

# Determination of iduronic acid and glucuronic acid in sulfated chondroitin/dermatan hybrid chains by $^1\text{H}$ -nuclear magnetic resonance spectroscopy

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**Abstract** The relative proportion of L-iduronic acid (IdoA) and D-glucuronic acid (GlcA) is of great importance for the structure–function relationship of chondroitin sulfate (CS)/dermatan sulfate (DS). However, determination of the isotypes of uronic acid residues in CS/DS is still a challenge, due to the instability of free uronic acid released by chemical degradation and its conversion to unsaturated uronic acid by digestion with bacterial eliminase.  $^1\text{H}$ -Nuclear magnetic resonance (NMR) spectroscopy is a promising tool with which to address this issue, but the traditional method based on the assignment of the ring proton signals of IdoA and GlcA residues still has drawbacks such as the serious overlap of signals in the  $^1\text{H}$ -NMR spectrum of CS/DS polysaccharides. We found that the proton signals of the *N*-acetyl group of *N*-acetyl-D-galactosamines in CS and DS could be clearly distinguished and accurately integrated in the one-dimensional (1D)  $^1\text{H}$ -NMR

spectrum. Based on this finding, here we report a novel, sensitive, and nondestructive 1D  $^1\text{H}$ -NMR-based method to determine the proportion of IdoA and GlcA residues in CS/DS hybrid chains.

**Keywords** Chondroitin sulfate · Dermatansulfate · L-Iduronic acid · D-Glucuronic acid ·  $^1\text{H}$ -NMR spectroscopy

## Abbreviations

1D	one-dimensional
CSase	chondroitinase
CS	chondroitin sulfate
DS	dermatan sulfate
GalNAc	<i>N</i> -acetyl-D-galactosamine
GlcA	D-glucuronic acid
IdoA	L-iduronic acid
$\Delta\text{HexA}$	4-deoxy-L-threo-hex-4-enopyranosyluronic acid
HPLC	high performance liquid chromatography
NMR	nuclear magnetic resonance

The contributions of Fuchuan Li and Shuhei Yamada should be considered equal.

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## Introduction

Chondroitin sulfate (CS)/dermatan sulfate (DS) are ubiquitously expressed at the cell surface and in the extracellular matrix as side chains of proteoglycans [1, 2]. Growing evidence suggests that CS/DS play crucial roles in various biological events such as development of the central nervous system, wound repair, infection, growth factor signaling, morphogenesis, and cell division [3, 4]. CS chains are composed of repeating disaccharide units of  $-4\text{GlcA}\beta 1-3\text{GalNAc}\beta 1-$ , where GlcA and GalNAc represent D-glucuronic acid and *N*-acetyl-D-galactosamine, respectively. DS chains, the isomer of CS chains, are formed

from precursor CS chains through the action of glucuronyl C5 epimerase [5]. CS and DS chains are endowed with enormous structural diversity, formed by multiple overlapping sequences constructed with various disaccharide units modified by the specific sulfotransferases at C-2 of GlcA/L-iduronic acid (IdoA) and/or C-4 and/or C-6 of GalNAc. Such diverse modifications are the structural basis of the various biological functions of CS/DS chains [6].

CS and DS are often found as CS/DS co-polymeric chains. CS/DS hybrid domains composed of both GlcA and IdoA in some CS/DS co-polymeric chains contain key functional sequences to interact with growth factors and to promote neurite outgrowth [7–11]. In particular, a series of octasaccharides, which contain a rare disaccharide D-unit GlcA(2-*O*-sulfate)–GalNAc(6-*O*-sulfate) and bind the heparin-binding growth factor pleiotrophin, have been isolated from CS/DS hybrid chains of embryonic pig brains [12]. Since they were released from the CS/DS chains using chondroitinase (CSase) B specific for IdoA, they must have been flanked by two IdoA-containing disaccharide units, such as iA [IdoA–GalNAc(4-*O*-sulfate)], where the lowercase “i” stands for iduronic acid. Thus, the combination of IdoA-containing units and oversulfated CS disaccharides such as D and E [GlcA–GalNAc(4,6-*O*-disulfate)] is critically important to the biological activity. Such structures are putative biomarkers for neural stem cells [13, 14]. Hence, determination of the proportion of IdoA and GlcA in CS/DS chains is of great importance for elucidating the biological functions of CS/DS chains from tissues.

