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Note

Anticoagulant activity of a sulfated chitosan

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Abstract

Chitin prepared from the shells of rice-field crabs (*Somanniathelphusa dugasti)* was converted into chitosan with a degree of acetylation of 0.21 and then sulfated with chlorosulfonic acid in *N*,*N*-dimethylformamide under semi-heterogeneous conditions to give 87% of water-soluble sulfated chitosan with degree of substitution (d.s) of 2.13. ^{1}H NMR revealed the sulfate substitution at C-2, C-3 and C-6. Gel filtration on Sepharose CL-6B of the sulfated chitosan gave three fractions with average molecular weights of 7.1, 3.5, and 1.9×10^4. The three sulfated chitosan preparations showed strong anticoagulant activities, with the same mechanism of action observed for standard therapeutic heparin. © 2002 Published by Elsevier Science Ltd.

Keywords: Chitosan; Sulfation; *Somanniathelphusa dugasti*; Rice-field crab shell; Anticoagulant activity

Chitin is a natural cationic polysaccharide found in fungus cell wall, crustacean shells, and insect cuticle.[1] It is biodegradable and biocompatible, and chemical modification of the amino and two hydroxyl groups can generate products of biological potential in relation to antithrombogenesis,[2] cell viability,[3] antitumor activity,[4] and blood compatibility.[5] Recently, applied research on chitin and its *N*-deacetylated derivative chitosan has been actively carried out in various industrial fields, including the production of medical materials.[6] Previous studies have focused mainly on chitin from marine crab and shrimp, but none on chitin and chitosan isolated from the shells of rice-field crabs.

Rice-field crabs (*Somanniathelphusa*) of four species, *S. germaini*, *S. sinensis*, *S. juliae* (Bott), and *S. dugasti* (Rathbun),[7] are widely distributed in Thailand. Over-infestation by *S. dugasti* in northern Thailand damages rice stalks, reducing rice production. Interest in the anticoagulant and antithrombotic action of chitin heparinoids prepared from bio-waste is motivated by an effort to reduce the risk of contamination by pathogens. This led us to modify chitosan, prepared from the shells of rice-field crabs, by sulfation under mild conditions without hazardous chemical reactions. The resulting sulfated chitosan possesses the high anticoagulant potency when compared to the standard therapeutic heparin.

The solubilization of chitin and chitosan in organic solvents is essential for effecting chemical modification.[8] We prepared chitosan from dried powdery crab shells by the method of Hackman,[9] and obtained 2-amino-2-deoxy-(1→4)-β-D-glucopyranan (**1**), in 10.3% yield with degree of acetylation of 0.21. This is in the range reported for similar preparations from marine crabs.[10] Factors influencing the sulfation process include the acetylation, sulfation agent, solvents, time, and reaction temperature.[11] We modified the method of Gamzazade[12] and used chlorosulfonic acid in *N*,*N*-dimethylformamide (DMF) as the sulfating complex in a two-phase system, using DMF as the solvent, at room temperature for 5 h, affording 2-deoxy-2-sulfoamido-3,6-di-*O*-sulfo-(1→4)-β-D-glucopyranan (**2**) in 87% yield (Scheme 1). The sulfation reaction typically involves swelling of the chitosan under semi-heterogeneous conditions for 1 h. Only a small amount of sulfation occurs at N-2 and O-3.[12] Our experiment was performed for 5 h, to achieve further substitution,[13] and we reduced the time for preparation of solvated chi-

