[Chem. Pharm. Bull. 36(12)4881—4890(1988)]

# Biochemical and Immunochemical Characterization of Proteodermatan Sulfate from Calf Skin

SATOSHI ONODERA

*Department of Clinical Chemistry, Showa College of Pharmaceutical Sciences, 5-1-8 Tsurumaki, Setagaya-ku, Tokyo 154, Japan*

(Received June 1, 1988)

A proteodermatan sulfate (PDS) was extracted from fresh calf skin with 3 M $MgCl_2$ in the presence of protease inhibitors and purified repeatedly by dimethylaminoethyl (DEAE)-cellulose chromatography. The average molecular weights were estimated to be 112000 for PDS and 56000 for core protein from chondroitinase ABC-treated PDS in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Unsaturated disaccharide of the glycosaminoglycan side chain (molecular weight = 20000) of PDS was found to be mainly composed (96.8%) of ΔDi-4S [2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-4-*O*-sulfo-D-galactose] as determined by high-performance liquid chromatography (HPLC).

Affinity-purified rat antibody against the core protein of PDS reacted specifically with PDS, but there was no cross-reaction with extracellular molecules such as A1-D1 proteoglycan from cartilage, fibronectin, laminin, and types I, II, III and IV collagens. Furthermore, high specificity of the antibody to PDS was also observed by immunoblotting after SDS-PAGE. Indirect immunofluorescence staining of anti-core protein antibody in tissues generally appeared along with interstitial collagen (types I, II and III). However, the fine reticular fiber composed of types I and III collagens in liver was not stained.

On the basis of these findings and of biochemical characterization of PDS, it is postulated that PDS possibly contributes to promotion of collagen fibrillogenesis and deposition in the extracellular matrix *in vivo*.

**Keywords**——proteoglycan; proteodermatan sulfate; core protein; glycosaminoglycan; unsaturated disaccharide; immunoblotting; collagen

## Introduction

Proteoglycans are major components of the ubiquitous extracellular matrix together with collagen in mammalian and avian tissues, and have been implicated generally in morphological interaction.[1] Since the development of an efficient procedure for the extraction of proteoglycans from connective tissues as described previously,[1b,2] it has become possible to isolate quantitatively proteoglycans from connective tissues in a more native state.

Recently, intensive studies on collagen fibril formation in the presence of various glycosaminoglycans have been performed.[3] The previous studies on the changes in the synthesis of glycosaminoglycans and collagen in embryonic chick skin showed that hyaluronic acid and chondroitin sulfate components decreased at an early stage, then dermatan sulfate components increased gradually together with accelerated deposition of collagen during development.[4] Parry *et al.*[5] reported that tissues with the largest diameter collagen fibrils (mass average diameter 200 nm) have a high concentration of dermatan sulfate. On the other hand, Scott and Orford[6] reported that dermatan sulfate rich in proteoglycan is associated with rat tail tendon collagen at the *d* band in the *gap* region by using an electron microscope. These observations led me to elucidate whether or not proteodermatan sulfate functions in promotion of collagen fibrillogenesis and deposition in the extracellular matrix of connective tissues. However, there is as yet little understanding of the function and

immunochemical properties of proteodermatan sulfate. For this reason, I prepared antibody against the core protein of proteodermatan sulfate and characterized the immunological properties of the antigen. This work is presented here.

## Materials and Methods

**Materials**——Fresh calf skin was obtained from a local slaughterhouse and was frozen at −20°C until used. Phenylmethanesulfonyl fluoride (PMSF), *N*-ethylmaleimide (NEM), pepstatin A and pronase E were purchased from Sigma. Dermatan sulfate (DS, from hog skin), chondroitin-4-sulfate (CS, from whale cartilage), hyaluronic acid (HA, from hog skin), heparin (HP, from hog intestine), chondroitinase ABC (from *Proteus vulgaris*) and AC II (from *Arthrobacter aurescens*), and unsaturated disaccharide units ΔDi-0S [2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-D-galactose], ΔDi-4S [2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-4-*O*-sulfo-D-galactose] and ΔDi-6S [2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-6-*O*-sulfo-D-galactose] were from Seikagaku Kogyo Co., Tokyo, Japan. Nitrocellulose membrane was from Bio-Rad Laboratories, California. Cellulose acetate membrane (Separax) was from Jookoo Sangyo Co., Ltd. Tokyo. Fluorescein isothiocyanate (FITC)-conjugated anti rat immunoglobulin G (IgG) rabbit IgG and horseradish peroxidase (HRPO)-conjugated anti rat IgG rabbit IgG were from MBL Company. Dimethylaminoethyl (DEAE)-cellulose (DE 32) was from Whatman. Fibronectin from human blood,[1b] laminin from human placenta,[7] cartilage proteoglycan A1-D1 monomer from bovine,[2] and collagens (types I, III and IV) from human placenta and type II from bovine articular cartilage[1b] were prepared as described previously.

