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Enzymatic Synthesis of Homogenous Chondroitin Sulfate Oligosaccharides

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Abstract

Chondroitin sulfate (CS) is a sulfated polysaccharide that plays essential physiological roles. Here, we report to utilize an enzyme-based method to synthesize a library of CS oligosaccharides consisting of 15 different CS oligosaccharides. The library covers 4-O-sulfated and 6-O-sulfated oligosaccharides with the size ranging from trisaccharide to nonasaccharide. We also demonstrate the synthesis of unnatural 6-O-sulfated CS pentasaccharides containing either a 6-O-sulfo 2-azido galactosamine or a 6-O-sulfo galactosamine residue. The availability of structurally defined CS oligosaccharides offers a novel approach to investigate the biological functions of CS.

TOC image

Keywords

chondroitin sulfate; sulfotransferase; oligosaccharides; enzymatic synthesis; carbohydrates

Chondroitin sulfates (CSs) are sulfated polysaccharides and widely present on the mammalian cell surface and in the extracellular matrix. CSs are known to be involved in cancer metastasis^[1], parasitic infections^[2] and neuron growth inhibition after injury^[3]. The sulfation patterns in CS dictate the binding affinity to the protein targets to manifest the selectivity in biological functions^[4]. A 6-O-sulfated *N*-acetyl galactosamine (GalNAc6S) containing CS facilitates the infection of *B. burgdorferi* to cause Lyme disease^[5], and 4-O-

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Conflict of interest

Authors declare no conflict of interest.

sulfated *N*-acetyl galactosamine (GalNAc4S) involves in *P. falciparum* infection that causes malaria^[2a]. A domain containing 4,6 disulfated *N*-acetyl galactosamine (GalNAc4S6S) residues is proved to be necessary to direct neuronal signaling and inhibit axon growth^[3c, 6]. CS in conjunction with glucosamine is prescribed as a treatment for osteoarthritis in Asia and Europe and available as a nutraceutical supplement in US; however, the efficacy remains controversial^[7]. Bikunan, a special form CS, exhibits defined saccharide sequences^[8]; however, the CS isolated from natural human or animal tissues are present in a mixture of different sulfated saccharides and sizes. To isolate a polysaccharide with a single sulfated saccharide sequence and defined length is technically demanding. The lack of structurally homogeneous or monodisperse CS is the major roadblock that hinders CS research. We plan to develop an effective method to synthesize structurally defined CS oligosaccharides to bridge the gap for investigating the structure–activity relationship.

CS contains the repeating disaccharide unit of $\beta 1 \rightarrow 3$ -linked glucuronic acid (GlcA) and *N*-acetylgalactosamine (GalNAc) disaccharide, $\rightarrow 4$)GlcA β (1 $\rightarrow$ 3) GalNAc β (1 $\rightarrow$). Both GlcA and GalNAc residues carry sulfo groups, giving rise to different types of CS. Chondroitin sulfate A (CS-A) contains 4-*O*-sulfated GalNAc (GalNAc4S) residues, chondroitin sulfate C (CS-C) contains 6-*O*-sulfated GalNAc (GalNAc6S), chondroitin sulfate D (CS-D) contains 2-*O*-sulfated GlcA and chondroitin sulfate E (CS-E) contains 4,6-*O*-disulfated GalNAc. Specialized CS sulfotransferases, including 4-*O*-sulfotransferase (CS4OST), 6-*O*-sulfotransferase (CS6OST), 2-*O*-sulfotransferase and GalNAc4S-6-*O*-sulfotransferase, participate in the biosynthesis of CS.

Chemical synthesis of structurally defined CS oligosaccharides^[4, 9] as well as CS oligosaccharide precursors^[10] has been described. As an alternative approach, we are investigating the feasibility to synthesize homogeneous oligosaccharides using CS biosynthetic enzymes with a goal to improve the synthetic efficiency. An enzyme-based method for synthesizing CS has been reported^[11], but the synthesis remained at the levels of preparing heterogeneous polysaccharides or oligosaccharides with limited quantities. Until now, the reported enzymatic method was clearly unable to prepare homogeneous CS oligosaccharides to support a wide range of biological evaluations.

