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Effect of oversulfation on the chemical and biological properties of chondroitin-6-sulfate

Ananda S. Amarasekara, Gloria Opoku, Xiangdong Qiu, Vasant Doctor *

Department of Chemistry, Prairie View A&M University, Prairie View, TX 77445, USA

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Abstract

Chondroitin-6-sulfate was oversulfated using chlorosulfonic acid–pyridine complex and isolated as the sodium salt. Infrared analysis of the native and oversulfated compound gave identical results in respect to the O—H stretching, hemiacetal stretching and S=O stretching. Absorption around 825 cm^{-1} was also present in both the compounds representing the sulfate group of galactosamine at equatorial C-6 position. The oversulfated compound showed a peak at 855 cm^{-1} representing the new sulfate group on axial C-4 position of galactosamine. ^1H NMR studies confirmed the IR results and further showed that the oversulfated compound was a mixture of 61% of chondroitin-4,6-disulfate and 39% of the native compound. The oversulfated compound showed a 2.8-fold increase in sulfate content and a significant anticoagulant activity by doubling the prothrombin time of normal citrated human plasma using 9.5 μg of the sulfated compound while the native compound was inactive even at 2000 μg level. During *in vitro* studies using 0.05 M Tris buffer pH 7.35 containing physiological concentrations of NaCl (0.9%) the oversulfated compound gave a 1.8-fold enhancement of the activation of glutamic plasminogen (Glu-Plg) by tissue plasminogen activator (t-PA) and a 3.2-fold enhancement of Glu-Plg activation by urokinase (u-PA) in comparison to the control while the native compound or the unfractionated heparin were less active.

Keywords: Chondroitin-6-sulfate; Plasminogen; Coagulation; t-PA; u-PA

1. Introduction

Sulfated polysaccharides constitute a large and complex group of macromolecules known to possess a wide range of important biological properties. In algae the carrageenans and fucoidans are mainly composed of sulfated galactose and fucose, respectively (Painter, 1983) while in the animal kingdom, the sulfated glycosaminoglycans are abundant in vertebrate tissues (Mathews, 1975). Invertebrate species are also a rich source of sulfated polysaccharides with novel structures (Pereira, Melo, & Mourao, 2002). Sulfated galactans obtained from egg jelly of sea urchins are reported to show *in vitro* anticoagulant properties (Farias, Valente, Pereira, & Mourao, 2000; Pereira et al., 2002). The anticoagulant heparin, a glycoaminoglycan obtained from cattle lungs or hog intestines is the most widely used anticoagulant in the prophlaxis and treatment of thrombosis (Kakkar & Hedges, 1989). Dermatan sulfate, designated earlier as chondroitin sulfate B or β-heparin is reported to show anticoagulant activity both *in vitro* and *in vivo* but of lower potency than heparin (Merton & Thomas, 1971; Thomas, Merton, & Borrowcliffe, 1989). In general oversulfation of the naturally sulfated polysaccharides is reported to enhance its anticoagulant action (Dace et al., 1997; Doctor et al., 1991; Soeda, Ohmagori, Shimeno, & Nagamatsu, 1993). The specific changes in the infrared spectra of sulfated polysaccharides following oversulfation is reported to be related to the introduction of new sulfate groups in key positions of fucoidan and kappa-carrageenan (Opoku, Qiu, & Doctor, 2006; Qiu, Amarasekara, & Doctor, 2006). In both of these reports,

* Corresponding author. Tel.: +1 936 261 3110; fax: +1 936 857 2546.
E-mail address: vmdoctor@pvamu.edu (V. Doctor).

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the oversulfated compounds showed significant enhancement of the anticoagulant activity and also a faster rate of *in vitro* activation of glutamic plasminogen (Glu-Plg) by the oversulfated compounds in comparison to the native compounds. In the present report chondroitin-6-sulfate was oversulfated using chlorosulfonic acid–pyridine complex and isolated as the sodium salts. The changes in the infrared and ^{1}H NMR spectra were investigated and the biological properties of the oversulfated compound were compared with the native compound and with unfractionated heparin.

