Purification, characterization and proteinase-inhibitory activity of a Clara-cell secretory protein from the pulmonary extracellular lining of rabbits

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A low- M_r Clara-cell secretory protein, CCSP, previously shown to be a major secretory product of Clara cells, was isolated from rabbit lung lavage effluents. CCSP accounted for $4.4 \pm 0.5\%$ of the protein in the soluble phase of cell- and surfactant-free pulmonary lavage effluents (LE). Purification of this protein from LE was achieved in two steps. First, the LE was acidified with HClO_4 and, secondly, CCSP was isolated by gel-exclusion chromatography on Sephadex G-50. Purified CCSP was homogeneous by SDS/polyacrylamide-gel electrophoresis (PAGE), consisting of a single major isoform with a pI of 6.0. The M_r of CCSP was 5800 according to SDS/PAGE under reducing conditions and 12600 under non-reducing conditions. However, by gel chromatography the M_r of the protein under non-reducing conditions was 12400 and under reducing conditions it increased to 15000. The discrepancy obtained by using these two techniques was attributed to anomalous electrophoretic mobilities of the protein in its reduced state. The molecule contained three half-cystine residues, but no free thiol groups, and tryptophan was not detectable. The first seven *N*-terminal amino acid residues were Gly-Ile-Xaa-Pro-Arg-Phe-Ala-. The third residue was not identified. CCSP showed inhibitory activity against the thiol proteinase papain (50% inhibition at $4 \mu\text{M}$ -CCSP), but only weak activities against human polymorphonuclear-leucocyte elastase, and bovine trypsin. The molecule was not digested by, and did not complex with, trypsin. CCSP was immunochemically different from surfactant apoprotein B (M_r 10000) present in rabbit lung surfactant. This study is the first partial characterization of the major secretory protein of rabbit lung Clara cells.

INTRODUCTION

The major secretory protein of bronchiolar Clara cells is a low- M_r protein (CCSP). We identified this low- M_r protein by examining the secretory products of Clara cells isolated from the lungs of rabbits (Patton *et al.*, 1986). We have also examined lavage effluents from the lungs of rabbits and identified a low- M_r protein that appears to be very similar to that which is synthesized and secreted by isolated Clara cells. The results of our studies are consistent with those of Singh *et al.* (1985), who identified a low- M_r protein in pulmonary lavage effluents (LE) from the lungs of rats that appeared to be immunohistochemically specific for Clara cells and which they believed to be secreted by those cells.

The functions of Clara-cell secretory proteins in the pulmonary extracellular lining are not known. However, several low- M_r proteins have been detected in bronchial secretions, some of which appear to have proteinase-inhibitory activities (Hochstrasser *et al.*, 1972; Ohlsson & Tegner, 1976). One of these low- M_r proteinase inhibitors has been localized immunohistochemically to non-ciliated cells of the bronchiolar epithelium (Mooren *et al.*, 1983; De Water *et al.*, 1986), and we speculate that CCSP might be such a proteinase inhibitor. Alternatively,

surfactant apoprotein B (Claypool *et al.*, 1984) is a small protein with an M_r of about 10000, and recent studies have also indicated that the Clara cell might contain surfactant apoproteins (Balis *et al.*, 1985). Unfortunately, the properties of Clara-cell secretory proteins are not known.

Examination of the physical and biochemical properties of CCSP could provide important information indicative of its functions *in vivo*. Isolation of CCSP from purified Clara cells using current cell separation techniques would be difficult because of the large number of cells required. Therefore, we have isolated a corresponding protein from the pulmonary extracellular lining. The present paper describes the purification and partial characterization of the Clara-cell secretory protein, CCSP, from the extracellular lining of the lungs of rabbits.

MATERIALS AND METHODS

Materials

The following materials were used: DTT, PMSF, Nbs₂, Bz-Arg-pNA, Suc-(Ala)₃-4NA, Bz-Arg-2Nap, Brij-35, Fast Garnet GBC, *p*-chloromercuribenzoic acid,

Abbreviations used: LE, lavage effluents; PAGE, polyacrylamide-gel electrophoresis; DTT, dithiothreitol; PMSF, phenylmethanesulphonyl fluoride; Nbs₂, 5,5'-dithiobis-(2-nitrobenzoic acid), Bz-Arg-pNA, benzoyl-DL-arginine *p*-nitroanilide; Suc-(Ala)₃-4NA, *N*-succinyl-L-alanyl-L-alanyl-L-alanine 4-nitroanilide; Bz-Arg-2Nap, benzoylarginine 2-naphthylamide; Tos-Phe-CH₂Cl, tosylphenylalanylchloromethane ('TPCK'); TEMED, *NNN'*-tetramethylethylenediamine; PBS, phosphate-buffered saline (0.15 M-NaCl buffered with 10 mM-sodium phosphate, pH 7.4); CCSP, Clara-cell secretory protein.

