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Studies on molecular weights of polysaccharides of *Auricularia auricula-judae*

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Abstract

Three D-glucans (A, C, and E) and two acidic heteropolysaccharides (D and B), isolated from the fruit bodies of *Auricularia auricula-judae* (which grows in Fangshan, Hubei, China) were analysed by paper chromatography, gas chromatography, and IR and ^{13}C NMR. Glucans A, C, and E consist mainly of a backbone chain of β-(1 → 3)-D-glucose residues, with various branched groups. Polysaccharides D and B contain residues of D-xylose, D-mannose, D-galactose, D-glucose, and D-glucuronic acid. The weight-average molecular weights M_w, number-average molecular weights M_n, intrinsic viscosities $[\eta]$, and Huggins constants k' of these polysaccharides were studied by light scattering, membrane osmometry, and viscometry. The values of M_w for samples of A, C, E, D, and B are 117×10^4, 144×10^4, 200×10^4, 30×10^4, and 50×10^4, respectively. Analysis of the M_w, M_n, $[\eta]$, and k' data indicates polysaccharides A and E to have stiff chains, and C, D, and B flexible chains in aqueous solutions.

Keywords: *Auricularia auricula-judae*; Polysaccharide; Molecular weight; Light scattering; Membrane osmometry; Intrinsic viscosity

1. Introduction

The fruit bodies of *Auricularia auricula-judae*, (family *Heterobasidiae*), have long been used as food and drugs. In recent years, non-cytotoxic and host-mediated antitumor polysaccharides have been obtained from various *Heterohasidiaes* [1–5]. In particular, antitumour, immunological, anti-inflammatory, and anticoagulant activities have been observed in the polysaccharides of *Auricularia auricula-judae* [2,3,6], and hence their structures have attracted much attention.

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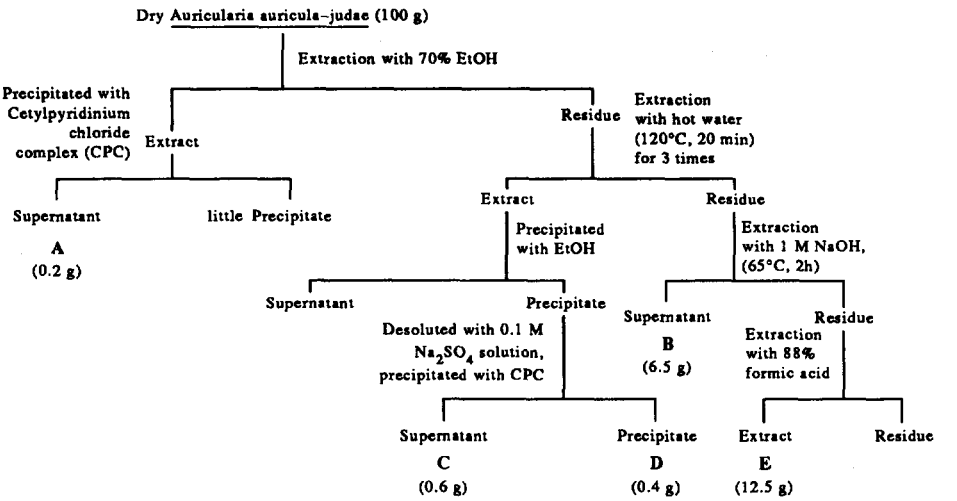
Sone et al. [7] isolated water-soluble and water-insoluble β-D-glucans, and two acidic heteropolysaccharides from the fruit body of *Auricularia auricula-judae*. They demonstrated that the water-soluble glucan consists of a backbone chain of β-(1 → 3)-linked D-glucose residues, two out of three glucose residues being substituted at O-6 by a single glucose group, and the water-insoluble glucan is also a β-(1 → 3)-D-glucan, but with an extremely branched structure. One acidic heteropolysaccharide contains D-xylose, D-mannose, D-glucose, and D-glucuronic acid (molar ratio 1.0 : 4.1 : 1.3 : 1.3), and consists of a backbone chain of (1 → 3)-linked mannose residues to which are attached D-xylose, D-mannose, and D-glucuronic acid residues at O-2 or O-6. Their molecular weights were determined by high-performance liquid chromatography to be 140×10^4 for the water-soluble D-glucan and 50×10^4 for the acidic heteropolysaccharide [2]. Ukai et al. [8] reported that two types of acidic polysaccharide could be isolated from the hot aqueous 70% ethanol and a hot water extract of the fruit body of this fungus. The acidic heteropolysaccharides have an α-(1 → 3)-linked D-mannose backbone, substituted at some of the O-2 positions by terminal β-D-glucuronic acid groups, and O-2 and O-6 positions by a short chain of β-D-xylose residues. Their molecular weights were estimated by sedimentation to be 37×10^4 and 30×10^4. In a previous paper [9], we studied the molecular weights and solution properties of an acidic heteropolysaccharide of *Auricularia auricula-judae* (cultivation by substitute), and reported 58.8×10^4 for the weight average molecular weight (M_w) and 12.1×10^{-18} cm^2 mol g^{-1} for unperturbed dimensions $\langle s^2 \rangle \theta/M$.

