Comparative Studies on Proteodermatan Sulfate of Bovine Gastrointestinal Tract

HITOSHI KAWASAKI

Department of Biochemistry, Hirosaki University School of Medicine, Hirosaki 036

Proteodermatan sulfate was extracted from four areas of the bovine gastrointestinal tract (esophagus, stomach, small intestine and colon) with 4 M guanidine-HCl and then purified by ion-exchange and gel filtration chromatography. Dermatan sulfate chains which made up proteodermatan sulfate from each area were separately prepared by Pronase P and endo-β-xylosidase digestion. The properties of the proteodermatan sulfate and dermatan sulfate chains were compared using electrophoresis and high-performance liquid chromatography. The molecular size of proteodermatan sulfate purified from each area was estimated to be greater than $8 \times 10^5$, and the molecular sizes of dermatan sulfate chains from esophagus, stomach, small intestine, and colon were 27,000, 24,500, 21,000, and 21,500, respectively. The dermatan sulfate chains from the esophagus were slightly undersulfated in comparison with the others. These results show that the molecular sizes of proteodermatan sulfate from different regions of the gastrointestinal tract are similar to each other, but are larger than those from other tissues. Dermatan sulfate chains differed from each other to a slight degree with respect to chain length and sulfation, that from the esophagus being the largest. These differences in proteodermatan sulfate structure seem to reflect the organ specificities of the gastrointestinal tract. ——— bovine gastrointestinal tract; proteodermatan sulfate; endo-β-xylosidase; extracellular matrix; glycosaminoglycan

Proteoglycans (PGs) are important structural macromolecules in the extracellular matrix (Antonopoulos et al. 1974; Vogel and Fisher 1986). Interest in PGs...
of the gastrointestinal tract has increased considerably in recent years, because of their relationship to physiological functions such as cell adhesion and recognition (Kraemer 1971; Dietrich et al. 1977) and to several diseases including cancers (Danishefsky et al. 1966; Takeuchi 1968; Sampaio et al. 1977; Iozzo et al. 1982; Nagai et al. 1985; Bouziges et al. 1990). The main component of PGs in the gastrointestinal tract is proteodermatan sulfate (proteo-DS) (Sekino et al. 1977; Sekino and Murata 1978; Iozzo et al. 1982; Nagai et al. 1985). However, only limited details of the chemical nature and physiological function of proteo-DS in the gastrointestinal tract are available (Sekino et al. 1977; Sekino and Murata 1978).

In order to elucidate the organ specificities of proteo-DS in each area of the gastrointestinal tract, it is necessary to purify the dermatan sulfate (DS) chains which compose of proteo-DS. Recently, endo-\(\beta\)-xylosidase, which specifically cleaves the xylosylserine linkage of PGs including proteo-DS, was isolated from the mollusc *Patinopecten* in our laboratory (Takagaki et al. 1988, 1989, 1990). As this enzyme was found to be an excellent tool for isolating the native glycosaminoglycan (GAG) chains from PG, the author attempted to elucidate the organ specificities of proteo-DS from the bovine gastrointestinal tract, comparing the DS chains liberated from PG using endo-\(\beta\)-xylosidase. This paper describes that the molecules of proteo-DS purified from each area of the bovine gastrointestinal tract differ slightly from each other.

**MATERIALS AND METHODS**

**Chemicals**

Sepharose CL-6B was purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. DEAE-cellulose (DE-32) was obtained from Whatman Chemical Separation Ltd., Maidstone, U.K., and Diaflow PM-10 membrane for ultrafiltration was purchased from Amicon Corp., Lexington, MA, USA. The following materials and enzymes were obtained from Seikagaku Kogyo Co., Tokyo: hyaluronidase from *Streptomyces hyalurolyticus*, chondroitinase ABC from *Proteus vulgaris*, chondroitinase AC-II from *Arthrobacter aurescens*, chondroitinase B from *Flavobacterium heparinum*, heparitinase from *Flavobacterium heparinum*, chondroitin 6-sulfate (Ch6S) from shark cartilage, chondroitin 4-sulfate (Ch4S) from whale cartilage, dermatan sulfate (DS) from pig skin, hyaluronic acid (HA) from human umbilical cord, heparan sulfate (HS) from bovine kidney, and 4,5-unsaturated disaccharides (\(\Delta\)Di-0S, \(\Delta\)Di-4S and \(\Delta\)Di-6S). Pronase P (from *Streptomyces griseus*) was obtained from Kaken Kagaku Co., Tokyo. Endo-\(\beta\)-xylosidase was prepared by the method described in a previous paper (Takagaki et al. 1990). Heparin (Hep) from porcine intestinal mucosa, guanidine-HCl (Gu-HCL), and urea were purchased from Wako Pure Chemical Ind. Co., Osaka. Other reagents were of analytical grade and were also obtained from commercial sources.