Although the CS/DS disaccharides produced by digestion with CSase ABC are readily analyzed by high-performance liquid chromatography (HPLC), the bacterial eliminases convert uronic acids into  $\Delta^{4,5}$ -unsaturated uronic acid, thus destroying the asymmetry distinguishing GlcA from IdoA [15, 16]. Chemical methods such as acidic hydrolysis [17–20], methanolysis [21], and hydrazinolysis after deamination [22] have also been used for the characterization of these uronic acid residues, but are associated with a considerable loss of these residues due to the difference in the sensitivity of glycosidic linkages of IdoA and GlcA to conditions for depolymerization and the instability of free uronic acid under neutral/acidic conditions [19, 21, 23, 24].  $^1\text{H}$ -Nuclear magnetic resonance (NMR) spectroscopy based on the integration of proton signals corresponding to GlcA and IdoA in CS/DS chains has been applied to overcome the difficulties [23], with solvolysis used to remove *O*-sulfate groups to simplify the complex spectrum caused by the variation in sulfation positions. Moreover, a large amount of sample (1–2 mg) is required for the spectroscopy, due to low sensitivity. Hence, the method not only destroys the original structural features as a result of the pretreatment for desulfation, but also has limitations when

analyzing samples from cells and tissues, which are only available in small quantities.

In this study, the chemical shifts of *N*-acetyl proton signals of GalNAc residues in CS were found to differ from those in DS, and to be separable and integrable in a mixture of CS and DS. Hence, a novel one-dimensional (1D)  $^1\text{H}$ -NMR-based method to determine the ratio of GlcA to IdoA in CS/DS chains was developed. This method is not only simple, rapid, and reliable but also more importantly, scatheless to samples.

## Materials and methods

**Materials** The following sugars and enzymes were purchased from Seikagaku Corp. (Tokyo, Japan): CS-A from whale cartilage, CS-B (DS) from porcine skin, CS-C and CS-D from shark cartilage, CS-E from squid cartilage, keratan sulfate from bovine cornea, heparan sulfate from bovine kidney, CSase ABC (EC 4.2.2.4) from *Proteus vulgaris*, CSase AC-I (EC 4.2.2.5) from *Flavobacterium heparinum*, CSase AC-II (EC 4.2.2.5) from *Arthrobacter aureescens*, hyaluronidase from *Streptomyces hyalurolytics*, keratanase II from *Bacillus* sp., and unsaturated disaccharides [ $\Delta\text{HexA}\alpha 1\text{--}3\text{GalNAc}$ ,  $\Delta\text{HexA}\alpha 1\text{--}3\text{GalNAc}(6\text{-}O\text{-sulfate})$ ,  $\Delta\text{HexA}\alpha 1\text{--}3\text{GalNAc}(4\text{-}O\text{-sulfate})$ ,  $\Delta\text{HexA}(2\text{-}O\text{-sulfate})\alpha 1\text{--}3\text{GalNAc}(6\text{-}O\text{-sulfate})$ ,  $\Delta\text{HexA}\alpha 1\text{--}3\text{GalNAc}(4,6\text{-}O\text{-disulfate})$ ,  $\Delta\text{HexA}(2\text{-}O\text{-sulfate})\alpha 1\text{--}3\text{GalNAc}(4\text{-}O\text{-sulfate})$ , and  $\Delta\text{HexA}(2\text{-}O\text{-sulfate})\alpha 1\text{--}3\text{GalNAc}(4,6\text{-}O\text{-disulfate})$ ]. CSase B (no EC number) was obtained from IBEX Technologies (Montreal, Canada). Hyaluronic acid from human umbilical cord was purchased from Sigma. CS/DS hybrid chains, SS-CS/DS from shark skin, E-CS/DS from embryonic pig brain, and A-CS/DS from adult pig brain were isolated and purified as reported previously [8, 9].

**Preparation of desulfated CS-C and CS-D** CS-C and CS-D preparations were desulfated as described previously [23]. Briefly, 0.5 mg of each CS preparation was dissolved in 1.5 ml of water and then converted from a sodium form to a hydrogen form using a cation-exchange Dowex 50-X2 column (hydrogen form, 1 ml). The acidic fraction was neutralized with pyridine and freeze-dried. The resultant residue was dissolved in 0.5 ml of dimethyl sulfoxide containing 10% (v/v) methanol and heated at 80°C for 5 h, and then the reaction mixture was neutralized with 0.1 M NaOH, dialyzed against water, and freeze-dried.

