Study of Sulfated Glycosaminoglycans from Porcine Skeletal Muscle Epimysium Including Analysis of Iduronosyl and Glucuronosyl Residues in Galactosaminoglycan Fractions

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A sulfated glycosaminoglycan fraction was isolated from porcine longissimus dorsi epimysium. It contained galactosaminoglycans with high iduronic acid content (78% of total uronic acid). Galactosaminoglycans were fractionated by precipitation with different concentrations (18, 25, 40, 50, and 75%) of ethanol to obtain five fractions (I−V). Fractions I−III, which accounted for 91% of total uronic acid recovered, were dermatan sulfates with varying proportions of iduronic acid (42−87% of total uronic acid). Fractions IV and V were galactosaminoglycans with high glucuronic acid contents. These results were supported by those from ELISA and immunohistochemical studies. Digestion with chondroitinase B or chondroitinase AC II showed that at least 70% of the epimysial galactosaminoglycans are copolymeric chains containing both iduronosyl and glucuronosyl residues. The composition of the remaining galactosaminoglycans, resistant to both enzymes is less certain.

Keywords: *Glycosaminoglycan; dermatan sulfate; proteoglycan; epimysium; muscle; pig*

INTRODUCTION

Tenderness, probably the most important factor determining the palatability of meat, is influenced by connective tissues (Bayley and Light, 1989). Epimysium, the connective tissue covering skeletal muscle, is largely composed of collagen fibers embedded in an amorphous ground substance containing proteoglycans and glycoproteins. Collagen, which accounts for more than 70% of dry weight of adult bovine and porcine epimysial tissues (Nakano and Thompson, 1980; Nakano *et al*., 1985), is the major protein providing the epimysium with tensile strength and stiffness. Epimysium contains primarily collagen fibers of genetic type I (Bailey and Light, 1989). Epimysial collagen has been studied in relation to animal age and meat toughness (Field *et al*., 1969; Nakano and Thompson, 1980; Nakano *et al*., 1985). There is relatively limited information available concerning noncollagenous constituents including proteoglycans in the epimysium. Proteoglycans are reported to have various physiological functions such as water retention, electrolyte control, and collagen fibrillogenesis (Kjellén and Lindahl, 1991; Vogel, 1994).

For many years, the extracellular matrix of connective tissues in muscle has been thought of as a static scaffold with limited ability to influence tissue structure, development, or gene expression (Velleman and Racela, 1994). Thus, much less attention has been given to connective tissue proteins (in particular proteoglycans and glycoproteins) than to myofibrillar proteins in the research of meat tenderness. Velleman and Racela (1994) recently reviewed and reinterpreted the role of proteoglycans in meat tenderness. They strongly suggested that connective tissue toughness in meat is not dependent on collagen alone but on the interaction among proteins including particularly collagen and proteoglycan. How the interaction influences the physical properties of meat remains to be investigated. It appears that proteoglycans are important molecules contributing to the texture of meat. However, we must understand the structure of proteoglycans before examining their potential roles in the meat texture. A proteoglycan molecule consists of a core protein to which one or more glycosaminoglycan (GAG) chains are covalently attached. A study of GAG structure is an important area to be investigated in this regard.

Glycosaminoglycans are straight-chain polysaccharides composed of repeating disaccharide units of hexosamine (D-glucosamine or D-galactosamine) and uronic acid (D-glucuronic acid or L-iduronic acid) or galactose (in keratan sulfate). With the exception of a nonsulfated GAG, hyaluronic acid, which exists as a free polymer, all GAGs are sulfated and covalently attached to core proteins to form proteoglycans (Muir and Hardingham, 1975; Rodén, 1980; Kjellén and Lindahl, 1991). Previous studies of bovine muscle connective tissues have shown that dermatan sulfate (DS) and hyaluronic acid are the major GAGs present in the epimysium with small amounts of chondroitin sulfate (CS) (Cormier *et al*., 1971; Nakano and Thompson, 1980). More recently, Eggen *et al*. (1994), in a study of bovine intramuscular proteoglycans, reported the presence of two distinct proteoglycans, decorin and a large molecular weight DS proteoglycan, in the perimysium and endomysium. They further reported that these proteoglycans carry predominantly DS chain. However, these studies provide little information on the composition of different chains of sulfated GAGs (galactosaminoglycans) including DS and CS.

Galactosaminoglycans in fibrous connective tissues have hybrid or copolymeric structures with varying uronic acid compositions, contrasting with CS of hyaline cartilage which consists entirely of D-glucuronosyl-*N*-acetylgalactosamine repeating disaccharide units in which the galactosamine is sulfated at C4 and C6. The major disaccharide repeating unit of pig skin DS is L-iduronosyl-*N*-acetylgalactosamine 4-sulfate, but small amounts of D-glucuronosyl-*N*-acetylgalactosamine 4-sul-

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S0021-8561(95)00688-1 CCC: $12.00

fate are also present. However, D-glucuronosyl residue rich galactosaminoglycan, in which L-iduronosyl residues are the minor components (CS/DS copolymers), occur in chicken comb and wattle (Nakano and Sim, 1992). Copolymeric structures of galactosaminoglycans including DS and CS/DS copolymers have been found in various tissues including bovine skin, porcine intestinal mucosa, human umbilical cord, human knee meniscus, horse aorta, and bovine periodontal ligament and sclera (Fransson and Rodén, 1967; Fransson, 1968a,b; Fransson and Havsmark, 1970; Habuchi *et al*., 1973; Inoue and Iwasaki, 1976; Cöster and Fransson, 1981; Gibson and Pearson, 1982; Pearson and Gibson, 1982).

To investigate the distribution of disaccharide in different chains, the intact galactosaminoglycan chains must be fractionated and then examined for the presence of copolymers. Using ethanol precipitation and enzymatic digestion, we have shown the presence of copolymeric galactosaminoglycans in rooster combs and wattles (Nakano and Sim, 1992).

The objectives of the present study were 1) to characterize whole galactosaminoglycans isolated from the porcine epimysium, (2) to fractionate whole galactosaminoglycans by ethanol precipitation and to determine the disaccharide composition in each fraction, and (3) to estimate the amounts of copolymeric galactosaminoglycans including DS and CS/DS copolymers in the epimysium. Tissue sections were also studied using histochemical and immunohistochemical techniques.

MATERIALS AND METHODS

Tissues. Samples of epimysium were collected from the dorsal surface of the lumbar longissimus lumborum muscle of four 5.5-month-old pigs. A central portion of each sample was cut sagittally into strips (approximately 0.5 × 2 cm), fixed in 4% formalin containing 0.5% cetylpyridinium chloride (Williams and Jackson, 1956), and used for light microscopy. The remaining tissues were dry-defatted using acetone and petroleum ether (boiling range 37.8−56.9 °C) and stored at 4 °C until analyzed.