Knowledge of the chemical structure, molecular weight, and chain conformation of these polysaccharides is important for understanding their bioactivities. However, the conformations and molecular weights of all of these polysaccharides have not yet been determined. Here, we determine molecular weights, radii of gyration, second virial coefficients, and intrinsic viscosities of five polysaccharides by light scattering, membrane osmometry, and viscometry, and discuss their conformation. The unusual solution behaviour of the acidic heteropolysaccharide in pure water is undoubtably due to its ionic character. In order to obtain reliable data, an investigation was made into suitable solvents for determining molecular weights and viscosities of these polysaccharides.

2. Experimental

Isolation of polysaccharides.—Dried fruit bodies of *Auricularia auricula-judae* (100 g), a commercial product cultivated in Fangshan (Hubei, China), was defatted with hot EtOAc and MeOH for 4 h. The residue was immersed in 70% aq EtOH overnight [8], pounded into pieces and then homogenized. The homogenized material was extracted with aq 1% NaCl and centrifuged. The overall process is outlined in Scheme 1. Components of low molecular weight and proteins in the polysaccharide solution were removed by dialysis and by the Sevag method, respectively. Each isolated polysaccharide was rotary evaporated under diminished pressure below 50°C and then lyophilized to afford the polysaccharide samples as colourless flakes (except for E).

Each polysaccharide, examined by UV spectroscopy (UV-240 Shimadsu, Japan)



Scheme. 1. Isolation of polysaccharides from the fruit bodies of *Auricularia auricula-judae*.

showed only a peak at 200 nm for polysaccharide; absorptions at 280 nm and 600 nm for protein and pigments were zero. Analysis of E by an amino acid analyser (Waters PICO TAG) showed no nitrogen-containing component.

Electrophoresis of 0.5% polysaccharide solutions (3 mÅ/tube) in 0.1 M triborate/24 mM EDTA buffer at pH 8.3–8.9 (indicator: 0.2% bromphenol blue, dye: 0.5% Alcian Blue in HOAc, decolourizer: 3% AcOH). Only a single band was detected for polysaccharide A.

Analysis of components.—Each polysaccharide (5 mg) was hydrolysed with 2 M H_2SO_4 (2 mL) in a sealed tube for 8 h at 100°C. After neutralization with $BaCO_3$ and filtration, the filtrate was concentrated to a syrup. Paper chromatography was performed with Xinghua filter paper, and developed with 4:1:5 1-butanol–AcOH–water. The sugars on paper chromatograms were detected with aniline–*o*-benzenedicarboxylic acid.