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neuraminidase (EC 3.2.1.18), Tox-Phe-CH₂Cl-treated trypsin (EC 3.4.21.4), papain (EC 3.4.22.2), Hepes and protein standards for gel filtration (carbonic anhydrase, cytochrome *c*, insulin and insulin β -chain oxidized) and electrophoresis (aprotinin) (Sigma Chemical Co., St. Louis, MO, U.S.A.); SDS, acrylamide, methylenebisacrylamide, ammonium persulphate, glycerol, TEMED and protein standards for electrophoresis (myosin, β -galactosidase, phosphorylase *b*, bovine serum albumin, ovalbumin, trypsin inhibitor and lysozyme) (Bio-Rad Laboratories, Richmond, CA, U.S.A.); human sputum elastase (EC 3.4.21.37) (Elastin Products Co., Pacific, MO, U.S.A.); Ampholine gels, pH 3.5–9.5 (LKB, Rockville, MD); Sephadex G-50 (Pharmacia Fine Chemicals, Piscataway, NJ, U.S.A.); Diaflo ultrafiltration membranes (Amicon Corp., Danvers, MA, U.S.A.).

Animals

Adult male rabbits of the New Zealand White strain, weighing 2–2.5 kg, were obtained from Dutchland Laboratory Animal, Denver, PA, U.S.A. They were maintained on standard diet and had free access to food and water.

Pulmonary-lavage procedure

Rabbits were killed by injection of 3 ml of a solution (50 mg/ml) of sodium pentobarbital in saline (0.9% NaCl) into the marginal ear vein. Pulmonary LE were obtained as described by Hook (1978). Briefly, the trachea and lungs were removed intact from the chest, cleared of extraneous tissues, and washed with 150 mM-NaCl/6 mM-KCl/5 mM-KH₂PO₄/5 mM-glucose, buffered with 25 mM-Hepes, pH 7.4, ('Hepes medium') to remove blood from the outer surfaces. The lungs were lavaged by filling them to capacity with Hepes medium at 4 °C without applying any pressure other than that exerted by the head of water present in the trachea. The lungs were then inverted and the lavage effluent collected in a beaker kept in ice. Contamination of lavage effluents with blood from the external surfaces of the lungs was prevented by wrapping the trachea in several layers of absorbent gauze. The procedure was performed six times.

Isolation of CCSP from LE

All procedures for the purification of CCSP were performed at 4 °C. Lavage effluents were centrifuged at 580 *g* for 10 min to remove cells and then at 227 000 *g* for 45 min to remove any particulate material, including surfactant. The supernatant was concentrated to 3–4 mg of protein/ml in Amicon cells fitted with YM-2 Diaflo ultrafiltration membranes. It was diluted once with 5 vol. of saline buffered with 10 mM-sodium phosphate, pH 7.4, and again concentrated to about 3–4 mg of protein/ml. The concentrated lavage effluents were stored at –70 °C until further use.

Concentrated LE (about 175 mg of protein) were treated with HClO₄ essentially by the procedure of Ohlsson *et al.* (1977). Concentrated LE was mixed slowly with HClO₄ (62%, v/v) to a final HClO₄ concentration of 5% (v/v). The solution was kept in ice for 1 h with stirring. The precipitate was sedimented by centrifugation at 40 000 *g* for 20 min. The supernatant was neutralized with KOH and again centrifuged at 40 000 *g* for 20 min

and then concentrated by ultrafiltration (YM-2 Diaflo ultrafiltration membrane in Amicon cell). The concentrated solution was diluted with 10 vol. of 0.5 M-NaCl buffered with 10 mM-sodium phosphate, pH 7.4 (elution buffer) and again concentrated. Dilution and concentration of the protein solution was repeated. The volume of the concentrated proteins was about 2.5 ml.

