Bulletin of Aichi Univ. of Education, 71 (Natural Science), pp. 90 − 99, March, 2022

Formation of a Highly Sulfated Nonreducing Terminal Sequence in Chondroitin Sulfate by Concerted Reactions of GalNAc 4-Sulfate 6-*O*-Sufotransferase and Uronyl 2-*O*-Sulfotransferase

Osami HABUCHI

Professor Emeritus of Aichi University of Education, Kariya 448-8542, Japan

Abstract

Chondroitin sulfate is a group of polysaccharides containing repeating disaccharide units composed of GlcA-GalNAc. Each disaccharide unit bears sulfate groups on the various position of the component sugars: position 4 of GalNAc residues in A unit; position 6 of GalNAc residues in C unit; position 6 of GalNAc residue and position 2 of GlcA residue in D unit; and position 4 and 6 of GalNAc residue in E unit. Specific sulfotransferases are involved in the synthesis of these sulfate groups. Of these sulfotransferases, GalNAc 4-sulfate 6-*O*-sulfotransferase (GalNAc4S-6ST) and uronyl 2-*O*-sulfotransferase (2OST) are responsible for the formation of E unit and D unit, respectively. Both GalNAc4S-6ST and 2OST recognize not only the kind and position of the sugar residues but also sulfation pattern around the targeted sugar residues. GalNAc4S-6ST efficiently transfers sulfate to position 6 of GalNAc(4-SO_4) residue in a nonreducing terminal sequence, GalNAc(4-SO_4)-GlcA(2-SO_4)-GalNAc(6-SO_4) in addition to position 6 of GalNAc(4-SO_4) residue in the repeating disaccharides units. On the other hand, 2OST preferentially transfers sulfate to position 2 of GlcA residue in a sequence, GalNAc(4-SO_4)-GlcA-GalNAc(6-SO_4). A highly sulfated nonreducing terminal sequence, GalNAc(4, 6-SO_4)-GlcA(2-SO_4)-GalNAc(6-SO_4) appears to be produced by the concerted action of these sulfotransferases.

Chondroitin sulfate (CS) is a linear polysaccharide attached to various proteoglycans. CS is composed of repeating disaccharide units bearing sulfate groups on the various position of the sugar residues: position 4 of GalNAc residues in A unit; position 6 of GalNAc residues in C unit; position 6 of GalNAc residue and position 2 of GlcA residue in D unit; and position 4 and 6 of GalNAc residue in E unit. Transfer of sulfate groups to the specific position of sugar residues are catalyzed by specific sulfotransferases[1]. C4ST transfers sulfate to position 4 of GalNAc residues; C6ST transfers sulfate to position 6 of GalNAc residues; GalNAc4S-6ST transfers sulfate to position 6 of GalNAc4S residues; and 2OST transfers sulfate to position 2 of GlcA residues. In this review I described specificities of GalNAc4S-6ST and 2OST, and showed that these sulfotransferases recognize not only the kind and position of the sugar residues but also sulfation pattern around the targeted sugar residues, and that the orchestrated reactions of these sulfotransferases could elaborate a unique sequence found in chondroitin sulfate. I provided experimental evidences concerning the specificity of each sulfotransferase as far as possible. Enzymatic and chemical reactions were briefly explained at the Appendix. Abbreviations were listed at the end of the review.

1. Substrate specificity of human *N*-acetylgalactosamine 4-sulfate 6-*O*-sulfotransferase (GalNAc4S-6ST)

1.1. Purification of squid GalNAc4S-6ST and cloning of human GalNAc4S-6ST

Chondroitin sulfate E (CS-E) initially found in squid cartilage contains GlcA-GalNAc4S6S units. A unique sulfotransferase was found in the squid cartilage that transferred sulfate to position 6 of GalNAc4S residues of CS and produced GalNAc4S6S residues[2]. About thirty years after this finding, this enzyme, *N*-acetylgalactosamine 4-sulfate 6-*O*-sulfotransferase (GalNAc4S-6ST) was purified to homogeneity[3]. A cDNA fragment of squid GalNAc4S-6ST was obtained by PCR using degenerated oligonucleotide primers which were designed according to

the partial amino acid sequences of the purified enzyme. On the basis of homology with the cDNA fragment, human GalNAc4S-6ST was identified[4]. The FLAG-human GalNAc4S-6ST fusion protein was extracted from COS-7 cells that were transfected with the cDNA, and affinity purified with an anti-FLAG monoclonal antibody-conjugated column[4, 5].

1.2. Substrate specificity of human GalNAc4S-6ST.

The rates of sulfation of CS-C and DS with human GalNAc4S-6ST were 1.5% and 14.5%, respectively, of the rate of sulfation of CS-A, meaning that CS-C is a poor acceptor for human GalNAc4S-6ST. When CS-A was sulfated with human GalNAc4S-6ST and [^{35}S]PAPS, and the ^{35}S-labeled products were digested with ChACII, a nearly equal amount of [^{35}S]GalNAc4S6S and [^{35}S]ΔDi-diS$_E$ were detected in SAX-HPLC. When ^{35}S-labeled products formed from DS was digested with ChABC, major radioactivity was obtained at the position of [^{35}S]GalNAc4S6S and only small radioactivity was detected at the position of [^{35}S]ΔDi-diS$_E$ in SAX-HPLC. When ^{35}S-labeled products formed from trisaccharide and pentasaccharide derived from CS-A were digested with ChACII, only [^{35}S]GalNAc4S6S was obtained in SAX-HPLC. These results suggest that human GalNAc4S-6ST preferentially transfer sulfate to GalNAc4S residues located at the nonreducing terminal of CS-A and DS[4].

2. Nonreducing terminal modification of chondroitin sulfate by human GalNAc4S-6ST

As described above, [^{35}S]GalNAc4S6S and [^{35}S]ΔDi-diS$_E$ were detected in the ChACII digests of the ^{35}S-labeled CS-A. When salt concentration used for gradient elution of SAX-HPLC was increased further, an additional radioactive peak ([^{35}S]Oligo A) was eluted later than ΔDi-diS$_E$. [^{35}S] Oligo A was not obtained when the ^{35}S-labeled products were digested with ChABC.

