STRUCTURAL ANALYSIS OF A LOW-SULFATED CHONDROITIN SULFATE CHAIN IN HUMAN URINARY TRYPsin INHIBITOR

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The low-sulfated chondroitin 4-sulfate(LSC) chain from human urinary trypsin inhibitor was purified and the structure was characterized. After hyaluronidase SD digestion of LSC, an oligosaccharide which contains the linkage region could be obtained. The structure of oligosaccharide was analyzed by HPLC and 500 MHz $^1$H-NMR spectroscopy. The analytical results revealed that 4-O-sulfo GaINAc residues were located in the neighborhood of the linkage region.

KEYWORDS trypsin inhibitor; chondroitin sulfate; glycosaminoglycan; proteoglycan; urine

Human urinary trypsin inhibitor(UTI) contains an N-linked oligosaccharide chain$^{1,2}$ and an O-linked low-sulfated chondroitin 4-sulfate(LSC) chain.$^{3-5}$ The structures of N-linked sialosyl and/or asialosyl oligosaccharides in UTI were determined in our previous report.$^{2}$ On the other hand, it has been reported that the LSC chain acts as cross-link moiety$^{5-7}$ between two fragments consisting of inter-α-trypsin inhibitor(ITI), which is currently considered the precursor of UTI. However, details of the sugar sequence of LSC are not clear, although characteristic features have been revealed by $^1$H-NMR.$^8$

In this paper, we report the study of the sequential structure of LSC chain in UTI.

UTI(75 mg) purified from pooled human urine by the method described in the previous paper$^4$ was subjected to β-elimination in 0.4 M NaOH and 0.3 M NaBH$_4$ for 24 h at 4°C. After neutralization with 1.2 M HCl, 4 volumes of ethanol were added and LSC was precipitated by standing for 24 h at 4°C.

Fig. 1. Chromatograms of Unasaturated Disaccharides Produced from LSC by Digestion with Enzymes

A, chondroitinase ABC digestion; B, chondroitinase ACII digestion. Peak 1, 2-acetamido-2-deoxy-3-O-β-D-glucopyranosyluronic acid)-D-galactose (ΔDi-0S). Peak 2, 2-acetamido-2-deoxy-3-O-(β-D-glucopyranosyluronic acid)-4-O-sulfo-D-galactose (ΔDi-4S).

Fig. 2. Gel Permeation Chromatography of LSC Digested with Hyaluronidase SD

About 450 μg of digested LSC was loaded on a column (Asahipak GS-320, 7.6 mm I.D. X 500 mm). Eluent, 10 mM NH$_4$HCO$_3$ (0.15 ml/min). Fraction size was 0.15ml. GalN and SO$_4$$^2$ were determined after hydrolysis in 6 N HCl at 100°C for 3 h.
The precipitate (crude LSC) was dissolved in 9 ml of 0.03 M NaCl. Then cetylpyridinium chloride precipitation and ethanol precipitation were repeated twice.

The disaccharide composition of the LSC was determined by fluorometric postcolumn HPLC with enzymatic digestion (Fig. 1). The difference of ΔDi-4S amount between chondroitinase ACII and ABC digestion arose from one disaccharide unit near to the linkage tetrasaccharide (GlcUA-Gal-Gal-Xylol), because chondroitinase ABC is unable to act on the unit. Consequently, LSC consists of about 10 units of Di-0S and 5 units of Di-4S (for abbreviations, see Fig. 5), calculated on the presumption that the difference corresponds to 1 mol per 1 mol LSC.

During a study on the sequence of LSC chain, we found that an oligosaccharide which contains the linkage region was produced after enzymatic digestion of LSC with hyaluronidase SD, which acts on hyaluronic acid and chondroitin, not on chondroitin sulfates. The hyaluronidase SD digest was fractionated by gel filtration on Asahipak GS-320 column (Fig. 2). The chromatogram shows two major peaks (oligosaccharide and ΔDi-0S) in a molar ratio of 6.0 : 9.4 as galactosamine. Most of the sulfates were detected in the oligosaccharide fractions (galactosamine to sulfate ratio is 6.0 : 4.9).

The 1H-NMR spectra of LSC and the oligosaccharide are presented in Fig. 3. It was revealed that a pair of signals of GalNAcH-4 was reduced, although signals arising from GlcUA and Gal of the linkage region and from GalNAc(4SO₃)H-4 remained, compared with spectrum of LSC.\cite{8}
Table I. Analysis of Unsaturated Disaccharides Produced from the Oligosaccharide

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>ΔDi-0S*</th>
<th>ΔDi-4S*</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABC</td>
<td>1.0</td>
<td>3.9</td>
<td>4.9</td>
</tr>
<tr>
<td>ACII</td>
<td>1.0</td>
<td>4.9</td>
<td>5.9</td>
</tr>
<tr>
<td>SD</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

*, Results are expressed as mol/mol of the oligosaccharide (deerease of ΔDi-4S amount between chondroitinase ABC and ACII digestion is presumed to be 1.0 mol per 1 mol of the oligosaccharide). ABC, chondroitinase ABC; ACII, chondroitinase ACII; SD, hyaluronidase SD.

The disaccharide composition of the oligosaccharide was determined by HPLC with enzymatic digestion (Table I). It was proved that 5 units of Di-4S are bonded to the linkage tetrasaccharide and the non-reducing end is ΔDi-0S (Fig. 4). ΔDi-0S at the non-reducing end was confirmed by observation of a differential NOE experiment (not shown). Taking all the results, LSC has 5 continuous units of Di-4S near the linkage tetrasaccharide and is followed by approximately 10 units of Di-0S (Fig. 5), though it is possible that a small number of Di-4S units are found in the region.

Recently, Enghild has shown that the heavy chain in PtxI is esterified, via the α-carbon of its C-terminal Asp, to C-6 hydroxyl group of a GalNAc of the LSC chain. The structure proposed in our study suggests that the point of ester linkage to heavy chains exist in the non-sulfated region of LSC due to steric hindrance from sulfate at the C-4 hydroxyl group of GalNAc. Further investigations are necessary to elucidate biosynthetic pathways and biological importance of the LSC chain.

Fig. 5. Structure of LSC

REFERENCES

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