**Isolation of proteo-DS**

Fresh bovine gastrointestinal tracts were obtained from a local abattoir in Hirosaki. The tissues used were the oral end of the esophagus, the first stomach as representative of the stomach, the jejunum as representative of the small intestine, and the anal end of the colon.
The extraction of PG was carried out according to the method described by Heinegard and Hascall (1974). The gastrointestinal tract tissue was immediately dissected free from surrounding tissue and perichondrium, cut into small pieces, frozen in liquid nitrogen, powdered with a Wiley Mill using a 20-mesh screen, and stored at −20°C. The powder of each tissue (50 g) was extracted with 200 ml of 4 M Gu-HCl in 0.05 M sodium acetate buffer (pH 5.8) containing protease inhibitors (10 mM EDTA, 0.1 M ε-amino-caproic acid, 10 mM N-ethylmaleimide, 5 mM benzamidine-HCl, and 1 mM phenylmethanesulfonyl fluoride) at 4°C with gentle shaking for 24 hr. The suspension was centrifuged at 15,000 × g for 30 min at 4°C. The supernatant was concentrated using an ultrafiltration system with a PM10 filter, dialyzed against 0.05 M Tris-HCl buffer (pH 6.8) containing 7 M urea and protease inhibitors, and passed through a DEAE-cellulose column (3.0 × 30 cm) equilibrated with the same buffer. The column was washed with the same buffer, and then proteoglycans were eluted with a linear gradient of 0 to 2 M NaCl in the same buffer at a flow rate of 40 ml/hr. The fractions, which were positive for the carbazole-\(\text{H}_2\text{SO}_4\) reaction (Bitter and Muir 1962) were collected and concentrated to 1/10 volume by ultrafiltration, and then applied to a column (0.9 × 94 cm) of Sepharose CL-6B equilibrated with 7 M urea in 0.05 M Tris-HCl buffer (pH 6.8). Elution was carried out with the same buffer at a flow rate of 10 ml/hr. Proteo-DS prepared as above was dialyzed against distilled water and lyophilized. The purified proteo-DS was stored at 4°C until use.

**Isolation of dermatan sulfate chains (DS chains)**

GAG chains were prepared by the method described in an earlier paper (Takagaki et al. 1990). In brief, proteo-DS fractions were digested with Pronase P in 0.1 M Tris-HCl buffer (pH 8.0) containing 10 mM CaCl₂ at 37°C for 24 hr. The peptido-DS obtained was digested with endo-\(\beta\)-xylosidase in 0.1 M sodium acetate buffer (pH 4.0). The reaction mixture was then treated with 10% trichloroacetic acid. After centrifugation, four volumes of ethanol saturated with NaCl were added to the supernatant. The resulting precipitates were washed with ethanol and ether, dried in vacuo and used as DS chains.

**Cellulose acetate membrane electrophoresis**

One-dimensional electrophoresis on cellulose acetate membrane was carried out using Separax (6 × 22 cm, Jookoo Co., Tokyo) in 0.47 M formic acid/0.1 M pyridine buffer (pH 3.0) at 1 mA/cm for 40 min. Two-dimensional electrophoresis on cellulose acetate membrane was carried out using Separax (10 × 10 cm) as reported previously (Yokoyama et al. 1986). The conditions were as follows: first dimension electrophoresis in 0.47 M formic acid/0.1 M pyridine buffer (pH 3.0) at 1 mA/cm for 60 min, and second dimension electrophoresis in 0.1 M barium acetate (pH 8.0) at 1 mA/cm for 4 hr. The resulting electrophoretograms were visualized with 0.1% alcian blue in 70% ethanol.

