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Structure and molecular weight of Asian lacquer polysaccharides

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Abstract

Structural analysis of Asian lacquer polysaccharides in Vietnam, Myanmar, Cambodia, Taiwan, and Japan was carried out by a combination of chemical and physical methods, and then their structures were compared with that of a Chinese lacquer polysaccharide reported previously. It was found that the structure of polysaccharides in China and Japan, Taiwan and Vietnam, Myanmar and Cambodia, was similar to each other. The polysaccharides in Myanmar and Cambodia had larger amounts of L-arabinose and L-rhamnose than those in other Asian lacquer polysaccharides. In addition, the degradation process of lacquer polysaccharide was revealed for the first time by the time-course of GPC measurements of polysaccharide in Aizu, Japan. The results suggest that the molecular weight of polysaccharide in lacquer tree had around $\bar{M}_n = 67 \times 10^3$ with narrow molecular weight distribution and then decreased gradually into two molecular weight fractions of $\bar{M}_n = 67 \times 10^3$ and 23×10^3 in the proportion of 25 and 75 mol% isolated after 3 weeks.

Keywords: Structure; Asian lacquer polysaccharides; Molecular weight; Sugar component

1. Introduction

Lacquer, originally produced in Asia, is the only natural product, which is polymerized by an enzyme, laccase, to give a beautiful coating surface (Kumanotani, 1995). In the sap of lacquer tree, there are three major components such as urushiols (3- or 4-alkenyl catechol derivatives) (Bartus et al., 1994; Du, Oshima, Iwatsuki, & Kumanotani, 1984a; Du, Oshima, & Kumanotani, 1984b; Du, Oshima, & Kumanotani, 1985; Niimura, Kamaya, Sato, Katano, & Miyakoshi, 1998), laccase (Reinhammar, 1970), and polysaccharides (Oshima & Kumanotani, 1984). Previously, it was revealed the partial structure of a Chinese lacquer polysaccharide by chemical methods such as sugar analysis, methylation analysis, and Smith degradation to afford a branched 1,3-β galactopyranan having 4-*O*-methyl glucuronic acid in the terminal of complex branches (Oshima & Kumanotani, 1984).

Recently, we reported the structural characterization of lacquer polysaccharide in the sap of Chinese lacquer tree (produced in Maoba, Hubei province, China) by means of high resolution NMR spectroscopy (Lu et al., 1999). Most of complex signals in the NMR spectra were assigned by a combination of two dimensional spectra, suggesting that galactopyranose in the main chain and glucuronic acid in the side chain had β-linkage, and L-arabinose and L-rhamnose in the terminal of branches had α-linkage by their coupling constants. These results suggest that the NMR analysis was a superior method for the structural analysis of lacquer polysaccharides having complex structures, because of short analysis time and simple procedures without degradation of polysaccharides.

On the other hand, we found that Chinese lacquer polysaccharides had specific biological activities such as blood coagulant-promoting and antitumor activities (Lu et al., 2000). After sulfation, sulfated lacquer polysaccharides had potent anti-HIV activity around 0.5 μg/ml as represented by the 50% protecting concentration (EC_{50}) of MT-4 cell (standard curdlan sulfate, $EC_{50} = 0.13$ μg/ml, which is one of the highest anti-HIV active polysaccharides (Yoshida et al., 1990)) and lower blood anticoagulant activity, 9–17 unit/mg, than that of a standard dextran sulfate, 22.7 unit/mg (curdlan sulfate, around 10 unit/mg). The lacquer was an interesting natural product not only in the coating material but also in a biological active material. However, there are no reports on the determination of

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structures of Asian lacquer polysaccharides. In this study, we wish to report the structural determination of lacquer polysaccharides in Asian countries. The structure was compared with that of the Chinese polysaccharide reported previously by the high resolution NMR spectroscopy. The Chinese lacquer polysaccharide has been known to have two molecular weight fractions around $\bar{M}_n = 90 \times 10^3$ and 30×10^3 in the proportion of roughly 25:75 mol%. The ratio of constitutional sugar residues was the same as the two fractions (Lu et al., 1999; Oshima & Kumanotani, 1984). However, from the results of GPC measurement of lacquer polysaccharide in Aizu, Fukushima prefecture, Japan, we found for the first time that the lacquer polysaccharide had one molecular weight fraction in the lacquer tree and then separate gradually into two fractions after collection. The degradation process of polysaccharides and the sugar compositions were determined by the GPC, HPLC, GC, and GC-MS measurements.

