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Anticoagulant Activities of the Chitosan Polysulfate Synthesized from Marine Crab Shell by Semi-heterogeneous Conditions

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ABSTRACT Chitosan from marine crab shell with a degree of deacetylation of 0.89 was sulfated using chlorosulfonic acid/dimethylformamide under semi-heterogeneous conditions. The resulting chitosan polysulfate exhibits high solubility in common organic solvents and, more importantly, dissolves well in water. Characteristic absorptions in the IR spectrum at 810 and 1240 cm^{-1}, due to sulfo groups, were assigned to C-O-S and S=O stretch bonds stretching, respectively. The elemental analysis showed C, H, N and S contents of 15.03%, 4.08%, 2.84% and 14.46%, respectively, with degree of substitution (ds.) of 1.45. The chitosan polysulfate was separated by gel filtration chromatography using Sepharose CL-6B to provide three different products with average molecular weights of 6.6, 3.5 and 1.8 x 10^4 daltons, follow with purification by FPLC. All three chitosan polysulfate preparations revealed strong anticoagulant activities. Moreover, it was shown that they inhibited Factor Xa and thrombin activity. This mechanism is the same as heparin. All results indicated that chitosan polysulfate modified by using semi-heterogeneous conditions with potent anticoagulant activity has been synthesized.

KEYWORDS: chitosan polysulfate, sulfation, anticoagulant activity.

INTRODUCTION

Over the past 30 years, chitosan, the (β-1,4)-linked D-glucosamine derivative of the polysaccharide chitin, has been promoted as a promising renewable polymeric material. Chitosan has wide ranging applications in many areas, including the wastewater treatment, food, agriculture, cosmetic/personal care, biotechnological and pharmaceutical industries.[1] Chitin and chitosan are natural cationic polysaccharides found in fungus cell walls, crustacean shells, and insect cuticle. The three forms, α-, β-, and γ-forms, exist with different chitin microfibril orientations.[2] Chitin and chitosan are biodegradable and biocompatible, and chemically modification of their amino and two hydroxyl groups can give rise to novel molecular biofunctionalities including antithrombogenesis[3], cell viability[4], antitumor activity[5], and blood compatibility.[6] Applied research on chitin and chitosan is being actively carried out in various industrial fields, including the production of medical materials.[7]

The correlation of physical and chemical factors to the biological activities shown by heparin is of interest because of the usefulness of this polysaccharides as a therapeutic agent of choice. Characteristic parameters under consideration in this relation have included degree of sulfation, molecular size and shape, dissociation of ionizable groups, and spatial considerations of N-sulfate groups. These are all depend on sulfate reagents, solvents, temperature and time of reaction in the process of sulfation .[8]

A variety of sulfating reagents and reaction media have been used[9]: sulfuric acid causes extensive degradation, even when employed under controlled conditions, as do chlorosulfonic acid and sulfur trioxide when applied alone. When combined with Lewis bases, these two reagents cause less degradation, and they have been widely used. Sulfation reactions of multi-functional polysaccharides are inevitably followed by the appearance of structural heterogeneity in a polymer chains. When chitosan is sulfated, a structural variety of products is obtained, which may be related to the various reactivities of the three functional groups of the parent polymer, leading to different degrees of completion in the individual groups. On the one hand this gives rise to uncertainty, but on the other hand,

some structures that emerge from the random distribution of modified groups along the chain can reveal new features of biological functions.

The increasing interest in the anticoagulant and antithrombotic actions of chitin heparinoids, has led us to prepare chemically modified chitosan from marine crab shell. The fine chemical structure of these modified chitosans have been determined and they have been tested in coagulation assays. This research is motivated by the recent increased interest in therapeutics prepared from non-mammalian sources, in an effort to reduce the risk of contamination by pathogenic agents.

In the present study, we report that chitosan from marine crab shell could be chemically modified by the introduction of sulfo groups. The resulting chitin heparinoids have distinguishable patterns and proportions of sulfate substitution. Moreover, they have been characterized and represent a valuable tool to understand the relationship between structure and anticoagulant activity.

