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CORRELATION BETWEEN THE ANTITUMOR ACTIVITY OF A POLYSACCHARIDE SCHIZOPHYLLAN AND ITS TRIPLE-HELICAL CONFORMATION IN DILUTE AQUEOUS SOLUTION

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Eight samples of a polysaccharide schizophyllan ranging in weight-average molecular weight M_w (in water) from 5×10^3 to 1.3×10^5 were prepared and their antitumor activity (expressed in terms of the tumor inhibition ratio) against Sarcoma 180 ascites, intrinsic viscosities $[\eta]$, and gel-filtration chromatograms in aqueous solution were determined. The tumor inhibition ratio was essentially unity for samples with M_w higher than 9×10^4, but reduced to zero or even to a negative value when M_w was lower than 10^4. The $[\eta]$ data combined with the chromatographic data showed that above $M_w \approx 9\times10^4$ the predominant species of schizophyllan in aqueous solution is the previously found rigid triple helix, whereas below $M_w \approx 9\times10^4$ both triple helices and single chains coexist in the solution and the fraction of triple helices decreases monotonically to zero as M_w is decreased to 5×10^3. From these findings it was concluded that the antitumor potency of schizophyllan in water is related to the amount of triple helices relative to that of single chains.

1. Introduction

Schizophyllan is a water-soluble, nonionic polysaccharide elaborated extracellularly by a fungus *Schizophyllum commune* [1]; it consists of a repeating unit [2] shown in fig. 1. Komatsu et al. [3] were the first to find that an aqueous solution of this polysaccharide has a host-mediated antitumor activity against Sarcoma 180. Later, Tabata et al. [4] showed that fragmentation of native schizophyllan by sonication does not impair the chemical structure and antitumor potency of the original sample if the molecular weights of fragmented samples (in water) are higher than about 2.4×10^5. At about the same time, Norisuye et al. [5] found that both native and sonicated samples of schizophyllan ranging in molecular weight from 10^5 to 6×10^6 dissolve in water as a trimer having a rigid triple-helical structure.

The present study was undertaken to see whether the antitumor activity and the triple-helical structure of schizophyllan in water are maintained when the molecular weight is decreased below 10^5 by extensive sonication. For this purpose, bioassay, sedimentation equilibrium, viscosity and gel-filtration experiments were performed on eight extensively sonicated samples.

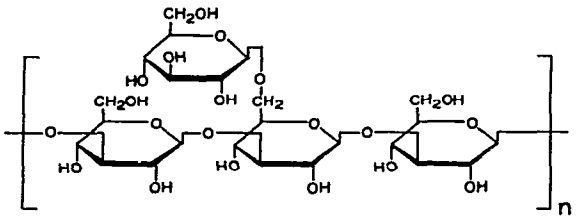

Fig. 1. Repeating unit of schizophyllan.

2. Experimental

2.1. Samples

From sonicated schizophyllan samples stored for clinical use at Taito Co., one with a molecular weight (in water) of 4.5×10^5 was chosen for the present study. Its aqueous solution (about 4% polymer) was exposed to 19.5 kHz sonic irradiation (Kaijo Denki, Model TA-6280N) for about 500 h, with pasteurization at suitable intervals of time. The jacket of the sonication vessel was kept below 20°C by circulating water at 10°C. The sonicated solution was passed through an 0.3 μm Millipore filter after being deionized with ion-exchange resins (Nippon Orugano, IRA 402 and IR 120B). The schizophyllan sample recovered from it was separated into 20 parts by repeating fractional precipitation with water as the solvent and acetone as the precipitant. Eight fractions designated below as U-1, U-4, U-6, U-11, U-15, U-16, U-17 and U-18 were chosen, and first freeze-dried from aqueous solutions and then vacuum-dried overnight. Methylation analysis and enzymic hydrolysis with *exo*-β-1,3-glucanase showed that all these fractions consisted of the same repeating units as that shown in fig. 1.

