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Biochemical and atomic force microscopic characterization of salmon nasal cartilage proteoglycan

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ABSTRACT

Biological activities of salmon nasal cartilage proteoglycan fractions are known, however, structural information is lacking. Recently, the major proteoglycan of this cartilage was identified as aggrecan. In this study, global molecular images and glycosaminoglycan structure of salmon nasal cartilage aggrecan purified from 4 M guanidine hydrochloride extract were analyzed using HPLCs and atomic force microscopy with bovine tracheal cartilage aggrecan as a control. The estimated numbers of sulfates per disaccharide unit of chondroitin sulfate chains of salmon and bovine aggrecans were similar (approximately 0.85). However, the disaccharide composition showed a higher proportion of chondroitin 6-sulfate units in salmon aggrecan, 60%, compared to 40% in bovine. Gel filtration HPLC and monosaccharide analysis showed the salmon aggrecan had a lower number (approximately one-third), but 1.5–3.3 times longer chondroitin sulfate chains than the bovine aggrecan. Atomic force microscopic molecular images of aggrecan supported the images predicted by biochemical analyses.

1. Introduction

The potential of cartilage proteoglycan (PG)[2] for industrial applications has been explored, with an emphasis on finding simple and efficient preparation processes and efficacious functions. In these studies cartilage from salmon was used to avoid the risk of animal diseases such as bovine spongiform encephalopathy (BSE). And PG fractions were prepared by a simplified procedure[3] using acetic acid (CH_3COOH) extraction with no protease inhibitors, an unusual but non-toxic extraction procedure, which provides adequate amounts of the PG fractions for investigations of functions. Salmon (*Oncorhynchus keta*) nasal cartilage PG fractions have been implicated in several biological activities. For example, they modulate cytokine responses stimulated by heat-killed bacteria in mouse macrophage cells as well as having therapeutic effects on experimentally induced colitis and autoimmune encephalomyelitis (Mitsui et al., 2010; Ota et al., 2008; Sashinami et al., 2012; Sashinami, Takagaki, & Nakane, 2006). An alteration of intestinal microbiota composition in PG-administered mice was investigated (Asano, Yoshimura, & Nakane, 2013). They also have important functions in hematological cells such as the cytokine-dependent promotion of *ex vivo* expansion of megakaryocytic progenitor cells (Kashiwakura, Takahashi, Monzen, Nakamura, & Takagaki, 2008; Kashiwakura, Takahashi, & Takagaki, 2007; Yoshino, Takahashi,

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[2] Abbreviations used are: PG, proteoglycan; GAG, glycosaminoglycan; *N*-terminal, amino terminal; *C*-terminal, carboxyl terminal; G1 domain, globular domain 1; IGD, interglobular domain; G2 domain, globular domain 2, G3 domain, globular domain 3; EGF, epidermal growth factor; GdnHCl, guanidine hydrochloride; HS, heparan sulfate; Hep, heparin; HPAEC-PAD, high performance anion exchange-pulsed amperometric detection; KS, keratan sulfate; ChS, chondroitin sulfate; HA, hyaluronan; GlcUA, glucuronic acid; GalNAc, *N*-acetylgalactosamine; GlcNAc, *N*-acetylglucosamine; GalN, galactosamine; GlcN, glucosamine; NeuAc, *N*-acetylneuraminic acid; NeuGc, *N*-glycolylneuraminic acid; Gal, galactose; Xyl, xylose; AFM, atomic force microscopy; HABP, HA binding protein; BSA, bovine serum albumin.

[3] Takagaki et al. (2002) A method for extraction and purification of cartilage type proteoglycan, Registered patent Nos. JPA37311150 (Japan), US 6,803,454 B2 (USA), and No. 2270023 (Russia).

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Monzen, & Kashiwakura, 2010). We recently established that the major PG isolated from salmon nasal cartilage belongs to the aggrecan family based on the analyses of its core protein and cDNA (accession number: AB571294 in the GenBank™/EMBL Data Bank, BAJ61837 for protein ID) (Kakizaki, Tatara, Majima, Kato, & Endo, 2011). However, information on the global molecular structure of this aggrecan as a native form and particularly the nature of the glycosaminoglycan (GAG) chains was still lacking. Additionally, the global structure of PG obtained by an unusual method using CH_3COOH extraction must be clarified because studies on functions have already proceeded.

Aggrecan is a large and complex glycoconjugate composed of a core protein and multiple chondroitin sulfate (ChS) chains, covalently linked to the core protein through a glucuronic acid-galactose-galactose-xylose-serine (GluUAβ1-3Galβ1-3Galβ1-4Xylβ-*O*-Ser) linkage (Heinegard, 2009). The GAG chains vary in length and sulfation pattern and core protein of mammalian aggrecan is composed of many functional domains. Starting from the amino terminus (*N*-terminus) there are the globular domain 1 (G1 domain), an interglobular domain (IGD), the globular domain 2 (G2 domain), GAG attachment domains, and the globular domain 3 (G3 domain) (Kiani, Chen, Wu, Yee, & Yang, 2002). The G1 domain has a hyaluronan (HA) binding site (Hascall, 1977) and an immunoglobulin subdomain. In cartilage, most aggrecan occurs as aggregates consisting of stable complexes formed by the interaction of the G1 domain with HA and link proteins. The G3 domain has epidermal growth factor (EGF)-like module(s), a lectin-like module, and a complement binding protein-like module. We previously established that the salmon aggrecan has the same functional domains as the mammalian aggrecan but with a shorter GAG attachment domain (Kakizaki et al., 2011) (Fig. S1).

The common procedure for extraction of PG uses 4 M guanidine hydrochloride (GdnHCl) (Sajdera & Hascall, 1969). The strong denaturing effect of 4 M GdnHCl facilitates breaking most intermolecular noncovalent bonds among extracellular matrix molecules, so the efficiency of extraction of PG from complex extracellular matrix molecular networks is highest among the various solvents (Heinegard & Sommarin, 1987). Also, in this strong protein denaturant, activities of proteases are attenuated. From these, GdnHCl extraction is expected to lead to a superior conservation of the PG structure compared to other solvents. However, to investigate biological activities in model cells and animals, salmon nasal cartilage PG fractions extracted with 4% CH_3COOH and dialysis have been used (Kashiwakura et al., 2008, 2007; Mitsui et al., 2010; Ota et al., 2008; Sashinami et al., 2012, 2006; Yoshino et al., 2010). It should be pointed out that PG fractions prepared with only non-toxic, edible materials (CH_3COOH, ethanol, and NaCl) are more suitable for biological assays for future medical applications than those prepared with toxic materials such as GdnHCl containing protease inhibitors.

In this study, we focused on the structural analysis of GAGs and the global molecular structure of salmon nasal cartilage aggrecan purified from 4 M GdnHCl-extract, as this aggrecan reflects the features of native aggrecan. Composition of GAG chains, size distributions of a whole PG, a core protein, and a GAG chain, and the number of GAG chains of PG were assessed by choromatographies including HPLCs after treatment with or without appropriate enzymes or reagent. In addition to investigating these parameters on PG, the global molecular image was investigated by atomic force microscopy (AFM). For comparison we also analyzed the bovine tracheal cartilage aggrecan purified from 4 M GdnHCl-extract and PG obtained by extraction of salmon nasal cartilage with 4% CH_3COOH. We established that the PG obtained from salmon by both extraction protocols is aggrecan, even though there are some differences in molecular structure. Also, there are differences in global molecular structure between the salmon and bovine aggrecans.

