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Preparation, Characterization and Anticoagulant Activity of Guar Gum Sulphate

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Guar gum was chemically modified by sulphonation using chlorosulphonic acid ($ClSO_3H$) as a reagent. Effects of molar ratio of $ClSO_3H$ to glucopyranosic unit ($ClSO_3H/GU$), reaction time and reaction temperature on the degree of sulphonation (DS) and molecular weight (Mw) of products were studied. The structures of guar gum sulphate were investigated by GPC, FT-IR and UV-Visible spectroscopy. Activated partial thromboplastin time (APTT) assay showed that the guar gum sulphate could inhibit the intrinsic coagulant pathway. The anticoagulant activity strongly depended on the DS and Mw of polysaccharides. DS $>$ 0.56 was essential for anticoagulant activity. The guar gum sulphate with the DS of 0.85 and the Mw of 3.40×10^4 had the best blood anticoagulant activity.

Keywords guar gum, sulphonation, anticoagulant activity

Introduction

Guar gum (GG) from the Indian cluster bean (*Cyamopsis tetragonolobus)* is a galactomannan polysaccharide having a 1,4-β-linked-D-mannopyranosyl main chain with a-D-galactopyranosyl units attached by (1 $\rightarrow$ 6) linkages (1, 2). Due to its unique high viscosity property, guar gum is an important food stabilizer used in a variety of food applications. It was indeed surprising during recent years to learn about the possible different uses of guar gum in the biomedical field, as a binder, cholesterol-lowering agent, and blood-glucose-lowering agent (3, 4). These findings indicate that guar gum will be a good basic component for new biomedical material development.

Heparin, a mucopolysaccharide with 5000–50,000 molecular weight, has been the drug of choice in clinical pre-surgical and post-surgical prophylaxis of thrombotic events (5, 6). However, because of its side effects, such as bleeding and other disadvantages (i.e., chemical inhomogeneity and the risk of contamination by animal proteins and pathogenic agents), many investigators have attempted to develop a new synthetic anticoagulant material having heparin—like structure that mimics heparin activity.

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Preeyanat, Warayuth, Dumrat and Prachya (7) reported strong anticoagulant activity of a sulfated chitosan in blood. Nishimura, Nishi and Tokura (8) and Huang, Du and Yang (9) suggested that inhibition of thrombin activity was increased upon the introduction of carboxyl groups in the sulfated chitin. The studies of Ferial, Mostafa, Corinne and Catherine (10) showed that inhibitory effects of fucans on both coagulation and cell proliferation are dependent on their sulphonation degree and the molecular size of fucan derivatives. Yasushi (11) reported that modification of silk fibroin by incorporation of sulfate groups could inhibit blood coagulation when the protein backbone derivatives contacted with blood. Lu et al. (12) and Yang, Du, Huang, Wan and Li (13) indicated that sulfated Chinese Lacquer polysaccharide could inhibit blood coagulation under *in vitro* and *in vivo* conditions; its degradation mechanism was explained by involving both dehydrolysis and hydrolytic degradation (14). These reports indicate definitively that sulfate incorporation into polymers affects polymer anticoagulant activity.

Although a variety of polymer backbones have been used for synthesis of sulphated polymers as an anticoagulant material, few reports are available concerning a sulphated guar gum. In the present study, we prepared various guar gum fractions having different sulfate content. Their anticoagulant activities were measured by APTT. The structures of guar gum derivatives were conferred by gel permeation chromatography (GPC), Fourier transform infrared (FTIR), and UV-Visible spectrometry.

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Experimental

Materials

Guar gum was provided from Danisco Cooperation (Denmark). Heparin sodium salt (150 IU/mg) was obtained from Shanghai Lanji Cooperation (Shanghai, China). APTT assay reagents and calcium chloride (0.025 mol/L) were purchased from Shanghai Sunbiotech Cooperation (Shanghai, China). Citrated normal human plasma was obtained from Wuhan blood center (Wuhan, China) and stored at -20°C until use. N,N-dimethyfomamide (DMF) was distilled under reduced pressure to remove water. All other chemicals used were of analytical grades.

The $ClSO_3H \cdot DMF$ sulphonation complex was obtained by dropping predetermined liquid $ClSO_3H$ in water-free DMF, under cooling in water-ice bath.