2.1. Characterization of [^{35}S]Oligo A

[^{35}S]Oligo A was eluted at the position of disulfated tetrasaccharide in gel chromatograpy. After digestion of [^{35}S]Oligo A with ChABC, [^{35}S]GalNAc4S6S was obtained, and after digestion with ChABC plus 6-Sase, $^{35}SO_4$ was obtained. When [^{35}S]Oligo A was digested with 6-Sase alone, an unidentified radioactive material was formed. This radioactive material was converted to [^{35}S]GalNAc4S6S by further digestion with ChABC. Because 6-Sase cleaves sulfate group from the reducing terminal GalNAc6S (see Appendix), [^{35}S]Oligo A was assumed to contain reducing terminal GalNAc6S. From these results, [^{35}S]Oligo A was deduced to be a trisaccharide containing GalNAc4S6S at the nonreducing end and GalNAc6S at the reducing end (Fig. 1). However the reason why [^{35}S]Oligo A was highly resistant to ChACII remained unclear.

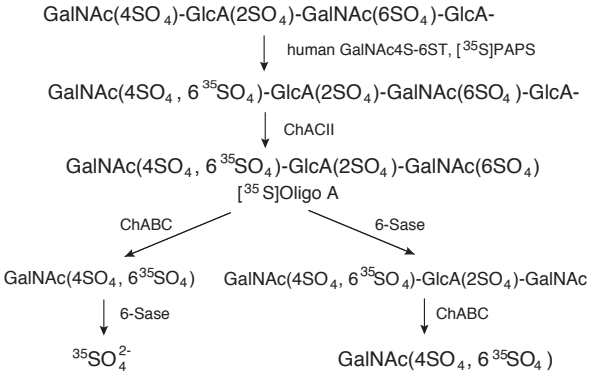

Fig. 1. Structural analysis of [^{35}S]Oligo A

2.2. Isolation and characterization of oligosaccharides obtained from the nonreducing terminal sequence of CS-A

As described above, [^{35}S]Oligo A was highly resistant to ChACII, but it was not known whether [^{35}S]Oligo A contained IdoA. To determine the structure of [^{35}S]Oligo A further, the nonreducing terminal structure of CS-A was investigated. The expected nonreducing terminal structure should serve as acceptor for GalNAc4S-6ST and be converted to a unique structure from which Oligo A was released after digestion with ChACII. In addition it was investigated whether or not the unique structure is present at the nonreducing end of CS-A itself. The strategy for detection of oligosaccharides released from the nonreducing end is based on the fact that oligosaccharides derived from the internal repeating units after ChACII digestion bear unsaturated uronic acid and hence show the absorption at 232 nm, but those derived from the nonreducing end exhibit no absorption at this wavelength. Instead, oligosaccharides released from nonreducing end of CS-A are able to be detected by the absorption at 210 nm. Two oligosaccharides which met such requirements were obtained from CS-A after ChACII digestion and were labeled as Oligo I and Oligo II. In SAX HPLC, Oligo I was eluted near ΔDi-diS$_D$, whereas Oligo II was eluted at the position of [^{35}S]Oligo A[5].

2.2.1. Characterization of Oligo I

When Oligo I was digested with ChABC, GalNAc4S and ΔDi-diS$_D$ were formed. After further digestion with 6-Sase, ΔDi-diS$_D$ disappeared and ΔDi-2S was formed. These results indicate that Oligo I was GalNAc4S-GlcA2S-GalNAc6S. Oligo I was completely resistant to ChACII digestion under

the conventional conditions, but was partially degraded under the strong conditions to GalNAc4S and ΔDi-diS$_D$. To determine whether the resistance of Oligo I to ChACII digestion was due to the presence of IdoA, Oligo I was hydrolyzed with 2 M trifluoroacetic acid at 100 ˚C for 4 h, and the hydrolysates were separated with thin layer chromatography. Under the hydrolysis conditions, GlcA, IdoA and GlcA were detected in the hydrolysates of CS-A, DS and Oligo I, respectively. From these results, it was concluded that Oligo I contained solely GlcA, and that the resistance of Oligo I to ChACII was attributable to the presence of GlcA2S. When Oligo I was sulfated with human GalNAc4S-6ST and [^{35}S]PAPS, the ^{35}S-labeled product was eluted at the position of [^{35}S]Oligo A, and was converted to [^{35}S]GalNAc4S6S after ChABC digestion. Unlike Oligo I, the ^{35}S-labeled product formed from Oligo I could be degraded completely to [^{35}S]GalNAc4S6S by ChACII under the strong conditions[5] (Fig. 2).

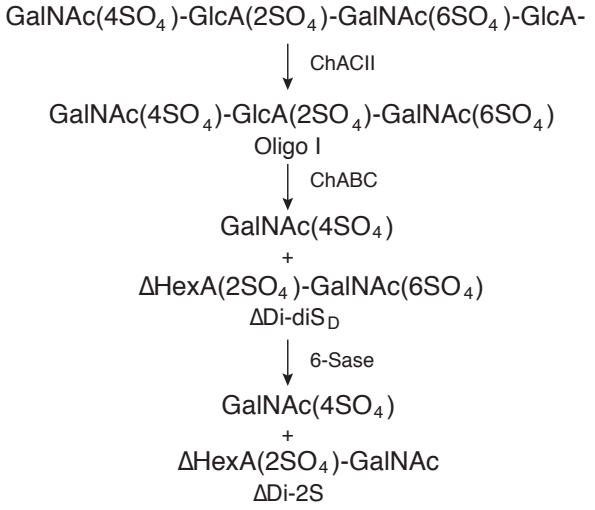

Fig. 2. Structural analysis of Oligo I

2.2.2. Characterization of Oligo II

When Oligo II was digested with ChABC, GalNAc4S6S and ΔDi-diS$_D$ were formed. After further digestion with 6-Sase, GalNAc4S and ΔDi-2S were formed. These results indicated that Oligo II was GalNAc4S6S-GlcA2S-GalNAc6S[5] (Fig 3).

