

ORIGINAL ARTICLE

CHARACTERIZATION OF DECORIN OF THE HUMAN YELLOW LIGAMENT

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Abstract Decorin, a component of the low-molecular-weight proteoglycans (PG) of yellow ligaments was purified, and the structures of the core protein and the glycosaminoglycan (GAG) chains were analyzed. The crude PG fraction extracted under dissociative conditions was subjected to DEAE-Sephacel ion-exchange chromatography, gel-filtration on Sepharose CL-4B, and Octyl-Sepharose CL-4B chromatography, and decorin was obtained. The amino-terminal amino acid sequence of decorin was found to be identical to those of human bone PG-II and human fetal membrane PG-II. The analyses of the GAG chains using two-dimensional electrophoresis on cellulose acetate membranes, affinity high-performance liquid chromatography (HPLC) with a hydroxyapatite column and gel-filtration HPLC after chondroitinase digestion, showed the presence of dermatan sulfate (DS) chains and chondroitin sulfate (CS). Further, it was suggested that CS located in the reducing termini and DS in the non-reducing termini in the hybrid chain.

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Key words: decorin; proteoglycan; glycosaminoglycan; yellow ligament, dermatan sulfate.

原 著

ヒト黄色靭帯のデコリンの性状

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抄録 低分子プロテオグリカン (PG) であるデコリンを黄色靭帯から抽出し、各種クロマトグラフィーで精製した後、そのコアタンパクおよびグリコサミノグリカン (GAG) 糖鎖の構造を解析した。コアタンパクのN末端アミノ酸配列はヒト骨PG-IIおよびヒト胎膜PG-IIのそれと一致していた。GAG糖鎖のセルロースアセテート膜二次元電気泳動、ハイドロキシアパタイトカラムを用いた親和性高速液体クロマトグラフィー (HPLC) およびコンドロイチナーゼ消化後のゲルろ過HPLCによる分析では、GAG糖鎖は還元末端側にコンドロイチン硫酸 (CS)、非還元末端側にデルマタン硫酸 (DS) が局在するハイブリッド型糖鎖であった。

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キーワード: デコリン; プロテオグリカン; グリコサミノグリカン; 黄色靭帯; デルマタン硫酸。

Introduction

Decorin, which is one of the low-molecular-weight proteoglycans (LMWPG), is a complex macromolecule

composed of a glycosaminoglycan (GAG) chain bound covalently to a core protein¹⁾. It is distributed in extracellular matrices (ECM) of various connective tissues. In recent years, the functions of decorin in ECM have been

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reported. For example, decorin can interact with transforming growth factor- β ²⁾ and epidermal growth factor³⁾. Decorin can also bind to type I, II, III, VI and XIV collagens⁴⁻⁶⁾, and it has been found that the PGs as decorin were involved in the regulation, stabilization and assembly of collagen fibers. Therefore, decorin would be considered to have various functions specific to the tissue.

The human yellow ligaments are a series of short ligaments that pass between the vertebrae along the length of the spine. Endochondral ossification may occur with aging in the ligaments⁷⁾, and patients with this disease present neurological symptoms such as myelopathy and radiculopathy, and easily sustain spinal cord injuries. The pathogenesis of ossification might involve many factors, such as genes, cytokines, hormones, and circumference, but the details of the ossification process remain uncertain. From past studies, we found that GAG chains change with aging from dermatan sulfate (DS) chains to chondroitin 6-sulfate near the protein-carbohydrate linkage region, and in the composition of GAG chains, Δ Di-6S instead of Δ Di-4S increases with aging and ossified ligaments⁸⁾. In addition, we found that decorin accounted for the major part of PGs from yellow ligaments⁹⁾.

By the present methods, it is impossible to sequence a GAG chain like a protein, or DNA. In this paper, we report the structure of GAG chains of decorin contained in human yellow ligament analyzed by using two-dimensional electrophoresis and the specific action of chondroitinase ABC and B.

