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ULTRASONIC DEGRADATION OF SCHIZOPHYLLAN, AN ANTITUMOR POLYSACCHARIDE PRODUCED BY *Schizophyllum commune* FRIES

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ABSTRACT

Schizophyllan, a water-soluble β-D-glucan elaborated by *Schizophyllum commune* Fries, was partially depolymerized by ultrasonic irradiation to a low-molecular-weight polysaccharide, designated "sonic-degraded schizophyllan. Both native and degraded polysaccharides exhibited essentially the same antitumor activities against Sarcoma-180 ascites. Both glucans are comprised solely of D-glucose residues and have a main chain of (1→3)-β-D-glucopyranosyl residues, one out of three glucose residues being attached as single, (1→6)-β-D-glucopyranosyl groups. Although both glucans have similar structural features, significant differences are observed in such physical properties as molecular weight and intrinsic viscosity. End-group analysis by using radioisotope-labeled glucans suggests that ultrasonic degradation occurs mainly by cleavage of glycosidic bonds of the main chain of schizophyllan. The molecular weights of the native and sonic-degraded schizophyllan were shown to be 75% of those of corresponding, original schizophyllan preparations, suggesting that there is no anomalous linkage sensitive to periodate oxidation, and ultrasonic irradiation may cause random hydrolysis of (1→3)-β-D-glucosidic linkages in the main chain.

INTRODUCTION

Glucans containing (1→3)-β-D-linkages, or containing both (1→3)-β-D- and (1→6)-β-D-linkages, have been found in fruiting bodies and culture broths of such fungi as *Sclerotium glucanicum*[1], *Auricularia auricula-judae*[2], *Pullularia pullulans*[3], and *Lentinus edodes*[4], and such bacteria as *Alcaligenes faecalis*[5].

Schizophyllan, elaborated by *Schizophyllum commune* Fries is likewise a β-D-

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glucan and its structure has been studied by Kikumoto and co-workers[6,7] It consists of a main chain of (1→3)-β-linked D-glucose residues with one (1→6)-β-linked D-glucosyl group for every three glucose residues

During investigations on the chemotherapeutic applications of schizophyllan as an antitumor or antiinfection agent[8,9], we became aware of difficulties in its preparation and administration for clinical use because of the high viscosity of its aqueous solution

It is well known that ultrasonic irradiation of polysaccharides causes depolymerization The resulting, depolymerized polysaccharides have been reported to have a lower molecular-weight limit regardless of the irradiation time, and the depolymerized product has a relatively narrow molecular-weight distribution, even if the native polymer has a wide one[10]

Our preliminary study on ultrasonic depolymerization of schizophyllan afforded a lower-molecular-weight degraded polysaccharide of low viscosity which exhibited biological activity essentially the same as or rather higher than, that of the native schizophyllan

This paper reports the mode of cleavage, and changes in physical properties, of schizophyllan caused by ultrasonic irradiation

RESULTS AND DISCUSSION

The native polysaccharide designated "native schizophyllan", isolated from the culture filtrate of *Schizophyllum commune* Fries by the method of Kikumoto *et al*[6,7] was essentially free from nitrogen (N <0 01 %) and gave only D-glucose by hydrolysis with acid (M sulfuric acid 6 h, 100°)

The purified native schizophyllan was ultracentrifugally homogeneous ($s_{25,w}$, 13 6 *S*). A 1 % aqueous solution was exposed to 19 5 kHz sonic irradiation at 25° for various times At suitable time-intervals, aliquots were taken and the intrinsic viscosities and release of reducing groups were measured As shown in Fig 1, the intrinsic viscosity of schizophyllan decreased during the course of ultrasonic irradiation, with an increase in the reducing-group content After 30 and 90 min of irradiation, respectively the degraded polysaccharides were subjected to fractionation into three fractions by addition of 20 and then 25 % (final concentration) of methanol, to give three fractions, namely, a high-molecular-weight fraction, an intermediate fraction, and one of low molecular-weight (supernatant) The intermediate molecular-weight fractions from the polysaccharides degraded by 30 and 90 min of irradiation were designated S-1 and S-2, respectively Fractions, S-1 and S-2 appeared to have rather narrower molecular-weight distributions than the native schizophyllan, as indicated by their gel-chromatographic patterns shown in Fig 2 All degraded-glucan preparations gave single peaks in centrifugal analysis, for instance, fractions S-1 and S-2 prepared by 30 and 90 min of irradiation of the native schizophyllan ($s_{25,w}$, 13 6 *S*) had $s_{25,w}$ 8 0 *S* and 6 8 *S*, respectively

