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Polysaccharides in Fungi. I. Purification and Characterization of Acidic Heteroglycans from Aqueous Extract of *Tremella fuciformis* Berk[1]

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Polysaccharides have been isolated from the fruit bodies of *Tremella fuciformis* Berk, which grows in Japan and China. Some chemical and physical properties of these polysaccharides (AC and BC: from Chinese fungus; AJI and BJ: from Japanese fungus) were examined.

The polysaccharides were homogeneous as judged by gelfiltration (Sepharose 4B), electrophoresis (Zone and Tiselius), and ultracentrifugal analysis. These polysaccharides are acidic heteroglycans composed mainly of xylose, glucuronic acid and mannose, and contain acetyl groups in their molecules (the molar ratios of them are given in Table II). The chemical properties of BC were closely similar to those of BJ in the numerical values of elemental analyses, component sugar content and acetyl content. However, significant differences were observed between BC and BJ in the physical properties, such as intrinsic viscosity, sedimentation coefficient and molecular weight.

The fruit bodies of *Tremella fuciformis* Berk[3] (Tremellaceae) have been used for food and medicine as the elixir of life in China. For the purpose of examining the nutritive value and the biological activities of this fungus, we reported in a previous communication[4] on the isolation and characterization of the polysaccharides prepared from aqueous- and alkaline-extracts of the fruit bodies. Also, our preceding paper[5] described the marked antitumor activities of these polysaccharides against Sarcoma 180 implanted in mice.

The present paper is concerned with the purification and characterization of the acidic heteroglycans obtained from the aqueous extract fraction of the fruit bodies of *Tremella fuciformis* Berk, which grows in Japan and in China.

The dried fruit bodies obtained from China were washed with hot methanol, and then the residue was extracted with hot water. The fresh fruit bodies harvested in Japan usually contained a large amount of water. Therefore, these were extracted directly with hot water without washing with methanol.

The polysaccharide fractions (AC and BC: Chinese fraction; AJ and BJ: Japanese fraction) were prepared from the aqueous extracts of Chinese and Japanese fungi by the process outlined in Chart 1.

Each fraction thus obtained was chromatographed on DEAE-Sephadex with 0.1N phosphate buffer (pH 6.1), followed by a gradient elution with aqueous sodium chloride (0 to 2M). On the chromatography with the phosphate buffer, carbohydrate was not detected in any of these fractions. By the gradient elution with aqueous sodium chloride, each of fractions AC, BC and BJ gave only one peak. Fraction AJ was divided into fraction AJI (major peak) and fraction AJII (minor peak) as shown in Fig. 1. These fractions (AC, BC, AJI, AJII

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2) Location: *492–36, Mitahora, Gifu.*
3) Japanese name: Shirokikurage; Chinese name: Ginji.
4) S. Ukai, K. Hirose, and T. Kiho, *Chem. Pharm. Bull.* (Tokyo), **20**, 1347 (1972).
5) S. Ukai, K. Hirose, T. Kiho, C. Hara, T. Irikura, T. Kanechika, and Y. Hasegawa, *Chem. Pharm. Bull.* (Tokyo), **20**, 2293 (1972).

the fruit bodies of *Tremella fuciformis* BERK

↓ extraction with hot water

extract | residue

↓ dialysis against running water

inner solution

↓ deproteinization by the Sevag method, centrifugation

aqueous phase

↓ concentration under reduced pressure, precipitation by addition of three volumes of ethanol

precipitate | supernatant

↓ reprecipitation

fraction AC[a] (AJ)[b] | fraction BC[a] (BJ)[b]

Chart 1. Fraction of Water-Soluble Constituents

a) AC- and BC-fraction from Chinese fungus
b) AJ- and BJ fraction from Japanese fungus

and BJ) purified by this procedure were dialysed against water, and then lyophilized to yield AC-, BC-, AJI-, AJII- and BJ-polysaccharides as colorless flakes. Each polymer was gradually dissolved in water to afford a viscous solution. The aqueous solution gave a positive carbazole test and precipitated a complex salt by addition of cetyltrimethylammonium bromide reagent. Further investigation on compound AJII was not performed in the present work, because of its low yield.

