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Mass preparation of oligosaccharides by hydrolysis of chondroitin sulfate polysaccharides with subcritical water microreaction system

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Abbreviations: CS, chondroitin sulfate; CSase, chondroitinase; GalNAc, N-acetyl-D-galactosamine; GlcA, D-glucuronic acid; GAG, glycosaminoglycan; \( \Delta \text{HexA} \), 4-deoxy-\( \alpha-L \)-threo-hex-4-enepyranosyluronic acid; 2S, 2-\( O \)-sulfate; 4S, 4-\( O \)-sulfate; 6S, 6-\( O \)-sulfate.

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Abstract

The biological functions of chondroitin sulfate (CS) are executed by the interaction of specific oligosaccharide sequences in the polysaccharide chain with effective proteins. Thus, CS oligosaccharides are expected to have pharmacological applications. Furthermore, the demand for CS in health food supplements and medication is growing. However, the absorbency of CS polysaccharides in the digestive system is very low. Since the activity of orally administered CS is expected to increase by depolymerization, industrial production of CS oligosaccharides is required. In this study, hydrolysis with subcritical and super-critical water was applied to the depolymerization of CS for the first time, and hydrolytic conditions for oligosaccharide production were examined. CS oligosaccharides principally containing an N-acetyl-D-galactosamine residue at their reducing ends were successfully obtained. No significant desulfation was found in CS oligosaccharides prepared under optimized conditions. The production of CS oligosaccharides by this method will have a strong influence on the CS-related materials market.

Keywords: chondroitin sulfate, hydrolysis, microreaction system, oligosaccharides, subcritical water.
1. Introduction

Chondroitin sulfate (CS) chains are composed of alternating units of N-acetyl-D-galactosamine (GalNAc) and D-glucuronic acid (GlcA), and their sugar backbones can be sulfated mainly at the C2 position of GlcA residues and at C4 and/or C6 positions of GalNAc residues, forming various disaccharide units [1]. CS chains have been demonstrated to play important roles in cytokinesis, cell proliferation, differentiation, migration, tissue morphogenesis, organogenesis, infection, and wound repair [1-4]. CS chains interact with a wide variety of functional proteins, such as growth factors, cytokines, chemokines, and adhesion molecules, via specific oligosaccharide domains within the polysaccharide chains that execute these functions. The biological significance of such interactions is gradually yet steadily emerging.

It has been shown that endogenous glycosaminoglycan (GAG) oligosaccharides generated by digestion with endo-type hydrolase, such as hyaluronidases, are involved in inflammation, tumor migration, and tumor apoptosis [5]. Some reports have shown that CS oligosaccharides have biological functions. Oligosaccharides derived from CS-E, but not intact CS-E, enhance CD44 cleavage and tumor cell motility [6]. Accumulating evidence has demonstrated the various biological functions of GAG oligosaccharides [7, 8]. Therefore, CS oligosaccharides are expected to have future pharmacological applications.

In addition, CS preparations are used commercially as health food supplements and for medication, and their demand is still growing. However, the molecular size of CS polysaccharides is too large for their absorption through the digestive system. Hence, the depolymerization of CS chains is expected to improve the absorbency of CS in the intestine such that the activity of orally administered CS may be more effectively utilized.

To prepare CS oligosaccharides, enzymatic as well as chemical methods are applicable for the depolymerization of CS chains. Chondroitinases (CSases) are often used to degrade CS polysaccharides. However, they are eliminases not hydrolases; therefore, degradation products contain an artificial unsaturated hexuronic acid at the
nonreducing end, contributing to strong antigenicity. Although hyaluronidases are hydrolases and depolymerize not only hyaluronan but also CS [9, 10], these enzymes are not suitable for the mass production of CS oligosaccharides because of their low-throughput. Major chemical methods for the preparation of CS oligosaccharides are acid hydrolysis [11-13] and methanolysis [14]. However, the hydrolysis of CS by these methods is accompanied by partial deacetylation and desulfation. Chemical synthesis of CS oligosaccharides has also been reported [15-17]. However, the cost of the industrial production of CS oligosaccharides by chemical synthesis is high.