Isolation and Fractionation of GAGs. The dry-defatted tissues (approximately 10 g) were digested with twice-crystallized papain (Sigma Chemical Co., St. Louis, MO) (4 μg/mg of tissue) in 10 volumes of 0.1 M sodium phosphate buffer, containing 0.005 M EDTA, 0.005 M cysteine hydrochloride, and 0.02% sodium azide, at 65 °C for 24 h (Scott, 1960). After proteolysis, trichloroacetic acid was added to the digests to a concentration of 7% (w/v) and the mixtures were held at 4 °C overnight. After the removal of the protein precipitates by centrifugation, the supernatants were quantitatively transferred to dialysis tubings previously boiled in distilled water for 10 min and dialyzed in running tap water for 24 h and then in cold distilled water for another 24 h. Cetylpyridinium chloride was added to the dialysate to precipitate GAG as a cetylpyridinium−GAG complex (Scott, 1960). The precipitate was sequentially washed with 0.4 M and 2.1 M NaCl to obtain nonsulfated and sulfated GAG fractions, respectively (Schiller *et al*., 1961). To each fraction was added potassium thiocyanate (Korn, 1959), and the resulting precipitate of cetylpyridinium thiocyanate was removed by filtration. The filtrate obtained was dialyzed in water and the dialysates were freeze-dried.

The 2.1 M NaCl soluble fraction was further fractionated by selective precipitation with various concentrations of ethanol in 5% (w/v) calcium acetate and 0.5 M acetic acid (Meyer *et al*., 1956). Five fractions (I−V) were collected at ethanol concentrations of 18, 25, 40, 50, and 75%, respectively.

Analytical Methods. Hydroxyproline was determined according to the method of Stegemann and Stalder (1967) on samples of tissue that had been hydrolyzed in 6 N HCl at 110 °C for 20 h. Sialic acid was determined by using the periodate−thiobarbituric acid reaction (Warren, 1959) after hydrolysis of tissues in 0.1 N sulfuric acid at 80 °C for 1 h. The chromophore formed was extracted using 1-propanol (Nakano *et al*., 1994) instead of cyclohexanone as used by Warren (1959). *N*-Acetylneuraminic acid (Sigma) was used as a standard. Uronic acid was determined by the original (Dische, 1947) and the modified (Kosakai and Yoshizawa, 1979) carbazole reactions using D-glucuronolactone as a standard. Iduronic acid was determined according to the periodate−Schiff method (DiFerrante *et al*., 1971) using hog skin DS (Miles Laboratories, Elkhardt, IN) as a standard. Schiff reagent was prepared from basic fuchsin (Serva Feinbiochemica GmbH & Co., Heidelberg, Germany) and used within 24 h. Standard chondroitin sulfates from whale cartilage (CSA; 4-sulfated disaccharide/6-sulfated disaccharide = 80:20) and from shark cartilage (CSC; 4-sulfated disaccharide/6-sulfated disaccharide = 10:90), both obtained from Miles Laboratories, gave negligible color yields in this reaction. The percentage of uronic acid present as iduronic acid was calculated by using the following formula:

$$\%\ \text{iduronic acid in total uronic acid} = \frac{\text{DS concn} \times 0.292 \times 0.8}{\text{total uronic acid concn}} \times 100$$

The factors 0.292 and 0.8 correspond to the proportions of the total uronic acid and iduronic acid, respectively, in the standard DS. The total uronic acid concentration was determined by the modified carbazole reaction (Kosakai and Yoshizawa, 1979). The proportion of iduronic acid was determined from the changes in absorbance at 232 nm (Saito *et al*., 1968) after digestion with chondroitinase ABC and chondroitinase AC II. The relative color yields obtained by the original (Dische, 1947) and the modified (Kosakai and Yoshizawa, 1979) carbazole reactions were used to confirm iduronic acid values obtained according to the periodate−Schiff method (DiFerrante *et al*., 1971). On the original carbazole reaction, the color yield is lower in iduronic acid than in glucuronic acid. On the other hand, the modified carbazole reaction essentially gives the same color yield in both uronic acids. Protein content was estimated according to the method of Lowry *et al*. (1951) using bovine serum albumin as a standard.

Enzymatic Digestion of GAG Fractions. Digestion with *Streptomyces* hyaluronidase (Calbiochem-Novabiochem. Co., San Diego) was carried out as previously described (Brandt *et al*., 1976). Digestions with chondroitinase ABC and chondroitinase AC II (both obtained from Seikagaku America Inc., Rockville, MD) were carried out following the procedure of Saito *et al*. (1968). Glycosaminoglycan fractions were also digested with chondroitinase B (Seikagaku America) according to the supplier's directions. Chondroitinase ABC cleaves the β-1,4-*N*-acetylhexosamidic linkage to either D-glucuronic acid or L-iduronic acid, whereas chondroitinase AC II cleaves the β-1,4-*N*-acetylhexosamidic linkage to D-glucuronic acid (Kresse and Glössl, 1987). Chondroitinase B cleaves the β-1,4-*N*-acetylgalactosamidic linkage to L-iduronic acid (Kresse and Glössl, 1987). The digests were examined by cellulose acetate electrophoresis, gel electrophoresis, and gel chromatography. The proportion of unsaturated disaccharides released after chondroitinase ABC digestion was determined using thin layer chromatography as previously described (Shimada *et al*., 1987).

All enzymes used were tested beforehand for activity against standard samples of the appropriate GAGs: human umbilical cord hyaluronic acid (Sigma), whale cartilage CSA, shark cartilage CSC, and hog skin DS. Chondroitinase B, which may be contaminated with chondroitinase AC (supplier's information), was incubated with whale CSA and shark CSC separately. Examination of the incubation mixtures using both cellulose acetate electrophoresis and gel chromatography on Sephacryl S-300 showed no appreciable digestion of these standards CS with chondroitinase B.

Cellulose Acetate Electrophoresis. Electrophoresis of GAGs on cellulose acetate strips (Sepraphore III, Gelman Sciences, Ann Arbor, MI) was carried out in 0.1 M pyridine−1.2 M acetic acid, pH 3.5 (Habuchi *et al*., 1973) and in 0.1 N HCl (Wessler, 1971). The mobility of the GAG band in 0.1 N

HCl reflects the content of sulfate groups (Wessler, 1971). The strips were stained in 0.1% (w/v) Alcian Blue 8GX in 0.1% acetic acid containing 0.02% sodium azide for 3 min, washed in 0.1% acetic acid, cleared in Sepra Clear II (Gelman Science), and scanned at 600 nm on a Gilford 252 spectrophotometer fitted with a linear transporter.

Gel Electrophoresis. Samples of GAG fractions treated with and without enzymes were electrophoresed on 12% polyacrylamide gels containing 0.1% sodium dodecyl sulfate in Tris−borate buffer, pH 8.6 (Scott *et al*., 1989).

