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Chemical modification and antitumour activity of Chinese lacquer polysaccharide from lac tree *Rhus vernicifera*

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Abstract

Lacquer polysaccharide from *Rhus vernicifera* was separated into two fractions with high and low molecular weights, LPH and LPL. LPL was degraded using dilute sulphuric acid. Besides molecular weight, products had only a little change in their contents of α-L-rhamnopyranose, α-L-arabinofuranose and α-D-galactopyranose. The side chains of LPH and LPL were partially removed with $NaIO_4$ oxidation. The structures of all polysaccharides were investigated by GPC, FT-IR, ^1H and ^{13}C NMR spectroscopy. S-180 ascites tumour in mice was used to assay the antitumour activity of lacquer polysaccharide and its derivatives using two doses (150 and 300 mg/kg/d). The antitumour activity was related to M_w and the branching structure of polysaccharide. Natural polysaccharides had significant activity, and LPL had the highest activity, its inhibitory rate by intraperitoneal injection for 7 d using a 150 mg/kg/d dose reached 63.5%. Decrease of M_w and removal of side chains led to reduction or loss of antitumour activity. Experimental results also showed that the low dose group had higher activity, and the bloody ascites was prevented by the polysaccharide groups.

Keywords: Lacquer polysaccharide; Chemical modification; Antitumour activity

1. Introduction

In recent years, studies of the antitumour of polysaccharides have been of particular interest. Lentinan and schizophyllan, which were (1→3)-β-D-glucans with the side chains at O-6, had significant antitumour activity (Bohn & BeMiller, 1995). Some acid heteropolysaccharides such as sodium alginate extracted from *Sargassum fulvellum* and pectic polysaccharides from *Feronia limonia* could also strongly inhibit tumour cell growth (Fujihara & Nagumo, 1992; Saima, Das, Sarkar, Sr, & Sur, 2000). It has been suggested that the activity of polysaccharide is strongly dependent on their structures, i.e. molecular weight, degree of substitution, the structure of main chain and branches, degree of branching, sugar components, mode of linkage, and high-order structures (Bohn & BeMiller, 1995; Kulicke, Lettau, & Thielking, 1997). However, the relationships between the structure and the activities are still ambiguous. Thus, at present, more effort is being expended in seeking to elucidate structure–activity relationships of antitumour polysaccharides.

Lacquer polysaccharide from the sap of lac tree (*Rhus vernicifera*) is an acidic heteropolysaccharide with a 1,3-linked β-D-galactopyranose 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-rhamnose (~5 mol%) and L-arabinose (~3 mol%). Terminal residues may occasionally be replaced by α-L-rhamnopyranose, β-D-glucopyranuronic acid, α-D-galactopyranose and α-L-arabinofuranose (Du, Kong,

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& Li, 1994; Du, yang, Kong, & Xiao, 1999; Oshima & Kumanotani, 1984). This water-soluble special structural polysaccharide had bioactivities in motivating the growth of leucocytes (Du et al., 1999; Zhang & Ding, 1995) and against leukopenia induced cyclophosphamide (Yang & Du, 2003). Lu et al. (2000) reported that lacquer polysaccharide also had the activity inhibiting the growth of the solid Sarcoma 180 tumour. Different from lentinan, lacquer polysaccharide had the antitumour activity not only by an intraperitoneal injection, but also by an oral administration. The relationship between special structure and activity was interesting. However, so far, the structure–activity relationship of lacquer polysaccharide was unclear yet. In this paper, lacquer polysaccharide was fractionated, and tailored using $NaIO_4$ oxidation and the dilute acid hydrolysis. The effect of the structures of lacquer polysaccharide, i.e. M_w, branches and sugar residues on ascitic Sarcoma 180 tumour cell growth inhibition was investigated.

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 is based on the process described in the literature (Du et al., 1994). Lacquer polysaccharide obtained was separated into two fractions (LPH and LPL) by a 100 kDa ultrafiltration membrane (Table 1). D-Glucuronic acid was purchased from Sigma Chemical Co. (St Louis, USA). All other chemicals used were of analytical grade.