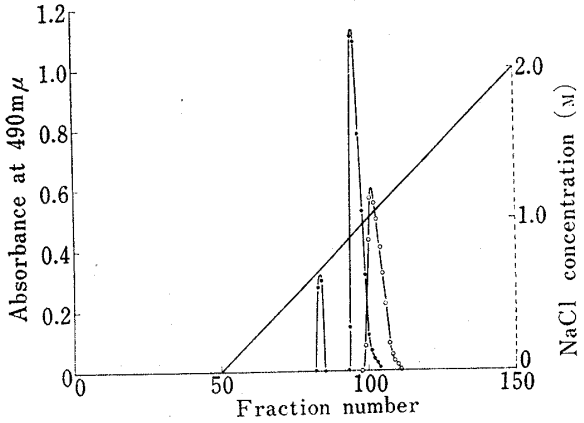

Fig. 1. Chromatograms on DEAE-Sephadex of Fraction AJ and BJ

—●—: fraction AJ (major peak: AJI; minor peak: AJII)
—○—: fraction BJ

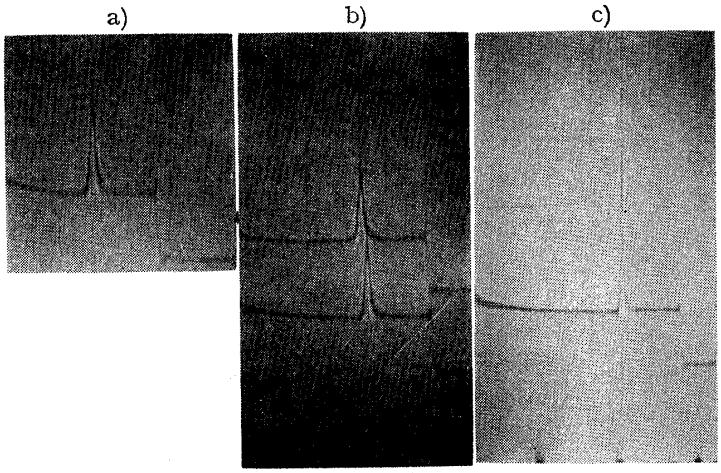

Fig. 2. Ultracentrifugal Pattern of 0.2% Polysaccharide in 0.3M NaCl–0.001M Sodium Phosphate Buffer (pH 7.0)

a) AJI, b) BJ (upper); AC (lower), c) BC
The photographs were taken 120 min after the start of the run at 51000 rpm (20°).

These polysaccharides were homogeneous on gel chromatography with Sepharose 4B and gave one spot on glass-fiber paper electrophoresis in an alkaline borate buffer of pH 12. Each polymer was found to be pure by the Tiselius electrophoresis in 0.05M phosphate buffer at pH 8.0 and by the ultracentrifugal analysis (Fig. 2).

The physical properties of the purified polysaccharides are shown in Table I. These physical data, except that of specific rotation, were determined in 0.3M sodium chloride–0.001M sodium phosphate buffer of pH 7.0 at 20° by standard means.

The optical rotation and the partial specific volume of the polysaccharides showed almost the same values. The intrinsic viscosity of Chinese compounds (AC and BC) revealed values

Table I. Some Physical Properties of Purified Acidic Heteroglycans from *Tremella fuciformis* Berk

Polysaccharide	Specific rotation $[\alpha]_D^{20}$ ($c=1$, H_2O)	Intrinsic viscosity (dl/g)	Partial specific volume (ml/g)	Sedimentation coefficient S_{20}	Molecular weight $\times 10000$
AC	+10	9.0	0.57	5.8	59
BC	+12	9.8	0.55	6.7	72
AJI	+12	6.3	0.55	5.3	41
BJ	+11	5.6	0.57	5.1	39

relatively higher than those of Japanese compounds (AJI and BJ). The sedimentation coefficient of BC showed the largest value (6.7 S) among all of these carbohydrates. Each molecular weight was calculated by the equation, which was based on sedimentation and viscosity data and was used by Eyring[6] and Pancake[7] in studying mucopolysaccharide. The molecular weight of BC was estimated to be approximately 7.2×10^5 by this method. This value is larger than those of the other polysaccharides (3.9 to 5.9×10^5).

