

Original Research Article

Characterization of Sulfate Groups and Assessment of Anti-Coagulant Activity of Glucomannan Sulfate Prepared from Konjac Glucomannan

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Abstract

Purpose: To determine the structure of Konjac glucomannan sulfate (KOGMS) including homogeneity, sulfate group composition as well as its anti-coagulant activity *in vitro*.

Methods: KOGMS was prepared using chlorosulfonic acid and N, N-dicyclohexyl carbodiimide (DCC) from konjac oligo-glucomannan (KOGM). Homogeneity analysis was confirmed by cellulose acetate membrane electrophoresis. Fourier transform infrared FT-IR spectra, Laser Raman spectra and ¹³C Nuclear Magnetic Resonance NMR spectra were obtained and used to analyze the sulfate groups. The anti-coagulant activity of KOGM and KOGMS was evaluated *in vitro* using activated partial thromboplastin time (APTT), prothrombin time (PT) and thrombin time (TT) as indicators.

Results: KOGMS was shown to be a homogenous carbohydrate with a relative molecular mass ranging from 5.8×10^3 to 6.2×10^3 Da and various degrees of substitution (DS) ranging from 1.28 to 1.81. The sulfate groups were inserted at C-2, C-6 and C-3 positions of KOGM. The APTT of human platelet-poor plasma containing KOGMS was 47.2 s which was close to that of heparin at a concentration of 0.5 mg/mL. Furthermore, the APTT of human platelet-poor plasma containing KOGM was only 15.6 s which is close to that of the negative control group. For KOGMS, the values obtained in all three tests were significantly higher than those of KOGM and the negative control group ($p < 0.05$).

Conclusion: Hydroxyl groups can be substituted efficiently by sulfated groups at C-2, C-6 and C-3 positions of KOGM with little degree of degradation. *In vitro* anti-coagulant activity data indicates that KOGMS has a significantly stronger anti-coagulant activity than KOGM due probably to the sulfated groups in the main chain of its molecule.

Keywords: Konjac, Oligo-glucomannan sulfate, Laser Raman spectra, Anticoagulant activity

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INTRODUCTION

It is well known that different kinds of sulfated polysaccharides exhibit biological activity as anti-coagulant, anti-thrombotic, anti-viral, anti-proliferative and anti-sclerotic compounds. [1]. One of the naturally-occurring sulfated polysaccharides is heparin, a widely used anti-coagulant which may increase the functional

activity of platelets [2]. Heparin has some side effects including osteoporosis, thrombocytopenia and other hemorrhagic complications [3]. Furthermore, since it is a preparation obtained from animal tissues, heparin may contain spore forms of bovine encephalitis [4]. A key research priority in modern medicine is to identify and design novel anti-coagulants with reduced clinical side effects.

Konjac is a plant of the genus *Amorphophallus* which has been used to isolate immune-modulating compounds and healthcare food products for a long time. The genus is native to warm sub-tropical and tropical Eastern Asia, including Japan, Southern China and Indonesia [5]. Mature konjac tubers are washed, sliced, dried and milled by Raymond mill to produce a mixed powder which is then separated to obtain konjac flour. Konjac flour contains large amounts of konjac glucomannan (KGM) at levels ranging from 51.3 to 96.9 % [6]. As the main component of konjac flour, KGM is a type of multi-functional natural polymer consisting of β -D-mannose and β -D-glucose residues (molar ratio 1.6:1) linked by β -1, 4-glycosidic bonds which has weak biological activities such as immunity enhancement, anti-oxidation and anti-tumor properties [7].

Konjac oligo-glucomannan sulfate (KOGMS) was prepared from konjac oligo-glucomannan (KOGM) by enzymolysis of KGM [8]. To introduce sulfate groups into KOGM, chlorosulfonic acid (CSA) was adopted with N, N-Dicyclohexyl carbodiimide (DCC) as a dehydration-condensation agent in the reaction system to increase the reaction rate [9]. The aim of this work was to investigate the structure and anti-coagulant activity of KOGMS.