Polysaccharides A (30 mg) and B (30 mg) were hydrolysed with 2 M H_2SO_4 in sealed tubes for 8 h at 100°C. After neutralization and centrifugation (3000 rpm), an aqueous solution of each hydrolysate was treated with $H_2NOH \cdot HCl$ (20 mg) and pyridine (1.5 mL) for 30 min at 90°C. The reduction product was acetylated with Ac_2O for 30 min at 90°C. Pyridine was removed by repeated evaporation with addition of $CHCl_3$ and gas chromatography of sugars was performed with a Model GC-7 Gas Chromatograph (Sichuan Analytical Apparatus Plant, China) in an open-chain crown ether capillary column (28 m × 0.25 mm) at 205°C.

Infrared (IR) spectra of the polysaccharides were recorded with a Nicolet FT-IR Spectrometer.

High resolution ^{13}C NMR spectra were recorded with a Bruker ARX-500 spectrometer operated at 125.769 MHz for ^{13}C and 500.139 MHz for ^{1}H. The samples were dissolved in Me_2SO-d_6 at 320 K. A ^{1}H 90° pulse-width and delay time were,

respectively, 12.5 μs and 10 s for one-dimensional quantitative ^{13}C NMR, and the spectra were accumulated 800 times by using 16K data points. The ^{1}H 90° pulse width and delay times were, respectively, 8.7 μs and 1.5 s for two-dimensional correlation $^{1}H-^{13}C$ NMR, and the spectra were accumulated 32 times.

Viscometry.—Viscosities of the polysaccharide solutions (C, D, B, and E) were measured at 30 ± 0.1°C by using a modified capillary viscometer supplied by the Institute of Industrial Science, Tokyo University. Polysaccharide A was examined at 25°C using a low-shear two-bulb capillary viscometer supplied by Beijing Chemistry Institute of Academia Sinica. Values of η_{sp}/c of B and D in pure water increased sharply with dilution at low concentration. This is attributed to uronic acid groups contained in the polysaccharide, which behaved as a polyelectrolyte, and hence aqueous 0.5 M NaCl/10% cadoxen and 0.5 M K_3PO_4/0.5 M NaH_2PO_4 were used as solvents for samples of B and D. Water, 10% cadoxen, and 88% formic acid were used as solvents for A, C, and E respectively. The kinetic energy correction was always negligible. Huggins and Kraeme plots were used to estimate the intrinsic viscosity $[\eta]$ and the Huggins constant k'.

Light-scattering measurements. — The light scattering intensities were observed with a dynamic light-scattering spectrophotometer (DLS-700, Otsuka Electronics Co.) at 633 nm in an angular range from 30 to 150° at 15° intervals at 25°C. The polysaccharide solutions were prepared with the aforementioned solvents and were used within 24 h after preparation. Optical clarification of the solutions was made by using a sand filter, with subsequent filtration through a 0.2 μm pore size filter (M-HJV) into the scattering cell. The refractive index increments (dn/dc) were measured with a double-beam differential refractometer (DRM-1020, Otsuka Electronics Co.) at 633 nm and 25°C. The acidic heteropolysaccharide (D and B) solutions were dialysed against solvent for 72 h. The values of dn/dc were 0.1363 $cm^3\ g^{-1}$ for A, 0.111 $cm^3\ g^{-1}$ for C in water, 0.136 $cm^3\ g^{-1}$ for both D and B in aqueous 0.5 M NaCl/10% cadoxen, and -0.232 $cm^3\ g^{-1}$ for E in 88% formic acid.

Osmometry.—Osmotic pressures (π) of the polysaccharide solutions in water, aqueous 0.5 M NaCl/10% cadoxen, and 88% formic acid were measured with an improved Bruss membrane osmometer by rapid static equilibrium [10] at 25°C. A regenerated cellulose membrane coded CN-8 with a pore size of 12 nm, prepared in our laboratory, was used.

3. Results and discussion

Components of polysaccharides.—The sugar component of polysaccharides D and B were identified as xylose, mannose, glucuronic acid, and glucose, and that of polysaccharides A, C, and E was glucose (paper chromatography). Gas chromatography demonstrated that B was composed of xylose, mannose, galactose, glucose/glucuronic acid (weight ratio: 1.8 : 75.6 : 1.7 : 21.1), and A contained mainly D-glucose (Table 1).

