Isolation and characterization of sheep α_1 -proteinase inhibitor

Rohitbhai MISTRY,* Phillip D. SNASHALL,* Nicholas TOTTY,† Abraham GUZ* and Teresa D. TETLEY*†

* Department of Medicine, Charing Cross and Westminster Medical School, Fulham Palace Road, London W6 8RF, and

† Ludwig Institute for Cancer Research, Courtauld Building, 91 Riding House Street, London W1P 8BT, U.K.

Sheep plasma proteinase inhibitor, analogous to human α_1 -proteinase inhibitor (α_1 PI), was isolated to homogeneity. Purification was achieved by using $(\text{NH}_4)_2\text{SO}_4$ precipitation, concanavalin A-Sepharose chromatography, Mono Q ion-exchange chromatography and PAGE. Sheep α_1 PI had an M_r of 56000, inhibited human leucocyte elastase, pig pancreatic elastase and bovine trypsin on a 1:1 molar basis and had a plasma concentration of 1.6 ± 0.21 g/l (mean $\pm$ s.d.). Amino acid/carbohydrate composition (15% glycosylated) was similar to that of human α_1 PI (16% glycosylated); *N*-terminal analysis to 31 residues revealed 48–52% identity between the human and sheep proteins. Sheep α_1 PI was susceptible to oxidative inactivation by chloramine-T. Re-activation with the use of methionine sulfoxide peptide reductase and dithiothreitol indicated the presence of a methionine residue at the active site. These results establish that sheep α_1 PI has functional and structural characteristics close to those of human α_1 PI.

INTRODUCTION

α_1 -Proteinase inhibitor (α_1 PI) is responsible for 80–90% of plasma leucocyte elastase-inhibitory and trypsin-inhibitory capacity in man [1]. Proteinases, particularly polymorphonuclear-neutrophil elastase, may be involved in lung damage, acutely in adult respiratory distress syndrome [2] and chronically in the development of emphysema [3]. Animal models are important in the study of these conditions. Sheep have been widely used in work on acute lung injury, because, in this species, it is possible to collect relatively pure lung lymph, which is considered to be a reliable sample of lung interstitial liquid [4]. In addition, lung lining liquid can be collected by bronchoalveolar lavage. This model is also proving very useful in the evaluation of the pharmacokinetics of α_1 PI augmentation therapy by inhalation of human α_1 PI [5]. However, there is at present little information available concerning the nature and metabolism of sheep α_1 PI itself. Recently, a study by Sinha *et al.* [6] questioned the use of this model to study α_1 PI and proteinase-antiproteinase interactions in lung disease. They reported that α_1 PI that they had isolated from sheep had a different *N*-terminal amino acid sequence and elastase-inhibitory activity from the human protein. Furthermore, this protein had an M_r of 62000, was resistant to inactivation by chemical oxidation and, although a potent inhibitor of trypsin (1:1 molar ratio), was a poor inhibitor of both human leucocyte elastase (HLE) and pig pancreatic elastase (PPE) (elastase/ α_1 PI molar ratios 0.07:1 and 0.13:1 respectively). This is unlike human α_1 PI, which inhibits all three proteinases on a 1:1 molar basis. This result is questionable in the light of findings that α_1 PI from rat [7], mouse [8], dog [9], rabbit [10] and goat [11] are chemically and functionally similar to the human protein, although none of the preparations cross-reacts serologically with the human protein.

As part of on-going studies into proteolytic mechanisms in endotoxin-induced acute lung injury in the sheep model, we purified α_1 PI from sheep plasma. Our preliminary findings (R. Mistry, P. D. Snashall, A. Guz & T. D. Tetley, unpublished work; poster presentation at a Biochemical Society Meeting in December 1988) disagreed with those of Sinha *et al.* [6], and since

then we have further characterized sheep α_1 PI. We find that sheep α_1 PI is analogous to human α_1 PI in terms of M_r , inhibition of elastase, amino acid/carbohydrate composition and plasma concentration.