The concentrated protein solution was loaded on to a column (1.6 cm × 100 cm) of Sephadex G-50 equilibrated with elution buffer. The rate of flow was 6 ml/h, and 2 ml fractions were collected. The column effluent was monitored at 225 nm. Selected fractions were subjected to SDS/PAGE under reducing conditions after overnight dialysis against distilled water in a microdialysis system (Bethesda Research Laboratories, Gaithersburg, MD, U.S.A.). The fractions containing purified low-*M_r* protein were pooled together and stored at –70 °C until required.

Isoelectric focusing and neuraminidase digestion

Analytical isoelectric focusing was performed on Ampholine gels (pH gradient 3.5–9.5) in a Multiphore electrophoresis apparatus (LKB, Bromma, Sweden) according to the directions supplied by the manufacturer (LKB application note no. 1804). The electrode strips were saturated with 1 M-NaOH/1 mM-H₃PO₄, and samples were applied with the aid of application strips. pI values were measured by excision of 0.5 cm pieces of focusing gel and extracted with distilled water at 37 °C or by using a surface pH electrode. Neuraminidase digestion of CCSP was performed for 20 h at 37 °C as described by Phelps *et al.* (1984). The freeze-dried protein (30 μ g) was treated with 5 units of neuraminidase in 0.5 ml of 0.1 M-sodium acetate, pH 5.0, containing 0.1 mM-PMSF. The samples were dialysed against distilled water, freeze-dried and used for isoelectric focusing.

M_r determination

The *M_r* of CCSP under non-reducing conditions was determined from its chromatographic behaviour on a column of Sephadex G-50 and its mobility on SDS/PAGE in the absence of β -mercaptoethanol. *M_r* values under reducing conditions were also determined by gel filtration and SDS/PAGE. Several conditions were used for reducing CCSP. First, the protein was incubated for 20 min at room temperature with elution buffer containing 10 mM-DTT. The protein was applied to the column (1.6 cm × 100 cm) of Sephadex G-50 and eluted with the elution buffer containing 0.1% DTT. Secondly, the protein was heated in a boiling-water bath for 5 min with elution buffer containing 5% (v/v) β -mercaptoethanol before loading it on to a Sephadex G-50 column equilibrated with elution buffer containing 0.5% β -mercaptoethanol. Thirdly, the protein was incubated for 4 h with 0.1 M-Tris/HCl, pH 8.0, containing 9 mM-urea, 0.2% EDTA and 10 mM-DTT. This solution was dialysed with elution buffer containing 0.1% DTT and chromatographed as in the first procedure. The protein markers used for calibrating the column were carbonic anhydrase (*M_r* 29 000), cytochrome *c* (12 400), insulin (6000) and insulin β -chain oxidized (3500), and for SDS/PAGE were myosin (200 000), β -galactosidase (116 250), phosphorylase B (92 000), bovine serum albumin (66 200), ovalbumin (45 000), carbonic anhydrase (31 000), trypsin inhibitor (21 500), lysozyme (14 400) and aprotinin (6500).

SDS/PAGE

SDS/PAGE was performed on 6–20%-(w/v)-polyacrylamide gradient gels by the method of Laemmli (1970). The samples were dissolved in SDS sample buffer with or without β -mercaptoethanol and heated for 3 min at 100 °C. Non-SDS/PAGE amide-gel electrophoresis was performed on PAA 4/30 gradient gels (Pharmacia Fine Chemicals, Uppsala, Sweden). A discontinuous buffer system was used, 0.05 M-Tris/0.05 M-glycine, pH 8.9, at the cathode, and 0.1 M-Tris/0.05 M-HCl, pH 8.1, at the anode. Non-SDS/PAGE for basic proteins was performed as described by Reisfeld *et al.* (1962). Gels were stained overnight with 0.125% Coomassie Brilliant Blue R-250 in methanol/acetic acid/water (5:1:4, by vol.) and destained with methanol/acetic acid/water (5:2:13, by vol.). The gels were stained by periodic acid/Schiff reagent (Fairbanks *et al.*, 1971) for carbohydrate detection.