Material and Methods

Materials

Chondroitin 6-sulfate (Ch6S, from shark cartilage), chondroitin 4-sulfate (Ch4S, from whale cartilage), dermatan sulfate (DS,

from pig skin), heparin (Hep, from bovine lung), chondroitinase ABC (from *Proteus vulgaris*) and chondroitinase B (from *Flavobacterium aurescens*) were purchased from Seikagaku Kogyo Co., Tokyo, Japan. Hyaluronic acid (HA, from human umbilical cord) and endo- β -xylosidase were prepared by the methods of Nakamura *et al.*¹⁰⁾ and Takagaki *et al.*¹¹⁾, respectively. Actinase E was purchased from Kaken Kagaku Co., Tokyo, Japan, and the immunoblotting ABC POD (R) Kit and 2-aminopyridine were purchased from Wako Pure Chemical Co., Osaka, Japan. DEAE-Sephacel, Sepharose CL-4B, Octyl-Sepharose CL-4B and the electrophoresis calibration kit were purchased from Pharmacia LKB Biotechnology AB, Uppsala, Sweden. Cellulose acetate membranes and 5-20% acrylamide gradient slab gels were purchased from Jookoo Co., Tokyo and Atto Co., Tokyo, Japan, respectively and anti-human decorin antibody (PG-40) was purchased from Gibco, Grand Island, New York, U.S.A. All the other reagents used were obtained from commercial sources.

Extraction and separation of PGs from human yellow ligaments

Normal human yellow ligaments (268g) were taken from 305 patients from teenagers to those in their eighties, at the time of operation for intervertebral disc herniation or lumbar spinal canal stenosis. This study was approved by The Committee of Medical Ethics of Hirosaki University School of Medicine, Hirosaki, Japan. All of the patients gave their informed consent.

The extraction of PGs from human yellow ligaments was carried out in 5 volumes of 4 M guanidine-HCl/50 mM sodium acetate buffer (pH 5.6) containing protease inhibitors at 4°C for 24 h, as described by Sajdera and Hascall¹²⁾. The extracts were collected

and designated the crude PG fraction. This fraction was dialyzed against 7 M urea/50 mM Tris-HCl buffer (pH 7.2) at 4°C for 48 h. After that, the dialyzed inner solution was applied to a DEAE-Sephacel column (1.0 x 15.0 cm) equilibrated with 7 M urea/50 mM Tris-HCl (pH 7.0), which was eluted with a linear concentration-gradient of NaCl (0-1.0 M) at a flow rate of 60 ml/h and 20-ml fractions were collected. Each fraction was assayed for uronic acid by the carbazole reaction¹³, and for protein by the protein-dye binding method¹⁴. The uronic acid-containing fractions were pooled. The precipitate by 80% ethanol was collected by centrifugation, and then soluted in 4 M guanidine-HCl/50 mM sodium acetate buffer (pH 5.6). These fractions were subjected to gel-filtration chromatography on a Sepharose CL-4B column (3.3 x 175 cm) at a flow rate of 60 ml/h, 20-ml fractions were collected, and the LMWPG fractions were separated.

For further purification, chromatography on an Octyl-Sepharose CL-4B column (3.4 x 29 cm) was carried out under the conditions described by Choi et al.¹⁵. The uronic acid-containing (LMWPG) fractions were dialyzed against 2 M guanidine-HCl/50 mM sodium acetate buffer (pH 5.6) at 4°C for 24 h. After that, the fractions were applied to the column, which was equilibrated with 2 M guanidine-HCl/50 mM sodium acetate buffer (pH 5.6), and eluted with a linear concentration-gradient of 2-6 M guanidine-HCl/50 mM sodium acetate buffer (pH 5.6) at a flow rate of 60 ml/h and 20-ml fractions were collected, which were designated the LMWPG fractions.

Enzymic digestions

To obtain the GAG chain, first, the core protein was digested with Actinase E (0.1 M Tris-HCl buffer, pH 8.0, containing 10 mM CaCl₂) which was one of the proteases.