**Proteoglycan Isolation**——After thawing, samples of calf skin (500 g wet weight) were freed of hair and fat, cut into small pieces and washed several times with 0.05 M Tris–HCl buffer (pH 7) containing 10 mM ethylenediaminetetraacetic acid disodium salt (EDTA), 10 mM NEM, 1 mM PMSF and 0.36 mM pepstatin A. The samples were extracted by gentle stirring for 48 h with 5 l of 3 M $MgCl_2$ in the same buffer and inhibitors, and furthermore the residue was extracted for 24 h with 2 l of the same solution and the two extracts were combined.

The extract was exhaustively dialyzed against water to precipitate proteoglycan and collagen complex. After centrifugation at 15000 × *g* for 20 min, the residue was dissolved in 7 M urea, 0.05 M Tris–HCl buffer (pH 7) containing inhibitors (buffer A). After removal of undissolved protein by centrifugation, the solution (about 1.5 l) was directly applied to a DEAE-cellulose column (2.6 × 24 cm) equilibrated with buffer A. After washing of the column with buffer A containing 0.15 M NaCl, the proteoglycan was eluted with buffer A containing 2 M NaCl. In this procedure, major collagen and other proteins were removed with 0.15 M NaCl solution. For futher purification, the 2 M NaCl eluate (alcian blue-positive) was dialyzed against 4 M urea, 0.15 M NaCl, 0.05 M Tris–HCl buffer pH 7 (buffer B), and rechromatographed on a DEAE-cellulose column equilibrated with buffer B; elution was done with the same solution containing 0.2 M NaCl, 0.25 M NaCl and 1 M NaCl. The proteoglycan recovered at 0.25 M NaCl was dialyzed against water, lyophilized (42 mg) and stocked at −20°C until used.

**Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)**——SDS-PAGE was performed according to the method of Laemmli.[8] A 10% acrylamide gel was prepared in 0.1% SDS, 0.375 M Tris–HCl buffer (pH 8.8), to which was applied a 3.6% acrylamide stacking gel in 0.125 M Tris–HCl buffer (pH 6.8). The running buffer was 0.38 M glycine, 0.1% SDS, 0.05M Tris–HCl buffer (pH 8.4). The sample buffer contained 1% SDS, 0.0006% bromphenol blue, and 16% glycine in 0.08 M Tris–HCl buffer (pH 6.8). SDS-PAGE was carried out at 3 mA/tube for 2.5 h at room temperature. The gels were removed and stained for 15 min in 0.1% Coomassie brilliant blue R-250 for protein or in 0.2% toluidine blue for proteoglycan (PG).[9] The gels were destained in 7% acetic acid, 25% methanol for protein or in 3% acetic acid for PG.

**Isolation and Analysis of Glycosaminogiycans (GAGs)**——PG solution was brought to 0.5 M NaOH by adding 5 M NaOH and kept at 4°C overnight. After adjustment of the pH to 6—8, an equal volume of 0.1 M Tris–HCl buffer (pH 7.8) containing 5 mM $CaCl_2$ was added, and the resulting solution was boiled for 30 min, then subjected to pronase digestion, followed by deproteinization with trichloroacetic acid, dialysis, and lyophilization to isolate GAGs. The isolated GAGs were subjected to two-dimensional electrophoresis on cellulose acetate membranes.[10] A cellulose acetate membrane (10 × 10 cm) kept in 25% methanol was soaked in 0.1 M pyridine–0.47 M formic acid buffer (pH 3) before sample application. In the first dimension, 2 to 5 μl of sample (about 1 μg of GAGs) was placed on the same line as a reference GAGs solution which contained 1 μg each of CS, DS and HA. An indigo tetrasulfonate as a guide marker was spotted at the same place as the reference GAGs. Electrophoresis was carried out at 10 mA until the guide marker reached within about 10 mm from the anode side of the membrane (about 1 h). The reference GAGs run was cut off for staining. In the second dimension, further reference GAGs (CS, DS and HP) were applied on the same line as the sample migrated in the first run. Electrophoresis was carried out with 0.1 M barium acetate as the running buffer at 1 mA/cm for 4 h. After electrophoresis, the membrane was stained for 15 min in 0.1% alcian blue in 0.1% acetic acid and destained in 0.1% acetic acid.

**Chemical Analysis and Enzymatic Treatment of PG or GAGs**——Uronic acids were measured by the borate–carbazole method with D-glucuronolactone as a standard.[11] Digestion of PG with chondroitinase ABC (0.1 unit) was

done at 37 °C for 4 h in 0.1 M sodium acetate, 0.1 M Tris–HCl buffer (pH 7.6) containing the following protease inhibitors: 10 mM EDTA, 10 mM NEM, 1 mM PMSF and 0.36 mM pepstatin A.[12] Digestion of GAGs with chondroitinase ABC or AC II was done under the same conditions but without inhibitors.[10] The resulting materials were subjected to electrophoresis.

**Amino Acid Analysis**——Samples (500 μg) were hydrolyzed in 0.5 ml of 6 M HCl in an evacuated tube at 110 °C for 24 h. After evaporation, amino acid analysis was performed on a JEOL 6AS autoanalyzer.