**Removal of hyaluronic acid and keratan sulfate in E- and A-CS/DS preparations** Since the E- and A-CS/DS preparations contained hyaluronic acid (approximately 8% and 19% (w/w), respectively) and keratan sulfate (approximately 1% and 10% (w/w), respectively), they were digested

with *Streptomyces* hyaluronidase and keratanase II. Each CS/DS preparation (1 mg) was incubated with 8 mIU of *Streptomyces* hyaluronidase in a total volume of 1 ml of 20 mM acetate–Na buffer, pH 6.0, at 60°C for 4 h. The digest was subjected to gel filtration HPLC on a column of Superdex™ peptide (Amersham Biosciences) at a flow rate of 0.4 ml/min using 0.2 M NH<sub>4</sub>HCO<sub>3</sub> as the eluent. The process was monitored by measuring ultraviolet (UV) absorbance at 232 nm. The flow-through fraction, which contained *Streptomyces* hyaluronidase-resistant polysaccharides, was collected and lyophilized. The hyaluronic acid-free CS/DS preparation was further digested with 5 mIU of keratanase II in a total volume of 500 µl of 10 mM acetate–Na buffer, pH 6.5, at 37°C for 3 h. The digest was subjected to gel filtration HPLC on a Superdex™ peptide column. The flow-through fraction was collected and lyophilized.

**Disaccharide composition analysis by HPLC** The disaccharide composition of CS/DS preparations was analyzed after digestion with CSase ABC [15] or AC-I [25]. Briefly, 1 µg of CS/DS sample was digested with 10 mIU of either CSase ABC or AC-I in a 50 mM Tris–HCl buffer, pH 8.0, containing 60 mM sodium acetate in a total volume of 40 µl at 37°C for 2 h. Each digest was heated at 100°C for 1 min to terminate the reaction and subsequently diluted to 400 µl with 16 mM NaH<sub>2</sub>PO<sub>4</sub>, and an aliquot (200 µl each) was analyzed by anion-exchange HPLC on a PA-03 column (YMC-Pack PA, Kyoto, Japan) as reported [26]. Identification and quantification of the resulting disaccharides were achieved by comparison with CS-derived authentic unsaturated disaccharides.

**Confirmation of the lack of IdoA in CS-C** The CS-C preparation (1 mg) in 250 µl of 50 mM Tris–HCl buffer (pH 7.3) was exhaustively digested with 330 mIU of CSase AC-I at 37°C for 5 h. The digest was fractionated on a column of Superdex™ Peptide (Amersham Biosciences), eluted with 0.2 M NH<sub>4</sub>HCO<sub>3</sub> at a flow rate of 0.4 ml/min with monitoring by a UV-detector at 232 nm. Peaks corresponding to individual different size fractions of oligosaccharides were collected and then desalted by repetitive freeze-drying. An aliquot (300 pmol) of the CSase AC-I-resistant tetrasaccharide fraction was further digested with 2 mIU of CSase AC-II in 50 mM acetate–Na buffer (pH 6.0) at 37°C for 1 h, or with CSase B in 50 mM Tris–HCl buffer (pH 8.0) at 37°C for 1 h. The digests were analyzed by anion-exchange HPLC under the conditions described above.

**<sup>1</sup>H-NMR spectroscopy** Glycosaminoglycan preparations (10–200 µg each) for NMR spectroscopy were fully-sodiated using a column (9×9 mm) of Dowex AG 50X-2

(Na<sup>+</sup> form; 200–400 mesh; Bio-Rad Laboratories, Hercules, CA, USA), and then repeatedly exchanged in <sup>2</sup>H<sub>2</sub>O (99.9% <sup>2</sup>H; C.E.A., Saclay, France) with intermediate lyophilization. The dried samples were reconstituted with 250 µl of <sup>2</sup>H<sub>2</sub>O (99.996 <sup>2</sup>H; Isotec Inc., Miamisburg, OH, USA) and then transferred to an NMR tube (3-mm o.d.×7 in.; Wilmad Glass Co., Buena, NJ, USA). The use of a 3 mm-o.d. NMR tube capable of working with a 250-µl volume provided enhanced sensitivity by reducing the total volume to increase the sample concentration. The 500-MHz <sup>1</sup>H-NMR spectra were recorded by a Varian UNITY INOVA-500 spectrometer at a probe temperature of 26°C as reported previously [27, 28]. Chemical shifts were given relative to sodium 4,4-dimethyl-4-silapentane-1-sulfonate but were actually measured indirectly of acetone (δ 2.225) in <sup>2</sup>H<sub>2</sub>O.