2. Materials and methods

2.1. Materials

Plasmin substrate *H*-D-Glu-Phe-Lys-pNA (S-2403) was purchased from DiaPharma Group Inc. (Westchester, Ohio, USA). Human glutamic type plasminogen, urokinase and plasmin were purchased from American Diagnostica (Greenwich, Connecticut, USA). Alteplase (t-PA) was obtained from Genentech Inc. (South San Francisco, California, USA). Citrated human plasma and thromboplastin were purchased from BioMerieux, Inc. (Durham, N.C). Chondroitin-4-sulfate, chondroitin-6-sulfate, unfractionated heparin and all other reagents were purchased from Sigma (St. Louis, Missouri, USA).

2.2. Sulfation of polysaccharide and IR and NMR studies

Three different batches of chondroitin-6-sulfate were sulfated by using chlorosulfonic acid–pyridine complex by the procedure described earlier (Rickets, 1952).The reaction mixture was neutralized with 40% NaOH at 0 °C and the residual pyridine and chlorosulfonic acid were removed by precipitation of the oversulfated compound by the addition of 2 volumes of ethanol followed by centrifugation. The supernatant was discarded and the sulfated compound was reconstituted in water. This step was repeated twice and the product was dialyzed, neutralized and lyophilized. The percentage of sulfate was determined by the AOAC procedure described earlier (Horwitz, 1980). Infrared analysis was performed using Thermo Nicolet (IR 200 spectrometer). A pellet was prepared by mixing 2 mg of the compound with 8 mg of potassium bromide using a vacuum press (Fig. 1). ^{1}H NMR was obtained on a Varian mercury plus spectrometer operating at 400 MHz. Samples for ^{1}H NMR were prepared by dissolving 10 mg in 0.6 ml of D_2O (Fig. 2). Spectra were recorded at 30 °C and 64 scans were collected with relaxation delay of 2 s. All ^{1}H NMR were performed using partially hydrolyzed samples of the compounds which were prepared as follows: 50 mg each of chondroitin-6-sulfate or the oversulfated compound were dissolved in 1.0 ml of 150 mM H_2SO_4 and heated at 100 °C for 1 h. The partially hydrolyzed samples were adjusted to pH 7.0 with 0.3 ml of ice cold 1.0 M NaOH and lyophilized.

2.3. Effect of sulfation on coagulation of human plasma

To determine the anticoagulant properties of the native and sulfated polysaccharides, prothrombin time (PT) was measured using three or more concentrations of the compounds dissolved in 0.1 ml of 0.05 M TES (*N*-tris-hydroxymethyl-2-aminomethance sulfonic acid) buffer pH 7.35. The concentrations were selected so that the clotting would occur above the doubling time or below the doubling time, since the clotting time was linear over a narrow range of concentrations of the polysacchrides. The compounds were mixed with 0.1 ml of normal citrated human plasma and after 2 min incubation of 37 °C, 0.1 ml of thromboplastin was added and the timer was started (Dace et al., 1997).

2.4. Effect of sulfation on activation of human plasminogen

Studies on the effect of native and sulfated chondroitin-6-sulfate or unfractionated heparin on the activation of Glu-Plg by t-PA or u-PA were carried out using a model Elx 800 well counter which was set at 405 nm. Incubations were carried out at room temperature and plasmin generation was measured using 0.36 mM chromogenic substrates S-2403 (Ranby, Norman, & Wallen, 1982). The reactions were performed in 700 μl of 0.05 M Tris buffer pH 7.4 containing 0.15 M NaCl in microfuge tubes. The concentrations of the reagents were as follows: native or oversulfated chondroitin-6-sulfate or heparin, 28.6 μg/ml; Glu-Plg 3.6 μM; t-PA 5.8 IU/ml; urokinase 41.4 IU/ml. Three hundred microliters of the reaction mixture were transferred to microplates and absorbancies were read at 2 or 4 min intervals. The results plotted are mean of three experiments. Control experiments were run using human plasmin to rule out enhancement by the reagents on the plasmin.