A total of 15 CS oligosaccharides were synthesized in this study at the scale of 4 to 30 mg (Fig 1 and Supplementary Table S1). The process covered both the synthesis of nonsulfated chondroitin backbones and sulfations of the backbones to yield the final products. The synthesis of nonsulfated chondroitin was accomplished using a bacterial glycosyltransferase from *E. coli* K4 strain, known as KfoC. The enzyme transfers both UDP-GalNAc and UDP-GlcA to the acceptor to form chondroitin. The recombinant KfoC was readily obtained through the expression in high yield, offering an effective way to synthesize chondroitin oligosaccharide backbones (Fig 2A)^[12]. One limitation, however, existed to prepare oligosaccharides in large quantity due to the cost of UDP-GalNAc, which is priced at ~\$16,000/mmol from a commercial source. Such price is prohibitively expensive to conduct gram-scale synthesis. To overcome the limitation, we synthesized UDP-GalNAc using *N*-acetylhexosamine kinase (NAHK)^[13] and mammalian GalNAc pyrophosphorylase AGX1^[14]. This success reduced the cost of UDP-GalNAc by >70-fold. Using the scheme

described in Fig 2A, we routinely synthesized gram-scale of chondroitin backbone to prepare different CS oligosaccharides.

KfoC enzyme is capable of transferring a GalNTFA residue from UDP-GalNTFA, where GalNTFA represented *N*-trifluoroacetyl galactosamine, into the chondroitin backbone to yield compound **4** (Fig 2C). The introduction of a GalNTFA residue into the backbone offered the flexibility to convert it to a GalNH₂^[15] or a 2-deoxy-2-azido- β -galactoside (GalN_{AZ}) residue as demonstrate in the synthesis of compound **6** and **8**, respectively. The conversion of a GalNH₂ residue to a GalN_{AZ} residue was accomplished using imidazole-1-sulfonyl azide following a method reported by Maza and colleagues^[16]. Neither GalNH₂ nor GalN_{AZ} residue was reported in CS isolated from natural sources, and these structures have not been synthesized previously. Our data demonstrate that the ability to synthesize structurally diversified chondroitin backbone structures by exploiting the substrate specificity of the enzyme.

4-*O*-sulfated oligosaccharides were successfully synthesized using CS4OST (Fig 2B). In this study, three oligosaccharides, including **3**, **11** and **15**, were synthesized. We obtained partially 4-*O*-sulfated products. Structural analysis of **3**, **11**, **15** by NMR revealed that the GalNAc residue that is near the nonreducing end was devoid of 4-*O*-sulfation (Supplementary Table S2 and Supplementary Fig S10–12, S42–S44 and S58–S60). The inability of sulfation at the GalNAc near the nonreducing end was attributed to the substrate specificity of CS4OST.

A total of nine different 6-*O*-sulfated oligosaccharides were synthesized, including **1**, **2**, **5**, **7**, **9**, **10**, **12**, **13** and **14** (Supplementary Table S1) using CS6OST. Depending on the positions of 6-*O*-sulfation, different synthetic schemes were used. Compounds **1**, **2**, **10** and **14** are fully 6-*O*-sulfated oligosaccharides ranging from tri-saccharide to nona-saccharide. The synthesis was achieved by incubating the chondroitin backbone (tri- to nona-saccharides) with CS6OST (Fig 2C). The syntheses of partially 6-*O*-sulfated heptasaccharides, including compounds **12** and **13**, were achieved using different starting 6-*O*-sulfated oligosaccharides. To synthesize compound **12**, a 6-*O*-sulfated trisaccharide (compound **1**) was exposed to elongation reaction using KfoC (Fig 2C). To prepare compound **13**, the starting material was compound **2**. The rearrangement of the sequence of enzymatic modification to achieve partially 6-*O*-sulfated oligosaccharides demonstrate the flexibility of the enzymatic synthesis to prepare structurally diversified CS-C oligosaccharides.