MATERIALS AND METHODS

Materials

PPE (type III), bovine pancreatic trypsin (type IX), Suc-Ala-Ala-Ala-NH-Np, MeO-Suc-Ala-Ala-Pro-Val-NH-Np, Suc-Ala-Ala-Pro-Phe-NH-Np, Bz-Arg-NH-Np, *p*-nitrophenyl *p*-guanidinobenzoate hydrochloride and BSA (fraction V) were purchased from Sigma Chemical Co. HLE was from Elastin Products, St. Louis, MO, U.S.A. Human cathepsin G was a gift from Dr. N. A. Roberts, Roche Pharmaceuticals (U.K.) and methionine sulfoxide peptide reductase (MS-PR) a gift from Dr. N. Brot, Roche Institute of Molecular Biology (U.S.A.). Concanavalin A-Sepharose, Mono Q ion-exchanger (HR 5/5 column) and Superose 12 (HR 10/30 column) were from Pharmacia. All other chemicals were of at least analytical grade.

Purification of sheep α_1 PI

$(\text{NH}_4)_2\text{SO}_4$ fractionation. Citrated plasma (50 ml) was fractionated by using solid $(\text{NH}_4)_2\text{SO}_4$ to a final concentration of 45% (w/v). After centrifugation (1800 *g* for 30 min), the supernatant was concentrated by ultrafiltration on an Amicon PM10 membrane under N_2 pressure at 4 °C. Buffer exchange was carried out by repeated reconstitution (four times) with 0.05 M-sodium acetate pH 6.0, containing 0.25 M-NaCl and 1 mM- Ca^{2+} , - Mg^{2+} and - Mn^{2+} (buffer A), and reconcentration.

Concanavalin A-Sepharose affinity chromatography. After the $(\text{NH}_4)_2\text{SO}_4$ fractionation the concentrated supernatant was further purified by concanavalin A-Sepharose chromatography using a modification of the method described by Abrams *et al.* [9]. Fractions containing PPE-inhibitory activity were pooled, then concentrated by ultrafiltration on an Amicon PM10 mem-

Abbreviations used: α_1 PI, α_1 -proteinase inhibitor; HLE, human leucocyte elastase; MS-PR, methionine sulfoxide peptide reductase; PPE, pig pancreatic elastase; Suc-, 3-carboxypropionyl-; Bz-, benzoyl-; -NH-Np, *p*-nitroanilide.

† To whom correspondence should be addressed.

brane, and buffer was exchanged with 20 mM-Tris/HCl buffer, pH 7.5, as described above.

Ion-exchange chromatography. The pooled sample was applied (0.5–1.0 mg/run) to a Mono Q column in conjunction with an f.p.l.c. system (Pharmacia). Fractions containing PPE-inhibitory activity were pooled and concentrated by ultrafiltration on an Amicon PM10 membrane.

Preparative PAGE. The sample obtained after the ion-exchange chromatography was electrophoresed through 10% polyacrylamide slab gels. Electrophoresis was carried out under non-denaturing conditions with 24.7 mM-Tris/191.8 mM-glycine buffer, pH 8.8, in both the anode and the cathode chambers as described by Hames [12]. After electrophoresis the slab gel was cut horizontally into 2.5 mm strips and the proteins were eluted by diffusion into 20 mM-Tris/HCl buffer, pH 8.0. Strip eluates containing PPE-inhibitory activity were stored at -70°C and used for the subsequent studies described below.

Measurement of proteinase-inhibitory activity

Samples from the purification procedure were screened for elastase-inhibitory activity against PPE by using a modification of the method of Bieth *et al.* [13] with Suc-Ala-Ala-NH-Np as substrate. One unit of inhibitory activity was defined as the amount of a preparation that completely inhibited 1 μg of active PPE. Specific activity was expressed as units of $\alpha_1\text{PI}$ activity/mg of protein.

The molar inhibitory capacity of sheep $\alpha_1\text{PI}$ towards PPE, HLE and cathepsin G was determined with Suc-Ala-Ala-NH-Np, MeO-Suc-Ala-Ala-Pro-Val-NH-Np and Suc-Ala-Ala-Pro-Phe-NH-Np respectively as substrates [14]; inhibitory capacity towards bovine trypsin was measured with Bz-Arg-NH-Np as substrate [15].

The relative activities of commercial HLE, cathepsin G and PPE were determined according to the method of Nakajima *et al.* [14], and bovine trypsin activity was determined by the method of Chase & Shaw [16] with *p*-nitrophenyl *p*-guanidinobenzoate hydrochloride as substrate.