Each polysaccharide showed characteristic IR absorption bands at 1733 and 1250 cm^{-1} [8], and D and B also showed IR absorption at 1611(CO_2^-) and 800 cm^{-1} (mannoside). Glucans A, C, and E showed IR absorption at 890 cm^{-1} indicative of the β-configuration.

Table 1
Components and yields of *Auricularia auricula-judae* polysaccharides

Sample	Polysaccharide	Component [a]	Content [a]	Yield (%)
		Paper chromatography	Gas chromatography	
A	β-D-Glucan	Glc	Glc:Gal = 97.2:2.8	0.2
C	β-D-Glucan	Glc		0.6
E	β-D-Glucan	Glc		12.5
D	Acidic heteropolysaccharide	Xyl, Man, Glc, GlcA		0.4
B	Acidic heteropolysaccharide	Xyl, Man, Glc, GlcA	Xyl:Man:Gal:Glc/GlcA = 1.8:75.6:1.7:21.1	6.5

[a] Glc, glucose; Gal, galactose; Xyl, xylose; Man, mannose; GlcA, glucuronic acid.

Figure 1 illustrates the ^{13}C NMR spectra of glucans A and C in Me_2SO-d_6 solution. The ^{13}C NMR spectra are very similar to that of *Auricularia auricula-judae* glucan reported by Misaki et al. [2]. Following peak assignments in the ^{13}C NMR spectra of various branched β-(1 → 3)-D-glucans [12], the peaks at 85 and 60 ppm are assigned to C-3 and C-6 respectively. The integral area ratio of C-3(1 → 3) to C-1(1 → 3,1 → 6), was 0.605 for glucan A and 0.501 for glucan C. Analysis of these data suggest that two out of three β-(1 → 3)-D-glucose residues of the backbone chain for A and three out of three β-(1 → 3)-D-glucose residues of the backbone chain for C are substituted at O-6 position by single D-glucosyl groups. Moreover, the two-dimensional ^1H–^{13}C NMR correlation spectrum of glucan C displayed that the signal intensity of C-1(1 → 3) was the same as that of C-1(1 → 6) (details will be reported elsewhere).

From the foregoing data it is evident that the basic structural features of the three glucans (A, C, and E) are similar: backbone chains of β-(1 → 3)-linked D-glucose residues substituted by multiple branches at O-6 [2]. The acidic heteropolysaccharides D and B are composed of xylose, mannose, galactose, glucose, and glucuronic acid. These results and the purity criteria already mentioned, showed that the five isolated polysaccharides were basically homogeneous.

Molecular weights. — Figure 2 illustrates the Zimm plot for sample B, where K is the light-scattering constant, and R_θ is the reduced Rayleigh ratio at angle θ. The measured values of M_w, root-mean-square radii of gyration $\langle s^2 \rangle^{1/2}$, and second virial coefficients A_2^{LS} of the polysaccharides are summarized in Table 2. The values of M_w for C and B were in good agreement with results reported by Misaki et al. [2]. The plots of π/c against c for each polysaccharide are shown in Fig. 3. Where R is the gas constant, T is the absolute temperature (K), and c is the polysaccharide concentration (g cm^{-3}), the values for the number-average molecular weight M_n and second virial coefficient A_2^{OS} are given by

$$\pi/c = RT\left(1/M_n + A_2^{OS}c + \cdots\right). \quad (1)$$

Observed values of M_n and A_2^{OS} are listed in Table 2. The results show that molecular weights of the five polysaccharides isolated from *Auricularia auricula-judae* are different, and their molecular-weight distributions are very broad.