2. Material and methods

2.1. Materials

Lacquer polysaccharides were isolated from an acetone powder of the sap of lacquer trees according to the same procedures of the previous report (Lu et al., 1999). The sap of Asian lacquer trees was stored for 3 years and then polysaccharides were isolated. Finally, the polysaccharides were purified by column chromatography packed with CM-Sephadex and subsequent Sephadex G-100.

Aizu lacquer polysaccharides were obtained from the sap of the lacquer tree in Aizu, Fukushima prefecture, Japan. The polysaccharide in Fig. 4D was separated from the sap of Aizu lacquer that had been kept for 7 years.

2.2. Measurements

NMR spectra were recorded at 40 °C in D_2O on a JEOL α-400 spectrometer by using a phase sensitive mode and a field gradient probe. Sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) was used as an internal standard at 0 ppm for ^1H and 0.015 ppm for ^{13}C spectra. The lacquer polysaccharides (60 mg) were freeze-dried several times from 99.96% D_2O before preparing sample for the NMR measurement. Infrared spectra were taken on a Shimazu FT-IR 8300 spectrometer by a KBr method. Specific rotations were measured at 25 °C on a JASCO DIP-140 digital polarimeter in water. Molecular weights were estimated at 40 °C by an aqueous phase GPC (column; TSK-gel $G2500PW_{XL}$, $G3000PW_{XL}$, and $G4000PW_{XL}$, 7.6 × 300 mm × 3; eluent, 66.7 mmol of phosphate buffer, pH 6.86) using pullulan standards. The GC and GC-MS measurements for methylation analyses were carried out by Shimazu GC-17A and QP-5000 apparatuses equipped with a fused silica capillary column (J and W DB-1, 0.25 mm × 30 m).

2.3. Sugar analysis

The sugar analysis was carried out by the two methods such as the reverse-phase HPLC with the TOSOH TSK-gel Amido-80 column and the anion exchange HPLC with the Dionex CarboPac PA-1 column. The typical procedure is as follows. Glucuronic acid in the lacquer polysaccharides was reduced with $NaBH_4$ by the method of Taylor (Taylor & Corrad, 1972). The reduced polysaccharide (10 mg) was hydrolyzed at 100 °C with 2N trifluoroacetic acid (1 ml) for 8 h. The solution was evaporated to dryness and then a mixture of methanol–water (1:1, 1 ml) was added to give a clear solution, which was evaporated again to dryness. This procedure was repeated several times to remove completely trifluoroacetic acid. Lastly, methanol was added to the hydrolyzed mixture and then the methanol solution was evaporated to give the hydrolyzed sugar sample.

The mixture of hydrolyzed sugar was applied at 80 °C to the reverse-phase HPLC on a Amido-80 column eluted with acetonitrile–water (8:2) solution at the flow rate of 1 ml/min. Furthermore, the hydrolyzed sugar was analyzed at room temperature by the ion exchange chromatography

