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VARIETY OF MOLECULAR SPECIES OF THE WATER-SOLUBLE POLYSACCHARIDE OF *Phellodendron amurense* RUPRECHT*

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ABSTRACT

The molecular weight of the water-soluble polysaccharide of *Phellodendron amurense* Ruprecht was found to differ with the sample used. The difference is considered to be due to different degrees of degradation of the polysaccharide chains, together with oxidation of galactose to galacturonic acid residues.

INTRODUCTION

The aqueous extract of the bark of *Phellodendron amurense* Ruprecht (Wobaku wood) has a high viscosity, because of the presence of a water-soluble polysaccharide having a high molecular weight[2]. The molecular properties and structural features have already been studied[1,2], and during these investigations, water extracts lacking high viscosities were encountered. In addition, the sugar compositions seemed to vary according to the time and place of harvest, the part of the tree, and the conditions and length of storage. It therefore became necessary to clarify the causes of these variations.

We now describe three kinds of polysaccharide sample, including one that had already been reported in this series of studies[1,2], that have been tested from this point of view.

RESULTS AND DISCUSSION

The analyses of sugar composition showed that the water-soluble polysaccharide obtained from dry, commercial bark of *P. amurense* harvested in Taiwan (L-PSWS) consisted of arabinose, rhamnose, galactose, and galacturonic acid residues in the ratios of 23.67, 29.64, 16.81, and 29.89%, and that from fresh bark harvested in

*Studies on the Water-soluble Polysaccharide of *Phellodendron amurense* Ruprecht, Part III. For Part II, see ref. 1.

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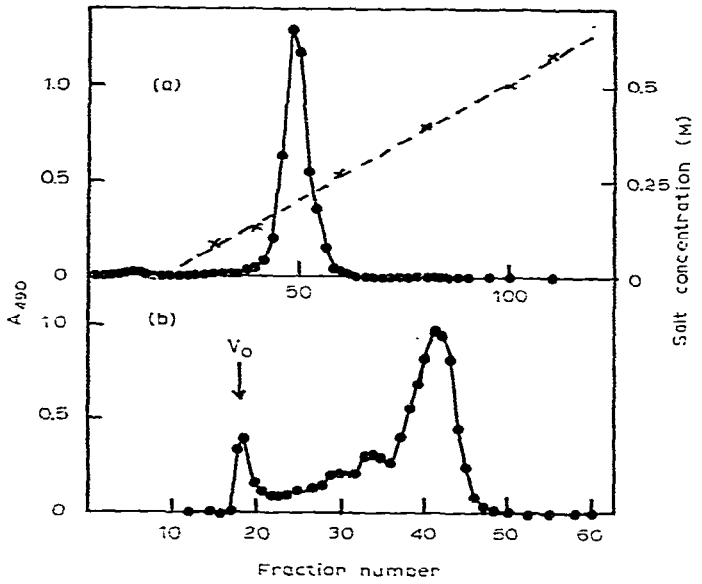

Fig. 1. Elution pattern of L-WSPS. [The results of column chromatography on DEAE-cellulose, and gel-filtration chromatography on Sepharose 6B, of L-WSPS are shown in a and b, respectively. The dotted line shows the concentrations of sodium chloride.]

Nara Pref., Japan (NA-WSPS) consisted of 21.87% of arabinose, 31.49% of rhamnose, 27.88% of galactose, and 18.77% of galacturonic acid. It had already been shown[1] that P-WSPS (harvested in Miyazaki Pref., Japan) has 22.45% of arabinose, 29.26% of rhamnose, 31.17% of galactose, and 17.11% of galacturonic acid. The three polysaccharides had almost the same proportions of arabinose (~22–23%) and rhamnose (30%), but the proportions of galactose and galacturonic acid varied with the polysaccharide, although the sums of the two hexoses were almost equal for the three polysaccharides. NA-WSPS and P-WSPS had similar compositions in this experiment, but there were large differences between L-WSPS and the others. Therefore, further studies were made on L-WSPS in comparison with P-WSPS.

L-WSPS was eluted in a single peak from a column of DEAE-cellulose with 0.19M sodium chloride (see Fig. 1a). The fact that L-WSPS was eluted by a lower concentration of salt than that (0.24M) needed for P-WSPS, despite its high content of galacturonic acid, showed that the molecular weight of L-WSPS was considerably smaller than that of P-WSPS.