Recently, hydrolysis with subcritical and super-critical water has been developed and is attracting much attention as it may replace the classical technologies of hydrolysis. The subcritical and super-critical water microreaction system has the advantages of a short reaction time, chemical-free hydrolysis, and mass production [18]. The hydrolysis of cellulose, hemicellulose, and alginic acid to monosaccharides or oligosaccharides by sub- and super-critical water has been well characterized [19-22]. Lignin degradation has also been accomplished by sub- and super-critical water [23]. The degradation products of a lignocellulosic biomass can serve as raw materials for bioethanol production. In this study, we have applied the subcritical water microreaction system to prepare oligosaccharides from CS polysaccharides on a large scale, and also investigated which glycosidic bonds of CS are cleaved during hydrolysis by subcritical water.

2. Results

2.1. Preparation of CS oligosaccharides by the subcritical water microreaction system

To examine optimal conditions for the preparation of CS oligosaccharides, CS polysaccharides were treated with subcritical and super-critical water. In the beginning, CS chains were treated at a very high temperature (250 °C) for less than one second, which is the reaction condition typical for hydrolysis with subcritical and super-critical water. However, under these conditions, severe desulfation of CS oligosaccharides was
observed (results not shown). Hence, in subsequent experiments, reactions were conducted at relatively lower temperatures for longer periods of time. We performed experiments using reaction times of 4.4 and 8.8 seconds, and the latter condition was found to be better for the preparation of CS oligosaccharides. Therefore, only data obtained by the 8.8 second treatment are shown in this paper.

CS chains from ray fish cartilage were treated with subcritical water for 8.8 seconds at various temperatures between 150 and 250 °C. The decomposition of GlcA residues in the CS preparation was analyzed by the carbazole method. As shown in Figure 1, no significant loss of GlcA occurred when CS chains were treated below 190 °C. However, the yield decreased above 195 °C, and the recovery of GlcA residues was less than 20% above 225 °C.

2.2. Analysis of degradation products by gel filtration HPLC

Degradation products were derivatized with the fluorophore 2-aminobenzamide (2AB), which specifically labels the aldehyde group, and derivatives were analyzed by gel filtration chromatography (Figure 2). Monitoring of generated CS oligosaccharides indicated that products contained an aldehyde group at their reducing ends and that CS depolymerization mainly took place via the cleavage of carbon-oxygen (ether) linkages during the subcritical water microreaction.

As shown in Figure 2, oligosaccharides were produced when treated at 180 - 200 °C. Compared with the elution positions of authentic 2AB-labeled even-numbered oligosaccharides, which were prepared by the partial digestion of CS polysaccharides with CSase ABC [24] as indicated by the numbered arrows, most peaks detected were eluted at similar positions to standard oligosaccharides, suggesting that major degradation products obtained by the subcritical water microreaction may be even-numbered oligosaccharides. Glucuronidic or N-acetylgalactosaminidic bonds appear to be predominantly hydrolyzed.

2.3. Disaccharide composition of degradation products
To examine whether these products still contained sulfate groups or were desulfated, the disaccharide compositions of CS preparations treated with subcritical water at various temperatures were analyzed. As representative chromatograms, the HPLC profiles of intact CS polysaccharides, as well as products treated at 195 °C after digestion with CSase ABC, are shown in Figure 3. Each disaccharide peak was identified by comparison of the elution position with that of standard disaccharides (indicated by the numbered arrows). The yield of each disaccharide was calculated based on the peak area. The compositions of disaccharides in CS preparations treated with subcritical water at various temperatures are summarized in Table 1 and Figure 4. A major disaccharide unit in the intact CS preparation from ray fish cartilage was ΔHexA-GalNAc(6S) (67.7%), with small proportions of other units, ΔHexA-GalNAc(4S) (22.0%), ΔHexA(2S)-GalNAc(6S) (6.5%), and ΔHexA-GalNAc (3.8%), also being detected. This disaccharide composition was compared with those of degradation products. No significant desulfation occurred below 190 °C, but the proportion of 4-O-sulfated GalNAc-containing disaccharide units and nonsulfated disaccharide units decreased and increased, respectively, to a certain degree above 195 °C (Table 1 and Figure 4), indicating that GalNAc 4-O-sulfate groups were partially hydrolyzed or decomposed above 195 °C.