2. Materials and methods

2.1. Materials

Frozen salmon nasal cartilage slices with skin were purchased from a local agency and bovine tracheal cartilage was obtained from a local slaughterer, both in Aomori, Japan. DEAE-Sephacel and Sepharose CL-4B were from GE Healthcare Japan (Tokyo, Japan). Mouse monoclonal antibody, 12/21/1-C-6 (which recognizes the G1 domain of rat chondrosarcoma PG) was from the Developmental Studies Hybridoma Bank of the University of Iowa, USA. Anti-rabbit polyclonal antibody against the synthetic peptide (^{2277}DGHPMQFENWRPNQPDN^{2293}) in the human aggrecan G3 domain was from Affinity BioReagents (Golden, CO, USA), and this peptide sequence of the antigen corresponds to ^{1182}DGSPLGFENWRPNQPDN^{1198} in the salmon aggrecan G3 domain (Kakizaki et al., 2011). Anti-salmon PG EGF was generated by immunizing rabbits with synthetic peptide (^{1069}RDLCEPNQCGTGTCSVQDGI^{1088}) of an EGF-like module of salmon nasal cartilage aggrecan with amino-terminal for conjugation with bovine thyroglobulin (IBL, Gunma, Japan). Peroxidase-conjugated rabbit anti mouse immunoglobulins and peroxidase-conjugated goat anti rabbit immunoglobulins were from Dako Japan (Tokyo, Japan). Actinase E (protease from *Streptomyces griseus*) was from Kaken Pharmaceutical Co. (Tokyo, Japan). Chondroitin ABC lyase (from *Proteus vulgaris*, EC 4.2.2.4), unsaturated disaccharide standards, HA lyase (from *Streptomyces hyalurolyticus*, EC 4.2.2.1), chondroitin AC-II lyase (from *Arthrobacter aurescens*, EC 4.2.2.5), and chondroitin B lyase (from *Flavobacterium heparinum*, EC 4.2.2) were from Seikagaku Biobusiness Co. (Tokyo, Japan). Heparin (Hep) lyase I (*from Flavobacterium heparinum*, EC 4.2.2.7) and Hep lyase II (from *Flavobacterium heparinum*, no EC number) were from Sigma–Aldrich (St. Louis, MO, USA). Cellulase (from *Aspergillu. niger*) was purchased from Sigma–Aldrich (St. Louis, MO, USA) and purified as described previously (Takagaki et al., 2002). HA (from *Streptococcus zooepidemicus*; average molecular weight, 1,200,000) was purchased from Food Chemifa Co., Ltd. (Tokyo, Japan). Other analytical grade reagents were obtained from commercial sources.

2.2. Extraction of PG from cartilage

The skin was removed from 1 kg of frozen salmon nasal cartilage slices, which were then minced and washed briefly with 0.85% NaCl. PG was then extracted by the following two protocols at 4 °C.

The first protocol was a modification of an established procedure using GdnHCl (Sajdera & Hascall, 1969). The minced cartilage was washed with acetone before and after delipidation by the method of Folch (Folch, Lees, & Sloane Stanley, 1957). PG was extracted from the delipidated and dried cartilage (33.5 g corresponding to 340 g of wet cartilage) with 30 volumes of 4 M GdnHCl in 50 mM sodium acetate buffer (pH 6.0) containing protease inhibitors (10 mM EDTA, 5 mM benzamidine hydrochloride, 10 mM *N*-ethylmaleimide, and 1 mM PMSF) as previously described (Kakizaki et al., 2011). The filtered extract was precipitated with ethanol and then dissolved in distilled water and re-precipitated to yield 15.6 g of lyophilized material, 1 g of which contained 375.0 mg uronic acid, 63.0 mg protein. The material was redissolved in distilled water, desalted by ultrafiltration using Amicon Ultra (Millipore, Billerica, MA, USA). Then aliquots containing about 10 mg uronic acid were lyophilized. For comparison, GdnHCl-extract of bovine tracheal cartilage were prepared by the same procedure from 30.2 g of delipidated and dried pieces of cartilage. The final yield of lyophilized GdnHCl-extract of bovine tracheal cartilage was 21.9 g, 1 g of which contained 48.6 mg uronic acid and 51.5 mg protein.

In the second protocol, PG was extracted from the salmon cartilage (200 g wet weight) by stirring for 48 h with 10 volumes of 4% CH_3COOH. It should be noted that in this procedure the cartilage is not washed with acetone or delipidated and that no protease inhibitors are used. The filtered extract was subjected to precipitation with ethanol as in the first protocol but the redissolved precipitate was dialyzed using cellulose ester membrane (exclusion limitation: M_r, 1,000,000) against distilled water to remove small PGs like decorin and lyophilized to yield 0.96 g of dried pellets. One gram of this PG preparation contained 420 mg uronic acid and 18 mg protein.

2.3. Purification of cartilage PG

2.3.1. DEAE-Sephacel anion-exchange column chromatography

The crude PGs (10 mg uronic acid) obtained by the two extraction protocols were each dissolved in 7 M urea in 50 mM Tris–HCl buffer (pH 7.4) containing protease inhibitors and applied to a DEAE-Sephacel column (1.8 cm × 15 cm) at a flow rate of 0.4 ml/min. The column was washed with 7 M urea in 50 mM Tris–HCl buffer (pH 7.4) containing protease inhibitors and eluted with a 500 ml of linear gradient (0–1.0 M) of NaCl followed by 2.0 M of NaCl in the same buffer, and 6.3 ml fractions were collected. Fractions positive both for uronic acid and protein, and whose elution position corresponded to around 0.5 M NaCl were pooled, desalted and concentrated by ultrafiltration to yield ChS-PG (pool I, IV, and VIII).

2.3.2. Sepharose CL-4B gel filtration column chromatography

The ChS-PG was further fractionated by gel filtration chromatography on a Sepharose CL-4B column (1.8 cm × 110 cm) using 4 M GdnHCl in 50 mM sodium acetate buffer (pH 6.0) containing protease inhibitors as the eluant. Fractions (3.3 ml) were collected and those positive for both uronic acid and protein were pooled (pool II, III, V, VI, VII, and IX) and concentrated by ultrafiltration to yield purified PG.

2.4. Immunological analysis

PG (0.2 μg of protein), which had been reduced-alkylated (Achur, Valiyaveettil, Alkhalil, Ockenhouse, & Gowda, 2000), was blotted to a PVDF membrane (Millipore, Billerica, MA, USA) using a slot blotter (Scie-Plas, Cambridge, UK), and probed with antibodies against aggrecan G1 domain, aggrecan G3 domain, or salmon aggrecan EGF-like module. Immunological analysis was performed according to the method of Towbin, Staehelin, and Gordon (1979) using the ECL system (GE Healthcare Japan, Tokyo, Japan) for detection.

2.5. Analytical methods

Uronic acid content was determined by the carbazole sulfuric acid method (Dische, 1947) and protein content determined by the method of Bradford (1976) or by monitoring the UV absorbance at 280 nm.

Amino acid analyses of purified PGs were performed on an amino acid analyzer (JEOL JLC-500/V, JEOL Co. Tokyo, Japan) after PGs (each 150 μg protein) were hydrolyzed with 6 N HCl at 110 °C for 24, 48 and 72 h (Moore & Stein, 1948). Since serine and threonine residues are sensitive to acid hydrolysis, they were obtained by extrapolating the values to zero time.

For analyses of the monosaccharides the purified PG was hydrolyzed with 4 N HCl at 100 °C for 3 and 6 h. The hydrolysates were dried, dissolved in H_2O and analyzed by high performance anion exchange-pulsed amperometric detection (HPAEC-PAD) on a Carbopac PA1 column (4 mm × 250 mm, Dionex, Sunnyvale, CA, USA). Elution was performed using 14 mM NaOH at 1.0 ml/min. Monosaccharide standards (Dionex, Sunnyvale, CA, USA) were used for calibration and calculations.

For the analysis of neuraminic acid, PG was hydrolyzed with 0.01 N HCl at 80 °C for 0.5, 1.0, 1.5, and 2.0 h (Svennerholm, 1958) and the hydrolysates after drying were dissolved in H_2O and analyzed by HPAEC-PAD on a Carbopac PA100 column (4 mm × 250 mm, Dionex, Sunnyvale, CA, USA). Elution was performed using 100 mM NaOH-150 mM sodium acetate at 1.0 ml/min. *N*-Acetylneuraminic acid (NeuAc) and *N*-glycolylneuraminic acid (NeuGc) (Wako, Tokyo, Japan) were used as standards.

GAG chains were liberated from PG by treatment with cellulase (from *Aspergillus niger*) by its endo-β-xylosidase activity (Takagaki et al., 2002). Prior to the cellulase treatment the core protein of PG was digested with actinase E. Then, the unsaturated disaccharide compositions of GAGs were determined by HPLC after digestion with chondroitin ABC lyase or double digestion with Hep lyase I and Hep lyase II, or other lyases (Sugahara, Okumura, & Yamashina, 1989; Yoshida, Miyauchi, Kikuchi, Tawada, & Tokuyasu, 1989).

All enzymatic digestions for analytical experiments were performed exhaustively under optimal conditions and reactions stopped by boiling at 100 °C for 3 min.

2.6. HPLC analyses

HPLC was performed on a Hitachi ELITE LaChrom system equipped with a model L-2420 UV–VIS detector and model L-2490 RI detector or model L-2485 fluorescence detector (Hitachi, Tokyo, Japan).

