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Effect of oversulfation on the chemical and biological properties of fucoidan

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Abstract

Fucoidan was sulfated using chlorosulfonic acid–pyridine complex and isolated as the sodium salt. Infrared analysis of the native and sulfated fucoidans gave identical results in respect to the O–H stretching, hemiacetal stretching and S=O stretching. Absorption around 840 cm^{-1} was also present in both the fucoidans representing the sulfate at the axial C-4 position except for a shoulder at 820 cm^{-1}, which was present only in the sulfated compound indicating the presence of sulfate groups at the equatorial C-2 position. The sulfated compound showed four times higher anticoagulant activity in doubling prothrombin time of normal citrated human plasma in comparison with native fucoidan. Earlier studies using native fucoidan showed that the activation of glutamic plasminogen (Glu-Plg) by tissue plasminogen activator (t-PA) or by urokinase (u-PA) was enhanced when the in vitro studies were conducted using 0.05 M Tris buffer (pH 7.4) and that the addition of physiological concentration of NaCl (0.9%) to the buffer reversed the activation. The results of the current studies showed that sulfated fucoidan gave higher stimulations of Glu-Plg activation by u-PA and by t-PA in comparison to native fucoidan when the in vitro studies are conducted using Tris buffer containing 0.9% NaCl.

Keywords: Fucoidan; 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. Fucoidan from *Fucus vesiculosus* is reported to show anticoagulant activity in vitro and in vivo (Bernardi et al., 1962) and it was reported (Church, Meade, Treanor, & Whinna, 1989) that the major antithrombic activity by fucoidan was mediated by heparin cofactor II. Fucoidan is also reported to mediate a variety of significant biological effects such as blocking sperm–egg binding in diverse species, blocking infection of human cell lines with HIV, herpes and cytomegalovirus, blocking cell–cell binding mediated by P- and L-selectin recognition of oligosaccharides and other molecular mechanisms by interfering with cell-to-cell recognitions (Patankar, Ochninger, Barnett, Williams, & Clarke, 1993). A revised structure for fucoidan was postulated to be primarily a polymer of α 1-3-linked fucose with sulfated groups substituted at the 4th position on some of the fucose residues. Fucose units were also involved in forming branch points, one for every 2–3 fucose residues within the chain (Patankar et al., 1993). Fucans sulfates obtained from another brown seaweed, *Ecklonia kurome*, were reported (Nishino, Nagumo, Kiyohara, & Yamada, 1991) to show potent anticoagulant activity and were structurally similar to the fucans obtained from *F. vesiculosus*. A relationship between sulfate content of these compounds and their anticoagulant activities was also reported (Nishino & Nagumo, 1991). Low molecular weight fucans isolated from the brown seaweed, *Ascophyllum nodosum*, were structurally different from the other brown seaweeds (Chevolot et al., 1999). The fucose units were found mainly sulfated at O-2, to a lesser extent at 0–3 and some 2,3-O-disulfate residues were observed for the first time and the anticoagulant activity was related not only to molecular weight, sulfate content but also to the levels of 2-O-sulfation and 2,3-O-disulfation. A highly sulfated fucoidan was prepared by using sulfurtrioxide–trimethylamine complex (Soeda, Sakaguchi, Shimeno, & Nagamatsu, 1992) and was reported to stimulate t-PA induced plasma clot lysis by protecting plasmin activity from α2-antiplasmin and by decreasing the rate of polymer formation (Soeda et al., 1992). Fucoidan purified by DEAE cellulose chromatography was reported

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(Doctor, Hill, & Jackson, 1995) to enhance t-PA or u-PA activation of glutamic plasminogen (Glu-Plg). These studies were performed using 0.05 M Tris buffer (pH 7.4) but not containing NaCl. Fucoidan obtained from another brown seaweed, *E. kurome*, was reported to enhance the activation of plasminogen by u-PA or t-PA (Nishino, Yamauchi, Horie, Nagumo, & Suzuki, 2000). In this study, the incubations were performed using subphysiological level (0.6%) of NaCl. In this paper, results are presented to show that sulfation of fucoidan using chlorosulfonic acid–pyridine complex doubled the sulfate content by introducing O-sulfate groups on carbon-2 of fucose units and that sulfation enhanced its anticoagulant properties by over 4-fold and unlike native fucoidan it stimulated by 5-fold the activation of Glu-Plg by t-PA and by 1.5-fold the activation of Glu-Plg by u-PA using 0.05 Tris buffer (pH 7.4) and containing physiological concentrations of sodium chloride.