**Enzymatic digestion**

GAGs were treated with hyaluronidase, chondroitinase AC-II, chondroitinase ABC, chondroitinase B, and heparitinase. The reactions with hyaluronidase were performed in 0.1 M acetate buffer (pH 8.0) (Ohya and Kaneko 1970), those with chondroitinase AC-II, chondroitinase ABC (Yamagata et al. 1968) and chondroitinase B (Michelacci and Dietrich 1975) in 0.1 M Tris-HCl buffer (pH 8.0), and those with heparitinase in 0.1 M Tris-HCl buffer (pH 7.0) (Linker and Hovingh 1972). The reaction mixture was treated with four volumes of ethanol saturated with NaCl. The resulting precipitates were washed with ethanol and ether and dried in vacuo. For unsaturated disaccharide analysis, samples were prepared as shown below. Each DS chain was digested with chondroitinase ABC, and four volumes of ethanol were added to the sample. After centrifugation, the resulting supernatant was used for analysis.
**High-performance liquid chromatography (HPLC)**

A high-performance liquid chromatograph (L-6200, Hitachi, Hitachi) equipped with a refractive index monitor (L-3300, Hitachi) and a UV monitor (L-4200, Hitachi) was used. Gel filtration of GAG was carried out with a Shodex OHpaK KB-802 column (0.8 × 30 cm, Shoko Co., Tokyo) and a Shodex OHpak KB-803 column (0.8 × 30 cm) using 0.2 M NaCl. The flow rate was 0.5 ml/min, and the column temperature was 30°C. The eluate was monitored by measurement of refractive index. Analysis of unsaturated disaccharides (ΔDi-0S, ΔDi-4S and ΔDi-6S), which were obtained from GAG chains by chondroitinase ABC digestion, was performed with two columns connected end to end with Shodex RS (Type DC-613, 0.6 × 15 cm, Shoko Co.) using acetonitrile/methanol/0.5 M ammonium formate buffer (pH 4.5) (13 : 3 : 4, v/v/v) (Murata and Yokoyama 1985). The flow rate was 0.9 ml/min and the column temperature was 70°C. The eluate was monitored at 232 nm.

**Chemical analysis**

Uronic acid was determined by the carbazole-H$_2$SO$_4$ method of Bitter and Muir (1962). Protein concentration was determined from the absorbance at 280 nm (Kalckar 1947).

**RESULTS**

**Properties of proteo-DS from the gastrointestinal tract**

PGs were extracted from whole layers of each part of the gastrointestinal tract with 4 M Gu-HCl. In this step, the amounts of GAG in each area (100 g) were compared in terms of uronic acid content (Table 1). The uronic acid content in each area ranged from 33 to 84 mg per 100 g wet tissue, the value for the small intestine being the highest.

To characterize the GAG components of PGs from the gastrointestinal tract, a portion of the proteoglycans from the crude extract step was digested successively with Pronase P and endo-β-xyllosidase, and subjected to two-dimensional electrophoresis on a cellulose acetate membrane (Fig. 1).

HA and DS were the main components of GAGs in the gastrointestinal tract. Hep was detected specifically in the small intestine. Ch4S or Ch6S, and HS were detected in the small intestine and colon. Each GAG was identified by sequential enzymatic digestion (hyaluronidase, chondroitinase ABC, chondroitinase AC-II, chondroitinase B, and heparitinase; data not shown). The results indicated that the main component of PGs from each area of the bovine gastrointestinal tract was proteo-DS. Small amounts of proteo-Hep were detected specifically only in the small intestine, and an intermediate amount of proteo-ChS and a small

<table>
<thead>
<tr>
<th>Table 1. Hexouronic acid contents of crude extract and purified proteo-dermatan sulfates from various regions of the bovine gastrointestinal tract</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexouronic acid (mg/100 g wet tissue)</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Esophagus</td>
</tr>
<tr>
<td>Crude extract</td>
</tr>
<tr>
<td>Purified proteo-DS</td>
</tr>
</tbody>
</table>
amount of proteo-HS were detected in the small intestine and colon.

