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PHYSICOCHEMICAL STUDIES OF THE WATER-SOLUBLE POLYSACCHARIDE OF *Phellodendron amurense* RUPRECHT*

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ABSTRACT

Purified, water-soluble polysaccharide of *Phellodendron amurense* Ruprecht (P-WSPS) was obtained in highly homogeneous state. The partial specific volume and intrinsic viscosity were, respectively, 0.56 and 11.68 dL/g. The S value and molecular weight depended strongly on the P-WSPS concentration, but extrapolation to infinite dilution gave $S^0_{w,20} = 10.67 \times 10^{-13} s^{-1}$ and $M^0 = 625 \times 10^3$ from velocity-sedimentation and equilibrium-sedimentation experiments, respectively, results that showed the highly branched structure of P-WSPS. The molecular weight calculated from the D^0 value (1.035×10^{-7}) and S^0 value agreed with that from the equilibrium-sedimentation experiment to within ~±9%, showing that the diffusion method is useful for rapid estimation of molecular weight.

INTRODUCTION

Phellodendron amurense Ruprecht (Wobaku wood) contains the yellow alkaloid berberine, and has been used as a material for yellow dyeing and as a drug for stomach disease[1,2]. In both cases, the hot-water extract of the bark was employed at the time of use. The extract contains a considerable proportion of polysaccharide[3], and this plays important roles in the color of dyed materials and the drug action, but very little research has been conducted thereon. Therefore, in order to understand the functions of this polysaccharide, it is necessary to clarify its characteristics and structure.

We now describe some physicochemical properties of the polysaccharide as the first step in a study thereof.

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*Studies on the Water-soluble Polysaccharide of *Phellodendron amurense* Ruprecht, Part I.

RESULTS AND DISCUSSION

The homogeneity of the purified, water-soluble polysaccharide (P-WSPS) obtained from *P. amurense* was examined by chromatography, both on a column of DEAE-cellulose and by gel-filtration. In the former (see Fig. 1a), it was eluted with 0.24M sodium chloride in a single, sharp peak; although very small quantities of