EXPERIMENTAL

Materials

KGM was purchased from Hubei Konson Konjac Co., Ltd. (China). KOGM was obtained from Chemical Engineering and Technology College, Wuhan University of Science and Technology, Hubei Province, China. DEAE Sepharose Fast Flow and Sephadex G-10 were purchased from Shanghai Pharmacia Co., Ltd (China). All other reagents were purchased from Sigma Co., Ltd (France).

Sulfated modification of KOGM

CSA was dripped in anhydrous pyridine (PD), under agitating and cooling in ice water bath [10]. The process lasted for 20 min to obtain sulfated reagent. KOGM (200 mg) was suspended in anhydrous DMF (20 mL) at room temperature with stirring for 30 min, and sulfated reagent was added dropwise. Thereafter, 50 mg DCC was added to ensure appropriate modification of polysaccharide. The mixture was stirred for 2 h at 50 °C. Subsequently, the mixture was cooled to room temperature and pH adjusted to 7 - 8 using 2.5 mol/L NaOH solution. The mixture was precipitated with ethanol, dissolved in water, and

dialyzed (molecular weight cutoff, 5 – 10 kDa) against distilled water for 48 h to remove pyridine, DCC, salt and potential degradation products. Four sulfated KOGMS batches (KOGMS-1 to KOGMS-4) with different DS were collected after lyophilizing.

Purification of KOGMS

The crude polysaccharide sulfate was dissolved in distilled water (50 mg/mL) and applied to a DEAE-Sepharose Fast Flow column [11]. The column was eluted with distilled water using a gradient of NaCl solution at a flow rate of 3 ml/min and fractions of 2 ml/tube. The polysaccharide sulfate in the eluted fractions was detected using the phenol sulfuric acid method [12]. The second positive peak eluted at 0.8 mol/L NaCl was pooled, dialyzed and lyophilized and the Sephadex G-50 was used for further purification. The fractions eluted at Kav of 0.45 were pooled and designated as KOGMS.

Homogeneity analysis

Samples (10 mg mL⁻¹, 8 μ L) were applied to cellulose acetate strips (Sepraphor III) [13] and electrophoresis conducted in a Gelman semi-micro bath containing ZnSO₄ buffer (0.2 M, pH 5.1) for 60 min at 6 mA and 100 V. Strips were stained with 1 % Alcian Blue and destained with 5 % aqueous acetic acid containing 10 % ethanol. Hyaluronic acid and chondroitin sulfate were used as standard charged polysaccharides.

Sulfate content and molecular mass

Sulfur content (S, %) of the sulfated derivatives were determined using an elemental analyzer (Vario EL, Elemental Co., Germany). DS was calculated which referred to the average number of sulfate group on each monosaccharide residue:

$$\text{DS} = \{(162 \times S)/(32 - 102)\}S \dots \quad (1)$$

The molecular mass of KOGMS was determined by gel permeation chromatography on a TSK 3000 sw column eluted with 0.7 % HAc-NaAc buffer solution at a flow rate of 1.0 mL/min at 35 °C. Elution was monitored by a refractive index detector.

Sulfate group analysis

FT-IR spectra were recorded on a Nexus FT-IR spectrophotometer (Thermo Nicolet Corporation, USA) from 4,000 to 400 cm⁻¹. Laser Raman spectra were recorded on a Laser Raman spectrophotometer (Thermo Nicolet Corporation,

USA) from 3,500 to 500 cm⁻¹. ¹³C NMR spectra of 40 mg/mL solution in D₂O was recorded at 40 °C with a Bruker 600 MHz spectrometer (Germany) and chemical shifts expressed in ppm relative to the resonance of internal standard DSS.

Human blood plasma presentation

Human blood plasma was collected from healthy individual donors into conical tubes with 2.5 % sodium citrate solution. The plasma was separated from blood cells by centrifuging at 5400 rpm at 4 °C for 20 min. The blood plasma was stored at -70 °C until used.

Anti-coagulant activity assay *in vitro*

Anti-coagulant activity was determined by activated partial thromboplastin time (APTT), prothrombin time (PT) and thrombin time (TT) coagulation assay.