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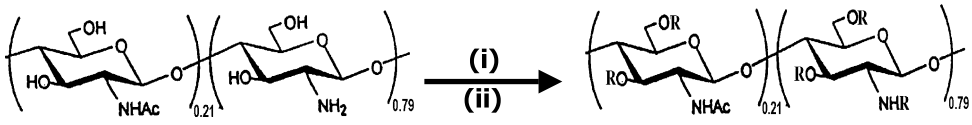
tosan from 12 to 1 h and performed the sulfation reaction in room temperature to avoid depolymerization of chitosan. This was desirable since our objective was to modify the original polymer and obtain an intact sulfated polysaccharide for subsequent size fractionation. The resulting product **2** was obtained in 87% yield as a white, fluffy, water-soluble solid. It was the sodium salt, as demonstrated by atomic absorption spectrometry. Characteristic absorptions in the IR spectrum at 800 and 1240 cm^{-1}, due to sulfo groups, were assigned to C–O–S and S=O bond stretching, respectively and the degree of substitution was 2.13. Moreover, ^1H NMR analysis showed the chitosan to be completely substituted. Separation of the ^1H signals for H-6S, H-3S, and H-2S was enhanced by recording the spectra at pD ~ 9, rather than at neutrality. The observed chemical shifts were δ 4.00, 4.60, and 3.41 ppm, respectively, with H-2 at δ 3.18 (Fig. 1). The data are comparable to those reported by Holme et al.[14] The major peak at δ 3.41 and a minor one at δ 3.18 indicated the substitution of H-2S and also confirmed the decrease of free amino groups in **2**, as compare to the starting chitosan. This decrease was confirmed by ninhydrin assay.[15] Separation into three fractions of different average molecular weights, using a Sepharose CL-6B column, provided P1–P3 with Mv values of 7.1, 3.5, and 1.9×10^4. These fractions were purified by Mono Q ion-exchange FPLC to exclude non-substituted polymers. The fractions were assayed by 1,9-Dimethylmethylene Blue (DMBA) binding[16] and only the sulfated polymers were collected, and dialyzed against distilled water for 48 h using a 3500 MW cut-off bag.

The anticoagulant activity of each fraction was compared to that of the standard therapeutic heparin and pentosan polysulfate (PPS, a synthetic sulfated polysaccharide having high anticoagulant activity). The Accuclot/Heptest of all three fractions showed the anticoagulant activity comparable to that of heparin and PPS. The Heparin/Accucolor, assay revealed the mechanism of action by showing that the product could inhibit FXa in the presence of antithrombin III. Moreover, in thrombin-time assays using normal human plasma, clotting times were prolonged in the presence of various concentrations of the product. The results (Table 1) indicated that the product interfered in the coagulation pathway mainly by enhancing the antithrombin III-mediated inhibition of FXa. This mechanism is consistent with the enhanced ratio of *N*-sulfo to *N*-acetyl groups in the product (7.5:2.1, by ninhydrin assay), which approaches the value of 7:3, demonstrated to be the optimal ratio for the regulation of the ability of heparin to form a complex with antithrombin III.[17] We excluded any interference of our product with the other coagulation factors in the extrinsic or intrinsic pathway or fibrin polymerization by performing assays of antithrombin activity, prothrombin time, and atroxin time (Table 2). To investigate whether the product directly inhibited FXa, we performed the Heparin/Accucolor in the absence of antithrombin III. The activity of FXa was found to be at normal levels, while heparin itself decreased the FXa activity (data not shown). These assays suggest that our product had two main effects on anticoagulant activity, inhibition of FXa activity through the interaction with antithrombin III, and direct inhibition of thrombin activity.

We concluded that the shells of rice-field crabs (*S. dugasti*) can be used to prepare sulfated chitosan by methods similar to those reported for chitin from other sources. The product inhibits the coagulation pathway



Scheme 1. (1) Chitosan (1 g); (2) sulfated chitosan (87%); (i) $HClSO_3$/DMF (4.5 mL/30 mL); (ii) rt, 5 h.

