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Effects of urea and sodium hydroxide on the molecular weight and conformation of α-(1→3)-D-glucan from *Lentinus edodes* in aqueous solution

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Abstract

The weight-average molecular weight (M_w) and intrinsic viscosity ([η]) of the α-(1→3)-D-glucan (L-FV-II) from *Lentinus edodes* in 0.5 and 1.0 M NaOH aqueous solution containing urea, were studied by light scattering and viscometry. The M_w value of the glucan decreased with increase of the urea and NaOH concentration. A strong intermolecular hydrogen bonding confers water-insolubility on the glucan, but NaOH and especially urea, broke this hydrogen bonding leading to enhanced water-solubility. Use of 1.0 M urea–1.0 M NaOH as solvent broke not only intermolecular hydrogen bonds but also partial covalent bonds of the α-glucan in aqueous solution, resulting in a decrease of M_w and [η]. The urea and NaOH concentrations, storage time with stirring, and mode of preparation of the polysaccharide in aqueous solution significantly affected the determination of M_w and [η]. The dependences of specific rotation and fluorescence emission ratio of a probe on urea concentration showed that a change in the molecular conformation of the α-glucan in 0.5 M NaOH aqueous solution containing urea occurred in the range 0.4–0.6 M urea. The 0.5 M urea–0.5 M NaOH aqueous solution is a suitable solvent for the glucan, and the M_w and [η] values obtained were 5.21×10^5 and 148 $cm^3\ g^{-1}$, respectively. Degradation of the glucan was obvious after storage for 15 months.

Keywords: *Lentinus edodes*; α-(1→3)-D-Glucan; Intrinsic viscosity; Molecular weight; Fluorescence; Conformation; Urea

1. Introduction

Polysaccharides have received attention as functional foods and a source for the development of natural drugs. Young and Jacobs [1] indicated that molecular conformation is an important factor in determining the biological activity of glucans. However, uncertainty in determination of molecular weight and molecular conformation of polysaccharides have often been encountered, owing to the abundance of hydrogen bonds complicating these analysis. The hydrogen bonding can be broken by using higher temperatures [2] or suitable solvents, such as urea [3,4], cadoxen [5], and dimethyl sulfoxide (Me_2SO) [6] or Me_2SO containing LiCl [7]. The choice of a proper solvent is essential for studies of molecular parameters and solution properties. It is noteworthy that pachyman, a water-insoluble β-D-glucan from *Poria cocos*, shows no antitumor activity, but after treatment with urea, it shows antitumor activity against sarcoma 180, and its water solubility increased [8]. Moreover, a conformational change in lentinan, a branched β-(1→3)-D-glucan from *Lentinus*

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edodes, occurred upon treatment with 8 M urea, because of interaction with urea [9], indicating that urea possibly plays some role in the solution properties and bioactivity of polysaccharides. Urea and NaOH are commonly needed in the isolation and post-treatment of polysaccharides [10,11], but their effects on molecular degradation and conformation have been little studied.

Most antitumor polysaccharides have a basic β-configuration, as in β-glucan, and exhibit a variety of biological and immunopharmacological activities, including the activation of macrophages through interaction with specific cell-surface receptors [12]. However, there are often antitumor polysaccharides with different chemical structures such as α-glucans [13,14] and α-glucan–protein conjugates [15]. In our previous work [16], the glucan isolated from fruiting bodies of *L. edodes* was investigated by high-performance liquid chromatography (HPLC), IR, ^{13}C NMR, light scattering, membrane osmometry and size exclusion chromatography (SEC), which identified it as an α-(1→3)-D-glucan, having 99.8% purity. Its solution in aqueous 0.5 M urea–0.5 M NaOH was prepared by dilution from 1.0 M urea–1.0 M NaOH, for use in molecular weight measurement. However, interpretation of the molecular weight data is complicated by the fact that the initial molecular weight and conformation of the glucan were unavoidably affected by urea, the NaOH concentration, and the mode of solution preparation. The purpose of this study was to clarify the effects of urea, NaOH, and the mode of solution preparation in water on hydrogen bonding, covalent bonds, and the molecular conformation of the polysaccharide.

2. Experimental

Materials.—The previously investigated α-(1→3)-D-glucan (L-FV-II) was isolated from fruiting bodies of *L. edodes* by extraction with 5% NaOH–0.05% $NaBH_4$, and then precipitation with 1 M AcOH, and subsequent washing with water and MeOH (five times), yield 3.2% [16]. It was stored in a dry container over silica for 15 months, and was used for the present study. Unless stated, all chemicals were of analytical grade.