Oxidation/reduction of sheep $\alpha_1\text{PI}$

Sheep $\alpha_1\text{PI}$ was exposed to 12 mM-chloramine-T or buffer (0.2 M-Tris/HCl buffer, pH 8.0) at 25°C for 30 min [17]. The solutions were then rapidly chilled to 4°C and the chloramine-T was removed from the $\alpha_1\text{PI}$ preparation by dialysing the solutions separately overnight against 0.2 M-Tris/HCl buffer, pH 8.0, at 4°C . The incubation medium for the reduction of $\alpha_1\text{PI}$ contained, in a total volume of 20 μl , 0.64 pmol of oxidized or native (buffer-treated) sheep $\alpha_1\text{PI}$ with buffer or 15 mM-dithiothreitol or 15 mM-dithiothreitol plus 40 pmol of MS-PR. The reaction mixture was incubated at 37°C for 1 h, after which its ability to inhibit PPE was assayed as described above.

Protein determination

Total protein was determined by the bicinchoninic acid protein assay [18]. BSA was used as standard.

M_r of sheep $\alpha_1\text{PI}$

The M_r of sheep $\alpha_1\text{PI}$ was determined on a 20% polyacrylamide gel in the presence of SDS and 2-mercaptoethanol by using the Pharmacia Phast electrophoresis system according to the manufacturer's instructions. The following M_r indicators were used: myosin (M_r 205000), phosphorylase *b* (M_r 94000), albumin (M_r 69000), human $\alpha_1\text{PI}$ (M_r 56000), ovalbumin (M_r 43000), carbonic anhydrase (M_r 30000) and soya-bean trypsin inhibitor (M_r 21000).

Rabbit antibody to sheep $\alpha_1\text{PI}$

Antiserum to sheep $\alpha_1\text{PI}$ was raised in New Zealand White rabbits as described by Mayer & Walker [19]. Antiserum with optimum reactivity (as judged by the density of precipitin lines using Ouchterlony immunodiffusion [20]) was used for further analysis of $\alpha_1\text{PI}$.

Immunoprecipitation techniques

Cross-reactivity between human and sheep $\alpha_1\text{PI}$ was determined by double-diffusion immunoprecipitation. In addition, the sheep $\alpha_1\text{PI}$ preparation was tested for albumin contamination with rabbit anti-(sheep albumin) serum. Homogeneity of sheep $\alpha_1\text{PI}$ was further checked by immunoelectrophoresis. Plasma concentration of sheep $\alpha_1\text{PI}$ was determined by rocket immunoelectrophoresis. The methods used were those described by Milford-Ward [20].

Immunoabsorbent column to sheep $\alpha_1\text{PI}$

An immunoabsorbent column to sheep $\alpha_1\text{PI}$ was prepared as described by Sugiura *et al.* [21] using the γ -globulin fraction from the antisera raised to sheep $\alpha_1\text{PI}$. The preparation from this column was typically greater than 98% pure as judged by SDS/PAGE. Contamination by minor proteins (non-specifically bound to the immunoabsorbent column) was removed by gel filtration on Superose 12 in conjunction with the Pharmacia f.p.l.c. system. This homogeneous preparation (as judged by SDS/PAGE) was used for further characterization of sheep $\alpha_1\text{PI}$.

Amino acid/carbohydrate composition of sheep $\alpha_1\text{PI}$

Protein samples were hydrolysed in 6 M-HCl in sealed tubes at 110°C for 24 h, 48 h and 72 h and were analysed on an LKB 4151 amino acid analyser. Cysteine and methionine residues were estimated by performic acid oxidation as described by Hirs [22]. The *N*-terminal sequence of purified sheep $\alpha_1\text{PI}$ was determined on 80–100 pmol of sheep $\alpha_1\text{PI}$ by using an Applied Biosystems 477A protein sequencer. Phenylthiohydantoin derivatives obtained from the sequence were identified by an on-line h.p.l.c. Neutral sugars were determined by g.l.c. (Varian 3300 gas chromatograph fitted with a Chrompack WCOT fused-silica CPsil 25 m $\times$ 0.32 mm column) according to the method of Clamp *et al.* [23]. Hexosamines were determined by using the LKB 4151 amino acid analyser according to the method of Allen & Neuberger [24].