Gel Chromatography. Samples of GAG fractions treated with and without enzyme or alkaline borohydride (Carlson, 1968), containing approximately 100 μg of uronic acid, were applied to a 1 × 110 cm column of Sephacryl S-300 equilibrated and eluted with 0.5 M sodium acetate containing 0.02% sodium azide, pH 5.8. Fractions (1 mL) collected were assayed for sulfated GAG using the dimethylmethylene blue dye binding method (Farndale *et al*., 1982). The partition coefficient (K_{av}) of GAG was calculated from the formula

$$K_{av} = \frac{V_e - V_0}{V_t - V_0}$$

in which V_e represents the volume of the peak fraction. V_o (void volume) and V_t (total column volume) were determined using blue dextran (Pharmacia Biotech Canada Inc., Baie d'Urfe, PQ) and tritiated water, respectively. Standard GAGs, including whale cartilage CSA, shark cartilage CSC, river sturgeon notochord CS (a gift from Dr. M. B. Mathews, University of Chicago; MW = 15 000), and hog skin DS, were also chromatographed on the same column.

ELISA. The antigenicity of sulfated GAG fraction was determined by the ELISA inhibition assay (Hamada *et al*., 1984). Fifty microliters of 5000 times diluted monoclonal antibody (MAb) CS-56 was incubated at 4 °C overnight with the same volume of each of different concentrations of sulfated GAG in a round-bottom microtiter plate. The incubation mixtures were then transferred to a flat-bottom microtiter plate coated with 1 μg/mL concentration of CS proteoglycan isolated from bovine fibrocartilage (Nakano and Scott, 1989a). Antibody binding was detected using alkaline phosphatase conjugated goat anti-mouse Ig (G + A + M) (Zymed Laboratories Inc., South San Francisco, CA) with *p*-nitrophenyl phosphate as the substrate. The percentage of inhibition was calculated using the following formula:

$$\% \text{ inhibition} = 100 - \frac{\text{abs in the presence of inhibitor}}{\text{abs in the absence of inhibitor}} \times 100$$

Light Microscopy. Fixed samples were dehydrated and embedded in paraffin by routine methods (Drury and Wallington, 1967). Five micrometer thick sections were cut and stained with hematoxylin and eosin, Gomori's trichrome, and Alcian Blue (Drury and Wallington, 1967). Sections were also stained with MAbs to DS proteoglycan, decorin (6D6; Pringle *et al*., 1985), and CS (CS-56; Avnur and Geiger, 1984; ICN Biomedicals Canada Ltd., Mississauga, ON). MAb 6D6 was raised against bovine skin decorin and was reactive to porcine decorin (Nakano *et al*., unpublished results). MAb CS-56 does not react with DS (Avnur and Geiger, 1984; Nakano and Scott, 1989b). All sections were pretreated for 30 min with 2% hydrogen peroxide in methanol to eliminate endogenous peroxidase activity. Sections to be incubated with 6D6 were treated with testicular hyaluronidase (type V from sheep testis, Sigma) (10 mg/mL in TBS: 0.05 M Tris-HCl/ 0.15 M NaCl, pH 7.6, at 37 °C for 1 h) to digest hyaluronic acid, which might otherwise mask tissue antigens. Sections to be incubated with CS-56 were not treated with hyaluronidase. Sections were then washed with TBS, soaked in normal rabbit serum (diluted 1 in 20 in TBS) for 30 min and incubated at 4 °C overnight with diluted hybridoma culture supernatant (6D6, 1:10) or ascites fluid (CS-56, 1:200). Control sections were incubated with and without normal ascites fluid derived from mouse myeloma cells or myeloma cell culture supernatant. Sections were washed with TBS and incubated for 45 min with rabbit anti-mouse Ig (G + A + M) (Zymed Laboratories, Inc.) diluted 1:50. Final incubation was carried out using mouse peroxidase−anti-peroxidase complex (Nordic Immunology, Tilberg, The Netherlands) diluted 1:300. Color was developed with diaminobenzidine tetrahydrochloride (Sigma) and hydrogen peroxide (Pringle *et al*., 1985).

RESULTS

Epimysium Composition. The hydroxyproline, sialic acid, and uronic acid contents, expressed as milligrams per gram of dry defatted tissue ± SD (four replicates), were 98.9 ± 3.4, 0.9 ± 0.1, and 1.2 ± 0.2, respectively.

Isolation of Sulfated GAG. An average of 81 ± 12 mg of dry weight/g of dry-defatted tissue was recovered as a papain digest which contained 11.5 ± 2.8 mg of uronic acid and 560.3 ± 20.3 mg of protein per gram of dry weight. The GAGs in the 0.4 and 2.1 M NaCl fractions obtained after fractionation of cetylpyridinium−GAG complex accounted for 58 and 42% (average of two experiments) of total uronic acid recovered, respectively. The 0.4 M NaCl fraction contained 106 μg of uronic acid and 202 μg of protein per milligram of dry weight, and the 2.1 M NaCl fraction contained 268 μg of uronic acid and 41 μg of protein per milligram of dry weight. The 0.4 M NaCl fraction had a band with mobility identical to that of standard hyaluronic acid on cellulose acetate electrophoresis in pyridine−acetic acid (Figure 1a). The band was completely digested by *Streptomyces* hyaluronidase (results not shown), an enzyme specific to hyaluronic acid (Ohya and Kaneko, 1970). This fraction was not analyzed further.

The 2.1 M NaCl fraction was found to have high iduronic acid content, accounting for 78% of total uronic acid. This was close to the iduronic acid content in the standard DS (80%, see Materials and Methods). On cellulose acetate electrophoresis in pyridine−acetic acid, the 2.1 M NaCl fraction gave a band broader than standard DS with mobility close to but slightly slower than that of DS (Figure 1b). When electrophoresed in 0.1 N HCl, the fraction gave a band with mobility similar to that of standard CS or DS (Figure 1c). Since the mobility of GAG in 0.1 N HCl reflects the content of sulfate groups (see Materials and Methods), the results suggested that the degree of sulfation is similar to those of CS and DS, in which the ratio of sulfate to uronic acid or hexosamine is close to 1 (supplier's information).

Enzymatic Digestion of the 2.1 M NaCl Fraction. Digestion with chondroitinase ABC resulted in complete elimination of the band of the 2.1 M NaCl fraction on cellulose acetate electrophoresis (results not shown), suggesting that the GAGs in the fraction contain galactosaminoglycans. The effect of chondroitinase AC II digestion on the size of band of the 2.1 M NaCl fraction was apparently small. However, the enzyme treatment gave a band with faster mobility than the untreated control (Figure 1d). This was unexpected. Since the mobility of CS is greater than that of DS on cellulose acetate in pyridine acetic acid, a reduced mobility of the chondroitinase AC II treated band was expected. Whether or not this was due to an increase in net negative charge was unknown.