**High Performance Liquid Chromatography (HPLC) Procedure**——The unsaturated disaccharides, products of chondroitinase ABC-digested GAGs, were analyzed by using the VYDAC 201TP ODS column (4.6 × 250 mm), which is a reversed phase column with a silica backbone, the average particle size being 10 μm. A suspension of 0.1 ml of DS (310 μg) in 0.1 M Tris–acetate buffer (pH 7.6) and 0.1 ml of 0.1 unit chondroitinase ABC in the same buffer were incubated at 37 °C for 16 h. The reaction mixture was added to 1 ml of water and then loaded on a Dowex 50W × 2 column (1 × 2 cm), washed three times with 1 ml of water, and lyophilized. The residues were suspended in 200 μl of 0.035 M tetrabutylammonium buffer (pH 7.5),[13] and then filtered through a Millipore filter (0.45 μm). An aliquot of the filtrate was injected into the HPLC column and the column was eluted with 0.035 M tetrabutylammonium buffer, the eluent being pumped at 0.6 ml/min. The ultraviolet (UV) absorption at 232 nm was recorded.

The gel filtration of GAGs was carried out using a TSK G3000SW column (7.5 × 600 mm, Toyo Soda), having an average particle size of 10 μm. The column was eluted with 0.1 M phosphate buffer (pH 7) at a flow rate of 0.5 ml/min. The detection of GAGs was performed by using the carbazole method.[11]

**Preparation of Core Protein as Antigen**——Purified proteodermatan sulfate (PDS) (10 mg) was dissolved in 5 ml of 0.15 M NaCl, 0.02% $NaN_3$, 0.05 M Tris–HCl buffer (pH 7.6) containing four protease inhibitors as described above and digested with 0.5 unit of chondroitinase ABC at 37 °C for 16 h to remove GAGs.[12] The core protein as an antigen was prepared by passing the reaction mixture through an anti bovine albumin antibody-Sepharose 4B column (1.5 × 5 cm) to remove albumin added to the enzyme as a stabilizer. The core protein was dialyzed against water and freeze-dried (4.7 mg).

**Immunodetection Procedure**——The specificity of anti core protein antibody was determined by using passive hemagglutination assay (PHA), the double immunodiffusion method[14] and enzyme-linked immunosorbent assay (ELISA). PHA was tested by using sheep red blood cells (SRBC), which were first fixed with glutaradehyde, then coated with PDS or core protein by the tannic acid fixation method as described by Herbert.[14] A suspension of the coated cells (0.5%, 25 μl) was incubated overnight at room temperature with 25 μl of a series of dilutions of antibodies. The PHA inhibition test was performed by preincubating a constant amount of antibody with 0.5—8.1 μg of test samples prior to the addition of coated cells. Immunodiffusion analysis was performed in 0.6% agarose in phosphate buffer (pH 8.6) by the Ouchterlony two-dimensional diffusion technique. ELISA was carried out by using the method described by Rennard *et al.*[15] Samples tested were bonded at 4 °C in the microtiter plates (96 wells) and incubated with anti core protein antibody diluted in 0.05% Tween 20, phosphate-buffered saline (PBS). The second antibody was peroxidase-conjugated anti rat IgG rabbit IgG. Activity of peroxidase was determined by the addition of substrate solution consisting of 0.01% *o*-phenylenediamine and $H_2O_2$. After 4 h at room temperature, the absorbance at 492 nm was recorded with a Multiscan plate reader.

**Preparation of Antibody to Core Protein**——Antibodies were raised in Sprague–Dawley rats. Approximately 0.4 mg of the core protein was suspended in 1 ml of 1/15 M PBS and 1 ml of complete Freund's adjuvant. The mixture was injected under the skin of the back of each rat.

Booster injections with incomplete Freund's adjuvant were given twice, after three weeks and six weeks. The rats were bled at intervals of one or two weeks and the presence of antibodies was monitored by using PHA. The serum was collected eight weeks after immunization. The antibody from the serum was purified by chromatography on a chondroitinase ABC–CH–Sepharose 4B column to remove antibody to the enzyme and on a PDS–CH–Sepharose 4B column (3 ml gel). The bound antibodies were eluted with 3 M NaSCN, 0.05 M Tris–HCl buffer (pH 7.6) and rapidly dialyzed against 0.05 M Tris–HCl buffer (pH 7.6) containing 0.02% sodium azide.

**Immunoblotting**——Samples were first subjected to SDS-PAGE (slab gel, 10%). Electroblotting[16] onto nitrocellulose using Bio-Rad Trans Blot equipment was done at 0.3 A for 16 h, and subsequent washing of the nitrocellulose was done in 1% bovine serum albumin (BSA). The sheet was then incubated for 4 h at 37 °C with anti core protein antibody diluted (1 : 100) in PBS containing 0.1% BSA, washed with PBS and incubated for 1 h with peroxidase-conjugated rat anti rabbit IgG diluted (1 : 1000) in PBS containing 0.1% BSA. The sheet was washed with PBS and incubated for 20 min with 0.06% 4-chloronaphthol and 0.01% $H_2O_2$, then washed with water.