2.2. Acid hydrolysis of lacquer polysaccharide

LPL was acid hydrolyzed in 0.05 M sulphuric acid at 90 °C for 2, 4 or 6 h. The reaction mixture was cooled to room temperature by an ice bath, and neutralized with 0.5 M sodium hydroxide, then dialyzed for 120 h against 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 penta-oxide under reduced pressure. The products were coded as LPL-2h, LPL-4h and LPL-6h, respectively.

Table 1
Contents of uronic acid and molecular weights of natural and modified lacquer polysaccharides

Polysaccharide	Molecular weight			Uronic acid (mol%)[a]
	$M_n\times10^{-4}$	$M_w\times10^{-4}$	PD[b]	
LPL	4.91	6.85	1.39	26.23
LPH	12.4	16.9	1.36	25.47
LPL-2h	3.09	4.67	1.51	28.08
LPL-4h	2.28	3.43	1.50	27.73
LPL-6h	2.00	2.98	1.49	27.47
LPLde	2.68	4.40	1.64	6.43
LPHde	7.11	11.18	1.57	6.10

[a] The total content of 4-*O*-methyl-D-glucuronic acid and D-glucuronic acid.
[b] Polydispersity, $PD=M_w/M_n$.

2.3. $NaIO_4$ oxidation of lacquer polysaccharides

Lacquer polysaccharide is an acidic polysaccharide that has a 1,3-linked β-D-galactopyranose main chain. Thus 1,6-linked side chains of LP may be cut off by sodium periodate oxidation. In our experiments, LPL and LPH were treated with sodium periodate oxidation. The experimental process was performed by Chihara's method (Chihara, Hmuro, Maeda, Arai, & Fukuoka, 1970).

2.4. Characterization

The number average molecular weight (M_n) and weight average molecular weight (M_w) of samples were measured by a gel permeation chromatography (GPC). GPC system incorporated in a TSP P100 instrument (USA). Two columns in series (TSK G5000-pw and TSK G3000-pw, Japan) were used. The eluent was 0.01 mol/l phosphate buffer solution containing 0.2 mol/l $NaSO_4$. 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 TOSHO pullulan of defined M_w ranging from 2.7 to 788 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 Simadz 1601 UV spectrophotometer (Japan). A calibration curve was constructed with D-glucuronic acid as standard.

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

^1H and ^{13}C NMR spectra were recorded on Bruker AMX-500 NMR (Germany) and Varian Mercury NMR-600 spectrometers (USA) at ambient temperature. The samples were dissolved in D_2O. Tetramethylsilane (TMS) was used as internal standard.

2.5. Assays of antitumour activities

At least 10 mice were used for each experiment. Mean values and SD were determined by standard methods. Significance of difference was estimated by the standard Student's *t*-test. Compared with the corresponding control group, significance levels were denoted as $^*P<0.05$, $^{**}P<0.01$.

Antitumour effects on ascites S-180 were assayed in normal Kunming mice (Body weight 20 ± 2 g). The test were made by observing the effect on the growth of the tumour in ascite form of a dose of 0.2 ml ($\sim 1 \times 10^7$ cells/mice) implanted subcutaneously at the abdomen of mice. After 24 h of the tumour implantation, the test samples dissolved in saline were provided once a day by intraperitoneal injection for 7 d, while 0.9% saline was provided for the control group. The mice were sacrificed on day 8 and the tumours were weighed. The rate of inhibition was calculated by using the following formula: Inhibition rate (%) $= 100 \times (C - T)/C$, where C is the average tumour weight of control group and T is the tumour weight of the treated sample group.