Characterization.—IR spectra of the glucan employed a Nicolet FTIR spectrometer. Test specimens were prepared by the KBr disc method.

The specific rotation, $[\alpha]_D^{20}$, at 589 nm wavelength was determined on an automatic polarimeter, (WZZ-2A model) with 0.5 M aq NaOH containing urea from 0 to 1 M as solvent at 20 °C. The sample concentration was adjusted to $\sim 1.0 \times 10^{-2}$ g mL^{-1}. The fluorescence spectra were recorded with a Shimadzu RF-5301 fluorescence spectrophotometer with a quartz cell (1 × 1 cm cross section) equipped with a 150 W xenon lamp and dual monochromator at 25 °C. The glucan was first dissolved in 0.5 M NaOH, a phenanthrene–MeOH stock solution was added as a fluorescence probe, and then the mixture was stirred with urea solutions from 0.2 to 1.0 M urea for 20 min. The polysaccharide solution and fluorescence probe concentration was adjusted to 1.26×10^{-3} g mL^{-1} and 5×10^{-6} M, respectively. Excitation and emission slits were set at 5.0 and 3.0 nm, with wavelengths of 252 and 364 nm, respectively. Rf-530X-PC software was used for data treatment.

Viscosity measurements.—The viscosity of the glucan solution was measured at 25 ± 0.1 °C by using a modified capillary viscometer, a gift from the Institute of Industrial Science in Tokyo University. Huggins plot was used to determine the intrinsic viscosity $[\eta]$ of the polysaccharide in aq soln. To study kinetic phenomena, such as the changes of $[\eta]$ of the samples with storage time, a one-point method was used to measure $[\eta]$. The Solomon–Ciuta equation was used to calculate $[\eta]$ as follows:

$$[\eta] = \sqrt{2(\eta_{sp} - \ln \eta_r)}/c \quad (1)$$

The methods for preparation of polysaccharide solutions were as follows. One procedure used a mixture of 0.5 M NaOH containing urea as solvent to dissolve the glucan directly with stirring over 24 h. This was coded as A mode. The other solution (coded as B mode) was prepared by dissolving the glucan first in 1.0 M NaOH containing urea for 15 min, and then diluting to one-half concentration by adding water.

Light scattering.—The light-scattering intensities were measured with multi-angle laser light scattering (MALLS) instrument (DAWN® DSP, Wyatt Technology Co.) at 633 nm over an angular range from 23 to 147° at 25 ± 1 °C. The glucan was dissolved directly in aq solns of 0.5 M NaOH, 0.5 M urea–0.5 M NaOH, and 1.0 M urea–0.5 M NaOH for 24 h, respectively, to obtain solutions denoted A-1, A-2, and A-3. The glucan was dissolved in aq 1.0 M urea–1.0 M NaOH with stirring for 15 min or 24 h, and then diluted to 0.5 M urea–0.5 M NaOH before the measurement, and solutions were coded as B-1 and B-2. Optical clarification of the solution was made by subsequent filtration through four 0.45 μm Millipore filters (Whatman, UK) into the scattering cell (SV mode). 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 polysaccharide solutions were dialyzed against solvent for 72 h, and the value of dn/dc was determined as 0.179 g^{-1} cm^3 in 0.5 M urea–0.5 M NaOH. The value of dn/dc in 0.5 M NaOH and aq 1.0 M urea–0.5 M NaOH were estimated according to the Wyatt Co. guide as follows:

$$(dn/dc)_{0.5\ M\ NaOH} = 0.179 + (RI_{0.5\ M\ urea-0.5\ M\ NaOH} - RI_{0.5\ M\ NaOH})$$

$$(dn/dc)_{1.0\ M\ urea-0.5\ M\ NaOH} = 0.179 + (RI_{0.5\ M\ urea-0.5\ M\ NaOH} - RI_{1.0\ M\ urea-0.5\ M\ NaOH})$$

The refractive indexes (RI) of 0.5 M NaOH, 0.5 M urea–0.5 M NaOH, and 1.0 M urea–0.5 M NaOH were measured by an Abbé refractometer as 1.3371, 1.3419, and 1.3460, respectively. The dn/dc values of the glucan in 0.5 M NaOH and 1.0 M urea–0.5 M NaOH were 0.184 and 0.174 g^{-1} cm^3, respectively.