For size estimation, purified PGs were analyzed by gel filtration HPLC using a Shodex OHpak SB-805 HQ column (8.0 mm × 300 mm, Shodex, Showa Denko K. K., Kawasaki, Japan) with 0.2 M NaCl as an eluent at a flow rate, 1.0 ml/min at 40 °C. Pullulan ($M_r = 78.8 \times 10^4$, 40.4×10^4, 21.2×10^4, 11.2×10^4, 4.73×10^4, 2.28×10^4, 1.18×10^4, and 0.59×10^4, Shodex, Showa Denko K. K., Kawasaki, Japan) were used as size standards. In addition, size standards of HA ($M_r = 190 \times 10^4$, 80×10^4, 30×10^4, 10×10^4, and 41×10^4, kindly supplied by Denki Kagaku Kogyo, Tokyo, Japan), dextran sulfate ($M_r = 50 \times 10^4$, $3.6–5 \times 10^4$, 0.5×10^4), and proteins (gel filtration calibration kit HMW, $M_r = 4.4 \times 10^4$ [Ovalbumin], 7.5×10^4 [Conalbmin], 15.8×10^4 [Aldolase], 44×10^4 [Ferritin], and 66.9×10^4 [Thyroglobulin], from GE Healthcare, Buckinghamshire, UK) were used. To analyze core proteins, purified PGs were treated with 0.5 M LiOH at 4 °C for 48 h to liberate GAG chains and other *O*-linked saccharides from the core protein (Heinegard, 1972). The GAG chains were further subjected to exhaustive digestion with chondroitin ABC lyase to break them down and avoid interference with the monitoring of the HPLC fractions for the core protein. To analyze GAG chains, core proteins of purified PGs were thoroughly digested with actinase E. The eluate was monitored by UV absorbance at 215 nm and/or 280 nm.

For size estimation of HA, Shodex OHpak SB-804 HQ column (8.0 mm × 300 mm, Shodex, Showa Denko K. K., Kawasaki, Japan) was used with 0.2 M NaCl as an eluent at a flow rate, 0.5 ml/min at 40 °C. HA content of each fraction was quantified using the HA Assay Kit (Seikagaku Biobusiness Co., Tokyo, Japan) as described in "assay 1" Section 2.9.

For unsaturated disaccharide analysis, a YMC-Pack Polyamine II column (4.6 mm × 250 mm, YMC Co., Kyoto, Japan) was used with a linear gradient of NaH_2PO_4 from 16 to 478 mM over 60 min at a flow rate of 1.0 ml/min at 30 °C. The eluate was monitored by UV absorbance at 232 nm.

For the analysis of sulfation degree, ion exchange HPLC was performed on a TSKgel DEAE-5PW column (7.5 mm × 75 mm, Tosoh., Tokyo, Japan) with a linear gradient of NaCl from 0 to 1.0 M for 60 min at a flow rate of 1.0 ml/min as reported previously (Iwafune

et al., 2004). The eluate containing pyridylaminated GAG (GAG-PA) chains was monitored by the fluorescence of PA at excitation and emission wavelengths of 320 and 400 nm, respectively. The calibration curve was prepared by using pyridylaminated standards of Ch4Ss desulfated for various times (0, 12, 24, 48, and 72 h) (Kantor & Schubert, 1957), and whose disaccharide compositions were known by unsaturated disaccharide analysis.

2.7. Aggregation experiments

PG aggregates were prepared by associative conditions according to the established procedure (Heinegard & Sommarin, 1987). Briefly, crude PG extracted with 4 M GdnHCl or 4% CH_3COOH was dissolved in 0.5 M GdnHCl in 50 mM Tris–HCl buffer (pH 8.0) containing protease inhibitors and fractionated by CsCl density gradient centrifugation under associative conditions (Faltz et al., 1979). The fractions in the bottom of the tube that were positive for both uronic acid and protein contained the PG aggregates with HA. These were dialyzed against 50 mM Tris–HCl buffer (pH 7.4) and then distilled water.

Distinct from the above samples, PG aggregates were reconstructed by incubation of chromatography-purified PG with exogenous addition of HA ($M_r = 120 \times 10^4$) in the ratio of 100:0.8 (by weight) in 50 mM Tris–HCl buffer (pH 7.4), at 4 °C for 18 h (Morgelin, Paulsson, Heinegard, Aebi, & Engel, 1995; Tang, Buckwalter, & Rosenberg, 1996). The resulting PG aggregates were dialyzed against distilled water. As a control bovine nasal cartilage PG aggregates (ICN Biomedical, Aurora, OH, USA) were used.

2.8. AFM imaging

AFM imaging was performed according to reports by Yeh et al. and Ng et al. (Ng et al., 2003; Yeh & Luo, 2004). Briefly, the surface of muscovite mica (Ladd Research Industries, Williston, VT, USA) was cleaved and used as a substrate to immobilize PG molecules for AFM imaging. The freshly cleaved mica surfaces (10 mm × 10 mm) were treated with 0.01% 3-aminopropyltriethoxysilane aqueous solution, to which 5% ethanol was added, for 30 min at room temperature, washed gently with distilled water, and dried using a spinner. PG solution (0.1 mg/ml) was put on the surface of the mica for 10 min, then, the surface of the mica was washed gently with distilled water, and spin-dried. The immobilized PGs were imaged with a Nanoscope IIIa Multimode AFM (Digital Instruments, Tonawanda, NY, USA) using silicon RTESP probes (Bruker AXS, Tokyo, Japan) with a 10 nm radius sharp pyramidal silicon tip on a rectangular micro cantilever. For topographic imaging of the HA molecules on mica surfaces, tapping mode was employed in ambient temperature and humidity. In the tapping mode, the cantilever was oscillated at about 330 kHz which was just below its resonant frequency. Scanning rate of the imaging was approximately 2 μm/s which was much slower than the tapping rate. Height and width of the immobilized molecules were measured from the AFM topographic profile.

2.9. HA content measurement

Assay 1: The HA in the cartilage-extract was recovered by chromatography on a DEAE-Sephacel column. The fractions 28–37 in Fig. 1A, a, fractions 29–33 in Fig. 1A, c, and fractions 29–33 in Fig. 1A, e eluted with about 0.2 M of the NaCl gradient (0–1.0 M), and where HA should elute, were combined as pools X, Y, and Z. The HA content of pools X, Y, and Z were quantified by an ELISA-like assay using HA binding protein (HABP) according to the manufacturers' instructions for HA Assay Kit (Seikagaku Biobusiness Co., Tokyo, Japan). The absorbance at 490 nm (control wavelength, 630 nm) of each well was measured by a microplate spectrophotometer xMark™ (Bio-Rad, Tokyo, Japan).

Assay 2: For the determination of HA content in PG aggregates obtained under associative conditions (*i.e.* HA content in PG aggregates without addition of exogenous HA), PG aggregates (0.05 μg/well) in 50 mM sodium carbonate buffer (pH 9.6) were coated on the microtiter-plate at 4 °C for 16 h. After washing with PBS, wells were blocked with 1% BSA in PBS at 4 °C, for 16 h. The wells were washed with PBS containing 0.1% tween-20 (PBS-T) followed by washing with PBS, then wells were incubated with PBS at 4 °C, for 16 h. HA contained in the aggregates was captured with biotinylated HABP at room temperature for 2 h, after washing with PBS-T, and detected using peroxisidase-conjugated streptoavidin and *o*-phenylenediamine. The absorbance at 492 nm of each well was measured using a microplate spectrophotometer xMark™. To generate a standard curve for estimating the content of endogenous HA associated with PGs, 0.5 μg/well of HABP were coated in the same way. The wells were blocked with 1% BSA in PBS, incubated with HA in PBS (0, 1.5, 5, 15, 50, 150, and 500 ng/well) 16 h at 4 °C, then the HA was captured with biotinylated HABP. Then wells were washed and detection was performed by the same procedure as described above.

3. Results and discussion

3.1. Purification and identification of salmon nasal cartilage PG

For the structural analysis, PG was purified from 4 M GdnHCl or 4% CH_3COOH-extract of salmon nasal cartilage or from 4 M GdnHCl-extract of bovine tracheal cartilage (Fig. 1A). Combined fractions 45–64 (pool I in Fig. 1A, a), fractions 45–61 (pool IV in Fig. 1A, c), and fractions 53–73 (pool VIII in Fig. 1A, e) of DEAE column chromatography were further fractionated by CL-4B column chromatography, respectively. The PGs eluting in fractions 28–30 (pool II) and 38–40 (pool III) in Fig. 1A, b, 38–40 (pool V), 53–55 (pool VI), and 63–65 (pool VII) in Fig. 1A, d, and 34–35 (pool IX in Fig. 1A, f) were collected respectively and characterized.