3. Results and discussion

3.1. Acid hydrolysis of lacquer polysaccharide and characterization

Lacquer polysaccharide is an acid heteropolysaccharide and has a complicated succession of *O*-glycosidic linkages. For these *O*-glycosidic linkages, their rate constants of hydrolysis are different, so the hydrolysis process of lacquer polysaccharide is complicated. Depending on the control of the reaction conditions, hydrolyzates with different molecular structures could be obtained. LPL was hydrolyzed using dilute sulphuric acid at 90 °C. Depending on different reaction times, the products with different M_w were obtained (Table 1). The M_w of the products decreased with the increase of reaction time, but the content of uronic acid in the polysaccharides increased slightly. This could be because the glycosidic linkages related to uronic acids were difficult to be hydrolyzed. The polydispersity of the hydrolyzates also only increased slightly. This indicated that the degradation of polysaccharide was homogeneous in an aqueous system.

Fig. 1 showed the FT-IR spectra of LPL, LPL-2h and LPL-6h. The profiles of the spectra of LPL, LPL-2h and LPL-6h were similar, and indicated these polysaccharides should have the similar structures. In these spectra, the absorbance band at 1616 cm^{-1} was due to asymmetrical COO^- stretching vibration, the absorbance band at 1419 cm^{-1} was due to symmetrical COO^- stretching vibration. Compared with the spectrum of LPL, the absorbance of these bands was almost unchanged, indicating that the content of uronic acids basically was not changed. In the FT-IR spectrum of LPL, the bands at about 1160–1030 cm^{-1} are dominated by the glycosidic linkage ν (C–O–C) contribution. The bands at 1155 and 1078 cm^{-1} belong to galactopyranose in the backbone and the side chains. The band at 1048 cm^{-1} is related to the arabinose and rhamnose constituents in the polysaccharides (Kačuráková, Capek, Sasinková, Wellner, & Ebringerová, 2000). Compared with the spectrum of

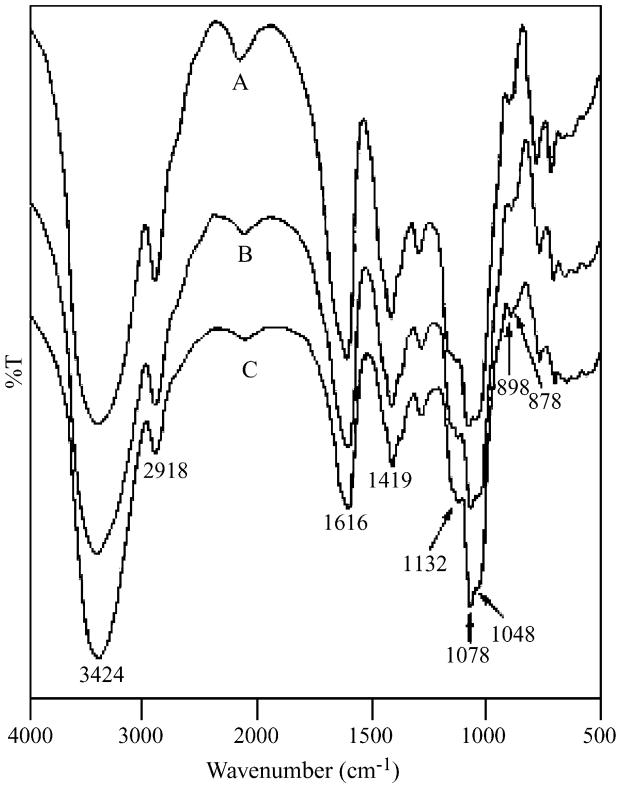

Fig. 1. FT-IR spectra LPL and its hydrolysis products (A) LPL, (B) LPL-2h, (C) LPL-6h.

LPL, the absorbance at 1048 cm^{-1} reduced gradually with the increase of the hydrolysis time, indicating that α-L-arabinose and α-L-rhamnose residues in the side chains of lacquer polysaccharide were partially removed. In addition, in the FT-IR spectrum of LPL, the characteristic band at 898 cm^{-1} belonged to the β-anomeric configuration, the characteristic band at 878 cm^{-1} was due to the pyranoid ring (Synytsya, Čopíková, Matějka, & Machovič, 2003; Zhbankov, Adnànov, & Marchewka, 1997). In the spectra of LPL-2h and LPL-6h, these bands still remained, indicating that the β-anomeric configuration and the pyranose ring of lacquer polysaccharide was not changed in the hydrolysis process.