**Immunofluorescence Staining**——Fresh normal bovine tissues and human skin were quickly frozen with *n*-hexane cooled with dry ice acetone. Sections (6 μm) sliced with a cryostat microtome were mounted on glass slides, air-dried and pretreated with acetone for 10 min. The sections were incubated with anti core protein antibody diluted 1 : 32 with 0.15 M NaCl in PBS, kept in a moist chamber for 1 h at room temperature, then rinsed in PBS, and subsequently stained with FITC-conjugated anti rat IgG rabbit IgG diluted 1 : 16 in PBS for 1 h, rinsed with PBS and mounted in 50% glycerol in PBS. The stained sections were observed under a Zeiss MC63 fluorescence microscope.

## Results

### Proteoglycan Isolation and Properties

Extraction of the calf skin with a buffer containing 3 M $MgCl_2$ resulted in solubilization of about 72% of the total uronic acid. The dialysis of the extracted solution against water resulted in precipitation of major PG (mainly PDS) and collagen. Its supernatant fluid mainly contained HA and other proteins. The precipitated material dissolved in 7 M urea was directly applied to a DEAE-cellulose column. Approximately 76% of the uronic acid-containing material was recovered in the fraction eluted from the first DEAE-cellulose column with 2 M NaCl. In this procedure, contaminants such as major collagen and glycoproteins in extracted solution were removed by washing with 0.15 M NaCl solution. Furthermore, to remove minor proteins, the 2 M NaCl fraction dissolved in 4 M urea was applied to the second DEAE-cellulose column. The 0.2 M NaCl and 0.25 M NaCl fractions were both alcian blue-positive, but significant quantities of uronic acid were present in the 0.25 M NaCl fraction. Amounts of proteoglycan obtained were approximately 42 mg (from about 1 kg of calf skin). Analysis of the GAG of the 0.25 M NaCl fraction by two-dimensional electrophoresis on a cellulose acetate membrane showed the presence of a single dermatan sulfate spot (Fig. 1, inset). This spot was sensitive to chondroitinase ABC, but not to chondroitinase AC.

To estimate the size of the proteoglycan, purified PDS and its core protein were subjected to SDS-PAGE without any reductant such as 2-mercaptoethanol. As shown in Fig. 2 (lanes 3 and 4), each SDS-PAGE showed a single band with an apparent molecular weight (Mr) of 112000 (PDS) or 56000 (core protein), which was stainable with Coomassie brilliant blue. The mobility of these bands was not substantially affected by reduction of the sample, indicating that they are not composed of disulfide-bonded subunits. The amino acid composition of PDS

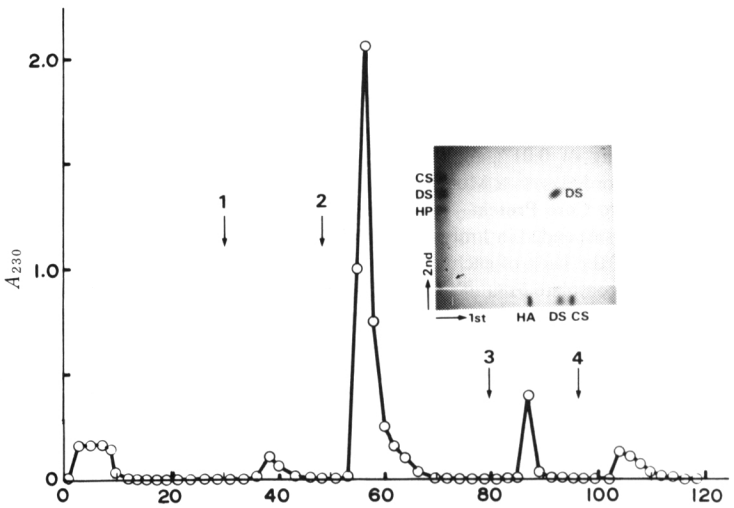


Fig. 1. DEAE-Cellulose Column Rechromatography of PDS from Calf Skin and Two-Dimensional Electrophoresis Patterns of GAGs

The 2 M NaCl eluate from the DEAE-cellulose column was dialyzed against 4 M urea, 0.15 M NaCl, 0.05 M Tris–HCl buffer (pH 7), and rechromatographed on a DEAE-cellulose column equilibrated with the same buffer. The column was eluted with the following NaCl concentrations, and 18 ml fractions were collected: 1, 0.2 M NaCl; 2, 0.25 M NaCl; 3, 0.3 M NaCl; 4, 1 M NaCl. A GAG preparation from the 0.25 M NaCl eluate was subjected to two-dimensional electrophoresis on a cellulose acetate membrane and was stained with 0.1% alcian blue (inset). HA, hyaluronic acid; DS, dermatan sulfate; CS, chondroitin 4-sulfate; HP, heparin. The small arrow indicates the origin.

Table I. Amino Acid Composition of PDS from Calf Skin

| Amino acid[a] | PDS | PPC(Gu)[b] |
|---|---|---|
| Hydroxyproline | 0 | — |
| Aspartic acid | 124 | 72 |
| Threonine | 51 | 59 |
| Serine | 60 | 113 |
| Glutamic acid | 105 | 139 |
| Proline | 72 | 100 |
| Glycine | 93 | 116 |
| Alanine | 49 | 74 |
| 1/2 Cystine | 14 | 10 |
| Valine | 58 | 65 |
| Methionine | 13 | 5 |
| Isoleucine | 55 | 35 |
| Leucine | 115 | 77 |
| Tyrosine | 24 | 18 |
| Phenylalanine | 31 | 36 |
| Hydroxylysine | 0 | — |
| Histidine | 24 | 22 |
| Lysine | 66 | 29 |
| Arginine | 46 | 36 |

*a*) The values of amino acid residues are given per 1000 amino acid residues. *b*) Cited from Table III in ref. 2.