2.4. Characterization of the hydrolysis of CS by the subcritical water microreaction

To investigate whether hydrolysis occurs at hexosaminidic or glucuronidic bonds in CS chains, the terminal sugar residue of oligosaccharides in the products was analyzed. Degradation products were labeled with 2AB and analyzed by anion-exchange HPLC after digestion with CSase ABC or both CSases ABC and AC-II (Figure 5). This strategy is summarized in Figure 6. Even-numbered oligosaccharides obtained from CS can be designated as [GlcA-GalNAc(S)]n or [GalNAc(S)-GlcA]n, where S represents 4-O- or 6-O-sulfate in GalNAc residues. It is possible to estimate the structures of the digests of the degradation
products with CSases based on differences in the substrate specificities of CSases ABC and AC-II [25]. When the 2AB-derivatives of \([\text{GlcA-GalNAc(S)}]_n\) oligosaccharides were digested with CSase ABC or a mixture of CSases ABC and AC-II, unsaturated tetrasaccharides, \(\Delta\text{HexA-GalNAc(S)-GlcA-GalNAc(S)-2AB}\), or unsaturated disaccharides, \(\Delta\text{HexA-GalNAc(S)-2AB}\), were yielded, respectively (Figure 6, left). In contrast, only unsaturated trisaccharides, \(\Delta\text{HexA-GalNAc(S)-GlcA-2AB}\), could be generated by the digestion of 2AB-derivatives of \([\text{GalNAc(S)-GlcA}]_n\) with either CSase ABC or a mixture of CSases ABC and AC-II (Figure 6, right).

When the 2AB-derivatized fraction was digested with CSase ABC only (Figure 5B), a major peak was detected near the elution position of the disulfated disaccharide unit, indicating that the digest was most likely disulfated. Upon digestion with CSase ABC and then AC-II, a major signal was observed at the elution position of \(\Delta\text{HexA-GalNAc(6S)-2AB}\). When co-chromatographed with authentic 2AB-disaccharide standards, it was co-eluted with \(\Delta\text{HexA-GalNAc(6S)-2AB}\) (data not shown). Based on these results, the major oligosaccharide in the degradation products appears to be the \([\text{GlcA-GalNAc(S)}]_n\)-type, and \(N\)-acetylgalactosaminidic bonds rather than glucuronidic bonds are predominantly hydrolyzed by the subcritical water microreaction.

3. Discussion

In this study, CS polysaccharides were subjected to hydrolysis using subcritical water for the first time, although the hydrolysis of plant polysaccharides including cellulose and hemicellulose by subcritical water microreaction has been well characterized [19]. We optimized reaction conditions for the microreaction system to prepare vast quantities of CS oligosaccharides. CS chains were treated by subcritical water at 180 - 190 °C for 8.8 seconds at a pressure of 25 MPa. \(N\)-Acetylgalactosaminidic bonds were specifically hydrolyzed rather than glucuronidic bonds, and no significant desulfation was observed in oligosaccharide products. Mainly tetrasaccharides to dodecasaccharides were obtained under the conditions used in this study.
Accumulating evidence has demonstrated the various biological functions of GAG oligosaccharides [7, 8]. Hyaluronan oligosaccharides have been demonstrated to be involved in inflammation, tumor migration, and tumor apoptosis [5]. The hyaluronan tetramer up-regulates Hsp72 expression and suppresses cell death under stress conditions [26]. The hyaluronan octamer and larger oligosaccharides are competitive inhibitors for the complex formation of serum-derived hyaluronan-associated proteins (SHAP) to inhibit cumulus cell-oocyte complex expansion [27, 28]. Some reports have shown that CS oligosaccharides also have biological functions. Chemically synthesized CS-E hexasaccharides enhanced CD44 cleavage and tumor cell motility in a CD44-dependent manner [6]. The CS-E tetrasaccharide was demonstrated to stimulate neurite outgrowths of dopaminergic neurons mediated through midkine-pleiotrophin/protein tyrosine phosphatase zeta and brain-derived neurotrophic factor/tyrosine kinase B receptor pathways [29]. Specific interactions between effective proteins and particular oligosaccharide sequences coded in polysaccharide chains are often considered to evoke the biological activities of GAGs [7, 30, 31]. Thus, CS oligosaccharides containing such active sequences bear great therapeutic potential and are expected to have pharmacological applications in the future.