Fig. 2 showed ^{13}C NMR spectra of LPL, LPL-2h and LPL-6h. In the ^{13}C NMR spectrum of LPL, the signals at 111.8, 107.1, 106.3, 105.2, 103.3 and 102.8 ppm attributed to the anomeric carbons of α-L-arabinofuranose, β-D-glucopyranosyluronic acid, β-D-galactopyranose, 4-*O*-methyl-glucopyranosyluronic acid, α-L-rhamnopyranose and α-D-galactopyranose, respectively (Lu et al., 1999; Oshima & Kumanotani, 1984). In the ^{13}C NMR spectra of LPL-2h and LPL-6h, the signals at 111.8, 103.3 and 102.8 ppm still existed, but their integration became weak. This indicated that α-L-arabinofuranose, α-L-rhamnopyranose and α-D-galactopyranose in the lacquer polysaccharide were only partially removed.

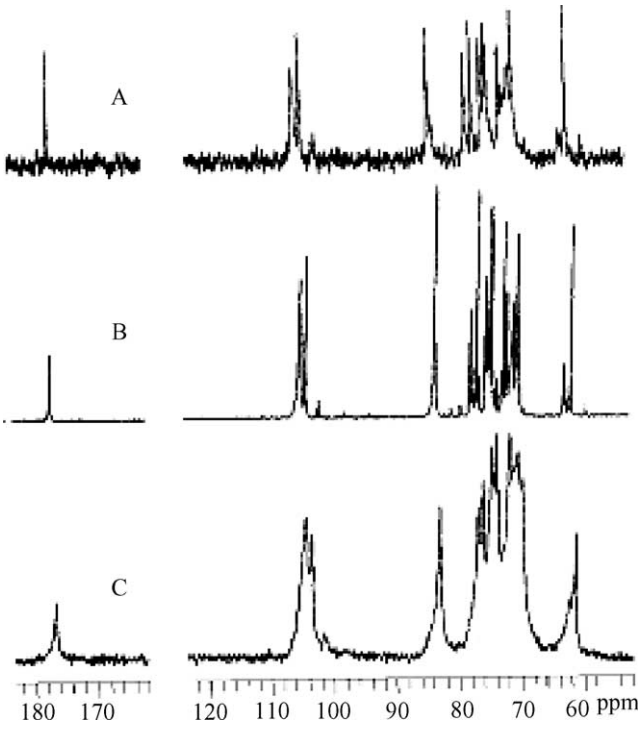

Fig. 2. ^{13}C NMR spectra of lacquer polysaccharide and its derivatives (A) LPL, (B) LPL-2h, (C) LPL-6h.

Fig. 3 showed ^{1}H NMR spectrum of LPL-2h, the peaks at 5.25 and 4.94 ppm were assigned to the H1 signals of terminal α-L-arabinofuranose and α-D-galactopyranose in the side chains, respectively. The double peaks at 1.20 ppm were assigned to the proton signals of the C-6 methyl group of terminal L-rhamnose residues (Lu et al., 1999). This indicated further that these terminal sugar residues still remained in the hydrolytic polysaccharides. In the hydrolysis process, α-L-arabinofuranose, α-L-rhamnopyranose and α-D-galactopyranose residues were not removed completely. This was because the α-D-glycosidic linkages were more difficult to hydrolysis (Ponder, 1998; Wu, 1987). This also suggested that for LPL and the hydrolysis products structures had few differences besides their molecular weights.

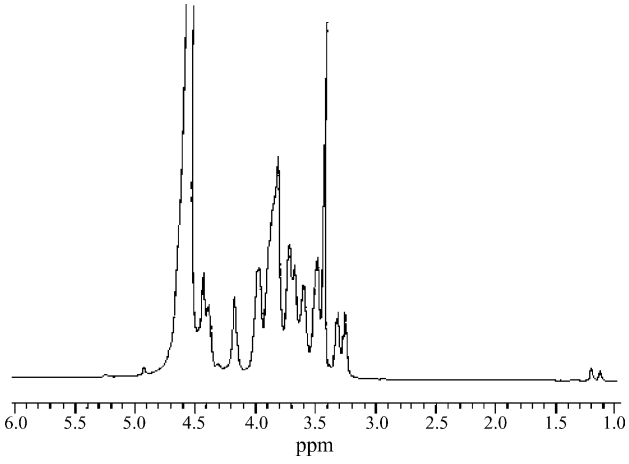

Fig. 3. ^{1}H NMR spectrum of LPL-2h.