2. Materials and methods

2.1. Materials

Plasmin substrate H-D-glu-phe-lys-pNA (S-2403) was purchased from DiaPharma Group Inc. (Westchester, OH, USA). Human glutamic type plasminogen, urokinase and plasmin were purchased from American Diagnostica (Greenwich, CT, USA). Alteplase (t-PA) was obtained from Genentech Inc. (South San Francisco, CA, USA). Citrated human plasma and thromboplastin were purchased from BioMerieux Inc. (Durham, NC). Fucoidan and all other reagents were purchased from Sigma (St Louis, MO, USA).

2.2. Purification of polysaccharide, sulfation and IR studies

Fucodan was purified by ultrafiltration and DEAE cellulose chromatography by the procedure reported previously (Doctor et al., 1995) and size exclusion chromatography gave a major peak with an average molecular weight of 80 kDa and gel electrophoresis showed one major peak (Dace et al., 1997). Purified fucoidan was sulfated using chlorosulfonic acid–pyridine complex and isolated as the sodium salt and percent sulfate was determined by the procedure described earlier (Dace et al., 1997; Doctor et al., 1991). The percent sulfate was increased from 24 to 48% by sulfation procedure. Infrared analysis was performed using Thermo Nicolet (IR 200 spectrometer). A pellet was prepared by mixing 1 mg of the native or sulfated fucoidan with 50 mg of anhydrous 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 compounds were mixed with 0.1 ml of normal citrated plasma and after 2 min incubation at 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 fucoidan 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 substrate S-2403. The reactions were performed in 700 μl of 0.05 M Tris buffer (pH 7.4) containing 0.9% NaCl in microfuge tubes. Three hundred microliters of reaction mixtures were transferred to microplates and absorbances were read at 405 nm at 10 min intervals. The results plotted are mean of three experiments. Control experiments were run using human plasmin to rule out the enhancement by the reagents on 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) fucoidan. In native fucoidan, the sulfate band around 840 cm^{-1} was reported (Bernardi et al., 1962; Percival et al., 1967) to be related to an axial C-4 position. It was further reported that if the equatorial C-2 position was sulfated that this would show up as a shoulder of absorption at 820 cm^{-1} (Patankar et al., 1993; Percival et al., 1967). On comparing our spectra of native (N-2) and sulfated (S-2) samples in Fig. 1, N-2 sample showed a sharp peak at 840 cm^{-1}, whereas S-2 showed the same peak at 840 cm^{-1} together with a shoulder at 820 cm^{-1}. This IR spectra clearly suggest that the native fucoidan was largely 4-substituted and that the sulfation procedure resulted in 2,4 disubstituted compound, since these were the only positions available for substitution. Both the fucoidans (N-2 and S-2) gave broad signals in the ^{1}H NMR spectrum recorded in D_2O with poor resolution.

3.2. Effect of sulfation on coagulation of human plasma

The anticoagulant properties of the two fucoidans were compared 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 sulfated fucoidan required one-fourth the concentration that of the native fucoidan, while heparin required twice the concentration of sulfated fucoidan. Earlier studies in our laboratory and by others (Dace et al., 1997; Soeda, Ohmagori, Shimeno, & Nagamatsu, 1993) have reported higher anticoagulant property by the sulfated fucoidan but the chemical changes taking place during sulfation using chlorosulfonic acid–pyridine complex have not been reported. The major antithrombic activity by fucoidan was reported (Church et al., 1989; Minix & Doctor, 1997) to be