In gel-filtration chromatography on Sepharose 6B (see Fig. 1b), L-WSPS gave a complicated elution-pattern having two peaks (Peak I, K_D 1.06; and Peak II, K_D 2.28). Small amounts of polysaccharides were also distributed continuously between the two peaks. P-WSPS was eluted at the same position as Peak I (ref. 2), showing the possibility that Peak I was the same polysaccharide as P-WSPS. The analyses of sugar composition of L-WSPS had already shown that the content of galacturonic acid increased markedly, although the sum of the galactose and galacturonic acid

TABLE I

SUGAR COMPOSITIONS (PERCENT) OF THE FRACTIONS SHOWN IN FIG. 1b

Fraction	*Arabinose*	*Rhamnose*	*Galactose*	*Galacturonic acid*
Peak I (Fr. 15-22)[a]	23.46	29.53	33.89	13.12
Fr. 25-35[a]	24.06	29.49	28.21	18.24
Peak II (Fr. 37-45)[a]	23.32	29.95	16.48	30.24
L-WSPS	23.67	29.64	16.81	29.89

[a]Fraction number as in Fig. 1b.

TABLE II

SUGAR COMPOSITIONS (PERCENT) AND [η] VALUES OF NA-WSPS AFTER STORAGE AT 37°

Storage period (days)	*Arabinose*	*Rhamnose*	*Galactose*	*Galacturonic acid*	[η] *(dL/g)*
0	23.03	29.97	26.44	20.55	18.2
25	24.01	29.69	21.01	25.21	11.5
50	23.89	29.11	18.32	28.68	5.38

was almost the same as that for P-WSPS. These results showed that Peak II and the polysaccharides distributed among Peaks I and II were degradation products of the material in Peak I. In order to confirm this, analyses of fractions 15–22 (Peak I), 25–35, and 37–45 (Peak II) were performed. The results are given in Table I. The sugar composition of Peak I was not the same as that of P-WSPS, but the proportions of arabinose and rhamnose were in good agreement with those for all three polysaccharide fractions. With the decrease in molecular weight, the content of galacturonic acid increased from 13.12 to 30.20%, confirming that the material in Peak I was degraded into that in Peak II, passing through the state of the polysaccharide lying between Peaks I and II.

An experiment on the storage of the fresh, wet bark of *P. amurense* was made in order further to confirm this conclusion. The fresh, wet bark of *P. amurense* was kept for 25 and 50 days at 37°, and then analyses of sugar composition were made, and gel-filtration chromatography and viscometry were performed; the results are given in Table II. The proportions of arabinose and rhamnose in all three polysaccharides did not change, but that of galacturonic acid increased with the length of storage.

The intrinsic viscosities ([η]) of the three polysaccharides were obtained by extrapolating the linear lines of the plots of $(\eta_{rel} - 1)/C$ *vs* C. NA-WSPS, which