3.2. Modification of side chains of lacquer polysaccharide and characterization

LPL and LPH were debranched by oxidative degradation using sodium periodate, reduction using sodium bonohydride and dilute sulphuric acid hydrolysis. The quantitative analysis showed that the content of uronic acid in the debranching polysaccharides, LPLde and LPHde, were 6.43 and 6.10 mol%, respectively, and were far less than those of LPL (26.23 mol%) and LPH (25.47 mol%) (Table 1). Because the uronic acids were at the terminal of the side chains, this indicated the side chains of lacquer polysaccharides were partially cut off. Removal of the side chains also resulted in the decrease of M_w. The M_w of LPLde was 4.40×10^4, and that of LPHde was 11.18×10^4.

Fig. 4 shows the ^{13}C NMR spectra of LPLde and LPHde. The anomeric carbons signals of α-L-arabinofuranose, β-D-glucuronic acid, α-L-rhamnopyranose and α-D-galactopyranose have disappeared. This suggested that these terminal sugar residues had been cut off. The signals at 61–64 and about 71 ppm were attributed to the primary hydroxyl groups. The integration of the signal of unlinked C-6 at 61–64 ppm increased, and that linked C-6 at about 71 ppm decreased. These indicated further that side chains of polysaccharides were partially cut off. Fig. 5 showed the FT-IR spectra of LPLde, LPHde and LPH. In the spectra of LPLde and LPHde, the absorbance of the bands at 1610 and 1420 cm^{-1} attributed to asymmetrical and symmetrical

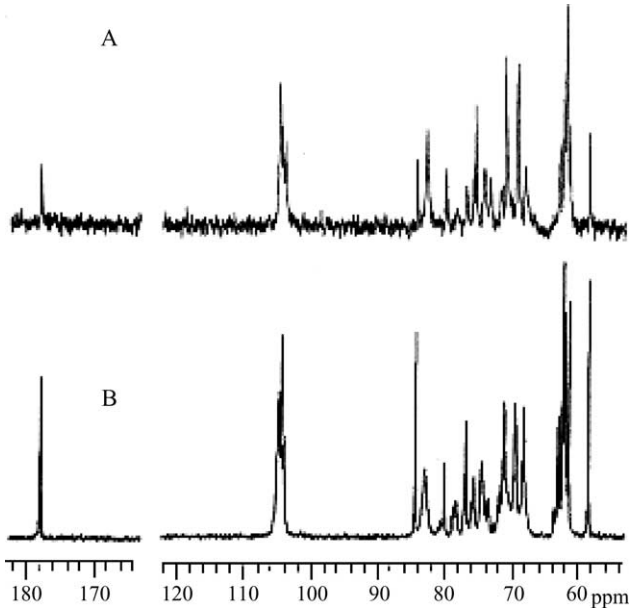

Fig. 4. ^{13}C NMR spectra of LPLde (A) and LPHde (B).

