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Sulfation of Chinese lacquer polysaccharides in different solvents

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Abstract

A branched ionic polysaccharide isolated from the sap of the Chinese lac tree (*Rhus vernicifera*) was chemically modified by sulfation using sulfur trioxide–pyridine (SO_3·Py) complex as a reagent. Effects of molar ratio of SO_3·Py complex to sugar unit, reaction time and reaction temperature on degree of sulfation (DS) and molecular weights of products were studied. Solvent was another important factor affecting sulfation reaction. In different solvents, when the other conditions remained constant, DS and molecular weights were in the following order: DMSO > DMF > FA (formamide) and DMSO < DMF < FA, respectively. The results depended on the nucleophilicity of the solvents used. The structures of sulfated polysaccharides were investigated by GPC, UV–Vis, FT-IR and FT-Raman spectroscopy. The results indicated that unsaturated bonds and anhydrogalactose residues were formed in lacquer polysaccharides due to the strong dehydration of SO_3·Py complex. Based on these, we deduced that degradation of polysaccharide in the sulfation reaction process involved both dehydrolysis and hydrolytic degradation.

Keywords: Sulfation; Lacquer polysaccharide; Solvent; Degradation mechanism

1. Introduction

Sulfation of polysaccharides has been reported for a variety of polysaccharides such as fucoidan (Soeda, Kozako, Iwata, & Shimeno, 2000), curdlan (Alban & Franz, 2000), fucan (Nishino & Nagumo, 1992), chitosan (Baumann & Faust, 2001), dextran and pullulan (Mähner, Lechmer, & Nordmeier, 2001). The reagents used were mainly $ClSO_3H$ in pyridine (Py) (Geresh, Mamontov, & Weinstein, 2002), piperidine *N*-sulfonic acid (Yoshida, Nakashima, Yamamoto, & Uryu, 1993) or sulfur trioxide complexes with pyridine (Wu et al., 1998), triethylamine (Soeda et al., 2000) or DMF (Alban, Schauerte, & Franz, 2002). The solvents used were usually formamide (FA), DMF, DMSO and pyridine. However, due to the structural complexity of polysaccharides, one sulfation method resulting in predictable derivatives of a certain polysaccharide was not easily applicable to another polymer. In addition, the different biological activities of sulfated polysaccharides, such as anticoagulation (Alban et al., 2000; Huynh, Chaubet, & Jozefonvicz, 2001), antithrombotic activities (Buchanan & Brister, 2000; Mourão et al., 2001), antiviral activities (Amornrut et al., 1999), antiinflammatory (Winkelhake, 1991), antiangiogenetic (Paper, Vogl, Franz, & Hoffman, 1995), antiproliferative (Hoffman & Paper, 1993) and so on, would strongly depend on their structure, i.e. the degree of sulfation (DS) (Alban et al., 2002), the molecular weight (MW) (Barbucci, Lamponi, Magnani, & Renier, 1998) and the glycosidic branchings (Mulloy, Mourão, & Gray, 2000; Yoshida et al., 1993). Therefore, it is important to establish an appropriate sulfated method for a certain polysaccharide.

Lacquer polysaccharide (LP) from the sap of lac tree (*Rhus vernicifera*) which was found to have bioactivities in motivating the growth of leucocytes (Zhang & Ding, 1995) and antitumor (Lu et al., 2000), is an acidic polysaccharide with a 1,3-β-linked-D-galactopyranosidic main chain having complex branches with 4-*O*-methyl-β-D-glucuronic acid in the terminal, containing D-galactose (~66 mol%), 4-*O*-methyl-β-D-glucuronic acid (~24 mol%), D-glucuronic acid (~3 mol%), L-rhanose (~5 mol%) and L-arabinose (~3 mol%) (Du, Yang, Kong, & Xiao, 1999; Oshima & Kumanotani, 1984). The branching heteropolysaccharide feature allows the preparation of sulfated compounds that