APTT was used to evaluate coagulation factors including VIII, IX, XI, XII and prekallikrein in the intrinsic blood coagulation pathway. PT was used to characterize the extrinsic coagulation factors and TT was an indicator of blood coagulation status as this transforms fibrinogen into fibrin [14]. Briefly, citrated normal human plasma (90 µL) was mixed with 10 µL of KOGMS at different concentrations and incubated for 1 min at 37 °C. APTT assay reagent (100 µL) was then added to the mixture and incubated for 5 min at 37 °C, followed by the addition of 0.05 mM CaCl₂ (100 µL) and the clotting time(s) recorded using a coagulometer.

For the PT assay, citrated normal human plasma (90 µL) was mixed with 10 µL of a KOGMS solution and incubated for 10 min at 37 °C. Pre-incubated PT assay reagent (200 µL) was then added and clotting time recorded. For the TT assay, citrated normal human plasma (190 µL) was mixed with 10 µL of KOGMS and incubated for 2 min at 37 °C. TT assay reagent (100 µL) was added and the clotting time recorded. Distilled water and heparin were used as experimental controls and standards respectively. The APPT assay and other determinations were performed with at least two replicates of samples and mean values used for calculations.

Statistical analysis

Statistical significance was determined using Student's t test; differences were considered as statistically significant at *p* < 0.05.

RESULTS

Homogeneity of KOGMS

Cellulose acetate electrophoresis of KOGMS (Fig. 1) showed one mobile band which appeared to indicate that KOGMS consisting of glucose and mannose was homogeneous.

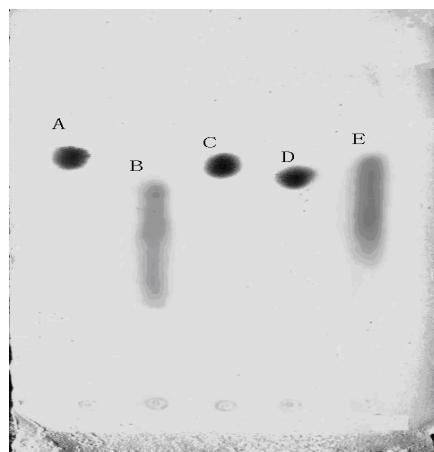


Figure 1: Cellulose acetate electrophoresis of KOGMS. (**Key:** A: Glucose, B: degradation of purified KOGMS, C: Mannose, D: sucrose, E: degradation of KOGM)

Component analysis of KOGMS

The molecular masses of KOGMS with different DS were investigated and the data presented in Table 1 showing the molecular masses and DS values of KOGMS increased from 5.8 to 6.2 kDa, 1.28 to 1.81 respectively. These data imply the hydroxyl groups were substituted efficiently by sulfated groups in the main chain of polysaccharide.