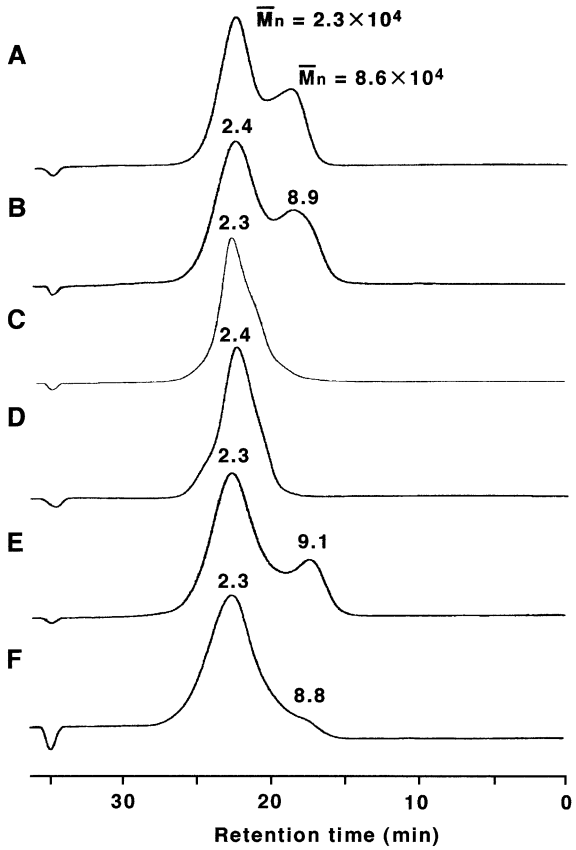

Fig. 1. GPC profiles of lacquer polysaccharides in Asian countries. (A) China, (B) Japan, (C) Taiwan, (D) Vietnam, (E) Myanmar, (F) Cambodia.

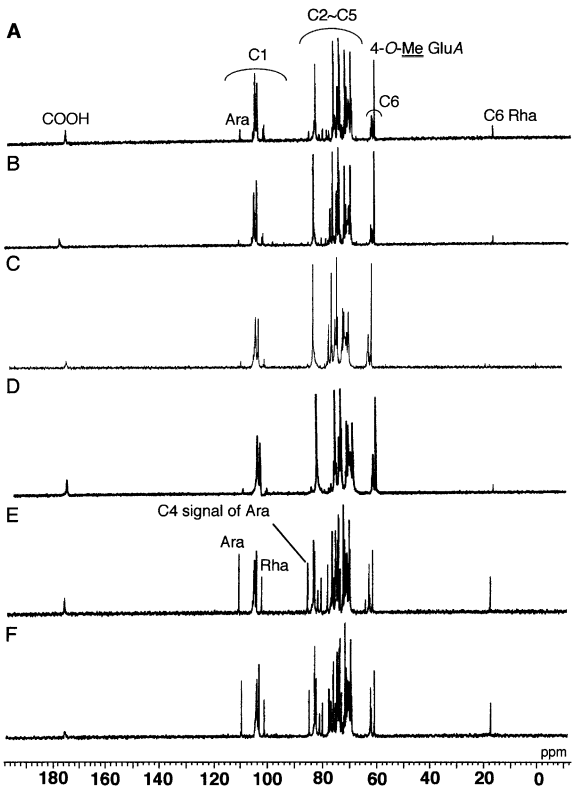

Fig. 2. 400 MHz ^{13}C NMR spectra of lacquer polysaccharides in Asian countries (37 °C, D_2O). (A) China, (B) Japan, (C) Taiwan, (D) Vietnam, (E) Myanmar, (F) Cambodia.

equipped with the anion exchange CarboPA-1 column eluted with 20 mmol NaOH solution at the flow rate of 1 ml/min.

2.4. Methylation analysis

The methylation analysis of the polysaccharides in Burma and Cambodia was carried out using methylsulfinylcarbanion in DMSO by the method of Hakomori (Hakomori, 1964). After hydrolysis, reduction, and acetylation, the partially methylated alditol acetates were analyzed by the GC and GC-MS spectroscopy.

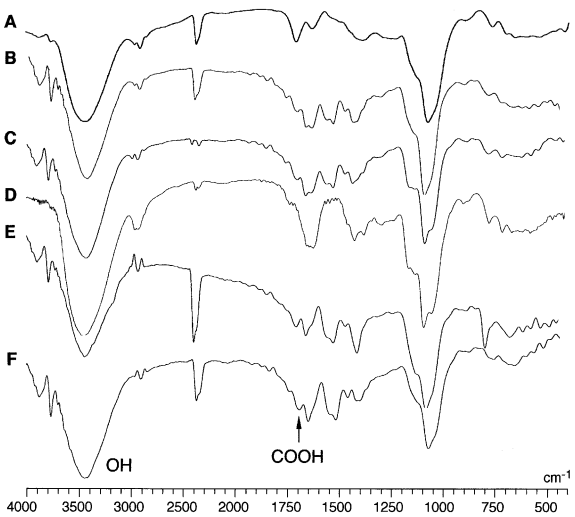

Fig. 3. FT-IR spectra of lacquer polysaccharides in Asian countries. (A) China, (B) Japan, (C) Taiwan, (D) Vietnam, (E) Myanmar, (F) Cambodia.

