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Preparation of low-molecular-weight polyguluronate sulfate and its anticoagulant and anti-inflammatory activities

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Abstract

Low-molecular-weight polyguluronate sulfate (LPGS) was prepared as a heparinoid by chemical sulfation of polyguluronic acid (PG), which was isolated from the hydrolysate of alginate by pH-fractionated precipitation. The structure of LPGS was elucidated based on FT-IR and 2D NMR spectroscopy. Sulfation was demonstrated to occur at the C-2 and C-3 positions of the guluronic acid residues and the degree of substitution (DS) was 1.53 per monosaccharide residue. The weight-averaged molecular weight of LPGS was 11.4 kDa determined by high performance gel permeation chromatography (HPGPC). The in vitro anticoagulant activity and in vivo anti-inflammatory activity of LPGS were evaluated. Results showed that LPGS significantly prolonged the whole blood clotting time and activated partial thrombinplastin time (APTT), and exhibited considerable anti-inflammatory activity in cotton pellet-induced granuloma in rats.

Keywords: Polyguluronate sulfate; Alginate; Anticoagulant activity; Anti-inflammatory activity

1. Introduction

Sulfated polysaccharides that are either of natural or semisynthetic origin, have shown a wide range of biological activities (Huang, Du, Yang, & Fan, 2003). Marine algae are a rich source of sulfated polysaccharides with novel structures, and a large number of sulfated polysaccharides have been isolated from green algae (Hayakawa et al., 2000; Lee, Hayashi, Maeda, & Hayashi, 2004), red algae (Farias, Nazareth, & Mourao, 2001; Zhou et al., 2004) and brown algae (Mourao, 2004; Senni et al., 2006). Apart from the anticoagulant and antithrombotic activities (Mourao, 2004; Trento, Cattaneo, Pescador, Porta, & Ferro, 2001), antitumor, antiproliferative, antiviral and anti-inflammatory activities have also been described for these sulfated polysaccharides (Riou et al., 1996; Senni et al., 2006; Witvrouw & De Clercq, 1997).

Alginate is a family of unbranched binary copolymers of β-(1→4)-linked D-mannuronic acid (M) and α-(1→4)-linked L-guluronic acid (G) residues that have been isolated from brown algae (Panikkar & Brasch, 1996). It is the only polysaccharide which naturally contains carboxyl groups in each constituent residue, and possesses various functional activities (Ikeda, Takemura, & Ono, 2000). There are three different polymeric segments in alginate molecule, namely polymannuronic acid segment (PM), polyguluronic acid segment (PG) and an alternating segment of M and G (PMG). PM and PG can be separated by pH fractionation when alginate was hydrolyzed (Haug, Larson, & Smidsrod, 1966). Sulfated alginate derivatives, owning to their blood-compatibility and structural similarity to that of heparin, have been prepared and their anticoagulant and antithrombotic activities were reported earlier (Huang, Du, & Yang, 2003; Yao et al., 2006). However, the composition (M/G ratio) and the hexuronic acid sequence of alginate vary considerably depending upon the alga species, the havest season, age,

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and the part of the plant used for extraction (Llanes, Ryan, & Marchessault, 2000). Alginate sulfated derivatives, just like most natural sulfated polysaccharides, are heterogeneous macromolecules showing wide variations in their structure and activities. This complicates the development of those polysaccharides into new drugs (Alban, Schauerte, & Franz, 2002; Han, Yao, Yang, Liu, & Gao, 2005). For this reason, we aimed to prepare structurally defined sulfated polysaccharides in order to establish structure–activity relationship as the basis for the development of macromolecular drugs.

Propylene glycol mannuronate sulfate (PGMS), a sulfated derivative of PM, has been previously prepared and used as an antithrombotic drug in China (Guan, 1997). Although M and G are epimers and only differ at C-5, they possess different conformations. In PM, all M residues take $^4C_1$ conformation and linked by β-1,4-glycosidic bond, while in PG, all G residues take $^1C_4$ conformation and linked by α-1,4-glycosidic bond. These features made their advanced structures different, for example, PG exhibits an egg-box like conformation, and it usually forms stiffer 2-fold screw helical chains (Atkins, Nieduszynski, Mackie, Parker, & Smolko, 1973) in water solution, while PM forms belt chains through intra-molecular hydrogen bond. Owning to their different structure characterization, the derivatives of PM and PG would exhibit different activities.