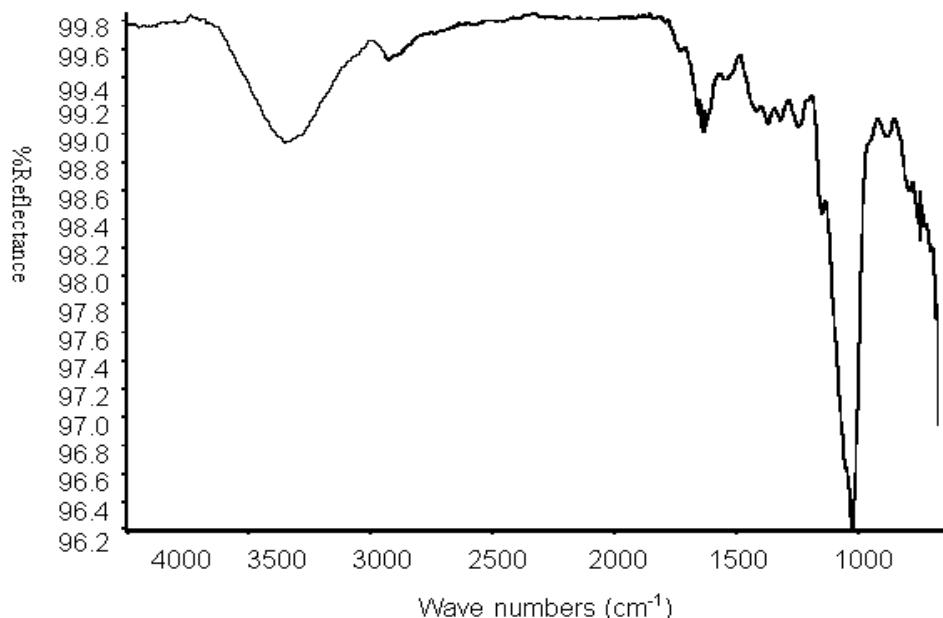
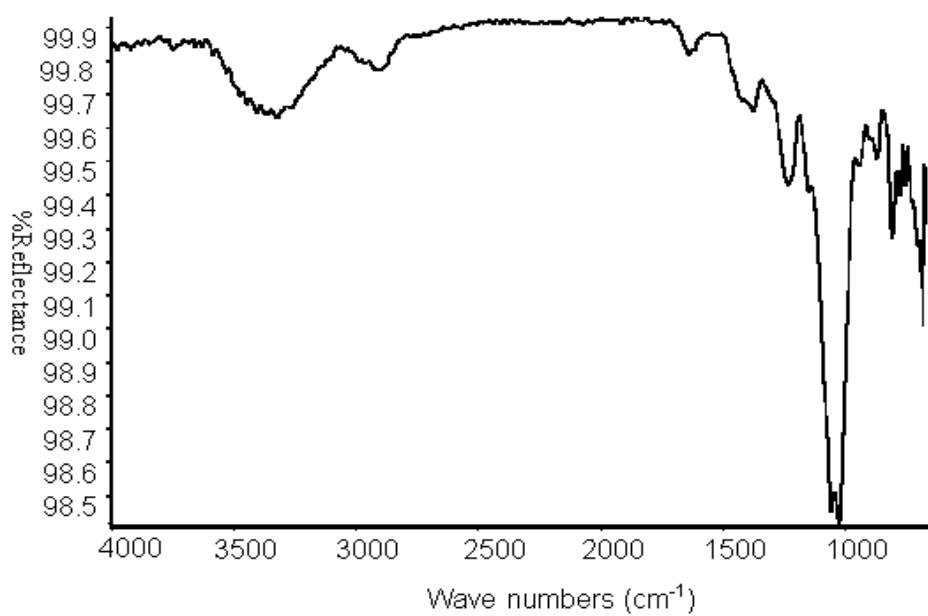
Sulfate group analysis of KOGMS

FT-IR spectra of native KOGM and KOGMS are shown in Figures 2 and 3. From Figure 3, the absorption peak at 3,200 - 3,700 cm⁻¹ which describes an asymmetrical OH stretching vibration is seen to be weaker than that shown in Figure 2, indicating the OH groups decreased in KOGMS because of its sulfation. Compared with KOGM, two characteristic absorption peaks appeared in the FT-IR spectra of KOGMS. One at 1,224 cm⁻¹ describing an asymmetrical S=O stretching vibration and the other at 808 cm⁻¹ representing a symmetrical C—O—S vibration, indicating that KOGMS may successfully sulfate.

Table 1: Component analysis of KOGMS

Sample	CSA:PD*	Time (h)	M (kDa)	Elemental analysis (%)			DS
				C	H	S	
KOGMS-1	1:5	2	5.80	21.04	3.95	14.54	1.28
KOGMS-2	1:4	2	5.85	19.97	3.87	15.96	1.48
KOGMS-3	1:3	2	6.05	19.06	3.69	17.08	1.67
KOGMS-4	1:2	2	6.20	18.65	3.74	19.33	1.81

*Volume ratio of chlorosulfonic acid to pyridine in sulfated reagent

**Figure 2:** FT-IR spectrum of KOGM**Figure 3:** FT-IR spectrum of KOGMS

The Laser Raman spectra of the native KOGM and KOGMS are shown in Figures 4 and 5. From Fig. 5, CH groups decreased in KOGMS due to its sulfation. the absorption peak had been enhanced at $1,059\text{ cm}^{-1}$ describing an

asymmetrical C-O stretching vibration of cyclic ether C-O-C in the spectra of KOGMS indicating that cyclic ether increased in KOGMS due to its sulfate reaction. There were a large number of absorption peaks at 580 cm^{-1} , 447 cm^{-1} , and 410 cm^{-1} .