Amino acid analysis and N-terminal determination

Desalted samples were hydrolysed with 5.7 M-HCl for 24 h at 110 °C *in vacuo* with and without prior performic acid oxidation. Samples were separately hydrolysed with 4 M-Mes to determine the number of tryptophan residues in the protein (Simpson *et al.*, 1976). N-Terminal sequence analysis were performed on a Sequemat Mini-15 and Applied Biosystems 470A gas-phase sequencer by the method of Edman & Begg (1967). Two separate analyses were performed and the results averaged.

Determination of free thiol groups with Nbs₂

The free thiol-group content was determined by titration of protein with Nbs₂ in 0.08 M-sodium phosphate buffer, pH 8.0, containing 2.0% SDS and EDTA (0.5 mg/ml) (Habeeb, 1972). It was also detected by spraying 0.1% Nbs₂ in ethanol/0.45 M-Tris buffer, pH 8.2 (1:1, v/v) on chromatograms (Glaser *et al.*, 1970).

Preparation of antiserum

Antiserum was developed in goats against highly purified Clara cells (93.2±3.3%; four separate preparations of Clara cells) isolated from the lungs of rabbits. Clara cells were prepared by using the procedure of Patton *et al.* (1986), except that the differential-adherence step was replaced with an overnight incubation in culture dishes coated with collagen I and fibronectin; during this period Clara cells attached to the matrix and were removed next day with trypsin. The antiserum was absorbed twice with rabbit liver acetone-dried powders.

Protein blotting and immunostaining

The electrophoretic transfer of proteins from SDS-containing gels to nitrocellulose sheets was based on Burnette's (1981) modification of the procedure described by Towbin *et al.* (1979), a Bio-Rad transblot apparatus being used. The electrode buffer was 25 mM-Tris/192 mM-glycine in aq. 20% (v/v) methanol, pH 8.3, and a constant current of 150 mA was passed for 16 h. The immunostaining was conducted by using a modification of the three-antibody procedure of Sternberger (1978) for paraffin sections. Electrophoretic blots were soaked for 1 h at 37 °C in buffer consisting of 10% (v/v) fetal-calf serum and 3% bovine serum albumin in PBS. Blots were then incubated with 1:50 dilution of IgG fraction of goat antiserum developed against rabbit Clara cells

(Patton *et al.*, 1986b) in the immunoblotting buffer for 1 h at room temperature. Blots were then treated with peroxidase-conjugated IgG fraction of rabbit anti-goat IgG (whole molecule) (1:40 dilution) and horseradish peroxidase-anti-(horseradish peroxidase) (antibody developed in goat) soluble complex in 1:100 dilution. The incubation with these antibodies was carried out for 30 min at room temperature. Every treatment was followed by three 10 min washings with PBS. Serum proteins on the blots were revealed by development of the peroxidase reaction for 5–10 min with 4-chloro-1-naphthol reagent. 4-Chloro-1-naphthol reagent was made by diluting a methanolic solution (3 mg/ml) of the compound with 5 vol. of PBS containing 0.03% H₂O₂. The blots were thoroughly washed with distilled water, air-dried on filter paper and then photographed.

Proteinase-inhibition assays

Proteinase-inhibitory activities against elastase, trypsin and papain were measured by estimating residual activity after incubation of enzyme with protein CCSP. Human sputum elastase, derived from polymorphonuclear neutrophils, was measured by the procedure of Balduyck *et al.*, (1985), with Suc-(Ala)₃-4NA as substrate. The enzyme (2 µg) was preincubated with CCSP for 15 min at 37 °C in 0.5 M-NaCl buffered with 0.05 M-Tris/HCl, pH 8.0. The reaction was started by the addition of substrate [2 mg of Suc-(Ala)₃-4NA]. The total volume of the reaction mixture was 1.0 ml. Trypsin activity was measured by the procedure of Erlanger *et al.* (1961). Tos-Phe-CH₂Cl-treated bovine trypsin (2 µg) was incubated for 25 min at room temperature, followed by 5 min at 37 °C with CCSP in 50 mM-Tris/HCl buffer, pH 8.0, containing 5 mM-CaCl₂. The reaction was started by adding Bz-Arg-pNA (800 µg) in 20 µl of dimethyl sulphoxide. Elastase and trypsin activities were measured for 10 min at 37 °C at 410 nm in a Gilford 2600 recording spectrophotometer. Papain activity was measured at 37 °C by using the procedure of Barrett (1972), with Bz-Arg-2Nap as substrate.