None of these polysaccharides had characteristic absorption spectra in the ultraviolet region. Moreover, nitrogen, phosphorus and sulfur could not be detected in any of these compounds by means of the elemental analyses as given in Table II. Consequently, none of these carbohydrates was contaminated by nitrogen-containing substances, like protein, peptide or nucleic acid.

Table II. Some Chemical Properties of Purified Acidic Heteroglycans from *Tremella fuciformis* Berk

		Polysaccharide			
		AC	BC	AJI	BJ
Elemental analysis	C	40.8	41.0	38.9	41.6
	H	6.3	6.3	6.1	6.3
	N	0	0	0	0
	S	0	0	0	0
	P	0	0	0	0
Molar ratio of component sugars	glucuronic acid	1.0	1.0	1.0	1.0
	xylose	1.5	1.5	1.2	1.5
	mannose	4.1	3.7	3.1	3.6
Acetyl content % (molar ratio ralative to glucuronic acid)		5.5(1.8)	9.9(3.2)	5.0(1.1)	9.7(3.5)

The infrared spectrum of each polysaccharide had absorption bands at 1725 cm^{-1} and 1250 cm^{-1}. These two bands disappeared by the treatment of these polymers with sodium hydroxide. This disappearance of the two bands suggests the presence of acyl groups in their molecules. Kind of the acyl groups was defined as follows:

The acid liberated by saponification of each polysaccharide was identified as acetic acid, because the ester obtained by the reaction of the acid with *p*-bromophenacyl bromide was characterized as *p*-bromophenacyl acetate by comparison with an authentic sample.[8] Thus the presence of O-acetyl groups was confirmed. The total acetyl contents of these polysaccharides were determined by the ferric hydroxamate method of Hestrin.[9] The contents of

6) E.J. Eyring and J.T. Yang, *J. Biol. Chem.*, **243**, 1306 (1968).
7) S.J. Pancake and M.L. Karnovsky, *J. Biol. Chem.*, **246**, 253 (1971).
8) W.L. Judefind and E.E. Reid, *J. Am. Chem. Soc.*, **42**, 1043 (1920).
9) S. Hestrin, *J. Biol. Chem.*, **180**, 249 (1949).

BC and BJ were larger than those of AC and AJI as shown in Table II. This finding is in accord with the result that the infrared spectra of BC and BJ had a stronger absorption band at 1725 cm^{-1} than those of AC and AJI.

Thin-layer chromatography of hydrolysate and gas-liquid chromatography of the trifluoroacetate of the reduction product of the hydrolysate indicated that the component sugars of each polysaccharide were xylose, glucuronic acid and mannose in addition to a small amount of glucose and a trace of fucose. The gas chromatographic analysis was carried out by a modification of the method of Imanari, *et al.*[10a] and Jones, *et al.*[10b] The hydrolysate above mentioned was prepared by heating each polymer with 2N sulfuric acid at 120° in a sealed tube for 1 hour. The main sugar components were determined by the procedures of Dische[11] and Bitter, *et al.*[12] The molar ratios of them are given in Table II. As is evident from the experiments, these polysaccharides are acidic heteroglycans composed mainly of xylose, glucuronic acid and mannose, and contain acetyl groups in their molecules.

