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Separation of lacquer polysaccharides and interaction with poly-L-lysine☆

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ABSTRACT

A naturally occurring acidic lacquer polysaccharide with glucuronic acid at the terminals of the complex branches has specific biological activities including promotion of blood coagulation and antitumor activities. The polysaccharide has two molecular weight fractions $\bar{M}_n = 10 \times 10^4$ and $\bar{M}_n = 3.0 \times 10^4$. In the present work, two pure fractions were isolated for the first time by Sephadex G-100 column chromatography. Then, each fraction was treated with diluted alkaline solution to decrease the molecular weights to $\bar{M}_n = 3.0 \times 10^4$ and $\bar{M}_n = 1.4 \times 10^4$, respectively. The NMR and IR spectra and specific rotations of the fractionated and original lacquer polysaccharides were almost identical, suggesting that the lacquer polysaccharides are an associated structure with several low molecular weight polysaccharides of $\bar{M}_n = 1.4 \times 10^4$. Interactions between each lacquer polysaccharide and poly-L-lysine, a model compound of proteins and peptides with positively-charged amino groups, were investigated by surface plasmon resonance (SPR) to elucidate the biological mechanism. The apparent dissociation-rate (k_d), association-rate (k_a), and dissociation constant (K_D) obtained by SPR indicate that the lacquer polysaccharides had weaker interactions with poly-L-lysine than sulfated polysaccharides and that the interaction depended on the molecular weight. These SPR results suggest that the specific biological activities of lacquer polysaccharides originate from electrostatic interaction.

1. Introduction

Naturally occurring lacquer (*urushi*) is the only natural product polymerized by the enzyme laccase; it has been used as a coating material for several thousand years in Japan and Asian countries (Lu, Yoshida, & Miyakoshi, 2013). The lacquer sap contains mainly urushiol (a general term for 3-alkenylcatechols), polysaccharides, and the enzyme laccase.

Fundamental and applied studies on natural lacquer have focused on the polymerization mechanism of urushiol, properties of the coating films, functionality of lacquer sap, and development of new lacquer paints. Kumanotani investigated the chemistry (*urushi*) for use in the future as a coating material (Kumanotani, 1995). The polymerization mechanism of urushiols was elucidated by the electron spin resonance (ESR) detection of a semiquinone radical and by the structure of the separated urushiol dimers (Oshima, Yamauchi, Watanabe, & Kumanotani, 1985). Natural lacquer requires a long time for complete polymerization; in general, it takes more than one month under humidity at 70% and a temperature around 25 °C. Miyakoshi et al. have developed hybrid lacquers that polymerize quickly at humidity below 50% and temperature below 20 °C by adding amine-functionalized organic silicone reagents to natural lacquer (Nagase, Lu, & Miyakoshi, 2004). As a coating material, the new natural lacquer with a precious metal colloid was found to give a beautiful and durable surface. The Au and Ag colloids produce lacquer films with homogeneous red and yellow colors, respectively (Lu, Ono, Suzuki, & Miyakoshi, 2006).

However, only limited studies on the other components of lacquer sap, lacquer polysaccharides, have been reported. Oshima and Kumanotani revealed the structure of lacquer polysaccharides by gel permeation chromatography (GPC), sugar analysis, methylation analysis, and Smith degradation, which suggest that the lacquer polysaccharide is a mixture of two fractions with the molecular weights of $\bar{M}_n = 2.3 \times 10^4$ and 6.7×10^4 and that it has a (1 → 3)-β-D-galactopyranosidic main chain with complex branches having glucuronic acid at the terminals (Oshima & Kumanotani, 1984). We also reported the structural analysis of the lacquer polysaccharides by high resolution nuclear magnetic resonance (NMR) measurements including 2D NMR spectroscopies, in which complex signals were assigned and the monosaccharide components were revealed to be D-galactose, 4-*O*-methyl-D-glucuronic acid, L-arabinose, and

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L-rhamnose (Lu et al., 1999). Furthermore, GPC studies of the time-course of the degradation of Japanese lacquer polysaccharides in the sap suggest that the molecular weight of polysaccharides in the lacquer sap was originally $\bar{M}_n = 6.7 \times 10^4$ with a narrow molecular weight distribution, and that then the lacquer polysaccharides separated gradually into two molecular weights after collection of the sap (Lu & Yoshida, 2003).