2.2. Assay of antitumor activity

Sarcoma 180 ascites (0.1 cm^3; about 2×10^6 cancerous cells) was implanted subcutaneously into the groins of thirty ICR-JCL mice (Clea Japan Co.) weighing 20–25 g. After 24 h, a physiological saline solution of a given schizophyllan sample (polymer concentration 0.5 wt.%) was intramuscularly injected into ten of these mice at an optimum dose [6,7] of 10 mg schizophyllan per kg of mouse. 31 days after the tumor implantation, all the mice were killed and dissected, and the tumor inhibition ratio ξ, defined by

$$\xi = (w_C - w_T)/w_C \quad (1)$$

was determined by weighing the tumors extirpated from the schizophyllan-treated and untreated mice. Here, w_T and w_C denote the average tumor weights of the schizophyllan-treated and untreated groups of mice, respectively. The value of w_C was 2.57 g with a standard deviation of 2.08 g. The complete regression, defined as the number of tumor-free mice * relative to that of the treated mice, was also determined.

2.3. Sedimentation equilibrium

Sedimentation equilibrium measurements were made on all samples in water and on samples U-6, U-15, U-17 and U-18 in dimethyl sulfoxide (DMSO), another solvent for schizophyllan, at 25°C using a Spinco Model E ultracentrifuge equipped with an electronic speed-control system. A Kel-F 12 mm double-sector cell was used, and the liquid column was adjusted to 1.2–2.5 mm. Rayleigh fringe patterns were read on a Nikon Shadowgraph Model 6 equipped with a digital micrometer. The weight-average molecular weight M_w of each sample was estimated by extrapolating the values of the apparent molecular weight M_{app} for different initial polymer concentrations to infinite dilution according to the well known equation [8]

$$M_{app}^{-1} = M_w^{-1} + 2A_2\bar{c} + \ldots \quad (2)$$

Here, A_2 is the second virial coefficient of the solution and $\bar{c}$ the mean concentration defined by $(c_a + c_b)/2$, with c_a and c_b being the equilibrium polymer mass concentrations at the meniscus and the bottom of the liquid column, respectively.

The specific refractive index increment $\partial n/\partial c$ for schizophyllan in water at 25°C was independent of molecular weight, being 0.145 cm^3 g^{-1} at 436 nm wavelength and 0.143 cm^3 g^{-1} at 546 nm. These values agreed with those [5] determined previously with higher molecular weight samples. For $\partial n/\partial c$ in DMSO and for the partial specific volumes in water and DMSO the previously determined values [5] were used.

2.4. Gel-filtration chromatography

Five schizophyllan samples U-1, U-4, U-11, U-16 and U-18 were dissolved in 0.05 M sodium acetate buffer and investigated by gel-filtration chromatography with a Sephadex G-100 column

* Mouse having a tumor weight less than 0.05 g.

of 2.5 cm diameter and 45 cm length. The polymer concentration of the loaded solution was 0.15 wt.%, and the flow rate 0.11 $cm^3\ min^{-1}$. The eluate (80 cm^3) was separated into 40 fractions, and the sugar content of each fraction was determined by the phenol-sulfuric acid method [9]. The gel-filtration chromatograms obtained were assumed to be the same as those in pure water, since virtually no viscosity difference was detected between pure water solutions and acetate buffer solutions.

2.5. Viscometry

Viscosities of schizophyllan in water and DMSO at 25°C were measured using capillary viscometers of the Ubbelohde suspended-level type. In converting the measured flow times to relative viscosities, correction for the solution density was made.

3. Results and discussion

3.1. Antitumor activity and molecular weight

Results from the bioassay are summarized in table 1, along with those from sedimentation equilibrium measurements. The values of ξ and the complete regression for samples U-1 and U-4 indicate potent antitumor activities against Sarcoma 180 ascites. These values are comparable to or even higher than those [4] determined previously for three higher molecular weight samples of schizophyllan, indicating that when M_w(in water) is above about 9×10^4, the antitumor potency of aqueous schizophyllan is independent of molecular weight. However, as M_w(in water) is decreased below 6×10^4, ξ decreases abruptly and even becomes negative at M_w(in water) $\approx 5 \times 10^3$. The complete regression also diminishes sharply in the same molecular weight region. Thus, it follows that for the dose of 10 mg kg^{-1}, schizophyllan almost loses its antitumor activity when sonicated to fragments having M_w(in water) of the order of 10^4.

3.2. Molecular species in aqueous solution

The ninth column of table 1 indicates that the molecular weight ratio Γ [$\equiv M_w$(in water)/M_w(in DMSO)] is fairly close to 3 for the two highest molecular weight samples U-1 and U-4, but decreases monotonically with decreasing M_w(in water) and approaches unity at M_w(in water) $\approx 10^4$. This leads to the conclusion that the predominant species of samples U-1 and U-4 in aqueous solution are a trimer, while those of the two lowest molecular weight samples U-17 and U-18 are a monomer, because, as shown previously [5,11], schizophyllan disperses in DMSO as single chains.