Portions of the 2.1 M NaCl fraction were also treated with and without chondroitinase B or chondroitinase AC II and examined using gel chromatography on Sephacryl S-300 and gel electrophoresis. This may be useful to determine if the minor disaccharides containing glucu-

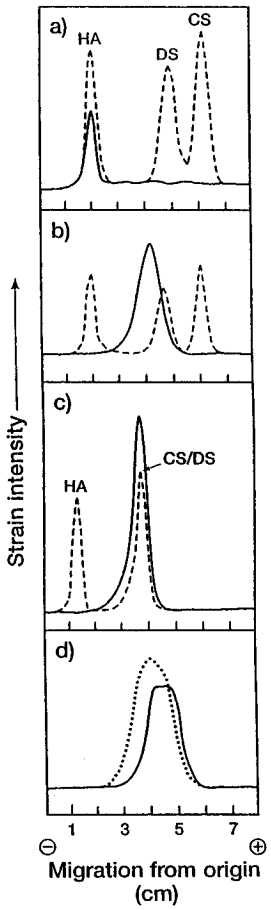

Figure 1. Cellulose acetate electrophoresis of porcine epimysium GAGs: (a) the 0.4 M NaCl fraction (—) and standard GAGs (- - -) in pyridine−acetic acid; (b) the 2.1 M NaCl fraction (—) and standard GAGs (- - -) in pyridine−acetic acid; (c) the 2.1 M NaCl fraction (—) and standard GAGs (- - -) in 0.1 N HCl; (d) the 2.1 M NaCl fraction incubated with (—) and without (· · ·) chondroitinase AC II in pyridine−acetic acid. HA, hyaluronic acid; CS, chondroitin sulfate; DS, dermatan sulfate. The experiment was repeated twice. The electrophoretograms shown were obtained from the first experiment. Similar results were obtained in the second experiment.

ronosyl residue are involved in a copolymeric chain. Sephacryl S-300 chromatography (Figure 2) showed that the average size of the 2.1 M NaCl fraction (K_{av} = 0.26, Figure 2a) was close to that of hog skin DS (K_{av} = 0.30, Figure 2b), larger than that of river sturgeon CS (K_{av} = 0.37, Figure 2c), similar to that of whale cartilage CSA (K_{av} = 0.26, Figure 2d), but smaller than that of shark cartilage CSC (K_{av} = 0.09, Figure 2e). These results, except for DS, were consistent with those obtained by gel electrophoresis (Figure 3). The DS had a significantly faster mobility than did the 2.1 M NaCl fraction.

The 2.1 M NaCl fraction was highly susceptible to chondroitinase B (Figure 2f). Most GAGs of the fraction (>90%) were digested by the enzyme. Chondroitinase AC II treatment (Figure 2g) showed that approximately 10% (eluting in tubes 40−54) of total GAG was resistant to, but more GAGs (probably most GAGs eluting after tube 54, which was the tube corresponding to V_e for the 2.1 M NaCl fraction) were susceptible to, the enzyme. These results were consistent with those obtained by gel electrophoresis (Figure 3) and suggested that the major GAGs in the 2.1 M NaCl fraction are copolymers having high iduronic acid content with glucuronic acid present as a relatively minor component. It may also be possible that the 2.1 M NaCl fraction contains CS chains with little iduronic acid present.

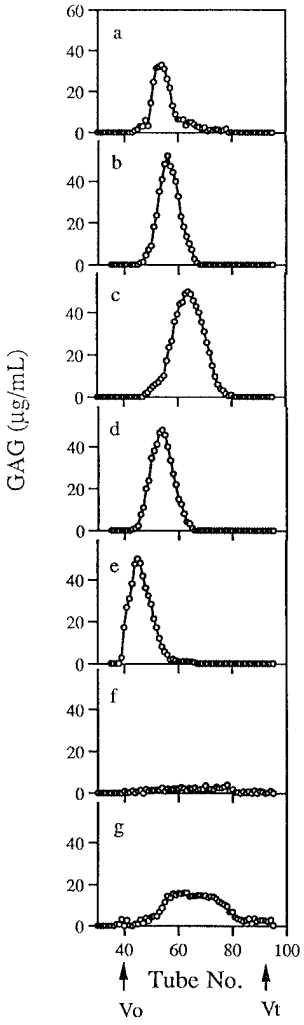

Figure 2. Sephacryl S-300 chromatograms of the 2.1 M NaCl fractions treated with and without enzymes and of standard GAGs: (a) the 2.1 M NaCl fraction not treated with enzyme (control); (b) hog skin DS; (c) sturgeon notochord CS; (d) whale cartilage CSA; (e) shark cartilage CSC; (f) the 2.1 M NaCl fraction incubated with chondroitinase-B; (g) the 2.1 M NaCl fraction incubated with chondroitinase-AC II.

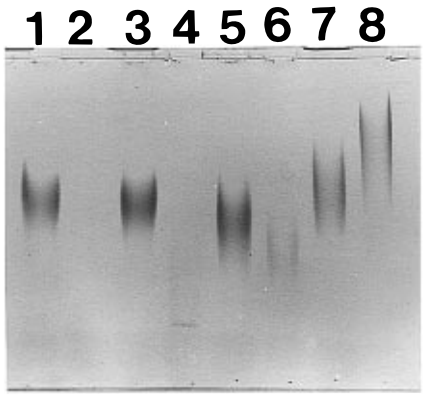

Figure 3. Gel electrophoresis (12% polyacrylamide) of the 2.1 M NaCl fraction and standard GAGs: (lanes 1 and 3) the 2.1 M NaCl fraction not treated with enzyme (controls); (lane 2) the 2.1 M NaCl fraction incubated with chondroitinase-B; (lane 4) the 2.1 M NaCl fraction incubated with chondroitinase-AC II; (lane 5) sturgeon notochord CS; (lane 6) hog skin DS; (lane 7) whale cartilage CSA; (lane 8) shark cartilage CSC.

Reactivity of Anti-CS MAb, CS-56, to the 2.1 M NaCl Fraction. The ELISA inhibition assay (Figure 4) showed a relatively low but significant antigenicity of the 2.1 M NaCl fraction to MAb CS-56. The hog skin

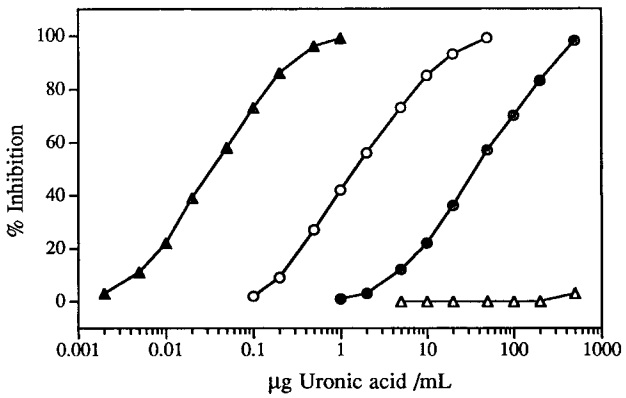

Figure 4. Inhibition of binding of the MAb, CS-56 to CS. Inhibitors used: shark cartilage CSC (▲); whale cartilage CSA (○); the 2.1 M NaCl fraction (●); hog skin DS (△).

DS showed no inhibition as reported previously (see Materials and Methods). The concentration of antigen in the 2.1 M NaCl fraction required for 50% inhibition of MAb binding (IC_{50}) was 37 μg of uronic acid/mL. This was 1121 and 25 times higher than the IC_{50} values of shark CSC (0.033 μg of uronic acid/mL) and whale CSA (1.485 μg of uronic acid /mL), respectively. This large difference in the antigenicity to CS-56 between CSA and CSC was not noted by Avnur and Geiger (1984), who produced the antibody. The proportion of CS-56 epitope in the 2.1 M NaCl fraction calculated using CSA as standard was 4.0% of total uronic acid. The CSA instead of CSC was used as standard because of its high proportion of 4-sulfated disaccharide in the epimysial galactosaminoglycans (see below).