Several biological activities of lacquer polysaccharides in vivo were reported, including the prevention of leukopenia induced by cyclophosphamide in mice and stimulating the growth of leucocytes (Du, Yang, Kong, & Xiao, 1999; Yang & Du, 2003). We also found that the lacquer polysaccharide promoted blood coagulation at the concentration of 0.016 mg/ml, shortening the coagulation time of bovine plasma by more than one minute in comparison with that of a blank. The lacquer polysaccharide also had anti-tumor activity in vivo; that is, oral administration of the lacquer polysaccharide to mice at the dose of 50 mg/kg halved the weights of sarcoma 180 tumors. In addition, after sulfation, sulfated lacquer polysaccharides had potent anti-HIV activity at concentrations as low as 0.1 μg/ml and moderate blood anticoagulant activity at 10 units/mg compared to those standard polysaccharides of curdlan and dextran sulfates (Lu et al., 2000).

In this paper, we report the separation of two lacquer polysaccharide fractions using Sephadex column chromatography to give pure polysaccharide fractions for the first time. The lacquer polysaccharides had two main molecular weights, $\bar{M}_n = 10 \times 10^4$, and 3.0×10^4, in the proportion of 25 mol% and 75 mol%. Then, structural analysis was carried out by high resolution NMR. Further, the fractions were treated with dilute alkaline solution to reduce the molecular weight of the polysaccharides to $\bar{M}_n = 3.0 \times 10^4$ and 1.4×10^4, respectively, with the same structure as each other. We then measured the interaction of these polysaccharides with poly-L-lysine using SPR to quantitatively elucidate their biological activities.

2. Experimental

2.1. Materials

Poly-L-lysine with a molecular weight of 1000–5000 was purchased from Sigma–Aldrich, Co. A CM5 sensor chip, an amine coupling kit, HBS-EP + 10X buffer (including 0.1 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 30 mM EDTA, and 0.5 v/v% polyoxyethylene (20) sorbitan monolaurate surfactant (surfactant P20)), and 50 mM NaOH solution were supplied by GE Healthcare Japan, Co., Ltd. The HBS-EP + 10X buffer was diluted 10 times with Milli-Q water.

2.2. Measurement

^{13}C NMR spectra were recorded on a JEOL JNM ECX-400 or JEOL ECX-600 spectrometer at 100 MHz or 150 MHz at 40 °C in D_2O or DMSO-d6 solvents, respectively. Chemical shifts are expressed as ppm downfield from 4, 4′-dimethyl-4-silapentane-1-sulfonate (DSS) as an internal standard. Molecular weights of polysaccharides were determined at 40 °C by an aqueous phase GPC column (Tosoh TSK-gel G2500PWXL, G3000PWXL, and G4000PWXL, 7.6 mm × 300 mm × 3 eluted with 66.7 mmol phosphate buffer, pH = 6.68) with a Tosoh RI detector using pullulan standards. Infrared spectra were taken on a Perkin Elmer Spectrum One FT-IR spectrometer using a KBr pellet method. Specific rotation was measured by using a JASCO DIP-140 digital polarimeter in H_2O at 25 °C in a water-jacketed 10 ml quartz cell. The surface plasmon resonance (SPR) spectrum was taken on a Biacore X100 instrument at 25 °C using a CM5 sensor chip.

2.3. Separation of lacquer polysaccharides by Sephadex G-100 column chromatography

Lacquer polysaccharides with a mixture of two molecular weight fractions ($\bar{M}_n = 10 \times 10^4$ and 3.0×10^4 in the proportion of 25 mol% and 75 mol%) were obtained from the acetone powder prepared from a Chinese lacquer sap according to the method of Reinhammar (1970). The mixture of lacquer polysaccharides (10 g) was dissolved in deionized water (20 ml) and then applied to a Sephadex G-100 column (2.4 × 40 cm). Phosphate buffer (66.7 mmol, pH = 6.68) was used as an elution solvent. The two light brown bands were separated and dialyzed by deionized water for 24 h, respectively. Each dialysate was freeze-dried to give pure lacquer polysaccharide fractions with molecular weights of $\bar{M}_n = 10 \times 10^4$ and 3.0×10^4, respectively.

