Isolation and Characterization of Acidic Structural Glycoproteins in Pulmonary Tissues

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1. Extraction of the pleural and parenchymal regions of bovine lungs with salt and organic solvents gave powders that contained glycoproteins in addition to collagen and elastin. The contents of these glycoproteins (g/100g of powder) was 4% in pleura and 15.5% in parenchyma. 2. Attempts were made to purify these glycoproteins from both tissues by various methods. 3. Both tissues contained heterogeneous mixtures that were similar to each other, and each tissue contained glycoproteins which, although differing in solubilities, had similar amino acid and carbohydrate compositions. 4. The compositions of the pulmonary glycoproteins resembled those isolated from other connective tissues by other workers.

A variety of connective tissues contain, in addition to the well-characterized fibrillar proteins collagen and elastin, other fibrous proteins. These proteins have been called non-collagenous (Bowes *et al.*, 1958) structural glycoproteins (Robert *et al.*, 1970), and acidic structural proteins (Timpl *et al.*, 1969). To avoid confusion these proteins will be referred to in the present study as acidic structural glycoproteins. They are insoluble under physiological conditions and are closely associated with collagen and elastic fibres. The protein impurities found in preparations of collagen (Steven *et al.*, 1968) and elastin (Gotte *et al.*, 1963) have been attributed to acidic structural glycoproteins.

The physiological function of these glycoproteins in connective tissue is unknown. Jackson & Bentley (1968) suggested that their function is in stabilizing the structure of collagen fibrils that are above a certain diameter. Ross & Bornstein (1969) isolated from elastic fibres proteins which were similar in amino acid compositions to acidic structural glycoproteins and which form microfibrillar structures around elastin in mature elastic fibres. During embryonic development these microfibrils form an aggregated structure before the amorphous elastin is secreted, indicating a primary role in the morphogenesis of the elastic fibre. The removal of acidic structural glycoproteins from elastin becomes more difficult with advancing age and the changes seen in the amino acid compositions and carbohydrate contents of 'elastins' isolated from the tissues of elderly subjects are due to contamination with these proteins (Lansing, 1954; John & Thomas, 1972).

Previous studies from this laboratory on the fibrous proteins in pulmonary tissues were concerned largely with elastin and collagen (John & Thomas, 1971; Francis & Thomas, 1975), but they indicated

also that pulmonary tissues contain substantial amounts of proteins similar in composition to acidic structural glycoproteins isolated from other connective tissues. In the present work proteins have been isolated from the visceral pleura and parenchyma of bovine lung by methods based on those described by Timpl *et al.* (1969) and Robert *et al.* (1970). The extracted proteins have been chemically characterized in terms of amino acid and carbohydrate compositions.

Materials and Methods

Materials

All reagents were obtained from BDH Chemicals Ltd., Poole, Dorset, U.K. (analytical grade where possible) unless otherwise stated. Trimethylchlorosilane and *N*-acetylgalactosamine were obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K. Hexamethyldisilazane, *N*-acetylneuraminic acid, collagenase (from *Clostridium histolyticum*, type III, fraction 'A') and trypsin (from bovine pancreas, type III: 2× crystallized) were from Sigma Chemical Co., St. Louis, Mo., U.S.A. Chromosorb (3% OV-1, W-AW-DMCS, 100–120) was from Hewlett-Packard Ltd., Slough, Bucks., U.K.

Isolation of acidic structural glycoproteins from bovine pulmonary tissues

Preparation of bovine pleura and parenchyma. Twelve lungs from adult cattle were obtained from the local abattoir immediately after slaughter. The visceral pleuras were stripped from the lungs and adhering tissue was removed. They were cut into small pieces, washed with water and homogenized in cold 1 M-NaCl (1:500, w/v) in a VirTis '45' homo-

genizer operated at top speed until a fine creamy suspension was obtained. The suspension was stirred overnight at 4°C and the insoluble material recovered by centrifugation at 15000g for 10min. Extraction with NaCl was repeated three times and the insoluble material was washed with water until salt-free. It was then defatted by treatment with 20vol. of cold chloroform-methanol (2:1, v/v) for 24h. After separation by filtration through sintered glass under suction, the preparation was washed successively with 250ml amounts of ethanol, acetone and ether, and dried in a desiccator to give a fine powder.

Parenchymal tissue was obtained from the main body of the lung, care being taken to dissect out blood vessels, airways and adhering pleura. The tissue was extracted as above except that the extraction with 1M-NaCl was repeated six times.