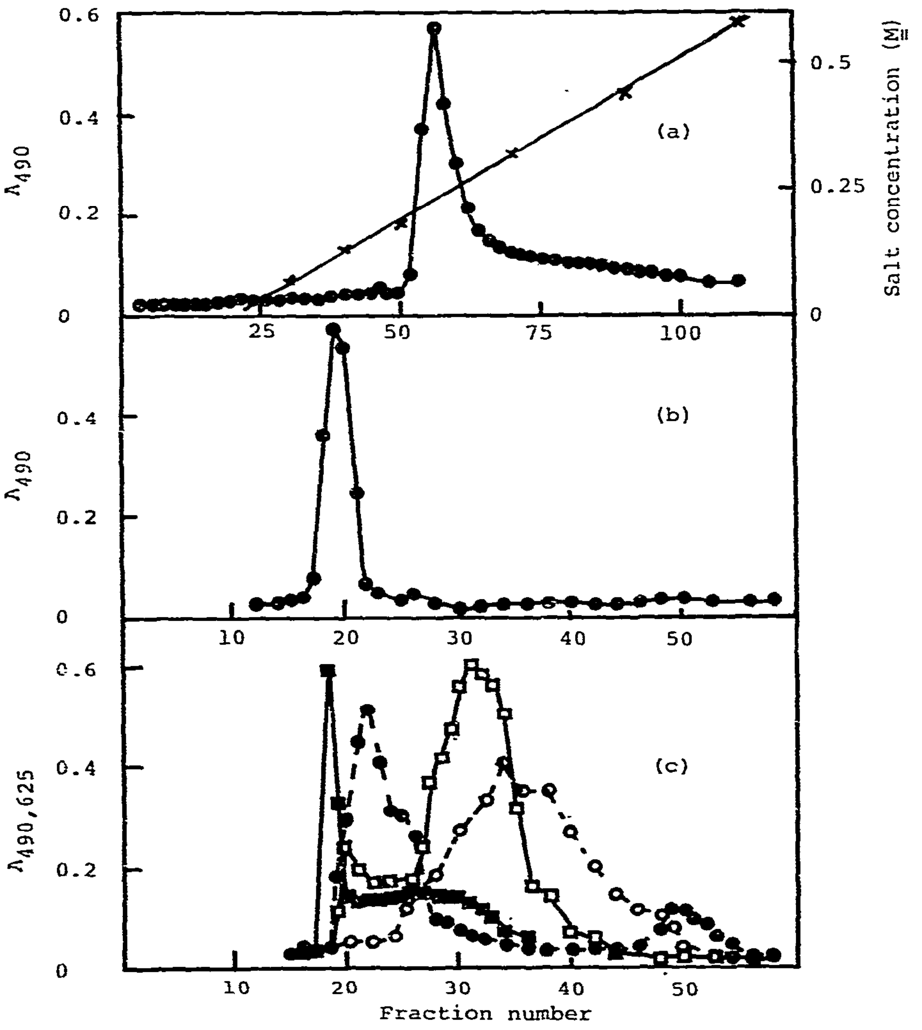

Fig. 1. Column-chromatographic patterns of P-WSPS and some other polysaccharides. [The elution patterns from a column of DEAE-cellulose and a column of Sepharose 6B are shown in a and b, respectively. The elution patterns of Blue Dextran T-2000 (■—■), gum tragacanth (○—○), gum arabic (□—□), and aliginic acid (○----○) from a Sepharose 6B column are given in c (as absorbances at 490 nm in the phenol method). The amounts of Blue Dextran T-2000 are expressed as absorbances at 625 nm. Salt concentrations are shown in the line (×—×).]

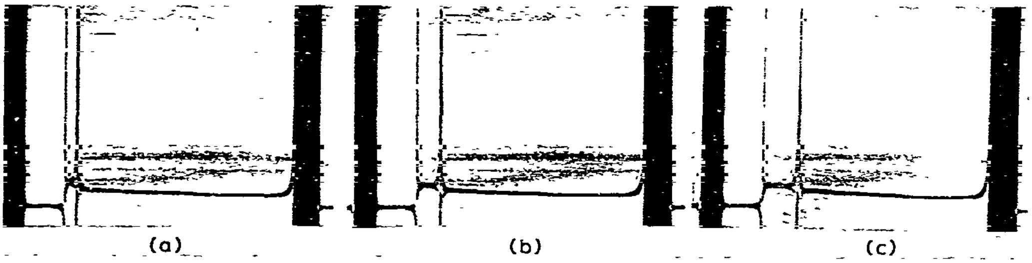

Fig. 2. Velocity-sedimentation patterns of P-WSPS. [Photographs were taken at (a) 24, (b) 56, and (c) 80 min after reaching 42.040 r.p.m. The centrifugation was made at 0.303% in 0.1M sodium chloride. Sedimentation is from left to right.]

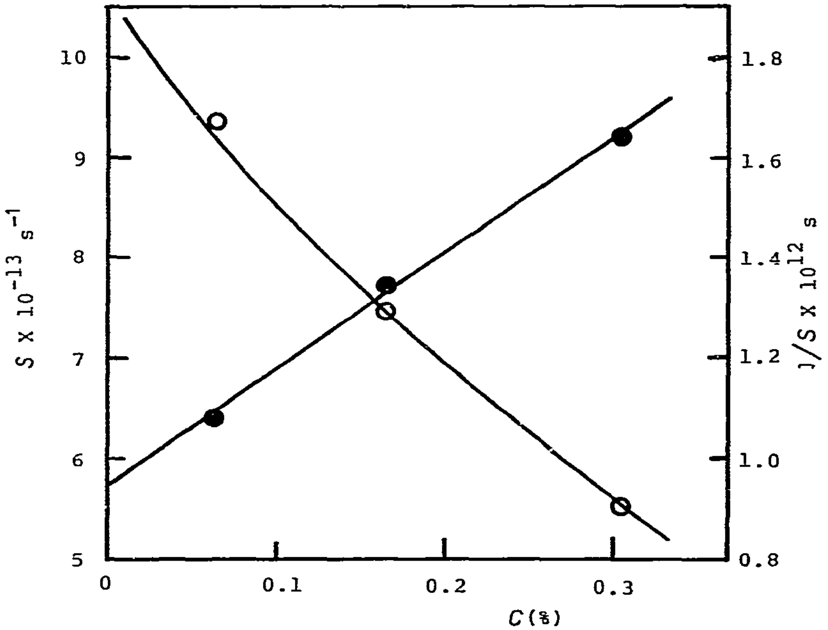

Fig. 3. The plots of S *vs* C (○—○) and $1/S$ *vs* C (●—●).

sugars were eluted with higher concentrations of salt, the result showed that the P-WSPS was highly homogeneous.