The chondroitin backbones containing a GalNTFA, GalNH₂ or GalN_{AZ} residue were employed to prepare the 6-*O*-sulfated oligosaccharides, compound **5**, **7** and **9** (Fig 2C). The success of synthesis of compound **5**, **7**, and **9** demonstrated that CS6OST displays the tolerance on the amino substitution of the GalNAc residue. However, the enzyme appears to be very specific towards the chondroitin backbone. Using a heparan hexasaccharide substrate, we found no reaction, where the repeating disaccharide unit is β -1,4-linked GlcA and *N*-acetylated glucosamine (GlcNAc), $\rightarrow 4$)GlcA β (1 $\rightarrow$ 4) GlcNAc α (1 $\rightarrow$.

Synthesis of CS-A and CS-C oligosaccharides requires access to high quality recombinant CS4OST and CS6OST. We obtained the enzymes from insect cells using baculovirus

expression approach. It is imperative to remove the viral chondroitinase contamination from enzyme preparations^[17]. The chondroitinase degrades chondroitin oligosaccharide backbones, the substrates used by both CS4OST and CS6OST. The expression of CS6OST in *E. coli* was unsuccessful possibly due to the fact that glycosylation is required for the sulfotransferase activity^[18].

The purity and structure of products were characterized by high resolution anion-exchange HPLC, electrospray ionization mass spectrometry (ESI-MS) and NMR. Majority of products displayed a single symmetric peak on HPLC, confirming the purities (Supplementary Table S1 and Supplementary Figs S1 to S60). One exception is compound **4** that had only 59% purity because the trifluoroacetyl group on residue d is labile, and the products were a mixture of compound **4** and detrifluoroacetylated compound **6** as demonstrated by ESI-MS analysis (Supplementary Fig S13). The structure of each compound was confirmed by 1D- and 2D-NMR with full assignments (Supplementary Table S2). Because compound **4** was impure, we did not carry out the NMR structural analysis.

One representative example for the structural analysis of compound **9** using heteronuclear multiple-bond correlation spectroscopy (HMBC) of 2D-NMR was shown in Fig 3. Cross signal of residue anomeric proton of residue C and carbon (C-4) of residue B was observed (blue double arrow in Fig 3); likewise cross signal of the proton (H-3) of residue B and anomeric carbon of residue C was also observed (pink double arrow in Fig 3). The cross peak signals suggest that the glycosidic linkage is 1→3 link. Using a similar approach, we proved that the glycosidic linkage between residue E and D is also 1→3 link. The anomeric proton-proton coupling constant ($^3J_{H-H}$) values of residue B, C, D and E were determined to be 8.5, 7.9, 8.2 and 7.9 Hz respectively from 1D-NMR analysis. The measured $^3J_{H-H}$ values suggest that the glycosidic linkages in compound **9** are in the β -form. Comparing the chemical shifts of H-6 from residues D and B in compound **9** and compound **8** allowed us to prove the presence of 6-*O*-sulfation in compound **9**. The chemical shifts of H-6 were changed to 4.19 ppm (residue B, compound **9**) from 3.72–3.74 ppm (residue B, compound **8**), and 4.14–4.15 ppm (residue D, compound **9**) from 3.68–3.72 ppm (residue D, compound **8**) (Supplementary Table S2). The 6-*O*-sulfation in compound **9** was also supported by the changes of ^{13}C chemical shifts at C-6 position in residue B and D comparing to compound **8**. The chemical shifts of C-6 in residue B changed from 63.8 ppm (compound **8**) to 70.3 ppm (compound **9**), and in residue D changed from 63.7 ppm (compound **8**) to 70.0 ppm (compound **9**) (Supplementary Table S2).