Sulphonation of Guar Gum

Sulphonation of guar gum was performed as follows (15): For activation, 162 mg of dried guar gum powder was finely suspended in 16.2 ml dry DMF and the mixtures were stirred for 30 min at rt. Sulphonation was carried out by the stepwise addition of a $ClSO_3H \cdot DMF$ sulphonation complex for various durations and/or temperatures. After the reaction, the mixtures were cooled to room temperature by an ice bath, and then precipitated with anhydrous alcohol. The precipitate was redissolved in distilled water and its pH was adjusted to 10–11 with 15% NaOH aqueous solution, then the solution was dialyzed against water for 72 h. The sulfated guar gum was collected after drying over phosphorus pentoxide in vacuum.

Characterization

The number average molecular weight (Mn) and weight average molecular weight (Mw) of samples were measured by a gel permeation chromatography (GPC). GPC system

incorporated a TSP P 100 instrument. Simple column (TSK G3000-pw) was used. The eluent was 0.01 mol/l phosphate buffer solution containing 0.2 mol/l Na_2SO_4.The flow rate was maintained at 1.0 ml/min. The temperature of the column was maintained at 30°C.The eluent was monitored with a RI 150 refractive index detector. The sample concentration was 0.4 mg/ml. The standards used to calibrate the column were TOSOH pullulan having molecular weights of 0.59×10^4, 1.18×10^4, 2.28×10^4, 4.73×10^4, 11.2×10^4. All data provided by the GPC system were collected and analyzed using the Jingshen Working software package.

The sulfate content was determined turbidimetrically by Dodgson and Price's method (16, 17). A calibration curve was constructed with sodium sulfate as a standard. The substitution degree with sulfate groups (DS) was established on the basis of the sulfur content determined using the following equations:

$$S\% \text{ (sulfur content)} = SO_4^{2-}\% \text{ (Sulfate groups content)}/3$$

$$DS = S\ (\%) \times 162/(3200\text{-}S\ (\%) \times 102)\ DS = S\ (5) \cdot (17)$$

UV-Visible absorption spectra were obtained with a dilute aqueous solution on a Shimadzu 1601 UV-Visible spectrophotometer.

FT-IR spectra were recorded with KBr pellets on a Nicolet FT-IR 360 spectrophotometer. Sixteen scans at a resolution of 4 cm^{-1} were averaged and referenced against air.

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Anticoagulant Activity of Guar Gum

The anticoagulant activity of the guar gum sulphate was investigated by the classical activated partial thromboplastin time (APTT) clotting tests. The analyses were carried out according to the manufacturer's specification as previously described (18). The polysaccharides diluted saline solutions were prepared with a concentration from 20 μg/ml to 60 μg/ml. In the contrast group, only the saline was used. The value of control assay for APTT was 51.1 s. All the data were expressed as means ± S.D. ($n = 3$).

Results and Discussion

Effects of Various Reaction Conditions on Sulfated Guar Gum

Sulfate group takes an important role in the bioactivities of polysaccharides. DS and Mw of polysaccharides are also important parameters for the bioactivities. In general, anticoagulant activity of polysaccharides sulphate is strongly dependent on their DS and Mw. Hence, the author has considered it of interest to test the effects of molar ratio of $ClSO_3H$/GU, reaction time and reaction temperature on the DS and Mw of guar gum sulphate in the DMF.

Pretreatment of Natural Guar Gum

Prior to sulphonation, treatment of natural polysaccharides with DMF was shown to be very important. Our studies show that DS of a pretreated guar gum derivative was higher than that of an unpretreated guar gum derivative (data not shown). This is because of the nucleophilicity of the solvent used in the experiments. When guar gum was pretreated with DMF, the hydrogen atom was pulled apart from the hydroxyl group in the sugar residues. So the oxygen atom of hydroxyl group had the higher electron density and made them more acces-

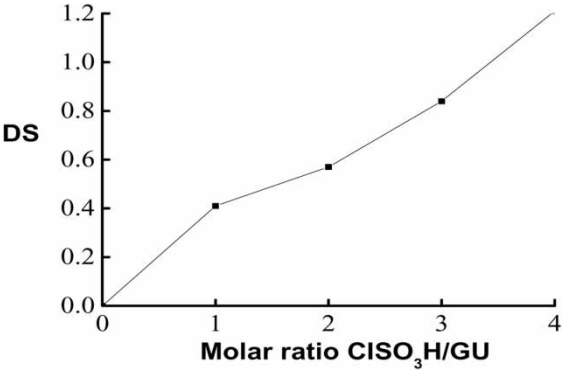

Figure 1. Influence of the molar ratio $ClSO_3H/GU$ on DS at 60°C for 3 h.

sible to $ClSO_3H \cdot DMF$ sulphonation complex. Pretreatment of starting materials was very important for achieving guar gum sulphate with high DS.

