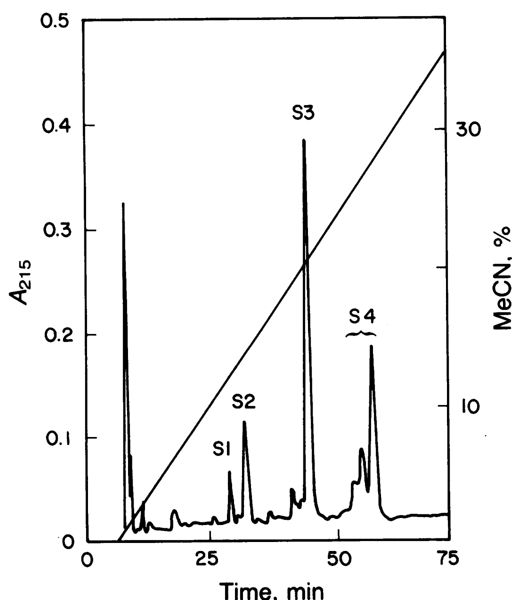


FIG. 4. Reverse-phase HPLC of a submaxillary protease digest of reduced carboxymethylated SLPI. Ten nanomoles (120 μg) of protein was dissolved in 80 μl of Tris·HCl (100 mM, pH 8.0) containing 2 M urea and treated with 0.8 μg of submaxillary protease for 18 hr at 25°C. The reaction mixture was loaded directly on the RP8 chromatography column.

The N-terminal sequence and amino acid composition of several fractions from the peaks labeled S4 in Fig. 4 showed them to be a single peptide. We have no information as to why this peptide runs so poorly, but we suspect that some modification of the amino acid side-chains occurred, either during purification of the protein or during preparation of the peptide. Ten nanomoles of this peptide gave good sequence information from Arg-59 to CMCys-92, with the exception of residues 82 and 84 (Fig. 3). Residues 82 and 84 were identified as Met and Gly, respectively, from peptide V2, described below. A tryptic digest of peptide S4 gave a small peptide of sequence Ser-CMCys-Val-Ser-Pro-Val-Lys-Ala that is not present in the sequence listed above and that was later found to be the C terminus of the protein. The amino acid compo-

Table 2. Amino acid composition of peptides used in sequencing SLPI

Residue	Composition, mol/mol of peptide			
	S2	S3	S4	V2
Ala	1.7	0	0.9	1.0
Asx	0.1	4.3	4.2	1.7
Arg	1.0	2.3	2.2	1.3
CMCys	1.8	(5.5)*	6.3	5.0
Glx	0.8	(3.0)*	2.1	0
Gly	2.1	2.5	5.7	3.5
His	0	0	0	0
Ile	0	1.0	0	0
Leu	0.8	1.3	3.0	1.0
Lys	3.4	5.0	6.7	3.9
Met	0	0	3.6	2.3
Phe	0.9	0	0.9	0
Pro	2.1	6.5	5.3	1.4
Ser	2.5	1.3	1.2	2.4
Thr	0.3	3.0	0.3	0
Tyr	0.2	1.3	0.9	0
Val	0.9	1.0	2.7	1.8

Figures represent moles of amino acid per mole of peptide. Where more than one set of near-integral values could be obtained, that closest to the composition expected from Fig. 3 is presented.
*Carboxymethylcysteine (CMCys) and Glu were not distinct enough to allow integration.