3. Results and discussion

3.1. Effect of sulfation on IR and NMR spectra

Fig. 1 shows the infrared spectra of native (N-2) and sulfated (S-2) chondroitin-6-sulfate. Both compounds exhibited a strong absorption at 1240–1250 cm^{-1} attributable to S=O stretching. The band at 825 cm^{-1}present in both the compounds is reported to represent sulfate group at C-6 position of galactosamine units. A comparison of infrared spectra of chondroitin-6-sulfate before and after desulfation showed that upon desulfation the band at 825 cm^{-1} disappeared (Hoffman, Linker, & Myer, 1958; Mathews, 1958). On comparing the spectra of native (N-2) and the sulfated (S-2) samples in Fig. 1, S-2 sample showed a new peak at 855 cm^{-1} representing the new sulfate at axial C-4 position of galactosamine. The desulfation of chondroitin-4-sulfate is also reported to result in the disappearance of the band of 855 cm^{-1} in the infrared spectra (Hoffman et al., 1958).

In Fig. 2, the ^{1}H NMR spectrum of partially hydrolyzed sample of oversulfated chondroitin-6-sulfate (a) showed

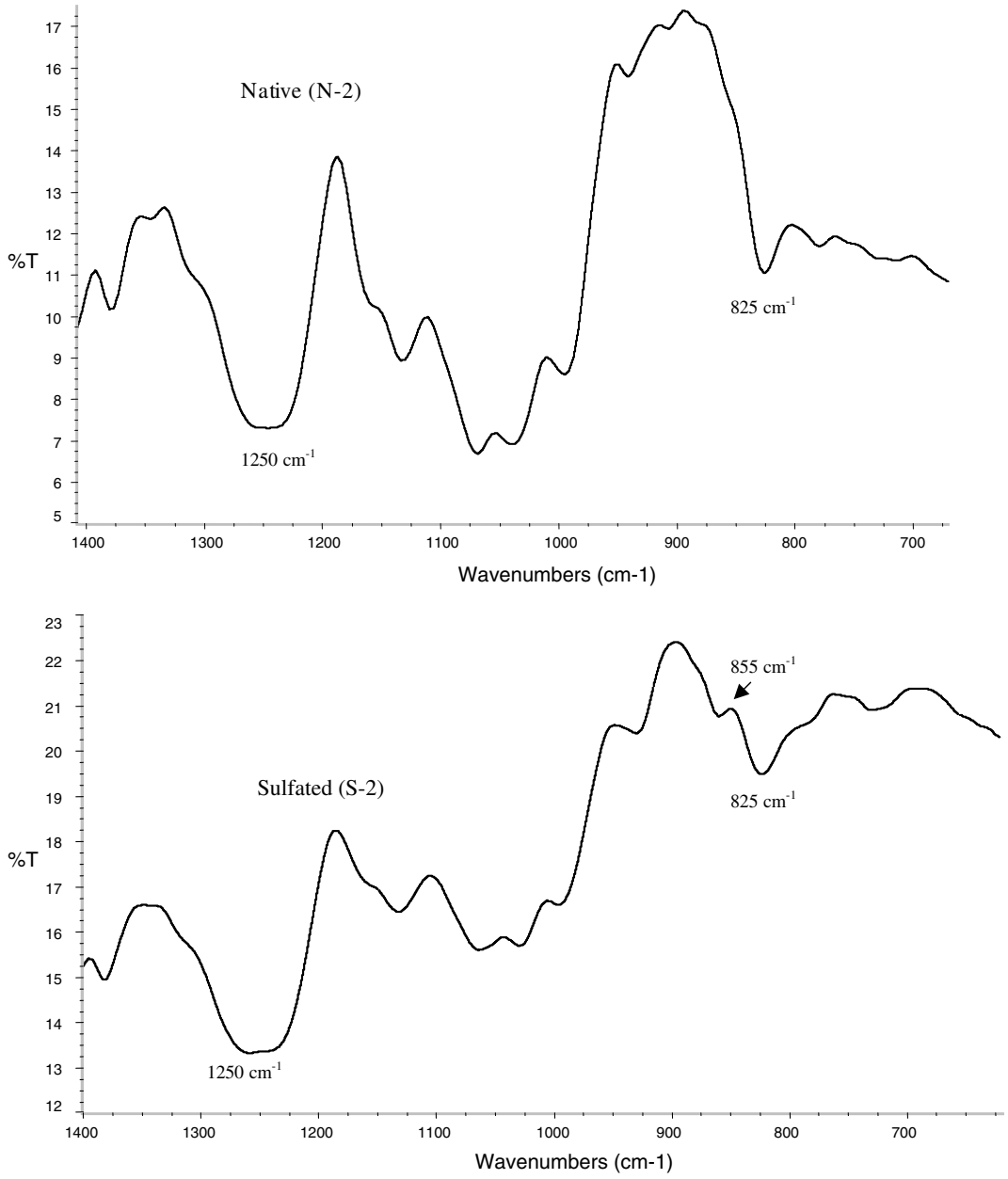

Fig. 1. Infrared analysis of native (N-2) and oversulfated chondroitin-6-sulfate (S-2). The compounds were scanned between 1400 and 600 cm^{-1}. The upper panel represents (N-2) and lower panel represents (S-2). Please see Section 2 for experiment details.