Materials and Methods

Materials

Chitosan from marine crab shell with a degree of deacetylation of 0.89 and average molecular weight (Mv) of 469 kDa was purchased from Sigma-Aldrich Chemical, St. Louis, MO, USA. All reagents and chemicals unless otherwise specified, were not purified, dried or pretreated. Dimethylformamide (DMF) was distilled under reduced pressure of calcium hydride (CaH_2) and stored over molecular sieves (3 A°). Heparin sodium (5000 IU/ml) was purchased from LEO Pharmaceutical Products Ballerup, Denmark. Sepharose CL-6B (MW cut-off 10-1000 kDa) and coagulation assay kits consists of Accuclot™ Heptest (CRS114), Heparin Accucolor™ (CRS106), Antithrombin Accucolor™ (CRS117), Thromboplastin-HS (T6540), Accuclot™ Thrombin Time (A8713) and Atroxin® (845-2) were purchased from Sigma-Aldrich Chemical. Dialysis tubing with a molecular weight cut-off of 3500 dalton (SpectraPore R1) was purchased from Fisher Chemical Co.

Method

Sulfation procedure for chitosan derived from marine crab shell

Sulfonation of chitosan was done based on the method of Gamzazade *et al* [10] with some modifications. Briefly, one gram of solvent including chitosan (chitosan in DMF) was added into the sulfating complex (4.5 ml. of $HClSO_3$/ 30 ml. DMF) and the reaction mixture was stirred for 5 hrs at room temperature. The chitosan, in a swollen state, was neutralized with 20% NaOH and precipitated with methanol. The resultant precipitate was dissolved and dialysed against distilled water for 48 hrs and then freeze-dried.

Gel filtration and determination of molecular weight

The crude chitosan polysulfate (200 mg. in 10 ml of phosphate bufferred saline (PBS), pH 7.2) was applied to a Sepharose CL-6B column (1.6 x 100 cm) equilibrated in PBS at pH 7.2. The column was eluted at a flow rate of 20 ml/hr. Fractions of 2 ml were collected and assayed by dye binding method using 1,9-dimethylmethylene blue.[11] The product was separated into 3 different molecular weight ranges at 0.16 and 0.52 KD cut-off values. The molecular weights of all three fractions were estimated from viscosity measurements using an Oswald type viscometer by applying the proposed equation for heparin ($[\eta] = 1.75 \times 10^{-5}\ Mv^{-0.98}$, 0.1 M NaCl at 25°C)[12] The starting material chitosan prepared from marine crab shell was estimated for its molecular weight by the proposed equation for chitosan ($[\eta] = 0.078\ Mv^{-0.76}$, 0.3 M acetic acid/ 0.2M sodium acetate at 25°C).[13]

Purification

Each fraction of sulfated chitosan prepared from marine crab shell (20 mg) was applied to a MonoQ column-FPLC (HR 5/5) (Amersham Pharmacia Biotech, Uppsala, Sweden) equilibrated in 20 mM Tris-HCl pH 8.0. The column was developed with a linear gradient of 0-2.0 M NaCl in the same buffer.[14] The flow rate of the column was 1 ml/min, and fractions of 1 ml were collected and assayed by the dye binding method.

Analytical methods

The degree of deacetylation was determined by Linear potentiometric titration. Optical rotations were determined with a Perkin-Elmer 343 digital polarimeter at 28 °C. IR spectra were recorded on a Jasco IR-810. X-Ray powder patterns of chitin, chitosan and chitosan polysulfate were measured at room humidity with a Rigaku Geigerflex X-ray diffractometer RAD-2R using Ni-filtered CuKα radiation generated at 35 kV and 30 mA. Degree of substitution was determined by CHNS elemental analysis.

Anticoagulant activity.