**Comparative studies of proteo-DS**

Proteo-DSs of the gastrointestinal tract were initially purified in 7 M urea by DEAE-cellulose column chromatography with NaCl gradient elution (Fig. 2). PGs (uronic acid-containing fractions) from the esophagus and small intestine were eluted as a single major peak, whereas those from the stomach and colon eluted as multiple peaks. Therefore, after digestion with Pronase P and endo-β-xylosidase, the GAG components of each fraction were checked using electrophoresis on cellulose acetate membrane. The DEAE-cellulose fractions which contained a large amount of DS were pooled as indicated by horizontal bars (Fig. 2) and further purified by chromatography on Sepharose CL-6B (Fig. 3). Upon electrophoresis after DEAE-cellulose, GAG spots which showed the same mobility
as the authentic DS were also detected in fractions 71 to 75 from the small intestine and in fractions 65 to 69 from the colon (Fig. 2). However, these GAG spots did not disappear after treatment with chondroitinase B. The results with

Fig. 2. Ion-exchange chromatography on DEAE-cellulose of proteoglycans from gastrointestinal tract. The column (3.0 × 30 cm) was eluted with a NaCl gradient in 7 M urea/0.05 M Tris-HCl buffer (pH 6.8) at 40 ml/h, and 15-ml fractions were collected. The fractions were determined for uronic acid (●) and absorbance at 280 nm (○), and each fraction was analyzed by one-dimensional electrophoresis. Fractions were pooled as indicated by horizontal bars. ChS, chondroitin 6-sulfate; DS, dermatan sulfate; HA, hyaluronic acid. A, esophagus; B, stomach; C, small intestine; D, colon.
Proteodermatan Sulfate of Gastrointestinal Tract

Sepharose CL-6B indicated that the molecular size of proteo-DS purified from esophagus and stomach was greater than $1 \times 10^6$, whereas the molecular size of those from the small intestine and colon were estimated to be about $8-9 \times 10^5$. Thus, the proteo-DS from the esophagus and stomach had a larger molecular size than that from the small intestine and colon.

As shown in Fig. 3, fractions were pooled as indicated by horizontal bars, dialyzed against distilled water, and lyophilized. The preparations were then used as purified proteo-DS. As shown in Table 1, the yield of purified proteo-DS ranged from 0.0013 to 0.005% wet tissue weight.

**Comparative studies of DS chains**

To obtain the native DS chains, each proteo-DS was digested successively with Pronase P and endo-β-xylosidase. The GAG components were identified
using two-dimensional electrophoresis on cellulose acetate membrane (Fig. 4). All the GAG fractions were composed of DS chains, and it was also confirmed that the purified PGs contained only DS chains. The molecular sizes of the DS chains were compared by HPLC using a Shodex OHpak KB-802 column and a OHpak KB-803 column for gel filtration (Fig. 5). The results indicated that the DS chains of the proteo-DS purified from esophagus, stomach, small intestine, and colon had molecular sizes of 27,000, 24,500, 21,000, and 21,500, respectively. Thus, the molecular size of DS chains of the proteo-DS purified from the upper gastrointestinal tract (esophagus and stomach) was larger than that of DS chains from the lower tract (small intestine and colon).

In order to determine the degree of sulfation of DS chains, they were digested with chondroitinase ABC, and the unsaturated disaccharides formed were analyzed by using HPLC on Shodex RS (Type DC-613). As shown in Fig. 6, complete separation and accurate quantitation of 4Di-0S, 4Di-4S and 4Di-6S were accomplished. The results are summarized in Table 2. For all samples, 4Di-4S was demonstrated to be the major product from the DS chains. Also a significant amount of 4Di-0S was found, derived from undersulfated DS chains. However, the percentage of 4Di-0S from esophagus preparations was higher than that from
other areas. This indicated that the degrees of sulfation of DS chains of the proteo-DS purified from stomach, small intestine, and colon were similar to each other. However, the DS chain of proteo-DS purified from the esophagus was slightly undersulfated in comparison with the others. No appreciable amount of ΔDi-6S was detected. Thus, the DS chains of proteo-DS purified from the gastrointestinal tract had no chondroitin sulfate (ChS) hybrid structures.