Gel-filtration chromatography with Sepharose 6B, in buffer *A* containing 0.1M sodium chloride, was performed on P-WSPS in order to check its homogeneity and to obtain a rough estimate of its molecular weight (see Fig. 1b). P-WSPS was eluted in a sharp, symmetrical peak, and contained no sugars of low molecular weight, showing that P-WSPS was homogeneous.

Despite the fact that the molecular weight of P-WSPS was ~623 × 10^3, as described later, it had a smaller K_D value (1.04) than expected; this was also observed in the chromatograms (see Fig. 1c) of acidic polysaccharides; gum tragacanth (M = 750 × 10^3)^4, gum arabic (M = 200–300 × 10^3)^4, and alginic acid (M = 32–200 ×

$10^3)^4$. These abnormalities in the K_D values were observed in chromatography with 0.5M sodium chloride, and therefore, are due both to the electronic forces of the carboxyl groups of uronic acid residues and to the network structure[5].

The velocity-sedimentation patterns, given in Fig. 2, showed a single, very sharp peak at all concentrations employed, indicating the high homogeneity of P-WSPS. The extremely sharp peaks were due to the self-sharpening effect of the polysaccharide solution, and showed that P-WSPS does not sediment in the free-diffusion state. Sedimentation coefficients at three different concentrations of P-WSPS (5.98 *S* at 0.303%, 7.43 *S* at 0.105%, and 9.37 *S* at 0.063%) revealed that there was a large dependence of the *S* values on the concentration. These results were in a good agreement with the strong, self-sharpening effect in the Schlieren pattern. Fig. 3 shows the results of velocity-sedimentation experiments. Because the plot of P-WSPS concentration (*C*) *vs* *S* value did not give a straight line, the S^0 value was obtained by extrapolation to infinite dilution of the *C* *vs* 1/*S* plot, which gave a straight line, and an S^0 value of 10.52×10^{-13} s^{-1}. The conversion of the S^0 value into the value under the standard conditions ($S^0_{20,w}$) was made by use of Eq. *1*,

$$S^0_{20,w} = \left(\frac{\eta_{20,\mathrm{solv}}}{\eta_{20,w}}\right) \frac{(1 - \bar{V}\rho)_{20,w}}{(1 - \bar{V}\rho)_{20,\mathrm{solv}}} S^0, \tag{1}$$

giving $S^0_{20,w} = 10.67 \times 10^{-13}$ s^{-1}. In this case, the following values were used: $(\eta_{20,\mathrm{solv}}/\eta_{20,w}) = 1.009$, $\bar{V}_{20,w} = \bar{V}_{20,\mathrm{solv}} = 0.56$, $\rho_{20,w} = 0.9982$, and $\rho_{20,\mathrm{solv}} = 1.0023$.

The molecular weight of the polysaccharide was roughly estimated from the S^0 value and the diffusion constant (D^0) at infinite dilution by use of Eq. *2*.

$$M^0 = \frac{RTS^0}{(1 - \bar{V}\rho)D^0}, \tag{2}$$

where M^0 is the molecular weight at infinite dilution, and $\bar{V}$ and ρ are the partial specific volume and the density of the solution. The measurement of the D values at various P-WSPS concentrations could be readily achieved in a short time, and, hence, the D values at three different P-WSPS concentrations were measured. It had been reported that the concentration-dependence of the D value is small[6]. The D values observed were very small (1.60×10^{-7} at 0.314%, 1.30×10^{-7} at 0.147%, and 1.17×10^{-7} at 0.073%), but showed dependence on the concentration. The value of D decreased with decrease in the concentration of P-WSPS, and the plot of D *vs* *C* gave a straight line (see Fig. 4), and $D^0 = 1.035 \times 10^{-7}$.

By measurement of the density, the partial specific volume of P-WSPS was determined to be 0.56. Then, by introducing D^0 and S^0 values, the molecular weight was calculated from Eq. *2* to be 565×10^3. The M^0 value obtained was ~9% smaller than that given by the equilibrium-sedimentation method (623×10^3), but it is considered that this method is useful in the rough estimation of molecular weight, because of the simplicity of measurement.