The gel-filtration chromatograms of some schizophyllan samples are shown in fig. 2. The curves

Table 1
Bioassay and sedimentation equilibrium data on schizophyllan samples

Sample	w_T (g) (±S.D.)	ξ	Complete regression	In water		In DMSO		M_w(in water)/M_w(in DMSO)
				$M_w(\times 10^{-4})$	$A_2(\times 10^4)$ ($cm^3\ mol\ g^{-2}$)	$M_w(\times 10^{-4})$	$A_2(\times 10^4)$ ($cm^3\ mol\ g^{-2}$)	
U-1	0.02 (±0.03)	0.992	9/10	13.4	2.6	4.7 [a]	–	2.9
U-4	0.01 (±0.02)	0.996	9/10	9.50	3.9	3.6 [a]	–	2.6
U-6	0.24 (±0.68)	0.91	9/10	7.40	2.3	3.08	10	2.4
U-11	0.53 (±0.85)	0.79	4/10	5.54	1.0	2.5 [a]	–	2.2
U-15	2.12 (±2.14)	0.18	3/10	4.08	–	1.96	15	2.1
U-16	2.14 (±1.73)	0.17	2/10	2.51	–	1.35 [b]	21 [b]	1.86
U-17	–	–	–	0.769	–	0.749	27	1.03
U-18	3.27 (±2.06)	−0.27	0/9 [c]	0.503	–	0.493	28	1.02

[a] Evaluated from $[\eta]$ (the intrinsic viscosity) using the relation between $[\eta]$ and M_w for schizophyllan in DMSO (see fig. 3).
[b] Taken from ref. 10.
[c] One mouse died of the ascites cancer 20 days after tumor implantation.

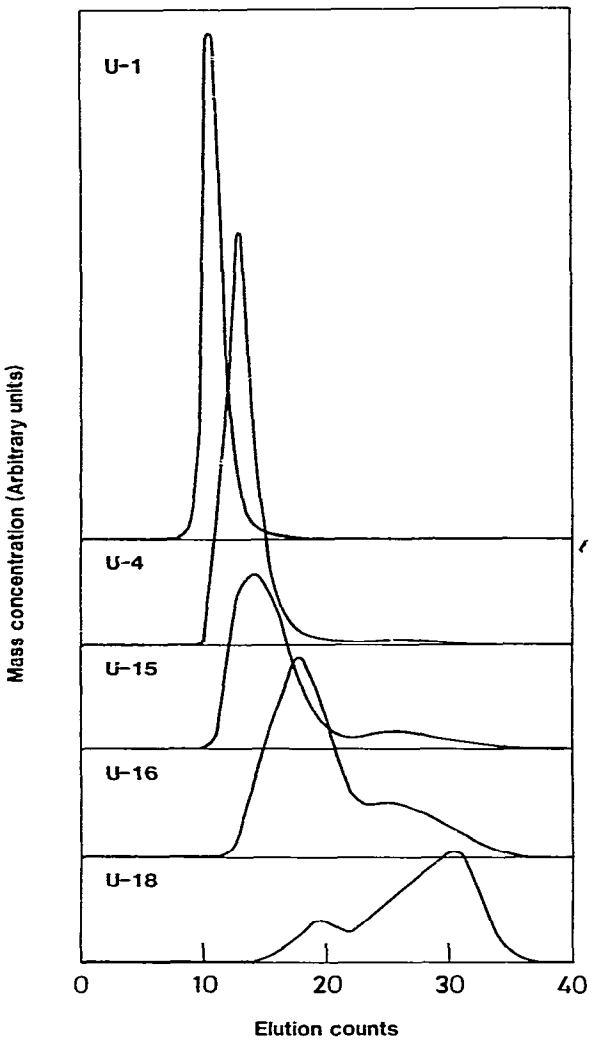

Fig. 2. Gel-filtration chromatograms of schizophyllan samples in 0.05 M aqueous sodium acetate.

for the two highest molecular weight samples U-1 and U-4 are essentially single peaked, but that for sample U-4 exhibits a broad tail in the region of large elution counts. As the molecular weight is lowered, this tail develops to a second peak, which eventually surpasses the first peak. Thus, except for U-1, the schizophyllan samples examined consisted of at least two species of different molecular weights, and the fraction of the lower molecular weight species increased with decreasing M_w of the sample. If this is combined with the above-mentioned conclusion that the predominant species of samples U-1 and U-4 in water are a trimer and that of sample U-18 is a monomer, it is reasonable to assign the first and second peaks in the chromatograms of fig. 2 to the trimer and the monomer, respectively.

