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Isolation and characterization of a novel chondroitin sulfate from squid liver integument rich in *N*-acetylgalactosamine(4,6-disulfate) and glucuronate(3-sulfate) residues

Ajaya Kumar Shetty [a,†], Takanari Kobayashi [a,†], Shuji Mizumoto [a], Masaki Narumi [b], Yoshiaki Kudo [b], Shuhei Yamada [a,*], Kazuyuki Sugahara [a,*]

[a] *Laboratory of Proteoglycan Signaling and Therapeutics, Hokkaido University, Graduate School of Life Science, Kita-ku, Sapporo 001-0021, Japan*
[b] *Biomatec Japan Inc., Kushiro 084-0925, Japan*

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This paper is dedicated to the 65 birthday of Professor Johannis P. Kamerling

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ABSTRACT

Novel chondroitin sulfate (CS) chains with an average molecular mass of 79.6 kDa were purified from squid liver integument. A compositional analysis of the CS chains using chondroitinases (CSases) ABC and AC-I revealed a range of variably sulfated disaccharides with GlcAβ1→3GalNAc(6-sulfate), GlcAβ1→3GalNAc(4-sulfate), and GlcAβ1→3GalNAc(4,6-disulfate) as the major ones, significant amounts of rare 3-sulfated GlcA-containing disaccharides, and a small amount of nonsulfated GlcAβ1→3GalNAc. The CS chains exhibited neurite outgrowth-promoting activity toward embryonic mouse hippocampal neurons, which was abolished completely by digestion with CSase ABC or AC-I. Consequently, whether these CS chains interact with heparin-binding growth factors was tested in a BIAcore system. All of the growth factors exhibited concentration-dependent and specific binding. CS chains from squid liver integument, with their unique composition and strong biological activities, may be a good candidate for therapeutic application.

1. Introduction

Glycosaminoglycans (GAGs), typically linked to a protein core-forming proteoglycans at the cell surface or in the extracellular matrix, are a family of linear, polydisperse polysaccharides that participate in a number of physiological phenomena including neuronal development, cell–matrix interactions, and activation of chemokines, enzymes, and growth factors.[1,2] The ability of GAGs to regulate these processes is attributed to their complex structure, which arises from extensive modifications of a nonsulfated precursor consisting of hexosamine and either hexuronic acid or galactose residues that are arranged in an alternating linear sequence by modifying enzymes such as sulfotransferases and epimerases.[3]

Heparan sulfate (HS) has attracted much attention because of its involvement in developmental processes and various signaling pathways.[4] Although chondroitin sulfate (CS) has attracted little attention, recent advances in the structural biology of CS chains suggest the importance of these molecules in various biological processes.[5,6] We and others have shown the importance of this class of molecules, from simple chondroitin involved in the cell division of a nematode[7,8] to differentially oversulfated CS-D and CS-E involved in neuroregulatory functions[9–11] and the binding of growth factors in mammalian systems.[12] CS has been isolated from various tissues obtained from a large number of animal species including both vertebrates and invertebrates.[13–19] Even though the structural organization of CS from the tissues of several vertebrates has been studied extensively,[18,20,21] the assessment of CS from the tissues of invertebrate organisms is rather limited. The main purpose of studies of CS chains from invertebrate tissues is to compare the chemical structure of CS chains from invertebrate

Abbreviations: SLI, squid liver integument; GAG, glycosaminoglycan; CS, chondroitin sulfate; HS, heparan sulfate; Hep, heparin; GalNAc, *N*-acetyl-D-galactosamine; GlcA, D-glucuronic acid; CSase, chondroitinase; HPLC, high performance liquid chromatography; 2AB, 2-aminobenzamide; $\Delta^{4,5}$HexA, 4,5-unsaturated hexuronic acid; 2S, 2-sulfate; 3S, 3-sulfate; 4S, 4-sulfate; 6S, 6-sulfate; P-ORN, poly-DL-ornithine; NOP, neurite outgrowth-promoting; E16, embryonic day 16; rh, recombinant human; MK, midkine; PTN, pleiotrophin; FGF, fibroblast growth factor; HGF, hepatocyte growth factor; HB-EGF, heparin-binding epidermal growth factor-like growth factor; $VEGF_{165}$, vascular endothelial growth factor-165; DMMB, dimethylmethylene blue; FACE, fluorophore-assisted carbohydrate electrophoresis; AMAC, 2-aminoacridone.