RESULTS

Purification of sheep $\alpha_1\text{PI}$

A summary of the purification procedure of sheep $\alpha_1\text{PI}$ is shown in Table 1. $(\text{NH}_4)_2\text{SO}_4$ precipitation removed most of the contaminating proteins, which probably included the equivalent of human α_2 -macroglobulin (M_r 720000), which also inhibits PPE. The affinity of the PPE inhibitor for concanavalin A (Fig. 1) confirmed that the inhibitor was a glycoprotein. Ion-exchange chromatography resulted in the elution of the PPE-inhibitory protein from the column between 0.15 M- and 0.25 M-NaCl (Fig. 2). Purification of this fraction by preparative PAGE resulted in a homogeneous PPE inhibitor (see below), which was used for subsequent studies. PPE-inhibitory activity was not detected in any other native PAGE protein eluates.

Characterization of sheep $\alpha_1\text{PI}$

Homogeneity of sheep $\alpha_1\text{PI}$. Sheep $\alpha_1\text{PI}$ migrated as a single band that ran parallel to human $\alpha_1\text{PI}$ in the presence of SDS and 2-mercaptoethanol (Fig. 3) and had an M_r of 56000. Homo-

Table 1. Purification of sheep α_1 PI

For experimental details see the text. One unit of inhibitory activity is defined as the inhibition of 1 μ g of PPE under standard assay conditions with Suc-Ala-Ala-Ala-NH-Np as substrate.

Step	Total protein (mg)	Total inhibitory activity (units)	Specific activity (units/mg)	Recovery (%)	Purification (fold)
1. Plasma	3150.0	10269	3.3	100.00	1.0
2. (NH ₄) ₂ SO ₄ (45 %) supernatant	342.0	2257	6.6	22.00	0.8
3. Concanavalin A-Sepharose	20.5	463	22.6	4.51	6.9
4. F.p.l.c. Mono Q	4.6	130	28.2	1.30	8.6
5. Preparative native PAGE	1.1	59	53.6	0.22	16.4

Fig. 1. Column chromatography of (NH₄)₂SO₄-fractionated sheep plasma on concanavalin A-Sepharose

The column (1 cm $\times$ 10 cm) was equilibrated with 0.05 M-sodium acetate buffer, pH 6.0, containing 0.25 M-NaCl and 1 mM-Ca²⁺, -Mg²⁺ and -Mn²⁺. After the sample (10 ml) was applied, the column was washed and elution continued with equilibration buffer containing 0.1 M-methyl α -D-glucopyranoside (fraction 100) without the bivalent metal ions. The flow rate was 12 ml/h, and 1 ml fractions were collected at 4 $^{\circ}$ C. $\bullet$, A_{280} ; $\circ$, PPE-inhibitory activity.

geneity was further checked by immunoelectrophoresis, which produced a single precipitin arc when tested against anti-(whole sheep) serum (Fig. 4a). Fig. 4(b) shows that sheep α_1 PI did not cross-react serologically with human α_1 PI and in addition did not react with rabbit anti-(sheep albumin) serum.

Sheep plasma concentrations of α_1 PI. The concentration of α_1 PI in sheep plasma was 1.69 ± 0.21 g/l (mean $\pm$ S.D., $n = 5$) and compared well with the concentration of α_1 PI in human plasma (1.9–3.9 g/l [25]).

Amino acid/carbohydrate composition of sheep α_1 PI. The chemical composition of sheep α_1 PI is shown in Table 2. The total amino acid content of sheep α_1 PI is in good agreement with that reported for human α_1 PI [8] as well as those reported for α_1 PI in other mammalian species [8–10]. Importantly, six residues of methionine/molecule of α_1 PI were detected and compares well with that observed in other mammalian species.

Amino acid sequencing from the N-terminus to 31 residues (Fig. 5) showed that sheep α_1 PI had 48–52 % identity with

Fig. 2. Ion-exchange chromatography of partially purified sheep α_1 PI on Mono Q

The concentrated PPE inhibitory sample from the concanavalin A-Sepharose column was applied to a Mono Q column (0.5 cm $\times$ 10 cm) equilibrated with 20 mM-Tris/HCl buffer pH 7.5. Starting at fraction 7, the column was eluted with a linear gradient of 0–0.35 M-NaCl in the equilibration buffer. The flow rate was 1 ml/min, and 1 ml fractions were collected at ambient room temperature. $\bullet$, A_{280} ; $\circ$, PPE-inhibitory activity; -----, [NaCl].