Trypsin digestion

The effects of trypsin on CCSP were determined by incubating purified protein with a 10-fold higher concentration of trypsin solution (1.2 mg/ml) in 0.05 M-Tris/HCl, pH 8.0, containing 5 mM-CaCl₂. Incubation was performed at 37 °C for 1 and 2 h. The purified protein and trypsin alone were also incubated at the same temperature as controls. The protein with and without treatment with trypsin and trypsin alone were subjected to polyacrylamide-gel electrophoresis with and without SDS. SDS/PAGE was performed on 3–27% (w/v) gradient gels as described by Laemmli (1970) and non-SDS/PAGE was performed on Pharmacia PAA 4/30 gradient gels.

Protein determinations

Protein was measured by the Bio-Rad protein assay method, with bovine serum albumin as standard.

RESULTS

Purification of CCSP

We refer to the protein with an apparent *M_r* of approx. 6000 (determined by using SDS/PAGE under reducing conditions), synthesized and secreted by Clara cells

Table 1. Purification of CCSP from rabbit lung LE

The total protein at three stages of purification was estimated by the Bio-Rad protein-assay procedure and the percentage of CCSP protein was calculated by densitometric scanning of Coomassie Brilliant Blue R 250-stained gels.

Purification step	Total protein (mg)	CCSP (%)	Total CCSP (mg)	Yield of CCSP (%)	Fold purification
1. Cell-free concentration LE	100	5	5.00	100	1
2. HClO ₄ extract	3.02	57	1.72	34.4	11.4
3. CCSP	0.65	95	0.62	12.4	19.0

Fig. 1. Purification of CCSP from rabbit lung LE

SDS/PAGE of proteins present in cell-free concentrated LE (a), HClO₄ precipitate (b) and HClO₄ supernatant (c). Samples were dialysed against distilled water, freeze-dried and subjected to SDS/PAGE on 6–20% gradient polyacrylamide gels in the presence of β -mercaptoethanol. Each lane contained 25 μ g of protein. The gels were stained with Coomassie Brilliant Blue R 250.

isolated from the lungs of rabbits, as 'CCSP'. However, our starting material for the purification of CCSP was the concentrated soluble phase of cell-free LE from the lungs of rabbits. Pulmonary surfactant was removed from the cell-free LE by centrifugation at high speed. CCSP accounted for approx. 5% of the total protein present in the concentrated LE.

Purification of CCSP from the LE was achieved in two steps as summarized in Table 1. HClO₄ treatment of the concentrated LE resulted in an 11.4-fold purification of CCSP, although approx. 66% of it was lost as a result of using this procedure. Further purification of CCSP on Sepharose G-50 resulted in a final product that consisted of at least 95% pure CCSP. The overall purification was about 19-fold and the yield was 12.4%. The purified protein was essentially homogeneous, although some preparations contained a few very minor impurities that we estimated, by using densitometry, to account for less than 5% of the total protein. These impurities could be removed by PAGE; however, this step necessitated treatment of the protein with SDS, which we considered undesirable and unnecessary for our purposes, and was therefore not routinely pursued.

The first step consisted of acidification of the concentrated LE with 5% HClO₄, which resulted in precipitation of about 66% of the total protein. From Fig. 1 it can be seen that most of the high- M_r proteins were precipitated during the acidification, but CCSP was retained in the soluble phase. The soluble phase also contained several minor proteins, but the major one was CCSP. The elution profile from the Sephadex G-50 column showed two major peaks of protein (Fig. 2). The proteins present in each of the fractions from the column were analysed by using SDS/PAGE under reducing conditions (Fig. 3). The void-volume peak contained most of the impurities, and the second peak consisted of almost pure CCSP. A few very minor contaminating proteins were detected in the second peak.

PAGE

The electrophoretic purity of CCSP was examined under a variety of conditions. The protein often ran as a heavy narrow band in association with a broad preceding band when electrophoresed under reducing conditions and in the presence of SDS (Fig. 3). In the presence of SDS, but without β -mercaptoethanol, the low- M_r protein electrophoresed as a single narrow band (results not shown). Non-SDS/PAGE performed under anionic conditions without reduction caused the protein to migrate as a broad band with two very minor preceding bands (results not shown). Under cationic conditions without SDS or reduction the low- M_r protein migrated as a single broad band (results not shown).

