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Effect of oversulfation on the chemical and biological properties of kappa carrageenan

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Abstract

Kappa carrageenan was sulfated using chlorosulfonic acid–pyridine complex and isolated as the sodium salt. Infrared analysis of the native and sulfated kappa carrageenans gave identical results in respect to the O–H stretching, hemiacetal stretching and S=O stretching. Absorption around 845 cm^{-1} was also present in both the compounds representing the sulfate at the axial C-4 position except for a peak at 811 cm^{-1} which was only present in the oversulfated compound representing C-6 position. The sulfated compound showed 30 times higher anticoagulant activity in doubling prothrombin time of normal citrated human plasma in comparison with native compound. Studies on the effect of addition of the two kappa carrageenans during the in vitro activation of glutamic plasminogen (Glu-Plg) by tissue plasminogen activator(t-PA) or by urokinase (u-PA) showed that sulfated kappa carrageenan gave a 3-fold enhancement of the initial rate of the activation in comparison to control without the compound while the native kappa carrageenan or the unfractionated heparin were less active.

Keywords: Kappa carrageenan; Plasminogen; Coagulation; t-PA; u-PA

1. Introduction

One abundant source of new anticoagulant polysaccharides is marine algae. They contain a variety of sulfated fucans (Pereira, Mulloy, & Mourao, 1999) and sulfated galactans (Farias, Valente, Pereira, & Mourao, 2000). These compounds are among the most abundant and widely studied of all the sulfated polysaccharides from nonmammalian sources. Recently, several sulfated α-L-fucans and sulfated α-L-galactans from invertebrates (mostly from the egg jelly of sea urchins) were isolated and characterized. These polysaccharides have simple linear structures composed of well-defined repeating units of oligosaccharides(Pereira, Melo, & Mourao, 2002). Sulfated galactans obtained from egg jelly of sea urchins are reported (Farias, Valente, Pereira, & Mourao, 2000; Pereira et al., 2002) to show in vitro anticoagulant properties. Carrageenans (kappa, lambda and iota) are linear polysaccharides of the Rhodophyceae family of red seaweed made up of galactans with alternating α(1→3) linkages and β(1→4) linkages and occasional 3,6-anhydro-D-galactose units. Kappa carrageenan is made up of α(1→4) D-galactose-4-sufate and β(1→3)3,6-anhydro-D-galactose. (Bayley, 1955; O'Neal, 1955; Weigl & Yaphe, 1966). Carragenans have been reported (Anderson & Duncan, 1965; Hawkins, 1962, 1963) to show anticoagulant activity and the effect was primarily on the thrombin time. In the present report kappa carrageenan was oversulfated using chlorosulfonic acid–pyridine complex and isolated as the sodium salt. The changes in the infrared spectra were investigated and the biological properties of the oversulfated carrageenan were compared with the native compound and the 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

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from BioMerieux, Inc. (Durham, NC). Kappa carrageenan, unfractionated heparin and all other reagents were purchased from Sigma (St Louis, Missouri, USA).

2.2. Sulfation of polysaccharide and IR studies

Four different batches of kappa carrageenan were sulfated by using chlorosulfonic acid–pyridine complex by the procedure described earlier (Doctor et al., 1991). 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 dialysed, neutralized and lyophilized. The percentage of sulfate was determined by the procedure described earlier (Dace et al., 1997). Infrared analysis was performed using Thermo Nicolet (IR 200 spectrometer). A pellet was prepared by mixing 8 mg of the compound with 2 mg of potassium bromide.

2.3. Effect of sulfation on coagulation of human plasma

To measure 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-hydroxy-methyl-2-amino-methance 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 concentration of the polysaccharide. 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).