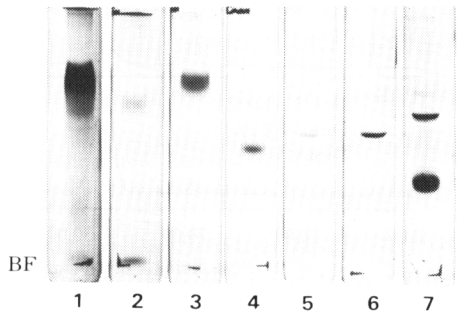


Fig. 2. SDS-PAGE Patterns of PDS and Its Core Protein from the Second DEAE-Cellulose Column

SDS-PAGE (10% gel) was performed according to the method of Laemmli.[8] 1, crude PDS from the first DEAE-cellulose column; 2, 0.2 M NaCl fraction from the second DEAE-cellulose column; 3, purified PDS; 4, core protein of PDS after treatment with chondroitinase ABC; 5, chondroitinase ABC alone; 6, bovine serum albumin (68000); 7, ovalbumin dimer (90000) and monomer (45000); BF, buffer front.

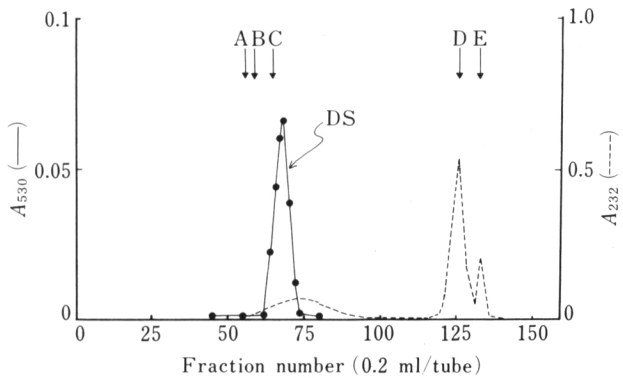


Fig. 3. Comparison of the Gel Chromatography Profiles of GAGs before and after Chondroitinase ABC Treatment on HPLC

The gel chromatography of GAGs before and after chondroitinase ABC treatment was carried out using a TSK G3000SW column (flow rate of 0.5 ml/min). GAGs were detected by the carbazole method ($A_{530}$).[11] Unsaturated disaccharides were detected by UV absorption measurement at 232 nm. A, A1-D1 monomer; B, HA; C, CS (37500); D, unsaturated disaccharide (458); E, buffer front. A and E indicate the void volume and total volume of the column, respectively.

is given in Table I. PDS is distinguished from the A1-D1 monomer from bovine articular cartilage[2] by high contents of aspartic acid, glutamic acid and leucine. The amino acid analysis of dried PDS indicated a protein content of 56%.

**GAGs Analysis on HPLC**

To estimate the size of GAG, PDS was treated with 0.5 M NaOH, digested with pronase, and subjected to HPLC as described under Materials and Methods (Fig. 3). DS was eluted as a peak with $K_{av}$ of 0.21. Comparison with GAG standards indicated that DS has an average

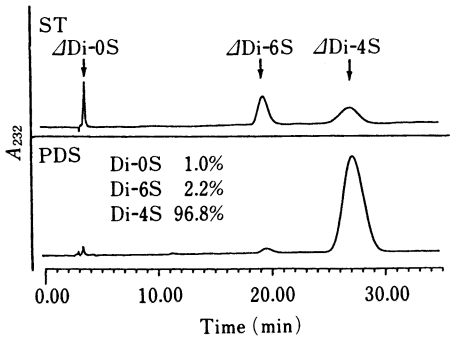


Fig. 4. Analysis of Unsaturated Disaccharide Components of DS on HPLC

Unsaturated disaccharide components obtained with chondroitinase ABC treatment of DS were analyzed by using a VYDAC 201TP ODS column (flow rate of 0.6 ml/min). The detection of each unsaturated disaccharide was done by measuring the UV absorption at 232 nm. ST, standard materials.

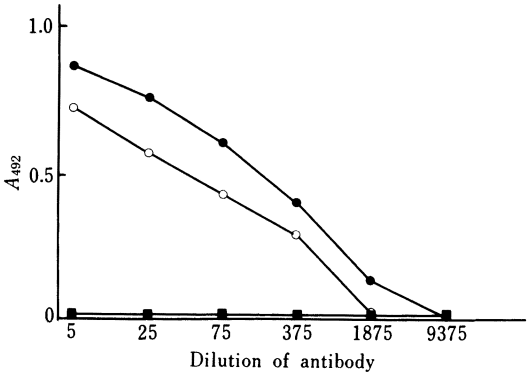


Fig. 5. Immuno-reaction of Anti Core Protein Antibody with PG and Collagens on ELISA

PDS (○); core protein (●); A1–D1 monomer and collagens (types I, II, III and IV) (■).