Extraction of acidic structural glycoproteins with urea. Dried pleura and parenchyma (20g amounts) were stirred separately in 500ml of 8M-urea at 4°C for 24h. The suspensions were centrifuged at 15000g for 10min, the residues re-extracted with 500ml of 8M-urea, washed with water, and dried with organic solvents, ethanol and acetone. Each supernatant and washings were combined, filtered through a glass sinter, concentrated to a small volume on a rotary evaporator at 40°C, dialysed exhaustively against cold water, freeze-dried and weighed.

Extraction of acidic structural glycoproteins with urea-NaBH₄, urea-2-mercaptoethanol and NaOH. The glycoproteins were extracted from the dried urea-insoluble residues of each tissue (5g amounts) by stirring separately in 250ml solutions of 8M-urea-0.1M-NaBH₄, pH10.0, 8M-urea-0.1M-2-mercaptoethanol, pH7.0, and 0.1M-NaOH at 4°C for 24h. The suspensions were centrifuged and the resulting supernatants filtered through a glass sinter under suction, dialysed against water and concentrated to a low volume on a rotary evaporator at 40°C. The solubilized proteins were then purified by a series of isoelectric precipitation steps. The pH of each extract was adjusted to 4.7 with 1M-HCl and the precipitates were recovered by centrifugation at 15000g for 10min. The great bulk of each precipitate was solubilized by suspension in 50ml of water, the pH being adjusted to 10.0 with 1M-NaOH. After centrifugation at 15000g for 10min the insoluble material was discarded and the supernatants were retained. The glycoproteins in the supernatants were purified by repeating the precipitation at pH4.7 two further times. The final resolubilized materials were freeze-dried and weighed.

Removal of collagen from urea-insoluble tissues before extraction of acidic structural glycoproteins. Because of the possibility of solubilization of part of the collagen during the extraction of glyco-

proteins (hence leading to contamination of the latter) it was thought necessary to remove collagen first. Two methods were used. In the first the collagen was solubilized with hot trichloroacetic acid by the procedure used by Robert *et al.* (1970). Urea-insoluble pleura (15g) was suspended in 5% (w/w) trichloroacetic acid (750ml) at 90°C for 30min. The insoluble material was removed by centrifugation at 15000g for 10min, washed with water and sequentially extracted with 8M-urea and 0.1M-NaOH as described above. In the second method each tissue (15g) was stirred separately in 5M-guanidine hydrochloride (750ml) at room temperature for 24h. The insoluble residue was recovered by centrifugation and washed several times with water. The residue was suspended in water (750ml) containing 1mM-CaCl₂ and the pH was adjusted to 7.4 with alkali. Collagenase (10mg) was added to the mixture, which was incubated at 37°C in the presence of toluene. After 48h further collagenase (10mg) was added and the incubation continued for a further 48h. The insoluble material was recovered by centrifugation, washed with water and dried with organic solvents. A portion of the dried residue was sequentially extracted with 8M-urea and 0.1M-NaOH as described above. Another portion (5g) was digested with trypsin by suspending it in water (200ml), and the pH was adjusted to 8.0. Crystalline trypsin (4mg) was added and the mixture was shaken gently at 40°C for 24h in the presence of toluene. The digest was centrifuged and the residue washed with water. The combined supernatant and washings were filtered through sintered glass under suction, concentrated to a small volume on a rotary evaporator at 40°C and freeze-dried.

Analytical methods

Amino acid analyses. Samples (10mg in 5ml of constant-boiling HCl) were hydrolysed in evacuated sealed glass tubes at 110°C for 24h. Amino acid analyses were performed on a Locarte autoanalyser by the procedure of John & Thomas (1971). Cystine was determined by measuring cysteic acid by the method of Moore (1963).

Determination of hexosamines. Weighed samples (10mg in 2ml of constant-boiling HCl) were hydrolysed in evacuated sealed glass tubes at 110°C for 20h. After removal of acid on a rotary evaporator, the hydrolysate was dissolved in 0.2M-sodium citrate-HCl buffer, pH2.2, and glucosamine and galactosamine were determined on 5mg samples by separation on a column (26cm×0.9cm) of sulphonated polystyrene on a Locarte autoanalyser. The eluting buffer system was 0.2M-sodium citrate-HCl, pH4.25 (for 120min), 0.35M-sodium citrate-HCl, pH5.28 (for 20min) and 1.0M-sodium citrate-HCl, pH6.65 (for 50min). Hexosamine values were corrected for losses during hydrolysis (<5%).

Determination of *N*-acetylneuraminic acid. This was determined by the colorimetric method of Warren (1959). The samples were hydrolysed in 0.05M-H₂SO₄ for 1 h at 80°C in sealed glass tubes. Crystalline *N*-acetylneuraminic acid was used as a standard and corrections were applied for any losses occurring during hydrolysis (<5%).