Molecular-weight distributions of the native and sonic-degraded schizophyllans

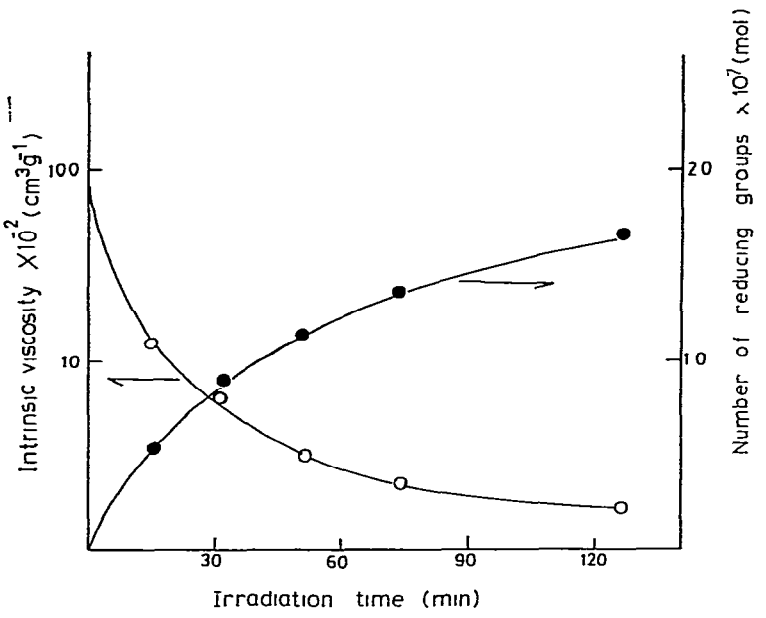


Fig 1 Time course of changes in intrinsic viscosity and number of reducing groups of schizophyllan solution during ultrasonic irradiation The number of reducing groups is expressed as moles of glucose in the sonicated solution (1 mL) ●——●, number of reducing groups, ○——○ intrinsic viscosity

by gel-filtration chromatography on a column containing Sephadex G-100 (lower layer), Sepharose 4 B (middle layer), and Sepharose 2 B (upper layer), are shown in Fig 2 It is apparent that depolymerization of the native schizophyllan by sonic irradiation causes degradation of the polysaccharide to give different fractions of lower molecular-weight, each fraction having a narrow molecular-weight distribution

Intrinsic viscosities of these fractions were 81 × 10^2 cm^3/g for the native schizophyllan (N-PS), 7 02 × 10^2 cm^3/g for S-1, and 1 66 × 10^2 cm^3/g for S-2, respectively These values correspond to molecular weights of 4 3 × 10^6 for N-PS, 5 6 × 10^5 for S-1, and 2 3 × 10^5 for S-2 as estimated from the relationship between the values of intrinsic viscosity and viscosity-average molecular weight of schizophyllan determined by Norisuye *et al* [11]

The foregoing polysaccharide fractions gave essentially identical i r spectra, each including an absorption band at 890 cm^{-1}, characteristic of the β-glucosidic linkage

Concerning the structure of schizophyllan, Kikumoto *et al* [6 7] established that it has a ramified structure consisting of a backbone chain of (1→3)-β-D-glucopyranosyl residues, one out of three D-glucose residues being substituted at O-6 with a single β-D-glucosyl group, as illustrated in Fig 3 This structure is confirmed in the present study by methylation and Smith-degradation techniques

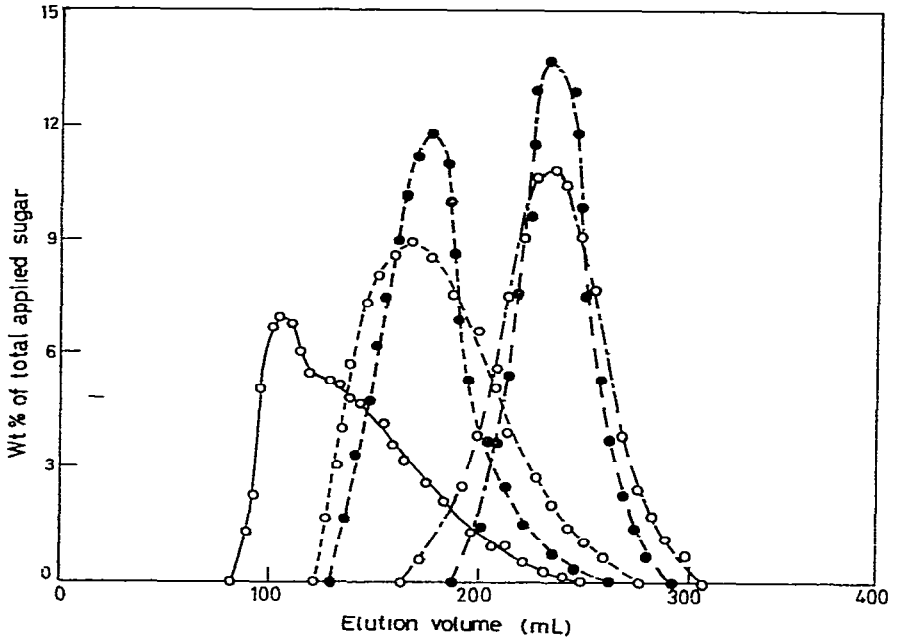


Fig 2 Gel-filtration patterns of schizophyllans on a connected column ○——○, N-PS, ●-----●, S-1 ●– – – ● S-2, ○-------○, schizophyllan preparation after 30-min of sonic irradiation, ○– – – ○ *schizophyllan preparation after 90-min of sonic irradiation*