The extraplations of η_{sp}/c and ln η_r/c againt c for solutions of polysaccharides C, D, B, and E are based on good linear relationships (Fig. 4), and the $[\eta]$ and k' values

Table 2
Experimental results of molecular weights and viscosities of polysaccharides

Sample	Solvent	$M_w \times 10^{-4}$	$\langle S^2 \rangle^{1/2}$ (nm)	$A_2^{LS} \times 10^4$ (cm^3 mol g^{-2})	$M_n \times 10^{-4}$	$A_2 \times 10^4$ (cm^3 mol g^{-2})	M_w / M_n	$[\eta]$ (cm^3 g^{-1})	k'
A	water	117	123.0	15	23.1	3.80	5.06	4900	0.49
	10% cadoxen	–	–	–	–	–	–	4050	0.41
C	water	144	120.0	1.47	23.4	4.20	6.15	265	0.21
	10% cadoxen	–	–	–	–	–	–	353	0.23
E	88% formic acid	200 [a]	–	0.50	33.2	1.60	6.02	750	0.49
D	0.5 M NaCl/10% cadoxen	30.0	55.5	9.33	4.6	8.26	6.50	72	0.20
B	0.5 M NaCl/10% cadoxen	50.0	76.5	3.45	16.6	8.10	3.01	114 [b]	–
	0.5 M K_2HPO_4 /0.5 M NaH_2PO_4	–	–	–	–	–	–	101	0.36

[a] The value was measured by low-angle laser light-scattering photometer (KMX-6).
[b] By one-point method.