2.3. Sulfation pattern of oligosaccharides affected the sensitivity of these oligosacchatides toward ChACII digestion

Oligosaccharides containing GlcA2S could be obtained from the nonreducing terminal of CS-A because these oligosaccharides were hardly digested with ChACII. The resistance of these oligosaccharides to ChACII was not due to the presence of IdoA (see 2.2.1). When Oligo I, Oligo II and Tri-46 were digested with ChACII under the

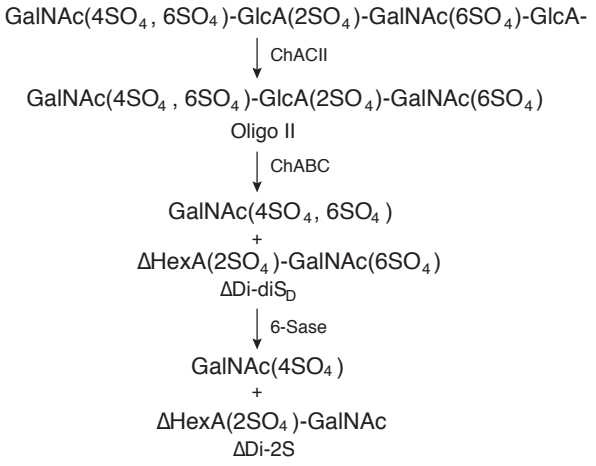

Fig. 3. Structural analysis of Oligo II

conventional conditions, less than 2%, 67% and 100%, respectively, were degraded, indicating that 2-*O*-sulfation of GlcA residue strengthened resistance to ChACII and 4,6-di-*O*-sulfation of GalNAc residue at the nonreducing side of GlcA2S residue weakened resistance to ChACII[5].

2.4. Modification of the nonreducing terminal structure of CS-A

Oligo II behaved in exactly the same way as [^{35}S]Oligo A in SAX-HPLC. When Oligo II was digested with ChABC, degradation products were the same as those from [^{35}S] Oligo A. Taken together, it was concluded that structure of [^{35}S]Oligo A was the same as that of Oligo II. Thus [^{35}S] Oligo A should be released after ChACII digestion from the nonreducing terminal structure, GalNAc4S6^{35}S-GlcA2S-GalNAc6S-, which was formed from the precursor sequence, GalNAc4S-GlcA2S-GalNAc6S-, by the reaction with GalNAc4S-6ST and [^{35}S]PAPS. The observation that both Oligo I and Oligo II were obtained from CS-A strongly suggest that such modification reactions should occur in vivo. Whale cartilage CS-A was used for the experiments described above. To determine whether such modification of the nonreducing terminal of CS could be observed in CS obtained from other sources, CS from bovine nasal cartilage, chick embryo cartilage and sturgeon notochord were incubated with GalNAc4S-6ST and [^{35}S]PAPS, and the ^{35}S-labeled products were digested with ChACII. From the ^{35}S-labeled products formed from CS of bovine nasal cartilage and chick embryo cartilage, [^{35}S]GalNAc4S6S, [^{35}S]ΔDi-diS$_E$ and [^{35}S]Oligo A were obtained as in whale cartilage CS-A. In contrast, from the ^{35}S-labeled products formed from sturgeon notochord CS, [^{35}S]GalNAc4S6S and [^{35}S]ΔDi-diS$_E$ were obtained but [^{35}S]Oligo A was not. These results suggest that the precursor sequence, GalNAc4S-GlcA2S-GalNAc6S-, may not be present at the nonreducing end of sturgeon notochord CS[5].

2.5. Specificity of human GalNAc4S-6ST toward oligosaccharides is favorable to the modification of nonreducing terminal of CS-A.

As described in 1.2, when trisaccharide derived from CS-A was sulfated with human GalNAc4S-6ST, sulfate was transferred to position 6 of GalNAc4S residue located only at the nonreducing terminal of trisaccharide. Values of Km of human GalNAc4S-6ST for Oligo I, Tri-44 and Tri 46 were 13 μM, 28 μM and 820 μM, respectively. These results indicate that the affinity for nonreducing terminal GalNAc4S was decreased in the presence of reducing terminal GalNAc6S and increased in the presence of GlcA2S[5].

3. Substrate specificity of squid GalNAc4S-6ST.

3.1. Specificity of the purified squid GalNAc4S-6ST

Purified squid GalNAc4S-6ST sulfated CS-A, CS-C and DS. The rate of sulfation of CS-C was 64% of the rate of sulfation of CS-A. Tri-, tetra- and pentasaccharides prepared from CS-A were sulfated at nearly the same rate as CS-A. When the ^{35}S-labeled products formed from CS-A and DS by the reaction with squid GalNAc4S-6ST and [^{35}S]PAPS were digested with ChACII and ChABC, respectively, and separated with SAX-HPLC, most of the ^{35}S-radioactivity was recovered in ΔDi-diS_E and only trace amount of the ^{35}S-radioactivity was detected at the position of GalNAc4S6S indicating that squid GalNAc4S-6ST transferred sulfate to GalNAc4S residues mainly located in the internal region of the polysaccharides. In contrast, when trisaccharide and pentasaccharide derived from CS-A were sulfated as above and digested with ChACII, nearly equal amounts of [^{35}S] GalNAc4S6S and [^{35}S]ΔDi-diS_E were recovered, indicating that squid GalNAc4S-6ST transferred sulfate to both nonreducing terminal GalNAc4S and internal GalNAc4S at the comparable rate. When tetrasaccharide derived from CS-A was sulfated as above and the ^{35}S-labeled product was digested with ChACII after removal of nonreducing terminal GlcA by ß-glucuronidase digestion, major radioactivity was recovered in ΔDi-diS_E, suggesting that nonreducing terminal GlcA residue of the tetrasaccharide appears to inhibit sulfation of penultimate GalNAc4S residue[3].

3.2. Specificity of the recombinant squid GalNAc4S-6ST

3.2.1. Preparation of the recombinant squid GalNAc4S-6ST

From the purified squid GalNAc4S-6ST, five peptide sequences were obtained. From two of five sequences, a cDNA fragment coding partial amino acid sequence of squid GalNA4S-6ST was obtained as described in 1.1. A cDNA coding the full open reading frame of squid GalNAc4S-6ST was obtained by 5'-RACE and 3'-RACE using the information of the partial cDNA fragment and the other amino acid sequences. The FLAG-squid GalNAc4S-6ST fusion protein was extracted from COS-7 cells that were transfected with the cDNA, and affinity purified with an anti-FLAG monoclonal antibody-conjugated column[6].