2.4. Treatment of lacquer polysaccharides with diluted alkaline solution

The each lacquer polysaccharide fraction (200 mg) was dissolved in 5% NaOH solution (20 ml) and the mixture was stirred for 12 h at 60 °C. After dialysis with deionized water for 24 h, the lacquer polysaccharide was obtained by freeze-drying. The molecular weights of the lacquer polysaccharides obtained were $\bar{M}_n = 10 \times 10^4$ and 3.0×10^4 in the proportion of 85 mol% and 15 mol% from the pure $\bar{M}_n = 10 \times 10^4$ fraction, and $\bar{M}_n = 3.0 \times 10^4$ and 1.4×10^4 in the proportion of 70 mol% and 30 mol% from the pure $\bar{M}_n = 3.0 \times 10^4$ fraction. The lacquer polysaccharide with the lower molecular weight of $\bar{M}_n = 1.4 \times 10^4$ was purified by Sephadex G-50 column chromatography.

2.5. SPR measurement

Poly-L-lysine in 10 mM sodium acetate buffer (pH 5.5) was immobilized on the CM5 sensor chip using an amine coupling kit according to the Biacore protocols (Fischer, 2010). The concentration was 5 mg/ml and the flow rate was 10 μl/min for 7 min. The value for the immobilized poly-L-lysine was 2000 response units (RU). A reference cell without poly-L-lysine was used. Lacquer polysaccharides (500 μg/ml) with different molecular weights were injected for 3 min (30 μl/min) over the poly-L-lysine immobilized sensor chip, and then the HBS-EP + running buffer solution was injected for 12 min to determine the association and dissociation rate constants, as shown in Fig. 4. A typical procedure for the weakly interacting sample was as follows. The lacquer polysaccharide solution ($\bar{M}_n = 10 \times 10^4$, 500 μg/ml) was prepared in the HBS-EP + buffer, and then the solution diluted to various concentrations with the HBS-EP + buffer was injected over the sensor chip at a flow rate of 30 μl/min for 3 min for association, and then dissociation was carried out with injection of only the HBS-EP + buffer for 12 min. For regeneration of the CM5 sensor chip, NaOH (50 mM) solution was used. The initial concentration of sulfated polysaccharides with high interaction was 5 μg/ml.

3. Results and discussion

3.1. Isolation and structure of lacquer polysaccharides

The lacquer polysaccharide was isolated from the acetone powder that was obtained by adding acetone to the Chinese lacquer sap according to Reinhammar's method (Reinhammar, 1970). In previous reports (Lu & Yoshida, 2003; Oshima & Kumanotani, 1984), the lacquer polysaccharide had two molecular weight fractions with number-average molecular weights ($\bar{M}_n$) of around $\bar{M}_n = 10 \times 10^4$ and 3.0×10^4, respectively, in the proportion of