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would be difficult to obtain by a synthetic approach. Although sulfation of LPs was reported by Lu et al. (2000), few papers involved the influence of reaction temperature, reaction time and the amount of reaction reagent on the structure of the obtained products. In this work, these factors affecting sulfation of LPs were studied with sulfur trioxide–pyridine complex (SO_3·Py) as sulfation reagent. In addition, polysaccharides usually show different solubility and conformation in different solvents (Azis, Chin, Deacon, Harding, & Pavlov, 1999; Kaliannan, Gromiha, & Elanthiraiyan, 2001; Srivastava & Kulshreshtha, 1989; Striegel, 1997), which should affect the sulfation of polysaccharides. This is due to different intermolecular interaction. In our knowledge, there are no reports on the influence of solvents on the sulfation of polysaccharides in details. Here, the influence of three solvents (DMSO, DMF and FA) on sulfation was also studied. The structures of sulfated polysaccharides were also investigated by GPC, UV–Vis, FT-IR and FT-Raman spectroscopy. Based on the results of analysis, degradation mechanism was discussed.

2. Experimental

2.1. Materials

The sap of Chinese lac tree from Maoba in Hubei province was supplied by Wuhan Chinese lacquer factory (Wuhan). The isolation process of Maoba lacquer was based on the process described in the literature (Du, Kong, & Li, 1994; Zhang, Qiu, Xu, Du, & Qian, 1992). The final LP (Mw 6.85×10^4) was obtained through the separation of 100 kDa ultrafiltration membrane. Sulfur trioxide–pyridine complex (SO_3·Py) was purchased from Tokyo Kasei Kegyo Co., Ltd (Tokyo, Japan). DMF, DMSO and FA were first treated with 5 Å molecular sieve to remove water, then, were distilled under reduced pressure. All other chemical used were of analytical grade.

2.2. Sulfation

Sulfation of LP was performed as follows: the LP powder (162 mg) was dissolved or suspended in dry solvent (16.2 ml) and the mixture was stirred at reaction temperature used for 30 min, then SO_3·Py complex was added. Various molar ratio of SO_3·Py complex to sugar residues and reaction duration were used. After reaction, the mixture was cooled to room temperature by an ice bath, neutralized with 15% NaOH solution, and then dialyzed for 120 h with distilled water in dialysis tubing. The dialysate was concentrated under reduced pressure below 45 °C, then, precipitated with anhydrous alcohol. The precipitate was collected after drying over phosphorus pentoxide in vacuum.

2.3. Characterization

The number average molecular weight (Mn) and weight average molecular weight (Mw) of sample were measured by a gel permeation chromatography (GPC). GPC system incorporated a TSP P100 instrument. Simple column (TSK G3000-pw) was used. The eluent was 0.01 mol l^{-1} 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 RI 150 refractive index detector. The sample concentration was 0.4 mg/ml. The standards used to calibrate the column were TOSOH pullulan of defined Mw ranging from 2.7 to 112 kDa. All data provided by the GPC system were collected and analyzed using the Jiangshen Workstation software package.

Uronic acid was determined colorimetrically using the method described in the literature (Bitter & Muir, 1962) with a Simadzu 1601 UV–Vis spectrophotometer. A calibration curve was constructed with glucuronic acid as standard. Sulfated content was determined colorimetrically by Antonopoulos' method (Antonopoulos, 1962). A calibration curve was constructed with sodium sulfate as standard.

UV–Vis absorption spectra were obtained with dilute aqueous solution on a Simadzu 1601 UV–Vis 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.

FT-Raman spectrum of the powder sample was recorded in 5 mm diameter glass NMR tube on Nicolet 670 FT-IR and FT-Raman on-line spectrophotometer with a Nd/YAG laser emitting at 1064 nm as excitation source. Power at the sample was maintained at 500 mW. An average of 80 scans was recorded at a resolution of 8 cm^{-1}.

3. Results and discussion

3.1. Solution behavior of lacquer polysaccharides in different solvents

The solvent in which the polysaccharide is immersed plays an important role in chemical reaction. LP is an acidic polysaccharide. In our studies, the uronic acids in LP exist in the form of sodium salt. The polysaccharides dissolve easily in water. The solubility parameter (δ) and polarity fraction (P) of H_2O are 23.2 and 0.819, respectively. LP is a non-crystalline state polar polymer. For this polymer, based on modified Hildebrand's formulation, only when the δ and P of the solvents approximate to those of the polymer, can the solvents make the polymer dissolve (He, Chen, & Dong, 1998). So the δ and P of LP approximate to that of water. The δ of FA, DMSO and DMF are 17.8, 13.4 and 12.1, and their polarity fractions are 0.880, 0.813 and 0.772, respectively (He et al., 1998). Thus, the capability to dissolve LP could be in the order of FA > DMSO > DMF.