Secondly, the residual core protein was eliminated from the GAG chains with endo- β -xylosidase (0.1 M sodium acetate buffer, pH 4.0)¹⁶ in the reducing termini. This enzyme catalyzes the eliminative cleavage of Xylose of the GAG chain linkages to Serine residue of the core protein. The GAG chains were digested with chondroitinase ABC (0.1 M Tris-HCl buffer, pH 8.0)¹⁷ which can be used for selective removal of the chondroitin sulfate or dermatan sulfate side chains, and chondroitinase B (0.1 M Tris-HCl buffer, pH 8.0)¹⁸ which can be used for selective removal of the dermatan sulfate side chains.

Electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out, as described by Rosenberg et al.¹⁹. SDS-PAGE was carried out on 5-20% acrylamide gradient slab gels at 4°C at 200 V for 4 h. The gels were stained successively with Coomassie brilliant blue R-250 followed by Alcian blue, as described by Fisher et al.²⁰.

Two-dimensional electrophoresis was carried out on cellulose acetate membranes (10 x 10 cm), as described by Yokoyama et al.²¹. The first dimension was obtained in 0.1 M pyridine/0.47 M formic acid buffer (pH 3.0) at 1 mA/cm for 1 h, and the second with 0.1 M barium acetate (pH 8.0) at 1 mA/cm for 4 h. The membranes were stained with 0.1% Alcian blue in 70% ethanol.

Immunoassay

Western blotting immunoassay for PGs using anti-human decorin antibody was carried out, as described by Towbin et al.²². Anti-human decorin antibody was from polyclonal antiserum of rabbits immunized with a peptide representing the carboxyl terminus of human decorin. It has specific activity against only decorin²³.