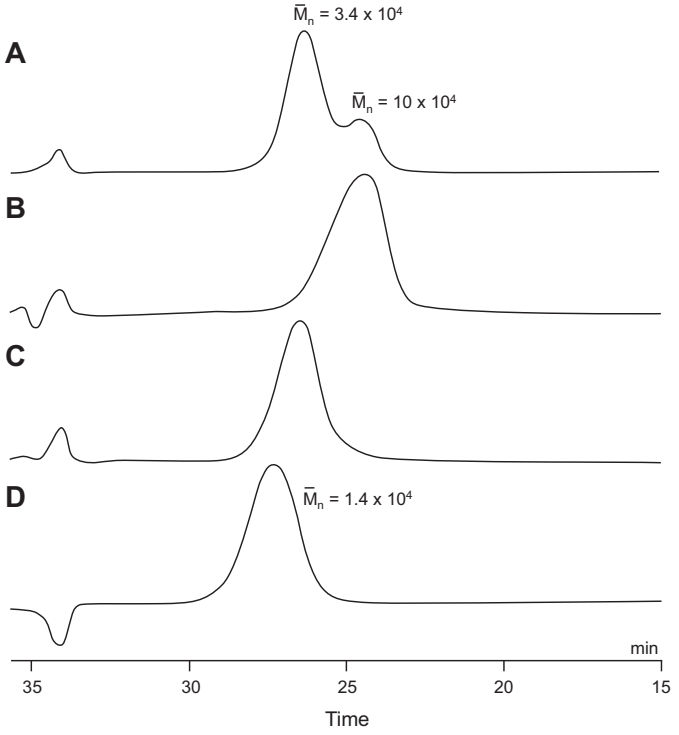

Fig. 1. Aqueous GPC profiles of Chinese lacquer polysaccharides. (A) The original lacquer polysaccharide ($[\alpha]_D^{25} = -4.13^\circ$ (c1, H_2O)) with two molecular weight fractions of $\bar{M}_n = 10 \times 10^4$ and $\bar{M}_n = 3.4 \times 10^4$ in the proportion of 25 mol% and 75 mol%, (B) and (C) pure larger ($[\alpha]_D^{25} = -3.63^\circ$ (c1, H_2O)) and smaller ($[\alpha]_D^{25} = -6.23^\circ$ (c1, H_2O)) fractions after Sephadex G-100 column chromatography of (A), and (D) pure fraction with $\bar{M}_n = 1.4 \times 10^4$ ($[\alpha]_D^{25} = -8.91^\circ$ (c1, H_2O)) after 5% NaOH treatment of (C).

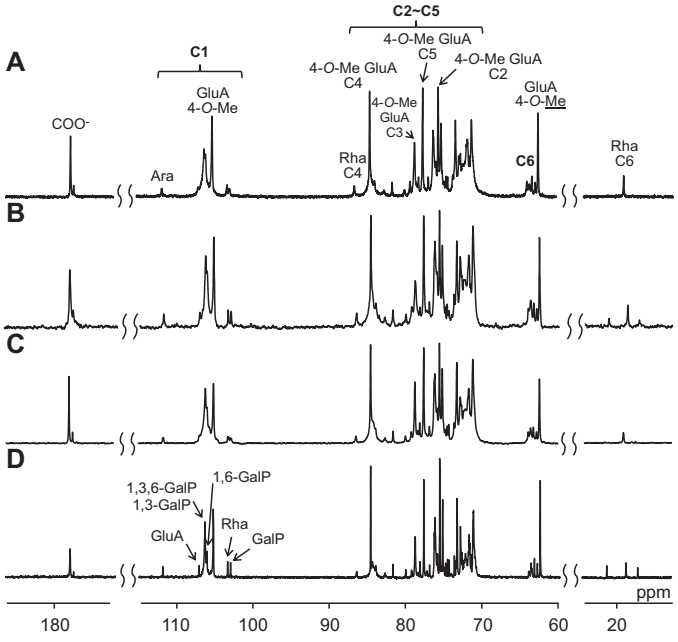

Fig. 2. ^{13}C NMR spectra of lacquer polysaccharides at 100 MHz for (A), (B), and (C), and at 150 MHz for (D) in D_2O at 40 °C.