Effects of the Molar Ratio $ClSO_3H/GU$ on DS and Mw

To investigate the effects of $ClSO_3H/GU$ molar ratio, diverse $ClSO_3H/GU$ molar ratio including 1.0, 2.0, 3.0, 4.0 were taken in this experiment. Figure 1 shows the influence of the molar ratio [$ClSO_3H/GU$] on DS. From the presented data, one may observe that, as the molar ratio $ClSO_3H/GU$ increased, DS of modified guar gum rose from 0.42 to 1.10. All samples were also studied by GPC. Because the Mark Houwink equation of pullulan could not be used for guar gum, these molecular weights determined only represent relative values. In Figure 2, on plotting the effect of the molar ratio $ClSO_3H/GU$ on Mw at 60°C for 3 h, one can observe that the mass of the sulphonation reagent greatly influenced the molecular weights of products. The more the sulphonation reagent was used, the lower the products' Mws were. Guar gum degraded rapidly with the increase of the $ClSO_3H/GU$ molar ratio. When the molar ratio was 4, the Mw dropped to 2.98×10^4. The rate of degradation in the presence of $ClSO_3H$ was quite high because of the acid environment. Under the reaction condition used, the fact that DS was low may be

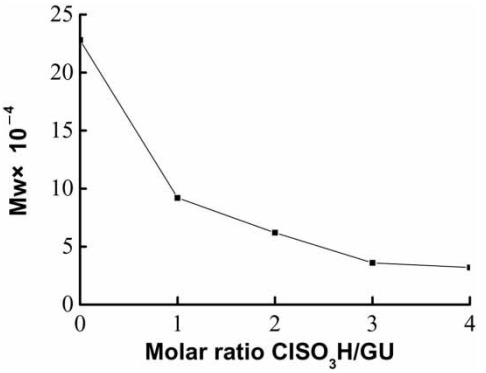

Figure 2. Effect of the molar ratio ClSO3H/GU on Mw at 60°C for 3 h.

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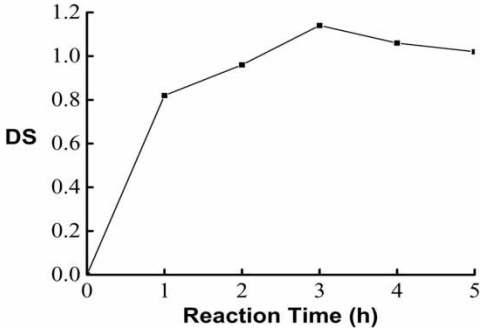

Figure 3. Influence of reaction time on DS with 4 : 1 molar ratio ClSO3H/GU at 60°C.

a result of the $ClSO_3H$ being less advantageous than SO_3 because the risk of chain scission or dehydratization is higher.

Effects of the Reaction Time on DS

In order to study the variation of DS with time, the reactions were conducted at 60°C using 162 mg of pretreated guar gum and 4 : 1 molar ratio of $ClSO_3H$/GU. Figure 3 plotted the influence of the duration of guar gum reaction with $ClSO_3H$ on DS. The increase of reaction time may contribute to the higher DS. For the sulphonation of guar gum, as expected, DS increased with reaction time, up to 3 h. However, a longer reaction time reduced the DS value. This indicated that the rate of sulphonation reaction was higher in the primary stage due to the higher concentration of $ClSO_3H$.