In fact, LPs can be dissolved to form a homogeneous solution in formamide and are insoluble in two aprotic polar solvents, DMF and DMSO. But powder LPs can be swollen to be transparent in DMSO and remain unchanged in DMF. Therefore, it is obvious that intermolecular forces contributing to solvent–polysaccharide interaction are in the order of FA > DMSO > DMF.

3.2. Effects of various reaction conditions on degrees of sulfation

Sulfate group takes an important role in the bioactivities of polysaccharides. DS of polysaccharides is also an important parameter for the bioactivities. In general, the bioactivities (e.g. anticoagulant activity) of sulfated polysaccharides are improved with increasing DS. So it is interesting to test the effects of molar ratio of SO_3·Py complex to sugar unit, reaction time and reaction temperature on the DS of LPs in the FA, DMSO and DMF, respectively.

Fig. 1 showed the influence of the molar ratio of SO_3·Py complex to sugar unit on DS. For every molar ratio, the reaction was conducted at 60 °C for 3 h. Irrespective of in DMSO or in DMF, DS of products increased with the molar ratio of SO_3·Py complex to sugar unit. DS was apparently proportional to the amount of SO_3·Py complex in DMSO, but was not in DMF. In DMF, only when the molar ratio reached 4, did DS rapidly increase. And DS of polysaccharide in DMSO was always higher than that in DMF. However, effects of reaction time and reaction temperature were different from that of the molar ratio (Figs. 2 and 3). The increase of reaction time and reaction temperature may contribute to the higher DS. For the sulfation of LP, as expected, DS increases with reaction time, up to 3 h, and with reaction temperature, up to 60 °C in DMSO and 80 °C in DMF, respectively. However, longer reaction time and higher temperature reduced the DS value. In the reaction system that FA acted as the solvent, DS of products increased very slightly with increasing of reaction time and temperature. When the temperature was up to 140 °C, DS was only 0.159. The results also suggested that effects of solvents were prominent in the sulfation.

In general, homogenous reaction was more rapid than heterogeneous reaction, however, it was obvious that for the sulfation of LP with the SO_3·Py complex, when the other reaction conditions remained constant, DS gradually decreased in different solvents by the following order: DMSO, DMF, FA. The results were not consistent with intermolecular forces contributing to solvent–polysaccharide interaction, but they were strongly dependent on the solvent effects in the sulfation reaction process. The studies with regard to the mechanism of sulfation reaction had showed that the controlling transition state has only weak N–S and O–S bonds and is symmetrical. Sulfate group transfer between nitrogen of SO_3·Py complex and oxygen is consistent with a concerted ‘in line’ sulfate group transfer or

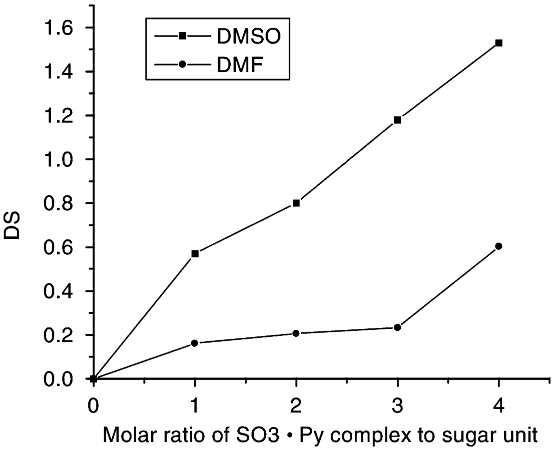

Fig. 1. Influence of the molar ratio of SO_3·Py complex to sugar unit on DS at 60 °C for 3 h.

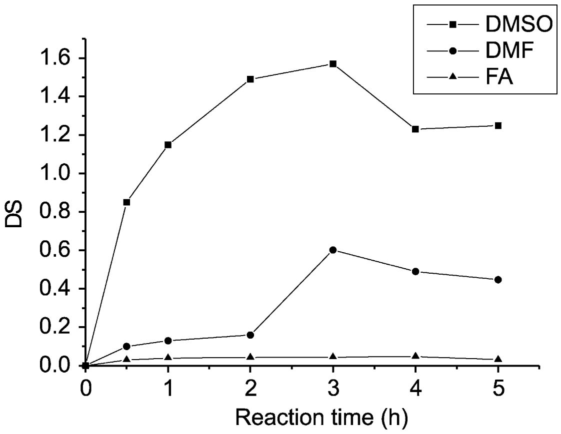

Fig. 2. Effects of reaction time on DS with 4:1 molar ratio of SO_3·Py complex to sugar unit at 60 °C.