Fig. 3. SDS/PAGE at each stage of the purification of sheep α_1 PI

Electrophoresis was carried out on 20 % polyacrylamide ultrathin gels using the Pharmacia Phast electrophoresis system. Sample preparation, running conditions and sensitive silver protein staining was according to the manufacturer's instructions. Lane 1, standards; lane 2, sheep plasma (2 μ g); lane 3, (NH₄)₂SO₄ supernatant (1 μ g); lane 4, concanavalin A fraction (0.6 μ g); lane 5, ion-exchange fraction (0.6 μ g); lane 6, sheep α_1 PI (0.1 μ g); lane 7, human α_1 PI (0.2 μ g); lane 8, sheep albumin (0.1 μ g).

Fig. 4. (a) Immunoelectrophoresis of sheep α_1 PI and plasma and (b) double-diffusion immunoprecipitation of sheep α_1 PI

(a) Electrophoresis was carried out for 1 h at 1.5 mA/cm in 45 mM-barbitone buffer, pH 8.6. The central trough was filled with 100 μ l of rabbit antiserum to sheep serum and immunodiffusion was allowed to continue for 48 h at room temperature in a humid chamber. Antigen-antibody complexes were detected with Coomassie Blue. Well 1, 0.9 μ g of sheep α_1 PI; well 2, 5 μ l of sheep plasma. (b) Sheep and human α_1 PI cross-reactivity was carried out in 1% (w/v) agarose gels. Albumin contamination was tested with antiserum to sheep albumin. Immunodiffusion was allowed to proceed for 48 h in a humid chamber, after which antigen-antibody complexes were detected with Coomassie Blue. Well 1, 2 μ g of sheep α_1 PI; well 2, buffer; well 3, rabbit anti-(human α_1 PI) serum; well 4, rabbit anti-(sheep α_1 PI) serum; well 5, rabbit anti-(sheep albumin) serum; well 6, 5 μ g of sheep albumin; well 7, 10 μ g of human α_1 PI; well 8, rabbit anti-(human α_1 PI); well 9, buffer; well 10, rabbit anti-(sheep α_1 PI).

human α_1 PI. In addition, there was no similarity (< 4% identity) between the inhibitor reported in this paper and that by Sinha *et al.* [6]. Neither was there any sequence similarity between the *N*-terminus of sheep α_1 PI and *N*-termini of human α_1 -antichymotrypsin (cathepsin G inhibitor), human antithrombin-III or ovalbumin.

Sheep α_1 PI had a higher total carbohydrate content (15%) than that reported by Takahara & Sinohara [8] for human α_1 PI (10%) and mouse α_1 PI (9.6%). However, there is good agreement between the carbohydrate content of sheep α_1 PI (15%) and the more detailed study on the oligosaccharide chains of human α_1 PI by Mega *et al.* [26], who reported that the carbohydrate content of human α_1 PI was 16%.

Inhibitory activity of sheep α_1 PI. The molar inhibitory capacity (mol of enzyme inhibited/mol of inhibitor) of sheep α_1 PI against PPE, HLE, bovine trypsin and cathepsin G is shown in Fig. 6. This shows that the inhibition of PPE, HLE and bovine trypsin occurred at a 1:1 molar ratio. However, complete inhibition of cathepsin G did not occur even with a 4-fold molar excess of sheep α_1 PI, and there was 34% residual cathepsin G activity.