Effects of the Reaction Temperature on DS and Mw

Several sulphonation reactions were conducted using 3 h time and 4 : 1 molar ratio of $ClSO_3H$ to sugar unit, but at varying temperatures. Pretreated guar gum used in each case was 162 mg. Figure 4 illustrated that DS of guar gum derivatives increased with temperature, up to 60°C, however, at 80°C, the lower DS was obtained. The influence of temperature (ranging from 20 to 100°C) on Mw was also investigated (Figure 5) with 4 : 1 molar ratio $ClSO_3H$/GU and 3 h duration. When the reaction temperature was less than

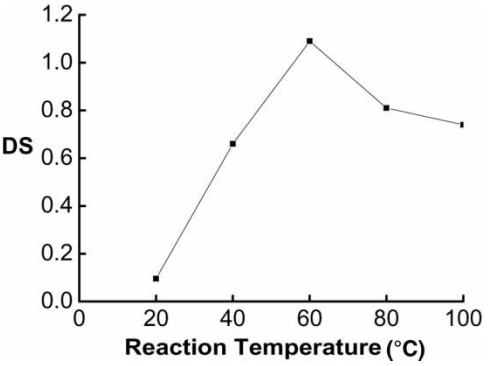

Figure 4. Effect of reaction temperature on DS with 4 : 1 molar ratio ClSO3H/GU for 3 h.

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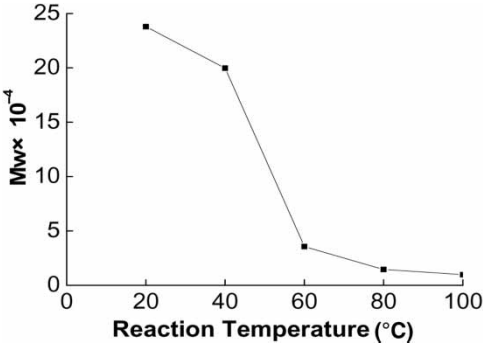

Figure 5. Influence of temperature on Mw with 4 : 1 molar ratio ClSO3H/GU for 3 h.

40°C, Mw didn't decrease basically, but when the temperature was higher than 40°C, Mw decreased significantly. At 100°C, the Mw of the product was only 9100 and the color of the reaction products changed from brown to black. The data clearly demonstrated that a high temperature enhanced the rate of degradation. Thus, reaction temperature was a major factor resulting in the degradation of polysaccharides.

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Characterization of Sulfated Guar Gum

FT-IR Spectra of the Sulfated Guar Gum. Figure 6 presented the FT-IR spectra of guar gum and guar gum sulphonate. The latter was prepared with 4 : 1 molar ratio $ClSO_3H$/GU in DMF at 60°C for 3 h. The frequencies of the guar gum at 810 cm^{-1} and 890 cm^{-1} indicated that the guar gum contained α-D-galactopyrannosyl units and β-D-mannopyranosyl units, respectively. The acid environment in the sulphonation would

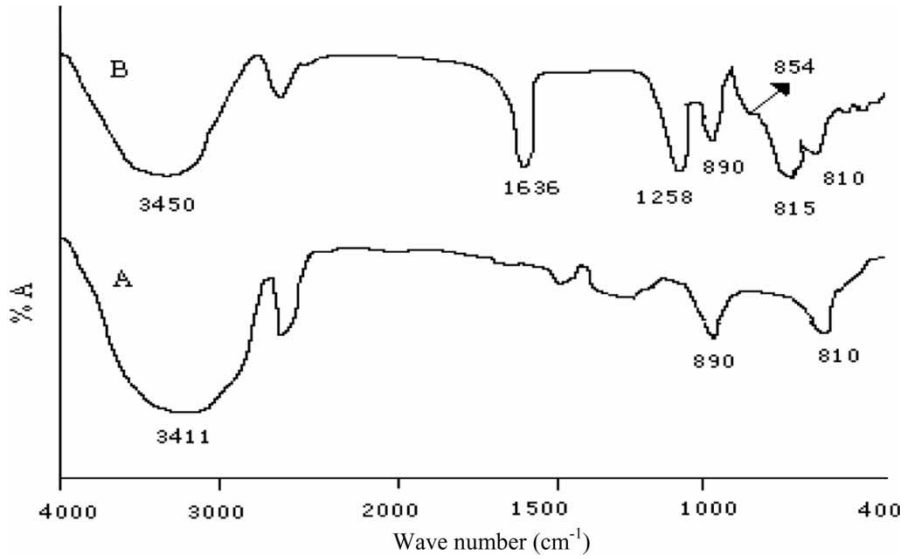

Figure 6. FT-IR spectra of guar gum (A) and sulfated guar gum (B). The sulfated guar gum was prepared by reaction for 1 h at 60°C using 4 : 1 molar ratio ClSO3H/GU.