3. Results and discussion

Degradability.—The method of storage for biochemicals isolated from natural products is always important, because some polysaccharides and proteins are easily denatured or degraded by heat or microorganisms. Fig. 1 shows the storage time dependence of $[\eta]$ for fresh (B-01) and storage (B-1) samples (the glucan) in aqueous 0.5 M urea–0.5 M NaOH prepared by mode B. The fresh sample indicates that it was used immediately for mea-

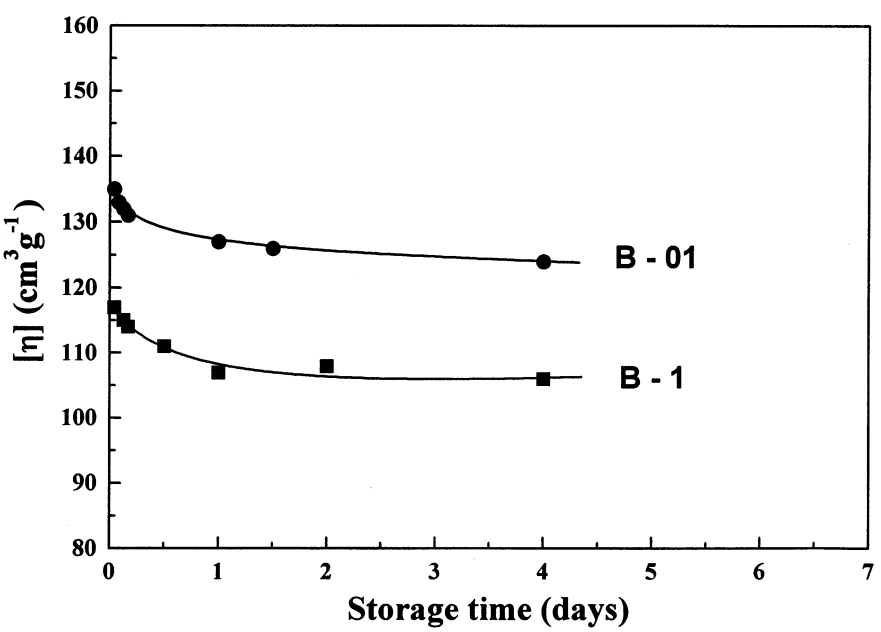

Fig. 1. Storage time dependences of $[\eta]$ for fresh glucan (B-01) and storage glucan in 0.5 M urea–0.5 M NaOH aqueous solution diluted from 1.0 M urea–1.0 M NaOH (B-1).

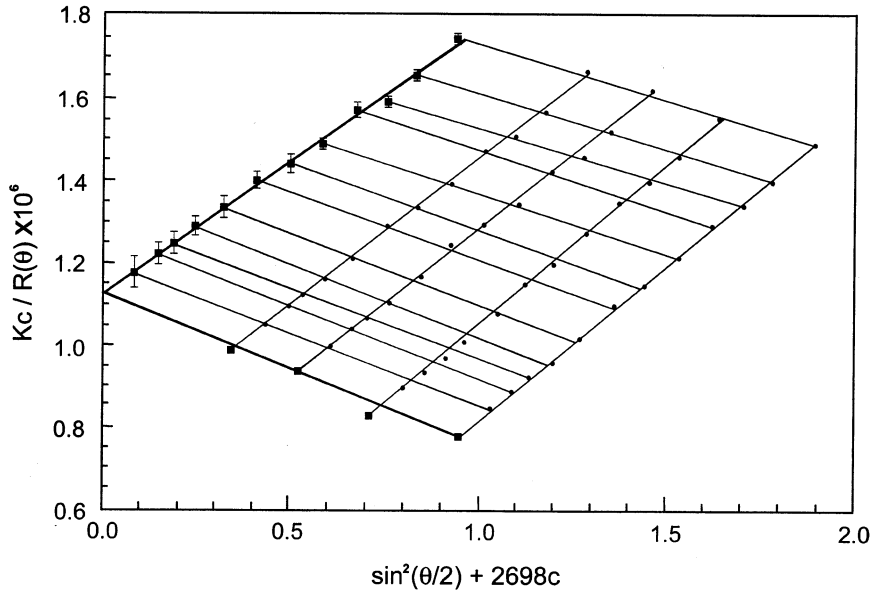

Fig. 2. Zimm plot of the glucan in 0.5 M NaOH aqueous solutions at 25 °C. (polysaccharide concentration: 1.276×10^{-4}; 1.924×10^{-4}; 2.591×10^{-4}; 3.521×10^{-4} g/g).