Fluoro-labeling

The prepared GAG chains were

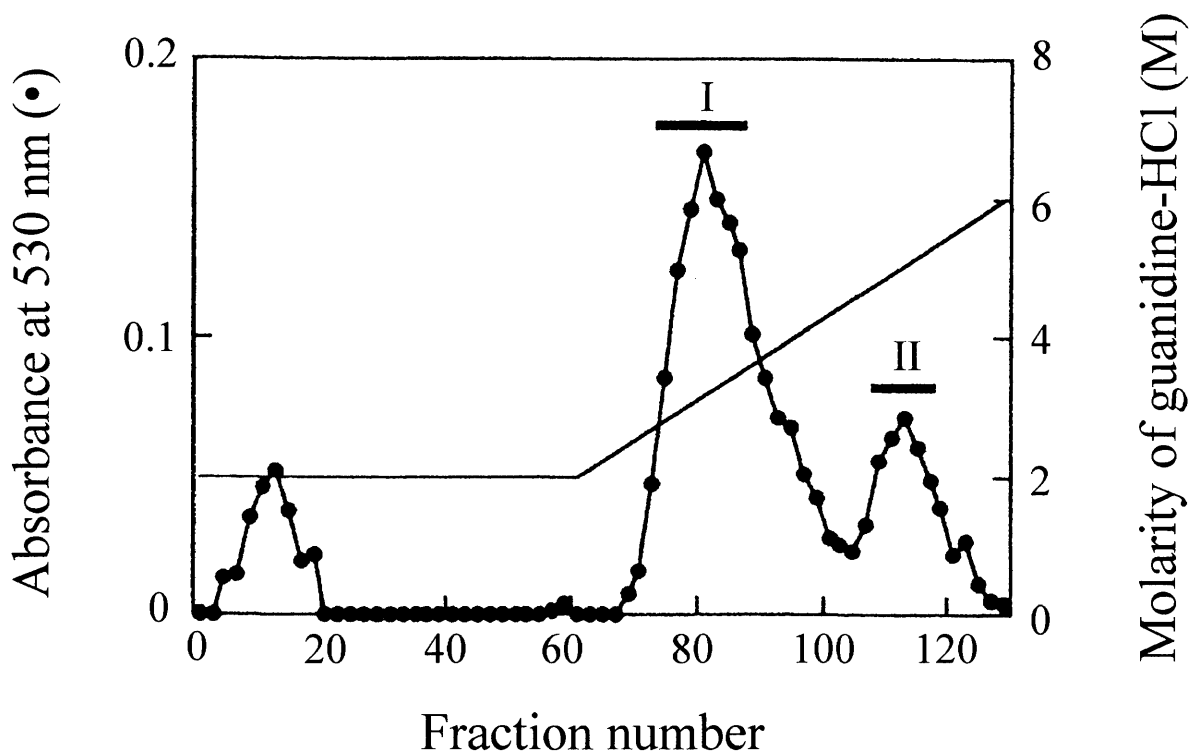


Figure 1 Separation of the low-molecular-weight proteoglycans by Octyl-Sepharose CL-4B. In order to separate the low-molecular-weight proteoglycans, chromatography was carried out on an Octyl-Sepharose CL-4B column (3.4 x 29 cm). The column was eluted with a linear concentration-gradient of 2-6 M guanidine-HCl/50 mM sodium acetate buffer (pH 5.6) at a flow rate of 60 ml/h and 20-ml fractions were collected. Each fraction was monitored for uronic acid (530 nm).

pyridylaminated at their reducing termini by the method of Kon *et al.*²⁴. The GAG chains were coupled with 2-aminopyridine with reductive amination to give pyridylaminated GAG chains (PA-GAG chains).

Analysis of the amino acid compositions and sequence

The amino acid compositions of the core protein were analyzed. The samples were hydrolyzed with 6 N HCl at 135°C for 3 h. This hydrolyzed solution was evaporated and soluted in distilled water again. This solution was analyzed with a Hitachi L-8500 amino acid automatic analyzing meter (Hitachi Co., Tokyo, Japan).

The amino-terminal sequence, and the inner sequence of PG-II core proteins followed by cyanogens bromide (BrCN) cleavage²⁵ were analyzed using a Hewlett Packard

G100A protein sequencer²⁶.

High-performance liquid chromatography (HPLC)

Affinity HPLC was carried out with a KB-0012 column (7.8 x 100 mm, Koken Co., Tokyo, Japan). The column was eluted with a linear gradient of increasing molarity (10-100 mM) of sodium phosphate buffer (pH 6.8) for 10-40 min at flow rate of 1.0 ml/min at 30°C²⁷.

Size-fractionation HPLC was carried out with a Shodex OHpak SB-803 HQ column (8 x 300 mm, Shoko Co., Tokyo, Japan) using 0.2 M NaCl. The flow rate was 0.5 ml/min and the column temperature was 30°C.

For detection, an excitation wavelength of 320 nm and an emission wavelength of 400 nm were used (Hitachi F-1050).