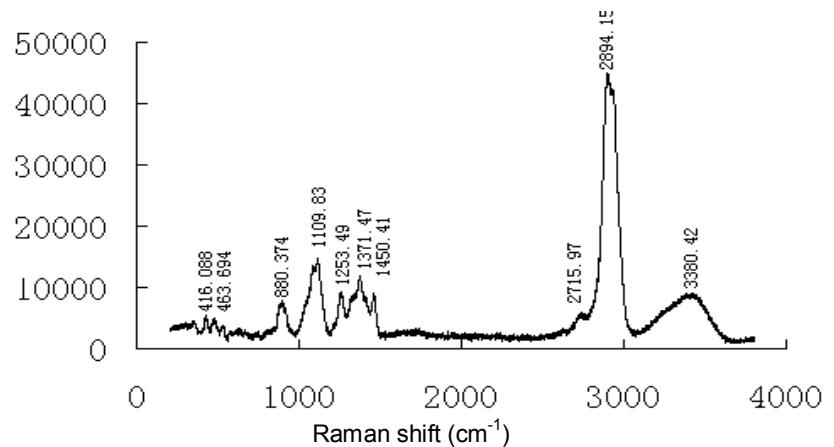


Fig 4: Laser Raman spectrum of KOGM

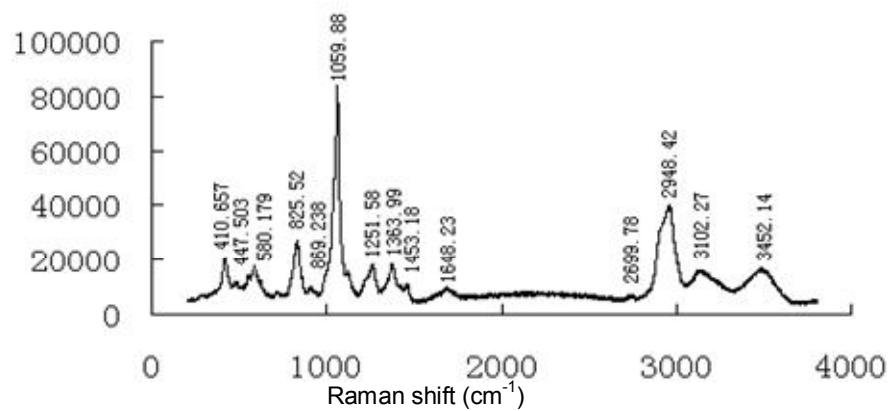


Fig 5: Laser Raman spectrum of KOGMS

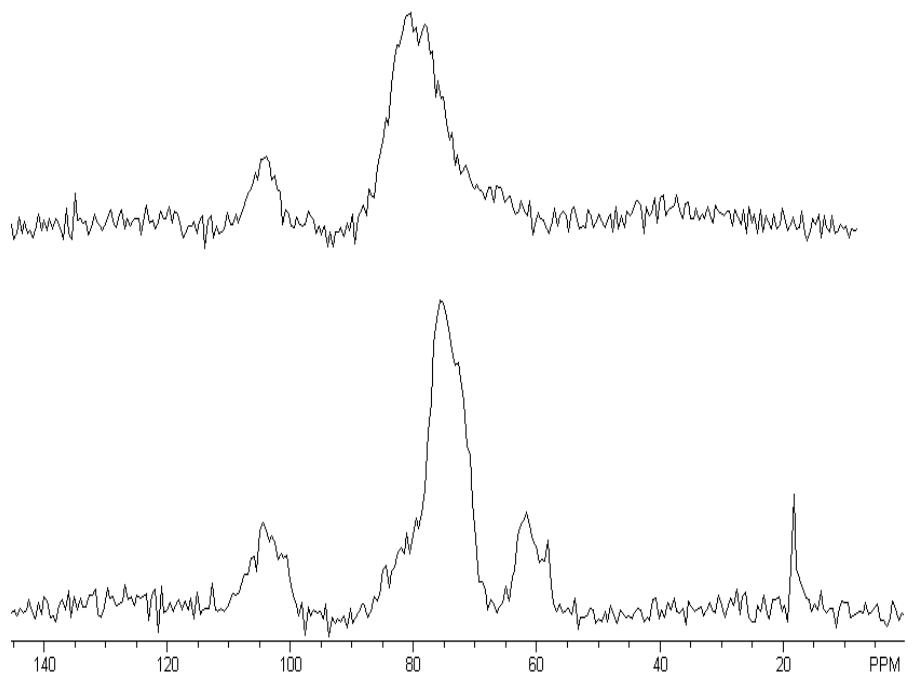


Figure 6: ¹³C NMR spectrum of KOGM (a), KOGMS (b) (600 MHz)

cm^{-1} describing a CH deformation vibration which could not give the definite sulfated position. At 825 cm^{-1} , this represents a CH deformation vibration of mono-substitution indicating that KOGMS was successfully sulfated in $-\text{OSO}_3$ combined with FT-IR analysis.

The sulfate position on the polysaccharide was determined by ^{13}C NMR spectrum. The ^{13}C NMR spectra for the native KOGM and KOGMS are shown in Figure 6. The ^{13}C NMR spectrum of the native KOGM exhibited six signals around 96.4, 78.2, 74.9, 73.1, 70.9, and 60.8 ppm, attributed to C-1, C-3, C-5, C-2, C-4, and C-6, respectively. In comparison, there are several new signals caused by sulfate groups in KOGMS. The peak at 66.8 was assigned to the signal of C-6s, the peak at 72.7 was assigned to the signal of C-2s. The peak at 61.6 was weakened which indicated that C-6 had been substituted by the sulfate group.

As their strong peaks existed in the NMR spectra, the peak at 72.7 for C-2s was much weaker than that of C-6s at 66.8. We can therefore conclude that the C-6 position was more active than the C-2 position due to the steric hindrance. Furthermore, a new peak appeared at 93.9 and the native C-1 peak at 96.4 became weaker in NMR spectra of KOGMS. New peaks at 65–75 ppm resulted in sulfating of

other positions in addition to the C-6 and C-2 positions.

In vitro anti-coagulant activities