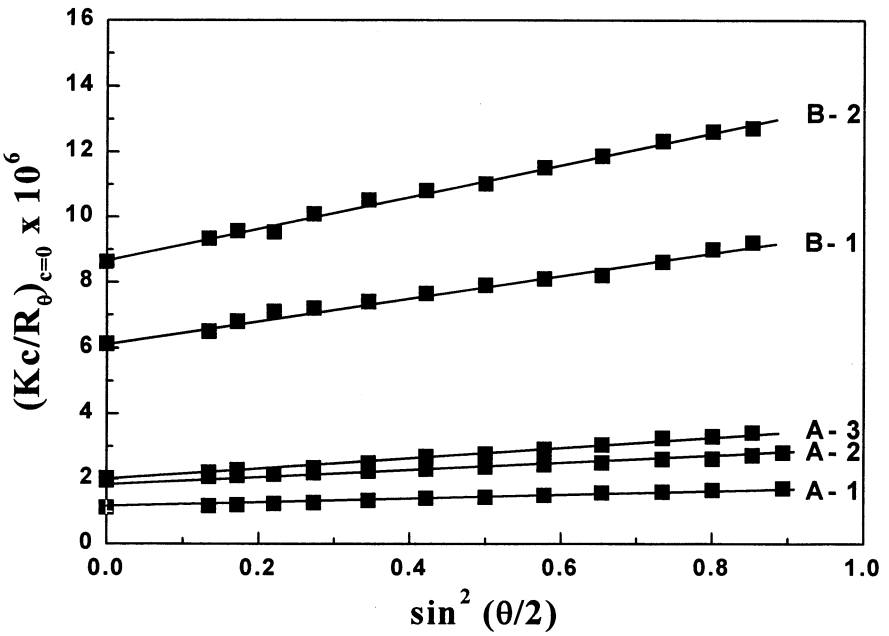

Fig. 3. Angular dependences of $(K_c/R_\theta)_{c=0}$ of the glucan in 0.5 M NaOH (A-1), 0.5 M urea–0.5 M NaOH (A-2), 1.0 M urea–0.5 M NaOH (A-3) aqueous solution. The stored glucan in aqueous 1.0 M urea–1.0 M NaOH for 15 min (B-1), and for 24 h with vigorously stirring (B-2), then were diluted, respectively, to 0.5 M urea–0.5 M NaOH aqueous solution at 25 °C for measurement.

surement after isolation from *L. edodes*. The $[\eta]$ value decreased from 135 $cm^3\ g^{-1}$ for fresh glucan to 117 $cm^3\ g^{-1}$ for the glucan with storage time of 15 months, indicating that $[\eta]$ of the glucan changed not only with storage time, but also with dissolving time, suggesting that covalent bonds have been broken. However, IR spectra suggested that its chemical structure did not change after 15 months.

Figs. 2 and 3 illustrate a Zimm plot and the angular dependence of $(K_c/R_\theta)_{c=0}$ of samples, where K is the light scattering constant, and R_θ is the reduced Rayleigh ratio at angle θ. The measured values of M_w, radii of gyration $\langle S^2\rangle^{1/2}$ and second coefficient A_2 of the glucan at 25 °C are summarized in Table 1. The error margin for the $\langle S^2\rangle^{1/2}$ and A_2 measurement was $\sim 8\%$. The solutions coded as 0.5 M

NaOH (A-1), 0.5 M urea–0.5 M NaOH (A-2), 1.0 M urea–0.5 M NaOH (A-3) were prepared by preparation mode A, namely by direct dissolution in the expected solvent. The polysaccharide solutions in aqueous 0.5 M urea–0.5 M NaOH with stirring times for 15 min (B-1) and 24 h (B-2) were obtained by mode B; namely their solutions in 1.0 M urea–1.0 M NaOH were diluted to 0.5 M urea–0.5 M NaOH for immediate measurement. The M_w value of the stored glucan in aqueous 0.5 M urea–0.5 M NaOH (B-1) is lower than that (2.41×10^5) of fresh glucan (B-01) similarly prepared [16], further proving that α-(1→3)-D-glucan (L-FV-II) is degraded on storage. Interestingly, the molecular weight of the glucan was degraded to 64% of the original glucan after storage for 15 months.