3.2.2. Sulfation of CS-C and CS-D with the recombinant squid GalNAc4S-6ST.

Using recombinant squid GalNAc4S-6ST, it was found that squid GalNAc4S-6ST could transfer sulfate to GalNAc4S residues of not only CS-A but also shark CS-C or CS-D. The rates of sulfation of CS-C and CS-D with recombinant squid GalNAc4S-6ST were 28% and 55%, respectively, of the rate of sulfation of CS-A, indicating that, unlike human GalNAc4S-6ST, recombinant squid GalNAc4S-6ST could sulfate CS-C efficiently as with the purified enzyme. When ^{35}S-labeled products formed from CS-C and CS-D by the reaction with recombinant squid GalNAc4S-6ST and [^{35}S] PAPS were digested with ChABC and separated with SAX-HPLC, the ^{35}S-radioactivity was found only in ΔDi-diS_E. In contrast, when the ^{35}S-labeled products were digested with ChACII, the ^{35}S-radioactivity was found in ΔDi-diS_E and an unidentified substance ([^{35}S]Oligo B) which was eluted later than Oligo II in SAX-HPLC. The radioactivity of [^{35}S]Oligo B was shifted to the position of ΔDi-diS_E after ChACII digestion under the strong conditions. When [^{35}S]Oligo B was digested with ChACII after the reaction with mercuric acetate, the radioactivity was shifted to the position of [^{35}S] Oligo A. These results suggested that [^{35}S]Oligo B was an unsaturated tetrasaccharide, ΔHexA-GalNAc4S6^{35}S-GalNAcGlcA2S-GalNAc6S[6] (Fig. 4).

To obtain direct evidences for the presence of GlcA2S-GalNAc6S unit in [^{35}S]Oligo B, unlabeled oligosaccharide identical to [^{35}S]Oligo B was prepared. CS-C was sulfated with recombinant squid GalNAc4S-6ST in the presence of 2 mM PAPS. When CS-C used for the substrate was digested with ChACII, ΔTetra AD (ΔHexA-GalNAc4S-GlcA2S-GalNAc6S) was obtained in addition to ΔDi-6S and ΔDi-4S. On the other hand when the sulfated CS-C was digested with ChACII, ΔTetra AD almost disappeared and a UV-absorbing peak (Oligo C) was obtained at the position of [^{35}S]Oligo B.

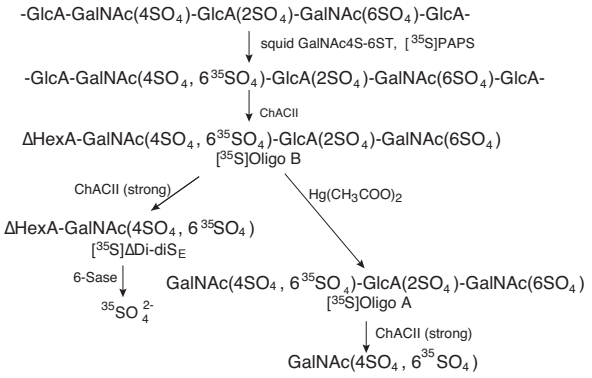

Fig. 4. Structural analysis of [^{35}S]Oligo B

When Oligo C was digested with ChACII under the strong conditions, equal amounts of ΔDi-diS_E and ΔDi-diS_D were formed. These results indicate that Oligo C was ΔHexA-GalNAc4S6S-GlcA2S-GalNAc6S (ΔTetra ED). From these observations, it became evident that squid GalNAc4S-6ST could convert -GlcA-GalNAc4S-GlcA2S-GalNAc6S- sequence to -GlcA-GalNAc4S6S-GlcA2S-GalNAc6S- sequence nearly quantitatively[6] (Fig. 5).

CS containing both D and E units (CS-DE) that was synthesized from CS-D by the sulfation with squid GalNAc4S-6ST inhibited thrombin in the absence of antithrombin; inhibition efficiency of CS-DE was comparable with that of CS-E from squid cartilage[7].

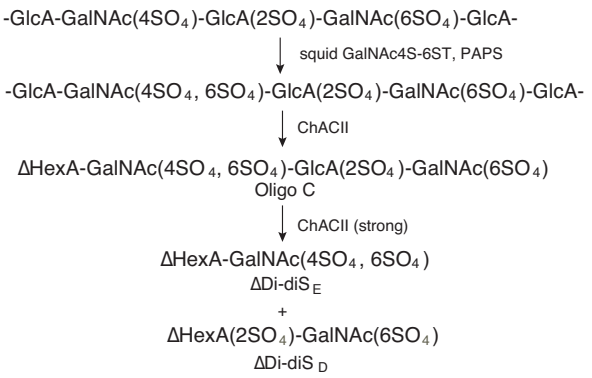

Fig. 5. Structural analysis of Oligo C

3.2.3. Sulfation of nonreducing terminal GalNAc4S residue with Squid GalNAc4S-6ST

When ^{35}S-labeled products formed from CS-A by the reaction with recombinant squid GalNAc4S-6ST and [^{35}S]PAPS was digested with ChACII, and separated with SAX-HPLC, the ^{35}S-radioactivity was found in [^{35}S]ΔDi-diS_E and [^{35}S]Oligo A but not in GalNAc4S6S. These results are quite different from the results obtained from human GalNAc4S-6ST described above, and suggest that squid GalNAc4S-6ST could efficiently sulfate nonreducing terminal GalNAc4S residues only if the GalNAc4S residues are included in GalNAc4S-GlcA2S-GalNAc6S. As described in 2.6, when sturgeon notochord CS was sulfated with human GalNAc4S-6ST and [^{35}S]PAPS and the ^{35}S-labeled products were digested with ChACII, [^{35}S] GalNAc4S6S and [^{35}S]ΔDi-diS_E were obtained but [^{35}S] Oligo A was not, suggesting that sturgeon notochord CS might be devoid of the precursor sequence, GalNAc4S-GlcA2S-GalNAc6S-. When sturgeon notochord CS was sulfated with squid GalNAc4S-6ST and [^{35}S]PAPS, and the ^{35}S-labeled products were digested with ChACII, the ^{35}S-radioactivity was found only in [^{35}S]ΔDi-diS_E. These results also supported the assumption that sturgeon notochord CS did not contain the nonreducing terminal sequence, GalNAc4S-GlcA2S-GalNAc6S-[6].

4. Substrate specificity of uronyl 2-*O*-sulfotransferase (2OST)

4.1. Cloning of 2OST

2OST transfers sulfate to position 2 of GlcA residues in CS and had been cloned as a homologous gene of HS2ST which transfers sulfate to position 2 of IdoA or GlcA of heparan sulfate and heparin[8]. 2OST should play important roles in the formation of the nonreducing terminal sequence, GalNAc4S-GlcA2S-GalNAc6S, described above; however, recognition of acceptor structures around the targeted GlcA by 2OST has not been sufficiently clarified. Affinity purified recombinant 2OST was prepared from COS7 cells transfected with FLAG-2OST cDNA and this enzyme was used to examine the substrate recognition[9]. Recombinant 2OST was also prepared from insect cells transfected with vaculovirus and purified with heparin Sepharose and 3',5'-ADP-agarose[7]. There were some differences in the substrate specificity between affinity purified recombinant 2OST[9] and crude extracts of the insect cells transfected with vaculovirus[8]. One of reasons for the apparent difference might be attributable to the methods used for the preparation of the enzyme; the crude extracts of insect cells transfected with bacurovirus were found to contained CS-degrading activity in addition to 2OST activity[7, 10]. The CS-degrading activity might disturb analysis of the sulfated products.