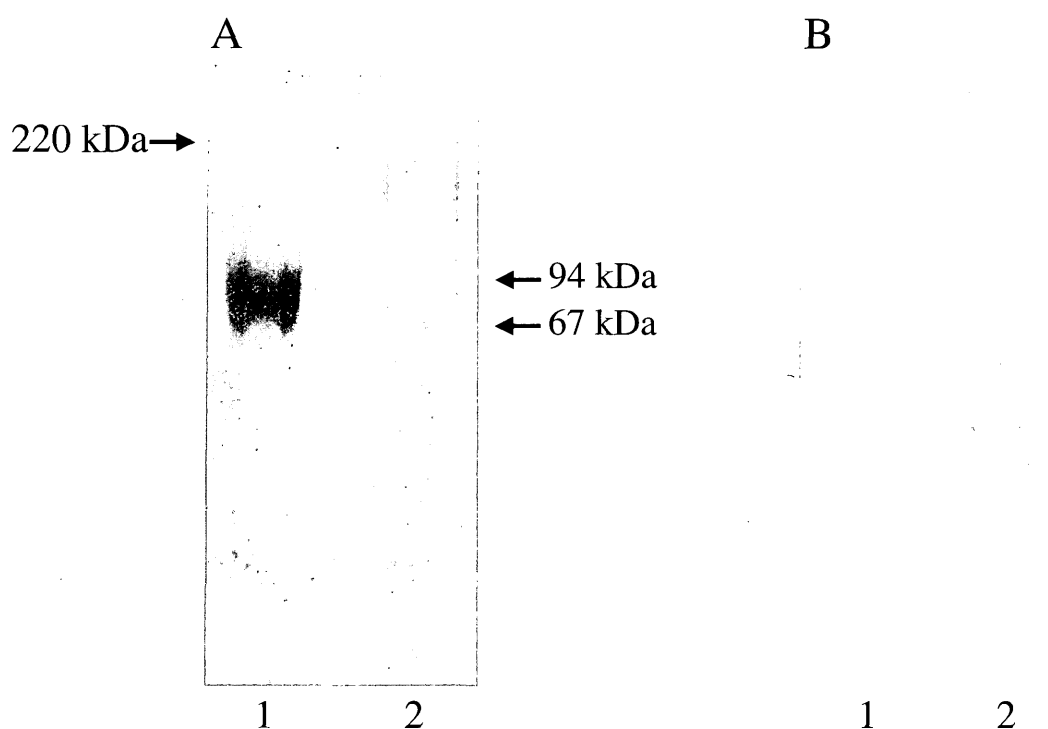


Figure 2 Analysis of the low-molecular-weight proteoglycans by SDS-PAGE and immunoassay. The proteoglycan fraction eluted from an Octyl-Sepharose CL-4B column (peak I and II in Figure 1) were collected and subjected to SDS-PAGE on 5 to 20% acrylamide gradient slab gels at 4°C at 200 V for 4 h, after which the gels were stained with Coomassie brilliant blue R-250 followed by Alcian blue (A). Western blotting immunoassay using anti-human decorin was carried out (B). Lane 1, peak I shown in Figure 1; lane 2, peak II shown in Figure 1.

Results

Separation of the LMWPGs

The crude PG fraction extracted with 4 M guanidine-HCl/50 mM sodium acetate buffer (pH 5.6) was applied to a DEAE-Sephacel column, and the eluted uronic acid-containing fractions using a NaCl concentration were collected and subjected to Sepharose CL-4B gel-filtration chromatography. The two peaks, which were reacted with the carbazole reaction, were found. They were pooled as the LMWPG fractions (figures are not shown).

For further separation, the LMWPG fractions were applied to an Octyl-Sepharose CL-4B column (Figure 1). The column was eluted with a linear-concentration gradient of 2-6 M guanidine-HCl/50 mM sodium acetate buffer (pH 5.6), and the uronic acid-containing

fractions were eluted at guanidine-HCl concentrations of about 3.35 M (peak I) and 5.0 M (peak II). The total amount of uronic acid which was contained in peak I was about 27 mg. It was suggested that more than two types of the LMWPGs which possess different hydrophobities of core protein are contained in human yellow ligaments.

Analysis by SDS-PAGE and immunoassay

The PGs contained in the fractions of each peak eluted from an Octyl-Sepharose CL-4B column were analyzed by SDS-PAGE (Figure 2-A). A single band appeared for the PG of peak I fractions at molecular weight of 70,000-95,000, while two bands appeared for those in peak II fractions at molecular weights of 70,000-95,000 and 180,000-200,000, and were designated PG-II, and PG-III and PG-I, respectively. These numbers of PG-I and

PG-II were the same as those of human bone²⁸⁾ and fetal membrane²⁹⁾. PG-III was the characteristic number of the present study.

Western blotting immunoassay for PG-I, PG-II and PG-III was carried out (Figure 2-B). Anti-human decorin reacted with PG-II, but not with PG-I and PG-III. It was suggested that PG-II was decorin, while PG-I and PG-III possess different core proteins from that of PG-II.