Sheep α_1 PI was inactivated by oxidation with chloramine-T (Table 3), unlike the oxidant-insensitive inhibitor isolated by

Table 2. Amino acid/carbohydrate composition of sheep α_1 PI

Residue	Composition (residues/1000)	Composition (residues/molecule)				
		Sheep*	Man	Pig	Rabbit	Mouse
Asp	119.1	48	42	52	35	41
Thr	63.7	25†	28	38	34	28
Ser	57.2	23†	19	42	28	28
Glu	107.6	43	48	54	79	50
Pro	38.9	15	20	25	27	18
Gly	57.9	23	22	47	20	19
Ala	82.7	33	23	30	39	28
CyS	4.2	2‡	2	—	3	2
Val	62.7	25	23	11	24	25
Met	15.3	6‡	8	8	7	8
Ile	45.1	18	17	20	10	18
Leu	129.4	52	43	48	50	46
Tyr	23.3	9	6	10	6	5
Phe	58.4	23	27	12	24	24
His	42.9	17	12	8	17	10
Lys	76.7	30	32	40	35	25
Trp	—	N.D.§	3	—	—	—
Arg	17.0	7	5	14	17	8
Mannose	23.3	9	9	—	—	6
Fucose	—	0	—	—	—	0.5
Galactose	13.3	5	5	—	—	5
Glucosamine	31.6	13	9	—	—	9
Galactosamine	—	—	—	—	—	—
Sialic acid	21.1	~8	6	—	—	4

* Based on M_r 56000.

† Extrapolated to zero hydrolysis time.

‡ Determined by performic acid oxidation.

§ Not determined.

Human	Glu-Asp-Pro-GLN-GLY-Asp-ALA-Ala-GLN-Lys-THR-ASP
Sheep	Gly-Val-Leu-GLN-GLY-His-ALA-Val-GLN-Glu-THR-ASP
Human	----THR-SER-HIS-His-Asp-Gln-Asp-His-Pro-Thr-Phe
Sheep	Asp-THR-SER-HIS-Gln-Glu-Ala-Ala-Ala-His-----
Human	Asn-LYS-ILE-Thr-PRO-ASN-LEU-ALA-Glu-PHE
Sheep	----LYS-ILE-Ala-PRO-ASN-LEU-ALA-Asn-PHE

Fig. 5. Comparison of *N*-terminal sequence of sheep α_1 PI with that of human α_1 PI

Upper-case letters indicate identical residues. Sequences have been interrupted (---) where necessary to increase the incidence of residue identity.

Sinha *et al.* [6]. Re-activation of oxidized sheep α_1 PI requires incubation with both MS-PR and dithiothreitol, as does human α_1 PI [17]. These results indicate that there is a methionine residue at the active site of sheep α_1 PI and that its oxidation inactivates the inhibitor.

DISCUSSION

We have isolated an inhibitor of elastase from sheep plasma, the characteristics of which demonstrate that it is the analogous molecule to human α_1 PI. In the following regards it is closely similar to the human protein: (1) it has an M_r of 56000; (2) it

Fig. 6. Inhibitory capacity of sheep α_1 PI towards HLE, PPE, cathepsin G and trypsin

PPE (●, 4 nmol), HLE (□, 4.25 nmol), trypsin (▲, 10 nmol) and cathepsin G (○, 5.38 nmol) were separately incubated with increasing amounts of purified sheep α_1 PI for 15 min at 25 °C, before addition of the appropriate substrate (see the Materials and methods section) and determination of residual proteolytic activity.

Table 3. Re-activation of oxidized sheep α_1 PI by MS-PR

In each case 0.64 pmol of sheep α_1 PI was incubated with 1.0 pmol of PPE.

Treatment	Amount of PPE inhibited (pmol)	
	Native α_1 PI	Oxidized α_1 PI
Buffer	0.24	0.00
Dithiothreitol	0.31	0.00
Dithiothreitol + MS-PR	0.41	0.08

inhibits PPE, HLE and trypsin on a 1:1 molar basis; (3) it has similar chemical properties (total amino acid/carbohydrate composition; (4) the plasma concentration [1.69 ± 0.21 g/l (mean $\pm$ s.d.)] compares well with that found in man (1.9–3.5 g/l [25]). Despite these similarities, the two molecules were found to be immunologically distinct, as is the case of α_1 PI isolated from the plasma of other mammalian species [9,11].