lead to hydrolysis of the guar gum. However, after sulphonation these frequencies changed little. According to this, it could be concluded that the hydrolysis showed no selectivity on the two different units. The spectrum of sulfated guar gum presented two characteristic absorption bands, one at $\sim 1258\,cm^{-1}$ describing an asymmetrical S═O stretching vibration and the other at $\sim 815\,cm^{-1}$ with a discernible shoulder at $\sim 854\,cm^{-1}$ indicating a symmetrical C–O–S vibration associated with a $C-O-SO_3$ group. The intensity of the OH absorption band decreased with incorporating sulfated groups, this result indicated that reaction with $ClSO_3H$ succeeded in incorporating sulfated groups in guar gum molecules. In addition to these, a new band appeared at $\sim 1636\,cm^{-1}$. It could be related to the unsaturated bond formed in the sulphonation process.

UV-Visible Absorption Spectra of the Sulfated Guar Gum

Presently, the degradation mechanism was regarded as hydrolysis. In our experiment, we found that the color of the guar gum sulphate gradually changed from slight yellow to deep brown with the increase of the sulphonation reagent's dosage. Figure 7 showed the UV-Vis absorption spectra of guar gum and guar gum sulphate. The latter was prepared with a 4 : 1 molar ratio $ClSO_3H$/GU in DMF at 60°C for 3 h. In the spectra of guar gum sulphate, two new broad absorption bands appeared at 260 and 375 nm, they may be due to the washing process of products. We could not indicate the origins of the two bands and further studies are currently well under way.

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Anticoagulant Activities of Sulfated Guar Gum

The activated thromboplastin time (APTT) is the most widely used clinical laboratory test for monitoring the heparin effect. By measuring the APTT, mainly the factor XII to VIII

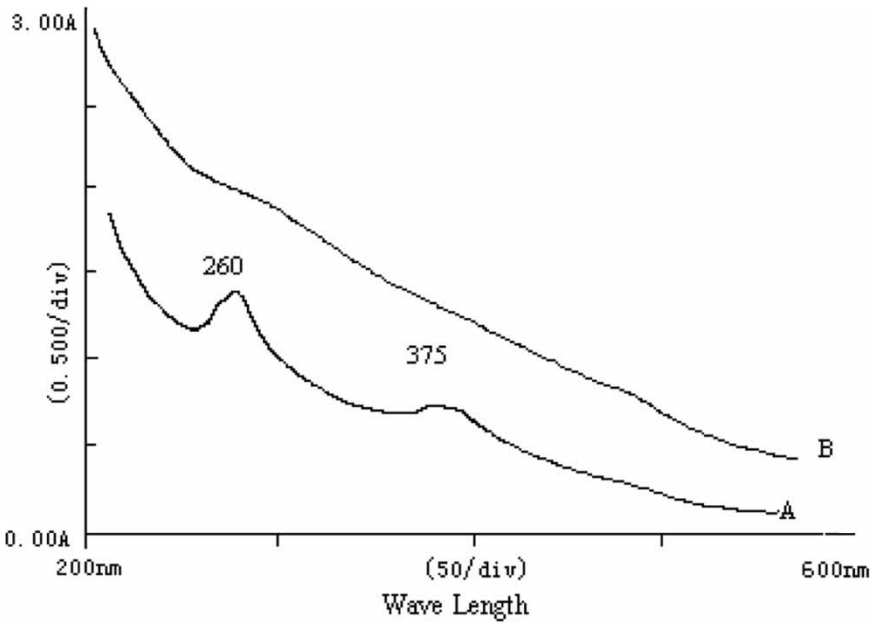

Figure 7. UV-Vis spectra of sulfated guar gum (A) and guar gum (B). The sulfated guar gum was prepared by reaction for 1 h at 60°C using a 4 : 1 molar ratio ClSO3H/GU.

are measured. The *in vitro* anticoagulant activities of initial guar gum and the previous sulfated guar gum were measured in APTT using normal human plasma. The physical-chemical characterization and anticoagulant activity of guar gums are reported in Table 1. The results showed that sulfated guar gum prolonged blood coagulant time at about 20 μg/ml of concentration, while unmodified guar gum did not show the effect. Prolongation of APTT suggested inhibition of the intrinsic coagulation pathway. However, anticoagulant activity was much less than that of heparin. At the same time, the anticoagulant activities of the sulfated guar gums strongly depended on their structure, i.e., the Ds and the molecular weight.