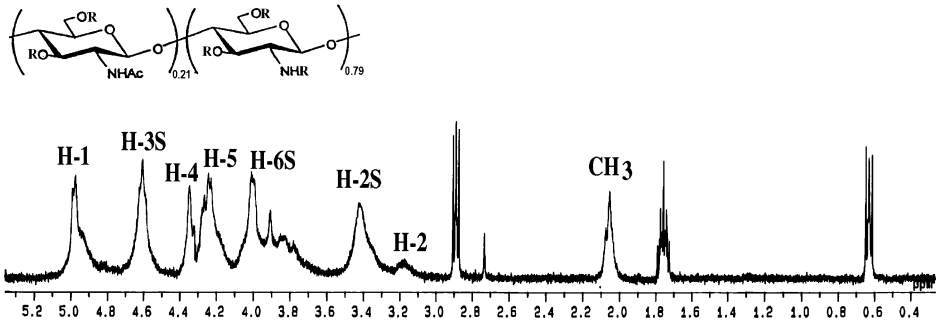


Fig. 1. 500-MHz ^1H NMR spectra of sulfated chitosan (2) $R = SO_3^-Na^+$.

Table 1
Assays of anti-FXa activity (Accuclot/Heptest, Heparin/Accucolor) and thrombin time of sulfated chitosan (equivalent to heparin: IU/mL)

Products	Accuclot/Heptest [a,c]	Heparin/Accucolor [b,d]	Thrombin time [a,c]
PPP	0	0	13.5
P1	0.46	0.05	21.6
P2	1.08	0.06	25.5
P3	0.62	0.05	23.2
Pentosan polysulfate	1.60	0.18	20.9
Heparin (0.05 IU/mL)			23.4

Each value represents the mean of duplicate of three independent determinations.
[a] Assay for the clotting time (s).
[b] Colorimetric assay.
[c] 1 μg/reaction.
[d] 2 μg/reaction; PPP, platelet-poor plasma.

Table 2
Effect of sulfated chitosan on antithrombin activity, prothrombin time and atroxin time

Products	Antithrombin activity (%) [a]	Prothrombin time (PT) (INR) [b]	Atroxin time (s) [c]
PPP	95	1.0	17
Control I (A4089)	ND	ND	18
P1	84	1.35	14
P2	80	1.35	15
P3	78	1.30	17
Pentosan polysulfate	83	1.16	ND
Heparin (0.05 IU/mL)	ND	0.91	ND
Heparin (0.1 IU/mL)	ND	0.96	ND
Heparin (0.5 IU/mL)	ND	ND	20

Each value represents the mean of duplicate of three independent determinations. Normal antithrombin activity = 79–125%; prolonged prothrombin time INR = 2.0–3.5.
[a] Product used in Antithrombin activity assay was 2 μg/reaction.
[b] Product used in PT assay was 1 μg/ reaction.
[c] Product used in Atroxin time was 10 μg/reaction; Control I (A4089), commercial normal control; PPP, platelet-poor plasma; ND, not done.

via a mechanism similar to that of standard therapeutic heparin and of pentosan polysulfate. However, heparin and PPS still have an adverse effect of anti-platelet activity, which causes abnormal bleeding in patients treated with those products.[18] Moreover, heparin is derived from mammalian sources and might be contaminated with animal proteins and pathogenic agents.

1. Experimental

Chitosan, 2-amino-2-deoxy-(1→4)-β-D-glucopyranan (**1**).—The method of Hackman[9] was slightly modified. Chitin was treated with 40% w/v aq NaOH containing $NaBH_4$ (0.1 g/500 mL) at 110 °C for 5 h under N_2. The resulting chitosan had $[\alpha]_D^{28}$ −19.0° (*c* 0.5, 2% v/v AcOH) with degree of acetylation of 0.21 as determined by potentiometric titration.

Sulfated chitosan, 2-deoxy-2-sulfoamido-3,6-di-O-sulfo-(1→4)-β-D-glucopyranan (**2**).—Based on the method of Gamzazade[12] with some modification, **1** was solvated by dissolving it in 1% AcOH and then precipitating it with 10% w/v aq NaOH. The precipitate was washed several times with water and then with MeOH and *N*,*N*-dimethylformamide (DMF). Solvated chitosan (1 g) was added dropwise to the sulfating complex (4.5 mL of $HClSO_3$ in cold DMF) and stirred for 5 h at rt. The polymer was neutralized with 20% aq NaOH and precipitated by cold MeOH. Finally it was resuspended and dialyzed against distilled water for 48 h at rt and then lyophilized to give **2** in 87% yield, $[\alpha]_D^{28}$ −9.5° (*c* 0.5, water); IR (KBr)ν_{max}:3500–3200 (OH, NH), 1650 (C=O), 1240 (S=O), and 800 (C–O–S) Anal. Calcd. for $[C_6H_8O_4N(C_2H_3O)_{0.21}(SO_3Na)_{2.13}(H)_{0.66}\cdot 3.11 H_2O]_n$: C, 16.59; H, 3.42; S, 15.70; Na, 11.29. Found: C, 17.45; H, 3.01; N, 3.23; S, 15.72; Na, 11.26. ^1H

