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Anticoagulant activity of a sulfated *Lachnum* polysaccharide in mice with a state of hypercoagulability

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ABSTRACT

A sulfated polysaccharide, designated as SLEP-1, was obtained after sulfation of the exopolysaccharide (LEP-1) which was isolated from *Lachnum*. The degree of substitution (DS) of sulfate group of SLEP-1 was 1.97. SEM images of SLEP-1 revealed laminated structure in mesh. UV, FT-IR and ^{13}C NMR spectra indicated that the LEP-1 was sulfated successfully. The result of the anticoagulant activity in vitro showed that both of LEP-1 and SLEP-1 could effectively prolong activated partial thromboplastin time (APTT) and thrombin time (TT) of the normal mice plasma, in which SLEP-1 was more effectively than those of LEP-1, and dose–effect relationships were found. According to the bleeding time (BT), clotting time (CT), APTT, PT, prothrombin time (TT), fibrinogen (FIB), AT-III activity and FXa concentration of the hypercoagulable mice, it indicated that SLEP-1 (30 mg·kg^{-1} and 90 mg·kg^{-1}) had strong inhibitory effect on intrinsic coagulation pathway, which could also enhance fibrinolytic activity. It may constitute an anticoagulant drug of interest in anticoagulant therapy.

Lachnum sp. can produce exopolysaccharide via submerged liquid fermentation. Exopolysaccharides from different strains of *Lachnum* sp. have diverse properties and bioactivities such as promoting effect on wound healing, lipid lowering and liver protecting effects and hypoglycemic activity.[1–3] Additionally, the bioactivities of these exopolysaccharides depend on their molecular weight, solubility, monosaccharide compositions, glycosidic bond types and flexibility of configuration, etc.[4,5] Different methods was applied to modify different *Lachnum* exopolysaccharides, including phosphorylation, sulfation and carboxymethylation, and the bioactivities of these modified exopolysaccharides were enhanced. Ye et al., (2013) reported a phosphorylated polysaccharide PLEP-1a which was prepared from LEP-1a obtained from a strain of *Lachnum* (YM120), and the antitumor activity in vivo of PLEP-1a was significantly higher than that of LEP-1a.[5] Wu et al., (2014) modified *Lachnum* YM281 exopolysaccharide LEP-1b by carboxymethylation, and after modification, the polysaccharide CLEP-1b could mitigate the chronic renal failure of mice.[6]

A complete coagulation, anticoagulation and fibrinolytic system can ensure the blood flowing in the normal state. The imbalance between coagulation and anticoagulation can lead to a hypercoagulable state or the formation of thrombus.[7] The coagulation-fibrinolysis imbalance may also induce the formation of thrombus, or lead to the instability of atherosclerosis plaque, or thus induce ischemic cardiovascular and cerebrovascular disease.[8,9] Commonly used clinical anticoagulation drugs are heparin, coumarin and anti-platelet drugs, where heparin is the most commonly used.[10] However, the application of heparin in anticoagulant treatment would be accompanied by side effects appearance such as hemorrhagic complications.[10,11] Currently, chondroitin sulfate B, sulfated fucoidan and semi-synthetic sulfated polysaccharides and other heparin alternatives have emerged in the market.[12] Vongchan et al., (2002) modified a *Somanniathelphusa dugasti* polysaccharide, and then obtained three sulfated polysaccharides, all of them exhibited strong anticoagulant activities.[13] It was reported that anticoagulant activity was an important bioactivity of sulfated polysaccharides.[14] Therefore, it's of great significance to evaluate the anticoagulant activity of sulfated derivatives of *Lachnum* polysaccharides .

The purpose of this research was to conduct sulfated modification of LEP-1, characterize the physical and chemical properties of the sulfated polysaccharide (SLEP-1), and reveal the effect of SLEP-1 in vivo and in vitro anticoagulant activity.

LEP-1 (1.50 g) was sulfated by sulfur trioxide pyridine complex and the sulfated polysaccharide was named as SLEP-1. Regression equation of the standard curve of sulfate anion (SO_4^{2-}) was $Y = 0.01847X + 1.9994$, $R^2 = 0.9947$. The regression equation was then used to calculate the content of $–SO_3H$ from SLEP-1

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(66.17%). According to the above calculation formula of the DS of $–SO_3H$, the DS of $–SO_3H$ in SLEP-1 was 1.97.

The Mw of sulfated polysaccharide is another important parameter influencing its bioactivities. HPLC was applied to determine the Mw of SLEP-1, calibration curve was obtained with the dextran standards. The profile of SLEP-1 appeared as a comparatively single peak. According to the standard curve between M_W and retention time. The average Mw of SLEP-1 was calculated about 1.58×10^7 g/mol and the retention times were 20.204 min. In the process of polysaccharides sulfation reaction, degradation was usually accompanied.[15,16] However, our result showed that the Mw of sulfated polysaccharide was higher than that of natural polysaccharide LEP-1, indicating that sulfated derivatives were produced successfully in this study without degradation, it was in accordance with the results of Liu et al.[17] and Li et al.[18], which may be due to the moderate sulfation reaction and the graft modification of polysaccharide which increased the functional groups. The HPLC profile of SLEP-1 and detailed method was provided in Supporting information.