* Corresponding authors. Tel./fax: +81 (0)11 706 9055 (K.S.).
E-mail addresses: tjohej@sci.hokudai.ac.jp (S. Yamada), k-sugar@sci.hokudai.ac.jp (K. Sugahara).

† These authors equally contributed to the work.

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tissues with the well-known CS chains that occur in vertebrate tissues, and to gain a deeper understanding of their structure with physicochemical and biological properties.

As a part of our continued exploration for sources of CS unique in structure and with therapeutic potential, in this study, we isolated CS chains with novel structural features and multiple biological activities from the integument of squid liver, an industrial waste with a good candidate to be exploited for pharmaceutical purposes.

2. Results

2.1. Preparation of the CS fraction from squid liver integument (SLI)

GAGs were extracted from SLI by protease digestion, and recovered by ethanol precipitation. This preparation was further fractionated by anion-exchange chromatography using an Accell™ Plus QMA cartridge, which was eluted stepwise with buffers containing 0.15, 0.5, and 2.0 M NaCl. Only trace amounts of CS/dermatan sulfate (DS) were detected in the fractions eluted with a buffer containing 0.15 M NaCl. Twelve and 56% of all GAGs were detected in the fractions eluted with buffers containing 0.5 and 2.0 M NaCl, respectively. The 0.5 M NaCl-eluted fraction lacked E-units (GlcAβ1→3GalNAc(4S,6S)), whereas the 2.0 M NaCl-eluted fraction was rich in E-units (21.6%) and further purified. Since the 2.0 M NaCl-eluted fraction showed trace amounts of HS disaccharides upon high performance liquid chromatography (HPLC) after digestion with heparitinase (data not shown), it was purified further by nitrous acid treatment[22,23] to remove HS. Subsequently, this preparation was subjected to alkali treatment to prepare peptide-free glycan chains,[24] and passed through a C_{18} cartridge to remove peptides (the final yield was 0.12% of the starting material). No significant amount of DS or keratan sulfate was found by anion-exchange HPLC in the digests of the SLI-CS preparations obtained with chondroitinase (CSase) B and keratanase-II, respectively (data not shown).

2.2. Determination of the molecular mass of SLI-CS

The molecular size of the purified SLI-CS was determined by gel-filtration HPLC (Fig. 1), using the calibration curve generated with standard polysaccharides. The elution profile of SLI-CS showed a broad peak with a molecular mass ranging from 37 to 122 kDa, giving an average molecular mass of 79.6 kDa. Notably, the molecular mass of SLI-CS was larger than that of the CS chains derived from squid skin (40 kDa).[25] In contrast, squid cartilage CS-E was larger than SLI-CS and eluted in the void volume of the Superdex 200™ column (Fig. 1), indicating that CS populations from various squid tissues may differ in structure and function.

2.3. Analysis of the disaccharide composition of SLI-CS

To determine its disaccharide composition, the SLI-CS preparation was digested with CSase ABC, labeled with a fluorophore 2-aminobenzamide (2AB) for high sensitivity and resolution, and analyzed by anion-exchange HPLC. The analysis of SLI-CS revealed a unique composition consisting of monosaccharides (mono- and di-sulfated GalNAc) and disaccharides with a diverse sulfation pattern as shown in Figure 2 and Table 1. SLI-CS was enriched with disulfated disaccharide Δ*E* ($\Delta^{4,5}$HexAα1→3GalNAc(4S,6S)),[‡] accounting for 21.6% of all its disaccharides, and contained significant proportions of monosulfated disaccharides of Δ*C* ($\Delta^{4,5}$HexAα1→3GalNAc(6S)) (14.9%) and of Δ*A* ($\Delta^{4,5}$HexAα1→3GalNAc(4S)) (41.8%). Its sulfate/disaccharide unit (S/unit) ratio was 1.18 (Table 1).

