Note

Analysis of Unsaturated Disaccharides from Glycosaminoglycans by High Performance Liquid Chromatography

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Classification of proteoglycans is based on the structure of the glycosaminoglycan (GAG) moiety, and the structure of GAGs can be identified by analyzing the unsaturated disaccharide units (ΔDi-Ss) which are produced by hydrolysis of GAGs with a particular enzyme, such as chondroitinase ABC. However, since the enzymic products contain generally the following different types of ΔDi-Ss: ΔDi-S non-sulfate (ΔDi-HA, ΔDi-OS), ΔDi-S monosulfate (ΔDi-4S, ΔDi-6S, ΔDi-UA2S), ΔDi-S disulfate (ΔDi-diSb, ΔDi-diSp), and ΔDi-trisulfate (ΔDi-triS), all of them must be identified. Yamagata et al. and Saamanen et al. reported the identification of ΔDi-OS, ΔDi-4S, and ΔDi-6S by paper chromatography and by thin-layer chromatography, respectively. Using HPLC, Ototani et al., Gherezgliler et al. and Macek et al. analyzed them within 25 min. Seldin et al. reported that non-sulfated, monosulfated, disulfated, and trisulfated ΔDi-S could be identified by adopting two kinds of mobile phase. Recently, Murata et al. have developed an analytical procedure for several ΔDi-Ss by HPLC using an isoacaric elution. However, this procedure does not completely resolve the following ΔDi-Ss: ΔDi-UA2S and ΔDi-diSb, ΔDi-diSp and ΔDi-triS, and ΔDi-OS, ΔDi-6S, and ΔDi-HA. In this paper, we describe an improved analytical method available for all of the ΔDi-Ss from GAG by HPLC using an isoacaric elution.

Nine kinds of ΔDi-S (ΔDi-HA, ΔDi-OS, ΔDi-4S, ΔDi-6S, ΔDi-UA2S, ΔDi-diSb, ΔDi-diSp, ΔDi-diSe, and ΔDi-triS), hyaluronic acid (HA), chondroitin (Ch), chondroitin 4-sulfate (Ch-4S) and dermatan sulfate (DS) were purchased from Seikagaku Kogyo Co., Ltd. Chondroitin 6-sulfate (Ch-6S) was purchased from P.L. Biochemicals Co., Ltd. Chondroitinase ABC (protease-free, EC 4.2.2.4) was purchased from Seikagaku Kogyo Co., Ltd.

These commercial GAGs (about 1 mg) were digested with chondroitinase ABC (100 μl, 0.2 U) in 0.05 M Tris-HCl buffer at pH 8.0 at 37°C for 4 hr, by the method of Saito et al. The enzymic products were centrifuged for 30 min at 1,800 rpm and 20 μl of the supernatant was taken for the HPLC analysis. HPLC of ΔDi-Ss was done under an isocratic elution as follows: liquid delivery pump, TOSOH CCPM; column, TSK gel Amide-80 (4.6 x 250 mm); mobile phase, acetonitrile-methanol-0.5 M ammonium formate buffer at pH 4.8 (70:5:25, v/v/v); flow rate, 0.5 ml/min; column temperature, 70°C; detection, absorbance at 232 nm using a TOSOH Model UV-8000. Retention times and areas of chromatographic peaks were measured by a Shimadzu Chromatopack C-R3A integrator.

The HPLC profile of a mixture of various ΔDi-Ss is shown in Fig. 1. It is thought that all of the ΔDi-Ss are precisely distinguished from each other by a single HPLC analysis. Especially, the resolution of ΔDi-diS and ΔDi-triS was better than that by other methods. The retention times of nine kinds of ΔDi-S were measured as follows: ΔDi-HA, 24.55 ± 0.32 min; ΔDi-4S, 27.36 ± 0.14 min; ΔDi-UA2S, 32.17 ± 0.12 min; ΔDi-6S, 35.20 ± 0.13 min; ΔDi-4S, 37.76 ± 0.19 min; ΔDi-diSb, 42.48 ± 0.29 min; ΔDi-diSp, 49.28 ± 0.19 min; ΔDi-diSe, 56.68 ± 0.15 min; ΔDi-triS, 74.53 ± 0.12 min (mean ± S.D., n = 3 ± 15). The optimum column temperature and pH of the ammonium formate buffer were 70°C and 4.8, respectively. Under the above conditions, the minimum