The treatment of CS polysaccharides with subcritical water under the conditions used in the present study resulted in the specific cleavage of N-acetylgalactosaminidic bonds, which appear to be more sensitive to hydrolysis than glucuronidic bonds in CS chains, which is consistent with the previous observation that glucuronidic bonds in GAGs are relatively resistant to acid hydrolysis [11]. When treated with subcritical water at a higher temperature, CS chains partially lost their sulfate groups. Desulfation was found to be more extensive in the GalNAc 4-O-sulfate than 6-O-sulfate residue. Cifonelli [11] also reported that chondroitin 4-sulfate lost more sulfate groups than chondroitin 6-sulfate during acid hydrolysis. For the preparation of oligosaccharides from the CS-A isoform, which is predominantly sulfated at the C4-position of GalNAc residues, milder conditions of hydrolysis with subcritical water may be adopted.
We also examined conditions of the subcritical water microreaction system for the preparation of far larger quantities of CS oligosaccharides at a manufacturing plant level using reciprocating positive displacement pumps (NIKKISO CO. LTD., Tokyo, Japan) (results not shown). The flow rate of the CS solution was adjusted to 10 L/h, and 200 g of the CS preparation could be treated by subcritical water per hour to prepare CS oligosaccharides. Thus, this method is very useful for the industrial production of CS oligosaccharides. Since only distilled water is used to hydrolyze CS chains and no chemicals such as hydrochloric acid are required, mass production of CS oligosaccharides can be accomplished by only lyophilization after the subcritical water microreaction. Therefore, CS oligosaccharides obtained by the microreaction system will be appropriate for health food supplements and medication, and the results obtained in the present research may have a strong influence on the CS-related materials market.

4. Experimental

4.1. Materials

CS from ray fish cartilage was prepared as reported previously [32]. CS-C from shark cartilage, chondroitinase (CSase) ABC (EC 4.2.2.20) from *Proteus vulgaris*, CSase AC-II from *Arthrobacter aurescens* (EC 4.2.2.5), and standard unsaturated disaccharides derived from CS were purchased from Seikagaku Corp. (Tokyo, Japan).

4.2. Operation of the subcritical water hydrolysis of CS

The subcritical water microreaction was performed using a continuous type system at various temperatures at a pressure of 25 MPa. CS preparations were dissolved in distilled water at a concentration of 20 mg/ml, and were injected directly into a reaction tube (i.d., 0.5 mm; length, 3,000 mm; volume 590 ml) using a high pressure feed pump (NP-KX-550, Nihon Seimitsu Kagaku Co. Ltd., Tokyo, Japan). Distilled water was degassed and loaded using an intelligent pump (PU-2086, JASCO Corp., Tokyo, Japan) into the reactor heated to precise temperatures. The ratio of the flow rate
of water: CS solution was 3:1, and the reaction time was 4.4 or 8.8 seconds. The reactor, purging lines, and sampling lines were fabricated using stainless steel SUS316.

4.3. HPLC analysis of degradation products

The degradation products of CS preparations by the subcritical water microreaction system were labeled with 2AB [33], and excess 2AB-derivatizing reagents were removed by extraction with chloroform [34]. An aliquot of 2AB-derivatives was analyzed by gel-filtration on a column of the Superdex™ peptide 10/300 GL column (GE Healthcare, Uppsala, Sweden) [35]. Eluates were monitored by measuring fluorescence with excitation and emission wavelengths of 330 and 420 nm, respectively.

4.4. Disaccharide composition analysis of degradation products

Samples treated by the subcritical water microreaction were digested with CSase ABC [36], and digests were analyzed by anion-exchange HPLC on an amine-bound silica PA-03 column (4.6 x 250 mm, YMC Co., Kyoto, Japan) using a linear gradient of NaH₂PO₄ from 16 to 538 mM over 60 min at a flow rate of 1 mL/min. The process was monitored by measuring UV absorbance at 232 nm.

4.5. Characterization of the glycosidic bonds cleaved by the subcritical water microreaction

The 2AB-derivatized oligosaccharides generated by hydrolysis with subcritical water were digested with CSase ABC and/or AC-II [9, 25, 37]. Digests were analyzed by anion-exchange HPLC as described above, and eluates were monitored by measuring fluorescence.

4.6. Analysis of uronic acid

Uronic acid was determined by the carbazole method [38].
Acknowledgments

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References


FIGURE LEGENDS

Figure 1. Analytical data of uronic acid in the degradation products prepared by treatment with subcritical water at various temperatures. Uronic acid was quantified by the carbazole method, and results are shown as relative amounts, taking the amount of uronic acid in the intact CS polysaccharide as 1.0. Values represent the mean ± SD (n = 3).

Figure 2. Gel filtration HPLC analysis of degradation products. An aliquot of 2AB-labeled products was subjected to gel filtration chromatography on a Superdex peptide column, and its elution profile was monitored by fluorescence intensity. Numbered arrows indicate the elution positions of CS oligosaccharides: 2, disaccharides; 4, tetrasaccharides; 6, hexasaccharides; 8, octasaccharides; 10, decasaccharides; and 12, dodecasaccharides. V₀, void volume; Vₜ, total volume. The large peak detected at around 50 min was derived from 2AB-labeling reagents.