Immunoblotting with Clara-cell antiserum

The electrophoretic and immunochemical similarity of the purified CCSP to that synthesized by Clara cells

Fig. 2. Chromatography of acid-stable proteins on Sephadex G-50

An HClO_4 extract of concentrated LE was neutralized and concentrated as described in the Materials and methods section and then applied to a Sephadex G-50 column equilibrated with 0.5 M-NaCl buffered with 10 mM-sodium phosphate buffer, pH 7.4. The absorbance of fractions were measured at 225 nm. Arrows indicate the peak positions of the protein standards: carbonic anhydrase (CA; M_r 29000), cytochrome c (CC; 12400), and insulin (I; 6000).

Fig. 3. SDS/PAGE of the fractions from gel-filtration chromatography show in Fig. 2

Selected fractions were subjected to electrophoresis on linear-gradient polyacrylamide gels in the presence of SDS and under reducing conditions as described in the Materials and methods section. Note the high purity of CCSP present in fractions 49–58. Fraction P is the purified protein obtained after combining fractions 45–58. Gels were stained with Coomassie Brilliant Blue R 250.

Fig. 4. SDS/PAGE and immunoblots of the protein purified from lavage effluents and proteins synthesized by isolated Clara cells

Autoradiograph of proteins synthesized by isolated Clara cells (the total activity loaded into lane was 50000 c.p.m.). CCSP is indicated by arrow. (b) Immunoblot of proteins synthesized by isolated Clara cells. Antiserum was developed in goats against highly purified Clara cells (the dilution of primary antiserum was 1:50). (c) Immunoblot of protein (1 μg) purified from pulmonary LE. The antiserum used was the same as that in (b).

isolated from the lungs of rabbits was investigated by using SDS/PAGE under reducing conditions followed by immunoblotting with antiserum developed in goats against rabbit lung Clara cells (Fig. 4). The purified protein from LE had the same M_r and reacted with the Clara-cell antiserum. These data confirm that the purified protein was immunochemically similar to, and had the same M_r as CCSP synthesized by Clara cells.

M_r determinations

SDS/PAGE under reducing conditions indicated an apparent M_r for the purified protein of 5800. In the absence of any reducing agent, but in the presence of SDS, on polyacrylamide gels the apparent M_r of the protein increased to 12600.

The M_r of CCSP was also determined by using gel-exclusion chromatography on Sephadex G-50 without SDS and in the presence or absence of reducing agents. The M_r of CCSP in the absence of reducing agent was about 12400 (Fig. 2). In the presence of a reducing agent the M_r of the protein determined on Sepharose G-50 did not decrease, as expected, but instead increased to 15000 (Fig. 5). The protein was reduced under mild and severe conditions and the M_r remained at 15000. Reduction was also performed with DTT in the presence of 9 M-urea without any change in M_r (results not shown).

Fig. 5. M_r determinations by gel-filtration chromatography on Sephadex G-50

CCSP was heated in boiling-water bath for 5 min with 5% β -mercaptoethanol in elution buffer. The column was equilibrated with the elution buffer containing 0.5% β -mercaptoethanol. The protein standards [carbonic anhydrase (CA; M_r 29000), cytochrome *c* (CC; 12400), and insulin β -chain oxidized (IB; 3496)], were run under the same conditions as CCSP.

Isoelectric focusing and the effect of neuraminidase

The purified protein was subjected to isoelectric focusing on Pharmacia Ampholine gels over a pH gradient of 3.5–9.5. CCSP consisted of a single major isoform with a pI of 6.0 (results not shown). The pI of CCSP was unaffected by neuraminidase, indicating that the protein did not contain sialic acid residues. The protein was not stained by periodic acid/Schiff reagent (results not shown).

Amino acid composition and *N*-terminal analysis

The results of amino acid analysis are shown in Table 2. The content of methionine and leucine were considerably higher in CCSP than the average for proteins in general. Tyrosine and histidine were considerably lower in CCSP than in proteins in general, and tryptophan was not found. There were three cysteine residues in the protein molecule, but none of these was present as free thiol groups. Integration of the experimental values suggested an M_r of about 12570 for the unreduced protein.

The *N*-terminal amino acid was glycine. The first seven residues of the *N*-terminal sequence was Gly-Phe-Xaa-Pro-Arg-Phe-Ala-. The phenylthiohydantoin derivative released in the third cycle was not identified.