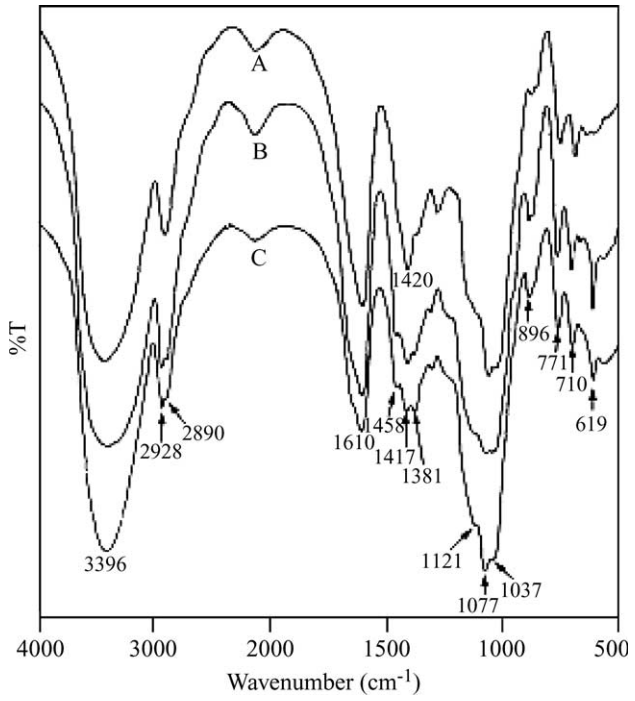

Fig. 5. FT-IR spectra of lacquer polysaccharide and its derivatives (A) LPH, (B) LPHde, (C) LPLde.

COO^- stretching vibration considerably weakened. This also indicated that most of the uronic acids residues were removed. The region of 1200–1000 cm^{-1} containing skeletal C–O and C–C vibration bands of glycosidic bonds and pyranoid ring changed, too. Especially, the band at 1048 cm^{-1} disappeared, and a new band at 1037 cm^{-1} appeared. This suggested that α-L-arabinose and α-L-rhamnose residues were removed. In addition, in the spectra of LPLde and LPHde, the characteristic bands at 896 and 878 cm^{-1} still remained. This indicated that the β-anomeric configuration and the pyranoid ring of lacquer polysaccharide were not changed in the modification process.

3.3. Antitumour activity

The antitumour activity of lacquer polysaccharide and its derivatives were assayed in ascitic Sarcoma-180 in mice. The results showed that LPH and LPL displayed significant antitumour activity with the two doses used. When the dose was 150 mg/kg/d, the inhibition rates of LPH and LPL reached 37.9 and 63.5%, respectively. However, when the dose was 300 mg/kg/d, those of LPH and LPL were 29.9 and 47.8%, and less than those of low dose. Besides the effect of the dose, M_w was also an important factor influencing the antitumour activity of the polysaccharides. (1→3)-β-D-Glucan, when the M_w is higher than 90,000, polysaccharide molecules appear to form helical structures in the aqueous solution and had high Sarcoma 180 growth-inhibiting activity (Bohn & BeMiller, 1995). It was also reported that a glucan with 20,000 of molecular weight exists in aqueous solution had no helical structures and did have significant antitumour activity (Blaschek, Schütz, Kraus, & Franz, 1987). LPH and LPL exist in aqueous solution as a dense random coil (Zhang et al., 1992). Although the M_w of LPH was higher than 90,000, its antitumour activity was less than that of LPL. This could be related to the special structure of lacquer polysaccharide.

LPL-2h, LPL-4h and LPL-6h were the products prepared using acid hydrolysis depending on the control of reaction time. They all exhibited the Sarcoma 180 growth-promoting activity at the 300 mg/kg/d dose, and had the antitumour activity at the 150 mg/kg/d dose. LPL-2h displayed the significant antitumour activity, but the inhibition rates of LPL-4h and LPL-6h were low. Their antitumour activity was also less than that of LPL. The order of the activity was in accord with the sequence of their M_w. It was similar to the antitumour activities of nonstarch polysaccharides (NSPs) from sclerotia of *Pleurotus tuber-regium*. The medium molecular weight fraction (M_w 9.8×10^4) of NSPs that has a random-coil chain conformation also exhibited the highest antitumour activities against Sarcoma 180 (Zhang, Cheung, & Zhang, 2001). In addition, the content and the type of sugar residues in the side chains also took important roles in the antitumour activity of the polysaccharides. Curdlan showed no antitumour activity. Curdlan modified with D-glucosyl, L-rhamnosyl and L-arabinosyl branch units significantly inhibited Sarcoma 180 tumours. However, only D-glucosyl modified lichenan showed significant antitumour activity; the other lichenan derivatives showed little or no antitumour activity and, in some cases, stimulated tumour growth (Bohn & BeMiller, 1995). For D-manno-D-glucan from *Microellobosporia grisea*, acid degradation decreased the antitumour activity, with increase in both the depolymerization and the elimination of the D-mannosyl groups (Inoue et al., 1983). The content of terminal α-L-arabinofuranose, α-L-rhamnopyranose and α-D-galactopyranose in the side chains of the LPL decreased gradually during the acid hydrolysis process. So these terminal sugar residues in the side chains could also be related to the antitumour activity of lacquer polysaccharide.