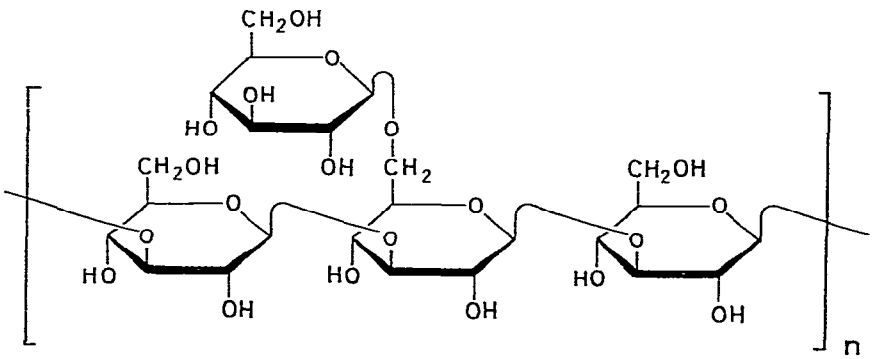
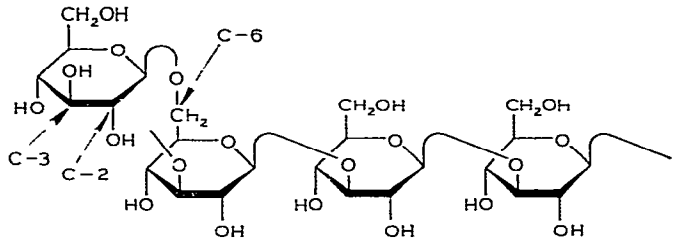
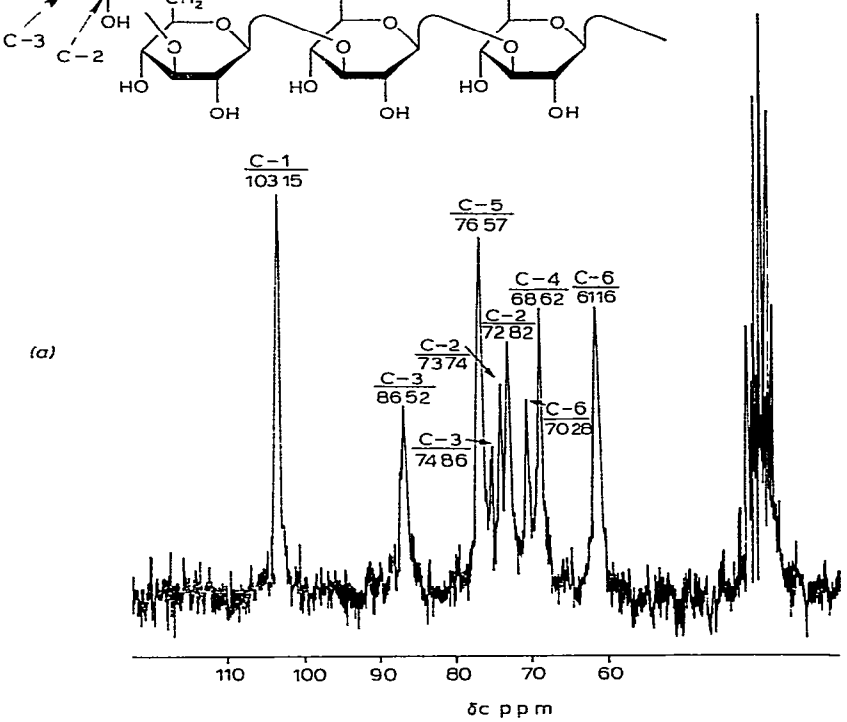


Fig 3 Structure of schizophyllan

To obtain information on any alteration in glycosidic linkages in the sonic-degraded schizophyllan, S-1 and S-2 were methylated by the method of Hakomori[12] The fully methylated polysaccharides were hydrolyzed by acid, and the partially methylated glucoses in the hydrolyzate compared with those from the methylated, native schizophyllan The molar ratios of 2,3,4,6-tetra-, 2,4,6-tri-, and 2,4-di-*O*-methyl-glucose were 1 00 2 10 1 03 for N-PS, 1 00 1 98 0 98 for S-1, and 1 00





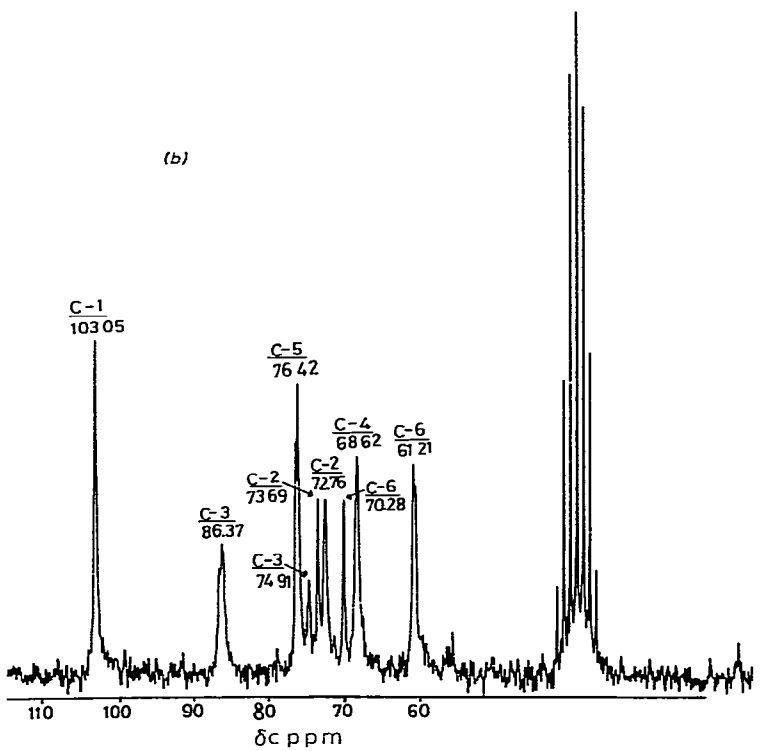


Fig 4 ^{13}C-N m r spectrum of N-PS (*a*) and S-2 (*b*) in Me_2SO-d_6 at 100° [Proton-noise-decoupled spectrum of each sample (100 mg/1 2 mL), 5,591 accumulations]