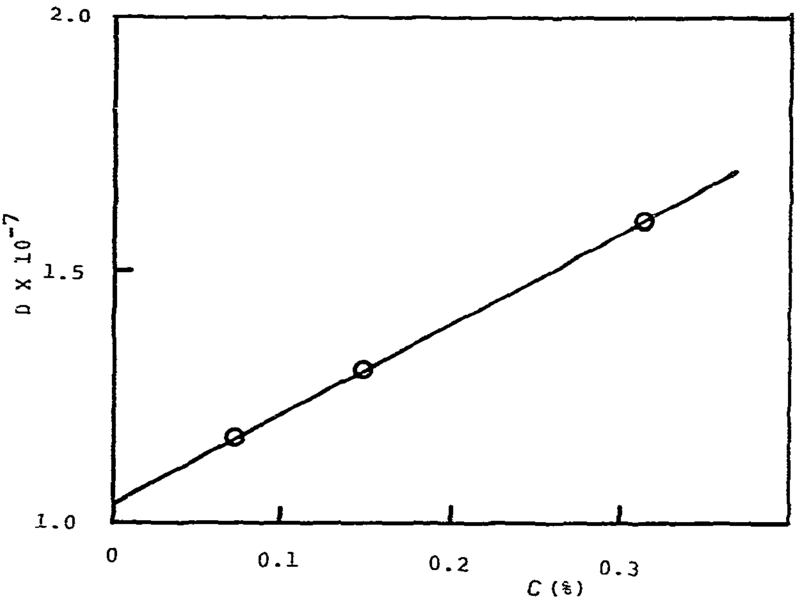

Fig. 4. The plot of D *vs* *C*.

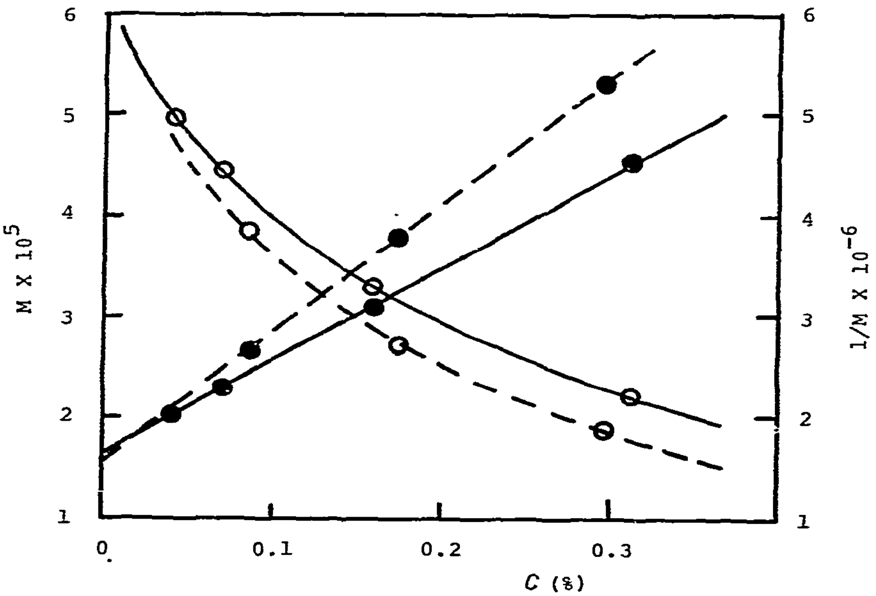

Fig. 5. The plots of M *vs* *C* and 1/M *vs* *C*. [The symbols (○—○) and (●—●) respectively represent the plots of M *vs* *C* and 1/M *vs* *C* in Exp. 1. The symbols (○---○) and (●---●) respectively represent the plots of M *vs* *C* and 1/M *vs* *C* in Exp. 2.]