The degree of branching also took an important role in the antitumour activity of the polysaccharide. Lentinan showed significant Sarcoma 180 growth-inhibiting activity, and partially debranching lentinan with sodium periodate had higher activity (Sasaki & Takasuka, 1976). Pachyman that consists of a 1,3-linked β-D-glucan backbone with 1,6-linked β-D-glucopyranosyl units as branches showed no antitumour activity. However, after the branches were partially removed through oxidative degradation using sodium periodate and mild acid hydrolysis, modified polysaccharide (pachyman) exhibited high antitumour activity (Chihara et al., 1970; Narui, Takahashi, Kobayashi, & Shibata, 1980). LPLde and LPHde were the products in which the branches had been partially removed through oxidative degradation using sodium periodate and mild acid hydrolysis. The M_w of LPHde was higher than that of LPL,

Table 2
Antitumour activity towards ascites Sarcoma 180 (intraperitoneal administration) of natural and modified lacquer polysaccharides

Sample	Dose (mg/kg/d)	Tumour weight (mean ± SD)	Inhibition degree (%)
LPH	150×7	1.70 ± 0.77	37.9*
	300×7	1.92 ± 1.37	29.9*
LPL	150×7	1.00 ± 0.61	63.5**
	300×7	1.43 ± 1.10	47.8*
LPL-2h	150×7	1.40 ± 1.21	48.8*
	300×7	3.33 ± 2.56	−21.6
LPL-4h	150×7	2.19 ± 2.1	20.1
	300×7	3.71 ± 1.60	−35.4
LPL-6h	150×7	2.11 ± 1.62	23.1
	300×7	4.60 ± 2.74	−67.9
LPLde	150×7	4.14 ± 2.25	−51.1
	300×7	3.08 ± 2.63	−12.4
LPHde	150×7	4.26 ± 2.47	−55.7
	300×7	2.85 ± 2.19	−4.01
Control		2.74 ± 1.30	

*$P < 0.05$, **$P < 0.01$.

and the M_w of LPLde was less than that of LPL. The results from the antitumour assay showed that LPHde and LPLde stimulated tumour growth within the scope of the dose studied (Table 2). This indicated the branching structures of lacquer polysaccharide take a very important role in the antitumour activity. At the same time, these debranching products of lacquer polysaccharide, lentinan and pachyman displayed different antitumour activities. This could be related to their different main chains and spatial structures.

In addition, it was found that all mice formed the bloody ascites in the control group, but for all lacquer polysaccharide and its derivatives, the ascites was not bloody. The result further showed that lacquer polysaccharide and its derivatives were effective against ascitic Sarcoma 180 tumour cell. But the relationship between the phenomenon and the antitumour activity of polysaccharide was unclear.

4. Conclusion

This study of the effect of the structure of lacquer polysaccharide and of its derivatives on ascitic Sarcoma 180 tumour cell growth inhibition showed that lacquer polysaccharide has significant antitumour activity. The activity was strongly dependent on the M_w, branch structure and sugar residue types of the polysaccharide. The polysaccharide with medium M_w had the highest activity. The removal of side chains caused reduction or loss of tumour cell growth inhibition. But in all lacquer polysaccharide and its derivatives groups studied, the bloody ascites which appears in controls did not appear. This suggested that the antitumour activity of lacquer polysaccharide with the complex side chains was related to several pathways of action. At present, detailed works on mechanism of action are now in progress.

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