4. 2. Specificity of 2OST toward various glycosaminoglycans

When CS-A and CS-C were sulfated with 2OST and [^{35}S] PAPS, and the ^{35}S-labeled products were digested with ChACII, only [^{35}S]ΔDi-diS_D was obtained, indicating that sulfate was transferred to position 2 of GlcA residue in GlcA-GalNAc6S unit in CS-A and CS-C. When DSDS was sulfated in the same manner, and the ^{35}S-labeled products were digested with ChABC, only [^{35}S]ΔDi-2S was obtained, indicating that sulfate was transferred to position 2 of IdoA

residue in IdoA-GalNAc unit in DSDS. The rate of sulfation of DS was very low compared to CS-A, CS-C and DSDS. But in the presence of ten-fold amounts of protamine, 2OST transferred sulfate to DS efficiently. When DS was sulfated with 2OST and [^{35}S]PAPS in the presence of ten-fold amounts of protamine, only [^{35}S]ΔDi-diS$_B$ was obtained after digestion of the ^{35}S-labeled products with ChABC, indicating that sulfate was transferred to position 2 of IdoA in IdoA-GalNAc4S units in DS. [^{35}S]ΔDi-diS$_B$ was confirmed by the observation that this material was converted to [^{35}S]ΔDi-2S after digestion with 4-Sase[9].

4.3. Identification of a unique sequence formed in CS-A by the sulfation with 2OST

Since ChACII exhibited varying degrees of resistance toward oligosaccharides containing GlcA2S as described in 2.3, it was expected that oligosaccharides containing GlcA2S should be released when the ^{35}S-labeled products formed from CS-A by the reaction with 2OST and [^{35}S]PAPS were subjected to limited digestion with ChACII. Actually a [^{35}S]tetrasacharide ([^{35}S]Oligo D) was obtained as a sole radioactive material after limited digestion with ChACII from the ^{35}S-labeled products. [^{35}S]Oligo D was converted to [^{35}S]ΔDi-diS$_D$ after ChACII digestion under the conventional conditions, and to [^{35}S]ΔDi-2S after further digestion with 6-Sase. When [^{35}S]Oligo D was digested with 6-Sase alone, the radioactivity was detected in an unidentified material and the unidentified material was converted to [^{35}S]ΔDi-2S after further digestion with ChACII. From these observations, it was strongly suggested that GlcA2S-GalNAc6S unit was present in the reducing side of [^{35}S]Oligo D. Structure of [^{35}S]Oligo D was analyzed by an alternative approach. After removal of ΔHexA in [^{35}S]Oligo D with mercuric acetate, [^{35}S]Oligo D was converted to a [^{35}S]trisaccharide eluted at the position of Oligo I in SAX-HPLC. When the [^{35}S]trisaccharide was sulfated with squid GalNAc4S-6ST and 2 mM PAPS, it was converted to

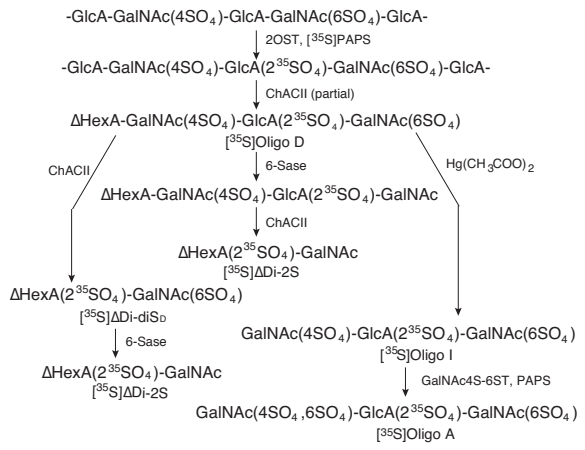

Fig. 6. Structural analysis of [^{35}S]Oligo D

[^{35}S]Oligo A, indicating that GalNAc4S residue should be present at the nonreducing terminal of the [^{35}S]trisaccharide. From these results, [^{35}S]Oligo D was deduced to be ΔHexA-GalNAc4S-GlcA2^{35}S-GalNAc6S (Fig. 6). Since GlcA2S-GalNAc6S unit was present in CS-A at about 0.5% of the total disaccharide units, the 2-*O*-sulfated sequence described above was expected to be present in CS-A itself. To confirm the structure of [^{35}S]Oligo D, CS-A was partially digested with ChACII and an oligosaccharide (Oligo E) that behaved identically with [^{35}S]Oligo D in SAX-HPLC was looked for in the digests. From 550 μmol CS-A, 0.4 μmol of Oligo E was obtained. When Oligo E was digested with ChACII, ΔDi-4S and ΔDi-diS$_D$ were obtained. After further digestion with 6-Sase, ΔDi-diS$_D$ was converted to ΔDi-2S, whereas after further digestion with 4-Sase, ΔDi-4S was converted to ΔDi-0S. When Oligo E was digested with 6-Sase alone, it was converted to the unidentified material that was obtained after 6-Sase digestion of [^{35}S]Oligo D. After further digestion of the unidentified material with ChACII, ΔDi-4S and ΔDi-2S were obtained. When Oligo E was treated with mercuric acetate, it was converted to Oligo I, which was further converted GalNAc4S and ΔDi-diS$_D$ after digestion with ChABC. From these results, Oligo E was confirmed to be ΔHexA-GalNAc4S-GlcA2S-GalNAc6S. Since [^{35}S]Oligo D was identical to Oligo E, it was concluded that 2OST transferred sulfate preferentially to position 2 of GlcA residue included in the sequence of GalNAc4S-GlcA-GalNAc6S, and produce GalNAc4S-GlcA2S-GalNAc6S[9] (Fig. 7).