Proteinase inhibition

On the basis of reports concerning the presence of low- M_r proteinase inhibitors in bronchiolar Clara cells (Mooren *et al.*, 1983; De Water *et al.*, 1986), we investigated the ability of CCSP to inhibit two serine proteinases and one cysteine proteinase. All three proteinases were inhibited by CCSP, but only when CCSP was present in relatively high concentrations (Fig. 6). Trypsin and elastase were inhibited by 13 and 20% respectively by 8 μ M-CCSP. CCSP was more effec-

Table 2. Amino acid composition of CCSP

The protein was hydrolysed for 24 h with and without prior performic acid oxidation. Half-cystine and methionine residues were determined as cysteic acid and methionine sulphone after performic acid oxidation. Tryptophan was analysed after hydrolysis with 4 M-Mes. The acidic and amidated forms of aspartic acid and glutamic acid were not distinguished.

Amino acid	Composition (residues/100 residues)	
	CCSP	General proteins*
Aspartate	10.31	9.9
Threonine	9.28	6.0
Serine	7.22	7.1
Glutamate	11.34	10.1
Proline	7.22	4.6
Glycine	5.15	7.5
Alanine	3.09	9.0
Valine	4.12	6.9
Methionine	6.18	1.7
Isoleucine	4.12	4.6
Leucine	11.34	7.5
Tyrosine	1.03	3.5
Phenylalanine	3.09	3.5
Lysine	9.28	7.0
Histidine	1.03	2.1
Arginine	3.09	4.7
Half-cystine	3.09	2.8
Tryptophan	0.00	1.1
Total residues ...	97	
<i>N</i> -Terminal residue ...	Glycine	

* From Chothia (1976).

Fig. 6. Inhibition of human polymorphonuclear-neutrophil elastase (2 μ g) (Δ), bovine trypsin (2 μ g) ($\blacksquare$), and papain (1 μ g) ($\circ$) by purified CCSP

tive against papain, producing 50% inhibition of the proteinase at an inhibitor concentration of 4 μ M. The inhibitory effect appeared to be specific insofar as albumin, at proteinase/albumin molar ratios at 1:100, had no effect on the proteinase activity.

CCSP was highly resistant to degradation by trypsin. Incubation of the protein with trypsin (trypsin/CCSP molar ratio = 5:1) at 37 $^{\circ}$ C for periods of up to 2 h did not result in the degradation of the protein or even in the

formation of complexes between CCSP and protease (results not shown).

Surfactant apoproteins

We purified the low- M_r (10000) surfactant apoprotein from rabbit lung surfactant by using the procedure of Claypool *et al.* (1984). This surfactant apoprotein did not react with antiserum reactive with CCSP (results not shown).

DISCUSSION

We have shown previously that CCSP is a major secretory product of Clara cells isolated from the lungs of rabbits (Patton *et al.*, 1986). On the basis of M_r similarities and immunochemical reaction with antiserum prepared against isolated Clara cells, this protein seems also to be present in LE from the lungs of rabbits. We assume that the protein present in LE arises, at least in part, from its secretion by Clara cells present in the airways. We cannot at this time exclude the possibility that CCSP may be synthesized and secreted by other cells of the airways and alveoli. This protein accounted for $4.38 \pm 0.52\%$ of the total soluble protein present in LE and it was not present in serum.

The extracellular functions of CCSP are not known at present, although its proteinase-inhibitory activity suggests the possibility of such a function within the pulmonary extracellular lining. The method used for the isolation of CCSP was similar to those used by others (Ohlsson *et al.*, 1977; Stockly *et al.*, 1984; Kramps & Klasen, 1985) for the purification of low- M_r proteinase inhibitors from human bronchial secretions. As with the low- M_r proteinase inhibitors, the effectiveness of the isolation procedure for CCSP depended primarily on its unusual acid-stability. This acid-stability permitted CCSP to remain soluble under conditions that precipitated most other proteins present in LE. In view of its airway epithelial source, its acid-stability, its low M_r and its ability to inhibit proteinases, CCSP appears very similar to proteinase inhibitors present in bronchial secretions.