Effects of DS on Anticoagulant Activity

The anticoagulant activities of guar gum derivatives depended on the sulfate content. DS of guar gum is also an important parameter influencing anticoagulant activity. It is obvious that a minimum degree of sulphonation is essential to obtain any anticoagulant activity. SGG1 and SGG2 contained sulfate groups, their DS were 0.09 and 0.42, respectively, and also showed the shortness of APTT. This effect was detectable even at low concentration (20 μg/ml). When the DS of sulfated guar gum such as SGG3, attained 0.56, it showed basic anticoagulant activity. This suggested that though the sulfate group was essential, certain DS must be above 0.56 for anticoagulant activity under the reaction conditions used. With increasing DS to 0.85, the biological effect rises to achieve a maximal anticoagulant time about 7.00 min at a concentration of 60 μg/ml, after which one can observe a decrease by APTT assay. In addition, as could be seen, compounds with DS higher than 0.56, prolonged blood coagulant time in a dose-dependent manner. The anticoagulant activity improved with increasing DS, indicating that sulfate esters played a major role in the biological activity.

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Table 1
Physical-chemical characterization and anticoagulant activity of guar gums measured by APTT test

	Sulfated guar gum		Activated partial thromboplastin time (s)			
No.	DS	MW $\times 10^{-4}$	0 μg/ml	20 μg/ml	40 μg/ml	60 μg/ml
GG0	—	22.2	51.1 ± 1.1	48.9 ± 0.3	49.6 ± 0.2	50.5 ± 0.4
SGG1	0.09	21.6	51.1 ± 1.1	49.2 ± 0.3	49.7 ± 0.6	50.1 ± 0.2
SGG2	0.42	9.75	51.1 ± 1.1	50.3 ± 0.6	50.4 ± 0.4	50.7 ± 0.1
SGG3	0.56	4.94	51.1 ± 1.1	60.1 ± 1.3	120.6 ± 2.6	174.6 ± 2.4
SGG4	0.72	4.12	51.1 ± 1.1	100.6 ± 2.4	162.4 ± 1.0	235.9 ± 1.6
SGG5	0.85	3.40	51.1 ± 1.1	180.6 ± 1.5	307.2 ± 1.7	420.0 ± 2.4
SGG6	0.97	3.02	51.1 ± 1.1	139.2 ± 1.2	275.1 ± 2.1	359.2 ± 2.6
SGG7	1.12	2.74	51.1 ± 1.1	124.2 ± 0.9	209.7 ± 0.4	297.5 ± 3.5
SGG8	1.09	1.98	51.1 ± 1.1	104.4 ± 2.3	147.9 ± 4.0	214.3 ± 2.6
SGG9	1.02	1.50	51.1 ± 1.1	89.4 ± 2.0	100.8 ± 1.6	178.3 ± 1.3
Heparin sodium salt			51.1 ± 1.1	369.2 ± 2.8	nd	nd

nd: not determined.

Effects of Molecular Weights on Anticoagulant Activity

Besides DS, the molecular weight of polysaccharide is another important parameter influencing anticoagulant activity. SGG5, SGG6, SGG7, SGG8, SGG9, all had high DS in the test. Prolongation of their APTT in the same concentration was in the order of SGG5 > SGG6 > SGG7 > SGG8 > SGG9, but DS was in the order of SGG5 < SGG6 < SGG7 > SGG8 > SGG9. This could result from their different molecular weights. Their Mw were in the order of SGG5 > SGG6SGG7 > SGG8 > SGG9. These data suggested that the anticoagulant activity improved with increasing the molecular weights, and the molecular weight of the sulfated polysaccharide was a more important parameter than DS. In our studies, the sulfated polysaccharide with the DS of 0.85 and the Mw of 3.40×10^4 was best in the prolongation of APTT.

Conclusion

Sulfate groups were introduced into guar gum molecules by sulphonation with $ClSO_3H$ and DMF complex; degradation of guar gum molecular occurred simultaneously with sulphonation. It was shown that the introducing of sulfate groups to guar gum increased the APTT. The sulfated guar gum with the DS of 0.85 and the Mw of 3.40×10^4, which was prepared under the reaction condition: molar ratio of $ClSO_3H/GU$ was 4 : 1, reaction time and temperature was 1 h and 60°C, respectively, had the best blood anticoagulant activity.

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