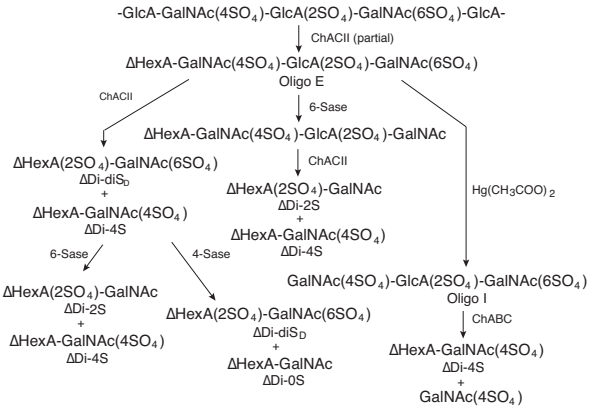

Fig. 7. Structural analysis of Oligo E

The specificity of 2OST described above appears to be supported by the observation that 2OST transferred sulfate efficiently to position 2 of the GlcA residue of the GlcA-GalNAc6S unit of the artificial CS containing both GalNAc4S and GalNAc6S, but poorly transferred sulfate at the same position of the artificial CS containing only GalNAc6S[11].

4.4. Substrate specificity of 2OST toward oligosaccharides

The rates of sulfation of Tetra-66, Tetra-44, and Tetra-64 with 2OST were 0.20-, 0.11- and 0.04-fold, respectively, of the rate of sulfation of Tetra 46, indicating that Tetra 46 was the best substrate among these tetrasaccharides. When Tetra-46 was sulfated with 2OST and [^{35}S]PAPS and the ^{35}S-labeled product was digested with ß-glucuronidase, the radioactivity was recovered at the position of Oligo I in SAX-HPLC, indicating that sulfate was transferred to the internal GlcA residue but not to the nonreducing terminal GlcA of Tetra-46. Such results appeared to be well coincided with the observation that 2OST transferred sulfate to GlcA residue included in the characteristic sequence of CS-A, GalNAc4S-GlcA-GalNAc6S.

Similar preference of 2OST was also observed in trisaccharides. The rates of sulfation of Tri-66, Tri-44, and Tri-64 were 0.19-, 0.16- and 0.05-fold, respectively, of the rate for Tri-46. As shown in 2.6, the nonreducing terminal sequence, GalNAc4S-GlcA2S-GalNAc6S-, could be modified to GalNAc4S6S-GlcA2S-GalNAc6S- with Gal NAc4S-6ST. Since 2OST transferred sulfate preferentially to Tri-46, the nonreducing terminal sequence, GalNAc4S-GlcA2S-GalNAc6S-, should be synthesized by 2OST from the precursor sequence, GalNAc4S-GlcA-GalNAc6S-[9].

4.5. 2OST is able to transfer sulfate to position 2 of GlcA residue not only in GlcA-GalNAc6S unit but also in GlcA-GalNAc4S unit in the presence of 2 mM PAPS.

CS-A was sulfated by the purified recombinant 2OST expressed in insect cells in the presence of 2 mM PAPS until GlcA2S-GalNAc6S unit reached 17% of total disaccharide units. When the resultant sulfated CS-A was digested with ChACII, not only ΔDi-diS_D but also ΔDi-diS_B was obtained, indicating that GlcA-GalNAc4S unit in CS-A could be converted to GlcA2S-GalNAc4S unit by 2OST[7]; however, it is not clear whether such reaction occurs in vivo, because GlcA2S-GalNAc4S unit has been rarely found in CS extracted from animal tissues.

5. A proposed mechanism by which the highly sulfated nonreducing terminal sequence is produced in CS-A

On the basis of the information described above, a possible mechanism by which the highly sulfated nonreducing terminal sequence is produced could be conceivable (Fig. 8). After or concurrently with elongation of CS polysaccharide chain, position 4 and position 6 of GalNAc residues appear to be sulfated nearly randomly with C4ST and C6S, respectively. A nonreducing terminal sequence, GalNAc4S-GlcA-GalNAc6S-, thus formed could be sulfated with 2OST. The resulting sequence, GalNAc4S-GlcA2S-GalNAc6S- could be further converted to GalNAc4S6S-GlcA2S-GalNAc6S- by the sulfation with GalNAc4S-6ST. According to this mechanism, organisms that are deficient in either GalNAc4S-6ST or 2OST would not produce this highly sulfated nonreducing terminal sequence.

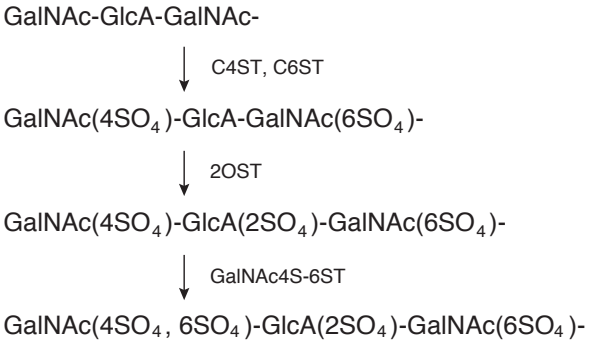

Fig. 8. A proposed mechanism by which the highly sulfated nonreducing terminal sequence is produced in CS-A

Perspectives

Unlike protein and DNA, glycosaminoglycans have no definite sequences over whole molecules. Nevertheless, some defined local sequences have been found in heparin/heparan sulfate which interact with specific proteins. GalNAc4S6S residues at the nonreducing terminal of CS have been reported[12-15]. The proportion of the nonreducing terminal GalNAc4S6S contained in the aggrecan has been reported to decrease in osteoarthritis[16]. In this review I described nonreducing terminal modification of CS by concerted reactions of C4ST, C6ST, 2OST and GalNAc4S-6ST. Whether the highly sulfated nonreducing terminal sequences described in this review are able to interact with specific proteins remains to be determined. The highly sulfated nonreducing terminal sequence was found in whale cartilage CS but not in sturgeon notochord CS. On the basis of the structural difference between whale cartilage CS and sturgeon notochord CS, it may be possible to identify proteins that interact with the sequence: proteins that bind to whale cartilage CS but not to sturgeon notochord CS might be candidates for the proteins that interact with the nonreducing terminal sequence. If oligosaccharide derivatives containing the highly sulfated nonreducing terminal sequence become available, those products would help our understanding of function of the unique structure

through binding experiments or antibody preparation.

GalNAc4S-6ST knockout mice generated in our laboratory have been used for the study concerning mast cell maturation[17], neurite outgrowth[18], liver fibrosis[19] osteoporosis[20], and binding of semaphorin-3A to CS in perineuronal net[21]. Since isoforms of GalNAc4S-6ST are not known, the nonreducing terminal highly sulfated structure in CS-A should disappear in GalNAc4S-6ST knockout mice, and hence some phenotypes of GalNAc4S-6ST knockout mice might be attributable to the absence of the highly sulfated nonreducing terminal structure in CS.