**Calculation of the relative intensity of individual N-acetyl proton signals in NMR spectra** The calculation for the quantification of N-acetyl proton signals of CS- and DS-like structures in <sup>1</sup>H-NMR spectra was performed using the Varian VNMR software according to the following procedure. For the observed multi-component spectra containing N-acetyl proton signals due to both CS- and DS-like structures, frequency, intensity, and a line width at half-height of each peak were measured. Using these parameters, multiple signals in the N-acetyl proton signal region were deconvoluted into individual Lorentzian signals and simulated. The resulting chemical shifts and integral scales of each N-acetyl proton signal were used to estimate the relative intensity of individual N-acetyl signals.

## Results and discussion

Five commercial CS/DS preparations (CS-A from whale cartilage, CS-C and CS-D from shark cartilage, CS-E from squid cartilage, and CS-B (DS) from porcine skin) as well as three CS/DS hybrid chain preparations (SS-CS/DS from shark skin, E-CS/DS from embryonic pig brain, and A-CS/DS from adult pig brain) were used as CS/DS reference samples in the present study. Their disaccharide composition was determined by HPLC after digestion with CSase ABC (Table 1), which is a bacterial eliminase that obliterates the stereochemical difference at C-5 between GlcA and IdoA by introducing unsaturation between C-4 and C-5 of the non-reducing terminal uronates of resultant disaccharides. Thus, the determination of unsaturated disaccharides provides no information about the ratio of GlcA and IdoA. Therefore, conventionally the IdoA content has been estimated by subtracting the amount of disaccharides released by CSase AC-I or AC-II specific for GlcA-containing disaccharides from that of disaccharides generated

**Table 1** Disaccharide composition of CS/DS preparations used in the present study<sup>a</sup>

CS/DS	% Composition					
	$\Delta O$	$\Delta C$	$\Delta A$	$\Delta D$	$\Delta E$	$\Delta T$
CS-A	N.D.	24.0	76.0	N.D.	N.D.	N.D.
CS-B	N.D.	2.4	97.6	N.D.	N.D.	N.D.
CS-C	N.D.	79.3	13.3	7.4	N.D.	N.D.
CS-D	9.7	44.9	22.3	23.1	N.D.	N.D.
CS-E	5.9	10.1	23.0	N.D.	61.0	N.D.
SS-CS/DS	10.5	32.8	41.2	3.0	12.5 <sup>b</sup>	N.D.
E-CS/DS	20.0	32.2	45.3	1.5	1.0	N.D.
A-CS/DS	4.3	9.4	83.0	1.8	1.5	N.D.

$\Delta O$   $\Delta$ HexA $\alpha$ 1–3GalNAc,  $\Delta C$   $\Delta$ HexA $\alpha$ 1–3GalNAc(6-*O*-sulfate),  $\Delta A$   $\Delta$ HexA $\alpha$ 1–3GalNAc(4-*O*-sulfate),  $\Delta D$   $\Delta$ HexA(2-*O*-sulfate) $\alpha$ 1–3GalNAc(6-*O*-sulfate),  $\Delta E$   $\Delta$ HexA $\alpha$ 1–3GalNAc(4,6-*O*-disulfate),  $\Delta B$   $\Delta$ HexA(2-*O*-sulfate) $\alpha$ 1–3GalNAc(4-*O*-sulfate),  $\Delta T$   $\Delta$ HexA(2-*O*-sulfate) $\alpha$ 1–3GalNAc(4,6-*O*-disulfate), N.D. not detected

<sup>a</sup> CS-A from whale cartilage, CS-B (DS) from porcine skin, CS-C from shark cartilage, CS-D from shark cartilage, CS-E from squid cartilage, CS/DS hybrid chains from shark skin (SS-CS/DS), CS/DS hybrid chains from embryonic pig brain (E-CS/DS), and CS/DS hybrid chains from adult pig brain (A-CS/DS) were digested with CSase ABC, and the unsaturated disaccharides were identified and quantified by HPLC as described under “Materials and methods”. For the nomenclature of the disaccharide units, see [29, 31]