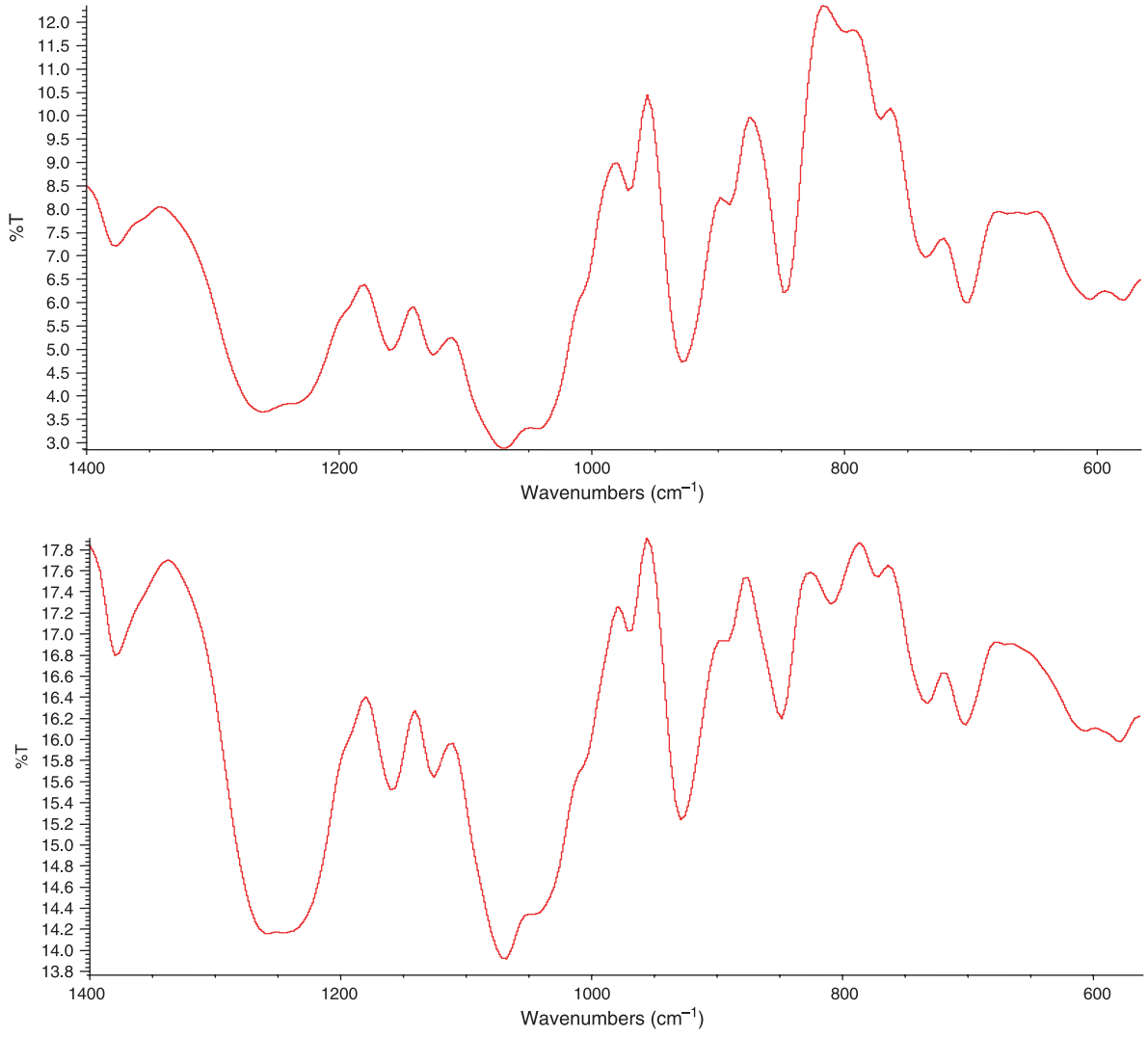

Fig. 1. Infrared analysis of native (N-2) and sulfated (S-2) kappa carrageenan. The carrageenans were scanned between 1400 and 600 cm^{-1}. The upper panel represents N-2 and lower panel represents S-2.

Table 1
Effect of oversulfation on the anticoagulant Properties of Kappa Carrageenan

Addition to the coagulation mixture[a]	% Sulfate[b]	Micrograms required to double prothrombin time[c]
Kappa carrageenan (N-2) native	16.0	600
Kappa carrageenan (S-2) oversulfated	28.5	20

[a] Please refer to the text for details of the coagulation mixture.
[b] The results are mean of three experiments.
[c] The figures were obtained from the graph of mean ±SD of five measurements for each compound against three different concentrations of the anticoagulants. Unfractionate heparin from porcine intestinal mucosa required 2.1 μg to double prothrombin time.