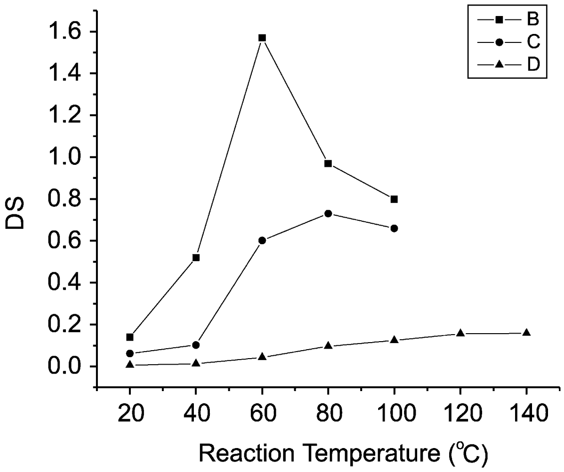

Fig. 3. Effect of reaction temperature on DS with 4:1 molar ratio of SO_3·Py complex to sugar unit for 3 h.

an open ‘exploded’ transition state (Hopkins, Day, & Williams, 1983). Thus the strong nucleophilicity of the oxygen atoms in the polysaccharides was necessary for high DS.

The nucleophilicity of three solvents used in the experiments is in the order of DMSO > DMF > FA (Reichardt, 1979). When polysaccharides were pretreated with the solvents, the hydrogen atom was pulled apart from the hydroxy group. The degree of deviation was in the order of DMSO > DMF > FA. So in DMSO, the oxygen atom of hydroxy group in the sugar residues had the highest electron density and the strongest nucleophilic ability, and easily attacked the electropositive sulfur atom of $SO_3 \cdot Py$ complex. In FA, the oxygen atom had the lowest electron density. At the same time, there are two active hydrogen atom linked to the nitrogen atom in FA. They could also form hydrogen bond with the oxygen atom of hydroxy group in the sugar residues. This lowered the nucleophilicity of the oxygen atom, and made it difficult to attack the electropositive sulfur atom of $SO_3 \cdot Py$ complex. Thus sulfation of polysaccharide in a homogeneous system did not favor the efficacy of substitution. In addition, the reactive activity was also related to the molar volumes of the solvents. The bigger molar volume of DMF compared with that of DMSO blocked the reaction between the hydroxy group and $SO_3 \cdot Py$ complex. So selecting an appropriate solvent was very important for high DS.

3.3. Change in molecular weights during the sulfation

The molecular weight of sulfate polysaccharide is another important parameter influencing the bioactivities. In the sulfation reaction process of polysaccharides, degradation was usually accompanied. Thus, to maintain the macromolecular features of the original polysaccharides during the sulfation reaction is an important research field.

Fig. 4 showed effect of the molar ratio of $SO_3 \cdot Py$ complex to sugar unit on Mw at 60 °C for 3 h. In DMSO, with the increase of unit molar ratio, LP degraded rapidly. When the molar ratio was 4, the Mw dropped to 2.58×10^4. However, in DMF, the Mw seldom changed, only when the molar ratio attained 4, was the Mw slightly reduced to 6.47×10^4. The rate of degradation of LPs was consistent with the nucleophilicity of the solvents used.

Fig. 5 showed effect of reaction time on Mw when 4:1 molar ratio of $SO_3 \cdot Py$ complex to sugar unit was used in all cases at 60 °C. In DMSO, with the increase of reaction time, Mw gradually decreased, and in the first hour, the Mw decreased promptly. It could be because the concentration of $SO_3 \cdot Py$ complex was higher in the primary stage, or the sulfated polysaccharide with high DS was difficult to be degraded. In the reaction process, the content of uronic acid was also investigated. Fig. 6 showed the correlation between the content of uronic acid and reaction time. The content of uronic acid, decreased in the beginning of 0.5 h, and increased from 1.0 to 2 h, then gradually decreased. Because the glycosidic linkage related to the uronic acid was difficult to be cleavaged, and the uronic acid was in the terminal of the side chains, these suggested that the distribution of the side chains could be heterogeneous along the main chains, and the structure of original polysaccharide changed in the sulfation. In DMF, with the increase of reaction time, Mw only slightly decreased. In FA, the Mw slightly increased.