The M_r of the protein was estimated by using both SDS/PAGE and gel filtration. Under non-reducing conditions, the same M_r value was obtained with both procedures. However, in the presence of reducing agents the apparent M_r values obtained by using SDS/PAGE and gel filtration differed considerably. Under reducing conditions the M_r increased from 12400 to 15000 with the gel-filtration procedure and decreased from 12590 to 5800 with SDS/PAGE. Several reasons for this apparent discrepancy are possible. First, the protein could consist of two identical subunits joined by a disulphide bond. This disulphide bond could be buried in the molecule and, therefore, be resistant to reduction. In the presence of SDS the molecule might be opened, permitting reduction of the disulphide bond by reducing agents. This possibility seems unlikely in view of the relatively harsh conditions actually used for its reduction by β -mercaptoethanol. Also, the presence of two subunits is not supported by the number of cysteine residues in the protein molecule. Amino acid analysis showed three residues in the protein molecule, suggesting the presence of at least one free thiol group, even in the non-reduced state of the protein. Examination of the protein for a free thiol group by using two different procedures failed to detect

the presence of free thiol groups. Two of the cysteine residues could be present as a disulphide bridge, but the third one must be present in a modified form. *N*-terminal sequence analysis of CCSP revealed an unknown residue at the third position that speculatively could be a cysteine derivative, perhaps similar to that reported in a thiol-proteinase inhibitor isolated from rat liver. This thiol-proteinase inhibitor from liver contained a cysteine residue with some special structure at the third position from the *N*-terminal (Wakamatsu *et al.*, 1984). Secondly, the apparent M_r of CCSP determined by using gel filtration under reducing conditions actually increased. The increase could be due to conformational changes in the tertiary structure of the protein in the presence of reducing agents (Kloppel & Brown, 1984). Also, reduction of the molecule in the presence or absence of urea did not result in a change in apparent M_r , suggesting that the molecule did not consist of subunits held together by disulphide bonds. Thirdly, the apparent decrease in M_r shown by SDS/PAGE could be accounted for if the electrophoretic mobility of the molecule was increased in the presence of SDS under reducing conditions (Swank & Munkres, 1971). Increased electrophoretic mobility could be achieved if binding of SDS were greater for the reduced, than for the native, protein. Increased electrophoretic mobilities have been reported for several proteins under reducing conditions in the presence of SDS. The electrophoretic mobilities of both bovine trypsin inhibitor (6200) and lysozyme (14300) increased by 15 and 12% respectively under reducing conditions, probably owing to increased amounts of SDS bound to the unfolded molecules (Swank & Munkres, 1971). Under reducing conditions the electrophoretic mobility of CCSP increased by 10%, a value similar to that exhibited by both bovine trypsin inhibitor and lysozyme.

The functions of CCSP *in vivo* are not known, although on the basis of reports of similar proteins in sputum and extrapulmonary tissues, we hypothesized that it might be a proteinase inhibitor. However, proteinase-inhibitory activity inhibited by CCSP was poor against trypsin and elastase. CCSP at micromolar concentrations appeared to inhibit papain, indicating that it might be a thiol-proteinase inhibitor. However, we do not know at this time against what normal pulmonary proteinases CCSP might be effective. Several low- M_r proteinase inhibitors from the lungs have been active against trypsin (Ohlsson *et al.*, 1977; Stockly *et al.*, 1984; Smith & Johnson, 1985).

Our low- M_r protein not only failed to show substantial inhibitory activity against trypsin, but it also failed to form a complex with the enzyme. A trypsin-resistant protein apparently of Clara-cell origin has also been reported by Singh *et al.* (1985) in the lungs of rats, although the M_r of that protein was 55000. Singh *et al.* (1985) detected three proteins of M_r 200000, 55000 and 12000 in rat lung lavage, which may be secreted by Clara cells. The low- M_r protein that we have isolated from the lungs of rabbits appears similar to the 12000- M_r protein detected by Singh *et al.* (1985) from the lungs of rats. CCSP was immunochemically distinct from the low (10000)- M_r surfactant apoprotein described by Claypool *et al.* (1984).

In summary, we have isolated, from the pulmonary extracellular lining, a protein that is immunochemically similar to the major CCSP. This protein is immuno-

chemically different from the low- M_r surfactant apoprotein present in rabbit lungs. CCSP may serve in the pulmonary extracellular lining as a thiol-proteinase inhibitor.

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