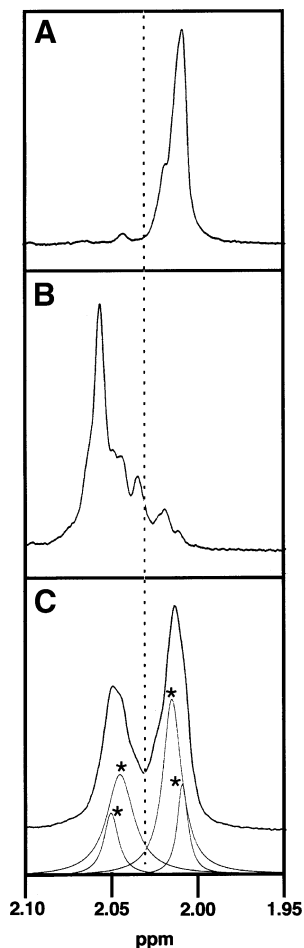
<sup>b</sup> This peak contains not only the  $\Delta E$ -unit but also the  $\Delta B$ -unit because of the incomplete separation of these two peaks

by CSase ABC, which can act on both GlcA-containing and IdoA-containing units.

In the <sup>1</sup>H-NMR spectra, H-1 and H-2 signals of uronate residues were considered to be used for determination of the ratio of GlcA and IdoA. Anomeric protons of GlcA and IdoA are observed distinctively at  $\delta$  4.5–4.8 and  $\delta$  4.9–5.2, respectively. However, a remarkably strong signal of HOD is present near this region. Presaturation for suppression of the HOD signal hinders the integration of these anomeric proton signals. H-2 signals for GlcA, IdoA, 2-*O*-sulfated GlcA, and 2-*O*-sulfated IdoA are observed at around  $\delta$  3.4, 3.6, 4.1, and 4.2, respectively, in the bulk region, and are influenced by sulfation rather than the type of uronate. Therefore, neither H-1 nor H-2 signals of uronate residues are suitable for determination of uronate types in GAG.

However, we found that the proton signals of the *N*-acetyl groups of GalNAc residues in CS and DS could be clearly distinguished. As shown in Fig. 1, the *N*-acetyl signals of CS-A are observed between 1.99 and 2.035 ppm (Fig. 1, A), whereas those of CS-B (DS) are located between 2.035 and 2.07 ppm (Fig. 1, B). Note that GlcA–GalNAc(4-*O*-sulfate) (A-unit) and iA-unit are predominant in CS-A and CS-B, respectively [29]. Significantly, in the <sup>1</sup>H-NMR spectrum of the mixture of CS-A and CS-B, the *N*-acetyl signals of CS-A and CS-B could be still clearly detected in the regions corresponding to CS-A (1.99–2.035 ppm) and CS-B (2.035–2.07 ppm), respectively (Fig. 1, C). These multiple signals were deconvoluted into four individual Lorentzian signals indicated by asterisks in Fig. 1 (C), and each peak was integrated. The sum of the integral scales of two peaks on the left side was compared with the sum of those on the right side. The relative

**Fig. 1** *N*-Acetyl signal region of 1D <sup>1</sup>H-NMR spectra of CS-A, CS-B, and a mixture of CS-A and CS-B. Two hundred micrograms of CS-A (A), CS-B (B), or a mixture of CS-A and CS-B in a ratio of 1:1 (C) was subjected to 1D <sup>1</sup>H-NMR spectroscopy as described under “Materials and methods”. The *N*-acetyl signal region of CS and DS is located on the right (1.99–2.035 ppm) and left (2.035–2.07 ppm) of the dotted line, respectively. Deconvolution of the signals of the mixture was performed, and individual Lorentzian signals indicated by asterisks are shown in panel C





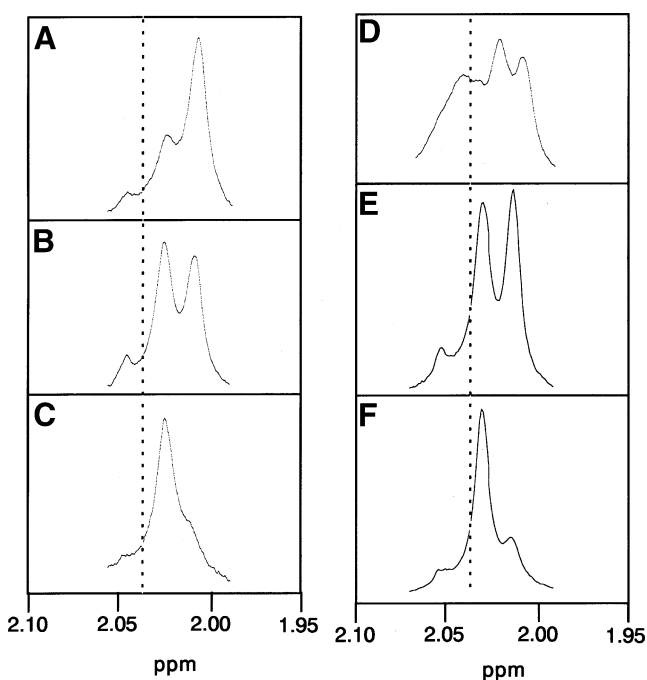
intensity of individual *N*-acetyl signals was consistent with the ratio of CS-A to CS-B in the mixture. These results indicated that the *N*-acetyl signals of GalNAc residues may well be used to determine the ratio of GlcA to IdoA in CS/DS hybrid chains.