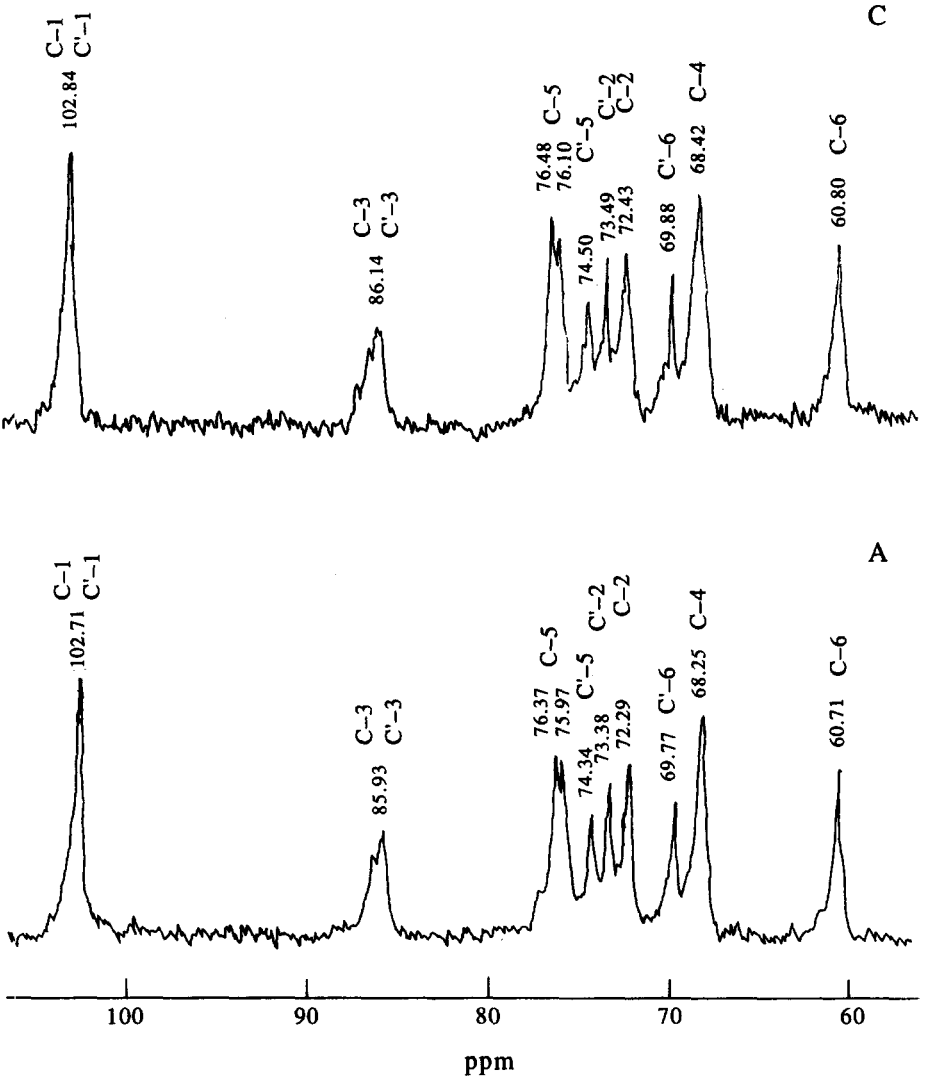


Fig. 1. ^{13}C NMR spectra of glucans A and C.

obtained are summarized in Table 2. The viscosity behaviour of glucan A in water is illustrated in Fig. 5. The value of k' for glucan A in water is close to 0.5, and the value of $[\eta]$ is far higher than that of glucan C or other flexible polymers. Moreover, it is noteworthy that, when glucan A was dissolved in 10% cadoxen, the value of $[\eta]$ decreased in a manner similar to the solution behaviour of double-stranded helix xanthan [12] or tri-stranded helix schizophyllan [13]. This result suggests that glucan A has a helical chain. Further evidence for a stiff chain is being sought. In contrast, the values of $[\eta]$ for glucan C in 10% cadoxen and for polysaccharide B in aqueous 0.5 M NaCl/10%

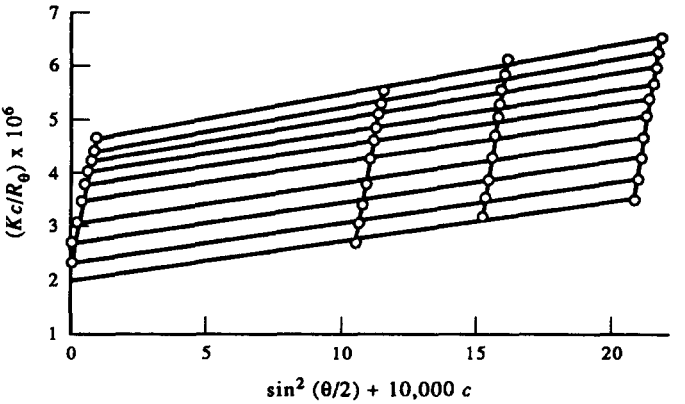


Fig. 2. Zimm plot of polysaccharide B in aqueous 0.5 M NaCl/10% cadoxen at 25°C.

cadoxen were higher than those of the solutions without cadoxen. This is probably attributable to the enhancement in solvent affinity for the sugar units by cadmium diaminoethane in cadoxen, and thus the polysaccharide chains are much expanded, thereby increasing the $[\eta]$ values. This behaviour is characteristic of flexible chains [9]. The values of $[\eta]$, k', and $\langle s^2 \rangle^{1/2}$ for glucan E in 88% formic acid are markedly higher than those of a general flexible polymer having the same molecular weight. The solution behaviour of glucan E differed greatly from that of flexible polymers, and this may be ascribed to the rigid or semiflexible rather than flexible macromolecules.

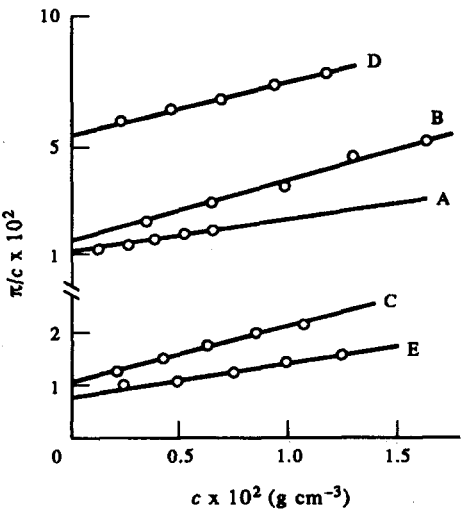


Fig. 3. Plots of $\pi/c - c$ for the polysaccharides in water (for A and C), 0.5 M NaCl/10% cadoxen (for D and B), and 88% formic acid (for E) at 25°C.