Acknowledgment

I thank many collaborators who participated in the research described in this review. I also thank colleagues who engaged together in the establishment, operation and maintenance of the isotope laboratory at Aichi University of Education. Our researches described in this review would not be achieved unless the isotope laboratory was available. Our studies were supported by Grant-in-Aid from Ministry of Education, Culture, Sports, Science and Technology, by Grants-in-Aid of Mizutani Foundation for Glycoscience, and by a special research fund from Seikagaku Corporation. Finally, I commemorate Dr. Yuki Ito's early death. Dr. Ito devoted her best efforts toward research on the squid sulfotransferase for three years in our laboratory. Her achievement became an important starting point for the research on GalNAc4S-6ST.

References

1. Habuchi O. Diversity and functions of glycosaminoglycan sulfotransferases. *Biochim Biophys Acta*. 2000; **1474**, 115-127. doi: 10.1016/s0304-4165(00)00016-7
2. Habuchi O, Yamagata T, Suzuki S. Biosynthesis of the acetylgalactosamine 4,6-disulfate unit of squid chond roitin sulfate by transsulfation from 3'-phosphoadenosine 5'-phosphosulfate. *J Biol Chem*. 1971; **246**: 7357-7365
3. Ito Y, Habuchi O. Purification and characterization of *N*-acetylgalactosamine 4-sulfate 6-*O*-sulfotransferase from the squid cartilage. *J Biol Chem*. 2000; **275**: 34728-34736. doi: 10.1074/jbc.M909633199
4. Ohtake S, Ito Y, Fukuta M, Habuchi O. Human *N*-acetylgalactosamine 4-sulfate 6-*O*-sulfotransferase cDNA is related to human B cell recombination activating gene-associated gene. *J Biol Chem*. 2001; **276**: 43894-438900. doi: 10.1074/jbc.M104922200
5. Ohtake S, Kimata K, Habuchi O. A unique nonreducing terminal modification of chondroitin sulfate by *N*-acetylgalactosamine 4-sulfate 6-*O*-sulfotransferase. *J Biol Chem*. 2003; **278**: 38443-38452. doi: 10.1074/jbc.M306132200
6. Yamaguchi T, Ohtake S, Kimata K, Habuchi O. Molecular cloning of squid *N*-acetylgalactosamine 4-sulfate 6-*O*-sulfotransferase and synthesis of a unique chondroitin sulfate containing E-D hybrid tetrasaccharide structure by the recombinant enzyme. *Glycobiology*. 2007; **17**: 1365-1376. doi: 10.1093/glycob/cwm103
7. Numakura M, Kusakabe N, Ishige K, Ohtake-Niimi S, Habuchi H, Habuchi O. Preparation of chondroitin sulfate libraries containing disulfated disaccharide units and inhibition of thrombin by these chondroitin sulfates. *Glycoconj J*. 2010; **27**: 479-489. doi: 10.1007/s10719-010-9293-2
8. Kobayashi M, Sugumaran G, Liu J, Shworak NW, Silbert JE, Rosenberg RD. Molecular cloning and characterization of a human uronyl 2-sulfotransferase that sulfates iduronyl and glucuronyl residues in dermatan/chondroitin sulfate. *J Biol Chem*. 1999; **274**: 10474-10480. doi: 10.1074/jbc.274.15.10474
9. Ohtake S, Kimata K, Habuchi O. Recognition of sulfation pattern of chondroitin sulfate by uronosyl 2-*O*-sulfotransferase. *J Biol Chem*. 2005; **280**: 39115-39123. doi: 10.1074/jbc.M508816200
10. Sugiura N, Setoyama Y, Chiba M, Kimata K, Watanabe H. Baculovirus envelope protein ODV-E66 is a novel chondroitinase with distinct substrate specificity. *J Biol Chem*. 2011; **286**: 29026-29034. doi: 10.1074/jbc.M111.251157
11. Sugiura N, Shioiri T, Chiba M, Sato T, Narimatsu H, Kimata K, Watanabe H. Construction of a chondroitin sulfate library with defined structures and analysis of molecular interactions. *J Biol Chem*. 2012; **287**: 43390-43400. doi: 10.1074/jbc.M112.412676
12. Otsu K, Inoue H, Tsuzuki Y, Yonekura H, Nakanishi Y, Suzuki S. A distinct terminal structure in newly synthesized chondroitin sulphate chains. *Biochem J*. 1985, **227**:37-48. doi: 10.1042/bj2270037
13. Shaklee PN, Conrad HE. Structural changes in the large proteoglycan in differentiating chondrocytes from the chick embryo tibiotarsus. *J Biol Chem*. 1985, **260**:16064-16067
14. Midura RJ, Calabro A, Yanagishita M, Hascall VC. Nonreducing end structures of chondroitin sulfate chains on aggrecan isolated from Swarm rat chondrosarcoma cultures. *J Biol Chem*. 1995; **270**: 8009-8015. doi: 10.1074/jbc.270.14.8009
15. Plaas AH, Wong-Palms S, Roughley PJ, Midura RJ, Hascall VC. Chemical and immunological assay of the nonreducing terminal residues of chondroitin sulfate from human aggrecan. *J Biol Chem*. 1997; **272**: 20603-20610.