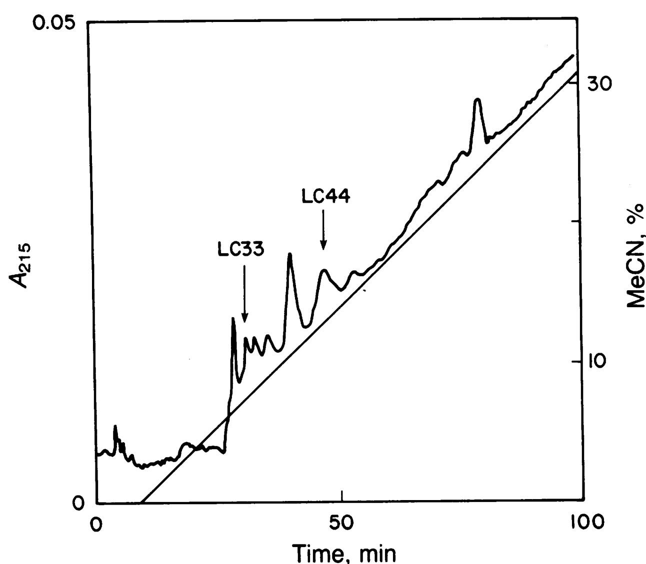


FIG. 5. Reverse-phase HPLC of an endoproteinase Lys-C digest of reduced carboxymethylated SLPI. A 0.7-nmol (8-μg) sample of protein was dissolved in 30 μl of 50 mM ammonium carbonate and treated with 0.06 unit of endoproteinase Lys-C for 15 hr at 25°C. The mixture was loaded directly onto the RP8 column.

sition of peptide S4 (Table 2) and the presence of sequences corresponding to residues 101–107 (Fig. 3) indicate that it continues to the C terminus of the protein.

(*iii*) An overlap between peptides S3C2 and S4 is provided by a peptide, LC44, isolated by reverse-phase HPLC of an endopeptidase Lys-C (22) digest of reduced carboxymethylated SLPI (Fig. 5). The sequence of this peptide suggests that it resulted from cleavage of the two Lys-CMCys bonds at positions 46–47 and 63–64, although the final Lys residue could not be detected on Edman degradation (Fig. 3). The sequence confirmed the presence of Thr at positions 53 and 57 and Pro at position 56 and showed that no sequence had been missed between Arg-58 and Arg-59.

(*iv*) The sequence of the protein was completed with peptides isolated from a V8 protease (24) digest of reduced carboxymethylated SLPI. Reverse-phase HPLC of this digest gave two sharp peaks corresponding to peptides V1 and V2 and a very broad peak, which was not analyzed further (Fig. 6). Peptides V1 and V2 correspond to the N and C termini of the protein, respectively. The sequence (Fig. 3) and composition (Table 2) of peptide V2 show that it results from cleavage of the Glu-Met bond at residues 81–82, in good accord with the known specificity of the protease. This peptide gave good sequence information up to Lys-106, but the Ala at position 107 could not be seen after Edman degradation. The presence of this residue is, however, required by the amino acid composition of the peptide.

(*v*) Further support for the view that residue 107 constitutes the C terminus of the protein comes from the isolation of an endoproteinase Lys-C peptide LC33 (Fig. 5), which results from cleavage of the Lys-Ser bond at residues 99–100. This peptide gave sequence data through to Ala-107 despite the presence of a Lys at position 106 (Fig. 3). Proteases of the trypsin type are known to be poor exopeptidases.

DISCUSSION

The complete amino acid sequence of SLPI, deduced from these and other peptides, is shown in Fig. 3. The calculated molecular weight is 11,726, which is in good agreement with that determined by gel filtration but is significantly smaller than that determined by $NaDodSO_4$/PAGE. The aberrant

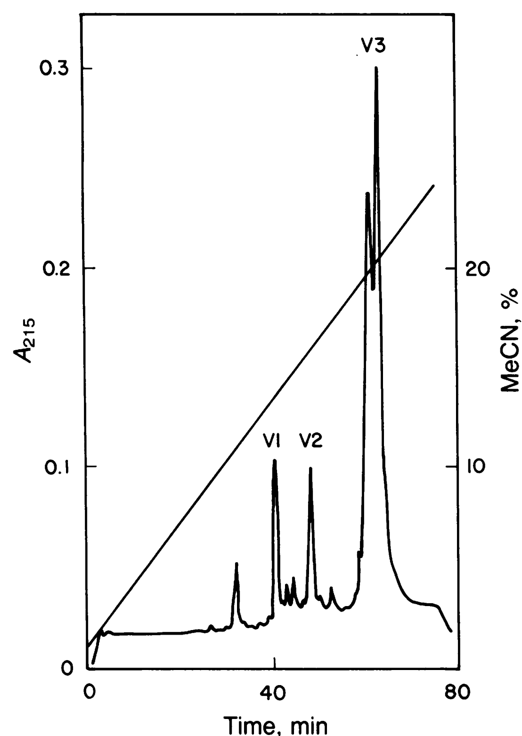


FIG. 6. Reverse-phase HPLC of a V8 protease digest of reduced carboxymethylated SLPI. Ten nanomoles (120 μg) of protein was dissolved in 100 μl of 100 mM ammonium carbonate and treated with 5 μg of V8 protease for 12 hr at 25°C. The mixture was loaded directly on the RP8 column.

result of the $NaDodSO_4$/PAGE experiment may be related to the overwhelmingly cationic nature of the protein.