To better illustrate the feasibility of the supposition, other CS/DS samples with different disaccharide compositions (Table 1) were investigated by 1D  $^1\text{H-NMR}$  spectroscopy. Figure 2 shows the *N*-acetyl signal region of  $^1\text{H-NMR}$  spectra of these CS/DS samples. The major *N*-acetyl signals of CS-C, CS-D, and CS-E, characterized by GlcA–GalNAc (6-*O*-sulfate) (C-unit), C- and D-units, and E-unit, respectively [29], were unambiguously assigned at 1.99–2.035 ppm (Fig. 2, panels A–C), which is the region of chemical shift characteristic of CS as observed for CS-A. More importantly, in the spectra of CS/DS samples (SS-CS/DS, E-CS/DS, and A-CS/DS) with the hybrid structure of CS and DS (Fig. 2, panels D–F), the *N*-acetyl signals were observed in the regions characteristic of CS (1.99–2.035 ppm) and DS (2.035–2.07 ppm), and could be integrated with quantitative accuracy after deconvolution. These results indicate that the chemical shifts of *N*-acetyl signals of GalNAc residues in CS/DS chains are mainly affected by the configuration of adjacent uronic acid residues rather than the pattern of sulfation of GalNAc and uronates. Since the conformational change by the epimerization brings the carboxyl group of uronate residues close to the *N*-acetyl group of the GalNAc residues on the

nonreducing side, the deshielding effect by magnetic anisotropy of the carbonyl group at the C6 position of uronate residues might result in the downfield shifts of the *N*-acetyl proton signals of neighboring GalNAc residues. Thus, *N*-acetyl signals of GalNAc residues seem to be useful for indirect determination of the ratio of GlcA and IdoA in CS/DS hybrid chains.

The uronate composition of CS/DS hybrid samples analyzed by the 1D  $^1\text{H-NMR}$  method is shown in Table 2. At the same time, the uronate composition of these samples was also determined by the conventional enzymatic method using CSase ABC having a broad substrate specificity and CSase AC-I specific for CS, but not for DS. The results from the 1D  $^1\text{H-NMR}$  method were comparable to those from the enzymatic method for most of the CS/DS samples (Table 2). The proportion of GlcA residues determined by the enzymatic method was always lower than that determined by the 1D  $^1\text{H-NMR}$  method. In fact, the value of GlcA from the enzymatic method is usually underestimated due to the frequent existence of CSase AC-I-resistant oligosaccharide fragments in CS chains, especially in the case of CS-D, because the galactosaminidic bond in GalNAc–GlcA(2-*O*-sulfate) is resistant to the action of CSase AC-I [30].

A small, yet appreciable signal was observed in the IdoA region (2.035–2.07 ppm) for commercial CS-C and CS-D samples by the 1D  $^1\text{H-NMR}$  method (Fig. 2 (A, B) and Table 2). However, no IdoA residue has been reported for CS-C or CS-D chains so far. To examine whether IdoA is indeed present in the CS-C preparation, CS-C was exhaustively digested with CSase AC-I and then analyzed by gel filtration on a Superdex™ Peptide column as described in “Materials and methods”. The results revealed a minor CSase AC-I-resistant tetrasaccharide peak (data not shown) besides the predominant disaccharide peaks. Based on the relative area of these peaks, the tetrasaccharide peak accounted for approximately 5% of all the disaccharides, which corresponds to the proportion of CSase AC-I-resistant disaccharides in CS-C estimated by the HPLC method (Table 2). Further digestion of the tetrasaccharide fraction with CSase AC-II showed that it could be cleaved into two major disaccharides,  $\Delta\text{HexA}\alpha 1\text{-3GalNAc}(4\text{-O-sulfate})$  and  $\Delta\text{HexA}(2\text{-O-sulfate})\alpha 1\text{-3GalNAc}(6\text{-O-sulfate})$ , whereas the tetrasaccharide fraction was resistant to CSase B. CSase AC-II specifically cleaves the *N*-acetylgalactosaminidic bonds linked to GlcA residues in CS chains including those linked to the 2-*O*-sulfated glucuronate, which are resistant to CSase AC-I [30], although CSase B specifically degrades DS chains. These results suggest that the CSase AC-I-resistant tetrasaccharides contained 2-*O*-sulfated GlcA, but no IdoA as the internal uronic acid residue. Thus, the IdoA content of the CS-C preparation is assumed to be below the detectable level.