In the present study, a low-molecular-weight polyguluronate sulfate (LPGS) was prepared by chemical sulfation of PG, and its structure was characterized by FT-IR and 2D NMR spectroscopy. The anticoagulant and anti-inflammatory activities of LPGS were also investigated.

2. Materials and methods

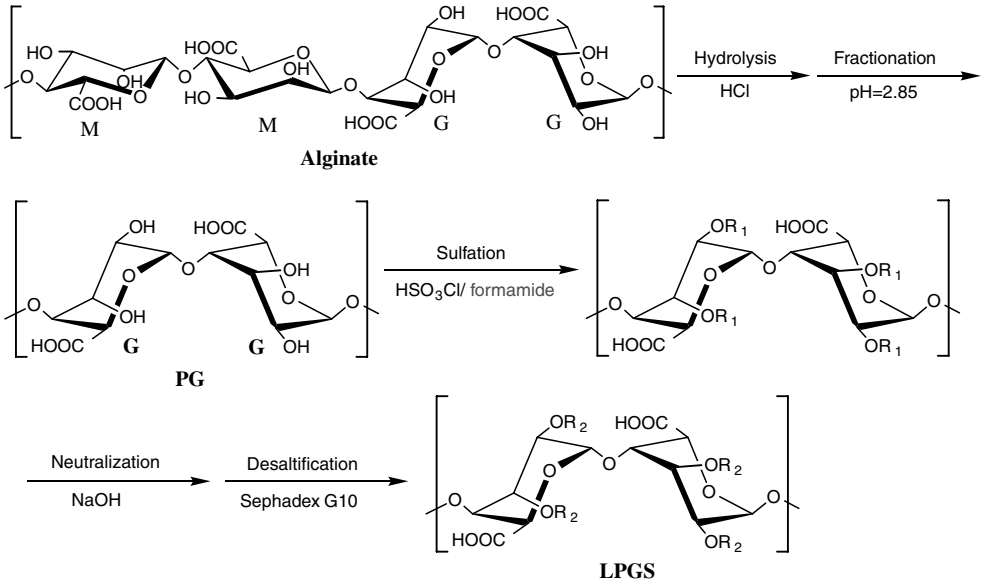
2.1. Materials

Alginate from *L. Japonica* and PGMS (the sulfur content is 12.14%, and the weight-averaged molecular weight is 12.6 kDa) were provided by Lantai Pharmaceutical industry (Qingdao, China). Sephadex G-10 was from Amersham Biosciences (Uppsala, Sweden). D_2O was from Cambridge Isotope Laboratories Inc. APTT assay kit was purchased from Shanghai Sun Bio. Corp. (Shanghai, China). Heparin (sodium salt, 204 IU/mg) was purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Dextran standards kit was purchased from Fluka Chemical Corp. All other commercial reagents were of analytical grade and were used without further purification.

Male New Zealand rabbits weighing 2.5–3.0 kg were purchased from the Centre for Laboratory Animal, Shandong University (Jinan, China). Male Wistar rats weighing 140–150 g were purchased from the Centre for Laboratory Animal, Academy of Military Medical Science (Beijing, China). All animals were housed in metabolic cages and had free access to pellet diet and tap water during the study.

2.2. Preparation of LPGS

The preparation of LPGS include separation of PG and sulfation of PG (Fig. 1). The separation of PG was carried out essentially as described by Haug and Larsen (1962). Briefly, thirty grams of alginate were hydrolyzed in 1000 ml of 0.5 mol/l HCl at 100 °C for 8 h, and PG were isolated from the hydrolysate by fractionated precipitation at pH



(R_1=H or SO_3H, R_2=H or SO_3Na)

Fig. 1. Preparation route of LPGS.

2.85. The sulfation of PG was carried out with the method described by Guan (1997). Briefly, PG was added slowly to a mixture of formamide and HSO_3Cl with stirring and cooling at 0–5 °C in ice-salt bath. After stirring for 3 h at 65–70 °C, the reaction mixture was cooled and precipitated with ethanol. The precipitate was dissolved in distilled water and then re-precipitated with ethanol. This process was repeated three times and the precipitate was redissolved in water and neutralized with 2 mol/l NaOH. Then the solution was desalted with a sephadex G10 column (2.6 × 80 cm), and the eluent was concentrated and lyophilized to yield a white fluffy solid of LPGS.