**DISCUSSION**

The present study involved purification of proteo-DS obtained from four different areas of the bovine gastrointestinal tract (esophagus, stomach, small intestine and colon), and comparison of the native DS chains which were prepared by endo-β-xylosidase digestion. Endo-β-xylosidase acts specifically only on the xylosylserine linkage between GAG chains and the protein moiety of PG. Thus, using this enzyme, it was possible to analyze native GAG chains by HPLC or electrophoresis, and to make comparisons of those with similar characteristics.

Proteo-DS has been isolated and characterized from connective tissue with
H. Kawasaki

elastic properties such as skin (Miyamoto and Nagase 1980; Damle et al. 1982), aorta (Kapoor et al. 1981), sclera (Cöster et al. 1987), articular cartilage (Cöster et al. 1987), and tendon (Vogel and Evanko 1987). These proteo-DSs ranged in molecular size from 80,000 to 285,000, and were classified as small PGs. However, there have been few studies on proteo-DS from gastrointestinal tract regions possessing elasticity (Sekino et al. 1977; Sekino and Murata 1978). In this study, the molecular size of proteo-DS from the gastrointestinal tract was estimated to be more than $8 \times 10^5$. This value is higher than those for most other proteo-DSs, but similar to that for PG from porcine ovarian follicular fluid (Yanagishita et al. 1979).

These results show that proteo-DS purified from esophagus is different from those of stomach, small intestine and colon, in terms of molecular size and degree of sulfation of the DS chain. The esophagus does not perform the function of digestion and absorption, but plays a role in transferring food by peristaltic

---

![HPLC of unsaturated disaccharides derived from DS by chondroitinase ABC digestion. Two Shodex RS (Type DC-613, 0.6×15 cm) columns connected end to end were used, and elution was carried out with acetonitrile/methanol/0.5 M ammonium formate buffer (pH 4.5) (13:3:4, v/v/v) at a flow rate of 0.9 ml/min and a column temperature of 70°C. A, esophagus; B, stomach; C, small intestine; D, colon. Arrows 1, 2, and 3 indicate ΔDi-6S, ΔDi-0S, and ΔDi-4S, respectively.]

### Table 2. Unsaturated disaccharide analysis of chondroitinase ABC-digested DS chains

<table>
<thead>
<tr>
<th></th>
<th>ΔDi-0S</th>
<th>ΔDi-4S</th>
<th>ΔDi-6S</th>
</tr>
</thead>
<tbody>
<tr>
<td>Esophagus</td>
<td>35</td>
<td>65</td>
<td>Trace</td>
</tr>
<tr>
<td>Stomach</td>
<td>11</td>
<td>89</td>
<td>Trace</td>
</tr>
<tr>
<td>Small intestine</td>
<td>7</td>
<td>93</td>
<td>Trace</td>
</tr>
<tr>
<td>Colon</td>
<td>7</td>
<td>93</td>
<td>Trace</td>
</tr>
</tbody>
</table>
movement. The main histological differences between the esophagus and other regions of the gastrointestinal tract are that the inner surface of the esophagus is mostly covered with squamous epithelium and that only a small number of glands are contained in the esophageal wall (Kapoor et al. 1981). Specific diseases of the esophagus, such as progressive systemic sclerosis (Fraser 1966), have been observed. This specific disturbance may be related to the constituents of the connective tissue including proteo-DS.

Acknowledgments

I thank Prof. M. Endo and Dr. K. Takagaki (Department of Biochemistry, Hirosaki University School of Medicine) for their helpful suggestions, and to emeritus Prof. K. Ono and Prof. M. Konn (Second Department of Surgery, Hirosaki University School of Medicine) for their encouragement during this work. The author is also indebted to Dr. K. Ishida (Institute for Animal Experiments, Hirosaki University School of Medicine) for his valuable advice.

References