Anticoagulant activity measured by Prothrombin Time (PT)

The method used to measure PT was as indicated by the manufacturer, Thromboplastin-HS. One hundred microliters of normal plasma (NHP) or NHP containing various concentration of chitosan or chitosan polysulfate was prewarmed for 5 minutes before adding of 200 μL of thromboplastin-HS reagent. The clotting time was recorded simultaneously.

Thromboplastin time was reported in International Normalized ratio (INR) by the equation of $INR=R^{ISI}$ where R= Mean Test PT/Mean normal PT and ISI is the lot specific International Sensitivity Index for reagent/ instrument system.

Determination of anticoagulant activity of chitosan polysulfate

The method used to was as indicated by the manufacturer, Accuclot ™ Heptest. Briefly, 100 µL of Factor Xa was added to 100 µL of prewarmed normal human plasma (NHP, citrated plasma pooled from more than 5 healthy adults) containing various concentration of heparin standard or chitosan or chitosan polysulfate. The mixture was incubated for exactly 120 seconds before adding 100 µL of prewarmed RECALMIX®, at which time recording of the clotting time was begun. The anticoagulant activity was reported in equivalents to therapeutic heparin by converting clotting time to heparin IU/ml plasma using a standard heparin calibration curve.

Inhibition of Factor Xa by antithrombin III in the presence of chitosan polysulfate

The inhibition of Factor Xa by antithrombin III was measured by the Heparin Accucolor™ method as indicated by the manufacture. Briefly, 25 µL of NHP containing various concentrations of heparin standard or chitosan or chitosan polysulfate were added into 200 µL of prewarmed human antithrombin III. The mixture was incubated for exactly 120 seconds before adding 200 µL of Factor Xa and the incubation extended for another 60 seconds. Finally, 200 µL of Factor Xa substrate was added, the mixture was mixed and incubated for 5 minutes before stopping the reaction with 200 µL of glacial acetic acid. The absorbance at 405 nm was recorded and determined for the activity in heparin equivalents (IU/ml) by interpolation from the heparin calibration curve.

Determination of antithrombin activity of chitosan polysulfate

Antithrombin activity was measured by the Accucolor™ Antithrombin (AT) method, as described by the manufacturer. Briefly, 200 µL of AT standard dilution or NHP containing various concentrations of chitosan or chitosan polysulfate were prewarmed for 5 minutes before adding 200 µL of Heparin/thrombin reagent. The reaction was mixed and incubated for exactly 120 seconds before adding the thrombin substrate and the incubation was extended for another 120 seconds. Glacial acetic acid (200 µL) was added to stop the reaction and the absorbance at 405 nm was recorded. The AT activity was determined by comparing absorbance to the AT standard curve.

Determination of Thrombin time

Normal human plasma was prewarmed in the presence or absence of various concentrations of chitosan polysulfate in a final volume of 75 µL. The thrombin reagent was then added in an equal volume and clotting time recorded in seconds using FibrinTimer II (Behring).

Determination of Atroxin time.

Normal human plasma was prewarmed in the presence or absence of chitosan polysulfate in a final volume of 200 µL. One hundred of Atroxin® reagent was added and clotting time was recorded in seconds.

Results and Discussion

Synthesis of chitosan polysulfate

Chitosan derivatives are largely studied due to the multitude of their bio-technological and biomedical applications. The sulfation reaction has been reported on chitosan[15] and several synthetic types of chitin heparinoids have also been reported.[12] A number of methods have been suggested for the sulfation, however, all of these methods suffer from one or more disadvantages, among which excessive degradation and incomplete substitution are the most common.[16] There was a report that complexes of certain neutral, highly polar compounds, in conjunction with sulfur trioxide are useful sulfating agents.[16] These agents produce a high degree of substitution, and cause little degradation, as indicated by viscosity measurements. N, N-dimethylformamide (DMF) was found most suitable, since it is readily available and because its complex with sulfur trioxide, as well as DMF itself, are quite stable. Also, DMF is an excellent solvent for a great number of polymers, polysaccharides, and polysaccharide derivatives.[16] If both the starting material and the reaction product, or at least the latter, are soluble or readily swollen in the reaction medium, a more homogeneous and complete reaction may be possible. There also has been reported that the reaction between the SO_3 complexes and polysaccharides presumably starts with the formation of an H-bond with polysaccharidic OH groups, which increases the electrophilicity of the S and the nucleophilicity of the OH group.[17] In the sulfation of pullulan using $HClSO_3$ /pyridine compared to sulfur trioxide/pyridine, the result showed that $HClSO_3$ may react as a sulfation agent alone or it can form a complex with organic donor bases.[18] However, the sulfation is influenced both by the complex used and by the reaction temperature. Sulfating reagent and suitable solvent, together with conditions of time and temperature led us to modify the sulfation of chitosan from marine crab shell with a degree of deacetylation of 0.89 in order to