doi: 10.1074/jbc.272.33.20603

16. Plaas AH, West LA, Wong-Palms S, Nelson FR. Glycosaminoglycan sulfation in human osteoarthritis. Disease-related alterations at the non-reducing termini of chondroitin and dermatan sulfate. *J Biol Chem*. 1998; **273**: 12642-12649. doi: 10.1074/jbc.273.20.12642
17. Ohtake-Niimi S, Kondo S, Ito T, Kakehi S, Ohta T, Habuchi H, Kimata K, Habuchi O. Mice deficient in *N*-acetylgalactosamine 4-sulfate 6-*O*-sulfotransferase are unable to synthesize chondroitin/dermatan sulfate containing *N*-acetylgalactosamine 4,6-bissulfate residues and exhibit decreased protease activity in bone marrow-derived mast cells. *J Biol Chem*. 2010; **285**: 20793-20805. doi: 10.1074/jbc.M109.084749
18. Brown JM, Xia J, Zhuang B, Cho KS, Rogers CJ, Gama CI, Rawat M, Tully SE, Uetani N, Mason DE, Tremblay ML, Peters EC, Habuchi O, Chen DF, Hsieh-Wilson LC. A sulfated carbohydrate epitope inhibits axon regeneration after injury. *Proc Natl Acad Sci U S A*. 2012; **109**: 4768-4773. doi: 10.1073/pnas.1121318109
19. Habuchi H, Ushida T, Habuchi O. Mice deficient in N-acetylgalactosamine 4-sulfate 6-*O*-sulfotransferase exhibit enhanced liver fibrosis and delayed recovery from fibrosis in carbon tetrachloride-treated mice. *Heliyon*. 2016; **2**: e00138. doi: 10.1016/j.heliyon.2016.e00138
20. Koike T, Mikami T, Shida M, Habuchi O, Kitagawa H. Chondroitin sulfate-E mediates estrogen-induced osteoanabolism. *Sci Rep*. 2015; **5**: 8994. doi: 10.1038/srep08994
21. Nadanaka S, Miyata S, Yaqiang B, Tamura J, Habuchi O and Kitagawa H. Reconsideration of the Semaphorin-3A Binding Motif Found in Chondroitin Sulfate Using Galnac4s-6st-Knockout Mice. *Biomolecules* 2020, **10**, 1499; doi:10.3390/biom10111499

Abbreviations

GalNAc	N-acetylgalactosamine
GlcA	Glucuronic acid
IdoA	Iduronic acid
ΔHexA	Δ4,5 unsaturated uronic acid
4S	4-SO_4
6S	6-SO_4
2S	2-SO_4
4S6S	4,6-diSO_4
C6ST	Chondroitin 6-sulfotransferase-1 (C6ST-1)
C4ST	Chondroitin 4-sulfotransferase-1 (C4ST-1)
GalNAc4S-6ST	*N*-Acetylgalactosamine 4-sulfate 6-*O*-sulfotransferase
2OST	Uronyl 2-*O*-sulfotransferase
ChABC	Chondroitinase ABC
ChACII	Chondroitinase ACII
6-Sase	Chondro-6-sulfatase
4-Sase	Chondro-4-sulfatase
CS	Chondroitin sulfate
DS	Dermatan sulfate
DSDS	Partially desulfated dermatan sulfate
PAPS	3'-Phosphoadenosine 5'-phosohosulfate (sulfate donor)
ΔDi-0S	ΔHexAß1-3GalNAc
ΔDi-4S	ΔHexAß1-3GalNAc4S
ΔDi-6S	ΔHexAß1-3GalNAc6S
ΔDi-2S	ΔHexA2Sß1-3GalNAc
ΔDi-diS_D	ΔHexA2Sß1-3GalNAc6S
ΔDi-diS_B	ΔHexA2Sß1-3GalNAc4S
ΔDi-diS_E	ΔHexAß1-3GalNAc4S6S
Tetra-44	GlcAß1-3GalNAc4Sß1-4GlcAß1-3GalNAc4S
Tetra-64	GlcAß1-3GalNAc6Sß1-4GlcAß1-3GalNAc4S
Tri-44	GalNAc4Sß1-4GlcAß1-3GalNAc4S
Tri-64	GalNAc6Sß1-4GlcAß1-3GalNAc4S
Tri-46	GalNAc4Sß1-4GlcAß1-3GalNAc6S
Tri-66	GalNAc6Sß1-4GlcAß1-3GalNAc6S
[^{35}S]Oligo A*	GalNAc4S6^{35}Sß1-4GlcA2Sß1-3GalNAc6S
Oligo I	GalNAc4Sß1-4GlcA2Sß1-3GalNAc6S
Oligo II	GalNAc4S6Sß1-4GlcA2Sß1-3GalNAc6S
[^{35}S]Oligo B*	ΔHexA-GalNAc4S6^{35}Sß1-4GlcA2Sß1-3GalNAc6S
Oligo C*	ΔHexA-GalNAc4S6Sß1-4GlcA2Sß1-3GalNAc6S
[^{35}S]Oligo D*	ΔHexA-GalNAc4Sß1-4GlcA2^{35}Sß1-3GalNAc6S
Oligo E*	ΔHexA-GalNAc4Sß1-4GlcA2Sß1-3GalNAc6S
SAX-HPLC	Strong anion exchange high performance liquid chromatography

*[^{35}S]Oligo A, [^{35}S]Oligo B, Oligo C, [^{35}S]Oligo D and Oligo E were described as [^{35}S]Oligo III, [^{35}S]Oligo X, unlabeled Oligo X, [^{35}S]Tetra A and tetrasaccharide that behaved identically with Tetra A, respectively, in the original papers.

Appendix

Chondroitinase ABC cleaves glycoside bonds between GalNAc and GlcA or IdoA in CS or DS by elimination reaction. Disaccharides or oligosaccharides having ΔHexA at the nonreducing terminal are formed.

Chondroitinase ACII cleaves glycoside bonds between GalNAc and GlcA in CS by elimination reaction. Disaccharides or oligosaccharides having ΔHexA at the nonreducing terminal are formed. The glycosyl bond between GalNAc and GlcA2S shows varying degree of resistance to this enzyme (see 2.3).

Chondro-6-sulfatase cleaves sulfate group from unsaturated disaccharide, ΔDi-6S. Under strong conditions, this enzyme is able to degrade monosaccharide, GalNAc4S6S, to yield GalNAc4S. 6-Sase cleaves sulfate groups from reducing terminal GalNAc6S residue but not from nonreducing terminal GalNAc6S residue of Tri-46, Tri-64, and Tri-66 (see ref. 5).

Chondro-4-sulfatase cleaves sulfate group attached to position 4 of GalNAc4S residue of ΔDi-4S and ΔDi-diS_B.

Nonreducing terminal ΔHexA attached to disaccharides or oligosaccharides are cleaved by the reaction with mercuric acetate.

Tetrasaccharides and trisaccharides bearing sulfate groups at different position were prepared from CS. CS was digested with testicular hyaluronidase and tetrasaccharide fractions were separated from the digests by gel chromatography. The tetrasaccharide fraction was separated into Tetra-44, Tetra-64 and a mixture of Tetra-46 and Tetra-66 with SAX-HPLC. The mixture of Tetra-46 and Tetra-66 was separated to Tetra-46 and Tetra-66 with Polyamine II-HPLC. Tri-44, Tri-46, Tri-64, and Tri-66 were obtained from the respective tetrasaccharides by ß-glucuronidase digestion.

Nonreducing terminal ΔHexA attached to disaccharides or oligosaccharides can be cleaved by the reaction with mercuric acetate.

(Received August 5, 2021)