Figure 3. Disaccharide composition analysis of CS preparations. CS preparations before (A) and after treatment with subcritical water were digested with CSase ABC. Each digest was analyzed by HPLC on an amine-bound silica column. As a representative, the chromatogram of the product treated at 195 °C is shown (B). Peaks marked by asterisks were due to the incubation buffer as well as unidentified impurities derived from the enzyme solution and eluted from the column resin. The elution positions of authentic disaccharides derived from CS are indicated by numbered arrows in panel A: 1, ΔHexA-GalNAc; 2, ΔHexA-GalNAc(6S); 3, ΔHexA-GalNAc(4S); 4, ΔHexA(2S)-GalNAc(6S); 5, ΔHexA-GalNAc(4S, 6S); and 6, ΔHexA(2S)-GalNAc(4S, 6S).
Figure 4. Comparison of the disaccharide compositions of degradation products. The width of each box corresponds to the proportion of each disaccharide unit. Variously shaded boxes for the repeating disaccharide region of CS indicate ΔHexA-GalNAc, ΔHexA-GalNAc(6S), ΔHexA-GalNAc(4S), and ΔHexA(2S)-GalNAc(6S), respectively, from the top. Note that boxes for the repeating disaccharide region represent the composition, but do not reflect the location or clustering of disaccharide units along the polysaccharide chains.

Figure 5. Anion-exchange HPLC of the 2AB-derivatives of degradation products after digestion with CSases. Degradation products were derivatized with 2AB and analyzed by anion-exchange HPLC on a column of amine-bound silica PA03 after digestion with CSase ABC (B) or ABC and then AC-II (C). The elution profile of the 2AB-derivatives of authentic CS disaccharides is shown in panel A. For the numbers of these peaks, see the legend to Figure 3. Peaks marked by asterisks were also detected in the chromatogram of the negative control and were due to an unidentified impurity derived from the CS preparation, enzyme solution, or 2AB reagent. The peak indicated by an arrow or an arrowhead is a major digestion product.

Figure 6. Strategy for the characterization of the reducing terminal residue of degradation products. CS polysaccharides were depolymerized by the subcritical water microreaction system (step 1). Hydrolysis occurred at the N-acetylgalactosaminidic or glucuronic bond in CS chains, yielding oligosaccharides with a GalNAc (left) or GlcA (right) residue, respectively, at their reducing end. Reaction products were derivatized with 2AB to introduce a fluorophore to the reducing terminus (step 2). 2AB-derivatized oligosaccharides were exhaustively digested with CSase ABC (step 3). An aliquot from the digest was further digested with CSase AC-II (step 4). Each digest was analyzed by
anion-exchange HPLC to identify the 2AB-labeled unsaturated oligosaccharides derived from the reducing terminus of degradation products. Closed hexagon, sulfated or nonsulfated GlcA; hatched hexagon; sulfated or nonsulfated ΔHexA; open hexagon, sulfated or nonsulfated GalNAc.
Table 1.

Disaccharide compositions of degradation products.

<table>
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<th>CS disaccharides</th>
<th>Products treated at the following temperatures (°C)</th>
<th>Proportion (%)</th>
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<tr>
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<td>Intact  150  175  180  185  190  195  200</td>
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<tr>
<td>ΔHexA-GalNAc</td>
<td>3.8      5.6   6.0   4.9   6.2   7.0   8.1   11.5</td>
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<tr>
<td>ΔHexA-GalNAc(6S)</td>
<td>67.7     67.2  66.9  67.3  67.3  67.4  68.9  69.1</td>
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<tr>
<td>ΔHexA-GalNAc(4S)</td>
<td>22.0     20.4  19.5  20.0  18.5  16.5  13.9  10.7</td>
<td></td>
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<tr>
<td>ΔHexA(2S)-GalNAc(6S)</td>
<td>6.5     6.8    7.6   7.8   8.0   9.1   9.1   8.7</td>
<td></td>
</tr>
<tr>
<td>ΔHexA-GalNAc(4S, 6S)</td>
<td>ND*a     ND    ND    ND    ND    ND    ND    ND</td>
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*aND, not detected.
Subcritical water microreaction

Derivatization with 2AB

Digestion with CSase ABC

Digestion with CSase AC-II