obtain the chitosan polysulfate with strong anticoagulant activity.

In our study, by semi-heterogeneous conditions using chlorosulfonic/DMF as a sulfating complex, chitosan polysulfate products were obtained. The degree of substitution (ds) calculated by elemental analysis was 1.45. As determined by the viscosity method, the resulting chitosan polysulfate could be separated into 3 different fractions with average molecular weights (P1-P3) of 6.6, 3.5 and 1.8 x 10^4 daltons, respectively. The synthesized sulfated products were further purified by strong anion-exchange column chromatography (Mono-Q FPLC). The percentage yields of the sulfated materials which were synthesized from each preparation and purified by Mono-Q FPLC column chromatography were 25%, 55% and 82%, respectively. In our work we performed the sulfation reaction at room temperature for 5 hrs, which was five fold longer than that recommended by Gamzazade *et al*[10], since there was a report that the reaction temperature has a significant influence on the ds in the sulfation[18], and reaction times longer than 5 hrs showed no significant different in ds.[19] Moreover, since the success of sulfation depends upon the surface condition of chitosan and it was found that a suitable surface activation apparently produced some purification and yielded a colloidal suspension readily susceptible to sulfation[17], in our application the reaction was stirred with occasionally stopping. We also modified the procedure by using 20% NaOH instead of 0.01 N NaOH in the precipitation step, since in this way a higher amount of chitosan polysulfate precipitate was obtained. Sulfation of chitosan was confirmed by infrared spectroscopy as was shown in Fig 1. Absorptions at 810 and 1240 cm^{-1} of chitosan polysulfate were assigned to the C-O-S and S=O stretch bonds, respectively. This indicated the polymer chains contained sulfate groups. Modification of chitosan by the above procedures yielded an amorphous product, isolated as a levorotatory sodium salt, which was a white, fluffy solid and was highly water soluble. This material shows excellent solubility for biological assays and in addition for preparation of various applications. The success of the modification was further supported by the X-Ray diffraction diagram of this material. Changes in the X-Ray diffraction diagrams of the product, in contrast with native crab chitin and chitosan, were evident (Fig 2). The original chitin powder showed three major crystalline peaks at $2\theta = 38.3^o$, 23.1^o and 19.3^o and two major peaks were observed at $2\theta = 19.9^o$ and 11.1^o in the case of chitosan with a degree of deacetylation of 0.89. On the other hand, the peaks in the X-Ray diffraction diagrams of chitosan polysulfate were apparently broader than those of chitin and chitosan, suggesting lower crystallinity or a less-

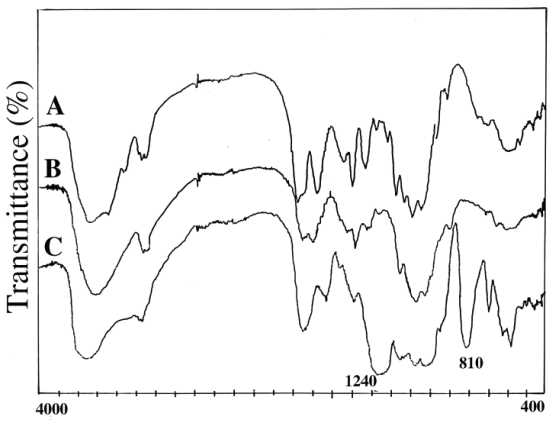

Fig 1. Infrared spectrua of marine crab shell chitin and its derivatives: (A) Chitin, (B) Chitosan, (C) Chitosan polysulfate