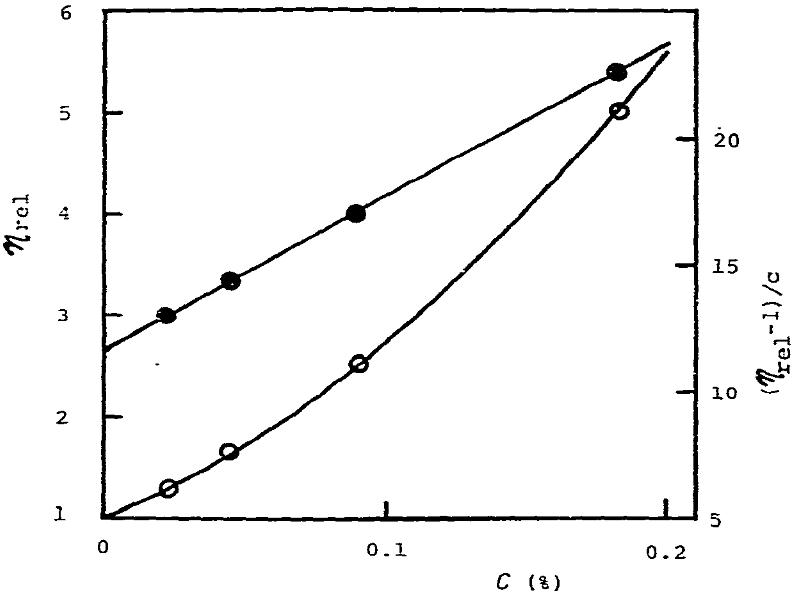

Fig. 6. The plots of η_{rel} (○—○) *vs* *C* and $(\eta_{rel}-1)/C$ *vs* *C* (●—●).

The molecular weight of P-WSPS was determined precisely by two independent, sedimentation-equilibrium experiments. The plots of ln*C* *vs* r^2 gave good linearity for all concentrations $<0.4\%$ of P-WSPS. The results (see Fig. 5) showed that these solutions were ideal, and that the molecular weights of P-WSPS were distributed in a relatively narrow range. The molecular weights at various concentrations of P-WSPS were somewhat different in two experiments, but showed strong dependence on concentration in both. The extents of the differences in molecular weight were larger at higher concentrations of P-WSPS, presumably because the differences in experimental conditions, centrifugal forces, and volumes of the sample solutions were stressed to a greater degree at high P-WSPS concentrations. In both experiments, the M *vs* *C* plots did not give straight lines, and hence, the M^0 values were obtained by extrapolation of the straight lines of plots of 1/M *vs* *C*. The M^0 values showed good agreement in both experiments (617 and 629 × 10^3 for Exp. 1 and 2), and were estimated to average 623 × 10^3, showing that the P-WSPS molecule is large, as in various kinds of polysaccharides[4]. The large molecular weight indicated that the P-WSPS molecule is not linear, but is highly branched.

One of the remarkable features of P-WSPS is that its solutions have a high viscosity, and the measurement of viscosity was conducted by using an Ostwald type of viscometer (see Fig. 6). The values of relative viscosity (η_{rel}) obtained at various concentrations of P-WSPS did not give a straight line in the plot of η_{rel} *vs* *C*, but gave good linearity in that of $(\eta_{rel} - 1)/C$ *vs* *C*. Extrapolation of the line gave an intrinsic viscosity ($[\eta]$) of 11.68 dL/g. The high value of $[\eta]$ is presumably due to the presence of ~17% of galacturonic acid[5] and a highly branched structure.

EXPERIMENTAL

Determination of concentrations of polysaccharide. — The concentrations of polysaccharide were determined by the phenol method[7]. Absorbances at 490 nm were measured, and expressed as concentrations of P-WSPS. The intensity of the color development of P-WSPS was ~63.7% of that of D-galactose.

Extraction and purification of water-soluble polysaccharide. — Water-soluble polysaccharide was obtained from ground bark (2 kg) of *P. amurense*, harvested in Miyazaki Pref., Japan, by the procedures described previously[3]. The yield was 105 g (~5%), of a brownish-black, fibrous mass. In order to purify this, a portion was dissolved in 0.05M Tris · HCl (pH 7.2; buffer *A*), and the solution was placed on a column of DEAE-cellulose. Colored materials were removed by washing the column with buffer *A*, and the water-soluble polysaccharide was recovered by elution with 0.3M sodium chloride in buffer *A*. Addition of two volumes of ethanol gave a white, fibrous mass (P-WSPS) which was used in all of the experiments in this study.