To determine whether the PGs purified above were aggrecan or not, we performed amino acid composition analysis, internal amino acid sequencing, and immunological analysis for specific domains of aggrecan. The amino acid compositions of purified PGs were both similar to that of salmon nasal cartilage aggrecan deduced from cDNA analysis (Table 1). Similar results were obtained for purified bovine PG. Internal amino acid sequencing using nano-LC/MS/MS revealed that the salmon PG purified from 4% CH_3COOH-extract was also aggrecan (Table S1) as was PG purified from 4 M GdnHCl-extract in our previous report (Kakizaki et al., 2011). Immunological analysis showed that the antibodies against the G1 and G3 domains reacted with all PGs (Fig. 1B). From these results, all PGs purified in this study were identified as aggrecan and we henceforth refer to these samples as "PG".

However, with both antibodies the intensity of the reaction with the 4% CH_3COOH-prepared PG was lower than with the 4 M GdnHCl-prepared PG. Further, both antibodies reacted more intensely with the larger molecules (pool II) than the smaller molecules (pool III) in the 4 M GdnHCl-extract. In the case of the 4% CH_3COOH-prepared PG, the middle-sized molecules eluting at the top of the peak (pool VI) reacted highest with the anti-G1 antibodies and lowest with anti-G3 antibodies compared to the larger molecules eluting in the leading edge of the peak (pool V) and smaller molecules eluting in the trailing edge of the peak (pool VII). This could be due to the omission of protease inhibitors during the 4% CH_3COOH-extraction. Although amino acids corresponding to some putative cleavage sites for proteases reported in mammalian aggrecan were displaced in salmon aggrecan, there may

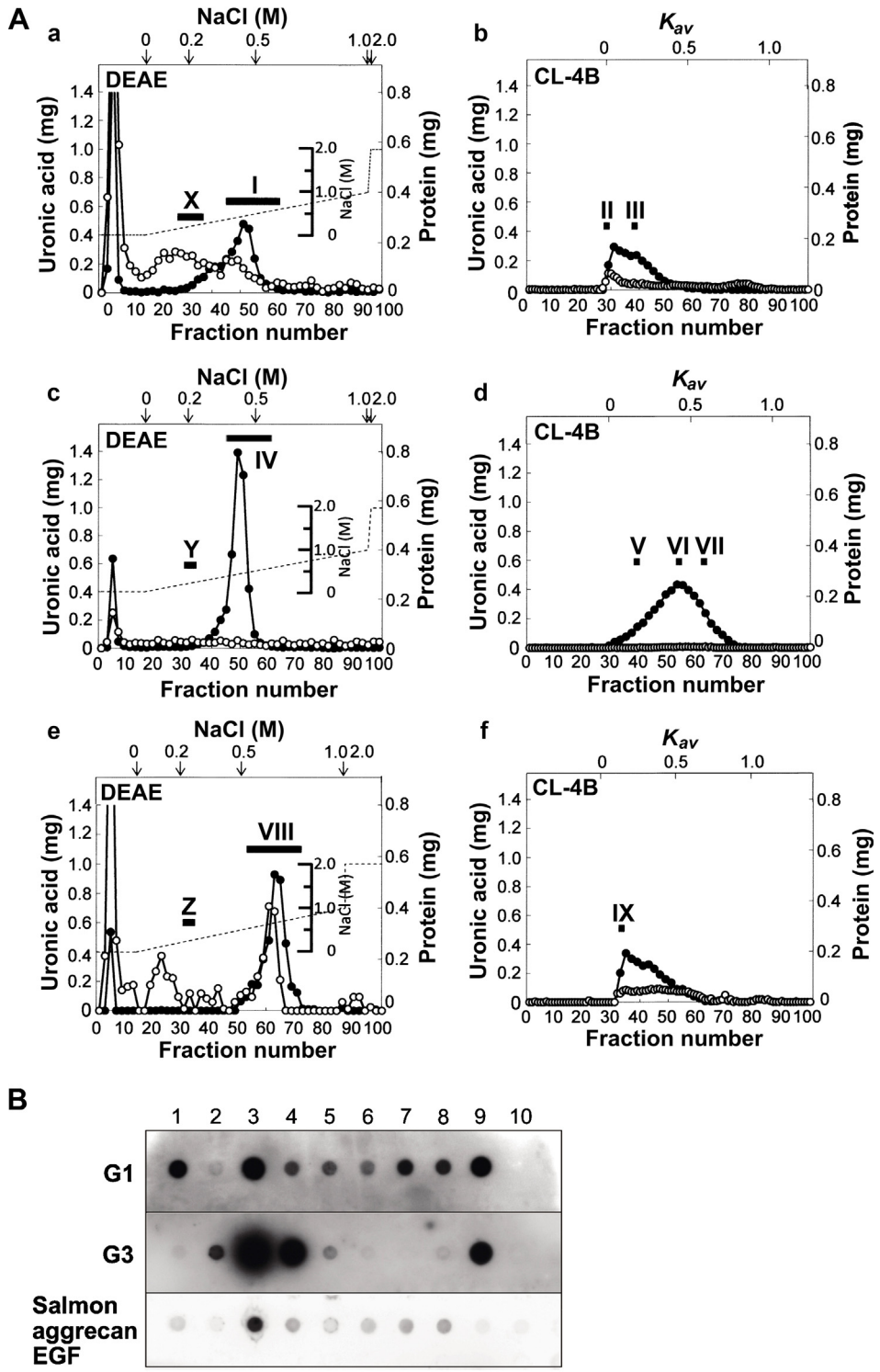

Fig. 1. DEAE-Sephacel and Sepharose CL-4B column chromatography of cartilage extracts (A) and immunological analysis of purified PG (B). (A) The 4 M GdnHCl-(a, b), 4% CH_3COOH-(c, d) extract of salmon nasal cartilage, or 4 M GdnHCl-extract of bovine tracheal cartilage (e, f) were fractionated by DEAE-Sephacel chromatography (a, c, e) followed by Sepharose CL-4B chromatography (b, d, f). Broken lines indicate the gradient curves of NaCl and arrows the specific NaCl concentrations. Solid circle, uronic acid content detected by carbazole sulfuric acid method; open circle, protein content detected by the method of Bradford (a, c, e) or protein content by monitoring the UV absorbance at 280 nm (b, d, f). Bars with Greek numerals indicate the fractions combined for recovery of material as Pools I–IX: pool I (Frs. 45–64), pool II (Frs. 28–30), pool III (Frs. 38–40), pool IV (Frs. 45–61), pool V (Frs. 38–40), pool VI (Frs. 53–55), pool VII (Frs. 63–65), pool VIII (Frs. 53–73), and pool IX (Frs. 34 and 35). Pools I, IV, and VIII from DEAE column chromatography were further fractionated by Sepharose CL-4B column chromatography. Pools II, III, V, VI, VII, and IX from Sepharose CL-4B chromatography were recovered and analyzed. The material indicated as pools X (Frs. 28–37 of a), Y (Frs. 29–33 of c), and Z (Frs. 29–33 of e) were each collected for the determination of the HA content by an ELISA-like assay (see Fig. 4). (B) Dot-blotted proteins (0.2 μg each), which had been reduced-alkylated, were probed with antibodies to aggrecan G1 domain, G3 domain, or salmon aggrecan EGF-like module. 1, HABP; 2, 4 M GdnHCl-extract of salmon nasal cartilage; 3, PG purified from 4 M GdnHCl-extract of salmon nasal cartilage (pool II in Fig. 1A, b); 4, PG purified from 4 M GdnHCl-extract of salmon nasal cartilage (pool III in Fig. 1A, b); 5, 4% CH_3COOH-extract of salmon nasal cartilage; 6, PG purified from 4% CH_3COOH-extract of salmon nasal cartilage (pool V in Fig. 1A, d); 7, PG purified from 4% CH_3COOH-extract of salmon nasal cartilage (pool VI in Fig. 1A, d), 8, PG purified from 4% CH_3COOH-extract of salmon nasal cartilage (pool VII in Fig. 1A, d); 9, PG purified from bovine trachea cartilage (pool IX in Fig. 1A, f); 10, BSA.

Table 1
Amino acid composition of purified salmon nasal cartilage proteoglycan (PG) isolated by two different extraction procedures and of bovine cartilage PG. Amino acid composition predicted by cDNA for the salmon and bovine PGs are also shown for composition. Values are given as residues per 1000.