Analysis of the amino acid compositions and sequence of the core protein of PG-II

The amino acid compositions of PG-II are shown in Table 1. The compositions of human yellow ligaments PG-II were not identical but were similar to those of human bone PG-II²⁸⁾ and fetal membrane PG-II²⁹⁾. The core protein contained 329 residues and its molecular weight was 36,400.

The amino-terminal sequence of PG-II is shown in Table 2. The sequence of human

yellow ligament PG-II was identical to those of human bone PG-II²⁸⁾ and human fetal membrane²⁹⁾. It was suggested that this LMWPG is possessed of the same core protein as the decorins previously reported.

Analysis of the GAG chains of PG-II

In order to identify the GAG chains of PG-II, GAG chains were first prepared by digesting PG-II with Actinase E and endo- β -xylosidase and then analyzed by two-dimensional electrophoresis on cellulose acetate membranes (Figure 3). It was found that DS, and Ch6S extended from DS were contained in the GAG chains of PG-II.

Secondly, after the GAG chains were fluoro-labeled at their reducing termini with 2-aminopyridine, they were analyzed by HPLC on a hydroxyapatite column (Figure 4). The PA-GAG chains were eluted as bimodal distribution with a linear gradient of increasing molarity (10-100 mM) of sodium

Table 1 Amino acid analysis residues/1000

	Human yellow ligament PG-II	Human bone ^a PG-II	Human fetal membrane ^c PG-II
Asp (D)	133	133	108
Thr (T)	41	52	60
Ser (S)	69	75	77
Glu (E)	88	122	101
Gly (G)	78	94	103
Ala (A)	45	50	62
Val (V)	60	51	66
1/2-Cys (C)	27	ND ^b	11
Met (M)	9	ND	14
Ile (I)	59	42	46
Leu (L)	126	109	99
Tyr (Y)	28	10	17
Phe (F)	32	29	34
Lys (K)	65	54	62
His (H)	26	20	34
Arg (R)	45	42	34
Try (W)	3	ND	ND
Pro (P)	67	70	72

^a Ref. No. [28].

^b ND, not determined.

^c Ref. No. [29].

Table 2 Amino acid sequences
X, no assigned amino acid; (), tentative amino acid assignment

	1	6	11	16
Human yellow ligament PG-II	NH2 DEASG	IGPEV	PDDRD	
Human bone PG-II ^a	NH2 (D) EAXG	I (A) PEV	PDD (R) (P)	FEPS
Human fetal membrane PG-II ^c	NH2 DEAXG	IGPEV	PDDR	

^a Ref. No. [28].

^b Ref. No. [29].