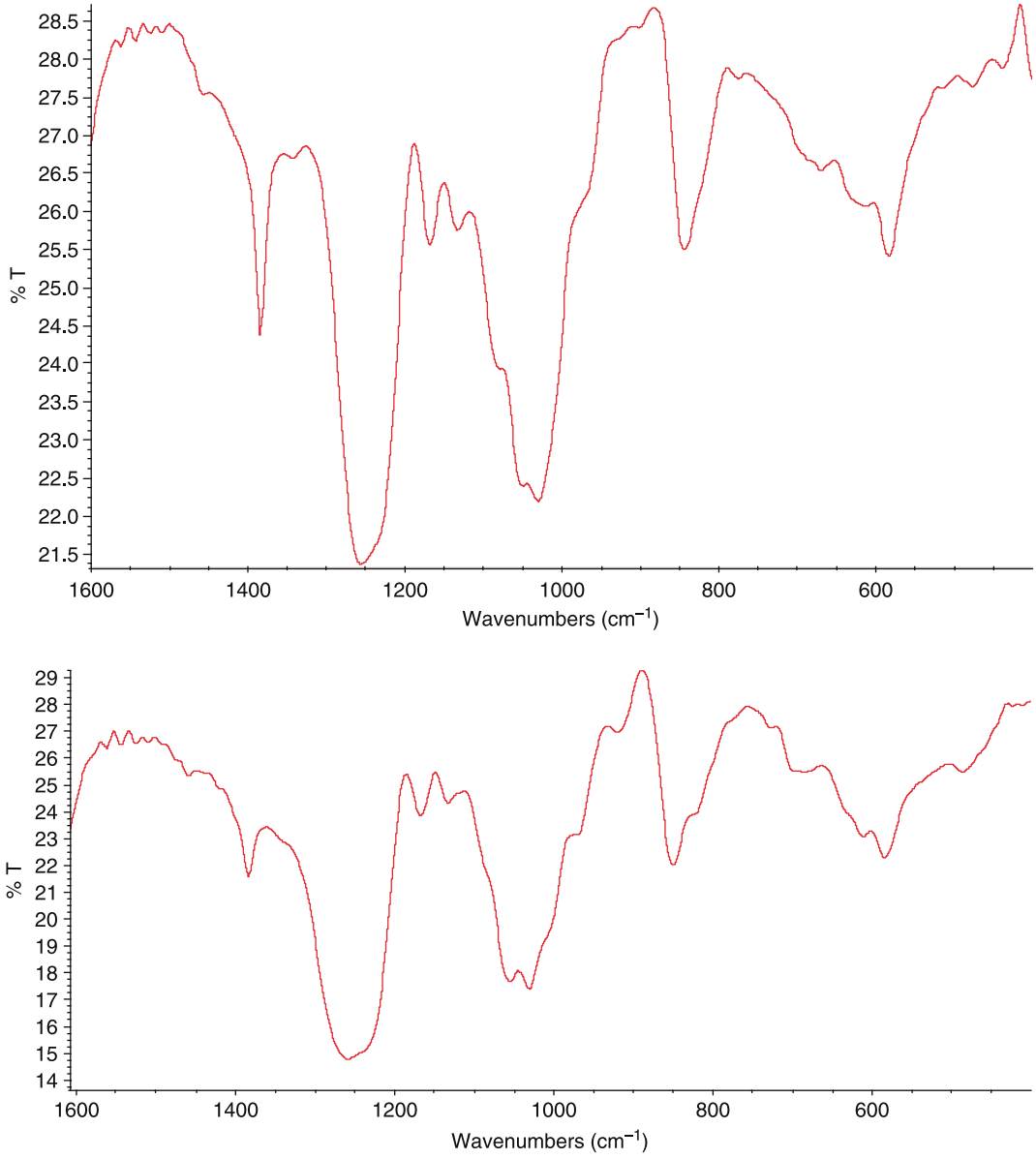

Fig. 1. Infrared analysis of native (N-2) and sulfated (S-2) fucoidan. The fucoidans were scanned between 1600 and 400 cm^{-1}. The upper panel represents N-2 and lower panel represents S-2.

mediated by heparin cofactor II. Patients receiving t-PA after heart attacks are also treated concomitantly with heparin in order to prevent coronary reocclusion. However, its effectiveness is limited as it promotes the binding of thrombin to fibrin polymer by forming a ternary complex thus lowering the inactivation of thrombin by antithrombin III (Hogg et al., 1989, 1990). On the other hand, fucoidan and other anticoagulants such as dermatan sulfate (Tollefson, Pestka, & Monafo, 1983) which inhibit thrombin mediated by heparin cofactor II will inactivate thrombin even on the fibrin surface.

Table 1
Effect of oversulfation on the anticoagulant properties of native fucoidan

Addition to the coagulation mixture[a]	% Sulfate[b]	Moles required to double prothrombin time[c]
Fucoidan native (N-2)	24	2×10^{-7}
Fucoidan sulfated (S-2)	48	5×10^{-8}
Heparin	31	9.4×10^{-8}

[a] Refer to the text for details of the coagulation mixture. The average molecular weight of N-2 was 80 kDa, S-2 was 100 kDa and heparin was 17 kDa.
[b] The results are mean of three experiments.
[c] The figures were obtained from a graph of mean ± SD of five measurements for each compound against three different concentrations of the anticoagulants.

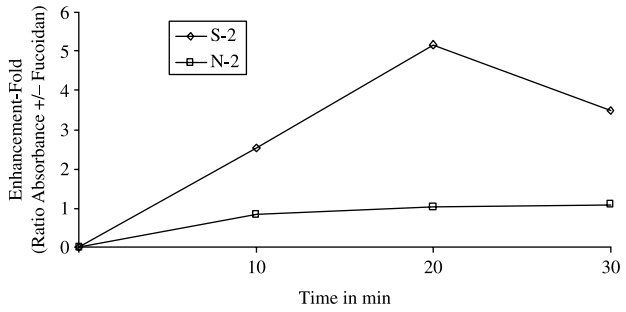

Fig. 2. Enhancement by native (N-2) and sulfated (S-2) fucoidans of activation of Glu-Plg by t-PA. The concentrations of the reagents were as follows: fucoidan (N-2) and sulfated fucoidan (S-2) 1.43 μg/ml, t-PA 5.8 IU/ml, and Glu-Plg 3.6 nM were incubated at room temperature using 0.05 M Tris buffer (pH 7.4) containing 0.9% NaCl. Please refer to Section 2 for details.