3. Results and discussion

3.1. GPC profiles and NMR spectra of Asian lacquer polysaccharides

The lacquer polysaccharides in Asian countries, Japan (Taiko, Ibaragi prefecture), Taiwan, Vietnam, Myanmar, and Cambodia, were isolated from their acetone powders, which are acetone-insoluble parts in the sap, according to the method of previous report (Lu et al., 1999). The Chinese lacquer polysaccharide had two fractions in the GPC profile having molecular weight of $\bar{M}_n = 93 \times 10^3$ and 29×10^3 in the proportion of 25:75 mol%, respectively (Lu et al., 1999; Oshima & Kumanotani, 1984). Therefore, we expected that the lacquer polysaccharides in other Asian countries should have two molecular weight fractions.

Fig. 1 shows the GPC profiles of Asian lacquer polysaccharides, which were found to have two molecular weight fractions. However, the shape and proportion in the GPC profiles were different from each other. Although the polysaccharides in Japan and Myanmar exhibited almost the same GPC profiles as that of Chinese one, the polysaccharides in Taiwan, Vietnam, and Cambodia had almost one fraction having molecular weight

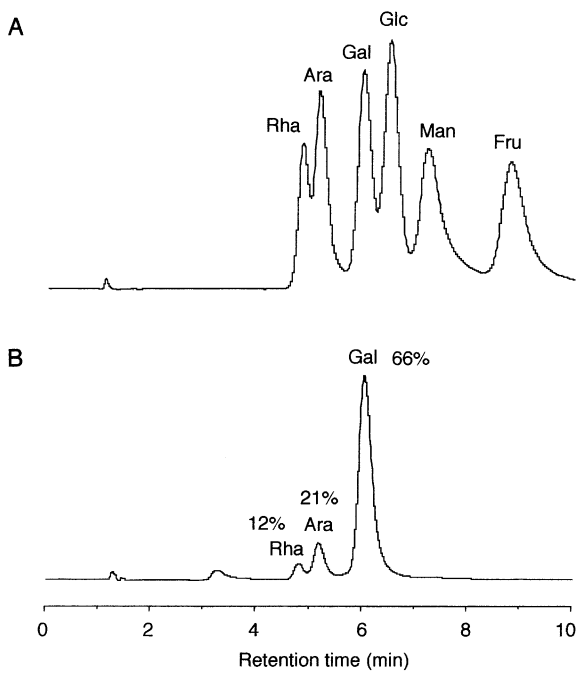

Fig. 4. HPAEC-PAD chromatograms of (A) monosaccharide standard and (B) monosaccharide residues after hydrolysis of polysaccharide in Myanmar at room temperature.

Table 1
Monosaccharide residues of lacquer polysaccharides in Chinese, Myanmar, and Cambodia by methylation and NMR analysis

Sugar	Chinese [a,b]	Myanmar		Cambodia	
		GC[b] (mol%)	NMR[c] (mol%)	GC[b] (mol%)	NMR[c] (mol%)
D-Galactose	66.1	52.0	49	54.0	51
4-*O*-Methyl-D-glucuronic acid	24.1	19.0	20	21.0	24
D-Glucuronic acid	3.0				
L-Arabinose	5.0	18.7	20	15.0	15
L-Rhamnose	3.1	10.0	10	9.4	9

[a] From Oshima and Kumanotani (1984).
[b] Calculated from TIC peaks of GC-MS spectrum.
[c] Calculated from the integration data of anomeric region in proton spectrum.

of $\bar{M}_n = 23 \times 10^3$ and showed a shoulder at higher molecular weight side of the main peak. These results suggest that the polysaccharides were degraded after collection. The detail of degradation is discussed in the later part.

Fig. 2 shows the ^{13}C NMR spectra of Asian lacquer polysaccharides, in which the complex signals appears. The Asian lacquer polysaccharides were found to be acidic polysaccharides because of appearing the carboxylic acid signal at 176 ppm due to 4-*O*-methyl glucuronic acid or glucuronic acid. It was revealed that the polysaccharides in Myanmar (E) and Cambodia (F) had larger amounts of arabinose and rhamnose in the terminal of branches than those in other polysaccharides by the intensity of NMR signals at 110 and 102 ppm due to C1 signals in L-arabinose and L-rhamnose residues, respectively. The signal at 17 ppm is assigned to C6 methyl group in L-rhamnose. From the results of Figs. 1 and 2, the polysaccharides in Japan, Taiwan, and Vietnam had almost the same structure as that of Chinese one. In the IR spectra shown in Fig. 3, the small absorption due to carboxylic acid (stretching vibration) appeared around 1730 cm^{-1}. The hydroxyl group and ether signals (C–O–C) in sugar units were absorbed as strong intensities around 3400 and 1050 cm^{-1}, respectively.