Our results are in direct contrast with the results reported by Sinha *et al.* [6], who claim to have isolated sheep α_1 PI; however, their protein has a higher M_r (62000) than human α_1 PI (52000–58000), inhibits elastase (PPE and HLE) poorly (α_1 PI/elastase molar ratio of 1:0.13 and 1:0.07 respectively) and is not susceptible to inactivation by chemical oxidation, indicating the absence of a methionine residue at the active site. Furthermore, *N*-terminal amino acid sequencing revealed that their inhibitor showed poor identity (22–35%) with the human protein compared with 48–52% for our protein. In addition, there was no sequence similarity (<4% identity) between the two inhibitors isolated from sheep, indicating that they are different proteins.

The difference between our results and those reported by Sinha *et al.* [6] (as described above) is most probably due to the difference between the proteinases used by Sinha *et al.* and ourselves to screen for α_1 PI. Since we are primarily interested in the role of neutrophil elastase and its inhibition by α_1 PI, in inflammatory lung diseases [27], we used PPE to screen for α_1 PI during our purification procedure because PPE is inhibited only by α_1 PI and α_2 -macroglobulin (removed early because of high M_r). In contrast, Sinha *et al.* [6] used bovine trypsin, which is susceptible to inhibition by other plasma proteinase inhibitors

such as α_2 -antiplasmin and antithrombin III, and α_1 PI is not the most likely inhibitor [28]. Furthermore, mammalian sera, including human [29], goat [11], horse [30], rabbit [10], rat [7] and mouse [8], have been reported to contain more than one proteinase inhibitor in the α_1 -globulin fraction, which can be separated by PAGE. Of particular relevance to this work are the studies where two of these inhibitors have been partially characterized [7,8,29,31] and which differ in their M_r and their inhibitory capacities towards elastase and bovine trypsin. In each case the proteinase inhibitor with the lower M_r (53000–58000) behaved like human α_1 PI and inhibited both elastase (leucocyte or pancreatic) and bovine trypsin at a molar ratio of 1:1. However, the proteinase inhibitor with a higher M_r (55000–65000) was required in a molar excess to inhibit 1 mol of elastase, but inhibited trypsin on a 1:1 molar basis. Since, in addition to using PPE in the purification protocol reported in this paper, we included PAGE as the final step to ensure that the inhibitor isolated has similar physicochemical properties to those of human α_1 PI, it is likely that the inhibitor isolated by us corresponds to the lower- M_r protein corresponding to α_1 PI and that the inhibitor isolated by Sinha *et al.* [6] corresponds to the higher- M_r trypsin inhibitor.

We did not observe an inhibitor similar to that isolated by Sinha *et al.* [6]. This may be because: (1) the $(\text{NH}_4)_2\text{SO}_4$ precipitation step ensured that only proteins that were present in a relatively large proportion in plasma remained in the supernatant (used for the subsequent purification step); (2) the void volume which contained poor PPE-inhibitory activity during the concanavalin A–Sephacryl chromatography procedure, was not used for the subsequent purification step. These two procedures probably removed any poor elastase inhibitor that may have been present.

In summary, our results show that sheep plasma contains a potent inhibitor of neutrophil elastase that is analogous to human α_1 PI in terms of M_r , chemical composition, activity towards HLE, PPE and trypsin, mechanism of inactivation (oxidation of methionine) and plasma concentration. This report on the isolation and partial characterization of sheep α_1 PI indicates that sheep α_1 PI may be expected to function in a similar way to human α_1 PI during an inflammatory response and thus sheep may be a good model in which to examine proteinase–antiproteinase mechanisms in lung injury.

This work was supported by a grant from the Northwest Thames Regional Health Authority (U.K.). We thank Dr. A. Allen and Mr. I. Moss, respectively of the Department of Biochemistry and the Department of Clinical Research, Charing Cross and Westminster Medical School, for assistance with the amino acid/carbohydrate analysis.

REFERENCES

- Heimburger, N. (1975) in *Proteases and Biological Control* (Reich, E., Rifkin, D. B. & Shaw, E., eds.), pp. 367–404, Cold Spring Harbor Laboratory, Cold Spring Harbor
- Wewers, M. D., Herzyk, D. J. & Gedek, J. E. (1988) *J. Clin. Invest.* **82**, 1260–1267
- Janoff, A. (1983) *Am. Rev. Respir. Dis.* **127**, S31–S38
- Staub, N. C., Flick, M., Perel, A., Landolt, C. & Vaughan, T. R. (1981) in *Tissue Fluid Pressure and Composition* (Hargens, A. R., ed.), pp. 113–123, Williams and Wilkins, Baltimore
- Smith, S. M., Traber, L. D., Traber, D. L. & Spragg, R. G. (1989) *J. Clin. Invest.* **49**, 1145–1154
- Sinha, U., Sinha, S. & Janoff, A. (1988) *Am. Rev. Respir. Dis.* **137**, 558–563
- Kuen, L., Rutschmann, M., Dahmann, B. & Reinauer, H. (1984) *Biochem. J.* **218**, 953–959
- Takahara, H. & Sinohara, H. (1982) *J. Biol. Chem.* **257**, 2438–2446