1.95 1 00 for S-2, respectively. This result confirmed that the linkage-mode in sonic-degraded schizophyllan is essentially the same as that of the native schizophyllan

On periodate oxidation with 0 01M sodium metaperiodate, N-PS, S-1 and S-2 all showed similar behavior. 0 54 mol of periodate per glucose residue was consumed with concomitant production of 0 23 mol of formic acid, in good agreement with the methylation data

The arrangement of D-glucose residues in the degraded schizophyllan was examined by Smith degradation as originally applied by Johnson *et al* for a similar type of β-D-glucan[13 14] The oxidized N-PS S-1, and S-2 were reduced with sodium borohydride, and each of the resulting glucan-polyalcohols was hydrolyzed completely with acid Paper chromatography revealed the presence of glucose and glycerol, their molar ratio was 3 0–3 15 1 0 in all glucans as analyzed by g l c , after borohydride reduction followed by conversion into the corresponding acetates The remainder of the glucan-polyalcohol was subjected to mild hydrolysis with 0 05M sulfuric acid for 2 days at 30° All glucans liberated glycerol, leaving the corresponding insoluble, β-(1→3)-linked degraded glucans, as revealed by methylation

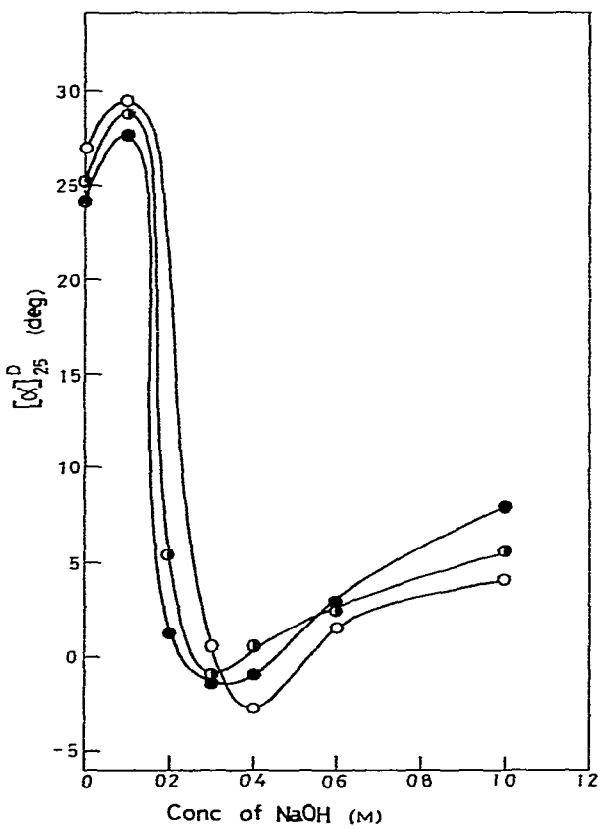


Fig 5 Dependence of specific optical rotation of N-PS, S-1, and S-2 at 589 nm on the concentration of sodium hydroxide ○——○ N-PS, ●——● S-1, ◑——◑ S-2

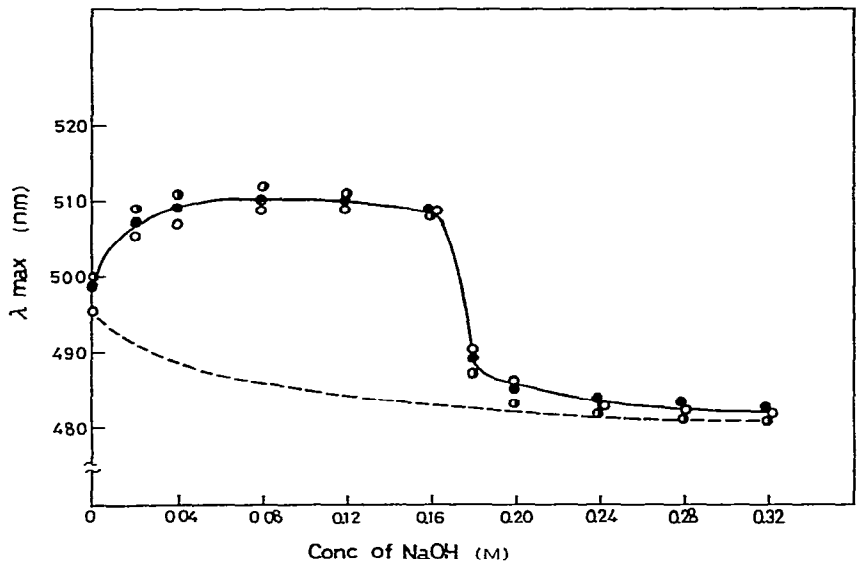


Fig 6 Change in the absorption maximum of the Congo Red–schizophyllan complex in various concentrations of sodium hydroxide ○——○, N-PS ●——●, S-1, ◑——◑ S-2 -------, Congo Red solution

analysis These results confirmed that all glucans have single, β-(1→6)-linked side-chains, one out of every three β-(1→3)-linked D-glucose residues There was no evidence for (1→4)-β-D-glucosidic linkages in sonic-degraded schizophyllan