3.2. Sugar and methylation analyses of polysaccharides in Myanmar and Cambodia

In the ^{13}C NMR spectra (Fig. 2) as mentioned above, the proportion of monosaccharide residues in Myanmar and Cambodia was different from the other Asian polysaccharides. Thus, the sugar and methylation analyses were carried out. Fig. 4 shows the High-pH anion-exchange chromatography (HPAEC)-pulsed amperometric detection (PAD) profile of (A) standard monosaccharides and (B) monosaccharide residues of polysaccharide in Myanmar. Taking into account the sensitivity of monosaccharides in PAD, the proportion of arabinose and rhamnose was calculated into 21 and 12 mol%, respectively, which was almost the same result as that of NMR measurements (Table 1). No glucose was detected in the HPAEC-PAD chromatogram.

To determine the structures in detail, the methylation analysis of polysaccharides in Myanmar and Cambodia were carried out and the results were compared to the Chinese one reported previously (Lu et al., 1999). Table 1 shows the proportion of monosaccharide residues of the polysaccharides in Myanmar and Cambodia. The GC-MS spectra of monosaccharide residues were in agreement with those of the previously reported data (Lu et al., 1999), indicating that arabinose, rhamnose, and 4-*O*-methyl

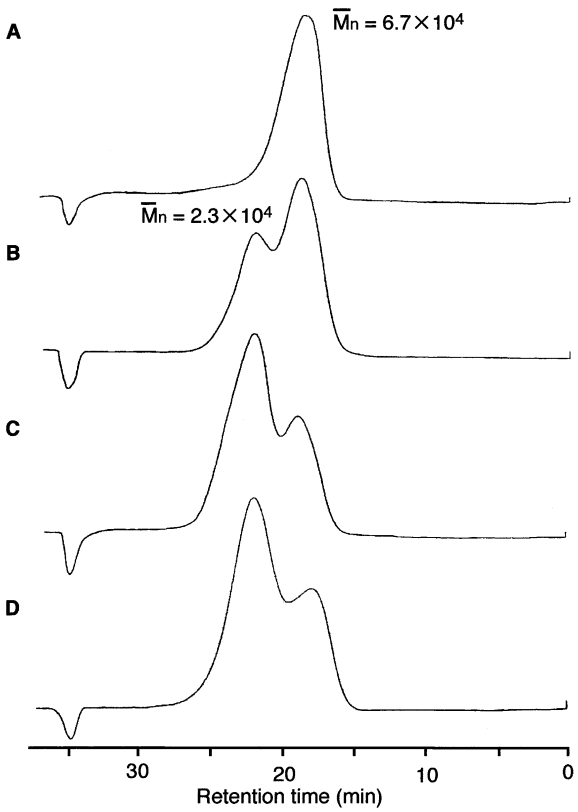

Fig. 5. GPC profiles of molecular weight changing of lacquer polysaccharide in Aizu, Fukushima prefecture, Japan. (A) Immediate isolation after collection of sap, (B) isolation after 3 days, (C) after 21 days, and (D) after 7 years.

glucuronic acid were located in the terminal of branches. Galactose with 1, 3-β-linkage should be in the main chain because of appearance of the largest signal due to 2,3,4-tri-*O*-methylated galactose.

3.3. Molecular weight changing of Japanese lacquer polysaccharide

The lacquer polysaccharides in China had two molecular weight fractions. For the Asian lacquer polysaccharides, however, the ratio of fractions seemed to be different in the GPC profiles (Fig. 1). In particular, polysaccharides in Taiwan, Vietnam, and Cambodia showed one molecular weight fraction.