Ethanol Fractionation of Galactosaminoglycans. To obtain more information about variations of uronic acid composition of sulfated galactosaminoglycans, the 2.1 M NaCl fraction was further fractionated by selective precipitation with various concentrations of ethanol. Trials with standard GAGs (CSA, CSC, and DS) gave fractionation patterns similar to those reported by others (Meyer *et al.*, 1956; Gibson and Pearson, 1982). The relative proportions and analytical data for each fraction are given in Table 1.

Fraction I contained most GAG uronic acid (>70% of total uronic acid recovered). Other fractions contained much less uronic acid (<10% of total uronic acid in each fraction). Iduronic acid content was highest in fraction I, higher in fraction II than in fraction III, and lowest (<10% of total uronic acid) in fractions IV and V. This was consistent with iduronic acid contents estimated by the uronic acid color yield ratio of original carbazole reaction to modified carbazole reaction. The color yield ratio was close to that of pig skin DS (0.45) in fractions I and II and increased in fractions III−V. The ratio was, however, lower than those of whale CSA (0.99) and shark CSC (1.02). From the iduronic acid values, it is suggested that fractions I−III are DS with various proportions of iduronic acid. The DS accounted for more than 90% of total uronic acid recovered. The remaining fractions were galactosaminoglycans with high glucuronic acid contents. Protein to uronic acid ratio was lowest in fraction I, increased approximately 2-fold in fractions II−IV, and was highest in fraction V. The ratio was 2.5-fold lower in fraction I than in the 2.1 M NaCl fraction, in which the protein to uronic acid ratio was 0.15 (see above).

Chondroitinase ABC digestion (Table 1) resulted in release of 4-sulfated unsaturated disaccharide only in both fractions I and II. The proportion of 4-sulfated unsaturated disaccharide decreased in fraction III, which contained both 6-sulfated and nonsulfated unsaturated disaccharides as relatively minor components with a much smaller proportion of the latter. In fractions IV and V, the proportion of 4-sulfated unsaturated disaccharide continued to decrease with a concomitant increase in both 6-sulfated and nonsulfated unsaturated disaccharides. Analysis of hexosamine by thin layer chromatography (results not shown) indicated that galactosamine is the only detectable amino sugar in fractions I, III, and IV. In fractions II and V, relatively weak bands, with mobilities similar to that of glucosamine, were seen in addition to a major band of galactosamine. Since no detectable band was seen for chondroitinase ABC digests of these fractions on cellulose acetate electrophoresis (results not shown), concentrations of glucosamine-containing GAGs (glucosaminoglycans), if any, were considered to be very low in the porcine epimysium. Further investigation of glucosaminoglycan was not carried out in this study.

From the values of iduronic acid and unsaturated disaccharide, the galactosaminoglycans in fractions I and II probably contain iduronosyl-*N*-acetylgalactosamine 4-sulfate as their major disaccharides with relatively small amounts of glucuronosyl-*N*-acetylgalactosamine 4-sulfate. The ratios of the two disaccharides were approximately 9:1 and 7:3 in fractions I and II, respectively. In fractions III−V, which had unsaturated nonsulfated, 4-sulfated, and 6-sulfated disaccharides of various proportions after chondroitinase ABC digestion, it was difficult to estimate which uronic acid is involved in each disaccharide. However, in fractions IV and V containing glucuronic acid as a dominant uronic acid, most disaccharides appeared to be glucuronosyl-*N*-acetylgalactosamine.

Cellulose Acetate Electrophoresis of Ethanol Fractions. Cellulose acetate electrophoresis in pyridine−acetic acid showed the presence of single bands in all fractions (Figure 5). The average mobility of each band was slower than that of standard DS. The

Table 1. Analysis of Porcine Epimysium Galactosaminoglycan Fractions[a] (2.1 M NaCl Fraction Fractionated with Different Concentrations of Ethanol)

ethanol fraction	uronic acid[b]	iduronic acid[c]	uronic acid color yield ratio[d]	protein:uronic acid ratio[e]	unsaturated disaccharide (%)		
					4-sulfated	6-sulfated	nonsulfated
I (18%)[f]	74.9	86.8	0.49	0.06	100	0	0
II (25%)	6.5	66.0	0.52	0.12	100	0	0
III (40%)	9.6	41.8	0.79	0.13	76	19	5
IV (50%)	5.2	8.9	0.93	0.13	39	36	25
V (75%)	3.8	7.6	0.84	0.35	35	45	20

[a] Values presented are the averages from two experiments. The results obtained in the first experiment were similar to those obtained in the second experiment. [b] Percent of total uronic acid. [c] Percent of total iduronic acid in total uronic acid. [d] Uronic acid color yield ratio of original carbazole (Dische, 1947) to modified carbazole (Kosakai and Yoshizawa, 1979). [e] Weight ratio. [f] Ethanol concentration.

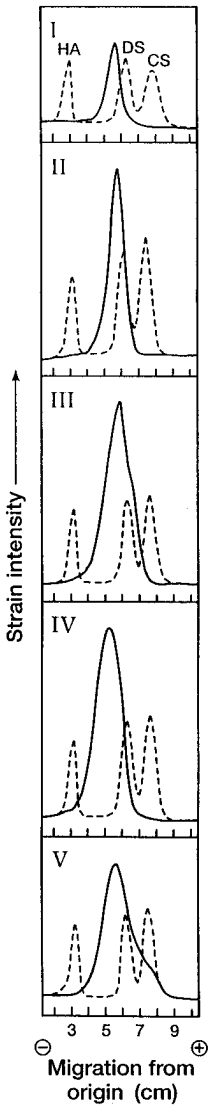

Figure 5. Cellulose acetate electrophoresis of epimysial galactosaminoglycans in pyridine−acetic acid: (—) fractions I−V, which were obtained by precipitation with different concentrations (18−75%) of ethanol; (- - -) standard GAGs as in Figure 1.

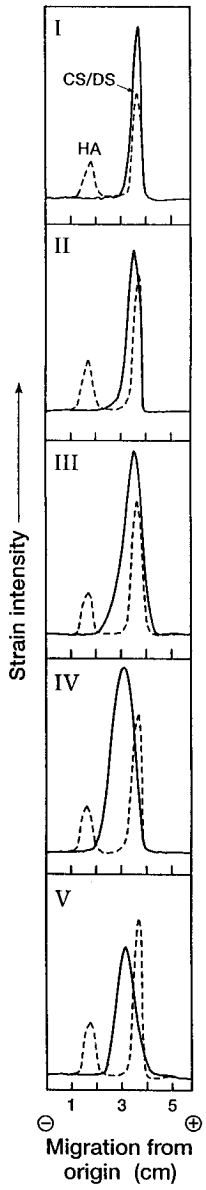

Figure 6. Cellulose acetate electrophoresis of epimysial galactosaminoglycans in 0.1 N HCl: (—) fractions I−V as in Figure 3; (- - -) standard GAGs as in Figure 1.

broadness of this band was similar to that of standard DS in fractions I and II and increased in fractions III−V.