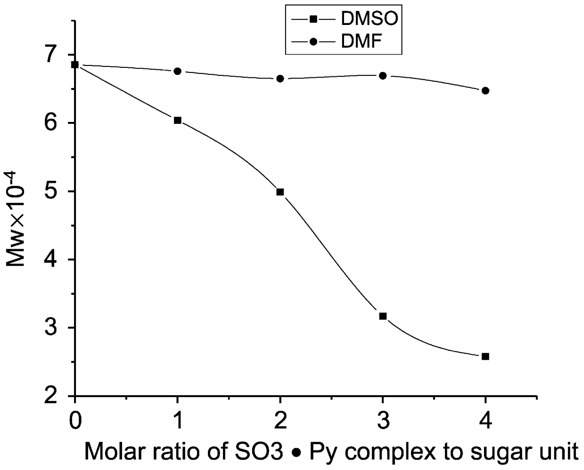

Fig. 4. Effect of the molar ratio of $SO_3 \cdot Py$ complex to sugar unit on Mw at 60 °C for 3 h.

The influence of temperature (ranging from 20 to 140 °C) on Mw was also investigated (Fig. 7). For every reaction temperature, the reaction was conducted with 4:1 molar ratio of $SO_3 \cdot Py$ complex to sugar unit for 3 h. In DMSO, when reaction temperature was less than 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 8200. In DMF, when the temperature reached 60 °C, the Mw only slightly decreased. But when the temperature was higher, degradation was severe. At 100 °C, the Mw was 1.14×10^4. In FA, at 100 °C, the sulfated polysaccharide has highest Mw, and the Mw of the product was higher than that of the original polysaccharide. When the temperature was higher than 100 °C, the Mw gradually decrease. The extent of degradation was

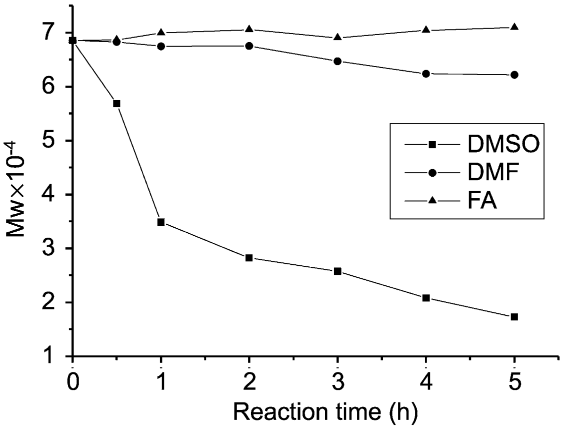

Fig. 5. Effect of reaction time on Mw with 4:1 molar ratio of $SO_3 \cdot Py$ complex to sugar unit at 60 °C.

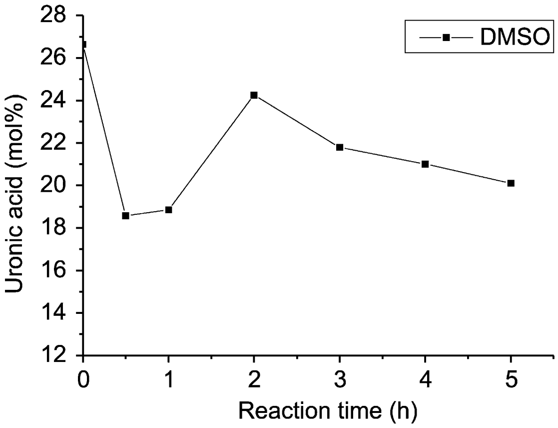

Fig. 6. Correlation between the content of uronic acid and reaction time with 4:1 molar ratio of SO_3·Py complex to sugar unit at 60 °C.

slight. The data clearly demonstrated that high temperature enhanced the rate of degradation. Thus reaction temperature was a major factor resulting in the degradation of polysaccharides.