Analysis of neutral monosaccharides and glucuronic acid. These were determined by g.l.c. by the procedure of Bhatti *et al.* (1970). The methyl glycoside derivatives of the bound monosaccharides in 12 mg samples of the glycoproteins were prepared by hydrolysis in dry methanolic 1.5M-HCl at 90°C for 24 h under N₂ in sealed glass tubes. The HCl was neutralized with Ag₂CO₃ and the hexosamines were re-acetylated by adding acetic anhydride and leaving overnight. The precipitated AgCl was removed by centrifugation and the supernatant evaporated to dryness on a rotary evaporator at 35°C and dried thoroughly over P₂O₅ in a desiccator. The trimethylsilylation of the dried samples was carried out and samples were injected into a Perkin-Elmer F 11 gas chromatograph. The glass column used in the gas chromatograph was 300 cm in length with an internal diameter of 0.32 cm and was packed with Chromosorb W-AW-DMCS, mesh 100-120, coated with 3% OV-1. The carrier gas was O₂-free N₂ at a flow rate of 50 ml/min. The gas chromatograph was temperature-programmed from 130-200°C at 1°C/min.

Results

Solubilization of acidic structural glycoproteins

The insoluble residues remaining after repeated extractions of visceral pleura and parenchyma from adult bovine lungs with 1M-NaCl and organic solvents contained collagen, elastin and acidic structural glycoproteins. Quantitative measurements (Francis & Thomas, 1975) showed that the residues from pleura contained 96% (w/w) collagen and elastin, whereas that from parenchyma contained 85.5%. The great insolubility of elastin and the great bulk of mature collagen presented technical problems for the purification of the glycoproteins from pulmonary tissues. The approach used was to solubilize selectively the glycoproteins by methods that did not cause degradation of collagen and elastin.

The extraction procedures and the yields of acidic structural glycoproteins expressed as % dried weight of tissues are summarized in Scheme 1. Extraction of defatted and salt-extracted tissues with cold concentrated urea solutions for 24 h dissolved 0.54% of pleura and 1.27% of parenchyma. The insoluble residues remaining from both tissues were further extracted with cold concentrated urea solutions containing the reducing agent 0.1M-NaBH₄ at the natural pH of 10.0 for 24 h. This solvent dissolved 3.34% of pleural residue and 14.65% of parenchymal residue and accounted for almost 100% of the

Scheme 1. Isolation of acidic structural glycoproteins from bovine pleura and parenchyma

The tissues were prepared by treatment with 1M-NaCl and organic solvents. After extraction with 8M-urea, the residues were extracted separately with 8M-urea-0.1M-NaBH₄, pH 10, 8M-urea-0.1M-2-mercaptoethanol, 0.1M-NaOH and enzymes (collagenase and trypsin). The amounts of acidic structural glycoproteins extracted are expressed as g/100 g of dried tissue. Results for parenchyma are shown in parentheses.

glycoproteins remaining after urea extraction. Extraction of the urea-insoluble pleural residue with cold concentrated urea solution containing mercaptoethanol at neutral pH dissolved smaller amounts of pleura (1.62%). When such residues were extracted with cold 0.1M-NaOH for 24h, 1.13% of pleura and 7.74% of parenchyma were dissolved. Attempts to extract further amounts of glycoproteins by prolonging the incubation time with alkali proved unsatisfactory because of chemical degradation of collagen.

The proteins extracted by the above procedures were insoluble at pH4.7 but the precipitates obtained redissolved at pH10.0. This isoelectric precipitation was used to purify all the glycoproteins extracted as described above.

Another procedure used to separate the glycoproteins from collagen and elastin was based on enzymic digestion. Collagen was first removed by treating the urea-insoluble residues with 5M-guanidine hydrochloride and incubating the insoluble portion with collagenase. The glycoproteins were then preferentially solubilized in partially degraded form from the collagen-free residue (mainly elastin) by digestion with trypsin. This enzymic procedure dissolved 1.77% of the urea-insoluble residue of the pleura and 5.9% of that of parenchyma.

Chemical compositions of acidic structural glycoproteins

The protein fractions solubilized from pleura and parenchyma by the procedures shown in Scheme 1 were analysed for their contents of amino acids and monosaccharides. Table 1 shows that the proteins dissolved from pleura by solutions of urea, NaBH₄ in urea at pH10, mercaptoethanol in urea at neutral pH, alkali and enzymes had overall amino acid compositions which were similar. These proteins also resembled similar fractions extracted from parenchyma. There were some differences in the fractions, especially in the glycine content. Those samples in which the glycine content was high also contained small amounts of hydroxyproline and hydroxylysine, indicating some contamination with collagen. The amino acid compositions of the protein fractions were very different from those of collagen and elastin, the main feature being the high contents of aspartic acid and glutamic acid. All samples contained cystine, the contents in the enzymically solubilized samples in both pleura and parenchyma being higher than in the other preparations.