2.3. Analytical methods

FT-IR spectra were recorded on a Nicolet Nexus 470 IR spectrometer with a KBr pellet. Samples for NMR analysis were dissolved in D_2O (99.96%) and freeze-dried twice to remove the exchangeable protons, before their final dissolution in D_2O (99.96%) for NMR analyses. All NMR spectra were performed on a JEOL JNM-ECP600 spectrometer at 20 °C using 4,4-dimethyl-4-silapentane-1-sulfonate (DSS) as internal standard. Sulfur content (S%) was determined by oxygen flask combustion method as reported previously (Zhao et al., 2001). Weight-averaged molecular weight (M_w) and distribution width of molecular weight (M_w/M_n) were determined by high performance gel permeation chromatography (HPGPC) with a column of TSKgel G3000PW$_{XL}$ (TOSOH, Japan) on an Agilent HP1100 chromatographic instrument. Aqueous Na_2SO_4 solution (0.7%, w/v) was used as the mobile phase and the flow rate was 0.5 ml/min. The temperature of column was maintained at 35 °C and detected by G1362A refractive index detector. Dextrans were used as standards to calibrate the column (Zhao, Miao, Fan, & Guan, 2000).

2.4. Anticoagulant activity of LPGS

The anticoagulant activity of LPGS was determined in vitro using a biological assay to compare its ability to delay the clotting of fresh rabbit blood with that of heparin calibrated in international units (IU). The assay was carried out essentially as the procedure described in Chinese Pharmacopoeia (2005 Edition). Briefly, heparin sodium or LPGS was diluted with 0.9% NaCl solution to contain accurately 5.00, 3.50, and 2.45 IU/ml (concentration ratio is 1:0.7, and the estimate potency of LPGS is 25 IU/mg). To each clean and dry tube add 0.1 ml of the appropriate dilution (either from heparin or LPGS) and 0.9 ml of fresh rabbit blood which was drawn from carotid artery. After each addition, mix immediately but do not allow bubbles to form. Transfer all the tubes to a water-bath at 37 °C, and the time between the addition and the onset of clotting was recorded in seconds. Transform the clotting times to logarithms, using the mean value for five tubes, and the potency of LPGS was calculated by parallel-line bioassay statistical methods (Iznaga et al., 1995).

The anticoagulant activity of LPGS was also measured by activated partial thrombinplastin time (APTT). For APTT assay, citrated rat plasma (100 μl) was mixed with sample solution (100 μl) at various concentrations and incubated at 37 °C for 1 min. Then, APTT assay reagent (100 μl) was added to the mixture and the solution was incubated at 37 °C for 5 min. Pre-warmed (at 37 °C) calcium chloride solution (100 μl, 0.025 M) was then added and the time to clot formation was measured (Yu et al., 2002).

2.5. Anti-inflammatory activity of LPGS

Male Wistar rats were randomly divided into six groups of 12 animals each as follows: One control group; One hydrocortisone group at the dose of 10 mg/kg; Four LPGS groups at the dose-levels of 12.5, 25, 50, and 100 mg/kg, respectively. All groups rats were fed with standard diet, and the LPGS groups rats were administered LPGS and the control group rats were administered distilled water at the same time. After 3 weeks, two sterile cotton pellets (10 mg) were implanted subcutaneously in the groin region of the rats, one in each side. Then the hydrocortisone group rats were started to administer hydrocortisone and all other groups rats were continued to administer LPGS or distilled water for seven consecutive days. Distilled water, LPGS and hydrocortisone were administered to rats by volume of 0.5 ml per 100 g body weight once a day. All animals were anaesthetized on the eighth day, and the cotton pellets were removed surgically and made free from extraneous tissues. The moist pellets were weighed and then dried at 60 °C until reach to constant weight. The increment in the dry weight of the pellets was regarded as measure of granuloma formation (Ismail, Gopalakrishnan, Begum, & Elango, 1997). Student's *t*-test was used for statistical comparison of data between groups.

3. Results and discussion

3.1. Preparation and structure determination of LPGS

Alginate is a linear polysaccharide composed of β-D-mannuronic acid (M) and α-L-guluronic acid (G) residues. Although two residues are epimers, they possess different physical–chemical properties. The pK_a of M is 3.38, and the pK_a of G is 3.65. PM was soluble in water at pH 2.85 but PG was insoluble under the same condition. Therefore, the two polysaccharide segments can be isolated by pH-fractionated precipitation following partial hydrolysis of alginate. PG was obtained with the yield of 32% (w/w) from alginate. LPGS was prepared by sulfation of PG using the chlorosulfonic acid/formamide method (Guan, 1997), and some small molecular impurities were removed with sephadex G10 column.