Based on the results regarding Mw and DS in various reaction conditions, it was also obvious that highly sulfated polysaccharides always had low molecular weights. So degradation and sulfation were a group of competing reactions. Seen in Figs. 2 and 3, after DS reached the maximum, with prolongation of reaction time and increase of reaction temperature, DS dropped gradually. It could be because highly sulfated sugar residues were cut off. If the products with high DS and Mw are demanded, control of the reaction conditions will be a major aspect.

3.4. Degradation mechanism

At present, the degradation mechanism was regarded as hydrolysis, so the removal of residual water became a necessary operating step in the sulfation of polysaccharides. However, Vogl, Paper, and Franz (2000) found that the molecular weight of product was not affected by the additional drying. Therefore, it seems unlikely that the low molecular weight is due to a hydrolytic process. In our experiment, we found that the color of the sulfated polysaccharide gradually changed from slight yellow to deep brown with the decrease of the molecular weight. Fig. 8 showed the UV–Vis absorption spectra of LP and LP-S. LP-S was a sulfated polysaccharide prepared with 4:1 molar ratio of SO_3·Py complex to sugar unit in DMSO at 60 °C for 4 h. In the spectrum of LP-S, two new broad absorption bands appeared at 258 and 373 nm. The former was due to $n \rightarrow \pi^*$ transition of sulfate, the latter could be due to the unsaturated bond formed in the sulfation process.

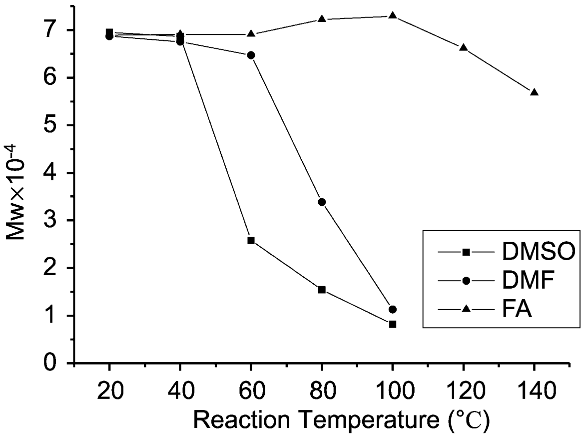

Fig. 7. Influence of temperature on Mw with 4:1 molar ratio of SO_3·Py complex to sugar unit for 3 h.

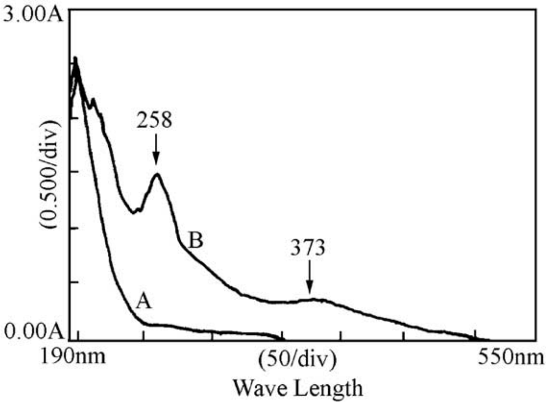

Fig. 8. UV–Vis spectra of LP (A) and LP-S (B).

Fig. 9 showed the FT-IR spectra of LP and LP-S. In the spectrum of LP, the absorb band at 1613 cm^{-1} was due to asymmetrical COO$^-$ stretching vibration, the absorb band at 1421 cm^{-1} was due to symmetrical COO$^-$ stretching vibration. In the spectrum of LP-S, the two band disappeared, and a new band appeared at 1734 cm^{-1} originating from C=O stretching vibration. The band disappeared when sulfated polysaccharides were treated with dilute NaOH solution, which indicated that it was due to the C=O stretching vibration from carboxylic acid. The spectrum of LP-S also showed two characteristic absorption bands, one at 1258 cm^{-1} describing an asymmetrical S=O stretching vibration and the other at 815 cm^{-1} with