2.4. Effect of sulfation on activation of human plasminogen

Studies on the effect of native and sulfated kappa carrageenan 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. The reactions were performed in 700 μl of 0.05 M Tris buffer pH 7.4 containing 1.54 M NaCl in microfuge tubes. Three hundred microlitres of the reaction mixture were transferred to microplates and absorbancies were read at 2 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 spectra

Fig. 1 shows the infrared spectra of native (N-2) and sulfated (S-2) kappa carrageenan. In the native compound the sulfate band around 840 cm^{-1} was reported (Bayley, 1955) to be related to axial C-4 position. A detail study of the infrared spectra comparing the absorbencies of kappa, lambda and iota carrageenans was reported (Thomas, 2005). It was shown that if the C-6 position of galatose was sulfated that this would show up as a peak in the 810–820 cm^{-1} range (Thomas, 2005). On comparing the spectra of the native (N-2) with the sulfated (S-2), the results of Fig. 1 showed that the sharp peak in both the compounds at 845 cm^{-1} was related to the sulfate on the axial C-4 position, while only the sulfated compound showed a peak at 811 cm^{-1} corresponding to C-6 substituted sulfate. Both the kappa-carrageenans (N-2 and S-2) gave broad signals in the ^{1}H NMR spectra recorded in D_2O with poor resolution.

3.2. Effect of sulfation on coagulation of human plasma

The anticoagulant properties of the two kappa carrageenans were compared with unfractionated heparin by measuring the concentrations of each required for doubling the prothrombin time. The results presented in Table 1 showed that in order to double prothrombin time, the concentration of the sulfated compound required was lowered by 30-fold upon sulfation, while the percentage of sulfate in the sulfated compound was increased by 78% during the sulfation procedure. A comparison with unfractionated heparin (UH) showed that the sulfated kappa carrageenan contained about the same percentage of sulfate as UH but was less effective than UH. Earlier studies in our laboratory, Dace et al. (1997) have reported higher anticoagulant property by the oversulfated kappa carrageenan but the structural changes taking place during sulfation procedure using chlorosulfonic acid–pyridine complex are being reported for the first time. There are no other published reports on the anticoagulant properties of the oversulfated carrageenans. However, laminarin, a β-D(1→3) linked glucan obtained from brown seaweed was sulfated to produce compounds which showed in vivo anticoagulant activity using rats and dogs (Hawkins & O'Neil, 1955).

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

Fig. 2 shows the comparison of the enhancement by native or sulfated kappa carrageenan or unfractionated heparin on the activation of Glu-Plg by t-PA. The results showed a 3-fold increase in the initial rate using the oversulfated carrageenan while the native compound or the unfractionated heparin were less effective. Similar results were also obtained during the in vitro activation of Glu-Plg by u-PA as shown in Fig. 3. In both the studies physiological concentration of 1.54 M of NaCl was added to 0.05 M Tris buffer pH 7.4.

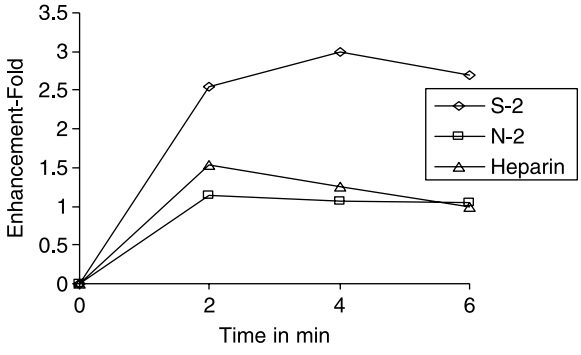

Fig. 2. Enhancement by native (N-2) and sulfated (S-2) kappa carrageenan of activation of Glu-Plg by t-pA. The concentrations of the reagents were as follows: carrageenan (N-2) sulfated and heparin carrageenan (S-2) 28.6 μg/ml, t-pA 5.8 IU/ml, and Glu-Plg 3.6 nM were incubated at room temperature using 0.05 M Tris buffer pH 7.4 containing 1.54 M NaCl. Please refer to the experimental procedure for details.