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

Fig. 2 shows the enhancement by native and the sulfated fucoidan of the activation of Glu-Plg by t-PA. The results showed a 5-fold increase in the initial rate while the native fucoidan was much less effective. Circular dichroism (CD) studies of Glu-Plg in the far ultraviolet (UV) range are reported (McCance & Castellino, 1995; Urano, Bakshy, Chibber, & Castellino, 1987) to show that addition of chloride ions induces a change in the conformation of Glu-Plg from a more relaxed to a more rigid T-conformation which was highly resistant to activation while addition of 6-aminohexanoic acid (6-AH) caused a reversal and induced a transformation to an expandable and flexible R-conformation. The results of affinity chromatography using fucoidan-sepharose showed a high degree of affinity between fucoidan and Glu-Plg (Minix & Doctor, 1997) and CD studies showed that addition of fucoidan increased its ellipticity and reversed the effects of NaCl on the secondary structure of Glu-Plg (Lang, Williams, Phillips, & Doctor, 2004). Similar results were also obtained during the activation of Glu-Plg by u-PA as shown in Fig. 3. The enhancement was 1.5-fold by addition of sulfated fucoidan while native fucoidan gave no enhancement when physiological concentration (0.9%) of NaCl was added to 0.05 M Tris buffer (pH 7.4).

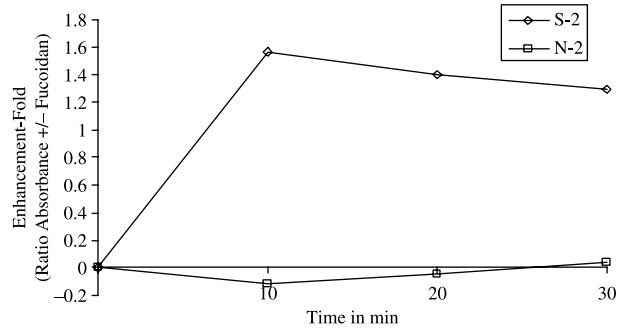

Fig. 3. Enhancement by native (N-2) and sulfated (S-2) fucoidans of the activation of Glu-Plg by urokinase. The concentration of the reagents were as follows: fucoidan (N-2) and sulfated fucoidan (S-2); 114 μg/ml, urokinase 43.7 IU/ml, and Glu-Plg 36 nM were incubated at room temperature using 0.05 M buffer (pH 7.4) containing 0.9% NaCl. Please refer to Section 2 for details.

4. Conclusion

This is the first report showing the nature of the chemical transformation of fucoidan upon sulfation using chlorosulfonic acid–pyridine complex. In a number of earlier studies from our laboratory and elsewhere (Dace et al., 1997; Doctor et al., 1991; Soeda et al., 1992, 1993) results were presented showing enhancement of anticoagulant properties of polysaccharides upon sulfation as measured by an increase in the prothrombin time of normal citrated human plasma. The anticoagulant activity of sulfated polysaccharides is reported (Chevolot et al., 1999) to be related to molecular weight and the levels of sulfation. Heparin is reported (Perlin, Mackie, & Dietrich, 1971) to contain three sulfate groups per disaccharide residue, native fucoidan contains one sulfate per fucose units (Patankar et al., 1993) while the sulfated fucoidan reported here may contain two sulfate per fucose units. Although sulfated fucoidan was not as effective as heparin on weight basis, its anticoagulant action is mediated by heparin, cofactor II instead by antithrombin III as reported for heparin (Church et al., 1989; Rosenberg & Damus, 1973). These differences limit the effectiveness of heparin since it promotes the binding of thrombin to fibrin polymer by forming a ternary complex thus lowering the inactivation of thrombin by antithrombin III (Hogg et al., 1989; Hogg et al., 1990). This is critical when heparin is used to prevent coronary reocclusion following clot formation. The treatment for recanalization following heart attack or ischemic stroke includes infusion of t-PA. The results of in vitro studies have shown that native fucoidan was effective in enhancing the activation of Glu-Plg by t-PA or u-PA (Doctor et al., 1995; Nishino et al., 2000). However, there was lower or no enhancement if physiological concentration of NaCl (0.9%) was added to the buffer. In this paper, we are reporting that sulfated fucoidan was effective in enhancing the activation of Glu-Plg by t-PA or u-PA using 0.05 M Tris buffer (pH 7.4) and containing physiological concentration of NaCl (0.9%).

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