TABLE II. PHA-Inhibition Test of Purified Anti-core Protein Antibody

| Inhibitor | Amount ($\mu$g) | Titer decrease ($-\log_2$)[a] |
|---|---|---|
| Core protein | 0.5 | 4 |
| | 1.0 | 5 |
| | 5.0 | 5 |
| | 10.0 | 5 |
| PDS | 0.4 | 3 |
| | 0.8 | 4 |
| | 4.0 | 5 |
| | 8.0 | 5 |
| DS | 8.1 | .0 |
| Unsaturated disaccharide[b] | 8.1 | 0 |

*a*) Reciprocal titer of the antibody employed: 5. *b*) Products from chondroitinase ABC-treated DS.

Mr = 20000. Furthermore, products of digestion of DS with chondroitinase ABC were eluted at the position of unsaturated disaccharide. Figure 4 shows unsaturated disaccharide analysis patterns of chondroitinase ABC-treated DS, based on HPLC with the UV detection at 232 nm. The proportions of ⊿Di-0S, ⊿Di-6S and ⊿Di-4S in DS were 1%, 2.2% and 96.8%, respectively. Uronic acid of ⊿Di-4S was entirely composed of iduronic acid (>92%), based on HPLC (preliminary data).

**Immunochemical Properties**

Specificity of anti PDS-core protein antibody was determined by PHA-inhibition test, ELISA, double diffusion and immunoblotting. The antibody purified by affinity chromatography showed a titer of 1 : 1024 on PHA. The Ouchterlony double diffusion using specific antibody showed a single precipitin line fused with both PDS and its core protein (data not shown). However, the antibody showed no precipitin line with bovine plasma fibronectin and human laminin. Furthermore, no immunoreaction was observed between the antibody and A1-D1 monomer or types I, II, III and VI collagens by ELISA (Fig. 5).

In order to elucidate the immunological determinants of the polyclonal antibody, I employed the PHA-inhibition test. As shown in Table II, remarkable inhibition was observed with PDS or core protein. However, neither DS (alkali- and pronase-treated PDS) nor unsaturated disaccharide (chondroitinase ABC-treated DS) inhibited the immunoreaction of anti core protein antibody with antigen. Furthermore, after tryptic digestion of PDS, inhibitory activity was not detected (data not shown). These results strongly suggest that the

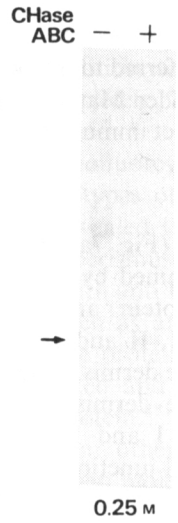


Fig. 6. Electroblotting and Immunodetection of PDS before and after Chondroitinase ABC Treatment

PDS before and after treatment with chondroitinase ABC was submitted to SDS-PAGE (10% gel) followed by electrotransfer to a nitrocellulose membrane.[16] Immunodetection was performed as described under Materials and Methods. An arrow indicates the position of the core protein after chondroitinase ABC treatment. Chase, chondroitinase ABC; 0.25 M, 0.25 M NaCl fraction from rechromatography on DEAE-cellulose.

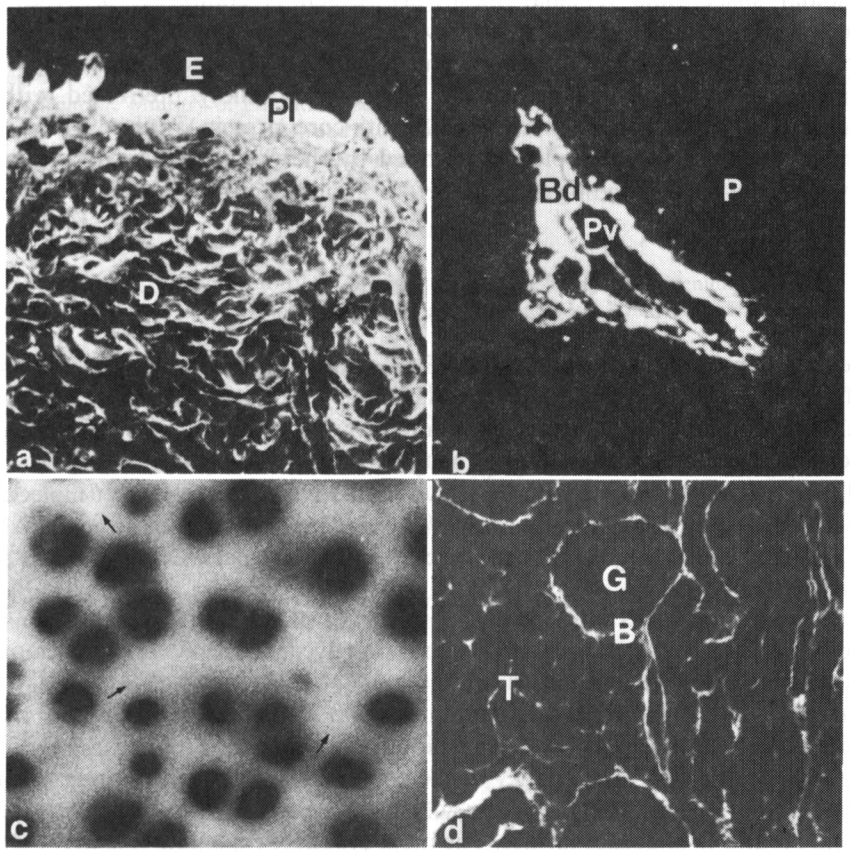


Fig. 7. Indirect Immunofluorescence Staining with Anti Core Protein Antibody in Tissues