a number of differences compared to the spectrum of hydrolyzed sample of chondroitin-6-sulfate (b). The most distinct difference is found in the peak at 4.178 ppm. This signal was identified as the 4-H of the 2-acetamido-2-deoxy-β-D-galactopyranoside unit by comparison with the reported spectrum of chondroitin-6-sulfate, in which this proton was reported as a broad singlet at 4.167 ppm (Welti, Rees, & Welsh, 1979) recorded under identical conditions. In the oversulfated compound, 2-acetamido-2-deoxy-β-D-galactopyranoside (spectrum a), integration of this peak at 4.178 was found to be relatively smaller compared to the relative integration of the same peak at 4.178 ppm in chondroitin-6-sulfate (spectrum b). Reduction in the area of the peak at 4.178 ppm can be explained as a result of partial sulfation of the 4-OH group of 2-acetamido-2-deoxy-β-D-galactopyranoside unit producing chondroitin-4,6-disulfate in the sulfation process. Further, it was reported (Welti et al., 1979) that 4-H of the 2-acetamido-2-deoxy-β-D-galactopyranoside unit of chondroitin-4-sulfate resonates at 4.723 ppm in the ^{1}H NMR showing a 0.556 ppm shift due to sulfaton of the —OH group attached to the same carbon. The peak at 4.7 ppm could not be observed in the spectrum as the 4.5–4.80 ppm region was masked by HDO solvent peak.

Broad unresolved peak at 4.232 ppm in the spectrum b corresponds to two methylene protons attached to C-6 of the 2-acetamido-2-deoxy-β-D-galactopyranoside unit of chondroitin-6-sulfate. The same peak in