Fractions I−V were also examined using cellulose acetate electrophoresis in 0.1 N HCl (Figure 6). Fraction I had a band with mobility similar to that of standard CS and DS. The mobility of the fraction II band was close to that of CS and DS, but the broadness of the band was slightly greater. The broadness of the band further increased in fractions III−V, among which the broadness of the bands was more or less similar. In these four fractions (II−V), the mobility of the fast moving front of each band was similar to that of fraction I. The Alcian Blue staining was almost homogeneous throughout the band, thus showing a symmetrical peak by densitometric scanning in fractions II and IV. In contrast, approximately half of the band had higher staining intensity than did the remaining half in fractions III (fast moving band > slow moving band) and V (slow moving band > fast moving band). These results suggest that there are variations in sulfate content among fractions. (The mobility of GAG in cellulose acetate in HCl reflects sulfate content as discussed above.) The sulfate contents were similar to those of standard CS and DS in fractions I and II (molar ratio of sulfate to uronic acid close to 1, see Materials and Methods). The proportion of undersulfated GAG increased in fractions III−V and appeared to be less in fractions III than in fraction IV and V.

ELISA Inhibition Assay. The reactivity of each fraction to anti-CS MAb CS-56 was examined using ELISA inhibition assay. The IC_{50}, the concentration of antigen required for 50% inhibition of MAb binding, was highest in fraction I and decreased rapidly with increasing ethanol concentrations (Table 2). The proportion of CS-56 epitope in each fraction was calculated using whale CSA as standard. The whale CSA, rich in 4-sulfated disaccharide (see Materials and Methods), was used instead of shark CSC because the majority of sulfated galactosaminoglycan fractions of porcine epimysium contained relatively high proportions of 4-sulfated disaccharide (Table 1). The proportions were 0.07, 0.61, 4.13, 5.71, and 9.90% of total uronic acid in fractions I, II, III, IV, and V, respectively. This increase in the content of CS-56 epitope was consistent with the decrease in the proportion of iduronic acid with increasing ethanol concentration (Table 1).

Table 2. Concentrations of Uronic Acid Required To Achieve 50% Inhibition of Binding of MA CS-56 to Chondroitin Sulfate[a]

ethanol fraction	concn (μg/mL)	ethanol fraction	concn (μg/mL)
I (18%)[b]	2050	IV (50%)	26
II (25%)	245	V (75%)	15
III (40%)	36		

[a] Values presented are the averages from two experiments. The results obtained in the first experiment were similar to those obtained in the second experiment. [b] Ethanol concentration.

Effects of Chondroitinase B and Chondroitinase AC II Treatments on Galactosaminoglycan Fractions. The results obtained so far showed distribution of different types of disaccharides in the five galactosaminoglycan fractions. However, these results do not provide any data on whether different types of disaccharide are involved in the same chain (copolymer) or in the different chains (homopolymers). To obtain this information, fractions I−IV were further analyzed using chondroitinase B and chondroitinase AC II. The susceptibilities of GAG chains to these enzymes were monitored by gel chromatography on Sephacryl S-300 (Figure 7). Control samples had broader peaks in fractions III and IV than in fractions I and II. Alkaline borohydride treatment of fraction I to remove peptides attached to GAG (β-elimination) resulted in no significant shift of peak (results not shown). No alkaline borohydride treatment was carried out for fractions II−IV. The average molecular size of GAG was smaller in fraction IV ($K_{av} = 0.43$, Figure 7-IVa) than in fractions I ($K_{av} = 0.26$, Figure 7-Ia), II ($K_{av} = 0.26$, Figure 7-IIa), and III ($K_{av} = 0.28$, Figure 7-IIIa). The K_{av} values for fractions I−III were larger than that of shark cartilage CSC (0.09), similar to that of whale cartilage CSA (0.26), close to that of hog skin DS (0.30), and smaller than that of river sturgeon CS (0.37) (see Figure 2 for the peak positions of these standard GAGs).

Most GAG chains (>90%) were degraded into smaller molecules by chondroitinase B in fractions I (Figure 7-Ib) and II (Figure 7-IIb). In contrast, approximately 73% of total GAG recovered were resistant to this enzyme in fraction III (tubes 39−55, Figure 7-IIIb). Little digestion was observed with chondroitinase B in fraction IV (Figure 7-IVb).

The proportions of GAG chains apparently resistant to chondroitinase AC II were 19, 8, and 22% in fractions I (tubes 39−54, Figure 7-Ic), II (tubes 39−54, Figure 7-IIc), and III (tubes 39−55, Figure 7-IIIc), respectively. All chains were degraded into small molecules by the enzyme in fraction IV (Figure 7-IVc). In a separate experiment, chondroitinase AC II resistant galactosaminoglycan from fraction I eluting between tubes 50−54 was prepared in larger amount and rechromatographed on the same column. A majority of the GAG applied was eluted as the enzyme resistant peak (results not shown), suggesting that the contribution of the enzyme susceptible GAG to the enzyme unsusceptible peak is negligible in this chromatographic technique.

Light Microscopic Histochemical and Immunohistochemical Staining. The porcine epimysium was highly eosinophilic with hematoxylin and eosin (Figure 8A) and had relatively strong staining for collagen with Gomori's trichrome (Figure 8B). Alcian Blue staining was weak (Figure 8C) indicating low content of GAG relative to collagen. Immunohistochemical staining showed that both 6D6 (anti-decorin MAb) and CS-56 (anti-CS MAb) epitopes are evenly distributed in the epimysium. Strong staining intensity was observed with 6D6 (Figure 8D). The staining intensity with CS-56 was relatively weak but positive (Figure 8E). No immunostaining was observed in the epimysium when primary antibodies were not included (results not shown). These findings were consistent with the results of chemical and immunochemical analyses showing that the porcine epimysium contains DS as the major galactosaminoglycan with glucuronic acid rich copolymers recognized by CS-56 as minor GAGs.