The structure of LPGS was elucidated based on FT-IR and 2 D NMR spectral analysis. In the IR spectrum of LPGS (Fig. 2), the bands at 1629.84 and 1421.11 cm^{-1} are attributed to the asymmetric and symmetric valence

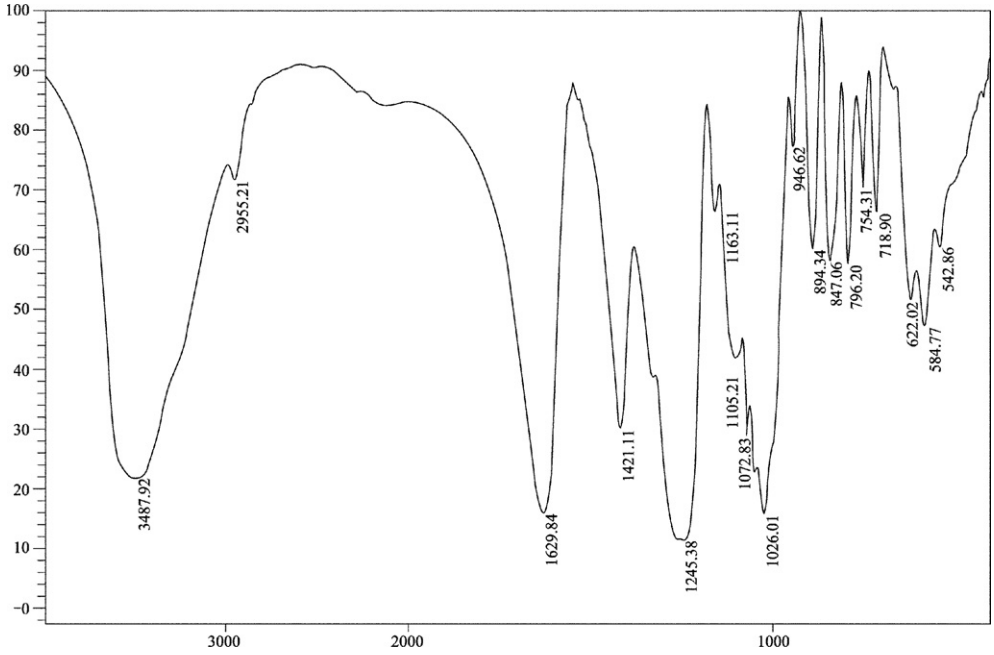


Fig. 2. The IR spectrum of LPGS.

vibrations of carboxyl group (COO^-), respectively, and the band at 796.20 cm^{-1} is from the characteristic α-anomeric C–H deformation vibration of PG (Chandia, Matsuhiro, & Vasquez, 2001). The bands at 1245.38 and 847.06 cm^{-1} are ascribed to the asymmetric S═O stretching vibration and symmetric C–O–S stretching vibration, respectively,