**Fig. 2** *N*-Acetyl signal region of 1D  $^1\text{H-NMR}$  spectra of CS-C (A), CS-D (B), CS-E (C), SS-CS/DS (D), E-CS/DS (E), and A-CS/DS (F). Each sample (200  $\mu\text{g}$ ) was subjected to 1D  $^1\text{H-NMR}$  spectroscopy as described under “Materials and methods”

**Table 2** Composition of uronate residues in CS/DS analyzed by NMR and HPLC

CS/DS	NMR <sup>a</sup>		HPLC <sup>b</sup>	
	<i>N</i> -Acetyl signals		Disaccharides	
	1.99–2.035 ppm (GlcA), %	2.035–2.07 ppm (IdoA), %	Sensitive to CSase AC-I, %	Resistant to CSase AC-I, %
CS-A	>99	<1	100	0
CS-B	7	93	6	94
CS-C	96	4 <sup>c</sup>	85	15
CS-D	91	9 <sup>c</sup>	63	37
CS-E	>99	<1	100	0
SS-DS	58	42	57	43
E-CS/DS	90	10	88	12
A-CS/DS	92	8	95	5

<sup>a</sup> The proportion of GlcA or IdoA was calculated based on the ratio of the peak area of the *N*-acetyl signals deconvoluted and observed at 1.99–2.035 or 2.035–2.07 ppm, respectively, to the total peak area of the *N*-acetyl signals

<sup>b</sup> The proportion of disaccharides sensitive to CSase AC-I was calculated based on the molar ratio of the unsaturated disaccharides released by CSase AC-I to those released by CSase ABC, whereas that of disaccharides resistant to CSase AC-I was calculated as follows: [(the amount (picomoles) of unsaturated disaccharides released by CSase ABC minus the amount (picomoles) of unsaturated disaccharides released by CSase AC-I)/the amount of unsaturated disaccharides released by CSase ABC] × 100 (%)

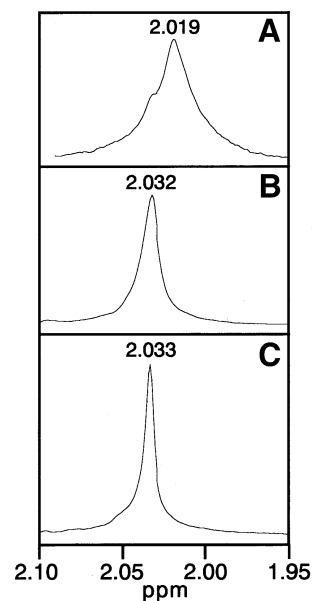
<sup>c</sup> The proportion of IdoA seems to be overestimated due to the consecutive disulfated disaccharide units, GlcA(2-*O*-sulfate)–GalNAc(4-*O*-sulfate) (B-unit) or GlcA(2-*O*-sulfate)–GalNAc(6-*O*-sulfate) (D-unit), in CS/DS chains, as described in the text

The absence of IdoA residues in CS-C and CS-D was confirmed by analyzing the desulfated CS-C and CS-D preparations using <sup>1</sup>H-NMR spectroscopy. The *N*-acetyl proton signals observed at 2.035–2.07 ppm in native CS-C and CS-D preparations and characteristic of DS disappeared after desulfation (data not shown), indicating that these signals were not derived from DS-like domains containing IdoA, but from a sulfated structure of CS chains. Taken together, it was assumed that the *N*-acetyl methyl proton signals of CS-C and CS-D observed in the region characteristic of DS (2.035–2.07 ppm) were attributed to the downfield shift of *N*-acetyl proton signals of GalNAc residues located in an unusual sequence. Actually, studies on structurally defined CS oligosaccharides by <sup>1</sup>H-NMR spectroscopy revealed that the *N*-acetyl signal of GalNAc in certain unique oligosaccharide sequences, such as –GlcA(2-*O*-sulfate)–GalNAc(6-*O*-sulfate)–GlcA(2-*O*-sulfate)– and –GlcA(2-*O*-sulfate)–GalNAc(4-*O*-sulfate)–GlcA(2-*O*-sulfate)–, showed a downfield shift into the region of an *N*-acetyl signal characteristic of DS (2.035–2.07 ppm; Kalayanamitra, K. *et al.* unpublished data), suggesting that the proportion of IdoA residues might be overestimated in CS/DS samples rich in GlcA(2-*O*-sulfate) residues. In the case of CS-D, only 9% of GlcA residues were assigned as IdoA residues by the 1D <sup>1</sup>H-NMR method, which is far lower than the AC-I-resistant disaccharides (37%) determined by the enzymatic method (Table 2). It appears that the *N*-acetyl signal of GalNAc shows a downfield shift only when the residue is located in consecutive D-units or B-