As shown in Table 2, APTT could reach 58.6 s at concentration of KOGMS about 2 mg/ml which was close to that of heparin and three times higher than that of KOGM (16.9 s). Also as shown in Tables 3 and 4, KOGMS increased prothrombin time and thrombin time. Interestingly, preparations exhibiting higher activity in the APTT test were also more active in the two later tests. However, detection of their inhibitory activity required higher concentrations.

DISCUSSION

We have successfully introduced the sulfate process as a technique for the conversion of KOGM into a potential therapeutic product that could be further characterized and developed as an anticoagulant from KGM. Characterization of the anti-coagulant activities in vitro indicated that sulfated derivatives showed much stronger ability than native polysaccharide. These phenomena indicated much stronger anti-coagulant ability of KOGMS than that of KOGM which might be attributed to a high property of sulfate group in KOGMS.

After sulfated modification, the molecular mass of polysaccharide had not been decreased

Table 2: APTT of human platelet-poor plasma containing KOGM, KOGMS and heparin

Sample	APTT(s) at different concentrations (mg/mL)		
	0.5	1.0	2.0
Negative control	14.3 \pm 0.6	—	—
KOGM	15.6 \pm 0.3	15.8 \pm 0.4	16.9 \pm 0.8
KOGMS	47.2 \pm 0.8	55.3 \pm 1.6	58.6 \pm 1.8
Heparin	60.6 \pm 1.3	68.4 \pm 1.5	—

Table 3: PT of human platelet-poor plasma containing KOGM, KOGMS and heparin

Sample	PT(s) at different concentrations (mg/mL)		
	0.5	1.0	2.0
Negative control	11.8 \pm 1.6	—	—
KOGM	12.3 \pm 0.5	13.8 \pm 0.6	15.2 \pm 1.4
KOGMS	15.8 \pm 1.5	17.9 \pm 1.6	19.3 \pm 1.2
Heparin	19.8 \pm 1.4	21.2 \pm 1.8	—