Amino acid	Salmon nasal cartilage PG			Bovine tracheal cartilage PG	
	Purified from 4 M GdnHCl-extract (pool II)	Purified from 4% CH_3COOH-extract (pool VI)	cDNA[a]	Purified from 4 M GdnHCl-extract (pool IX)	cDNA[b]
residues/1000 residues					
Asp, Asn	95	77	88	69	59
Thr	57	54	74	58	67
Ser	109	134	94	112	135
Glu, Gln	110	103	112	140	139
Gly	161	175	108	126	118
Ala	51	62	60	71	67
Cys	35	21	27	6	12
Val	62	56	81	68	68
Met	16	8	20	5	4
Ile	47	34	36	38	37
Leu	94	71	60	77	75
Tyr	28	23	37	20	20
Phe	24	30	37	35	35
Lys	13	53	21	21	16
His	15	17	30	12	15
Arg	29	28	49	38	35
Pro	54	54	66	104	98

[a] Accession number: AB571294 in the GenBank™/EMBL Data Bank.
[b] Accession number: NM_173981 in the GenBank™/EMBL Data Bank.

Table 2
Molecular weight estimated by gel filtration HPLC. PG samples were analyzed with a Shodex OHpak SB-805 HQ column. Various sizes of pullulan were used as size standards.

Calculated molecular weight	Purified salmon nasal cartilage PG		Purified bovine tracheal cartilage PG
	From 4 M GdnHCl-extract (pool II)	From 4% CH_3COOH-extract (pool VI)	From 4 M GdnHCl-extract (pool IX)
(A) Non-treated	360,000–6,600,000 (1,850,000)[c]	130,000–3,300,000 (540,000)[c]	205,000–5,800,000 (1,550,000)[c]
(B) Deglycosylated[a] (core protein)	25,000–290,000 (104,000)[c]	11,000–500,000 (86,000)[c]	14,500–360,000 (80,000)[c]
(C) Peptide-degraded[b] (GAG chain)	49,000–920,000 (176,000)[c]	34,000–66,000 (135,000)[c]	18,000–400,000 (53,000)[c]

[a] Treated with LiOH followed by chondroitin lyase ABC digestion.
[b] Digested with Actinase E.
[c] Molecular weight of material eluting at the peak position.

be some sites on IGD or GAG attachment domains, and acid proteases are active in 4%-CH_3COOH where the pH is 2.3. We also examined whether salmon PGs have an EGF-like module (Fig. 1B). Both purified salmon PGs reacted with an antibody against EGF-like module of salmon aggrecan. Especially, it is worthy of attention that PG fragments in 4% CH_3COOH-extract retained EGF-like modules although most of them lacked the G3 domain. This result supports the data[4] regarding EGF-like activity of salmon PG in 4% CH_3COOH-preparation from the structural aspect.

3.2. Molecular size distribution of salmon nasal cartilage PG

It is not possible to determine the absolute molecular size distribution of large PGs such as aggrecan or even of its GAG moiety, because of the lack of appropriate size standards. In order to assess the relative size distributions of PGs (pools II, VI, and IX) we performed gel filtration HPLC using various size standards (Fig. 2). Size distributions of PGs (sizes of whole, core protein, and one GAG) estimated from pullulans as size standards are shown in Table 2. Even though pullulans are neutral polysaccharides and are unlike the polyanionic GAGs, we used them since these standards are available in a relatively wide range compared to other size standards.

The number of repeating disaccharide units was calculated from the estimated size of a GAG chain, (C) in Table 2, and shown in Table 3. Results using size standards other than pullulan (HA, dextran sulfate, or proteins) are shown in Tables S2–S7. Calculated values were different depending on the choice of size standards. However, in all cases the size of the GAG chains of 4 M GdnHCl-extracted salmon PG was largest and that of 4 M GdnHCl-extracted bovine PG was the smallest with that of 4% CH_3COOH-extracted salmon PG being intermediate. From Tables 2 and 3 and Tables S2–S7, it was seen that overall GAG chains of salmon PG are1.6–3.3 times longer than those of bovine PG recovered from the peak of the HPLC chromatogram (Fig. 2). GAG chain length of PG in pool VI was 77% longer than that in pool II.

The calculated values for molecular weight of a core protein based on the cDNA-predicted amino acid sequence are 143,276 for salmon aggrecan (AB571294), and 240,709 for bovine aggrecan (NM_173981). The molecular size distribution of core protein for salmon PG calculated by gel filtration HPLC was relevant to the above value from cDNA. However, the core protein size of bovine PG calculated by HPLC was one third of that from cDNA and lower than that for salmon PG. A possible reason for the discrepancy could be that in our non reduced-alkylated protein samples for HPLC analysis, bovine PG assumed a compact conformation in solution due to the higher order structure compared to salmon.

Numbers of GAG chains in the PGs were also calculated (Table 3) using data presented in Table 2 as follows: The total M_r of the

[4] Nakamura et al. (2013) A novel pharmacological application for salmon nasal cartilage proteoglycan, Registered patent No. JPA5194253 (Japan).

Table 3
Calculated values of glycosaminoglycan chains from Table 2.

	Purified salmon nasal cartilage PG		Purified bovine tracheal cartilage PG
	From 4 M GdnHCl-extract (pool II)	From 4% CH_3COOH-extract (pool VI)	From 4 M GdnHCl-extract (pool IX)
Number of repeating disaccharide units	121.7–2313.7 (441.3)[a]	83.9–1659.4 (338.1)[a]	43.7–1005.0 (131.77)[a]
Number of GAG chains	1.9–35.9 (9.9)[a]	0.9–20.7 (3.4)[a]	3.6–102.6 (27.7)[a]

[a] Peak top data from standard size pullulans.

carbohydrate portion (GAG chains) was obtained by subtracting the estimated M_r of a core protein, (B) in Table 2, from the estimated M_r of the non-treated PG, (A) in Table 2. Numbers of GAG chains in the PGs were obtained by dividing the total M_r of carbohydrates by M_r of one GAG chain, (C) in Table 2. For example, the number of GAG chains in the salmon nasal cartilage PG purified from 4 M GdnHCl-extract (pool II) was obtained by the following formula: (1,850,000–104,000)/176,000 = 9.9. The calculated number of GAG chains in salmon was approximately one-third of those of bovine, that is relevant to the calculated sizes of their GAG attachment domains from cDNA: 41870.14 for salmon and 141604.81 for bovine (Fig. S1).

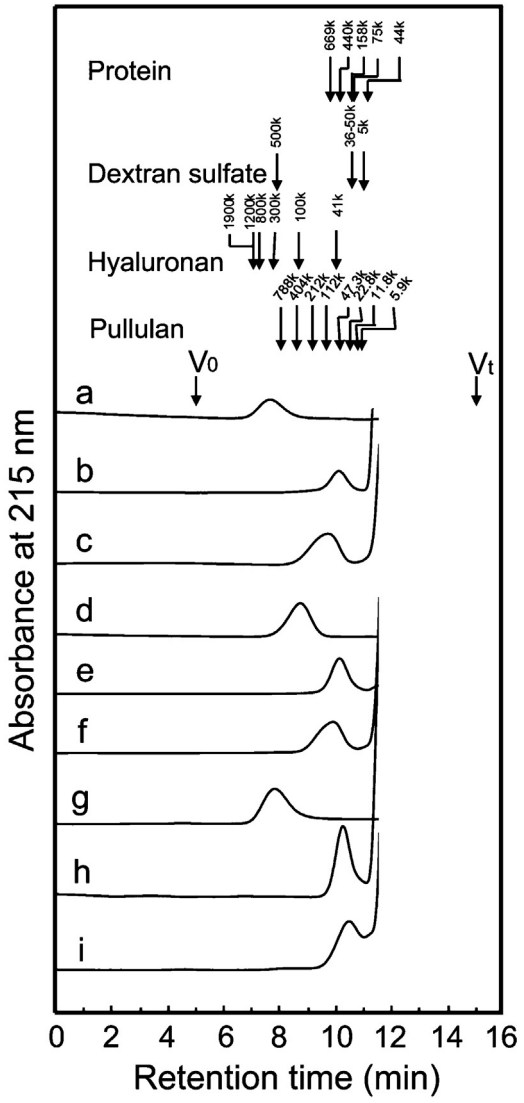

Fig. 2. Gel filtration HPLC of salmon nasal cartilage PG. PGs were analyzed by Shodex OHpak SB-805 HQ gel filtration column before treatment (a, d, g) or after treatment with LiOH followed by chondroitin lyase ABC (b, e, h) or after treatment with actinase E (c, f, i). a, b, c, PG (pool II) purified from 4 M GdnHCl-extract of salmon nasal cartilage; d, e, f, PG (pool VI) purified from 4% CH_3COOH-extract of salmon nasal cartilage; g, h, i, PG (pool IX) purified from bovine tracheal cartilage. Arrows indicate the elution positions of size standards of proteins, dextran sulfate, HA, and pullulan with defined approximate molecular weight.