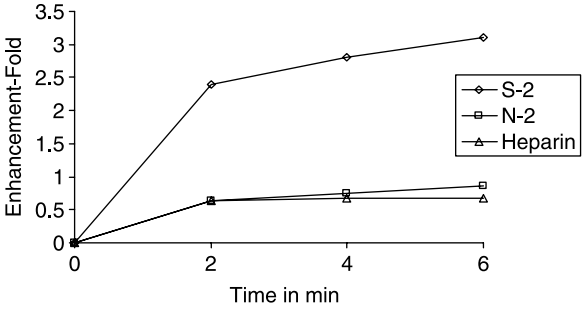

Fig. 3. Enhancement by native (N-2) sulfated (S-2) carrageenans of the activation of Glu-Plg by urokinase. The concentration of the reagents were as follows: carrageenan (N-2) sulfated carrageenan and heparin (S-2): 28.6 μg/ml; urokinase 41.4 IU/ml and Glu-Plg; 36 nM were incubated at room temperature using 0.05 M Tris pH 7.4 containing 1.54 M NaCl. Please refer to the experimental procedure for details.

4. Conclusion

This is the first report showing the nature of the chemical transformation of kappa carrageenan upon oversulfation using chlorosulfonic acid–pyridine complex. Kappa carrageenan is made up of 4-sulfated α(1→4)D galactose and alternating β(1→3)3,6-anhydro-D-galactose units. Oversulfation introduced a sulfate group in 6-position with the formation of 4, 6-disulfated compound. It was reported (Qiu, Amarasekara, & Doctor 2006) that fucoidan, a C-4 sulfated fucan upon oversulfation introduced a new sulfate group in the C-2 position with the formation 2,4-disulfated compound. The oversulfated fucoidan was reported (Qiu et al., 2006) to show 4-fold higher anticoagulant activity in doubling prothrombin time of normal citrated human plasma in comparison with the native fucoidan. Kappa carrageenans showed lower percentage of sulfate compared to fucoidan (Qiu et al., 2006) because it contained one sulfate per disaccharide unit while fucoidan is reported (Patankar, Ochinger, Barnett, Williams, & Clarke, 1993) to be primarily a polymer of α(1→3) linked fucose units with sulfate group in 4 position for each fucose unit. Structural requirements for interaction of sulfated galactan or sulfated fucans from invertebrate animals with coagulation inhibitors were reported (Pereira et al., 2002). The results showed that 2-*O*-sulfated 3-linked α-L-galactan was a potent inhibitor of thrombin mediated by AT-III or by heparin cofactor II (HC-II) while the 2,4-di-*O*-sulfated 3-linked α-fucan enhanced thrombin inhibition mediated by AT-III and the 4-*O*-sulfared fucan enhanced thrombin inhibition by HC-II suggesting a stereospecific nature of the interactions between the cofactors and target proteases.

Considerable attention has been focused recently on the therapeutic value of t-PA as a thrombolytic agent. Although t-PA activates preferentially fibrin-bound Plg with the potential of sparing fibrinogen, one possible problem of t-PA therapy is the rapid reocclusion of the affected artery. Therefore, patients treated with t-PA are treated concomitantly with an anticoagulant heparin. This has been shown to have some clinical benefits but some reports suggest that heparin cannot prevent coronary reocclusion in patients treated with t-PA (Gold et al., 1986; Williams et al., 1986). A possible explanation for the limited efficacy of heparin is that it promotes the binding of thrombin to fibrin polymer by forming a ternary complex (Hogg & Jackson, 1990), resulting in the lowering of the efficacy of thrombin in activation by heparin antithrombin III (AT-III) by 300-fold (Hogg & Jackson, 1989). Therefore, a search for an ideal anticoagulant is continuing. Oversulfated kappa carrageenan was found to show dual properties of inhibiting coagulation and also significantly enhancing the in vitro activation of Glu-Plg by t-PA. Unfractionated heparin on the other hand was not as effective as oversulfated kappa carrageenan in enhancing the activation of Glu-Plg by t-PA.

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References

Anderson, W., & Duncan, J. G. (1965). The anticoagulant activity of carrageenan. *The Journal of Pharmacy and Pharmacology*, *17*, 647–654.