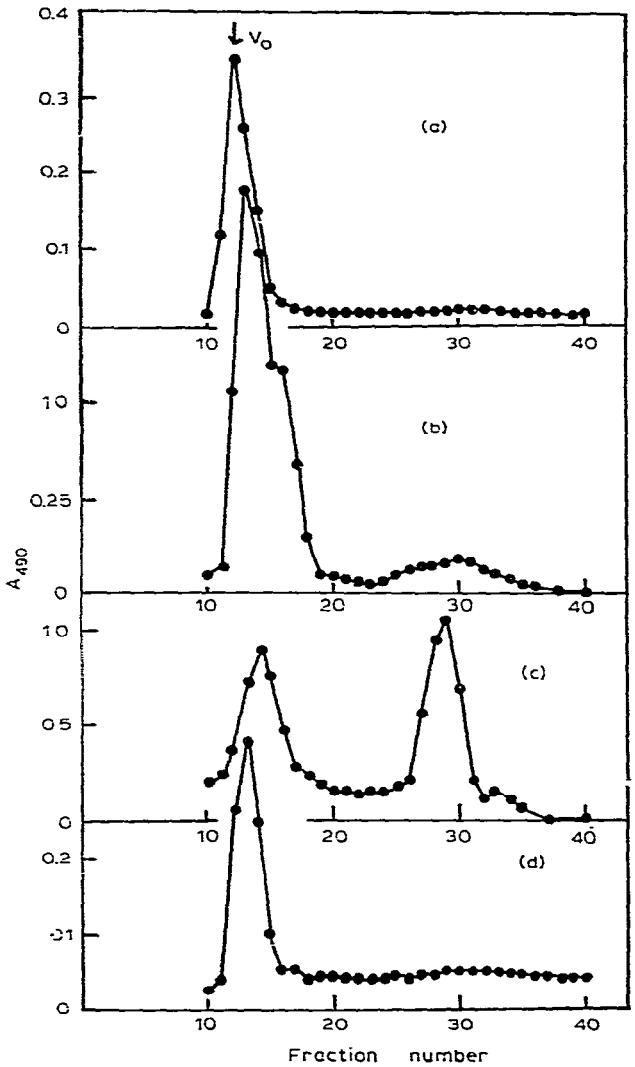

Fig. 2. Gel-filtration chromatography of the stored NA-WSPS. [NA-WSPS stored for (a) 0, (b) 25, and (c) 50 days at 37°, and (d) for 50 days at −20° were chromatographed on a small column (1 × 14 cm) of Sepharose 6B].

had a large $[\eta]$ value (18.20 dL/g), was degraded during storage at 37°, to give an $[\eta]$ of 5.58 dL/g after 50 days.

The results of gel-filtration chromatography showed the degradation of polysaccharide (see Fig. 2). NA-WSPS was excluded from Sepharose 6B gel, showing that NA-WSPS had a larger molecular weight than P-WSPS. Most of the NA-WSPS was slightly degraded by storage for 50 days at −20°; it was eluted at K_D 1.07, the same K_D value as that of P-WSPS. After 25 days at 37°, NA-WSPS had been degraded to the molecule eluted at K_D 1.07, and a small shoulder (K_D 1.3) was also detected.

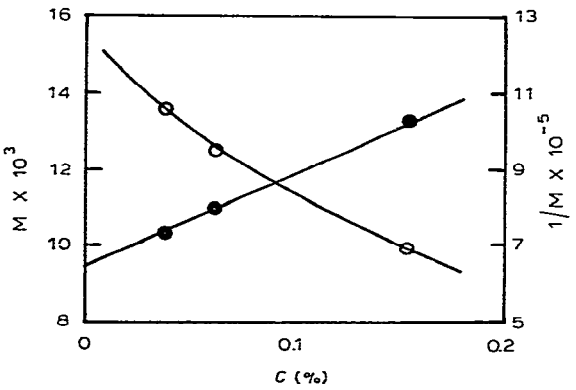

Fig. 3. The plots of S *vs* C (○——○) and $1/S$ *vs* C (●——●).

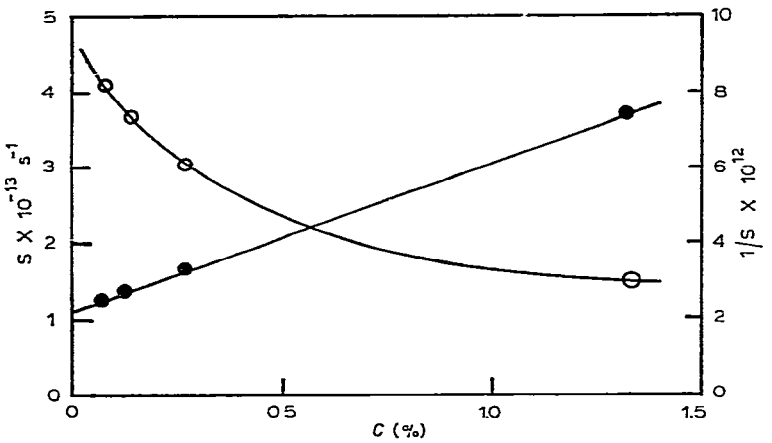

Fig. 4. The plots of M *vs* C (○——○) and 1/M *vs* C (●——●) in the sedimentation-equilibrium experiment.

The presence of a small proportion of the polysaccharide of low molecular weight was detected after storage for 25 days and the proportion increased to over a half after 50 days. These results showed that the water-soluble polysaccharide of *P. amurense* was degraded to a smaller one, together with oxidation of galactose to galacturonic acid residues, and that degradation occurred at random.

The intrinsic viscosity decreased with the period of storage at 37°, in good agreement with the results of gel-filtration chromatography. It has been reported[3] that the equation relating molecular weight (M) and intrinsic viscosity ($[\eta] = KM^{\alpha}$) is effective only for linear polysaccharides, but the molecular weight of NA-WSPS was presumed to be $>1 \times 10^6$, as the $[\eta]$ value was larger than that of P-WSPS (623×10^3) and the results of gel-filtration chromatography on Sepharose 6B confirmed this. From the large K_D value and the small $[\eta]$ value, the molecular weight of L-WSPS is quite small.