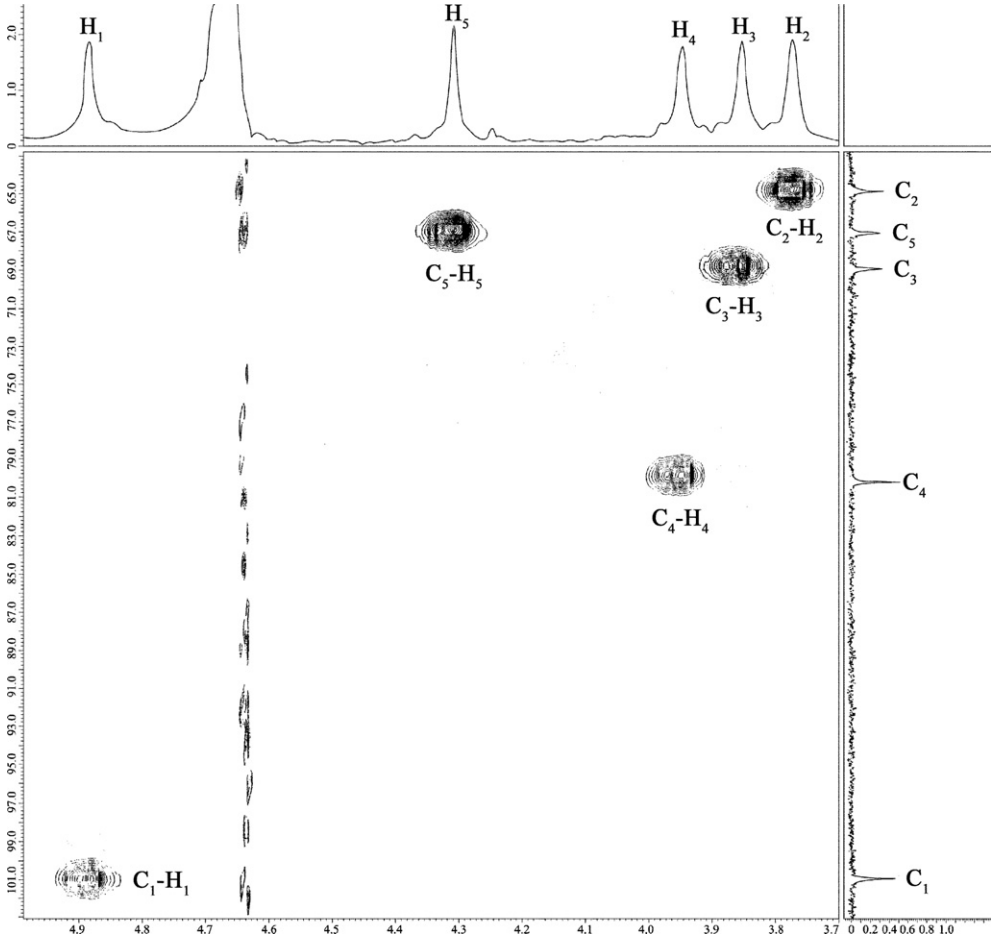


Fig. 3. The HMQC spectrum of PG.