Comparisons of the ^{13}C-n m r spectra of N-PS and S-2 in dimethyl sulfoxide-d_6 at 100° are shown in Fig 4 Both glucans showed nine peaks, and the chemical shifts for S-2 are very close to those of N-PS, suggesting that both glucans have the same covalent sequence of D-glucose residues

The conformational structures of (1→3)-β-D-glucans have been discussed by Ogawa[15] and by Sasaki *et al* [16], who reported changes in specific rotation and visible absorption spectra of the complexes formed with Congo Red, at various concentrations of alkali

N-PS, S-1, and S-2 showed similar abrupt changes in their specific rotations over the range of 0 18–0 24M sodium hydroxide, as shown in Fig 5 Fig 6 shows the formation of complexes of these fractions with Congo Red in low concentrations of alkali (below 0 18M) These results also confirm that the sonic-degraded schizophyllan has the same conformation as that of the native schizophyllan

The foregoing findings suggest that the (1→6)-β-branching linkages in schizophyllan are not sensitive to ultrasonication under the conditions employed This presumption was also confirmed by the fact that no appreciable release of glucose, which might have been derived by cleavage of (1→6)-β-linked, single branches during ultrasonic irradiation, was observed An aqueous solution of the borohydride-reduced, native schizophyllan (0 92%) was exposed to ultrasonic irradiation at 19 5 kHz at 25°, and any glucose released, and reducing end-groups in the irradiated

TABLE I

CHANGES IN CONTENT OF GLUCOSE AND REDUCING END-GROUPS IN SCHIZOPHYLLAN SOLUTION DURING ULTRASONIC IRRADIATION[a]

Ultrasonic irradiation time (min)	*Glucose content (wt % of schizophyllan)*	*Reducing end-groups (wt % as glucose in schizophyllan)*
0	0	0 072
10	0 085	0 476
30	0 108	0 839
90	0 281	1 532

[a]Glucose content and reducing end-groups were determined by the D-glucose oxidase method and Somogyi–Nelson method respectively

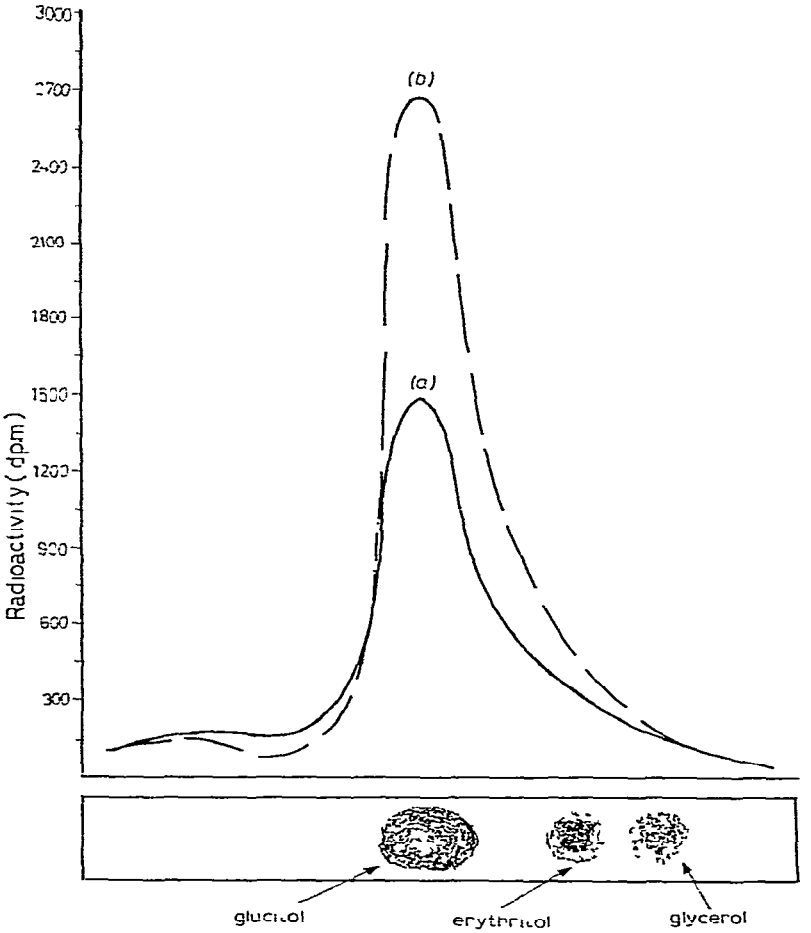


Fig 7 Paper chromatogram of the sugar fractions in hydrolyzates of tritiated schizophyllans (*a*), N-PS, (*b*), S-2

solution, were determined by the D-glucose oxidase method[17] and the Somogyi–Nelson method[18], respectively The amount of glucose was negligible in comparison with the increase in reducing end-groups, indicating that neither essentially no cleavage of (1→6)-β-linkages that are attached as single side-chains, nor extensive cleavage of the (1→3)-β-D-glucosidic linkage in the main chain, occur during ultrasonic treatment (Table I)