DISCUSSION

The results obtained in the present study suggested that collagen is the major constituent of the porcine epimysium accounting for approximately 72% of the dry-defatted tissue, which was calculated using a factor of 7.25 (Goll *et al.*, 1963) to convert from hydroxyproline content. The content of GAG in the epimysium can be

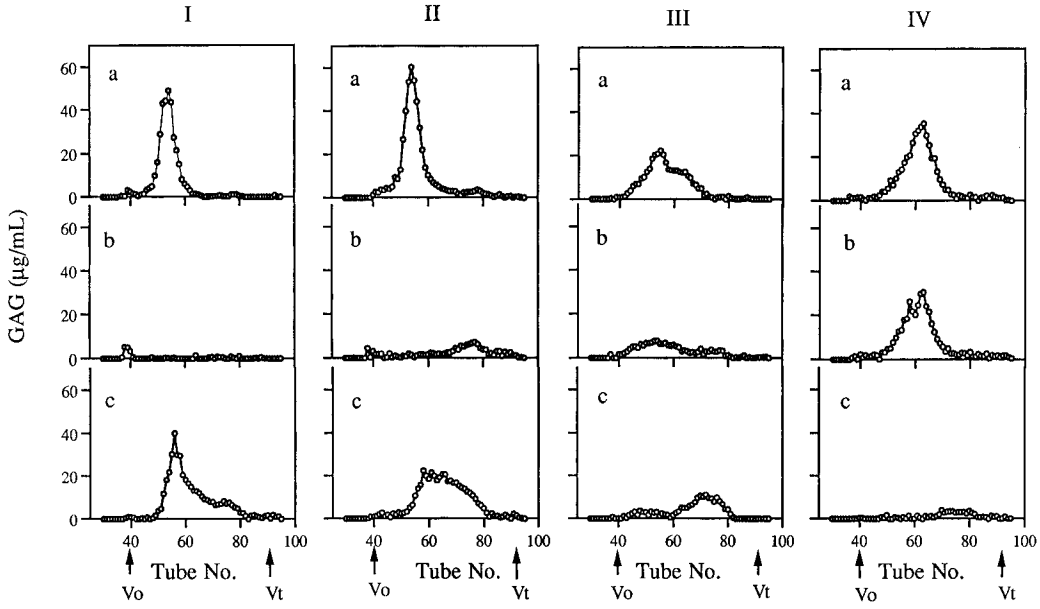

Figure 7. Chromatography on Sephacryl S-300 (1 × 110 cm). Epimysial galactosaminoglycan fractions I−IV were treated with chondroitinase B or with chondroitinase AC II: (a) not treated with enzyme (control); (b) incubated with chondroitinase B; (c) incubated with chondroitinase AC II.

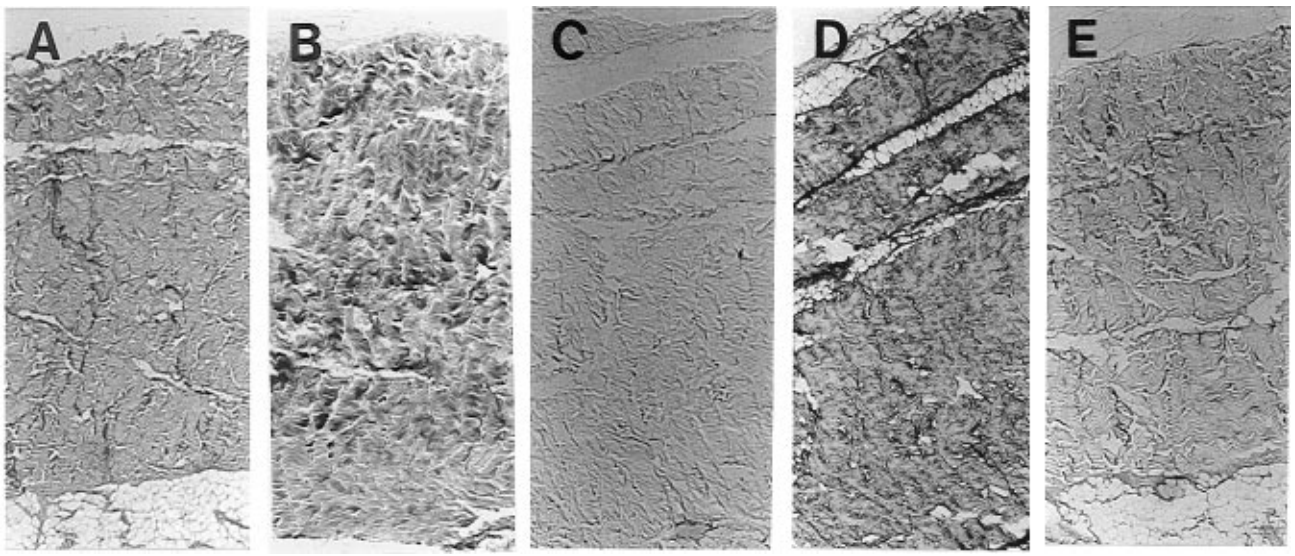

Figure 8. Staining of sections of porcine lumbar longissimus lumborum epimysium. Sections were stained with hematoxylin and eosin (A), Gomori's trichrome (B), Alcian Blue (C), anti-decorin MAb 6D6 (D), and anti-CS MAb CS-56 (E). Magnification: 34×.

calculated to be approximately 0.35% using a factor of 3 (Nakano and Scott, 1989b) to convert from uronic acid content. These values are close to those of collagen and GAG concentrations in bovine epimysium (Nakano and Thompson, 1980). Limited information is available on sialic acid concentration in epimysium. Nakano and Thompson (1980), in a study of bovine epimysium composition, reported the presence of small amounts of sialic acid in a heat labile fraction (<0.2 μg/mg dry-defatted tissue). Total sialic acid content was, however, not reported by these authors. The sialic acid concentration in the porcine epimysium determined in this study was approximately half that of calf joint cartilage (1.7−2.8 μg/mg, Pal *et al.*, 1981) and chicken comb (2.3 μg/mg, Nakano *et al.*, 1994).

The 2.1 M NaCl fraction had iduronic acid values and cellulose acetate electrophoresis patterns similar to those for hog skin DS, suggesting that the majority of the fraction consists of DS. However, a difference was observed in the antigenicity to CS-56 (anti-CS MAb) between these galactosaminoglycan preparations (Figure 4). The ELISA inhibition assay of ethanol fractions showed that 58% of total CS-56 epitope (calculated on the basis of the proportion of uronic acid shown in Table 1) is in non-DS fractions (IV and V), and 34% of the CS-56 epitope is in fraction III containing DS. It is likely that the reactivity of the MAb to the 2.1 M NaCl fraction is largely contributed by the non-DS fractions. Further work to examine the antigenicity of pooled DS fraction (I + II + III) without non-DS fractions was not pursued in this experiment.

The faster electrophoretic mobility of the band in hog skin DS than in the 2.1 M NaCl fraction (Figure 3), which was inconsistent with the results of gel chromatography (Figure 2), is difficult to explain. The difference in the chain composition between the two samples (*e.g.*, difference in the content of CS-56 epitope) may be involved. It appears that gel chromatography in which separation is by molecular sieve and not influenced by charge or disaccharide composition is a more reliable method to determine the molecular size of DS.

The slower electrophoretic mobility of the band of fraction I compared to that of DS on cellulose acetate in pyridine−acetic acid may be due to the difference in iduronic acid content, but not due to the difference in sulfation. The iduronic acid content was higher in fraction I than in the hog DS, while the sulfate content was shown to be similar between the two galactosaminoglycans by cellulose acetate electrophoresis in 0.1 N HCl.

The increased broadness of the band in higher ethanol fractions on cellulose acetate electrophoresis in both pyridine−acetic acid (Figure 5) and HCl (Figure 6) suggests increased variations in galactosaminoglycan composition including the contents of iduronic acid and/or sulfate. Undersulfation is consistent with increased proportions of unsaturated nonsulfated disaccharide in the chondroitinase ABC digests from fractions III−V (Table 1).