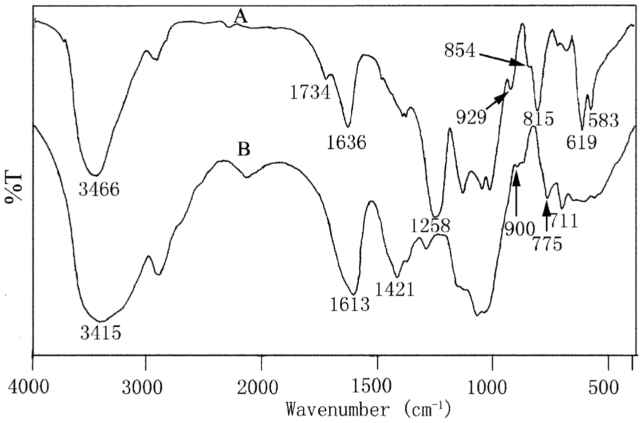

Fig. 9. FT-IR spectra of LP (B) and LP-S (A).

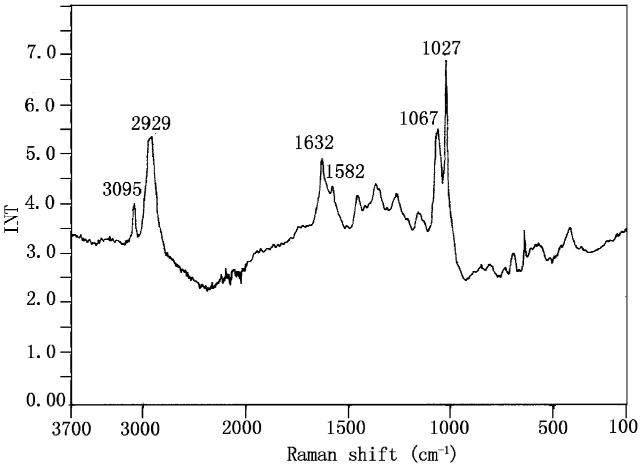

Fig. 10. FT-Raman spectrum of LP-S.

a discernible shoulder at 854 cm^{-1} indicating a symmetrical C–O–S vibration associated to a C–O–SO_3 group. Besides these, a new band appeared at 1636 cm^{-1}. It could be related to the unsaturated bond formed in the sulfation process, and could be due to the band of O–H deformation vibration of H_2O at about 1640 cm^{-1}, too (Chatjigakis et al., 1998). Fig. 10 showed the FT-Raman spectrum of LP-S. In the FT-Raman spectrum, the absorption band still occurred at 1632 cm^{-1} and there was also an absorption band at 3095 cm^{-1}, indicating the presence of the unsaturated bond (Edwards, Falk, Sibley, Alvarez-Benedi, & Rull, 1998). Because SO_3 has the strong dehydration, under such a reaction condition, it was possible that the polysaccharide with hydroxy groups was dehydrated. In addition, in the FT-IR spectrum of LP-S, another new absorption band occurred at 929 cm^{-1} indicating the presence of AnGal residues (Liao et al., 1996). This further confirmed that dehydrolysis happened in the sulfation of LP. At the same time, Alban et al. (2002) found that when the sulfated products of pullulan were resulfated, further degradation was not observed. This could contribute to the decrease of the hydroxy groups in the sulfated pullulan. On account of dehydrolysis, hydrolytic degradation of polysaccharides was inevitable during the sulfation. Therefore, degradation of polysaccharide in the sulfation reaction process likely involved both dehydrolysis and hydrolytic degradation.

4. Conclusion

In the sulfation of LPs, it was very important to choose an appropriate solvent. In DMSO, highly sulfated polysaccharides were obtained. However, the products with high DS were often accompanied with low Mw. Based on the experimental results, degradation of polysaccharides involved dehydrolysis of SO_3·Py complex, so it was a major operating step to control the reaction temperature. If sulfated polysaccharides with high DS and Mw were demanded, the appropriate temperature range would be 40–60 °C. In DMF, sulfated polysaccharides with moderate DS and Mw could be obtained. Optimum reaction temperature range was 60–80 °C. In FA, highly sulfated products were difficultly prepared using SO_3·Py complex as a sulfate reagent. Previous reports indicated that high degrees of sulfation could be reached with $ClSO_3H$ in FA (Mähner et al., 2001). In our laboratory, preliminary experiments indicated that the incorporation of sulfate groups into LP was successful with $ClSO_3H$ in FA under mild reaction conditions, and Mw of original polysaccharide could be basically retained. Further studies are currently well under way.

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