It has been found that acidic polysaccharides show abnormal behavior in gel-

filtration chromatography, and give excessively large molecular weights[2]. Therefore, velocity-sedimentation and sedimentation-equilibrium experiments were conducted on L-WSPS.

Although a component of large molecular weight (Peak I) was observed in gel-filtration chromatography, fast-sedimenting material was not observed. The sedimentation pattern was relatively broad, indicating heterogeneity of L-WSPS. L-WSPS had a small sedimentation-coefficient, and showed weak concentration-dependence (see Fig. 3). The S^0 value was obtained by extrapolation of the plot of $1/S$ *vs* C, to give 4.65×10^{-13} s^{-1}, which was converted into $S^0_{20,w}$ 4.72×10^{-13} s^{-1}.

Fig. 4 shows the results of the equilibrium sedimentation. The deviations of ln$C$ values from the straight line for the plot of ln$C$ *vs* r^2, where r is the distance from the center of rotation, were larger than those of P-WSPS, reflecting the heterogeneity in molecular weight of L-WSPS. The molecular weights obtained at three different concentrations of L-WSPS were small and showed weak concentration-dependence, but the plot of M *vs* C did not give a straight line. Hence, the M^0 value was obtained from the plot of 1/M *vs* C, to give 15.6×10^3, a very small value that showed that the polysaccharide was degraded to an extremely large extent.

A methylation analysis of L-WSPS was made, in order to study the structural relationship between the large and the small polysaccharides. Completely methylated L-WSPS was reduced (MR-L-WSPS), converted into the alditol acetates, and analyzed by g.l.c.–m.s. (in comparison with P-WSPS). The results are given in Fig. 5. An analysis of remethylated MR-L-WSPS (MMR-L-WSPS) was also conducted. There was no essential difference between the chromatograms of MR-L-WSPS and MR-P-WSPS (see ref. 1), except for the proportions of 2,3,6-tri- and 2,3- and 2,4-di-*O*-

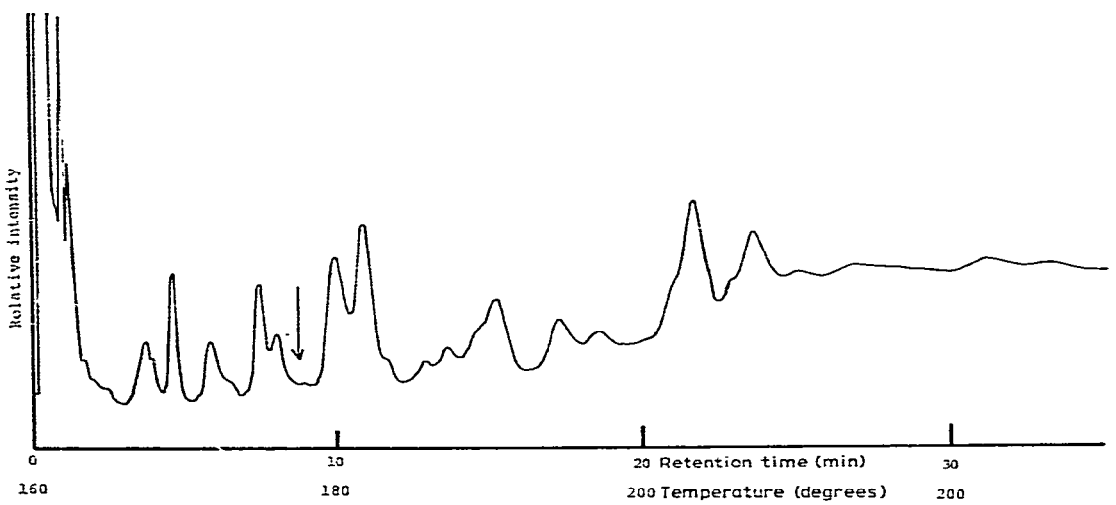

Fig. 5. G.l.c. patterns of MR-L-WSPS. [The temperature was increased from 160 to 200° at the rate of 2°/min. After reaching 200°, the temperature was kept thereat. A column (1.5 m) of 3% of ECNSS-M was used in this experiment. The arrow indicates the position of R_T 1.00.]

methylgalactose derivatives, reflecting the differences in galacturonic acid and galactose content. Degradation of P-WSPS should produce nonreducing end-groups, but change in the proportion of each peak was not observed in the spectrum; presumably, the increase in nonreducing end-group was $<1\%$, according to a calculation based on the molecular weights of the two polysaccharides, and hence, it cannot be detected unless the degradations take place at specific linkages, suggesting that the degradations occur at random.