Table 2 shows that all the above protein fractions solubilized from pleura and parenchyma by various procedures contained carbohydrate. The fractions

Table 1. *Amino acid compositions of acidic structural glycoproteins isolated from bovine pleura and parenchyma*

Salt-extracted defatted tissues were extracted with 8M-urea and solubilized materials analysed. Insoluble materials were extracted separately with 8M-urea-0.1M-NaBH₄, 8M-urea-0.1M-2-mercaptoethanol, 0.1M-NaOH and enzymes (collagenase and trypsin) and solubilized materials analysed. Results are expressed as residues/1000 amino acid residues.

Treatment	Pleura					Parenchyma			
	Urea	Urea-NaBH ₄	Urea-2-mercaptoethanol	NaOH	Trypsin	Urea	Urea-NaBH ₄	NaOH	Trypsin
4Hyp	14.0	0	14.0	0	Trace	0	Trace	0	Trace
Asp	105.5	102.0	103.5	119.0	95.0	103.5	105.5	105.0	95.0
Thr	39.1	43.0	42.0	48.5	45.2	50.5	50.2	55.7	48.5
Ser	59.0	61.5	57.2	55.5	64.5	65.0	61.0	69.0	61.0
Glu	111.0	123.5	110.5	117.0	110.5	159.0	137.0	130.0	115.5
Pro	62.9	53.5	90.0	57.8	62.0	47.2	54.2	63.6	65.2
Gly	139.0	91.8	156.0	85.5	146.0	69.2	113.0	73.5	119.0
Ala	81.0	68.3	74.2	65.5	76.0	80.5	74.5	73.4	70.9
Cys/2	24.3	15.5	54.4	11.5	37.4	12.8	14.2	13.1	38.6
Val	59.4	63.5	48.3	63.5	53.2	52.5	64.0	67.2	56.6
Met	12.9	14.6	12.3	5.4	12.8	16.8	15.9	17.1	14.4
Ile	35.6	45.3	34.4	49.6	38.4	42.0	44.5	42.8	33.7
Leu	80.2	97.0	59.4	101.0	66.5	100.8	94.9	77.4	74.5
Tyr	25.4	37.0	21.2	30.4	25.4	28.5	17.4	27.7	21.3
Phe	41.8	61.5	31.0	52.0	37.8	37.2	29.8	35.0	26.2
5Hyl	Trace	0	Trace	Trace	Trace	0	Trace	0	Trace
His	15.5	14.8	12.1	20.4	15.2	14.3	19.8	23.7	20.5
Lys	42.8	48.6	35.8	55.9	49.5	50.0	47.4	58.9	71.0
Arg	50.7	59.1	43.6	62.5	65.0	69.4	56.4	67.2	68.5
Total	1000.1	1000.5	1000.0	1001.0	1000.3	999.2	999.7	1000.3	1000.4

Table 2. *Carbohydrate compositions of acidic structural glycoproteins isolated from bovine pleura and parenchyma*

The salt-extracted defatted tissues were treated with 8M-urea and the insoluble residues treated with 8M-urea-0.1M-NaBH₄, 0.1M-NaOH and enzymes (collagenase and trypsin). The materials solubilized were analysed and the results expressed as g/100g. The difference between the carbohydrate content and 100% is equal to the amount of protein. The results for parenchyma are shown in parentheses.

Treatment	Urea	Urea-NaBH ₄	NaOH	Trypsin
Monoraccharide				
Fucose	0.20 (0.12)	0.22 (0.09)	0.19 (0.11)	0.28 (0.13)
Mannose	1.01 (0.61)	1.27 (0.59)	1.25 (0.62)	0.98 (0.62)
Galactose	1.20 (0.86)	1.51 (1.36)	1.50 (0.82)	1.27 (1.16)
Glucose	0.81 (0.63)	0.83 (1.04)	0.90 (0.28)	0.59 (0.46)
N-Acetylglucosamine	1.21 (0.96)	1.41 (0.55)	1.49 (1.02)	1.01 (0.91)
N-Acetylgalactosamine	Trace	1.37 (Trace)	3.20 (0.82)	Trace
N-Acetylneuraminic acid	0.69 (0.62)	0.68 (0.48)	0.58 (0.59)	0.73 (0.45)
Total	5.12 (3.80)	7.29 (4.11)	9.11 (4.26)	4.86 (3.73)

Table 3. *Amino acid compositions of proteins extracted from bovine pleura with 8M-urea and 0.1M-NaOH after pre-treatment with 5% trichloroacetic acid*

Results are expressed as residues/1000 amino acid residues.

