IDENTIFICATION OF PROTEOGLYCANS IN PERIMYSIUM OF BOVINE M. SEMIMEMBRANOSUS

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SUMMARY

In the present study proteoglycans were extracted from perimysium of bovine M. semimembranosus by use of 4 M guanidin-HCl in 0.05 M acetate buffer pH 6.
The extract was purified by ultracentrifugation in a CsCl gradient and gelfiltration. Cellulose acetate electrophoresis and chondroitinase ABC and AC treatment showed that the extract contained hyaluronic acid, chondroitin 4-sulfate and dermatan sulfate. The glycosaminoglycans were constituents of at least two different groups of proteoglycans PG-L and Pg-Sm as judged by gelfiltration and ELISA.

INTRODUCTION

Connective tissue is found throughout the body, like in muscle sheaths surrounding the entire muscle (epimysium), the bundles of fibres (perimysium) and the individual fibres (endomysium). The main components of connective tissue are collagen and elastic fibres and the ground substance. The latter consists mainly of glycoconjugates. Several years ago it was suggested that postmortem aging of meat might involve changes in the glycoconjugates of the ground substance (McIntosh, 1967, Dutson and Lawrie, 1974). However, very little information is known about the chemical composition of the ground substance in muscular connective tissue. Carbohydrates in cartilage, bone, sclera, aorta and skin are found to be covalently bound to protein as proteoglycans (PG), and several populations of PGs have been isolated and characterized according to size and chemical composition such as large proteoglycans (PG-L) of mol.w. $> 10^6$ and small proteoglycans (PG-Sm) of mol.w. $< 10^5$ (for review Heinegaard, 1987).
The aim of the present study was to establish a method for extraction and purification of proteoglycans from perimysium of bovine M. semimembranosus to elucidate some aspects of the composition of the ground substance.

MATERIALS AND METHODS

Extraction:

M. semimembranosus of steers was obtained at the abattoir and brought to the laboratory. The perimysium was dissected, homogenized and incubated in a solution of 4 M guanidin-HCl in 0.05 M sodium acetate buffer pH 6 with a final solid liquid ratio of 1:12 (w/w) (Sajdera and Hascall, 1969). 6-aminohexanoic acid (0.1 M), Na_2 EDTA (0.01 M) and phenylmethylsulfonylfluorid (1 mM) were included in the extraction solution as protease inhibitors. The suspension was left for 16 hr. at 4 °C under gentle shaking. The suspension was then centrifuged, the sediment was reincubated in fresh extraction buffer and the treatment was repeated. The supernatants were pooled and clarified by centrifugation at 20.000 g at 4 °C for 25 minutes. The sediment was washed with distilled water, and samples from the supernatant were dialyzed against distilled water. Then the material was freeze-dried and used for chemical analyses.

Ultracentrifugation:

The supernatant was applied to isopycnic ultracentrifugation in a density gradient of CsCl. The CsCl was added directly to the supernatant to get a starting density of 1.5 g/ml. The gradient was achieved by centrifugation at 120.000 g for 65 hr. at 10 °C in a Beckman Ultracentrifuge LS-75. The gradients were harvested from the bottom and divided into four fractions called D1, D2, D3 and D4. D1 represented the bottom fraction, D4 the top fraction. Samples from D1, D2, D3 and D4 were dialyzed, freeze-dried and used for chemical analyses. Aliquot of the D1 fraction was dialyzed against buffer (see under gelfiltration).

Chemical analyses:
The content of protein was estimated according to the method of biuret (Gornall, 1949). The contents of total neutral sugar and uronic acid were measured after the method of Dubois (1956) and Bitter and Muir (1962). The protein content was monitored during gelfiltration by measuring the absorbance at 280 nm.

Gelfiltration:
The D1 fraction was examined by gelfiltration on a Sepharose Cl-4B column (90 x 1.5 cm). The elution buffer consisted of 2 M guanidin-HCl in 0.5 M sodiumacetate pH 7 including the same protease inhibitors as described above. The eluate was collected in 4 ml fractions at a flow rate of 0.4 ml/min. The uronic acid containing fractions were pooled, dialyzed and freeze-dried.