units [GlcA(2-*O*-sulfate)–GalNAc(4-*O*-sulfate)], and mixed sequences of these units. Because of the low frequency of such unique sequences including consecutive GlcA(2-*O*-sulfate)-containing disaccharide units, the error from the 1D <sup>1</sup>H-NMR method is often insignificant compared with that from the enzymatic method.

To investigate the effects of possible contamination by other glycosaminoglycans on the discrimination of GlcA and IdoA in CS/DS preparations, <sup>1</sup>H-NMR spectra of

**Fig. 3** *N*-Acetyl signal region of 1D <sup>1</sup>H-NMR spectra of hyaluronic acid (A), keratan sulfate (B), and heparan sulfate (C). Each sample (200 μg) was subjected to 1D <sup>1</sup>H-NMR spectroscopy as described under “Materials and methods”. The chemical shift of *N*-acetyl signals is shown in each panel



hyaluronic acid [ $-4\text{GlcA}\beta 1-3\text{GlcNAc}\beta 1-]_n$ , keratan sulfate [ $-4\text{GlcNAc}(6-O\text{-sulfate})\beta 1-3\text{Gal}(6-O\text{-sulfate})\beta 1-]_n$ , and heparan sulfate [ $-4\text{GlcA}\beta/\text{IdoA}\alpha 1-4\text{GlcNAc}\alpha 1-]_n$  were measured in this study, and the proton signals of an *N*-acetyl group of GlcNAc in hyaluronic acid, keratan sulfate, and heparan sulfate were observed at 2.019, 2.032, and 2.033 ppm, respectively (Fig. 3). Since the *N*-acetyl signals in hyaluronic acid are present in the region characteristic of CS (1.99–2.035 ppm), contamination from hyaluronic acid in a CS/DS preparation will cause the proportion of GlcA in the sample to be overestimated. Although the *N*-acetyl signals in keratan sulfate and heparan sulfate do not completely overlap those of CS/DS, they interfere with the accurate integration of proton signals corresponding to GlcA and IdoA residues in CS/DS chains. Thus, removal of hyaluronic acid, keratan sulfate, and heparan sulfate from CS/DS preparations is required for the 1D  $^1\text{H-NMR}$  method to determine the proportion of IdoA and GlcA residues in CS/DS hybrid chains.

The 1D  $^1\text{H-NMR}$  method successfully circumvents the problem of overlapping signals in the assignment of ring protons of CS/DS samples, because the *N*-acetyl methyl signals of GalNAc residues are in the upfield far from the region of ring proton signals of monosaccharide residues and are little affected by the variation of sulfation positions in CS/DS chains, as shown in the NMR spectra of CS/DS variants with different sulfation patterns (Fig. 2). Hence, CS/DS samples can be directly used to analyze uronates by 1D  $^1\text{H-NMR}$  spectroscopy without any destructive modifications. In addition, the sensitivity of the spectroscopy has been improved to 10–50  $\mu\text{g}$  by using a 3-mm-o.d. NMR tube and the far stronger signals of the *N*-acetyl protons as compared with the signals of other protons in the  $^1\text{H-NMR}$  spectrum of CS/DS.

In conclusion, we have developed a novel 1D  $^1\text{H-NMR}$  method to determine the proportion of IdoA and GlcA residues in CS/DS hybrid chains. Compared with methods reported previously, the 1D  $^1\text{H-NMR}$  spectroscopy for analyzing the uronate composition of CS/DS preparations is simple, rapid, and reliable. This method will considerably facilitate the study of the structure–function relationship of CS/DS hybrid chains.

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