![Fig. 1. Typical HPLC Profiles of a Mixture of Nine Kinds of ΔDi-S.](image-url)
detectable amount of ΔDi-HA was 0.12 nmol. It was also found that the relationship between the peak area (Y, μV·sec) integrated and the sample amount (X, nmol) could be described as following a regression equation within 0.12~2.5 nmol: \( Y = 92600X - 3520 \) \( (r^2 = 0.997) \), where \( r^2 \) is a correlation coefficient. These mean that our HPLC analysis can measure with a sensitivity of detection as low as 0.12~2.5 nmol. If the analysis of ΔDi-triS may be omitted, more rapid analysis (about 25 min) than the analysis described above can be done using a mobile phase of acetonitrile–methanol–0.5 M ammonium formate buffer at pH 4.8 (60:25:15, v/v/v), at a flow rate of 1 ml/min. The HPLC profiles of ΔDi-Ss from several DSs by Murata et al. indicated that peaks of ΔDi-diSα and ΔDi-diSβ overlapped ΔDi-UA2S and ΔDi-triS, respectively, and that the resolution of ΔDi-HA, ΔDi-6S and ΔDi-0S was not complete. For instance, the resolution index (Rs) of ΔDi-6S and ΔDi-0S can be calculated as 0.64 according to the following equation\(^{11}\):

\[ Rs = \frac{2(\text{tr}^B - \text{tr}^A)}{W^A + W^B} \]

where \( \text{tr}^A \) and \( \text{tr}^B \), and \( W^A \) and \( W^B \) are the retention times and the peak base widths of components A and B, respectively. Since \( Rs = 1.0 \) and \( Rs = 1.5 \) indicate the separation with an overlap of only \( 2\% \) and no overlap respectively, 0.64 is very poor separation. In the case of our HPLC method, this \( Rs = 4.43 \), and even the lowest \( Rs = 1.27 \) (ΔDi-6S and ΔDi-4S in Fig. 1), indicating the excellent separation.

The HPLC profiles of the enzymic products of five kinds of commercial GAG are shown in Fig. 2. Their ΔDi-S compositions (mole percent) are shown in Table I. It was confirmed that the ΔDi-S of HA was only composed of ΔDi-HA, that is, no ΔDi-S except for ΔDi-HA could be detected. In the case of DS, the contents of ΔDi-4S and ΔDi-diSα amounted to about 99% of all ΔDi-Ss, while very small amounts of ΔDi-0S and ΔDi-diSβ (0.2% and 0.1% respectively) were also analyzed. The major ΔDi-S of Ch-4S was ΔDi-4S (about 77%), while ΔDi-6S contained as much as about 21% Ch-6S had the most complicated ΔDi-S composition among the five GAGs. It contained about 49% ΔDi-6S, 32% ΔDi-4S, 15% ΔDi-diSβ, and four kinds of minor ΔDi-S. In the case of Ch, it was recognized that about 5% ΔDi-monoS remained because of incom-

**Table I. Unsaturated Disaccharide Compositions of Products by Chondroitinase ABC-Digestion of Several GAGs (mol%)**

<table>
<thead>
<tr>
<th>ΔDi-HA</th>
<th>ΔDi-0S</th>
<th>ΔDi-UA2S</th>
<th>ΔDi-6S</th>
<th>ΔDi-4S</th>
<th>ΔDi-diSα</th>
<th>ΔDi-diSβ</th>
<th>ΔDi-diSγ</th>
<th>ΔDi-triS</th>
</tr>
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<tr>
<td>HA</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>DS</td>
<td>0</td>
<td>0.2</td>
<td>0</td>
<td>1.0</td>
<td>91.1</td>
<td>0.1</td>
<td>7.7</td>
<td>0</td>
</tr>
<tr>
<td>Ch-4S</td>
<td>0</td>
<td>0.7</td>
<td>0</td>
<td>20.8</td>
<td>76.6</td>
<td>1.4</td>
<td>0</td>
<td>0.3</td>
</tr>
<tr>
<td>Ch-6S</td>
<td>0.6</td>
<td>1.1</td>
<td>0</td>
<td>49.3</td>
<td>32.2</td>
<td>15.2</td>
<td>0.4</td>
<td>1.2</td>
</tr>
<tr>
<td>Ch</td>
<td>0</td>
<td>95.4</td>
<td>0</td>
<td>1.6</td>
<td>3.0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

![Fig. 2. HPLC Profiles of Chondroitinase ABC-Digestion Products of Several GAGs.](image)

(1) = HA; (2) = DS; (3) = Ch-4S; (4) = Ch-6S; (5) = Ch. Peak number in each panel, see Fig. 1.

Thus, it was confirmed that nine kinds of ΔDi-S can be quantitatively analyzed by a single procedure under the above conditions.

References

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