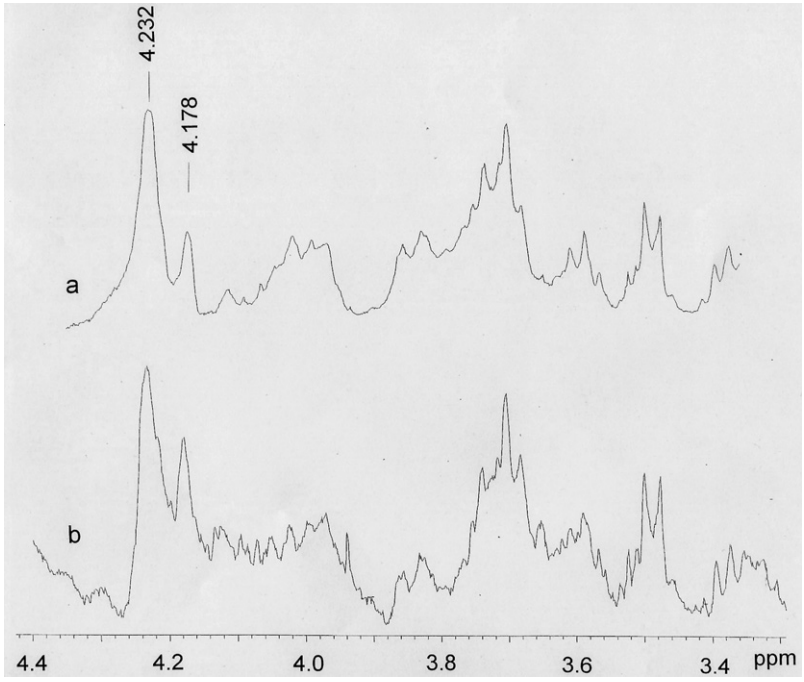

Fig. 2. ^{1}H NMR analysis of the oversulfated (S-2) and native (N-2) chondroitin-6-sulfate. The upper panel (a) represents the oversulfated compound and the lower panel (b) represents the native compound. Please see Section 2 for experiment details.

spectrum a, of the oversulfated sample corresponds to the contributions from the mixture of chondroitin-6-sulfate and chondroitin-4,6-disulfate. Composition of the oversulfated chondroitin-6-sulfate was calculated by comparison of the peak areas of the 4.232 and 4.178 ppm peaks of the two spectrums. Oversulfated sample was found to be a mixture of 61% chondroitin-4,6-disulfate and 39% natural chondroitin-6-sulfate. The percentages of the two compounds were calculated by using the formula shown below in spectrum "a".

$$\frac{\text{Peak area at 4.232 ppm}}{\text{Peak area at 4.178 ppm}} = \frac{[\text{chondroitin-6-sulfate} + \text{chondroitin-4-6 disulfate}]}{\text{chondroitin-6-sulfate}}$$

3.2. Effect of sulfation on coagulation of human plasma

The anticoagulant properties of the two chondroitin-6-sulfates were compared by measuring the concentrations of each required for doubling the prothrombin time. The figures were obtained from the graph of mean ISD of five measurements for each compound against three different concentrations of the anticoagulants. The results presented in Table 1 showed that the sulfation of chondroitin-6-sulfate increased the % sulfate from 10% to 28% and in order to double prothrombin time, oversulfated chondroitin-6-sulfate required 9.25 μg while the native compound did not show anticoagulant activity even after addition of 2000 μg. Unfractionated heparin was more effective on weight basis in doubling prothrombin time and required 2.5 μg and contained 33% sulfate. There are no recent published reports on the anticoagulant properties of chondroitin-6-sulfate but dermatan sulfate is reported (Merton & Thomas, 1971) to show weaker anticoagulant property compared to heparin.

3.3. Effect of sulfation on the activation of Glu-Plg by t-PA or u-PA

Figs. 3 and 4 show the comparison of the enhancement by native and oversulfated chondroitin-6-sulfate and unfractionated heparin on the activation of Glu-Plg by t-PA and u-PA, respectively. Calculations of enhancement

Table 1
Effect of oversulfation on the anticoagulant properties of chondroitin-6-sulfate

Addition to the coagulation mixture[a]	Sulfate[b] (%)	Micrograms required to double prothrombin time
Chondroitin-6-sulfate (N-2)	10.0	>2000
Oversulfated chondroitin-6-sulfate (S-2)	28.0	9.5
Unfractionated heparin	33.0	2.5

[a] Refer to the text for details of the coagulation mixture.
[b] The results are mean of three experiments.