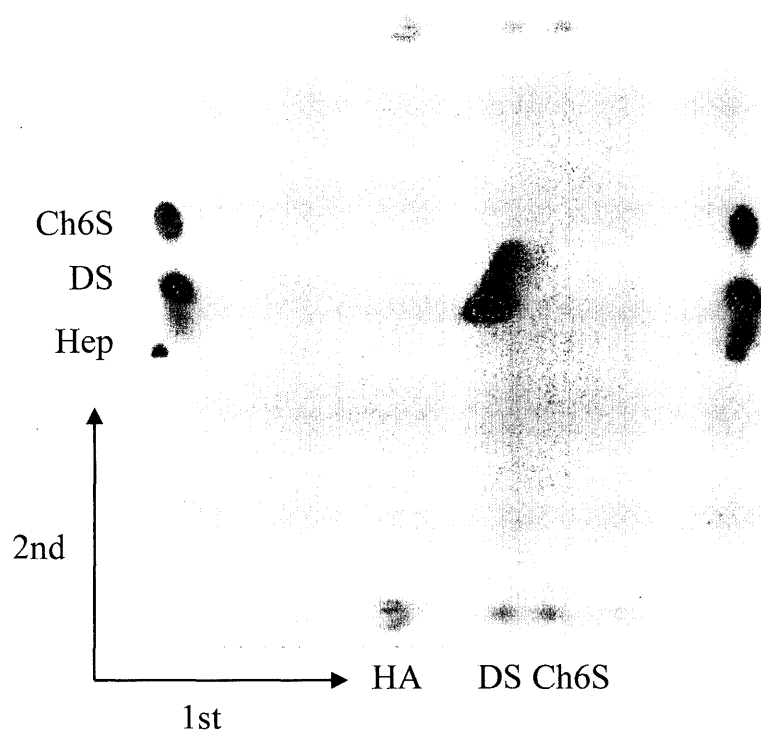


Figure 3 Two-dimensional electrophoresis on cellulose acetate membranes of the glycosaminoglycan chains of PG-II. The glycosaminoglycan chains from PG-II obtained by Actinase E and endo- β -xylosidase digestions were subjected to electrophoresis on cellulose acetate membranes (10 x 10 cm) in the first dimension with 0.1 M pyridine/0.47 M formic acid buffer (pH 3.0) at 1 mA/cm for 1 h, and in the second with 0.1 M barium acetate (pH 8.0) at 1 mA/cm for 4 h. The membranes were stained with 0.1% Alcian blue in 70% ethanol. DS, dermatan sulfate; Ch6S, chondroitin 6-sulfate; Hep, heparin; HA, hyaluronic acid.

phosphate buffer (pH 6.8). The analysis of the GAG chains showed that those of decorin were distributed from DS to CS compared with standards. It was suggested that the GAG chains contained in this decorin are composed of DS and CS.

Next, in order to examine the existing form of DS and CS in the GAG chains, the chondroitinase ABC and chondroitinase B digests of the PA-GAG chains were analyzed by HPLC on a Shodex SB 803 HQ gel-filtration column (8 x 300 mm) (Figure 5). After chondroitinase ABC treatment, the GAG chains were completely digested to PA-tetrasaccharides and PA-hexasaccharides, however, after chondroitinase B treatment, some were only partially digested. That is to say, the GAG chains, which possess the parts of CS and resist the chondroitinase B

digestion, were present.

It was suggested that two types of GAG chains exist in decorin of yellow ligaments, one of which is a major component, a DS chain, and the other a hybrid chain of DS and CS. Further, it was suggested that part of CS is located in the reducing terminal side of the part of DS in the hybrid chains.

Discussion

Three types of LMWPGs could be separated from human yellow ligaments in the current study. PG-II was identified as decorin, because the core protein of PG-II reacted with anti-human decorin antibody. The authors analyzed the structure of decorin, which is considered to be a major component in the connective tissue of human yellow ligaments. N-Terminal amino acid sequence

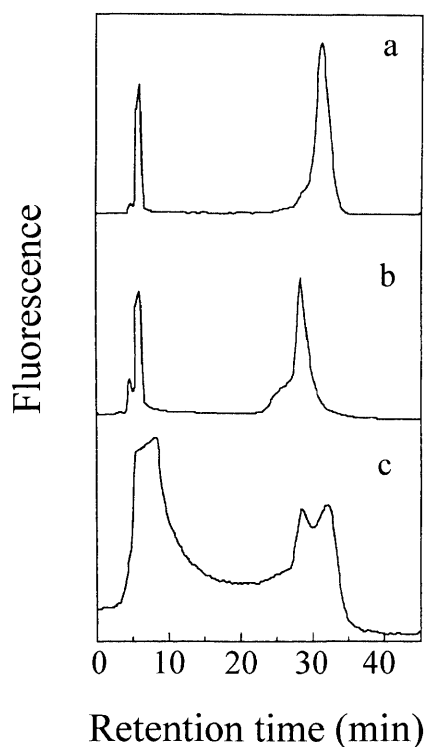


Figure 4 Affinity HPLC of the PA-glycosaminoglycan chains of PG-II on a hydroxyapatite column. The PA-glycosaminoglycan chains were examined with HPLC using a KB-0012 hydroxyapatite column (7.8 x 100 mm). The column was eluted with a linear gradient of increasing molarity (10-100 mM) of sodium phosphate buffer (pH 6.8) for 10-40 min at a flow rate of 1.0 ml/min at 30°C. For detection, an excitation wavelength of 320 nm and an emission wavelength of 400 nm were used. a, PA-dermatan sulfate; b, PA-chondroitin 6-sulfate; c, PA-glycosaminoglycan chains from PG-II.