Protease treatment:
Lyophilized samples obtained after ultracentrifugation in CsCl and gelfiltration were solubilized in 0.1 M tris-HCl buffer pH 7.5 including 5 mM $CaCl_2$ at a protein concentration of 4 mg/ml. 100 µl was then added aliquot protease type XIV (Sigma) solubilized in a similar buffer at an enzyme concentration of 10 mg/ml. The samples were incubated at 55 ^{0}C for 18 hr.

Identification of glycosaminoglycans:
Enzyme digestion techniques by use of chondroitinases were performed on the protease treated samples from ultracentrifugation (D1, D2, D3 and D4) to identify glycosaminoglycans as described by Waddington and Embery (1987).

Cellulose acetate electrophoresis:
Cellulose acetate electrophoresis was carried out according to the method of Stanburry and Embery (1977). A standard mixture of glycosaminoglycans containing hyaluronic acid (HA), heparan sulfate (HS), dermatan sulfate (DS), chondroitin 4-sulfate (C4S) and chondroitin 6-sulfate (C6S) (Sigma chemical Co Ltd) with a concentration of 0.05 mg/ml was always run together with the samples on the electrophoresis sheets.

ELISA
ELISA (enzymlinked immunosorbent assays) were performed by use of specific polyclonal antibodies raised in rabbits and directed against epitopes on the protein core of large or small proteoglycans from bovine sclera. The experiments were performed as described by Heinegaard et al (1985). The antibodies were kindly given by Professor Anders Malmstrøm and Dr. Lars Cøster, University of Lund.

RESULTS
The major proportion of the proteglycans in the perimysium was extracted by the use of 4 M guanidin -HCl as shown by the measured content of uronic acid in the supernatant compared to the sediment after extraction (table I).

Table 1.

Uronic acid in the supernatant:

1. steer	2. steer	3. steer
87 %	92 %	80 %

The results of the chemical analyses obtained by centrifugation in CsCl are shown in figure 1. The bottom fraction called D1 contained the highest content of uronic acid and the lowest content of protein.
Cellulose acetate electrophoresis of D1, D2, D3 and D4 after protease treatment confirmed that the major proportion of glycosaminoglycans were present in the D1 fraction (figure 2). The presence of hyaluronic acid, chondroitin 4-sulfate and dermatan sulfate was established by chondroitinase ABC and chondroitinase AC treatment. Chondroitinase ABC digests dermatan sulphate (DS), chondroitin 4-sulfate (C4S), chondroitin 6-sulfate (C6S) and, at a reduced rate, hyaluronic acid (HA). Chondroitinase AC digests the same glycosaminoglycans except dermatan sulfate (figure 3, a and b).
The proteoglycans from the D1 fraction were separated by gelfiltration on a Sepharose Cl-4B column by use of

an acetate buffer containing 2 M guanidin-HCl into several peaks as shown by the uronic acid profile (figure 4). Quantitation of the total carbohydrate content gave similar results (results not shown). The eluate was pooled into 4 major fractions according to the diagram (figure 4). Cellulose acetate electrophoresis of lyophilized and protease treated samples from the four major fractions named I, II, III and IV (see figure 4) showed that peak I and II were rich in hyaluronic acid and chondroitin 4-sulfate whereas peak III and IV showed a high content of dermatan sulfate (figur 5a and b). ELISA experiments showed that peak I and II contained proteoglycans with similar antigenic epitopes on the protein core as large proteoglycans (PGL) from bovine sclera. Peak III and IV contained proteoglycans (PG-Sm) obtained from bovine sclera. Proteoglycans in peak I and II showed a very weak reaction in the assay for small proteoglycans whereas proteoglycans in peak IV did not react in the assay for large proteoglycans.

DISCUSSION

The present study has shown that 4 M guanidin-HCl was an efficient extractant for proteoglycans in perimysium of muscle in the same way as for proteoglycans in tendon, sclera and cartilage (Antonopolous, 1974). Defatting of the dissected perimysium by ethanol and aceton treatment did not increase the yield and was subsequently abandoned. Whether the uronic acid content of the sediment represents similar proteoglycan populations as the supernatant is not established at present.