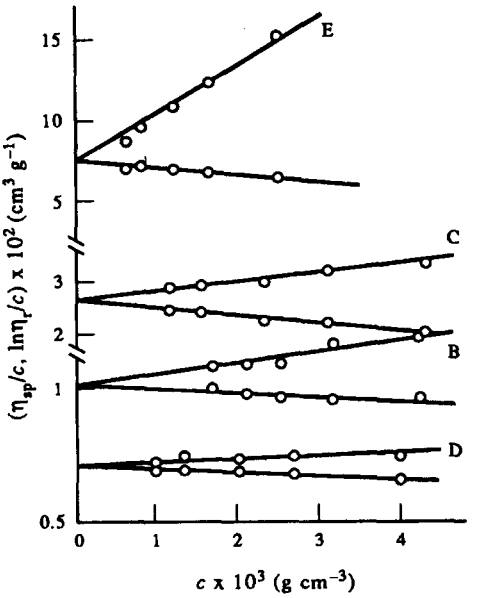


Fig. 4. Plots of $\eta_{sp}/c - c$ and $\ln \eta_r/c - c$ for the polysaccharides in water (for C), 0.5 M NaCl/10% cadoxen (for D), 0.5 M K_2HPO_4/NaH_2PO_4 (for B), and 88% formic acid (for E) at 30°C.

The good linear relationships of $\eta_{sp}/c - c$ and $\ln \eta_r/c - c$ of polysaccharides D and B (Fig. 4), and low values of k' suggests that the repelling effects caused by their ionic character (uronic acid) are inhibited, and that normal solution behaviour is exhibited by the two acidic polysaccharides in aqueous NaCl and K_2HPO_4 solutions. Analysis of

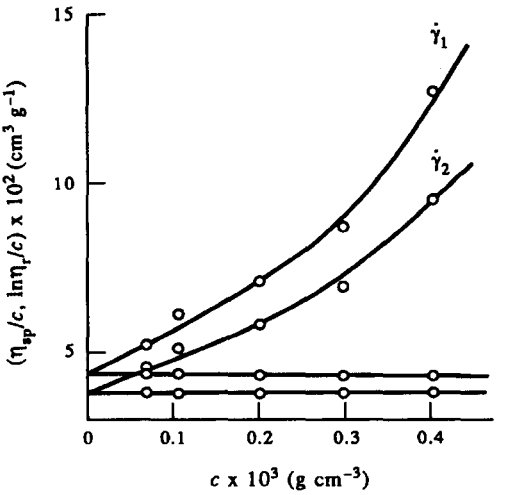


Fig. 5. Plots $\eta_{sp}/c - c$ and $\ln \eta_r/c - c$ for glucan A in water at 25°C.

M_w, M_n, A_2^{QS}, $[\eta]$ and k' values indicates that aqueous 0.5 M NaCl/10% cadoxen is a good solvent for polysaccharides D and B. The molecular weight and viscosity data of the acidic polysaccharide B are in agreement with results recently reported by us [9], where we concluded that these polysaccharides behave as slightly stiffened flexible chains in aqueous solution.

Summarizing the foregoing data analysis, we conclude that the *Auricularia auricula-judae* fruit contains at least five polysaccharides having different molecular weights and structure. The molecular weights were $M_w = 200 \times 10^4$, 144×10^4, 117×10^4, 50.0×10^4, and 30.0×10^4 for polysaccharides E, C, A, B, and D, respectively. Analysis of molecular weight and viscosity data suggests that glucans A and E have stiff chains, and polysaccharides D, C, and B have flexible chains in aqueous solutions.

Acknowledgments

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