3.3. Analysis of salmon nasal cartilage PG sugar chains

The natures of the GAG chains were determined by analyzing the unsaturated disaccharides generated by treatment with chondroitin lyases. The disaccharide composition showed a higher proportion of ΔDi-6S in salmon PG, 60%, compared to 40% in bovine, regardless of the extraction method (Table 4). This compositional difference in cartilage PG may be due to the differences between animal species. It was determined that the origin of ΔDi-4S was from chondroitin 4-sulfate only since chondroitin B lyase treatment did not yield any unsaturated disaccharides, indicating absence of dermatan sulfate (data not shown). This was supported by the combinations of unsaturated disaccharide analyses with chondroitin ABC lyase and chondroitin AC-II lyase (data not shown). Unsaturated disaccharides from heparan sulfate (HS) or Hep were not detected after digestion with Hep lyases (data not shown). Also, unsaturated disaccharides from HA were not detected after *Streptomyces* hyaluronidase digestion (data not shown). Cellulose acetate membrane electrophoresis (Fig. S2) and ion-exchange HPLC analysis on a TSKgel DEAE-5PW column (Table 5) revealed that salmon and bovine GAGs had similar degrees of total sulfation.

In order to estimate the number of repeating disaccharide units and the molecular weight of a ChS chain by a method other than gel filtration HPLC, monosaccharide analysis was performed on PGs (Table 6). In aggrecan, the major portion of *N*-acetylgalactosamine (GalNAc) is from the ChS chains and a very minor portion is from the linkage regions through which keratan sulfate (KS) chains and *O*-linked oligosaccharides are attached to the core protein. After acid hydrolysis GalNAc and *N*-acetylglucosamine (GlcNAc) are detected as GalN and GlcN, respectively (Campo, Campo, Ferlazzo, Vinci, & Calatroni, 2001). The approximate number of repeating disaccharide units of a ChS chain can therefore be calculated from the ratio of GalN to Xyl. Then, the molecular weight of a ChS chain was calculated as 46,848 for purified PG from 4 M GdnHCl-extract of salmon nasal cartilage (pool II), 41,273 for purified PG from 4% CH_3COOH-extract of salmon nasal cartilage (pool VI), and 31,976 for purified PG from 4 M GdnHCl-extract of bovine tracheal cartilage PG (pool IX). ChS chain length of salmon nasal cartilage PG was longer by 1.5 folds than that of bovine tracheal cartilage. ChS chain length of PG in pool VI was 88% longer than that in pool II. These data supported the results of gel filtration HPLC.

GlcNAc is a component of KS/*O*-linked oligosaccharides, HA, or HS/Hep and is not contained in ChS chains. Since HA and HS/Hep were not detected in any of our purified PG described above, the presence of GlcN in PG suggests the presence of KS/*O*-linked oligosaccharide. The values of GlcN suggests that salmon nasal

Table 4
Unsaturated disaccharide compositions of GAG moiety of salmon nasal cartilage PG. Unsaturated disaccharide compositions of GAGs were determined by HPLC on a YMC-Pack Polyamine II column after digestion with chondroitin ABC lyase. The results are expressed as a percentage of each detected unsaturated disaccharide to total unsaturated disaccharides.

Unsaturated disaccharide	Unsaturated disaccharide (%)		
	Purified salmon nasal cartilage PG		Purified bovine tracheal cartilage PG
	From 4 M GdnHCl-extract (pool II)	From 4% CH_3COOH-extract (pool VI)	From 4 M GdnHCl-extract (pool IX)
ΔDi-0S[a]	11.7 ± 2.5[b]	15.3 ± 2.2	6.29 ± 0.2
ΔDi-6S	62.1 ± 1.3	62.4 ± 0.6	40.9 ± 1.1
ΔDi-4S	24.8 ± 1.6	21.6 ± 2.7	52.3 ± 1.3
ΔDi-diSD	1.31 ± 0.2	0.68 ± 0.1	0.27 ± 0.1
ΔDi-diSE	N.D.[c]	N.D.	N.D.
ΔDi-triS	N.D.	N.D.	N.D.

[a] ΔDi-0S, ΔGlcUA-β1–3GalNAc; ΔDi-4S, ΔGlcUA β1–3GalNAc(4S); ΔDi-6S, ΔGlcUAβ1–3GalNAc(6S); ΔDi-diSD, ΔGlcUA(2S) β1–3GalNAc(6S); 2S, 4S, and 6S, represent 2-*O*-sulfate, 4-*O*-sulfate, and 6-*O*-sulfate.
[b] Values presented are mean ± SD obtained from four independent experiments.
[c] Not detected.

Table 5
Number of sulfates per disaccharide unit estimated by anion exchange HPLC on a TSKgel DEAE-5PW column. Values presented are mean ± SD obtained from three independent experiments.

	Purified salmon nasal cartilage PG		Purified bovine tracheal cartilage PG
	From 4 M GdnHCl-extract (pool II)	From 4% CH_3COOH-extract (pool VI)	From 4 M GdnHCl-extract (pool IX)
Number of sulfates per disaccharide unit	0.86 ± 0.01	0.85 ± 0.01	0.82 ± 0.01

Table 6
Monosaccharide analysis of salmon nasal cartilage PG.

Monosaccharide	nmols monosaccharide per 1000 nmol uronic acid		
	Purified salmon nasal cartilage PG		Purified bovine tracheal cartilage PG
	From 4 M GdnHCl-extract (pool II)	From 4% CH_3COOH-extract (pool VI)	From 4 M GdnHCl-extract (pool IX)
GalN[a]	469.0 ± 40.5[b]	415.1 ± 34.4	453.9 ± 64.6
GlcN	7.7 ± 2.1	5.3 ± 1.7	82.0 ± 13.8
Gal	16.2 ± 10.9	12.8 ± 6.7	21.3 ± 0.8
Xyl	5.2 ± 2.1	5.2 ± 2.6	6.1 ± 1.6
GalN/Xyl	116.3 ± 82.7	102.2 ± 65.4	78.8 ± 26.5

[a] GalN, galactosamine; GlcN, glucosamine; Gal, galactose; Xyl, xylose.
[b] Values presented are mean ± SD obtained from four independent experiments.

cartilage PG has KS/*O*-linked oligosaccharide although the content is much lower (less than one-tenth) than that of purified bovine PG. KS/*O*-linked oligosaccharides chains of mammalian cartilage aggrecan occasionally have neuraminic acid(s) at the non-reducing terminal (Bhavanandan & Meyer, 1968; Funderburgh, 2000; Stuhlsatz et al., 1989). The total neuraminic acid content of salmon PG was about one-tenth of that in bovine PG (Table 7). This observation is in agreement with our previous conclusion from cDNA analysis that salmon aggrecan does not have the KS attachment domain observed in mammalian aggrecan (Kakizaki et al., 2011). *N*-Linked oligosaccharide chains also have some GlcNAcs however, mannose, one of the components of *N*-linked oligosaccharides, was detected in only trace amounts or not at all in salmon aggregan by monosaccharide analysis (data not shown). Therefore, once again salmon cartilage aggrecan has, if any, very few KS/*O*-linked oligosaccharides and *N*-linked oligosaccharides.

3.4. Molecular image of PG

We compared the molecular images of salmon nasal cartilage PG monomers to those of bovine cartilage PG monomers prepared under the dissociative condition by AFM and found them to be different (Fig. 3). The core proteins of salmon PGs were shorter (197.7 ± 13.4 nm) than those of bovine PGs (297.5 ± 99.4 nm). In accordance with the length of a core protein the number of GAG chains of salmon PG was much lower than those of bovine PG. Conversely, the GAGs of salmon PG were longer (58.5 ± 13.6 nm) than those of bovine PG (29.1 ± 7.7 nm). The maximal number of GAGs (35.9 in salmon, 102.6 in bovine) shown in Table 3 was similar to the value predicted by cDNA analysis and alkaline β-elimination experiment on salmon aggrecan in our previous reports (Kakizaki et al., 2011). The number of Ser residues involved in *O*-glycosidic linkages of saccharide chains including GAG is about 45 in salmon

Table 7
Monosaccharide analysis (neuraminic acid analysis) of salmon nasal cartilage PG.

nmols monosaccharide per 1000 nmol uronic acid			
Monosaccharide	Purified salmon nasal cartilage PG		Purified bovine tracheal cartilage PG
	From 4 M GdnHCl-extract (pool II)	From 4% CH_3COOH-extract (pool VI)	From 4 M GdnHCl-extract (pool IX)
NeuAc[a]	2.2 ± 0.10[b]	1.4 ± 0.02	21.8 ± 0.33
NeuGc	N.D.	N.D.	0.16 ± 0.06

[a] NeuAc, *N*-acetylneuraminic acid; NeuGc, *N*-glycolylneuraminic acid.
[b] Values presented are mean ± SD obtained from three independent experiments.