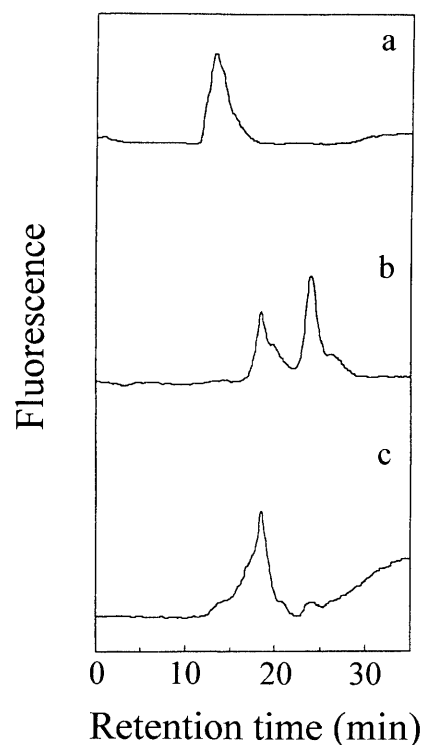


Figure 5 Size-fractionation HPLC of the PA-glycosaminoglycan chains of PG-II before and after chondroitinase digestion. The PA-glycosaminoglycan chains after digestion without or with chondroitinase ABC and chondroitinase B were analyzed by HPLC using a Shodex SB803 HQ column (8 x 300 mm). The column was eluted with 0.2 M NaCl at a flow rate of 0.5 ml/min at 30°C. For detection, an excitation wavelength of 320 nm and an emission wavelength of 400 nm were used. a, PA-glycosaminoglycan chains before digestion; b, after chondroitinase ABC digestion; c, after chondroitinase B digestion.

of the core protein of decorin was identical to those from human fetal membrane and human bone. These results were also identical to the results reported by Yukawa *et al.*³⁰⁾

The authors analyzed the composition of the GAG chain from decorin by the methods of two-dimensional electrophoresis and HPLC using PA-GAG chains. From these results, it was demonstrated that the GAG chain of decorin was composed of two kinds of GAG, which were DS and CS, and were found to be a single molecular size. Further,

from observation of digestion patterns of PA-GAG, the locations of CS and DS in GAG chains were speculated. PA-GAGs of decorin were digested with chondroitinase ABC, and two peaks were found indicating that GAG chains were completely digested to tetra- and hexasaccharides. While, after chondroitinase B digestion, the peak was shifted to the location of hexasaccharides, PA-GAGs were not completely digested and more than hexasaccharides were contained. This finding indicated that the GAG chains were hybrid, in which CS was located in the reducing terminal

site and DS was located in the non-reducing terminal site because chondroitinase B is an exo type enzyme. Recently, Yukawa et al. reported on the structures of the GAG chains and core proteins of LMWPGs³⁰. They analyzed decorin by HPLC analysis after chondroitinase digestion and demonstrated that part of CS is located in the reducing terminal side of part of DS. In the present study, we analyzed the composition of the GAG chains of decorin by different methods than theirs, and demonstrated that the GAG chains were hybrid GAG chains.

Okada et al., in analyzing the LMWPGs from yellow ligaments, reported that the GAG chains were mainly composed of DS in young patients and Ch6S in elderly patients⁹. Takeda et al. reported that the GAG chains in normal yellow ligaments were DS and those in ossified yellow ligaments were CS¹⁰. In this study, it was demonstrated that these changes of GAG chains from DS to CS with age and ossification were the changes of inner structure of the GAG chains, since the hybrid GAG chains composed of DS and CS were present. Decorin accounted for the major part of PGs from yellow ligaments. That is to say, these changes of the GAG chains of decorin were related to the ossification of yellow ligaments.

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