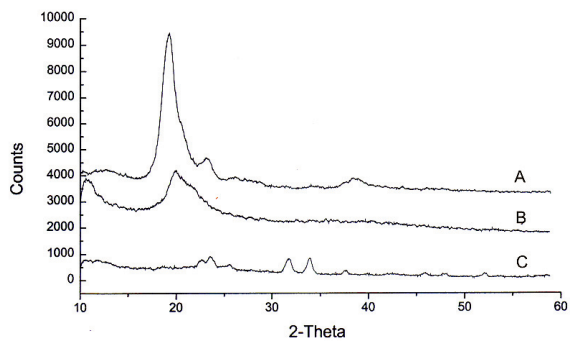

Fig 2. X-ray powder diffraction patterns of marine crab shell chitin and its derivatives:(A) Chitin, (B) Chitosan, (C) Chitosan polysulfate

ordered structure. These results indicated that destruction of the rigid crystalline structure of chitin and chitosan is of great importance for successful solubilization, and was introduced through the systemic sulfation strategy under semi-heterogeneous conditions, using chlorosulfonic acid in dimethylformamide as a key material.

Reaction with sulfating complex in dimethylformamide proceeded smoothly in semi-heterogeneous solution, and the crude product obtained was purified by dialysis and FPLC to afford P1, P2, and P3, respectively. Based on the elemental analytical data (C 15.03%, H 4.08%, N 2.84%, S 14.46%), the calculated degree of substitution (ds) is 1.45. The specific rotation of chitosan polysulfate at 28°C was $[\alpha]^{28}_D = -7.6$, which revealed that its nature was L-form. Taken together, we concluded that the chitin and chitosan prepared from marine crab shell could be sulfated in semiheterogeneous conditions using chlorosulfonic acid and dimethylformamide as a solvent.

Table 1. The anticoagulant activity of sulfated chitosan synthesized from marine crab shell.

Sample (1 μg)	ACCUCLOT/Heptest Equivalent to heparin (IU/ml)*	ACCUCLOT/ Heparin Equivalent to heparin (IU/ml)*
Chitosan	0	0
Pre-Sepharose CL-6B	0.05	0.55
P1	0.039	1.2
P2	0.046	0.57
P3	0.035	0.12
Pentosan polysulfate	0.18	1.8

* The clotting times were recorded as described under "Materials and methods." The activity is expressed in equivalents to heparin using a standard curve based on the International Heparin Standard (100 IU/mg). Each value represents the mean of duplicate of three independent determinations. P1: fraction with Mv of 6.6 x 10^4 dalton, P2: fraction with Mv of 3.5 x 10^4dalton, P3: fraction with Mv of 1.8 x 10^4 dalton, IU: International Unit

Table 2. Effect of sulfated chitosan of marine crab shell on thrombin time.

Products (1 μg)	Thrombin time (TT)*(second)
Normal human plasma	13.5
Control I (A4089)	10.4
Chitosan	14.1
Pre-Sepharose CL-6B	29.7
P1	27.8
P2	22.2
P3	22.4
Pentosan polysulfate	20.9
Heparin (0.1 IU/ml)	23.4
Heparin (0.2 IU/ml)	48.5
Heparin (0.4 IU/ml)	145.0

* The clotting times were recorded as described under "Materials and methods." The activity is expressed in equivalents to heparin using a standard curve based on the International Heparin Standard (100 IU/mg). Each value represents the mean of duplicate of three independent determinations. P1: fraction with Mv of 6.6 x 10^4 dalton, P2: fraction with Mv of 3.5 x 10^4dalton, P3: fraction with Mv of 1.8 x 10^4 dalton, IU: International Unit, Control I (A4089): Commercial normal control plasma (Sigma-Aldrich Chemical)