The chemical properties of BC were closely similar to those of BJ in the numerical values of elemental analyses, component sugar content and acetyl content. However, significant differences between BC and BJ were observed in the physical properties, such as intrinsic viscosity, sedimentation coefficient and molecular weight. The differences in the physical properties of the polysaccharides obtained from Japanese and Chinese fungus are of interest on chemotaxonomy because both fungi are of the same origin. Possible cause of the differences in the physical properties is the difference in the place of growth of these fungi. Some structural changes of AJ- and BJ- polysaccharides to AC- and BC- polysaccharides might have occurred in the drying process of the fruit bodies. Closer studies will be made in the future on the differences in the physical properties.

In the previous paper,[5] we reported the effect of these polysaccharides on the growth of sarcoma 180 implanted in mice for four weeks. The paper described that the inhibition ratio (80.8%) of the BC- polysaccharide was higher than those (45.9%, 67.9% and 61.5%) of the AC-, AJI- and BJ- polymers. This result indicates that a certain relationship is present between the antitumor activities and the physical properties of these polysaccharides. Thus, the antitumor activity is possibly related with higher-order structure of the molecules of polysaccharides, as reported by Sakaki, *et al.*[13]

The AC-, AJI-, BC- and BJ-polysaccharides are similar in components to the polysaccharides produced by strains of other species of *Tremella* genius[14] cultured on glucose-containing media. Both the polysaccharides consist of xylose, glucuronic acid, mannose and acetyl groups. However, the AC-, AJI-, BC- and BJ-polysaccharides widely differ from the polysaccharides prepared from strains of other *Tremella* species, in the molar ratios of the components and in the optical rotations. The specific rotations of the former had positive values as shown in Table I, whereas those of the latter showed negative values.

The structural elucidation of these polysaccharides prepared from the fruit bodies of *Tremella fuciformis* Berk will be reported in following papers.

Experimental

Ultraviolet (UV) absorbances were taken on a Shimadzu MPS-50L automatic recording spectrophotometer. Infrared (IR) spectra were recorded on a Japan Spectroscopic Co., Model IRA-I spectrometer. Specific rotations were measured by a JASCO Model DIP-S automatic polarimeter.

10) *a*) T. Imanari, Y. Arakawa, and Z. Tamura, *Chem. Pharm. Bull.* (Tokyo), **17**, 1967 (1969); *b*) T.M. Jones and P. Albersheim, *Plant Physiol.*, **49**, 926 (1972).
11) *a*) Z. Dische, *J. Biol. Chem.*, **181**, 379 (1949); *b*) Z. Dische, L.B. Shettles, and M. Osnos, *Arch. Biochem.*, **22**, 169 (1949).
12) T. Bitter and H.M. Muir, *Anal. Biochem.*, **4**, 330 (1962).
13) T. Sakaki, Y. Arai, T. Ikekawa, G. Chihara, and F. Fukuoka, *Chem. Pharm. Bull.* (Tokyo), **19**, 821 (1971).
14) M.E. Slodki, L.J. Wickerham, and R.J. Bandoni, *Can. J. Microbiology*, **12**, 489 (1966).

Materials——The fruit bodies of *Tremella fuciformis* Berk growing in Ssuchuan (China) were obtained as dry material. The fresh fruit bodies, which contain 95% of water, of this fungus were collected in June of 1971 in Gifu Prefecture (Japan).

Isolation and Purification——The fruit bodies growing in China were crushed and then washed with hot methanol. The material was extracted with 20 volumes of hot water (90° to 100°) for 5 hr and centrifuged at 5000 rpm for 20 min. This process was repeated 3 times. The combined extracts were dialyzed against deionized water for 2 days. The internal solution was deproteinized by the Sevag method.[15] This procedure was repeated until gelatinous substances no longer formed in the mixture. After centrifugation, the supernatant was concentrated to a small volume under reduced pressure, and added with 3 volumes of EtOH. The resulting precipitate was collected by centrifugation at 12000 rpm for 20 min, dispersed into a small amount of water, again added with 3 volumes of EtOH, and then centrifuged. The product purified by the reprecipitation was dissolved in a small amount of water, and lyophilized to give fraction AC as colorless flakes (yield: 27%).