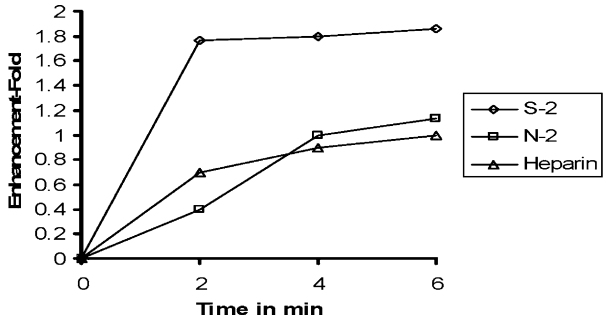

Fig. 3. Enhancement by native (N-2) and oversulfated chondroitin-6-sulfate (S-2) of activation of Glu-Plg by t-pA. Please see Section 2 for experiment details.

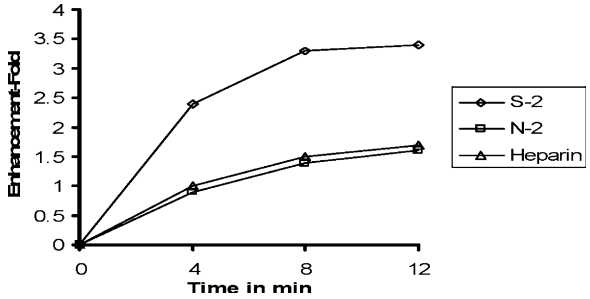

Fig. 4. Enhancement by native (N-2) and oversulfated chondroitin-6-sulfate (S-2) of the activation of Glu-Plg by urokinase. Please see Section 2 for experiment details.

fold were based on the ratios of absorbances for each of the three samples over the control sample at the three time intervals. The results for oversulfated compound showed a 1.8-fold enhancement of the activation of Glu-Plg by t-PA and a 3.2-fold enhancement of Glu-Plg activation by u-PA in comparison to the control while native compound or unfractionated heparin were less active or not active. In both the studies physiological concentration of 0.15 M of NaCl was added to 0.05 M Tris buffer pH 7.4.

4. Conclusion

This is the first report showing the nature of the chemical transformation of chondroitin-6-sulfate upon oversulfation using chlorosulfonic acid–pyridine complex. Chondroitin sulfate obtained from bovine tracheal cartilage was overulfated to produce polysulfated compound for treatment of arthritis in dogs and marketed under trade name Adequan^{R} Canine. However no reports are available on the characterization of the product. The structures of chondroitin-6-sulfate and chondroitin-4-sulfate have been identified. These are linear chains composed of alternate β-D-glucopyranosyluronic acid and 2-acetamido-2-deoxy-β-D-galactopyranosyl monosulfate units linked $(1 \rightarrow 3)$ and $(1 \rightarrow 4)$, respectively. Chondroitin-4-sulfate has the sulfate group esterified to hydroxyl at position C-4 of galactosamine unit, while the sulfate group of chondroitin-6-sulfate was located at C-6 (Wolfrom & Juliano, 1960). The infrared absorption curves of both are reported to show characteristic bands at 855 cm^{-1} for chondroitin-4-sulfate and at 825 for chondroitin-6-sulfate (Lloyd, Dodgson, Price, & Ruse, 1961; Mathews, 1958; Orr, Harris, & Sylven, 1952). Oversulfation of chondroitin-6-sulfate introduced a new sulfate group in the axial C-4 position of galactosamine as shown by the appearance of a peak at 855 cm^{-1} at the same wavelength as that reported earlier for chondroitin-4-sulfate (Hoffman et al., 1958). ^{1}H NMR results confirmed the conclusions of the IR study and further showed that the oversulfated samples contained 61% of chondroitin-4, 6-disulfate following sulfation. The oversulfated chondroitin-6-sulfate showed significant anticoagulant activity and also acted as an effective cofactor in enhancing the activation of plasminogen by t-PA or u-PA. Further studies of the biological properties of oversulfated glycosaminoglycans may lead to the development of a new generation of dual function compounds.

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