In addition, SLI-CS had small yet appreciable proportions of GalNAc(4S), GalNAc(6S), and GalNAc(4S,6S), indicating the presence of GlcA(3S)-containing disaccharides as revealed in our recent studies, where we identified mono- and disulfated GalNAc as stable end products of the degradation of GlcA(3S)-containing disaccharides

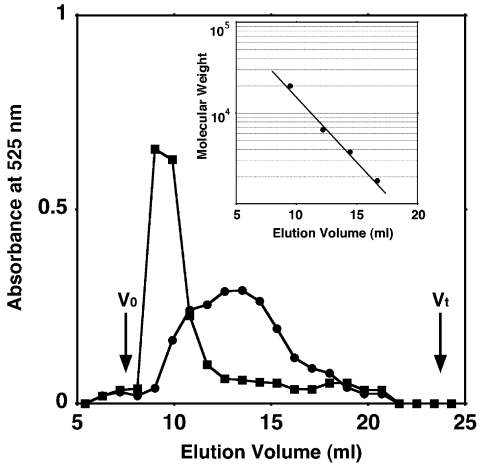

Figure 1. Determination of the average molecular mass of SLI-CS by gel filtration. The CS preparations (10 μg as GlcA) from SLI and squid cartilage (CS-E) were individually loaded onto a Superdex 200™ column calibrated with molecular mass markers as described under 'Section 4'. Fractions were monitored with the dimethylmethylene blue (DMMB) dye, and the average molecular mass was estimated using the calibration curve (*inset*). The void volume (V_o) and total volume (V_t) were determined using dextran (average mass: 200 kDa) and NaCl, respectively. The *circles* and *squares* indicate the elution profiles of SLI-CS and CS-E, respectively. Note that the CS-E preparation is devoid of HS and keratan sulfate as demonstrated by digestion experiments carried out using either heparinases or keratanase II, respectively.

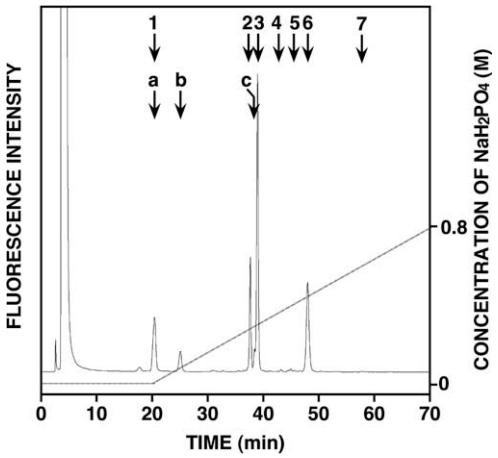

Figure 2. Anion-exchange HPLC of the digest of the SLI-CS preparation obtained with CSase ABC. The SLI-CS preparation was digested with CSase ABC. After 2AB-labeling, the digest was analyzed by HPLC on an amine-bound silica PA-03 column using a liner gradient of NaH_2PO_4 as indicated by the *dashed line*. The peaks before 10 min were derived from 2AB-derivatizing reagents. Arrows indicate the elution positions of the mono- and disulfated GalNAc as well as CS disaccharides: (a) GalNAc(6S); (b) GalNAc(4S); (c) GalNAc(4S,6S); 1, $\Delta^{4,5}$HexAα1→3GalNAc; 2, $\Delta^{4,5}$HexAα1→3GalNAc(6S); 3, $\Delta^{4,5}$HexAα1→3GalNAc(4S); 4, $\Delta^{4,5}$HexA(2S)α1→3GalNAc(6S); 5, $\Delta^{4,5}$HexA(2S)α1→3GalNAc(4S); 6, $\Delta^{4,5}$HexAα1→3GalNAc(4S,6S); 7, $\Delta^{4,5}$HexA(2S)α1→3GalNAc(4S,6S).

[‡] Treatment with bacterial lyases including CSases converts the original structures of internal uronic acid residues, β-D-GlcA and α-L-IdoA, in the GAGs into a common product, namely 4-deoxy-α-L-*threo*-hex-4-enepyranosyluronic acid (α-L-ΔHexA).