The weight fraction f of trimers in a given aqueous solution may be calculated from the relation

$$f = \tfrac{1}{2}(\Gamma - 1) \qquad (3)$$

provided that M_w of the trimer species in the solution is 3-times as large as that of the monomer species. The trimer fraction may also be estimated from the gel-filtration chromatogram in fig. 2 if the curve can be decomposed correctly into the chromatograms of the two species. For example, a reasonable decomposition of the curve for sample U-18 gives f a value of between 0.1 and 0.2. However, this is much larger than 0.01 evaluated from Γ using eq. 3. A similar discrepancy was found for sample U-4, for which the values of f from Γ and the chromatogram were 0.8 and 0.9, respectively. At present, no reasonable explanation

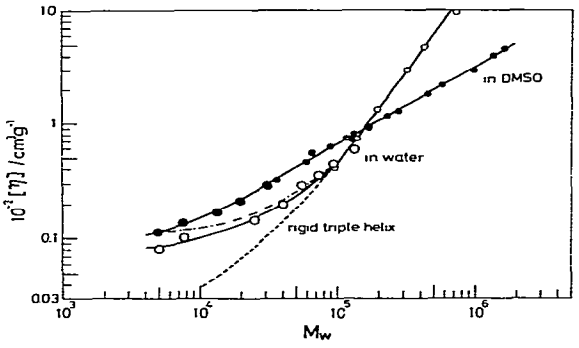

Fig. 3. Double-logarithmic plots of $[\eta]$ vs. M_w for schizophyllan in water and DMSO at 25°C. The smaller circles indicate previous data [5,11,12], and the dashed and dotted-dashed lines represent, respectively, the calculated values for the schizophyllan triple helix with a pitch (per residue) of 0.30 nm and a diameter of 2.6 nm and for polymer mixtures consisting of triple helices and single randomly coiled chains.

can be made for these discrepancies. Here, the values of f from Γ are assumed to be correct and used for subsequent data analysis.

The intrinsic viscosities $[\eta]$ in water and DMSO at 25°C are plotted double-logarithmically vs. M_w in fig. 3. Here, the smaller circles indicate previous data [5,11,12] for higher molecular weight schizophyllan in these two solvents, and the dashed line represents the theoretical values calculated for the schizophyllan triple helix (pitch per main chain residue = 0.30 nm and diameter = 2.6 nm [12]) using the theory of Yoshizaki and Yamakawa [13] for straight rods. This line approximately fits the data points for the three highest molecular weight samples U-1, U-4 and U-6 in water, indicating that the trimers of these samples in water have the same rigid triple-helical structure as that found previously. Another significant fact is that $[\eta]$ for aqueous solutions begins to deviate upward from the dashed line at $M_w \approx 7 \times 10^4$ and the deviation becomes more appreciable as M_w is decreased.

The intrinsic viscosity of a polymer mixture consisting of trimers and monomers is given by

$$[\eta] = f[\eta]_3 + (1-f)[\eta]_1 \quad (4)$$

regardless of the chain length distributions of the two species. Here, $[\eta]_3$ and $[\eta]_1$ denote the intrinsic viscosities of the trimer and monomer, respectively. If it is assumed that the trimers and monomers of schizophyllan in water are, respectively, the rigid triple helices and the same randomly coiled chains * as those [5,11] in DMSO, then $[\eta]$ of a given schizophyllan sample in water may be calculated from eq. 4 with the aid of the dashed and DMSO lines in fig. 3; for a given value of M_w(in DMSO), the value of $[\eta]$ at $3M_w$(in DMSO) interpolated from the dashed line may be taken as $[\eta]_3$. The values of $[\eta]$ thus calculated are shown by the dotted-dashed line in fig. 3. In the range of M_w between 7×10^4 and 10^4, this line is quite close to the experimental points for aqueous solutions, confirming that the schizophyllan trimer in this molecular weight range maintains the rigid triple-helical structure, while the monomer in water assumes a random-coil conformation similar to that in DMSO.