25:75 mol%. However, we had observed that only the larger fraction was present in the sap isolated immediately after collection of Aizu lacquer from Fukushima Prefecture, Japan (Lu & Yoshida, 2003). The smaller fraction was contained only in Taiwan and Vietnam lacquer saps. In the present work, two lacquer polysaccharide fractions were isolated from Chinese lacquer sap for the first time by using the Sephadex G-100 column chromatography to give two pure polysaccharides with different molecular weights and the same structure as each other according to the results of NMR measurements, as described below. Fig. 1 shows the GPC profiles of (A) the original lacquer polysaccharides in the proportion of 25 mol% (larger fraction) and 75 mol% (smaller fraction) isolated from the Chinese lacquer sap, and pure fractions with the molecular weights of $\bar{M}_n = 10 \times 10^4$(B), and 3.0×10^4(C). The specific rotations of the three polysaccharides were almost the same, $[\alpha]_D^{25} = -4.13^\circ, -3.63^\circ$, and -6.23° (c1, H_2O) for the original, larger, and smaller polysaccharides, respectively, as shown in Fig. 1. In addition, the separated lacquer polysaccharides with $\bar{M}_n = 10 \times 10^4$ and 3.0×10^4 were treated with 5% NaOH solution for 12 h at 60 °C to give two pure polysaccharides with $\bar{M}_n = 3.0 \times 10^4$ and 1.4×10^4 with homogeneous molecular weight distributions after separation by Sephadex G-50 column chromatography. Fig. 4D shows the GPC profile of the lacquer polysaccharide with $\bar{M}_n = 1.4 \times 10^4$ and $[\alpha]_D^{25} = -8.91^\circ$. Therefore, we assumed that the lacquer polysaccharide had an associated structure with the smaller molecular weight polysaccharide, but further treatment of the smaller lacquer polysaccharide with the diluted alkaline solution gave no lower molecular weight polysaccharides. These results do not contradict our previous results that the lacquer polysaccharide was one molecular weight fraction with $\bar{M}_n = 6.7 \times 10^4$ after immediate isolation from the fresh sap (Lu & Yoshida, 2003). After the sap was stored for a long time, a new fraction with the smaller molecular weight of $\bar{M}_n = 2.7 \times 10^4$ was observed.

Fig. 2 shows the ^{13}C NMR spectra of lacquer polysaccharides that correspond to those in Fig. 1. Previously, we assigned most of the complex signals (Fig. 2A) by high resolution NMR measurements involving two dimensional DQF-COSY, TOCSY, HMQC, and HMBC experiments. The intensity and chemical shifts of the carbon spectra in Fig. 2 were similar to each other. In the IR spectra of these fractions (Fig. 3), absorption bands at 1610 cm^{-1}, 1250 cm^{-1}, and

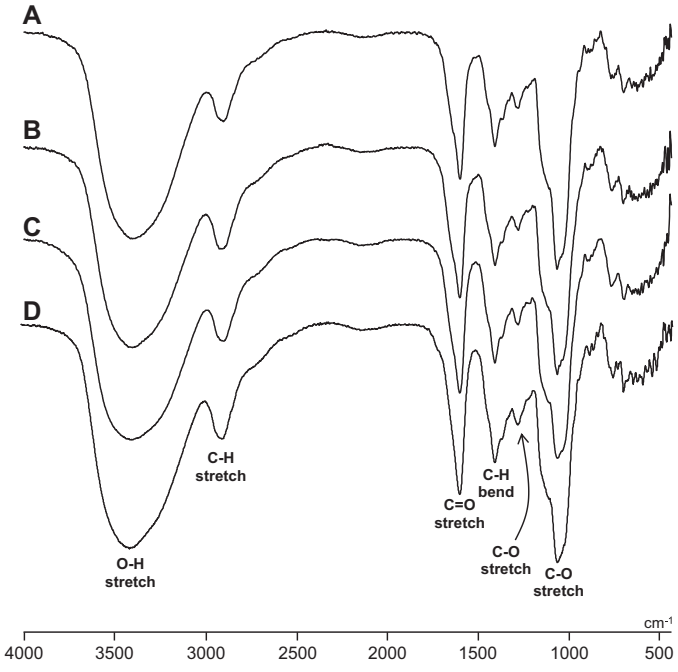

Fig. 3. FI-IR spectra of lacquer polysaccharides (KBr method). (A) Original, and (B), (C), and (D) pure fractions with $\bar{M}_n = 10 \times 10^4$, and 3.4×10^4, 1.4×10^4, respectively, corresponding to those in Fig. 1.