Treatment	Urea	NaOH
4Hyp	4.7	5.0
Asp	58.4	61.4
Thr	30.2	33.5
Ser	36.4	33.9
Glu	70.3	73.1
Pro	98.1	91.5
Gly	196.6	222.5
Ala	146.7	141.9
Cys/2	12.4	15.5
Val	100.4	96.3
Met	8.1	9.7
Ile	35.3	37.7
Leu	72.9	67.0
Tyr	17.1	20.7
Phe	35.8	33.8
5Hyl	0	0
His	9.4	9.8
Lys	35.6	20.1
Arg	32.2	26.1
Total	1000.6	999.5

obtained from parenchyma contained 4% (w/w) of carbohydrate, whereas those from pleura were more variable in content covering a range from 5-9%. All fractions from both tissues contained fucose, mannose, galactose, glucose, N-acetylglucosamine and N-acetylneuraminic acid in relatively similar proportions. However, the content of N-acetylgalactosamine was very variable, being hardly detected in some fractions and very high in the pleural urea-NaBH₄-soluble and NaOH-soluble fractions.

Removal of collagen before extraction of glycoproteins

The presence of hydroxyproline and hydroxylysine in some of the above extracts indicated contamination with collagen. Attempts were therefore made by two methods to solubilize collagen before extraction of glycoproteins. In the first method the salt-extracted and defatted pleura were treated with hot trichloroacetic acid. The insoluble residue was then sequentially extracted with 8M-urea and 0.1M-NaOH. The amino acid compositions of the urea- and alkali-soluble proteins are shown in Table 3. The high content of proline, glycine, alanine and valine, and the presence of trace amounts of demosine and isodesmosine, indicated contamination with elastin. Control experiments carried out on purified preparations of elastin showed that hot trichloroacetic acid caused some dissolution. In the second method, digestion of salt-extracted and defatted pleura and parenchyma with collagenase was used. Most of the collagen was solubilized but about 15% remained insoluble as judged by hydroxyproline measurements. Extraction of the insoluble residues by sequential extraction with solutions of 8M-urea and 0.1M-NaOH dissolved proteins that were similar in amino acid compositions to those obtained without pre-treatment with collagenase. The presence of small amounts of hydroxyproline indicated the incomplete removal of collagen by collagenase. Also, the prolonged incubation with collagenase seemed to cause some proteolysis of the glycoproteins, as shown by the lower yields obtained.

Discussion

It has been demonstrated that the visceral pleura and the parenchyma of bovine lungs contain fibrous proteins that are different from collagen and

elastin. Because of their high contents of aspartic acid and glutamic acid, their carbohydrate contents and their insolubilities in salt solutions these proteins have been designated acidic structural glycoproteins. They make up about 4% of the dry weight of pleura and about 16% of that of parenchyma. About 10% (w/w) of the total present in pleura and parenchyma could be solubilized with concentrated solutions of urea. Further amounts could be dissolved by alkaline solutions or solutions containing reducing agents. To dissolve the maximum amount from the tissues it was necessary to use alkaline solutions containing a reducing agent and concentrated urea. The great insolubility of the main connective-tissue proteins, collagen and elastin is due to the formation of covalent cross-links between the side chain groups of lysine and hydroxylysine residues in the polypeptide chains. However, there is no evidence for the presence of similar cross-links in the acidic structural glycoproteins. Their insolubility could be due partly to a physical trapping within the fibrous network of collagenous and elastic fibres and partly to the presence of disulphide cross-bridges of cystine formed between the polypeptide chains in the glycoproteins.

The extraction conditions used did not dissolve elastin. However, some solubilized glycoprotein fractions were contaminated with collagen as shown by the presence of trace amounts of hydroxyproline and hydroxylysine and the high glycine content. Attempts to remove collagen from the tissues before extraction of glycoproteins were unsuccessful. Treatment with hot trichloroacetic acid caused some elastin to become soluble in urea and dilute alkali. Incubation with collagenase did not remove all the collagen and caused some dissolution of glycoproteins.

The pulmonary proteins studied here have certain

similarities in composition to those proteins isolated by analogous methods from other connective tissues by other workers, e.g. skin (Timpl *et al.*, 1969), ligament (Ross & Bornstein, 1969), aorta (Barnes & Partridge, 1968) and cornea (Robert *et al.*, 1970).

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