* Since the molecular chains of our samples are very short (for example, about 46 in main-chain residues for M_w(in DMSO) $\approx 10^4$), a difference in excluded-volume effect between water and DMSO solutions may be ignored, if any occurs.

3.3. Effects of extensive sonication

Both the viscosity and chromatographic data presented above have shown that our sonicated samples of schizophyllan in water are a mixture of triple helices and single chains when their M_w(in water) values are lower than about 10^5 and that the relative amount of single chains increases with decreasing M_w(in water). It is reasonable to consider that single chains were dissociated from triple helices not in dissolving a given schizophyllan sample in water for either viscosity or chromatographic measurement but during the sonication of the original sample. The extensive sonication employed in this work is quite likely to weaken interchain hydrogen bonds of triple helices by its generation of localized heat and stress, and eventually dissociate the helices to single chains when it proceeds to the point where the fragmented sample has a molecular weight lower than a certain value. Our experimental data indicate that such a critical molecular weight (in water) is about 10^5. As M_w(in water) is decreased further by a more extensive sonication, there should appear more single chains in the solution, in agreement with our experimental finding.

3.4. Correlation between antitumor activity and helix fraction

Besides schizophyllan, there are several β-1,3-D-glucans with or without β-1,6-D-glucose side chains [14–17] which are known to have host-mediated antitumor activities against Sarcoma 180. However, little is known about the molecular mechanism of their tumor inhibition or even what is likely to be the primary factor for antitumor activities. The backbone chemical structure cannot be such a factor, because pachyman (β-1,3-glucan with β-1,6-linked side chains) [18] and laminaran (β-1,3-glucan with no side chain) [15,16] have no antitumor activity.

Sasaki et al. [17,19] found that curdlan (β-1,3-glucan with no side chain) and lentinan (β-1,3-

glucan with β-1,6-linked side chains) lost their antitumor power when the molecular weight was lowered down to about 8×10^3 (for curdlan) and about 5×10^3 (for lentinan). These authors [19] and also Saito et al. [20] investigated conformations of lentinan fractions in dilute aqueous NaOH by visible absorption and ^{13}C-NMR spectroscopy, and concluded that the loss of the antitumor potency of lentinan is associated with an ordered-to-disordered conformation change of the glucan accompanying the decrease in molecular weight. On the basis of this conclusion along with some other pieces of information [15,18,21,22], Chihara [16] hypothesized that a certain ordered structure of a glucan in aqueous solution is primarily responsible for its antitumor activity. However, the 'ordered' structure remains unspecified.

Our bioassay has shown that for the chosen dose schizophyllan loses the antitumor potency when M_w in water is lowered to about 10^4. This critical molecular weight is comparable to those found by Sasaki et al. [17,19] for curdlan and lentinan. The present study has also shown that the predominant species of schizophyllan in water changes from a trimer having a rigid triple-helical structure to a single separated chain as the molecular weight is decreased from 10^5 to 10^4. Thus, it is likely that the triple helix is the ordered structure invoked by Chihara [16] as the primary factor for the antitumor activity of a β-1,3-glucan.

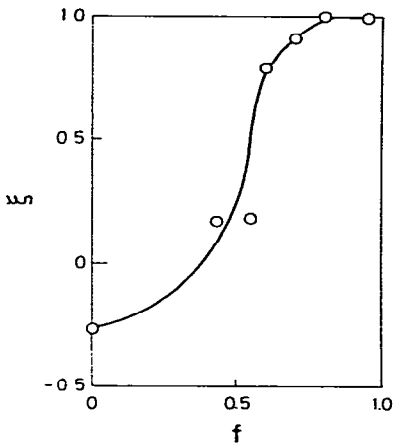

Fig. 4. Relation between tumor inhibition ratio and the weight fraction f of triple helices for schizophyllan.

In fig. 4, the values of ξ given in table 1 are plotted vs. the weight fraction of triple helices evaluated from eq. 3. It may be concluded from the smooth curve fitting the data points that the loss of the antitumor potency of schizophyllan in water is related to the decrease in the amount of triple helices relative to that of single separated chains in the solution and that an aqueous schizophyllan containing less than about 50% triple helices has virtually no potent antitumor activity.

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