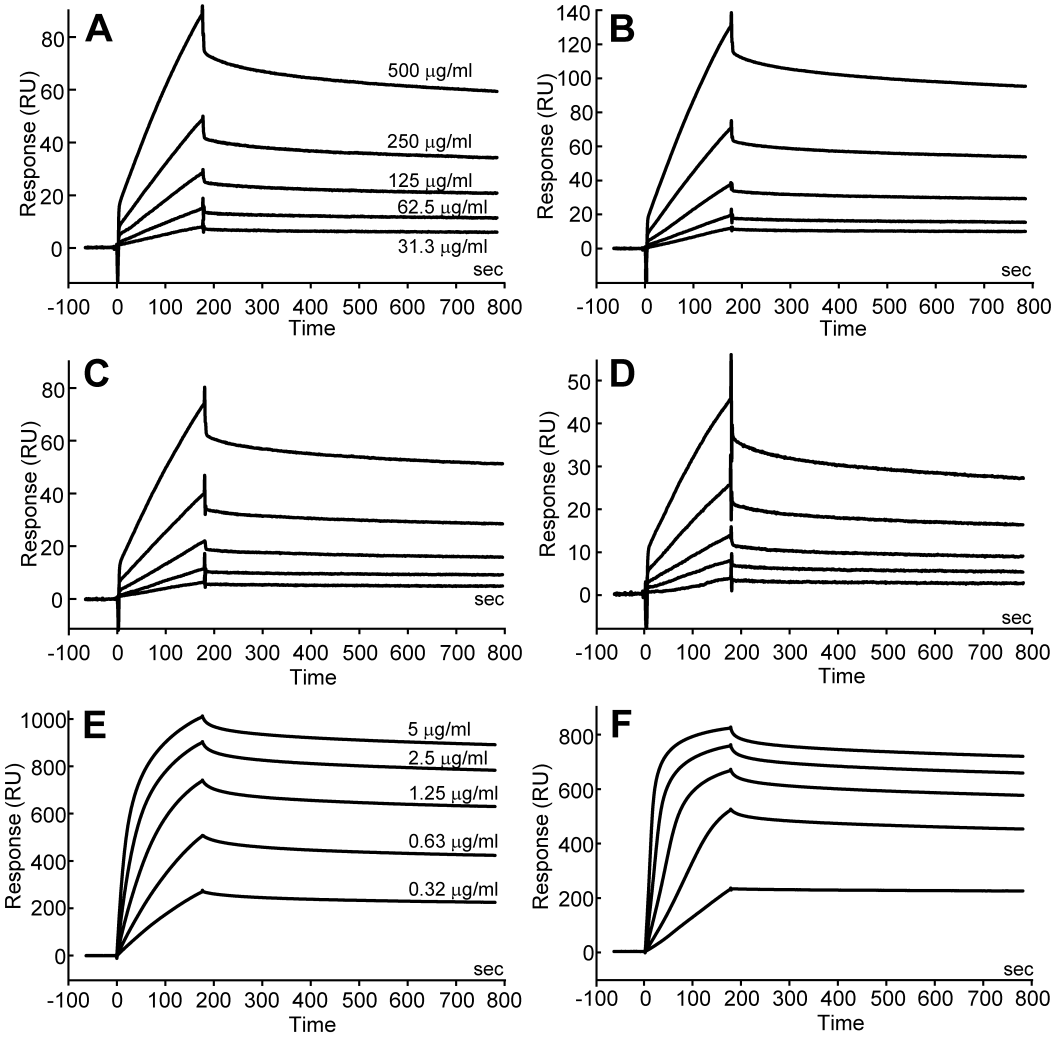

Fig. 4. Binding curves of lacquer polysaccharides with $\bar{M}_n = 10 \times 10^4$ to immobilized poly-L-lysine. Binding of (A) original, and (B), (C), and (D) pure molecular weight fractions with $\bar{M}_n = 10 \times 10^4$, 3.4×10^4, and 1.4×10^4, respectively, corresponding to those in Fig. 1. (E) Binding of sulfated lacquer polysaccharide with $\bar{M}_n = 0.7 \times 10^4$ and (F) dextran sulfate with $\bar{M}_n = 1.3 \times 10^4$. Lacquer polysaccharide (90 μl) was injected for 180 s at a flow rate of 30 μl/min of a HBS-EP running buffer at 25 °C, and then the running buffer was further flowed for 600 s. Concentrations of lacquer polysaccharide were 500, 250, 125, 62.5, and 31.3 μg/ml. For sulfated polysaccharides, the concentrations were 5.0, 2.5, 1.25, 0.62, and 0.31 μg/ml.