a, human skin; b, bovine liver; c, bovine articular cartilage; d, bovine kidney; E, epidermis; D, dermis; Pl, papillary layer; Pv, portal vein; Bd, bile duct; P, perisinusoidal space; G, glomerulus; B, Bowman's capsule; T, tubule. Magnification a, b and d, × 100; c, × 200.

epitope of the antibody against PDS-core protein is proteinaceous, not a linkage region containing unsaturated carbohydrates attached to the core protein.

When the proteins separated on SDS-PAGE were transferred to nitrocellulose paper and stained with antibody against core protein as described under Materials and Methods, the PDS and its core protein on the blots were detected by indirect immunochemical staining with antibody (Fig. 6).

**Immunofluorescence Staining**

The tissue distribution of PDS in normal human skin (Fig. 7a), bovine liver (Fig. 7b), articular cartilage (Fig. 7c) and kidney (Fig. 7d) was examined by using indirect immunofluorescence staining. The staining with anti core protein antibody in tissues was generally observed along with interstitial collagen (types I, II and III). Anti core protein antibody in skin (Fig. 7a) reacted throughout all levels of the dermis, though the staining was apparently more prominant in the papillary layer of the dermis, and PDS was widely distributed in bundles and whirlpools along with types I and III collagens. However, epidermis, the basement membrane of the dermis-epidermal junction and hair follicles were not stained. The staining of the liver (Fig. 7b) showed a clear delineation of the interstitium of a central vein and a portal space surrounded by connective tissue, but not intralobularly in the perisinusoidal space (fine reticular meshwork which is composed of types I and III collagens).[17] In the kidney (Fig. 7d), PDS was codistributed with types I and III collagens (data not shown) in the connective tissue, but not in the basement membrane of glomeruli. Furthermore, when an articular cartilage section was mildly pretreated with testicular hyaluronidase, it was strongly stained with anti core protein antibody (Fig. 7c). These fluorescence stainings were completely inhibited by the preincubation of anti core protein antibody with the corresponding antigen before application to the tissue section (data not shown).

## Discussion

The first step of the purification of PDS was based on the fact that PDS is precipitated with collagens (especially types I and III collagens) by means of dialysis against water. Next, the precipitate containing PDS–collagens complex was dissolved in 7 M urea and applied to a DEAE-cellulose column (Fig. 1). This method was advantageous to separate negatively charged proteoglycan from the solution. Two kinds of PDS with distinct molecular weights were obtained by stepwise elution with various NaCl concentrations from DEAE-cellulose, Mr = 112000 and 98000 (Fig. 2). The presence of low-molecular PDS in skin was reported by Matsunaga and Shinkai.[18]

As shown in Fig. 4, DS was entirely composed of $\Delta$Di-4S. However, it is considered that the ratios of $\Delta$Di-4S and $\Delta$Di-6S in glycosaminoglycans will alter in developing tissues, especially during aging and various disorders.

In recent studies, proteoglycans containing DS have been isolated and characterized from numerous tissues.[19] However, the biophysical functions of these PG are not well understood. The apparent discrepancy in the conclusions described in these reports may be due to the heterogeneity of PDS molecules, which are composed of core protein, glycosaminoglycans, and N- and O-oligosaccharides. The most probable explanation is that PDS (interacts differently with extracellular matrix components such as types I and III collagens in various tissues.

Generally, it is considered that the main immunological determinants of PG are at three sites: the linkage regions between the core protein and the glycosaminoglycan (revealed by chondroitinase ABC treatment),[21] the glycosaminoglycans[22] and the core protein.[23,24] In

this paper, it is shown that neither DS peptides nor unsaturated disaccharide obtained from chondroitinase ABC-digested PDS inhibited the interaction of antibody with PDS or its core protein (Table II). In addition, when PDS was pretreated with trypsin for 1 h, its inhibitory activity disappeared completely. These results suggest that polyclonal antibody against PDS recognized a core protein.

The present immunofluorescence studies were performed for comparison with the distributions of various types of collagen in tissues stained with antibody raised against collagens. The results revealed that PDS is apparently codistributed with types I and III collagens. However, the perisinusoidal space composed of types I and III collagens in bovine liver[17] was not stained with anti PDS-core protein antibody (Fig. 7). Furthermore, we found that hyaline cartilage such as articular and bronchial cartilage was well stained with the antibody against the core protein of PDS after pretreatment with testicular hyaluronidase (Fig. 7C), and we isolated and characterized the cartilage PG immunoreactive with the antibody to PDS-core protein.[25]

As PDS is smaller than other PG[1] and does not interact with hyaluronic acid, PDS may easily interact with collagen molecules as described previously.[6] These observations suggest that PDS may be an important factor in collagen fibrogenesis and collagen deposition in tissues. The antibody described above will be useful for biochemical analysis and for studies on the variation of PG structure and function in connective tissues.