A variety of glycosaminoglycans including hyaluronic acid, chondroitin 4-sulfate and dermatan sulfate were identified as constituents of the perimysium. The molecular size profile obtained by gelfiltration of D1 revealed a number of fractions of different molecular size and glycosaminoglycan composition. Hyaluronic acid and chondroitin 4-sulfate were found predominately in the large molecular size range whereas dermatan sulfate was the major glycosaminoglycan in the intermediar and low molecular size range. Whether the fractions represent mixed or separate proteoglycan populations cannot be established at present. Further work is needed to clarify that aspect. The ELISA experiments detected the presence of two different proteoglycan populations, large proteoglycans (PG-L) and small proteoglycans (PG-Sm). The antibodies used in the present study were directed against PG-L and PG-Sm from sclera. PG-L from sclera, tendon, cartilage and aorta are known to form large aggregates with hyaluronic acid. The aggregates have an unique water binding capacity and play an important role for the consistency of the tissue. PG-Sm has a different role. Several studies have shown a very close relationship to the collagen fibres (Scott *et al.*, 1981) and a decisive role for fibrillogenesis (Lowther *et al.*, 1970). Perimysium contained proteoglycans which may play an important role for the quality of meat. Any study on postmortem degradation of the ground substance should therefore not be restricted to the hexosamine or glycosaminoglycan contents as in the past but include the total proteoglycan population. Until then no conclusions can be drawn on the process of meat tenderization post mortem.

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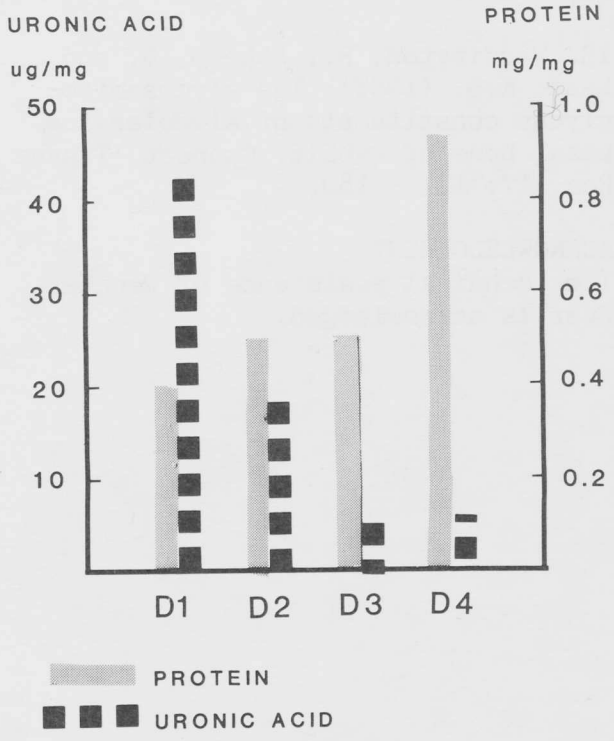

Figure 1.
Distribution of uronic acid and protein in the gradients (D1, D2, D3 and D4) harvested after ultracentrifugation in CsCl.