Further information on the ultrasonic degradation of this glucan was obtained by paper chromatography of the neutral-sugar fraction from the tritiated N-PS and S-2 As shown in Fig 7, acid hydrolysis of both tritiated glucans yielded only labelled glucitol, with quantitative recovery of all radioactivity This result indicates that the possibility of such anomalous cleavage as scission between carbon–carbon bonds, may be ruled out under these ultrasonic condition This result confirms that (1→3)-β-D-glucosidic linkages in the main chain of schizophyllan are cleaved preferentially by ultrasonic energy This outcome would be consistent with the fact that the (1→3)-β-D-glucosidic linkage is the most sensitive among various types of β-D-glucosidic linkages, toward acid hydrolysis

The presence of a small proportion of anomalous linkages other than (1→3)-glucosidic linkages in the long main-chain of schizophyllan cannot be established by the foregoing structural investigations If the main chain contains some anomalous linkages, selective cleavage may be possible at these anomalous linkages by ultrasonic treatment The presence of any bond other than the (1→3)-glucosidic linkage in the main chain was examined by comparing the molecular weights of N-PS, S-1, and S-2 before, and after, periodate-oxidative cleavage Following mild, Smith degradation of N-PS, S-1, and S-2, each resulting water-insoluble, degraded glucan was methylated and the methylated sugar fragments were analyzed Both methylated S-1 and S-2 gave only 2,4,6-tri-*O*-methylglucose plus, in the case of methylated N-PS, a trace of 2,3,4,6-tetra-*O*-methylglucose When these glucans were hydrolyzed with an exo-β-(1→3)-glucanase[19] from *Basidiomycetes* AM-806 only glucose was detected in the product These results indicate that the Smith-degraded polysaccharide is a linear (1→3)-β-D-glucan

TABLE II

RESULTS FROM VISCOMETRY ON SCHIZOPHYLLAN SAMPLES IN Me_2SO AND THE CORRESPONDING SMITH-DEGRADED SCHIZOPHYLLANS IN CADOXEN–WATER MIXTURE

	Before Smith-degradation			*After Smith-degradation*		
	Intrinsic viscosity $\times 10^2$ *cm³/g*	*Huggins constant*	*Molecular weight* $\times 10^4$	*Intrinsic viscosity* 10^2 *cm³/g*	*Huggins constant*	*Molecular weight* $\times 10^4$
N-PS	4 32	0 331	151	1 20	0 365	105
S-1	0 98	0 335	17 7	0 55	0 343	11 7
S-2	0 59	0 336	8 3	0 36	0 340	6 8

Recent work by Norisuye *et al* has shown that native and sonic-degraded schizophyllan are molecularly dispersed in dimethyl sulfoxide, whereas they exist as a triple helix in water[11] In the present study, the molecular weights of N-PS, S-1, and S-2 in dimethyl sulfoxide were computed from the intrinsic viscosity vs molecular weight relation measured by Norisuye *et al*[11] The water-insoluble, Smith-degraded glucans were dissolved in mixtures of water and triethylenediamine–cadmium hydroxide (Cadoxen) and the intrinsic viscosities were measured, and compared with data obtained for curdlan in Cadoxen[20] All viscosity-average molecular weights of N-PS, S-1, and S-2 after mild Smith degradation were estimated to be ~75% of those before the degradation, as shown in Table II As these values coincide with those expected from the removal of all (1→6)-β-linked side-chains the main chain should thus consist solely of (1→3)-β-D-glucosidic linkages which are stable to periodate cleavage This result also supports the findings of Norisuye *et al*[11] that schizophyllan adopts a rod-like conformation in aqueous solution, where the exponent in the Houwink–Mark–Sakurada viscosity vs molecular weight relationship is close to 1 7

As regards biological activity of schizophyllan host-mediated antitumor activity has been already reported[8] However, native schizophyllan has not yet been put into clinical use as a chemotherapeutic agent because of the high viscosity of its saline solution This problem of high viscosity might be overcome by controlled, ultrasonic depolymerization, as indicated by the foregoing experimental data The sonic-degraded schizophyllan has the same chemical structure as native schizophyllan except for its lower molecular weight Table III shows a comparison of the antitumor activities of sonic-degraded schizophyllans (S-1 and S-2) with that of the native schizophyllan no difference is evident between these polysaccharide preparations in their activities Ultrasonic depolymerization also gives the advantage that the degree of depolymerization of the degraded polysaccharide can be adequately controlled It appears, therefore that ultrasonic depolymerization may provide a more-useful preparation for chemotherapeutic application

TABLE III

ANTITUMOR ACTIVITIES OF N-PS S-1 AND S-2 AGAINST SARCOMA-180 (SOLID FORM) IN MICE

	Dose mg/kg × times	*Tumor weight (g)* Mean ± S E	*Inhibition ratio (%)*[a]	*Complete regression*[b]
N-PS	1 0 × 10	0 25 ± 0 143	95 1	6/10
S-1	1 0 × 10	0 06 ± 0 021	98 5	7/10
S-2	1 0 × 10	0 08 ± 0 056	98 1	7/10
Control	—	4 11 ± 0 327	0	0/10

[a]The inhibition ratio (%) = (1 − T/C) × 100 where C is the average tumor-weight of the control group, and T is that of the treated group [b]Ratio of number of mice showing complete regression to number of mice tested

EXPERIMENTAL

General methods — Specific optical rotations and i r spectra were recorded at 25° with a JASCO DIP-SL automatic polarimeter and a Digilab Model FTS-20 B spectrometer, respectively Sedimentation analysis was performed at 48,000 r p m with a Beckman Spinco Model E ultracentrifuge equipped with an electronic speed-control system and Schlieren optics regulated at 25° Paper chromatography was performed on Whatman 3 MM paper with 10 3 3 (v/v/v) 1-butanol–pyridine–water as solvent