**Acknowledgement** This work was supported in part by a Grant-in-Aid (62771923) for Encouragement of Young Scientists from the Ministry of Education, Science and Culture , Japan. The author is grateful to Dr. H. Konomi, National Center for Nervous, Mental, and Muscular Disorders, and Dr. J. Sano, Tokyo University School of Medicine, for advice on immunohistochemical staining.

## References

1) *a*) W. J. Lennarz (ed.), "The Biochemistry of Glycoproteins and Proteoglycans," Plenum Press, New York and London, 1980, p. 267; *b*) L. W. Cunningham and D. W. Frederiksen (eds.), "Methods in Enzymology," Vol. 82, Academic Press, New York, 1982, p. 453.
2) S. W. Sajdera and V. C. Hascall, *J. Biol. Chem.*, **244**, 77 (1969).
3) *a*) M. B. Mathews, *Biochem. J.*, **96**, 710 (1965); *b*) B. P. Toole and D. A. Lowther, *Arch. Biochem. Biophys.*, **128**, 567 (1968); *c*) B. P. Toole, *Nature* (London), **222**, 872 (1969); *d*) T. R. Oegema, J. J. Lailaw, V. C. Hascall and D. D. Dziewiatkowski, *Arch. Biochem. Biophys.*, **170**, 698 (1975).
4) T. Nakamura and Y. Nagai, *J. Biochem.* (Tokyo), **87**, 629 (1980).
5) D. A. D. Parry, M. H. Flint, G. C. Gillard and A. S. Craig, *FEBS Lett.*, **149**, 1 (1982).
6) J. E. Scott and C. R. Orford, *Biochem. J.*, **197**, 213 (1981).
7) S. Onodera, in preparation.
8) U. K. Laemmli, *Nature* (London), **222**, 680 (1970).
9) C. A. McDevitt and H. M. Muir, *Anal. Biochem.*, **44**, 612 (1971).
10) R. Hata and Y. Nagai, *Anal. Biochem.*, **45**, 462 (1972).
11) T. Bitter and H. M. Muir, *Anal. Biochem.*, **4**, 330 (1962).
12) Y. Oike, K. Kimata, T. Shinomura, K. Nakazawa and S. Suzuki, *Biochem. J.*, **191**, 193 (1980).
13) N. Ototani, N. Sato and Z. Yoshizawa, *J. Biochem.* (Tokyo), **85**, 1383 (1979).
14) D. M. Weir (ed.), "Handbook of Experimental Immunology," 3rd., Blackwell Scientific Publications, Oxford, 1978.
15) S. I. Rennard, R. Berg, G. R. Martin, J. M. Foidart and P. G. Robey, *Anal. Biochem.*, **104**, 205 (1980).
16) H. Towbin, T. Staehelin and J. Gordon, *Proc. Natl. Acad. Sci. U.S.A.*, **76**, 4350 (1979).
17) H. Konomi, J. Sano and Y. Nagai, *Acta Pathol. Jpn.*, **31**, 973 (1981).
18) E. Matsunaga and H. Shinkai, *J. Invest. Dermatol.*, **87**, 221 (1986).
19) *a*) T. Nakamura, E. Matsunaga and H. Shinkai, *Biochem. J.*, **213**, 289 (1983); *b*) B. Öbrink, *Biochim. Biophys. Acta*, **264**, 354 (1972); *c*) I. Miyamoto and S. Nagase, *J. Biochem.* (Tokyo), **88**, 1793 (1980); *d*) L. Cöster and L. Å. Fransson, *Biochem. J.*, **193**, 143 (1981); *e*) N. Uldbjerg, A. Malmström, G. Ekman, J. Sheeham, U. Ulmsten and L. Wingerup, *ibid.*, **209**, 497 (1983); *f*) J. C. Anderson, *Biochim. Biophys. Acta*, **379**, 444 (1975); *g*) L. C. Rosenberg, H. U. Choi, L. H. Tang, T. L. Johnson, S. Pal, C. Webber, A. Reiner and A. R. Pool, *J. Biol. Chem.*,

**260**, 6304 (1985).
20) H. Keiser and J. DeVitro, *Connect. Tissue Res.*, **2**, 272 (1974).
21) J. E. Christner, B. Caterson and J. R. Baker, *J. Biol. Chem.*, **255**, 7102 (1980).
22) R. B. Jenkins, T. Hall and A. Dorfman, *J. Biol. Chem.*, **256**, 8279 (1981).
23) *a*) G. Loewi and H. M. Muir, *Immunology*, **9**, 119 (1965); *b*) B. R. Champion, A. Reiner, P. J. Roughley and A. R. Pool, *Collagen Rel. Res.*, **2**, 45 (1982).
24) G. A. Pringle, C. M. Dodd, J. W. Osborn, C. H. Pearson and T. R. Mosmann, *Collagen Rel. Res.*, **5**, 23 (1985).
25) S. Onodera and Y. Nagai, *Biochem. Biophys. Res. Commun.*, **129**, 95 (1985).