1050 cm^{-1} due to COO^- stretching vibration and the large absorption at 3400 cm^{-1} due to OH stretching vibration appeared. These characteristic absorptions as well as small bands in the finger print region gave the same shapes and intensities as the four fractions. The associated structure might be constructed by the alkaline metal and/or earth ions, for example, Ca^{2+} and/or Fe^{2+} (Aspinall, 2003; Klemm, 2010). With passing of time after collection, the association decreased and they divided into smaller polysaccharides. Further detailed investigations of the associated structure of the lacquer polysaccharides are in progress.

3.2. Binding of lacquer polysaccharides to poly-L-lysine

We found previously that lacquer polysaccharides have specific biological activities such as promoting blood coagulation and antitumor activities. After sulfation, sulfated polysaccharides have potent anti-HIV and moderate blood anticoagulant activities (Lu & Yoshida, 2003). Therefore, we predicted that the electrostatic interaction of negatively charged carboxylic or sulfated groups of lacquer polysaccharides and positively carged amino groups of the cell surface glycoproteins between polysaccharides and cell surface glycoproteins play an important role in the biological activities (Jeon, Katsuraya, Kaneko, Mimura, & Uryu, 1997; Jeon et al., 2000; Uryu et al., 1992). The affinity of lacquer polysaccharides to poly-L-lysine as a model compound of proteins and peptides was examined to elucidate the interaction mechanism of the biological activities using surface plasmon resonance (SPR) measurement (Schasfoort & Tudos, 2008). We found that lacquer polysaccharides interacted weakly with poly-L-lysine. The RU values decreased with decreasing molecular weight of lacquer polysaccharides. The lacquer polysaccharide with the higher molecular weight of $\bar{M}_n = 10 \times 10^4$ gave the higher association response of about 130 RU (Fig. 4B) compared to the lower molecular weight polysaccharides with $\bar{M}_n = 3.4 \times 10^4$ and $\bar{M}_n = 1.4 \times 10^4$ (Fig. 4C and D) at the concentration of 500 μg/ml, indicating that the interaction was dependent on the molecular weights of lacquer polysaccharides. Lacquer polysaccharides are acidic polysaccharides with glucuronic acids at the terminals of the branches, but their affinity to poly-L-lysine was weaker than that of sulfated polysaccharides (Fig. 4E and F). These results are comparable with the biological activities of lacquer polysaccharides, the increased anti-HIV and blood coagulant activities we reported previously (Lu et al., 2000).

Table 1 shows the apparent kinetic constants of lacquer polysaccharides to poly-L-lysine calculated by the global fitting curves

Table 1
Kinetic results of lacquer polysaccharides.[a,b]

	$\bar{M}_n$ ($\times 10^4$)	S (%)	k_a (1/M)	k_d (1/s)	K_D (M)
Original lacquer polysaccharide[c]	5.1	–	4.99×10^2	3.54×10^{-4}	7.10×10^{-7}
Lacquer polysaccharide 1	10	–	8.23×10^2	2.94×10^{-4}	3.58×10^{-7}
Lacquer polysaccharide 2	3.4	–	3.02×10^2	3.08×10^{-4}	1.02×10^{-6}
Lacquer polysaccharide 3	1.4	–	1.69×10^2	4.75×10^{-4}	2.81×10^{-6}
Sulfated lacquer polysaccharide	0.7	11.0	4.31×10^4	1.74×10^{-4}	4.04×10^{-9}
Dextran sulfate	1.3	18.4	2.27×10^6	3.73×10^{-4}	1.64×10^{-10}

[a] Lacquer polysaccharide was injected 90 μl for 180 s at a flow rate of 30 μl/min of a HBS-EP running buffer at 25 °C and then the running buffer was further flowed for 600 s. Concentration of lacquer polysaccharide was 500, 250, 125, 62.5, and 31.3 μg/ml, respectively. For sulfated polysaccharides, the concentration was 5.0, 2.5, 1.25, 0.62, and 0.31 μg/ml, respectively.
[b] k_a; Association-rate, k_d; dissociation-rate constants, and dissociation constant $K_D = k_d/k_a$.
[c] Original lacquer polysaccharide had two molecular fractions with $\bar{M}_n = 10 \times 10^4$ and $\bar{M}_n = 3.4 \times 10^4$ in the proportion of 25 and 75 mol%.