9. Abrams, W. R., Kimbel, P. & Weinbaum, G. (1978) *Biochemistry* **17**, 3556-3561
10. Dubin, A., Koj, A., Klein, A. & Kurdowska, A. (1985) *Folia Biol. (Krakow)* **33**, 65-72
11. Hercz, A. (1982) *Can. J. Biochem.* **60**, 28-35
12. Hames, B. D. (1984) in *Gel Electrophoresis of Proteins: A Practical Approach* (Hames, B. D. & Rickwood, D., eds.), pp. 23-63, IRL Press, Oxford and Washington
13. Bieth, J., Speiss, B. & Wermuth, C. G. (1974) *Biochem. Med.* **11**, 350-357
14. Nakijima, K., Powers, J. C., Ashe, B. M. & Zimmerman, M. (1979) *J. Biol. Chem.* **254**, 4027-4032
15. Erlanger, B. F., Kokowski, N. & Cohen, W. (1961) *Arch. Biochem. Biophys.* **95**, 271-278
16. Chase, T. & Shaw, E. (1970) *Methods Enzymol.* **19**, 21-27
17. Carp, H., Janoff, A., Abrams, W., Weinbaum, G., Drew, R. T., Weissbach, H. & Brot, N. (1983) *Am. Rev. Respir. Dis.* **127**, 301-305
18. Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. K., Olsen, B. J. & Klenk, D. C. (1985) *Anal. Biochem.* **150**, 76-85
19. Mayer, R. J. & Walker, J. H. (1980) in *Immunochemical Methods in the Biological Sciences: Enzymes and Proteins* (Treherne, J. E. & Rubery, P. H., eds.), pp. 5-33, Academic Press, London
20. Milford-Ward, A. (1981) in *Techniques in Clinical Immunology* (Thompson, R. A., ed.), pp. 1-17, Blackwell Scientific Publications, Oxford
21. Sugiura, M., Hayakawa, S., Adachi, T., Yoshimasa, I., Hirano, K. & Sawaki, S. (1981) *Biochem. Biophys. Methods* **5**, 243-249
22. Hirs, C. H. W. (1956) *J. Biol. Chem.* **219**, 611-621
23. Clamp, A. K., Bhatti, T. & Chambers, R. E. (1972) in *Glycoproteins: Their Composition, Structure and Function* (Gottschalk, A., ed.), pp. 300-321, Elsevier, London
24. Allen, A. K. & Neuberger, A. (1975) *FEBS Lett* **60**, 76-80
25. Tetley, T. D., Smith, S. F., Winning, A. J., Foxall, J. M., Cooke, N. T., Burton, G. H., Harris, E. & Guz, A. (1989) *Eur. Respir. J.* **2**, 802-810
26. Mega, T., Lujan, E. & Yoshida, A. (1980) *J. Biol. Chem.* **255**, 4053-4061
27. Mistry, R., Pai, V., Briggs, B., Guz, A., Snashall, P. D. & Tetley, T. D. (1989) *Am. Rev. Respir. Dis.* **139**, A46
28. Travis, J. & Salvesen, G. S. (1983) *Annu. Rev. Biochem.* **52**, 655-709
29. Yoshida, A. & Mega, T. (1979) *Arch. Biochem. Biophys.* **195**, 591-595
30. Dubin, A., Potempa, J., Kurdowska, A., Pajdak, W. & Koj, A. (1986) *Comp. Biochem. Physiol. B* **83**, 375-380
31. Koj, A. & Regoeczi, E. (1981) *Int. J. Pept. Protein Res.* **17**, 519-526

Received 2 July 1990; accepted 16 August 1990