Preparation of native and sonic-degraded schizophyllan — Native schizophyllan was prepared according to the method described in the previous reports[6,7] It was dissolved in water, deionized with a mixed bed of Amberlite IR-120 B and IRA-402 resins, and dialyzed and precipitated again with methanol, to afford N-PS The latter was dissolved in water at a concentration of 1%, and 200 mL of the solution was exposed to 19 5 kHz sonic irradiation at 25° The intrinsic viscosity and reducing-group content changed, as shown in Fig 1 The two sonic-degraded schizophyllan solutions obtained by ultrasonication for 30 and 90 min were fractionated, each into three parts, by precipitation with methanol to afford the two intermediate molecular-weight fractions, S-1 and S-2

Component sugars of native and sonic-degraded schizophyllan — Each glucan (10 mg) was hydrolyzed with M sulfuric acid (1 mL) for 6 h at 100° and the hydrolyzate was made neutral with barium carbonate The sugars were reduced with sodium borohydride (50 mg) in the dark with stirring for 24 h at room temperature The excess of borohydride was decomposed by careful addition of acetic acid and evaporation of methyl alcohol from the product was performed five times to ensure removal of any exchangeable borate The resulting alditols were acetylated by heating with 1 1 pyridine–acetic anhydride (0 2 mL) for 2 h at 100°, and the products analyzed by g l c with a column (2 m) of 3% ECNSS-M on Gaschrom Q at 190°

Methylation of native and sonic-degraded schizophyllan — Methylation was performed by the method of Hakomori[12] Each preparation (20 mg) was dissolved in dimethyl sulfoxide (2 mL) with stirring under nitrogen The solution was treated with methylsulfinyl carbanion (0 5 mL) for 4 h at room temperature, and then with methyl iodide (1 5 mL) for 1 5 h at 20° The methylation procedure was repeated three times, and the fully methylated glucan (5 mg) was then hydrolyzed with 90% formic acid (0 4 mL) for 12 h at 100°, and then heated with 2M trifluoroacetic acid (0 5 mg) for 7 h at 100° The methylated sugars were acetylated by heating with 1 1 pyridine-acetic anhydride (0 2 mL) for 2 h at 100°, and the products analyzed by g l c with a column (2 m) of 3% ECNSS-M on Gaschrom Q at 190° All methylated samples gave three peaks, which were identified as 2,3,4,6-tetra- 2,4,6-tri- and 2,4-di-*O*-methyl-glucose, and their molar ratio was calculated

Periodate oxidation — Each glucan (80 mg) was oxidized with 0 01M sodium metaperiodate (50 mL) at 5° in the dark The production of formic acid and consumption of periodate were periodically determined by titration with 0 01M sodium

hydroxide and by the Fleury–Lange method[21], respectively After completion of the oxidation (16 days) the reaction was stopped by addition of ethylene glycol (10 mL) and the mixture was dialyzed in water for 24 h The resulting, oxidized glucan was reduced with sodium borohydride (50 mg) in the dark, with stirring for 18 h at room temperature A portion (3 mg) of each glucan-polyalcohol was hydrolyzed with 3M sulfuric acid (1 mL). and the hydrolyzate made neutral with barium carbonate (complete Smith degradation) Paper chromatography of the resulting hydrolysis products revealed two spots for each sample one corresponding to glycerol and the other to glucose The other portion (12 mg) of each glucan-polyalcohol was hydrolyzed with 0 05M sulfuric acid (1 mL) for 2 days at 30° (mild Smith degradation), and the degraded glucan was collected by centrifugation Only glycerol could be detected by g l c of the alditol acetates of the products obtained from the supernatant solution Furthermore, paper chromatography of the resulting sugars showed one spot corresponding to glycerol in each sample

Gel-filtration chromatography — The distributions of the native and sonic-degraded schizophyllan preparations were investigated by gel-filtration chromatography on a column (diameter 2 6 cm) packed with a lower layer of Sephadex G-100 (length. 16 cm) a middle layer of Sepharose 4B (length. 20 cm) and an upper layer of Sepharose 2B (length 36 cm) after equilibration with 50mM sodium acetate buffer, and subsequent elution with the same buffer The eluate was collected in 5-mL portions and the content of carbohydrate in each fraction measured by the phenol–sulfuric acid method[22] The elution-profile of each sample is shown in Fig 2

Determination of intrinsic viscosity and number of reducing end-groups during the course of ultrasonication — An aqueous solution (1%, 250 mL) of N-PS was reduced with sodium borohydride (100 mg) with stirring for 24 h at room temperature The excess of borohydride was decomposed by careful addition of acetic acid and the reduced product was dialyzed in water for 2 days, and the nondialyzable solution was dried *in vacuo*

The reduced schizophyllan was dissolved in water (180 mL) at a concentration of 1% and the solution exposed to 19 5 kHz sonic irradiation at 25° At suitable time-intervals. the intrinsic viscosity was measured, and the number of reducing end-groups was determined by the Somogyi–Nelson method[18]