according to the 1:1 binding model. Dextran sulfate ($\bar{M}_n = 1.3 \times 10^4$, $S = 18.4\%$), which has potent anti-HIV and blood anticoagulant activities, was used as a positive reference compound. Although the lacquer polysaccharide is an acidic polysaccharide with carboxylic acids, the association (k_a) and dissociation-rate (k_d) constants were low because of the low affinity to poly-L-lysine. These results are consisttent with the weak anti-HIV and blood coagulation promoting activities of lacquer polysaccharides (Lu et al., 2000). After sulfation, sulfated lacquer polysaccharides ($\bar{M}_n = 0.7 \times 10^4$, $S = 11.0\%$) had higher association (k_a) and lower dissociation- (k_d) rate constants, indicating that sulfated lacquer polysaccharides had a high affinity to poly-L-lysine by the electrostatic interaction of the negative charges of sulfate groups and positive charges of amino groups. Therefore, the SPR measurement was found to support the elucidation of biological activities.

Fig. 5 shows the relationship between the kinetic properties of lacquer polysaccharides and sulfated polysaccharides in which the affinity to poly-L-lysine differed with the molecular weights and acidic functional groups. The polysaccharides had almost the same dissociation rate constant (k_d), but the association rates (k_a) differed with their functional groups and molecular weights. The association rate of lacquer polysaccharides increased gradually with increasing molecular weights. On the other hand, the sulfated lacquer polysaccharide showed high affinity to poly-L-lysine, indicating that sulfated lacquer polysaccharide had potent biological activities due to the interaction between the sulfate groups and amino groups.

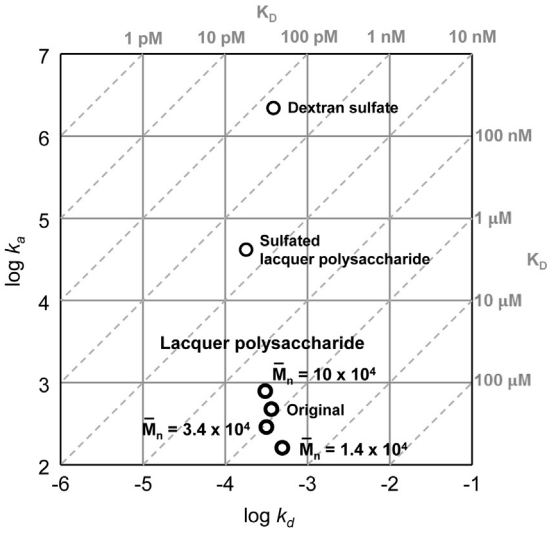

Fig. 5. Relationship between association- (k_a) and dissociation- (k_b) rate constant of lacquer polysaccharides. The dotted line shows the dissociation constant, K_D.

4. Conclusion

We obtained three pure polysaccharide fractions of a Chinese lacquer polysaccharide with $\bar{M}_n = 10 \times 10^4$, 3.0×10^4, and 1.4×10^4 by Sephadex column chromatography and dilute alkaline treatment of the fractionated lacquer polysaccharides. The structures of these polysaccharides were almost the same according to NMR, IR, and specific rotations, assuming that the low molecular weight polysaccharides structures were associated through Ca^{2+} and/or Fe^{2+}. In addition, the present work showed the interaction of these lacquer polysaccharides with poly-L-lysine using SPR measurement to calculate the apparent kinetic constants such as the k_a, k_d, and K_D constants, suggesting that lacquer polysaccharides had a weak affinity to poly-L-lysine compared to sulfated polysaccharides. These kinetic data support the hypothesis that the antitumor and blood coagulation-promoting activities are due to electrostatic interaction. However, sulfated lacquer polysaccharides have a high affinity to poly-L-lysine according to the SPR measurements, results that correspond to the potent anti-HIV and anticoagulant activities we reported previously. The mechanism of action is under further investigation to elucidate the specific biological activities of lacquer polysaccharides.

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