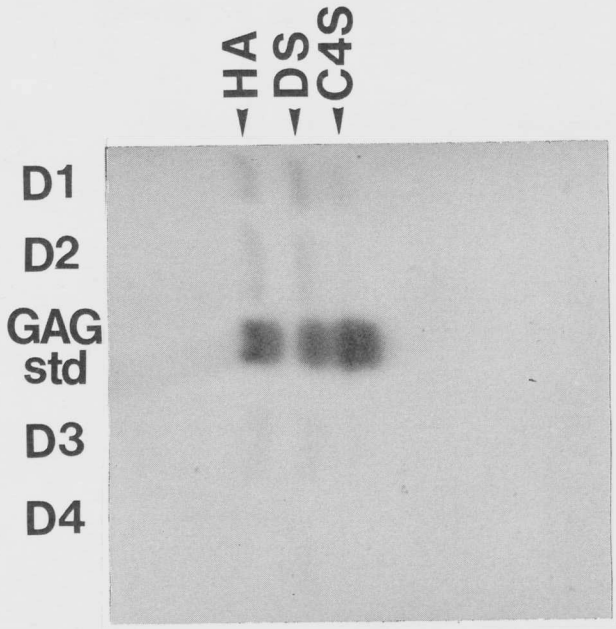

Figure 2.
Electrophoretic patterns obtained by cellulose acetate electrophoresis of protease treated samples from D1, D2, D3 and D4. A standard mixture containing hyaluronic acid (HA), heparan sulfate (HS), dermatan sulfate (DS), chondroitin 4-sulfate C4S and chondroitin 6-sulfate (C6S) were run together with the samples.

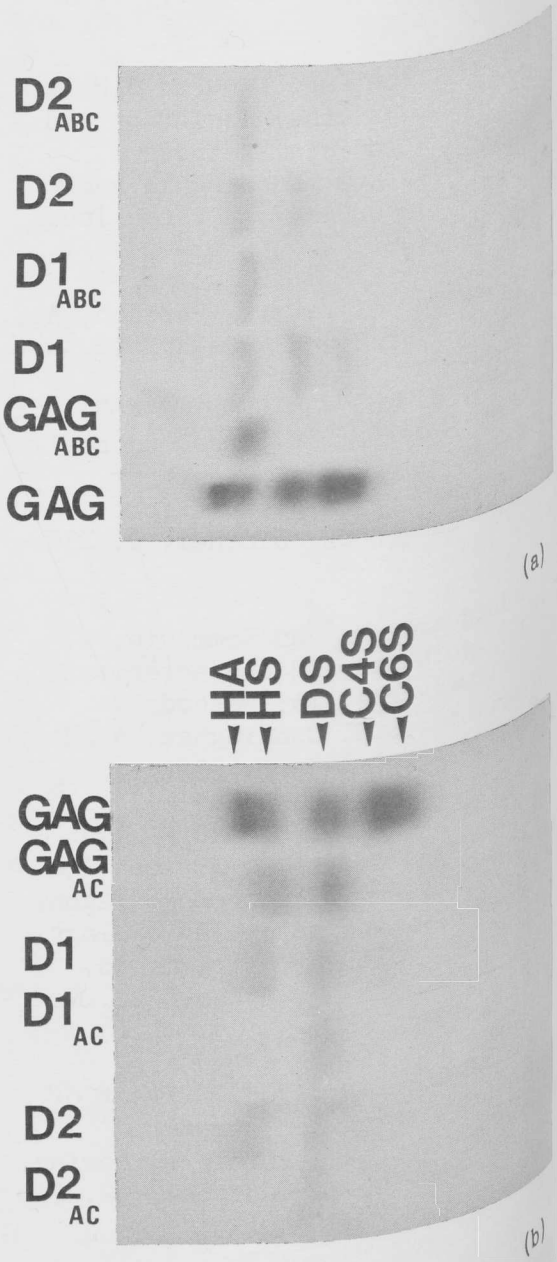

Figure 3.

Electrophoretic patterns obtained after chondroitinase ABC digestion (a) and chondroitinase AC digestion (b) of protease treated samples from D1, D2, D3 and D4, and the std. mixture.