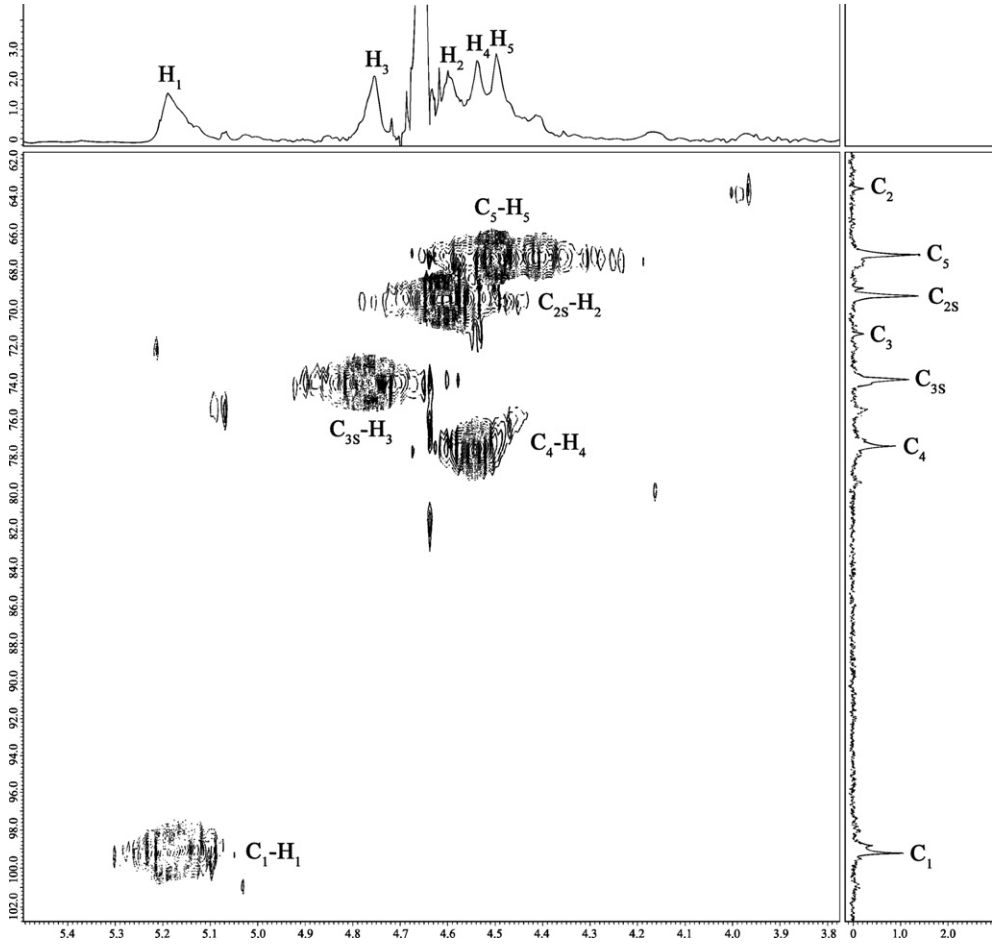


Fig. 4. The HMQC spectrum of LPGS.

indicating that the sulfation reaction had actually occurred (Han et al., 2005).

Through the correlative signal analysis of each carbon with hydrogen in the HMQC spectra of PG (Fig. 3) and LPGS (Fig. 4), the NMR data of PG and LPGS are listed in Table 1. In the ^{1}H NMR spectrum of PG, the signal of anomeric proton (H-1) of PG appeared at 5.03 ppm and signal of H-1 of PM in the range of 4.5–4.6 ppm (Grasdalen, Larson, & Smidsrod, 1979; Heyraud et al., 1996) was absent, indicating a good purity for the PG obtained. In the ^{1}H NMR spectrum of LPGS, the signals of H-2 and H-3 shift downfield significantly from 3.91 to 4.70 ppm and from 3.99 to 4.86 ppm, respectively, indicating that the C-2-OH and C-3-OH of PG were substituted by sulfate groups. There are 6 carbon signals appeared in the ^{13}C NMR spectrum of PG (Table 1), and the peak at 102.45 ppm was ascribed to the resonance of C-1, the peaks at 66.42 and 70.51 ppm were ascribed to the resonances of C-2 and C-3, respectively. The absence of carbon signals in the range of 72–78 ppm for the potential C-3 and C-5 of PM (Grasdalen, Larson, & Smidsrod, 1981; Heyraud et al., 1996; Schurks, Wingender, Flemming, & Mayer, 2002), further indicating that the test sample was mainly made up of PG. All carbon signals of PG that we prepared were in accordance with the data which reported by Heyraud et al. (1996). In the ^{13}C NMR spectrum of LPGS (Fig. 5), the signals of C-2 and C-3 shift downfield sharply from 66.18 to 71.44 ppm and from 73.91 to 76.40 ppm, respectively, further supporting that the sulfation occurred at the C-2 and C-3 positions of PG. There are about 81% of C-2-OH and about 77% of C-3-OH were substituted by sulfate groups based on the peak-height ratio of C-2S/C-2 and C-3S/C-3 signals measured (namely, the degree of sulfate substitution is about 1.6 per monosaccharide residue). The signal of C-2S is only a little higher than that of C-3S, indicating that the sulfation reaction has no significant regioselectivity between C-2-OH and C-3-OH.

Table 1
The NMR data of PG and LPGS[a]

Compound	^{13}C NMR							
	C-1	C-2	C-2S	C-3	C-3S	C-4	C-5	C-6
PG	102.45 (101.84)	66.42 (66.47)		70.51 (70.35)		81.72 (81.08)	68.66 (68.44)	177.02 (–)
LPGS	101.29	66.18	71.44	73.91	76.40	80.02	69.73	176.63
	^{1}H NMR							
	H-1	H-2		H-3		H-4	H-5	
PG	5.03	3.91		3.99		4.09	4.45	
LPGS	5.29	4.70		4.86		4.64	4.61	

[a] The chemical shifts in bracets are data of Heyraud et al. (1996).