Table 4: TT of human platelet-poor plasma containing KOGM, KOGMS and heparin

Sample	TT(s) at different concentrations (mg/mL)		
	0.5	1.0	2.0
Negative control	6.5 \pm 1.6	—	—
KOGM	7.6 \pm 0.9	8.4 \pm 0.8	8.8 \pm 0.6
KOGMS	25.8 \pm 1.3	28.3 \pm 1.6	32.2 \pm 1.7
Heparin	44.3 \pm 1.5	49.3 \pm 1.8	—

Note: All the data are mean \pm SEM, n = 3; — indicates that values were not measured

obviously. It might be the reason of adding DCC in synthesis reaction, and making the reaction achieve dynamic equilibrium fast, which decreased the depolymerization of polysaccharide. DCC worked as a dehydration agent to afford dicyclohexylurea in the reaction.

The sulfated position on the polysaccharide was usually determined by ^{13}C NMR spectrum. It is known that the signal of C-1 splits if the hydroxyl group on C-2 was functionalized. It could be explained by the fact that C-2 had been substituted which could influence the adjacent C-1 to split into two peaks. As a consequence of a heterogeneous reaction, sulfated groups were distributed unevenly.

To investigate the anticoagulant properties of the sulfated polysaccharides, APTT was measured using normal human plasma. The results show that APTT is powerfully prolonged by KOGMS at 0.5mgmL^{-1} . Usually, prolongation of APTT indicates inhibition of the intrinsic or common pathway [15]. In the present study, KOGMS inhibited the intrinsic or common pathways of coagulation.

Although lower than heparin, KOGMS exhibited much stronger anti-coagulant activity than KOGM, with increasing concentration in tests which differed significantly from the negative control and KOGM groups. APTT, PT, TT data indicate that KOGMS may possess anti-coagulant activity correlating with the extrinsic coagulation process. However, KOGM had little anti-coagulant activity as per APTT, PT and TT data. The results suggest that the difference in the potency of thrombin inhibition may be due to the sugar residue, sulfation content and their structural features.

The relationship between structure and anticoagulant activity has been investigated in detail for galactan and fucans [16]. These observations show eight different sulfated polysaccharides isolated from *Chlorophyta* exhibit thrombin inhibition through a heparin cofactor II –dependent pathway, and their effects on the inhibition of thrombin are more potent than those of heparin or dermatan sulfate. Melo *et al* [17] also report the paradigm of heparin–antithrombin interaction cannot be extended to other sulfated polysaccharides, and each type of polysaccharide may form a particular complex with the plasma inhibitor and the target protease. The investigations suggest that the structural requirements for the interaction of sulfated galactans with coagulation inhibitors and their target proteases are not merely a consequence of their charge density, and the structural basis of

this interaction is complex, and depends on the distribution of sulfate groups and on monosaccharides composition [18]. Further structure characteristic of the sulfated polysaccharides isolated from *U. conglobata* will aid understanding of the mechanism of heparin cofactor II activation by the sulfated polysaccharides, and may ultimately lead to the development of novel antithrombotic agents.

In previous studies, a polysaccharide of fucosylated chondroitin sulfate (FucCS) isolated from sea cucumber was reported to be a potent inhibitor of P- and L-selectin which bound to immobilized cells [19]. Functional inhibition was also exhibited in a concentration dependent manner. Interestingly, removal of the sulfated fucose branches on the FucCS abolished the inhibitory effects both *in vitro* and *in vivo*. Our results were consistence with these findings which further confirmed sulfate group to play an important role in anti-coagulant biological activity [20].

CONCLUSION

The findings of this study indicate that hydroxyl groups are substituted efficiently by sulfate groups on the polysaccharide with a little degradation. KOGMS exhibits much stronger anti-coagulant activity than KOGM and rises with increase in APTT concentration, suggesting that KOGMS may express anti-coagulant activity that correlates with extrinsic coagulation process. The mild anti-coagulant activity of these compounds suggests they may be useful for therapeutic purposes, particularly as potential anti-thrombotic drugs.

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