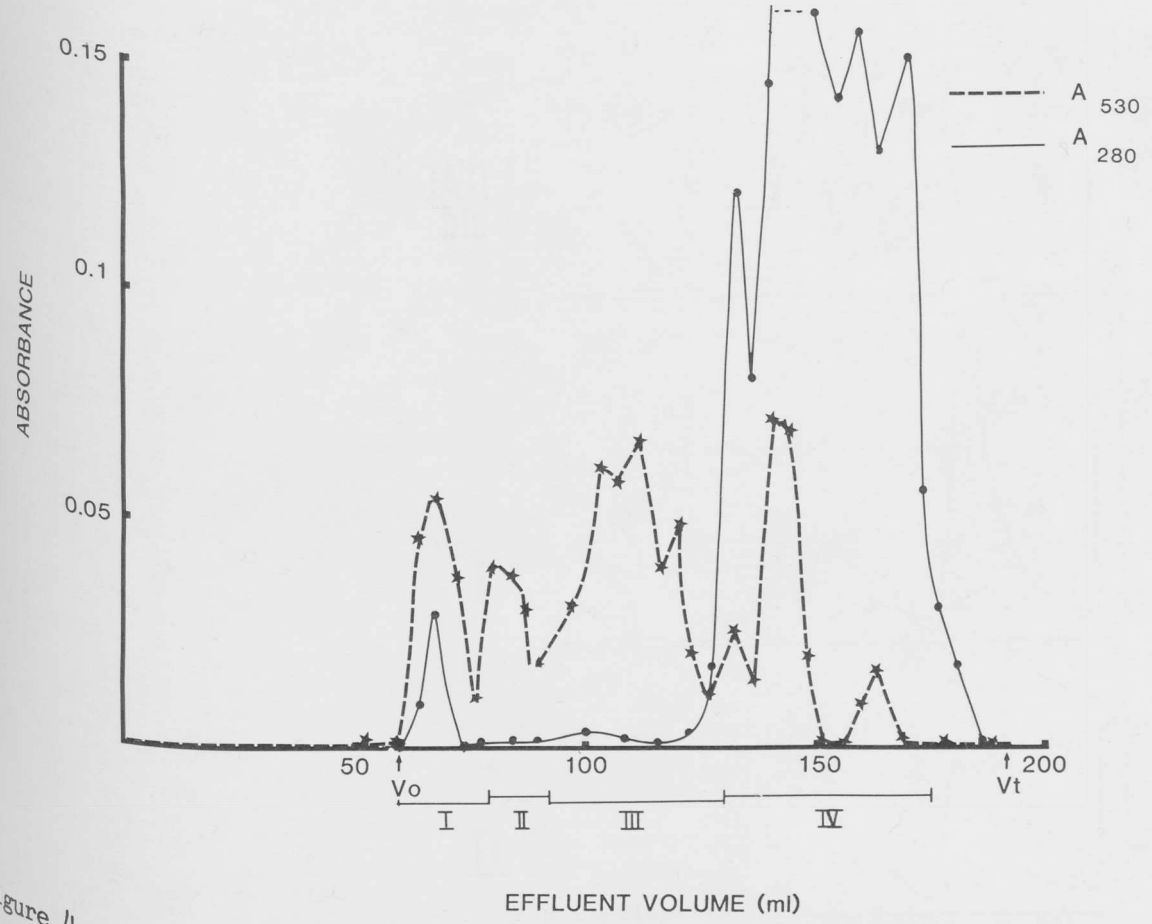

Figure 4.
Elution diagram of D1 run on a Sepharose C1-4B column. The eluate was collected in 4 ml fractions and monitored for protein at 280 nm (solid line) and uronic acid at 530 nm (dotted line). The uronic acid containing samples were pooled into 4 fractions, I, II, III and IV as illustrated.

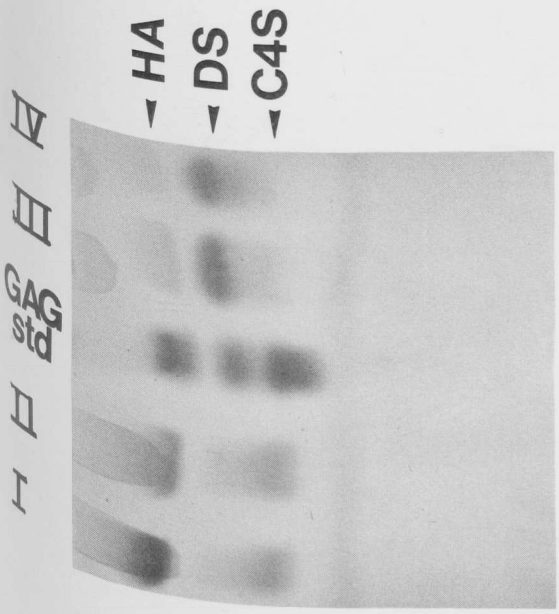

Figure 5a.
Electrophoretic patterns of protease treated samples of I, II, III and IV on cellulose acetate sheets.