The sequence suggests several interesting hypotheses about the domain structure and the evolutionary development of the protein. There is strong evidence for the presence of two distinct domains. If residues 1–54 are compared with residues 55–107 and their homology is maximized by an insertion between positions 66 and 69 and a deletion at position 79, we find 19 residues that are identical in the two proposed domains. These conserved residues are boxed in Fig. 3. They include all the Cys residues, which might be expected to be highly conserved in the two domains since two sections of the polypeptide must come sufficiently close at this point to form a cystine crosslink.

Each of the putative domains of SLPI is about the same size as the better characterized "Kunitz" and "Kazal" domains of other protease inhibitors (25). However, neither bears any strong resemblance to the two classical domains at the amino acid sequence level. A slight homology exists between residues 20–29 and 72–81 of SLPI and the consensus sequence for the active site of Kazal inhibitors (26) (Fig. 7), suggesting that residues 20–29 and 72–81 of SLPI may interact with the proteolytic enzyme. These postulated active sites fit with the known activity of SLPI against both trypsin-like and chymotrypsin/elastase-like proteases. The Arg-20 from the first domain would be in the correct position to interact with the specificity pocket of trypsin, and Leu-62, Met-63, or Leu-64 would be in the correct position to occupy the specificity pocket of chymotrypsin and leukocyte elastase. Limited proteolysis of native SLPI confirms that residues 20–22 and 72–74 are on the surface of the protein and are exposed to proteolytic enzymes (R.C.T. and K. Hale, unpublished results).

A number of other protease inhibitors having properties similar to those of SLPI have been isolated from mucus secretions and seminal plasma. The elastase/chymotrypsin/trypsin inhibitors from seminal fluid (HUSI-1), cervical

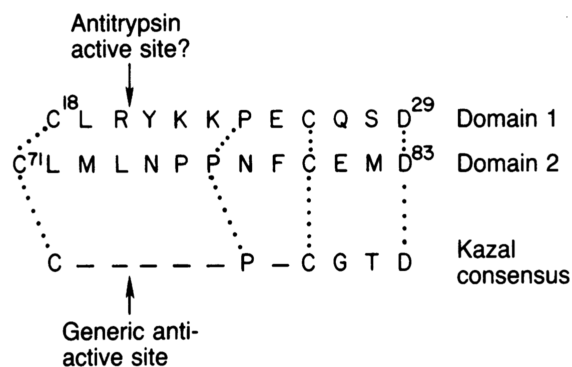


FIG. 7. Homology between residues 18-29 and 71-83 of SLPI and the consensus active site region of Kazal protease inhibitors.

mucus (CUSI), and bronchial mucus have amino acid compositions similar to the composition of SLPI, with the exception that they are reported to lack tryptophan. However, a partial amino acid sequence of the seminal plasma inhibitor (8) indicates that there are significant differences between SLPI and HUSI-1. More recently, a similar inhibitor has been isolated from bronchial mucus by Smith and Johnson (5). This protein has tryptophan, like SLPI, but it is reported to be a glycoprotein. We have failed to find any sugar residues in the course of sequencing SLPI and, in particular, Asn-55, the only Asn present in a consensus N-glycosylation sequence in the protein, is found in the expected yield in peptides S3C2 and LC44. Another protein that is partially homologous to SLPI has been isolated by Klassen and Kramps from bronchial mucus (4). These workers have published the sequence of 26 N-terminal amino acids from this protein. It appears to have a different sequence from SLPI at positions 1, 4, 15, and 26. Obviously, our knowledge of the true relationship of these protease inhibitors to SLPI will have to await their further characterization.

The amino acid sequence of SLPI has provided information essential for elucidating the physiological role of this inhibitor. In addition, it will allow cloning of the gene for SLPI and the production of large quantities of the protein for examining its potential therapeutic value in preventing the excessive proteolysis catalyzed by leukocyte proteases in the course of emphysema and septic shock.

Note Added in Proof. After submission of this manuscript, a revised sequence of the elastase inhibitor from seminal plasma (8) was published (27). It is now clear that the seminal plasma inhibitor is a proteolyzed form of SLPI.

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