NMR (DSS, D_2O): δ 4.98 (d, 1 H, H-1), 4.60 (br t, 1 H, H-3S), 4.35–4.25 (m, 2 H, H-4, H-5), 4.00 (br d, 2 H, H-6S), 3.41 (br t, 1 H, H-2S), 3.18 (br t, 1 H, H-2), 2.08 (s, 3 H, CH_3).

Separation and purification.—Sulfated chitosan (**2**) was applied to a column (1.6 × 100 cm) of Sepharose CL-6B equilibrated in phosphate-buffered saline, pH 7.2 (PBS) and the column was eluted at a flow rate of 20 mL/h. Fractions of 2 mL were collected and assayed by the 1,9-Dimethylmethylene Blue (DMBA) binding assay. All fractions showing positive DMBA were separated into three fractions (P1–P3) at K_d 0.16 and 0.58. Following dialysis against distilled water and lyophilization, each fraction was purified on a MonoQ column-FPLC (HR5/5) (Amersham Pharmacia Biotech) equilibrated in 20 mM Tris–HCl, pH 8.0. Fractions of 1 mL were eluted with a linear gradient of 0–2.0 M NaCl in the same buffer. Finally, all DMBA positive fractions were dialyzed against distilled water.

General methods.—^{1}H NMR spectra were recorded on a Jeol JNM-A500 instrument with DSS as the internal reference. IR spectra were recorded on a Jasco IR-810 instrument as KBr discs. Optical rotations recorded on a Perkin–Elmer 343 digital polarimeter at 28 °C. The average molecular weights of **1** and **2** were determined viscometrically using an Oswald type viscometer and applying the Mark–Houwink equation $[\eta] = 7.8 \times 10^{-3} Mv^{0.76}$ (0.3 M AcOH–0.2 M NaOAc, 25 °C)[19] and $[\eta] = 1.75 \times 10^{-5} Mv^{0.98}$ (0.1 M NaCl, 25 °C),[17] respectively. CHNS analyses were performed on a PE2400 Series 2 Perkin–Elmer instrument.

Anticoagulant assays.—These were performed with citrated human platelet-poor plasma (PPP) pooled from five healthy adults. PPP was obtained according to the guidelines for preparing citrated plasma for hemostaseological analyses. The PPP was supplemented with various final concentrations of **2**, fraction 1–3 (P1–P3), in comparison with pentosan polysulfate (PPS) and standard therapeutic heparin (Heparin sodium (5000 IU/mL), LEO Pharmaceutical Products, Ballerup, Denmark). The assays were performed according to the manufacturers' instructions. The clotting time was recorded in seconds. The specific anticoagulant activities of P1–P3 and PPS were evaluated by means of a standard curve of heparin. The following reagents were used: Accuclot™ Heptest (CRS114) for the determination of anti-FXa activity, Heparin Accucolor™ (CRS106) for the study of inhibition of FXa in the presence of antithrombin III, Antithrombin Accucolor™ (CRS117) for antithrombin activity assay, Thromboplastin-HS (T6540), for prothrombin time (PT), ACCUCLOT™ Thrombin Time (A8713) for Thrombin time (TT), and Atroxin® reagent (Cat. No 845-2) for the determination of anti-fibrin polymerization activity. All assays were performed in duplicate and repeated at least three times on different days ($n = 3$). In general, the standard deviations were less than 3% of the mean.

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