Effects of urea and NaOH on molecular weight.—The glucan is water-insoluble because of the presence of strong intermolecular hydrogen bonds, but is soluble in 1.0 M urea–1.0 M NaOH aqueous solution [16]. Data in Table 1 indicates that the M_w of the glucan decreased with an increase of concentration of urea or NaOH, suggesting that urea and NaOH can break the covalent bonds of this polysaccharide. However, the glucan was dissolved with difficulty in 0.5 M NaOH aqueous without urea, so that dissolution with stirring required over 1 day and the M_w values (A-1) obtained were higher than others because of the presence of microgels, due to poor solvent. This suggests that adding urea can break intermolecular hydrogen bonds more easily than NaOH without urea in the aqueous solution. The M_w value decreased slightly when the concentration of urea changed from 0.5 to 1.0 M in 0.5 M NaOH aqueous solution. The values of $\langle S^2 \rangle^{1/2}$ indicated that the molecular dimensions increased with increase of urea concentration. Comparing solutions A-3 with B-2, the M_w value of the glucan in B-2 solution decreased when the concentration of NaOH changed from 0.5 to 1.0 M in 1.0 M urea aqueous solution, implying that NaOH more readily breaks covalent bonds of the polysaccharide. Interestingly, when the polysaccharide solution (B-2) in 1.0 M urea–1.0 M NaOH was vigorously stirred for 24 h and then diluted to aqueous 0.5 M urea–0.5 M NaOH, this resulted in a very low M_w value (1.15×10^5), indicating that stirring may promote the cleavage of covalent bonds.

Effects of solvent and preparation mode on viscosity.—The intrinsic viscosities [η] of the glucan in different solvents and by different preparation modes are shown in Fig. 4 and Table 1. The higher [η] value of the sample dissolved directly in 0.5 M NaOH containing 0.5 M urea suggested this to be a good solvent. Increasing the urea and NaOH concentrations caused significant decrease in [η], owing to some breaking [see the [η] value in 1.0 M urea–1.0 M NaOH (B-2-1 solution)]. In order to evaluate the effect of air oxidation in 0.5 M NaOH aqueous solution, purging by N_2 (g) was equipped. The result of B-2-2 in Fig. 4 shows that oxidative action did not cause a significant change in this case. The [η] value of the glucan in the 0.5 M urea–0.5 M NaOH aqueous solution prepared by the A mode, differed greatly from that in 0.5 M urea–0.5 M NaOH obtained from diluting its solution in 1.0 M urea–1.0 M NaOH (B mode), suggesting a effect of method of solution preparation.

Fig. 5 shows that [η] of the glucan in aqueous 0.5 M urea–0.5 M NaOH is significantly higher than for the other urea concentrations, indicating that molecular chains are more extended in this solvent. The shrinking

Table 1
Experimental results of M_w, $\langle S^2 \rangle^{1/2}$, A_2 and [η] at 25 °C for the glucan in various solvents and solution preparation mode

Solution	A-1	A-2	A-3	B-01	B-1	B-2
$M_w \times 10^{-5}$ (g mol^{-1})	8.85	5.21	4.85	2.41	1.54	1.15
$\langle S^2 \rangle^{1/2}$ (nm)	65	66	74	51	63	58
$A_2 \times 10^4$ (cm^3 mol g^{-2})	−5.2	−8.3	−5.8	−8.8	−14	−19
[η] (cm^3 g^{-1})	108	148	119	135	117	95