The first supernatant obtained by the addition of EtOH was concentrated to a small volume under reduced pressure, and lyophilized to afford fraction BC as colorless flakes (yield: 10%).

The fruit bodies harvested in Japan were disintegrated and extracted immediately with 3 volumes of hot water (90° to 100°) for 5 hr. The aqueous extract was treated in the same manner as with Chinese fungus. Thus, fractions AJ and BJ were prepared. Fraction AJ was not pure at this stage.

Each polysaccharide fraction (AC, BC, AJ, and BJ) was further purified by column chromatography on DEAE-Sephadex A-25 (Pharmacia Co.), which was previously treated with 0.1 N phosphate buffer at pH 6.1. The column (2.6×100 cm) was eluted with the buffer, followed by gradient elution with aq. NaCl (0 to 2 M). Fractions of 5 ml were collected, and an aliquot of each was assayed for carbohydrate by the phenol–sulfuric acid method.[16] These fractions (AC, BC, AJI, and BJ) purified by this procedure were dialyzed against deionized water and then lyophilized to yield AC-, BC-, AJI-, and BJ- polysaccharides as colorless flakes.

Gel Filtration on Sepharose 4B Column——Sepharose 4B (Pharmacia Co.) was washed repeatedly with water, followed by washing with 0.3 M sodium chloride–0.001 M sodium phosphate buffer of pH 7.0. After preparation of a column (2.6×100 cm), each polysaccharide (1 mg) dissolved in the buffer (2 ml) was applied to the column and eluted with the buffer at a flow rate of 10 ml/hr. Fractions of 5 ml were collected, and an aliquot of each was assayed for carbohydrate by the phenol–sulfuric acid method.[16]

Zone and Tiselius Electrophoresis——Zone electrophoresis was carried out on Whatman GF 81 glass fiber paper in the following system: alkaline borate buffer (0.1 N NaOH: 0.25 M borax; 1: 1) 10 V/cm, 2 hr. The spots were detected by spraying α-naphthol–sulfuric acid reagent.[17]

Tiselius electrophoresis was performed with a Hitachi Model HID-1 boundary electrophoresis apparatus in 0.05 M phosphate buffer at pH 8.0 for 1 hr.

Some Physical Properties of the Purified Polysaccharides (AC, BC, AJI and BJ)——The physical measurements described below were made in 0.3 M NaCl–0.001 M sodium phosphate buffer (pH 7.0).

Viscosity: The viscosities were measured with an Ubbelohde viscometer at five concentrations in the range of 0.05 to 0.25 g each polysaccharide per dl at 25°. The values of intrinsic viscosity were determined by extrapolating the reduced viscosities to zero concentration.

Partial Specific Volume: The partial specific volumes were calculated from density measurements made at 20° with a 10 ml density bottle.

Sedimentation: The sedimentation velocities were determined at 51000 rpm at 20° by a Hitachi Model UCA-I analytical ultracentrifuge with a schlieren optical system. Sedimentation coefficients (S_{20}) were obtained at three concentrations (0.1%, 0.15%, nd 0.2%) for each polysaccharide. Extrapolation to infinite dilution was given by plotting $1/S_{20}$ against concentration.

Molecular Weight: The molecular weight (M) was calculated by the equation, $M^{2/3}=N[\eta]^{1/3}\eta_0 S/\Phi^{1/3}\rho^{-1}(1-\bar{v}\rho)$, used by Eyring[6] and Pancake.[7] Here N is Avogadro's number, $[\eta]$ the intrinsic viscosity, η_0 the solvent viscosity, S the sedimentation coefficient, $\bar{v}$ the partial specific volume, and ρ the solvent density. A value of 2.5×10^6 was used for the Flory function, $\Phi^{1/3}\rho^{-1}$. This value was close to the value experimentally obtained for flexible, non-ionic polymers in general and for most flexible polyelectrolytes.[7]