Chromatography on a column of DEAE-cellulose. — A solution of P-WSPS (100 mg) in buffer *A* (10 mL) was added to a column (2 × 10 cm) of DEAE-cellulose equilibrated with buffer *A*. The absorbed materials were eluted with a linear gradient of 0→0.5M sodium chloride in buffer *A*, the concentrations of sodium chloride being measured by the Mohr method[8].

Gel-filtration chromatography of P-WSPS on Sepharose 6B. — P-WSPS in buffer *A* containing 0.1M sodium chloride was placed on the top of a column (3 × 42 cm) of Sepharose 6B, and elution was conducted with the same buffer. In the case of chromatography with 0.5M sodium chloride in buffer *A*, a small column (1 × 16 cm) was employed. The void volumes of these columns were determined by chromatography of Blue Dextran T-2000.

Velocity sedimentation. — Velocity-sedimentation analysis of P-WSPS was performed at 20° in a Spinco, model E, analytical ultracentrifuge at 42,040 r.p.m. (0.303%) and 52,640 r.p.m. (0.105 and 0.063%). All samples were dialyzed five times against 0.1M sodium chloride, with 1-day intervals, and all dilutions were made with this solution. The concentration of P-WSPS was measured for each sample.

Determination of the diffusion constant. — The diffusion constant (D) was determined by using three samples (0.314, 0.147, and 0.073%) of P-WSPS in 0.1M sodium chloride. Centrifugation was conducted at 20° in a Spinco, model E, analytical ultracentrifuge at 6,569 r.p.m. by using double-sector, synthetic-boundary cells, DF (SB-II)-1. All samples were dialyzed completely against 0.1M sodium chloride. The concentration of P-WSPS was measured for each sample. Calculation of diffusion constants was made by plotting $(A/H)^2$ *vs* t according to Eq. *3*, because of the small value of $2\omega^2St$,

$$(A/H)^2 = 4\pi \mathrm{D}t, \qquad (3)$$

where A and H are the area and the height of the Schlieren pattern.

Sedimentation equilibrium. — The precise molecular weight was determined by two independent experiments employing the sedimentation-equilibrium method. A solution (0.4%) of P-WSPS was prepared by dissolving P-WSPS in 0.1M sodium chloride (2 mL). The solution was dialyzed five times against 0.1M sodium chloride at 5° with 1-day intervals. After dialysis, the suspension was centrifuged at 10,000 r.p.m., to remove insoluble materials. The resultant, clear solution was diluted with the outer solution from the dialysis, and the concentration of P-WSPS was determined for each sample. Five samples, containing various concentrations of P-WSPS, were centrifuged at 20° in a Spinco, model E, analytical ultracentrifuge at 5,784 r.p.m. for 50 h (Exp. 1). Another experiment (Exp. 2) was performed at 20° in a Hitachi, model 282, analytical ultracentrifuge at 5,000 r.p.m. for 48 h (three samples). In both cases, initial concentrations were measured in the centrifuge by means of synthetic-boundary cells and Raleigh interference optics. Column lengths of the sample solutions varied from 1.2 to 1.5 mm (Exp. 1) and from 2.3 to 2.6 mm (Exp. 2). Calculations of molecular weight were based on Eq. *4*.

$$(\mathrm{Mw})_r = \frac{2\mathrm{RT}}{(1 - \bar{V}\rho)} \cdot \frac{\mathrm{dln}\ C}{\mathrm{d}r^2}, \tag{4}$$

where (Mw) is the weight-average molecular weight, ρ is the density of the solution, and r is the distance from the center of rotation.

Partial specific volume. — A 50-mL pycnometer was employed at 20° to measure the densities of solution having various P-WSPS concentrations. The solvent used was 0.1M sodium chloride. The partial specific volume was estimated from the plot of C *vs* density, according to Eq. *5*.

$$\rho_{\mathrm{soln}} = \rho_{\mathrm{solv}} + (1 - \bar{V}\rho_{\mathrm{solv}})C, \tag{5}$$

where ρ_{soln} and ρ_{solv} are the densities of the solution and solvent, respectively.

Viscometry. — Viscometry was conducted in an Ostwald viscometer. The flow time at 20° was measured with an accuracy of ±0.1 s. The fine particles in the suspensions were removed by filtration through glass filter No. 3. The concentration of P-WSPS was measured for each sample. The flow time for the solvent (0.1M sodium chloride) was 42.0 s.

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