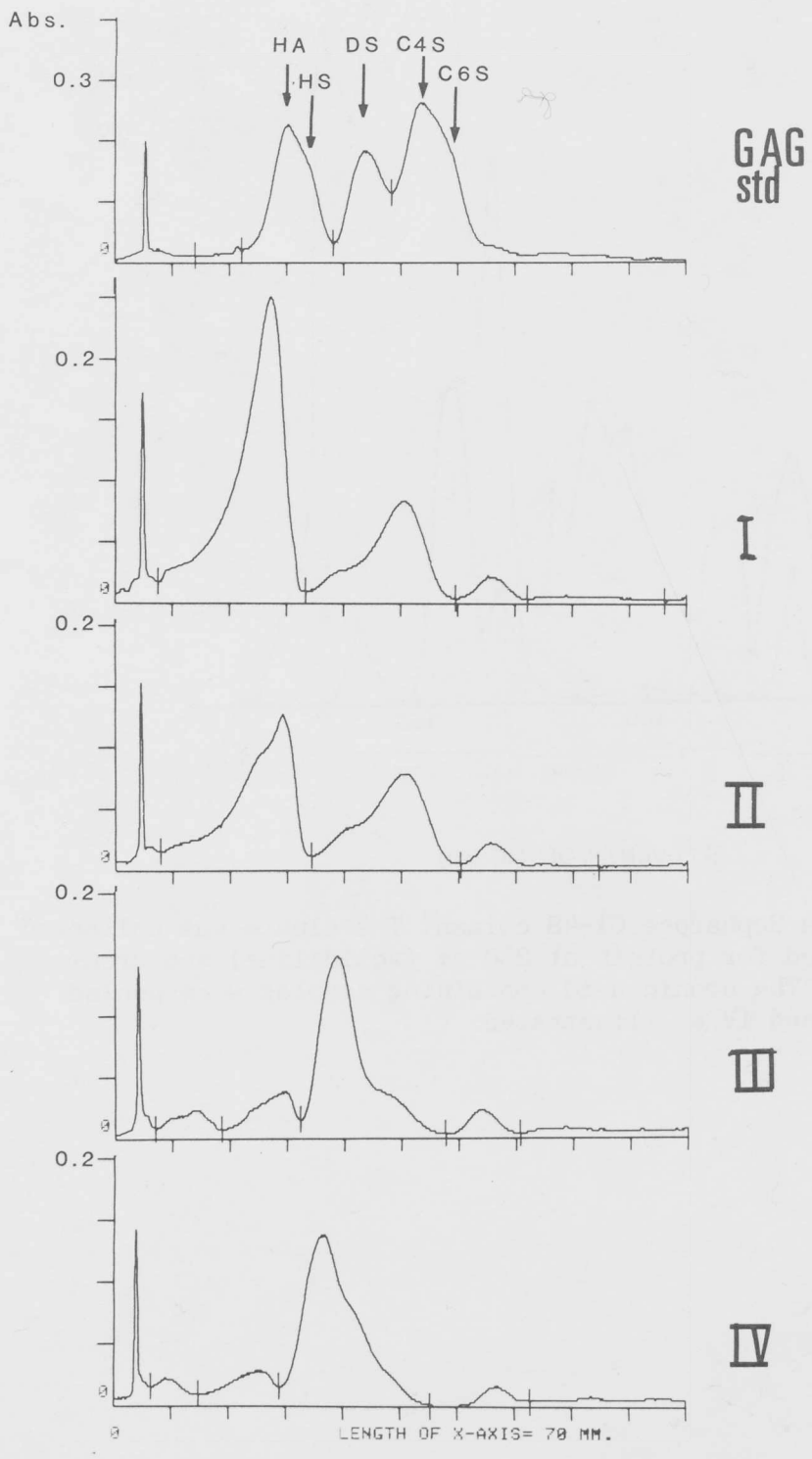

Figure 5b.
Densitometric scan of the cellulose acetate sheets illustrated in 5a.