Identification of O-Acetyl Groups in the Molecules of the Polysaccharides——Each polysaccharide (1 g) was dispersed in 40 ml of 0.25 N NaOH. The mixture was allowed to stand for 24 hr at room temperature and then added with 3 volumes of EtOH. The resulting precipitate was separated off by centrifugation, and washed with 3×5 ml of 70% aq. EtOH. The supernatant and the washings were combined. The combined solution was evaporated to dryness under reduced pressure. The residue was dissolved in 5 ml of H_2O and acidified slightly with 3% ethanolic HCl. 5 ml of 2% ethanolic *p*-bromophenacyl bromide was added to the slightly acidic solution. The mixture was refluxed for 3 hr and then poured onto ice–H_2O to

15) M.G. Sevag, *Biochem. Z.*, **273**, 419 (1934).
16) M. Dubois, K.A. Gilles, P.A. Rebers, and F. Smith, *Anal. Chem.*, **28**, 350 (1950).
17) M. Geldmacher-Mallinckrodt and H. Wienland, *Hoppe-Sey. Z.*, **292**, 65 (1953).

afford a colorless precipitate. The product was collected by filtration and removed from the excessive reagent by means of column chromatography on silica gel with $CHCl_3$. The *p*-bromophenacyl ester thus obtained was crystallized from petroleum ether to give colorless leaves, mp 85°. The ester was identified with an authentic *p*-bromophenacyl acetate[8] by a mixed melting point test, IR spectral comparison and TLC on Silica gel G with $CHCl_3$.

Acetyl Determination——The total acetyl contents of these polysaccharides were determined by the ferric hydroxamate method of Hestrin.[9] The quantities of O-acetyl groups in their molecules of the polymers were calculated by comparison with the standard curve obtained from the analysis of β-D-glucose pentacetate.

Qualitative Analyses of Component Sugars——Each polysaccharide was hydrolyzed with 2 N H_2SO_4 in a sealed glass tube at 120° for 1 hr, neutralized with $BaCO_3$, and then filtered. The filtrate was passed through a column of Amberlite CG-120 (H^+ form) and concentrated under reduced pressure.

Thin-Layer Chromatography (TLC): Thin-layer chromatography of these hydrolysates was performed on Avicel SF cellulose with the solvent system AcOEt: pyridine: AcOH: H_2O (5: 5: 1: 3) and on Kieselguhr G with the solvent system BuOH: pyridine: H_2O (70: 15: 15). Sugar spots were detected with *p*-anisidine hydrochloric acid reagent[18] for Avicel SF cellulose TLC and with naphthoresorcinol–sulfuric acid reagent[19] for Kieselguhr G TLC.

Gas–Liquid Chromatography: The hydrolysates were reduced with $NaBH_4$ to give alditols and aldonic acid. The aldonic acid was separated from the alditols with Amberlite CG-400 (acetate form) ion exchange resin, which bound the aldonic acid. The alditols, which did not bind, were washed out from the resin and then trifluoroacetylated with trifluoroacetic anhydride. The aldonic acid was eluted from the resin with HCl. The HCl solution of the aldonic acid was evaporated to dryness under reduced pressure, converting the aldonic acid to aldonolactone. The aldonolactone was reduced with $NaBH_4$ to the corresponding alditol, dried and trifluoroacetylated.

The resolution of the trifluoroacetate derivatives was carried out by a JEOL Model JGC-1100 gas chromatograph equipped with a hydrogen flame detector in a glass column (2 m long × 0.4 cm inner diameter), which was packed with 2% XF-1105 on Chromasorb P (80 to 100 mesh), at 145° with a flow of 37 ml per min of N_2.

Determination of Component Sugars——The component sugars were estimated on each of the 0.01% polysaccharide solutions by the method of Dische (for pentose[11a] and hexose[11a,b]) and Bitter–Muir[12] (for uronic acid).

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18) L. Hough, J.K.N. Jones, and W.H. Wadman, *J. Chem. Soc.*, **1950**, 1702.
19) G. Pastuska, *Z. Analyt. Chem.*, **179**, 427 (1961).