Fraction I comprised at least two groups of DS chains including those susceptible and unsusceptible to chondroitinase AC II. The former group of DS is probably composed of copolymers containing iduronosyl-*N*-acetylgalactosamine-4-sulfate as a major disaccharide with glucuronosyl-*N*-acetylgalactosamine-4-sulfate as minor disaccharide. The latter group of DS may be composed of homopolymers containing iduronosyl-*N*-acetylgalactosamine-4-sulfate only. However, in this experiment, it was not tested whether or not a single unit of glucuronosyl-*N*-acetylgalactosamine, if present in the DS chain, is recognized by chondroitinase AC II. It has been suggested that testicular hyaluronidase, which degrades hyaluronic acid and CS, does not cleave a single disaccharide of glucuronosyl-*N*-acetylgalactosamine (Fransson and Rodén, 1967; Cöster *et al.*, 1975; Gibson and Pearson, 1982). Thus, the evidence of entire homopolymer of DS is not conclusive.

Most DS chains (but not all) in fraction II were susceptible to chondroitinase AC II and thus were considered to be copolymers composed of iduronosyl-*N*-acetylgalactosamine-4-sulfate and glucuronosyl-*N*-acetylgalactosamine-4-sulfate with a higher proportion of the former. A majority of GAG chains are copolymers containing both iduronosyl and glucuronosyl residues in fraction III, in that more GAGs were digested with chondroitinase AC II than with chondroitinase B. This is consistent with the lower content of iduronic acid compared to that of glucuronic acid in fraction III (Table 1). It is uncertain whether or not small proportions of GAGs apparently undigested with chondroitinase AC II and chondroitinase B, respectively, are related to homopolymers. Most GAG chains in fraction IV appear

to be CS homopolymers. However, if, for example, one iduronosyl residue is involved in glucuronic acid rich GAGs, it is uncertain whether chondroitinase B can digest an iduronosylgalactosamine sulfate. Nonsulfated iduronosylgalactosamine sulfate, which may be present in this undersulfated GAG fraction, is also undigested by chondroitinase B (supplier's information). Thus, as discussed above for the occurrence of DS homopolymers in fraction I, the occurrence of homopolymers in fractions III and IV is uncertain. This would only be clarified by preparing synthetic galactosaminoglycans with known amounts of sulfate and iduronosyl or glucuronosyl residues.

The uronic acid composition of fraction V, which was not studied using enzymatic digestion because of shortage of sample, is less certain. This fraction, which resembled fraction IV in its iduronic acid content, electrophoretic pattern, and concentration to inhibit binding of CS-56 to CS, probably contains galactosaminoglycans highly susceptible to chondroitinase AC II.

The ratio of DS with high iduronic acid content to DS with low iduronic acid content varies among tissue sources. For example, the ratio in the porcine epimysium obtained in this study is similar to that in bovine skin (Gibson and Pearson, 1982) but higher than those in rooster comb and wattle (Nakano and Sim, 1992) and periodontal ligament (Gibson and Pearson, 1982). These differences are difficult to explain in relation to physiological functions of individual tissues.

Chondroitin sulfate chains are the biosynthetic precursors of copolymeric galactosaminoglycans. Iduronic acid is formed by epimerization of glucuronic acid of the CS chain, catalyzed by a glucuronosyl epimerase (Malmström *et al*., 1975). It is not well understood how some CS chains are affected by this enzyme to a relatively small extent, whereas others are altered drastically to form DS with high iduronic acid contents. The present results suggest the presence in the porcine epimysium of galactosaminoglycans with iduronic acid contents ranging from 0 to 100% of total uronic acid. At least 70% of galactosaminoglycans have copolymeric chains containing both iduronosyl and glucuronosyl residues. (The percentage was calculated on the basis of the proportions of uronic acid and of enzyme susceptible fractions in total uronic acid recovered.)

Anti-CS MAb CS-56, reported by Avnur and Geiger (1984), recognizes CS but not DS. It is interesting to know the reactivity of the antibody to the epimysial galactosaminoglycans containing various amounts of iduronic acid. The results obtained with the ELISA inhibition assay in this study showed that fractions I and II had little antigenicity to CS-56 and that most (92%) of the CS-56 epitope in the epimysial galactosaminoglycans is in fractions III−V. The epitope accounted for 1.2% of total galactosaminoglycan uronic acid. From our previous study of rooster comb and wattle (Nakano and Sim, 1992), an average proportion of CS-56 epitope is calculated to be approximately 3% of total galactosaminoglycan uronic acid.

Christner *et al*. (1980) raised MAbs specific to the oligosaccharide stubs of chondroitinase ABC treated proteoglycans, which have been used by many researchers to immunohistochemically localize DS and CS with different sulfation [*e.g*., Fine and Couchman (1988) and Byers *et al*. (1992)]. These antibodies were considered to be inappropriate for the present study of copolymeric GAGs in the epimysium, in that carbohydrate stubs resulting from chondroitinase ABC digestion do not reflect disaccharide composition of the whole GAG chain.

Since GAGs were isolated by tissue proteolysis in this study, it is unknown which proteoglycan DS chains were derived from. The high staining intensity with MAb 6D6 observed in the porcine epimysium confirms the presence of DS in the epimysium and suggests that DS chains were derived at least in part from decorin.

Decorin is a low molecular weight proteoglycan (MW $\sim$ 100 000), which can bind to collagen fibrils and transforming growth factor β (TGF-β) (Kresse *et al*., 1994). Roles of decorin in regulating collagen fibrillogenesis and neutralizing the effect of TGF-β have been suggested (Kresse *et al*., 1994). Decorin may indirectly affect background toughness of meat (Bailey and Light, 1989). Recently, Nishimura *et al*. (1995) studied ultrastructure of intramuscular connective tissues in relation to post-mortem tenderization of meats. They found disappearance of the staining for the Cuprolinic Blue positive proteoglycan (probably decorin) on electron micrographs of the collagen fibrils of the perimysium during aging. The removal of the proteoglycan may be related to the weakening of collagen fibers.

No attempt was made in this study to detect biglycan and versican in the epimysium. Biglycan and versican are proteoglycans containing DS and/or CS produced by fibroblasts (Kresse *et al*., 1994; Zimmermann and Ruoslahti, 1989). It has been reported that GAG composition of decorin and biglycan from a same type of cells is similar (Choi *et al*., 1989; Fransson *et al*., 1991).

The present study provides a basis for more detailed investigation of the structure of GAGs and proteoglycans in the skeletal muscle connective tissues. Studies of muscle connective tissue GAGs and proteoglycans in relation to age, breeds, anatomical locations, muscle fiber types, nutrition, anticoagulant activity in blood, and pathological conditions are important.

ACKNOWLEDGMENT

We thank Brian Turner for photography and the Department of Oral Biology, University of Alberta, for the monoclonal antibody to bovine decorin and for the use of the densitometer.

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Received for review October 19, 1995. Revised manuscript received February 29, 1996. Accepted April 9, 1996.[⊗]

JF950688C

[⊗] Abstract published in *Advance ACS Abstracts,* May 15, 1996.