Here, we report a novel method to synthesize structurally homogeneous 4-*O*-sulfated and 6-*O*-sulfated CS oligosaccharides. The approach is highly divergent to prepare a range of oligosaccharides merely using a small number of enzymes and cofactors. The short and simple scheme is ideal for the preparation of structurally diversified CS oligosaccharide library. The enzymatic synthesis of CS was perceived unable to prepare sufficiently large quantity of structurally defined CS oligosaccharides for biological evaluation. We overcame the limitation by improving the accessibility of recombinant CS biosynthetic enzymes and reducing the production cost for UDP-GalNAc. In addition to synthesize natural CS oligosaccharides, we demonstrate the possibility of preparing unnatural 6-*O*-sulfated CS oligosaccharides. The synthesis was accomplished in multi-milligram scales, allowing

complete structural characterization by MS and NMR. All synthesized CS oligosaccharides in the present study contain a pNP group at the reducing end. The pNP-group has a characterized $UV_{\max}$ at 310 nm which facilitates product detection after the enzymatic modifications. In addition, the introduction of pNP-group allows the oligosaccharides to bind to the C_{18} resin for product purification. Furthermore, the pNP-group can be further derivatized to an azido or an amine group to affix the oligosaccharides on the chips for glycan microarray analysis^[19]. The pNP-glucuronic acid residues can be selectively cleaved when treatment with periodate oxidation followed by Smith degradation or alkaline elimination. The method has been successfully used in synthesis of heparin oligosaccharides^[20]. However, this method may be unsuitable to remove the pNP group from chondroitin sulfate oligosaccharides as numerous GlcA residues are in CS oligosaccharides.

In addition to CS-A and CS-C, CS-D and CS-E also exist. The synthesis of both CS-D and CS-E requires additional CS sulfotransferases, CS 2-*O*-sulfotransferase (to prepare CS-D) and GalNAc4S-6-*O*-sulfotransferase (to prepare CS-E). The efforts for obtaining high expression of both 2-*O*-sulfotransferase and GalNAc4S-6-*O*-sulfotransferase are underway with a goal to expand the capability of the synthesis of more diversified CS oligosaccharides. The demonstration of the enzymatic synthesis of structurally defined CS oligosaccharides offers an essential tool to investigate the biological functions of CS.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Experimental Section

Experimental procedures and compound structural characterization are presented in Supporting Information.

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Fig 1.

Chemical structures of 15 CS oligosaccharides synthesized in this study

Fig 2.

Synthetic scheme of CS oligosaccharides using enzymes. Panel A shows the scheme for the synthesis of chondroitin backbones. Panel B shows the conversion of backbone to CS-A targets. Panel C shows the pathway for synthesis of CS-C oligosaccharides. Step a is an elongation step to add a GalNAc residue involving KfoC, a glycosyltransferase from *E. coli* K4. Step b is also an elongation step to add a GlcA residue using KfoC. Step c is a 6-*O*-sulfation step involving chondroitin sulfate 6-*O*-sulfotransferase (CS6OST) and sulfate donor 3'-phosphoadenosine 5'-phosphosulfate (PAPS). Step d is a 4-*O*-sulfation step involving chondroitin sulfate 4-*O*-sulfotransferase (CS4OST) and PAPS. KfoC is also able to transfer a GalNTFA residue to a trisaccharide backbone to yield a tetrasaccharide, where the trifluoroacetyl group (TFA) is in red color. The yield from each reaction step is indicated. ISAH represents imidazole-1-sulfonyl azide.

Fig 3. HMBC spectrum of compound 9

Top indicates the structure of compound **9**, and the anticipated cross-peak signals are indicated by either blue or pink double arrows. The results from HMBC analysis confirmed that the linkages between residue E and D as well as the linkage between residue C and B are 1→3 links. No signals were detected between 85 to 100 ppm, and thus this region has been truncated.