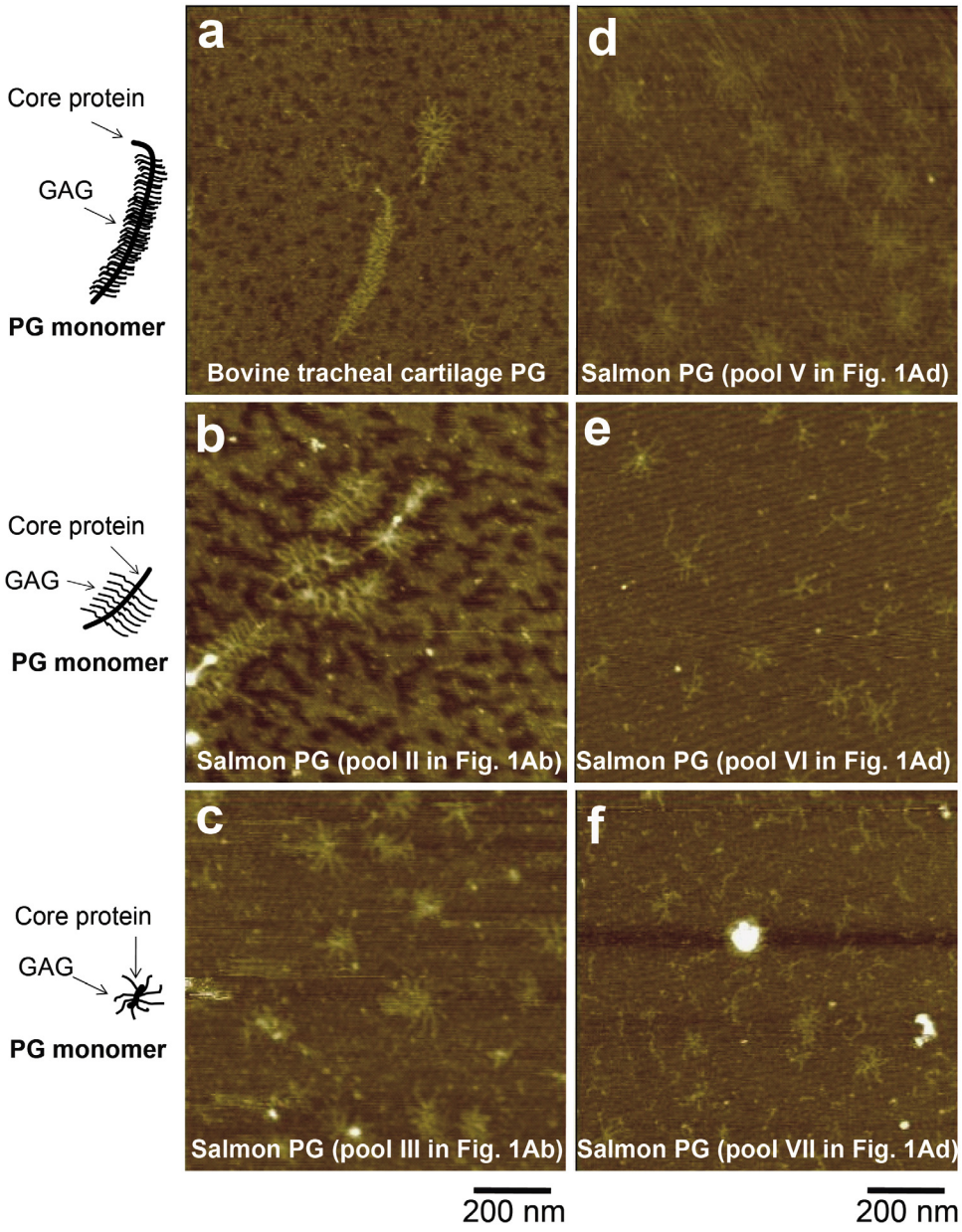

Fig. 3. AFM images of salmon cartilage PG purified under dissociative conditions. PG pools purified by Sepharose CL-4B chromatography under dissociative condition were dissolved in distilled water, desalted and examined by AFM. a, PG purified from 4 M GdnHCl-extract of bovine tracheal cartilage PG (pool IX in Fig. 1A, f); b, PG purified from 4 M GdnHCl-extract of salmon nasal cartilage (pool II in Fig. 1A, b); c, PG purified from 4 M GdnHCl-extract of salmon nasal cartilage (pool III in Fig. 1A, b); d, PG purified from 4% CH_3COOH-extract of salmon nasal cartilage (pool V in Fig. 1A, d); e, PG purified from 4% CH_3COOH-extract of salmon nasal cartilage (pool VI in Fig. 1 A, d); f, PG purified from 4% CH_3COOH-extract of salmon nasal cartilage (pool VII in Fig. 1 A, d).

(AB571294) and 160 in bovine (NM_173981) as estimated from the cDNA-predicted amino acid sequence. The intermediate numbers of GAG chains (9.9 in salmon, 27.7 in bovine) shown in Table 3 are lower than those values. The visualized number of GAG chains on AFM imaging is more in agreement with the intermediate number of GAG chains determined by gel filtration HPLC, although it is unclear whether ChS chains are overlapped in AFM or not. Structural differences between salmon and bovine cartilage PG are assumed to be due to the differences of animal specie PG sources.

Also, the AFM images of salmon PG of different preparations were compared. PGs in the CL-4B-peak top fractions (pool II) purified from 4 M GdnHCl-extract (Fig. 3A, b) were larger than that in the CL-4B-peak top fractions (pool VI) purified from 4% CH_3COOH-extract (Fig. 3A, e). PGs in pool II from 4 M GdnHCl-extract were larger than those in pool III. In the case of purified PGs from 4% CH_3COOH-extract, pool V contained the largest PG (105.5 ± 18.7 nm) and pool VI, the smallest (60.6 ± 12.5 nm), with pool VII having intermediate size molecules (97.0 ± 4.3 nm). PGs in pool III from 4 M GdnHCl-extract and pool V from 4% CH_3COOH-extract had similar sizes and molecular images. A large number of the salmon PGs in 4% CH_3COOH-extract were fragmented and lacked the G3 domain at the C-terminus but retained the G1 domain and/or EGF-like module. This probably depends on the extraction condition of minimizing of protease activities based on the strength of denaturation, pH, and presence or absence of protease inhibitors in extraction solvent. In addition to 4% CH_3COOH extraction, alternative extraction methods are currently being developed that will be even more simple, low-cost, large-scale, and non-toxic for preparing PG for possible industrial application research and the functions of PG fractions thus obtained are being studied (Goto

et al., 2011; Goto, Yamazaki, Kato, Yamamoto, & Katagata, 2012). Therefore, analysis of the relationships between structures and functions of PG prepared by different methods will become more important. As reported previously (Kashiwakura et al., 2008, 2007; Mitsui et al., 2010; Ota et al., 2008; Sashinami et al., 2012, 2006; Yoshino et al., 2010), 4% CH_3COOH-prepared PG fractions showed effective activities. This suggests that PG with incomplete structure has the possibility to show enough activities or sometimes more effective activities compared to PG with complete structure in specific pharmacological activities, thus preparation methods should be selected in accordance with the intended applications.

The PGs under associative conditions (*i.e.*, 1, PGs prepared by CsCl density gradient centrifugation; 2, chromatography-purified PGs with added exogenous HA) were also scanned by AFM. Images of the PG aggregates were different between salmon and bovine. Bovine samples appeared to be representative PG aggregates that just lack visible HA. Apparent images of PG aggregates with visible HA (Hascall, 1977; Heinegard & Hascall, 1974; Rosenberg, Hellmann, & Kleinschmidt, 1975) were hardly observed in any samples even in the control (Fig. 4, d) because independent filaments of HA are so thin that we were not able to observe them under our conditions of AFM. Therefore, the images indicated by arrows in Fig. 4, d and h, which were only observed occasionally, are not considered to be single HA filaments, but are bundles of multiple HA filaments. A typical single HA filament is reported to be about 0.7 nm in height and 4 nm in width (Cowman, Li, & Balazs, 1998; Toole, 2004). In fact, sizes for bundles of multiple HA filaments observed in similar conditions of AFM were assessed to be 0.7–1.9 nm in height and 20–35 nm in width (Fig. S3). Like a control (Fig. 4, d), under associative conditions PG monomers seemed to form an aggregation in all samples (Fig. 4 a–c). The size of the aggregates appeared to become larger with the addition of exogenous HA (Fig. 4 e–g). This suggests that under physiological conditions all current PG samples, including salmon PG prepared by 4% CH_3COOH-extraction, are able to interact with HA *via* their G1 domain.