The differences in the molecular weights and galacturonic acid contents of the water-soluble polysaccharides of *P. amurense* presumably derive from different extents of degradation of a polysaccharide that, *in vivo*, had been synthesized to have almost the same molecular weight. However, other conditions, such as the age, seem to be in part concerned with the formation of different molecular species, because of the exceptional relationship among molecular weight, viscosity, and galacturonic acid content that was present.

EXPERIMENTAL

Determination of polysaccharide concentrations. — The concentrations of polysaccharides were determined by the phenol method[3], and are expressed as the concentrations of P-WSPS, or as the absorbances at 490 nm.

Extraction of water-soluble polysaccharide. — Water-soluble polysaccharides were extracted with hot water as described previously[2]. Three polysaccharides were used in this study. One was P-WSPS (see refs. 1 and 2) which was prepared from dried, commercial bark harvested in Miyazaki Pref., Japan. Another was prepared from dried bark harvested in Taiwan (L-WSPS). The third was prepared from fresh, wet bark immediately after harvesting it in Nara Pref., Japan (NA-WSPS). The yield of the former two was $\sim 5\%$, and that of NA-WSPS was $\sim 8.6\%$ (for dry barks). All three samples were purified to the state that gave white, fibrous precipitates on addition of an excess of ethanol, and by chromatography in a column of DEAE-cellulose as described previously[2], and these were used in all the experiments in this study.

Analysis of sugar composition. — Sugar compositions were analyzed by g.l.c. as described in refs. 1 and 4.

Column chromatography on DEAE-cellulose. — A solution of the polysaccharide (~ 10 mg) in 5 mL of 0.05M Tris-HCl, pH 6.2 (buffer *A*) was placed on a DEAE-cellulose column (2 × 14 cm), and developed with a linear gradient of sodium chloride as described[2].

Gel-filtration chromatography. — An aliquot of a solution of polysaccharide in buffer *A* containing 0.1M sodium chloride was placed on a column (3 × 42 cm) of Sepharose 6B, and developed with buffer *A* as described[2].

Sedimentation velocity. — Sedimentation-velocity analysis was conducted at 20° in a Spinco Model E ultracentrifuge at 52,640 r.p.m. (0.065, 0.131, and 0.26%) and 59,780 r.p.m. (1.33%). All samples were dialyzed completely against 0.1M

sodium chloride, and the concentration of L-WSPS was measured for each sample by the phenol method[5].

Sedimentation equilibrium. — The molecular weight of L-WSPS was determined by sedimentation-equilibrium centrifugation as described[2]. An 0.157% solution of L-WSPS in 0.1M sodium chloride (2 mL) was dialyzed against 0.1M sodium chloride, and then centrifuged at 10,000 r.p.m. for 10 min, to remove suspended materials. The resultant clear, colorless solution was diluted with 0.1M sodium chloride. The concentrations of L-WSPS were measured for each sample. The centrifugation was conducted at 20° in a Spinco Model E analytical ultracentrifuge at 21,740 r.p.m. for 24 h. Initial concentrations were measured in the centrifuge with synthetic boundary-cells, and Raleigh interference optics were employed. Column lengths of the solutions were ~1.9 mm. For the calculation of molecular weight, $\bar{V}$ 0.56, as estimated for P-WSPS, was used.

Viscosity. — Viscosity was measured in an Ostwald viscometer at 20°, as described[2]. The concentrations of L-WSPS were measured for each solution. The flow time for the solvent was 41.9 s.

Tracing of changes in the molecular properties of the polysaccharides during storage. — Fresh bark of *P. amurense*, harvested in Nara Pref., was separated into two parts. One part was kept at −20° and the other at +37°. Aliquots were withdrawn after 25 and 50 days, and the polysaccharide was extracted, and analyzed by gel-filtration chromatography, and viscometry; also, the sugar compositions were determined by g.l.c.

Methylation analysis. — L-WSPS (500 mg) was methylated with methylsulfinyl carbanion and methyl iodide[6], and additional methylations were performed by the Kuhn method until hydroxyl absorption was not observed in the i.r. spectrum. The product was reduced (MR-L-WSPS, 310 mg), hydrolyzed, converted into alditol acetates, and these analyzed by g.l.c.–m.s. according to the method of Björndal *et al.*[8], as described[1].

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