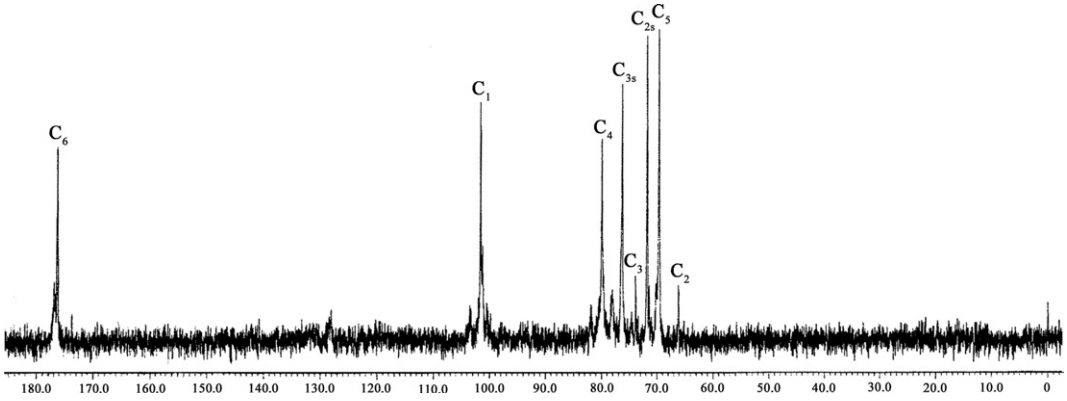


Fig. 5. The ^{13}C NMR spectrum of LPGS.

3.2. Sulfur content and weight-averaged molecular weight

Oxygen flask combustion is a rapid and accurate method to determine the sulfur content and it has been conventionally used for the determination of sulfur present in a sulfated polysaccharide. The sulfur content (S%) of LPGS was 13.87% (w/w) measured by oxygen flask combustion followed by titration with barium perchlorate. The degree of sulfate substitution was calculated as 1.53 per monosaccharide residue, and it is corresponding with the estimated value of sulfate substitution based on the peak-height ratio of C-2S/C-2 and C-3S/C-3 signals in the ^{13}C NMR spectrum of LPGS (Fig. 5). The weight-averaged molecular weight (M_w) of LPGS determined by HPGPC was 11.4 kDa, and the ratio of weight-to-number average molecular weight (M_w/M_n) was 1.37, indicating that LPGS has a narrow molecular weight distribution.

3.3. Anticoagulant and anti-inflammatory activities of LPGS

It was reported that inflammation and coagulation are two factors involved in the pathology of cancer and cardiovascular diseases, and there is accumulating evidence that anti-inflammatory and anticoagulant agents may be useful for the prevention of such diseases (Walston et al., 2002; Zacharski, 2002; Zhao, Yu, Hu, & Yuan, 2003), so we are focused on evaluation of the anticoagulant and anti-inflammatory activities of LPGS in the present study.

LPGS is a low-molecular-weight linear polyanionic molecule containing both sulfate groups and carboxyl groups, its structure is similar to that of heparin, which has been widely used as anticoagulant therapy for more than 50 years. Results of clotting assay showed that LPGS significantly prolonged the clotting time of fresh rabbit blood (Fig. 6). The anticoagulant potency of LPGS determined using a biological assay to compare with heparin was 26.52 IU, with 95% confidence limits between 24.84 and 28.38 IU, and the fiducial limit is 6.67%. This result was approximate to the anticoagulant potency of PGMS, which was 23.07 IU, with 95% confidence limits between 21.64 and 24.56 IU. The structure–activity relationships of sulfated polysaccharides showed that, in general, the presence of

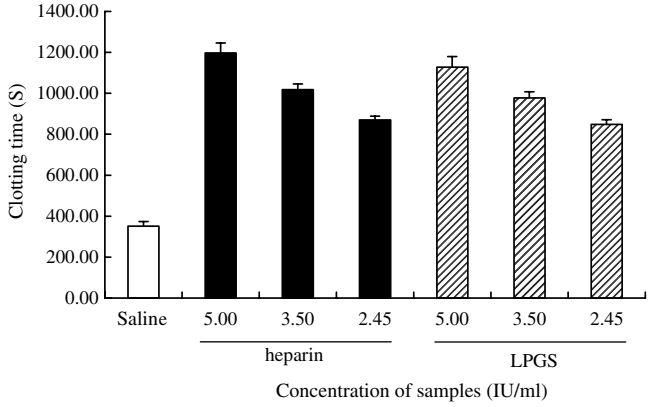


Fig. 6. Anticoagulant activity of LPGS with respect to clotting time.

sulfate groups is an essential requirement for the anticoagulant activity (Huang et al., 2003). PG has no anticoagulant activity, but LPGS, which prepared by sulfation of PG, showed significantly anticoagulant activity. This result further supports that the anticoagulant activity of polysaccharides was closely depended on the sulfate groups.