Bayley, S. T. (1955). X-ray and infrared studies on carrageenan. *Biochimica et Biophysica Acta*, *17*, 194–205.

Dace, R., McBride, E., Brooks, K., Gander, J., Buszko, M., & Doctor, V. M. (1997). Comparison of the anticoagulant action of sulfated and phosphorylated polysaccharides. *Thrombosis Research*, *87*, 113–126.

Doctor, V. M., Lewis, D., Coleman, M., Kemp, M. T., Marbley, E., & Sauls, V. (1991). Anticoagulant properties of semisynthetic polysaccharide sulfates. *Thrombosis Research*, *64*, 413–425.

Farias, W. R. L., Valente, A. P., Pereira, M. S., & Mourao, P. A. (2000). Structure and anticoagulant activity of sulfated galactans. Isolation of a unique sulfated galactan from red algae *Botryocladia accidentalis* and comparison of its anticoagulant action with that of the sulfated galactans from invertebrates. *The Journal of Biological Chemistry*, *275*, 29299–29307.

Gold, H. K., Leinbach, R. C., Garabedian, H. D., Yasuda, T., Johns, J. A., Crossbard, E. B., et al. (1986). Acute coronary reocclusion after thrombolysis with recombinant human tissue-type plasminogen activator. Prevention by a maintenance infusion. *Circulation*, *73*, 347–352.

Hawkins, W. W. (1962). Antipeptic and antithrombic properties of carrageenan. *The Journal of Laboratory and Clinical Medicine*, *60*, 641–648.

Hawkins, W. W. (1963). Antithrombic activity of carrageenan in human blood. *Canadian Journal of Biochemistry and Physiology*, *41*, 1325–1327.

Hawkins, W. W., & O'Neil, A. N. (1955). The anticoagulant action in blood of laminarin. *Canadian Journal of Biochemistry and Physiology*, *33*, 545–552.

Hogg, P. J., & Jackson, C. M. (1989). Fibrin monomer protects thrombin from inactivation by heparin–antithrombin III: Implications for heparin efficacy. *Proceedings of the National Academy of Sciences USA*, *86*, 3619–3623.

Hogg, P. J., & Jackson, C. M. (1990). Heparin promotes binding of thrombin to fibrin polymer. *The Journal of Biological Chemistry*, *265*, 241–247.

O'Neal, A. N. (1955). 3, 6-anhydro-D-galactose as a constituent of k-carrageenan. *Journal of the American Chemical Society*, *77*, 6324–6326.

Patankar, M. S., Ochinger, S., Barnett, T., Williams, R. L., & Clarke, G. F. (1993). A revised structure for fucoidan may explain some of its biological activities. *The Journal of Biological Chemistry*, *268*, 21770–21776.

Pereira, M. S., Melo, F. R., & Mourao, P. A. S. (2002). Is there a correlation between structure and anticoagulant action of sulfated galactans and sulfated fucans? *Glycobiology*, *12*, 573–580.

Pereira, M. S., Mulloy, B., & Mourao, P. A. S. (1999). Structure and anticoagulant activity of sulfated fucans: Comparison between the regular, repetitive and linear fucans from echinoderms with the more heterogeneous and branched polymers from brown algae. *The Journal of Biological Chemistry*, *274*, 7656–7667.

Qiu, X., Amarasekara, A., & Doctor, V. M. (2006). Effect of oversulation on the chemical and biological properties of fucoidan. *Carbohydrate Polymers*, *63*, 224–228.

Thomas, W. (2005). Personal communication. Data published in bulletin by Kelco, C. P. under 'Genu Carrageenan; molecular structure'.

Weigl, J., & Yaphe, W. (1966). The enzymatic hydrolysis of carrageenan by *Pseudomonas carrageenovara*: Purification of kappa-carrageenase. *Canadian Journal of Microbiology*, *12*, 939–947.

Williams, D. O., Borer, J., Braunwald, E., Chesebro, J. H., Cohen, L. S., Dalen, J., et al. (1986). Intravenous recombinant tissue-type plasminogen activator in patients with acute myocardial infarction. *Circulation*, *73*, 338–346.