Identification of the point of initiation in the schizophyllan molecule — Sodium [^{3}H]borohydride (3 8 mg, 25 mCi) was dissolved in *N*,*N*-dimethylformamide (2 mL) and the solution was mixed with non-radioactive borohydride solution (0 2 mL) and 0 05M sodium hydroxide (4 mL). and then added to aqueous solutions (15 mg/mL) of N-PS and of S-2 The solutions were incubated for 48 h at 25°. and the reactions were stopped by adding 1 mL of acetic acid The acidic mixtures were then evaporated to dryness and the residues dissolved in 0 5 mL of M acetic acid and again evaporated The procedure was repeated five times to ensure the removal of any exchangeable tritium as [^{3}H]H_2O After hydrolysis with 2M trifluoroacetic acid (1 mL) for 1 h at 120°, each hydrolyzate was dried, dissolved in water, and then passed through a column of Amberlite IR-120 B (H^+) Following concentration, the labeled samples

were spotted onto Whatman No 50 paper and a mixture of authentic specimens (D-glucitol, erythritol, and glycerol) was also spotted on the edge of the paper which was then developed with 6 4 3 (v/v/v) 1-butanol–pyridine–water Detection was effected with alkaline silver nitrate The appropriate bands were extracted with 0 2 mL of water, and 4 mL of ethanol was added 1 mL of scintillator solution [2 5-diphenyloxazole, 7 g and 1,4-bis(2-methylstyryl)benzene 1 g in toluene] was added to the extract and the radioactivities were measured with a Packard Model 3320-Tri-CARB scintillation spectrometer

Determination of viscosity-average molecular weights of native and some-degraded schizophyllan and the Smith-degraded polysaccharide — N-PS S-1, and S-2 (400 mg of each) were subjected to periodate oxidation with 0 01M sodium metaperiodate (200 mL) at 5° in the dark After complete oxidation (7 days) each solution was mixed with ethylene glycol (10 mL) and the solution was then dialyzed against water for 24 h The dialyzate was reduced with sodium borohydride (50 mg) in the dark with stirring for 24 h at room temperature, and the resulting glucan-polyalcohol was subjected to mild acid hydrolysis with 0 05M sulfuric acid for 2 days at 25° The water-insoluble, degraded schizophyllan was collected by centrifugation and washed thoroughly with distilled water A portion of each dried, degraded schizophyllan was methylated by the Hakomori method[12] hydrolyzed with acid, and the hydrolyzate examined by glc as before, revealing only 2 4,6-tri-*O*-methyl-glucose together with a trace of 2,3,4 6-tetra-*O*-methyl-D-glucose Another portion of each degraded schizophyllan was hydrolyzed with exo-(1→3)-*β*-D-glucanase prepared[19] from a culture of *Basidiomycetes* QM-806 The purified enzyme-preparation was incubated with solutions (2 mL, 0 5%) of each degraded schizophyllan in 0 05M McIlvaine buffer (pH 4 0) at 50° At suitable time-intervals the reducing power of the solution was determined by the Somogyi–Nelson method[18]

When the reaction was complete (2 days) the mixture was heated for 10 min in boiling water and, after addition of acetone (2 mL) to the incubation mixtures the turbid solution was centrifuged The supernatant was passed through a column of Amberlite IR-120 B (H^+), and the eluates were evaporated and resolved by paper chromatography as already described Alkaline silver nitrate revealed only one spot, corresponding to glucose The results of methylation analysis and of enzymic analysis indicate the structure of degraded-schizophyllan to be the same as that of curdlan a straight-chain (1→3)-*β*-D glucan

The remainder of each degraded schizophyllan (250 mg) was dissolved in a mixture of water and Cadoxen [1 1 (v/v) 25 mL] and the intrinsic viscosity of each degraded schizophyllan was measured From the intrinsic viscosity vs viscosity-average molecular weight relation for curdlan in Cadoxen, as measured by Hirano *et al*[20], the viscosity-average molecular weights of the degraded schizophyllans were determined Viscosity-average molecular weights of the native and semi-degraded schizophyllan (N-PS, S-1, and S-2) were also determined from the intrinsic viscosity vs viscosity-average molecular-weight relationship in dimethyl sulfoxide, as measured by Norisuye *et al*[11]

^{13}C-N m r spectra — ^{13}C-N m r spectra were recorded with a JEOL PFT-100/EC-100 spectrometer operating at 25 03 MHz in the pulsed Fourier-transform mode Free-induction decays were accumulated with a 45° pulse (11 μs) All spectra were recorded in dimethyl sulfoxide-d_6 at 100° by using 8000 data points and a spectral width of 5 kHz ^{13}C-Chemical shifts are expressed in p p m downfield from internal tetramethylsilane

Complex-formation of N-PS S-1, and S-2 with Congo Red — The complexation of N-PS, S-1, and S-2 with Congo Red was evaluated from the shift in the visible absorption of the dye induced by the glucans in sodium hydroxide at concentrations between 0–0 32M according to the method of Ogawa *et al* [15]. A Hitachi 124 spectrophotometer was employed

Assay of antitumor activity — Seven-day-old Sarcoma-180 ascites (0 1 mL, 2 × 10^6 cells) were transplanted subcutaneously into the left groins of ICR-JCL mice (weight, about 20 g) The test samples were dissolved in saline solution, sterilized for 20 min at 120°, and then injected intramuscularly every other day for 20 days starting 24 h after tumor implantation The growth of tumors was charted daily for 31 days At the end of the 31st day, the mice were killed, and the tumors extirpated and weighed

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