Therefore, we assumed that polysaccharides in the sap of lacquer tree had originally one fraction having high molecular weights. After collection of the sap and then contact with air during preservation, the polysaccharides were degraded gradually into lower molecular weights. To confirm this estimation, we collected directly the sap of lacquer tree in Aizu lacquer field of Fukushima prefecture, Japan, and then the sap was poured immediately onto acetone to isolate the polysaccharide. After the sap was kept for 3 and 21 days, the polysaccharides were also isolated. In addition, a polysaccharide was extracted from another sap that was kept for 7 years in Aizu. Fig. 5 shows the GPC profiles of polysaccharides isolated (A) immediate (within 1 min), (B) after 3 days, (C) after 21 days, and (D) after 7 years. In Fig. 5A, the polysaccharide was revealed to have one fraction having molecular weight of $\bar{M}_n = 67 \times 10^3$. After 3 days, the polysaccharide was separated into two fractions with $\bar{M}_n = 67 \times 10^3$ and 23×10^3 in the proportion of 30:70, respectively, in Fig. 5B. The proportion was further changed into 75:25 isolated after 21 days of collection (Fig. 5C). After 7 years in Fig. 5D, the polysaccharide had the same proportion as that stored for 21 days. Therefore, we resulted that the lacquer polysaccharide had originally one molecular weight fraction in the lacquer tree. The molecular weight decreased gradually with an elapse of stored time after collection. More than 21 days, the proportion of molecular weights was not changed at least for 7 years. In addition, we found that the isolated polysaccharides had high stability without degradation for several years. After collection, the sap was fermented in the air and the baubles of carbon dioxide were gushed out to change the pH of sap. Since some polysaccharides in Fig. 1 had almost one fraction having lower molecular weight, the degradation might occur by the difference of stored conditions of the sap.

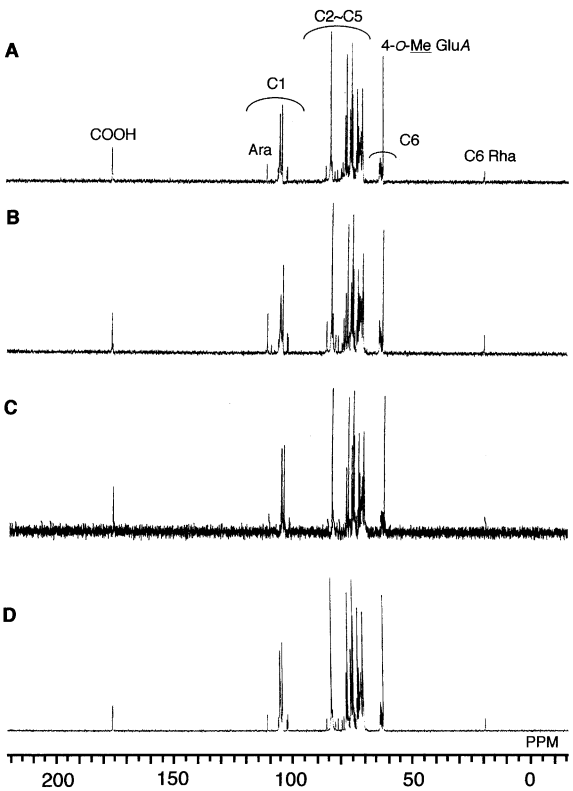

Fig. 6. 400 MHz ^{13}C NMR spectra of lacquer polysaccharide in Aizu, Fukushima prefecture, Japan. (A) Immediate isolation after collection of sap, (B) isolation after 3 days, (C) after 21 days, and (D) after 7 years.

Fig. 6 shows the ^{13}C NMR spectra of Aizu lacquer polysaccharides, which corresponded to those in Fig. 5. The polysaccharides in the two molecular weight fractions had the same structure, because of no change of the NMR spectra, even though the degree of degradation was different. Thus, one of the reasons why the degradation of lacquer polysaccharides occurred might be due to the pH changing of the sap solution into acidic side. The elucidation of degradation mechanism in detail is under investigation.

In conclusion, the characterization of Asian lacquer polysaccharides was carried out with NMR, GPC, and GC-MS analyses, suggesting that the polysaccharides in Myanmar and Cambodia had large amounts of arabinose and rhamnose residues. The degradation process of polysaccharides was examined by the GPC measurements using Aizu lacquer polysaccharides. It was found that the lacquer polysaccharides had originally one fraction having high molecular weights in the tree. After collection, the molecular weight decreased gradually with the stored time of the sap.

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