The morphology of LEP-1 and SLEP-1 were presented in Figure 1. LEP-1 had irregular multi-layer flakes with a flat compact surface (Fig. 1A) accompanied by slight wrinkles (Fig. 1B), which indicated the existence of intermolecular cross-linking and stronger intermolecular interactions of polysaccharide molecules. Moreover, morphology feature of LEP-1 might also related to its high molecular weight.[19] SLEP-1 had multi-layer reticular structure with different mesh sizes, the layers of SLEP-1 were bigger than those of LEP-1 (Fig. 1C). Such reticular structure of polysaccharide could demonstrated a good biocompatibility which might beneficial to cell adhesion.[20] Along with an increase in magnification of SEM images, the SLEP-1 also showed lamellar structure (Fig. 1D) which was similar with that of LEP-1. Therefore, the generation of meshes in SLEP-1 was probably due to changes in physical appearance of polysaccharide during the process of chemical modification.

Compared with LEP-1 UV spectrum, a big absorption band around 264 nm was appeared in SLEP-1 UV spectrum (Fig. 2A), which indicated the successful sulfate modification of LEP-1. It was the characteristic absorption peak of $-S-O-/-SO_3H$ caused by the $n \rightarrow \pi^*$ electron transition of the sulfate.[21]

The characteristic peaks of carbohydrate rings changed after sulfated modification. As shown in Fig. 2B, absorption peaks at 3400 cm^{-1} for the hydroxyl groups stretching vibration of SLEP-1 was weaker than that of LEP-1 which could be attributed the conversion of hydroxyl groups to sulfate groups. Compared with the FT-IR spectrum of LEP-1, stretching vibration of C—H in methylene at 2930 cm^{-1} and variable angle vibration of C—H in methyl and methylene at 1410 cm^{-1} of SLEP-1 were obviously shifted lower (Fig. 2B), which might be the substitution at C-6 position in the monosaccharide residues, because the hydroxyl bond at C-6 position of polysaccharide was relatively active and had a big influence on methylene.[22] In addition, as shown in SLEP-1′s spectrum (Fig. 2B), the new absorption peak at 1220 cm^{-1} was caused by asymmetry stretching vibration of S=O of $C-O-SO_3$ group.[23] which was also found to be corresponding to ester sulfate groups. The absorption peak at 818 cm^{-1} was corresponding to the symmetry stretching vibration of C-O-S.[4] In fact, the absorptions at region 800–850 cm^{-1} was used to infer the position of the sulfate group in sulfated polysaccharide.[24] Therefore, the successful sulfation of LEP-1 was confirmed by FT-IR results.

As shown in the ^{13}C NMR spectrum (Fig. 3A), the carbon signals of SLEP-1 were overlapped and relatively weak, it was probably due to the existence of the sulfate group in SLEP-1.[25] The position of sulfation occurred to polysaccharide can be further determined by NMR. In comparison with the ^{13}C NMR spectrum (Fig. 3B) of LEP-1, the C-6 signal at 62.555 ppm of →1)-β-D-Glc*p* of SLEP-1 was almost disappeared in Figure 3A, suggesting C-6 of →1)-β-D-Glc*p* was modified by $–SO_3H$. The carbon signals at 64.206 ppm were significantly weakened, suggesting hydroxyl groups at C-6 of →2)-α-Man*p*-(6→ were substituted by sulfate groups. When the carbon atom was directly attached to an electron group, its signal peak would move to a lower field position in NMR spectrum.[18,25] Therefore, the signal at 79.901 ppm and 80.170 ppm, which were respectively generated by the chemical shifts of C-3 in →2)-α-Man*p*-(6→ and →1)-β-D-Glc*p*-(6→ of LEP-1 in Fig. 3B,

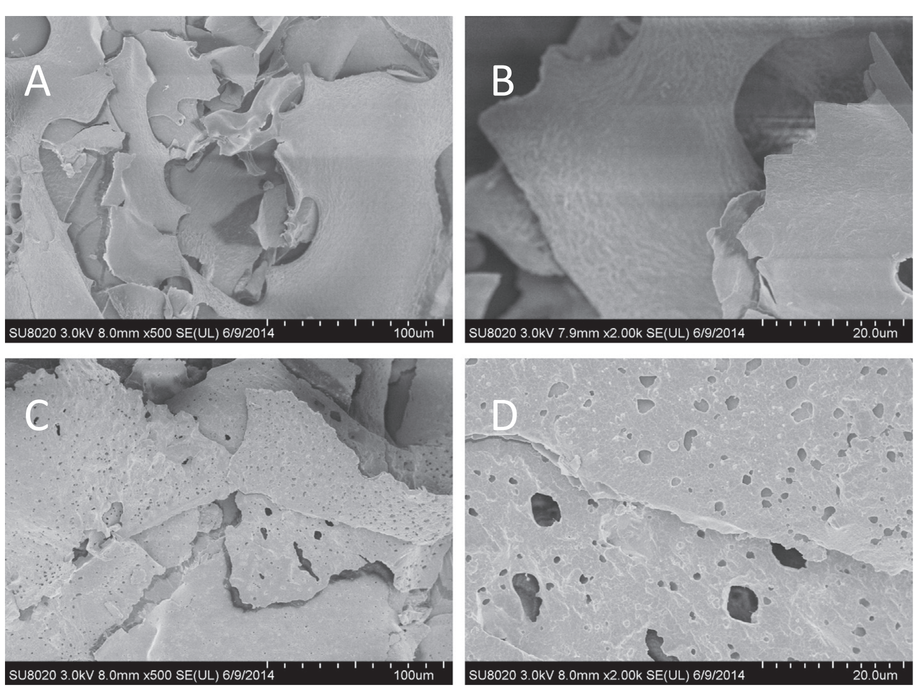

Figure 1. Scanning electron micrographs of LEP-1 and SLEP-1: A. LEP-1 (500×), B. LEP-1 (2.00k×), C. SLEP-1 (500×), D. SLEP-1 (2.00k×).