Studies of the anticoagulant activities of chitosan polysulfate

The anticoagulant activity of the resulting chitosan polysulfate was evaluated on human blood clotting time. The ACCUCLOT/Heptest assay showed that the pre-Sepharose CL-6B preparation and all 3 post-MonoQ preparations (P1, P2 and P3) with different mean molecular weights have strong anticoagulant activity, however, less than pentosan polysulfate and standard therapeutic heparin (Table 1). The ACCUCLOT/Heparin assay revealed that chitosan polysulfate inhibits Factor Xa by promoting antithrombin III activity. Moreover, the chitosan polysulfate also showed the prolonged thrombin time (TT) similar to heparin (Table 2). The results showed that our products might be heparin-mimetic compounds. In order to investigate whether, they have any inhibition effects on extrinsic factors or fibrin polymerization, antithrombin activity, prothrombin time (PT) and atroxin time were also performed, as shown in Table 3. The normal values for antithrombin activity, PT and atroxin time revealed that our heparin-mimetic chitosan polysulfate inhibits only FXa and thrombin activity. It has no effect on extrinsic factors and does not interfere with fibrin polymerization.

As chitin has a similar skeletal structure to heparin, the biomedical properties of chitin have received considerable attention. Chitosan with different degrees of deacetylation has been used to prepared heparin-like derivatives, because it can be chemically modified under homogeneous conditions in few reaction steps.[15] Several synthetic types of chitin heparinoids using different sources of chitin and chitosan have also been reported.[12] However, the influence of molecular weight and/or substitution degree of sulfated chitosan, together with different methods used, including sulfate generating materials, solvent, temperature and time of reaction, on their biological activity is considerable in the majority of works involving the anticoagulant or antiviral properties of these substances.[20] We have reported the anticoagulant activities of chitosan polysulfate obtained from the synthesis in mild and semi-heterogeneous conditions.

Conclusion

It was found that marine chitosan from crab shell with a degree of deacetylation of 0.89 could be modified by sulfation in mild conditions at room temperature using chlorosulfonic acid as a sulfate donor reagent and dimethylformamide as a solvent. This product with ds of 1.45 could be separated into 3 different size fractions with average molecular weights of 6.6, 3.5 and 1.8 x 10^4 daltons, respectively. These chitosan polysulfate fractions were well dissolved in water. It

Table 3. Effect of sulfated chitosan on antithrombin activity, prothrombin time, and atroxin time.

Products (μg)	Antithrombin activity[a](%)	Prothrombin time (PT) [b](INR)	Atroxin time[c](second)
Normal human plasma	95	1.0	17
Control I (A4089)	ND	ND	18
Chitosan	93	1.0	18
Pre-Sepharose CL-6B	90	1.39	17
P1	86	1.25	ND
P2	84	1.35	17
P3	84	1.39	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

The clotting times were recorded as described under "Materials and methods." The activity is expressed in equivalents to heparin using a standard curve based on the International Heparin Standard (100 IU/mg). Each value represents the mean of duplicate of three independent determinations. P1: fraction with Mv of 6.6 x 10^4 dalton, P2: fraction with Mv of 3.5 x 10^4dalton, P3: fraction with Mv of 1.8 x 10^4 dalton, IU: International Unit Control I (A4089): Commercial normal control plasma (Sigma-Aldrich Chemical), Normal antithrombin activity = 79-125%, Prolong prothrombin time INR = 2.0-3.5, [a]Product used in Antithrombin activity assay was 2 μg/reaction of 150 μL, [b]Product used in PT assay was 2 μg/reaction of 300 μL, [c]Product used in Atroxin time was 10 μg/reaction of 300 μL.

was also found that the mechanism of their anticoagulant activities is the same as heparin, which indirectly inhibits Factor Xa activity through antithrombin III activity and directly inhibits thrombin activity.

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