3.5. HA content and HA size distribution in the salmon nasal cartilage PG extract

Chromatography-purified PGs in this study did not contain HA as described in subsection 3.3. In order to determine whether the 4 M GdnHCl-extract of salmon cartilage contained HA, which will interact with PG and form aggregation in cartilage, combined fractions 28–37 (pool X) were concentrated and HA content was measured (by assay 1 in Section 2.9). The HA content in the 4 M GdnHCl-extract from salmon cartilage was 39.2 ± 0.8 μg per 1 mg total uronic acid, thus, the ratio of HA (by ELISA like assay) to uronic acid (by carbazole sulfuric acid method) is 1:25.5. The HA content was estimated to be roughly 674 μg per 1 g wet salmon nasal cartilage. In the same way, HA content was determined as 13.7 ± 1.0 μg and 42.7 ± 0.3 μg per 1 mg total uronic acid in 4% CH_3COOH-extract of salmon cartilage, and 4 M GdnHCl-extract of bovine cartilage, respectively. The ratios of HA to uronic acid of them were 1:73.0 and 1:23.4, respectively. To examine the size distribution of HA, the above samples were fractionated by gel filtration HPLC using a Shodex OHpak SB-804 HQ column and the HA content of the fractions analyzed (Fig. 5). Size distribution of salmon cartilage HA in the 4 M GdnHCl-extract was found to be broad, from 400 to 850,000 with the peak at 70,000. These values were from 400 to 850,000 with the peak at 36,000 in the 4% CH_3COOH-extract, and from 3,000 to 850,000 with the peak at 70,000 in the 4 M GdnHCl-extract of bovine cartilage. From these, it is suggested that salmon cartilage contains similar amounts and sizes of HA compared to bovine cartilage.

In another approach, the HA contents of PG aggregates (*i.e.*, CsCl density gradient centrifugation preparation), were also measured

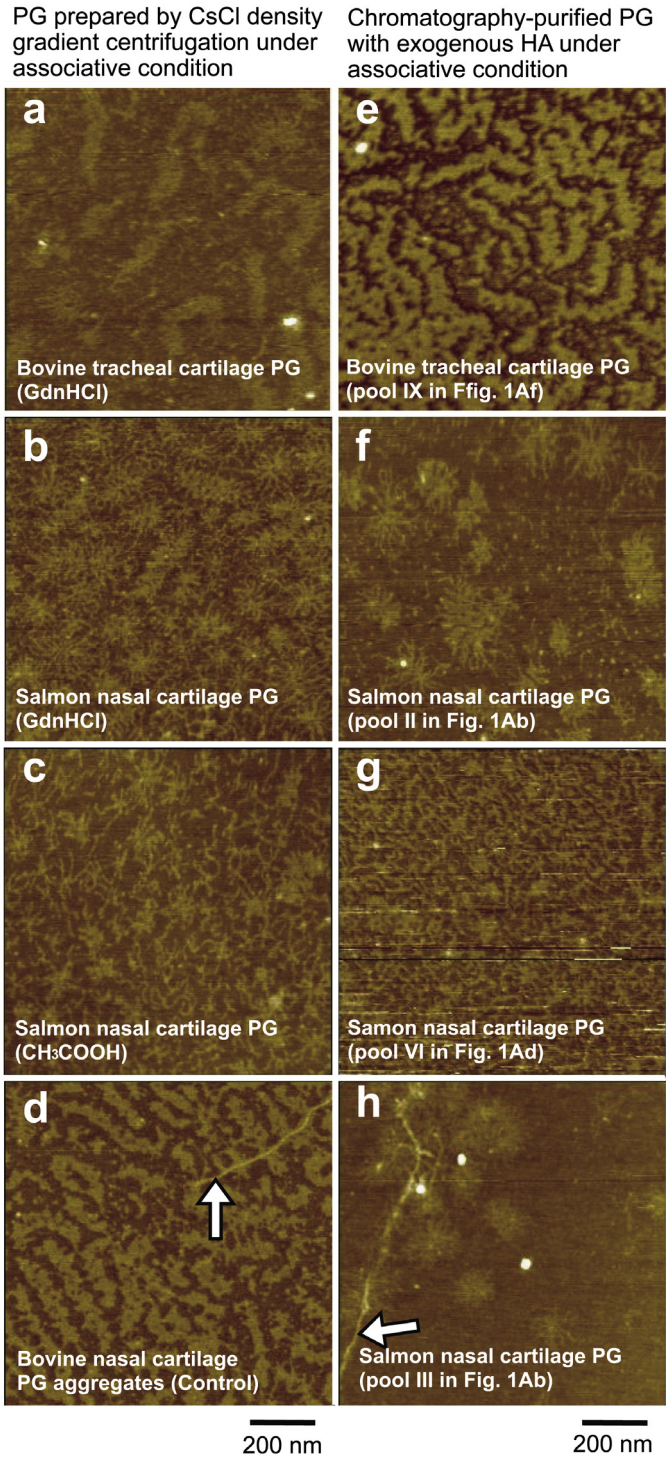

Fig. 4. AFM images of salmon cartilage PG purified under associative conditions. (a, b, c) PG aggregates prepared by CsCl density gradient centrifugation under associative conditions. (e, f, g) PG aggregates reconstructed by addition of exogenous HA to CL-4B-purified PG. a, PG aggregates from 4 M GdnHCl-extract of bovine tracheal cartilage; b, PG aggregates from 4 M GdnHCl-extract of salmon nasal cartilage; c, PG aggregates from 4% CH_3COOH-extract of salmon nasal cartilage; d, Bovine nasal cartilage PG aggregate (ICN Biomedical) as a control; e, reconstructed PG aggregates of purified bovine tracheal cartilage PG (pool IX in Fig. 1A, f); f, reconstructed PG aggregates of purified salmon nasal cartilage PG from 4 M GdnHCl-extract (pool II in Fig. 1A, b); g, reconstructed PG aggregates of purified salmon nasal cartilage PG from 4% CH_3COOH-extract (pool VI in Fig. 1A, d); h, PG purified from 4 M GdnHCl-extract of salmon nasal cartilage (pool III in Fig. 1A, b) without adding exogenous HA. Arrows indicate the possible images of a bundle of multiple HA filaments. Magnification bar represents 200 nm. Height, 5,000 nm.

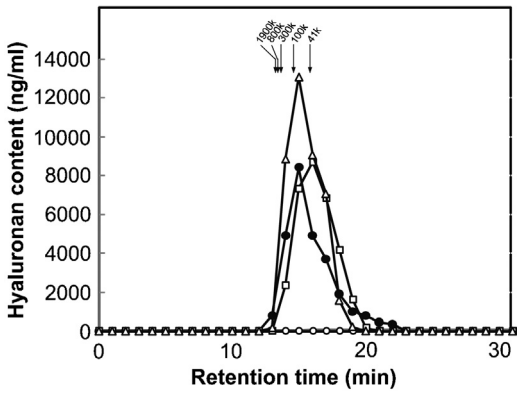

Fig. 5. Gel filtration HPLC of HA contained in extracts from cartilage. The combined fractions indicated as pools X, Y, and Z in DEAE chromatogram Fig. 1A were each concentrated and fractionated by Shodex OHpak SB-804 HQ gel filtration column (8.0 mm × 300 mm). Fractions (1.0 ml/fraction) were collected and HA content in each fraction was measured by an ELISA-like assay (assay 1 in Section 2.9). Solid circle, pool X without HA lyase (from *S. hyalurolyticus*) treatment; open circle, pool X treated with HA lyase (from *S. hyalurolyticus*); open squre, pool Y without HA lyase treatment; open triangle, pool Z without HA lyase treatment. Arrows indicate the elution positions of size standards of HA.

(by assay 2 in Section 2.9). The amount of HA, in PG aggregates from 4 M GdnHCl-extract of salmon cartilage, 4% CH_3COOH-extract of salmon cartilage, and bovine cartilage were 92.2 ± 1.6 μg, 16.8 ± 1.0 μg, and 122.0 ± 5.0 μg per mg PG (uronic acid), respectively. HA content of salmon PG aggregates in 4 M GdnHCl-extract but not in 4% CH_3COOH-extract was comparable to that of bovine PG aggregates in 4 M GdnHCl-extract, suggesting that all PGs have the ability to form aggregates with HA.

4. Conclusions

Through this study, we now know the molecular images of both native and incomplete forms of salmon cartilage aggrecans which are used in several biological studies. For future applications, it will be increasingly important to analyze the relationships between structures and functions of the PG from different preparations. Therefore, analytical strategy used in our studies will be essential to assess structures, specifically overall molecular images, core proteins and GAG moieties, in variously prepared PG fractions.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2013.12.083.

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