APTT is the most widely used clinical laboratory test for monitoring the heparin effect on the anticoagulant activity and it is related to the intrinsic coagulation phase in plasma (Han et al., 2005; Martinichen-Herrero, Carbonero, Sassaki, Gorin, & Iacomini, 2005). LPGS prolonged APTT at concentration of 0.5 μg/ml and the prolongation rate was almost linearly correlated to the concentration of LPGS

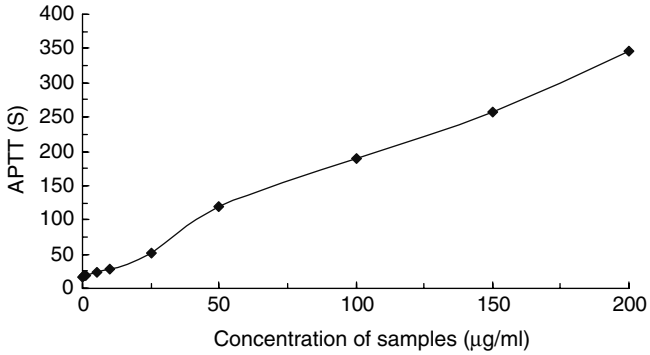


Fig. 7. Anticoagulant activity of LPGS with respect to APTT.

Table 2
Effect of LPGS on cotton pellet granuloma in rats

Group	Dose (mg/kg)	Animal (number)	Granulation tissue (mg)		Inhibition[c] (%)
			Wet weights	Dry weights	
Control		12	131.7 ± 26.5	33.0 ± 6.50	–
LPGS	12.5	12	112.2 ± 19.7	28.4 ± 7.34	13.9
LPGS	25	12	110.2 ± 19.5[a]	26.1 ± 5.91[a]	20.9
LPGS	50	12	102.5 ± 13.7[b]	21.9 ± 2.68[b]	33.6
LPGS	100	12	94.7 ± 20.0[b]	19.7 ± 3.62[b]	40.3
Hydrocortisone	10	12	88.4 ± 16.9[b]	17.8 ± 3.37[b]	46.1

Values are expressed as means ± SD.
[a] $p < 0.05$.
[b] $p < 0.01$ (vs. control).
[c] Calculated by dry weights of granulation tissue.

($r = 0.9978$) in rat plasma (Fig. 7). Compared to PGMS, an interesting phenomenon was observed, that is, LPGS showed weaker anticoagulant activity at lower concentrations (<25 μg/ml) and stronger anticoagulant activity at higher concentration (>25 μg/ml) than that of PGMS, for example, the clotting time of LPGS and PGMS was 28.87 and 42.49 s at 10 μg/ml, but was 188.67 and 144.89 s at 50 μg/ml, respectively. The significant effect of LPGS on APTT suggested an inhibition of the intrinsic and/or common coagulation system.

The cotton pellet granuloma method is widely used to evaluate the transudative and proliferative components of the chronic inflammation (Buyukokuroglu, 2002). The fluid absorbed by the pellet influences the wet weight of the granuloma and the dry weight correlates well with the amount of granulomatous tissue formed (Ismail et al., 1997; Swingle & Shideman, 1972). As shown in Table 2, LPGS exhibited considerable anti-inflammatory activity in the cotton pellet-induced granuloma in rats, both the wet weight and the dry weight of the granuloma were reduced considerably at dose-levels of 25, 50 and 100 mg/kg, when compared to the control group. This reflected its efficacy to inhibit the proliferative phase of the inflammation process.

4. Conclusions

LPGS, with a define heparinoid structure, was prepared by chemical sulfation of PG. The structure of LPGS was characterized by FT-IR and 2D NMR spectroscopy, and the sulfation was demonstrated to occur at the C-2 and C-3 positions of PG. Owning to the presence of 2,3-*O*-disulfate groups and the structural similarity to heparin, LPGS exhibited significant anticoagulant activity in vitro. The significant effect of LPGS on APTT suggested an inhibition of the intrinsic and/or common coagulation system. The considerable anti-inflammatory activity of LPGS in cotton pellet-induced granuloma in rats reflected its efficacy to inhibit the proliferative phase of the inflammation process. Further study is ongoing for preparation of LPGS with different molecular weights and different degrees of sulfate substitution, and investigation of the influence of these structural variations in LPGS on its anticoagulant and anti-inflammatory activities.

Acknowledgements

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