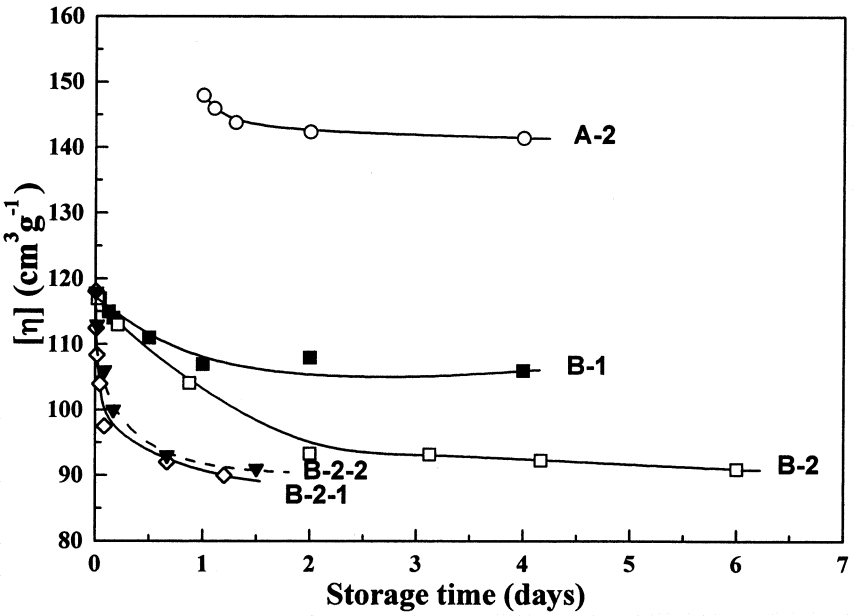

Fig. 4. Storage time dependences [η] for the storage glucan in aqueous 0.5 M urea–0.5 M NaOH by different preparation methods (see text) at 25 ± 0.1 °C.

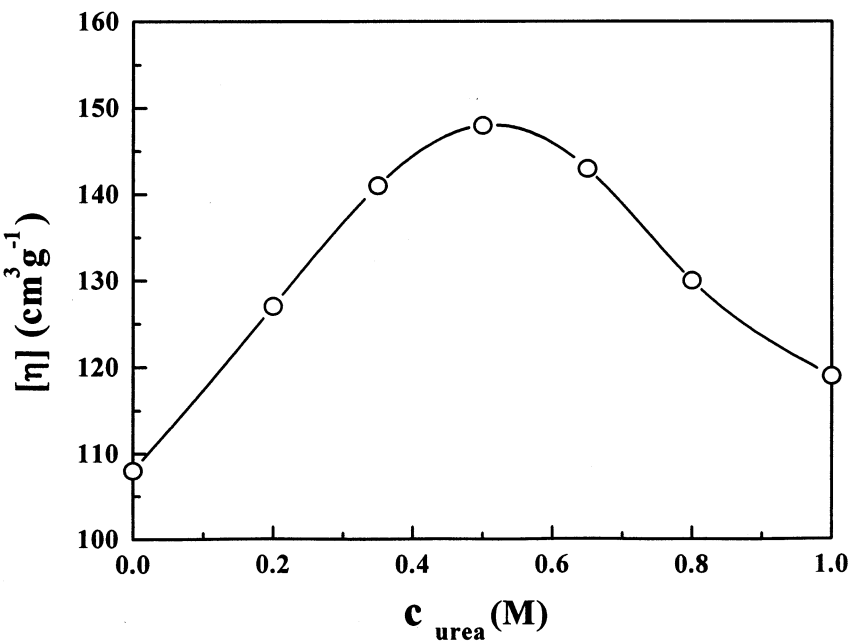

Fig. 5. Dependence of [η] for the glucan in aqueous urea–0.5 M NaOH on urea concentration from 0 to 1.0 M at 25 ± 0.1 °C.

of the molecular dimension in 0–0.4 M urea–0.5 M NaOH is consistent with $\langle S^2 \rangle^{1/2}$ results from light scattering (Table 1). The $\eta_{sp}/c \sim c$ curves of the fresh glucan in aqueous 0.5 M NaOH containing urea, are shown in Fig. 6 [16], where the solutions were obtained by dissolution in 1.0 M urea–1.0 M NaOH, and then diluting to one-half concentration. The [η] values of the glucan in aqueous 0.5 M urea–0.5 M NaOH were more than others, suggesting a better solvent. Based on information mentioned already, 0.5 M urea–0.5 M NaOH is suitable as a solvent for the glucan.

Effects of urea on molecular conformation.—Fig. 7 shows urea-concentration (c_{urea}) dependence of $[\alpha]_D^{20}$ for the glucan in aqueous 0.5 M NaOH containing 0–1.0 M urea. With increasing c_{urea} from 0.3 to 0.6 M, the $[\alpha]_D^{20}$

decreased sharply. This finding implies a conformational transition of the polysaccharide [17,18] and shows an increase in dimensions of the polysaccharide chain with the increase of urea concentration. However, when c_{urea} was more than 0.6 M and lower than 0.4 M, the molecular weight of the glucan decreased.

The fluorescence intensity of a probe should be sensitive to the microenvironment in polymer solution [19,20]. Phenanthrene was employed as a probe in polysaccharide solution to detect the intermolecular interaction [21]. The effect of urea concentration in aqueous 0.5 M NaOH on fluorescence–emission ratio

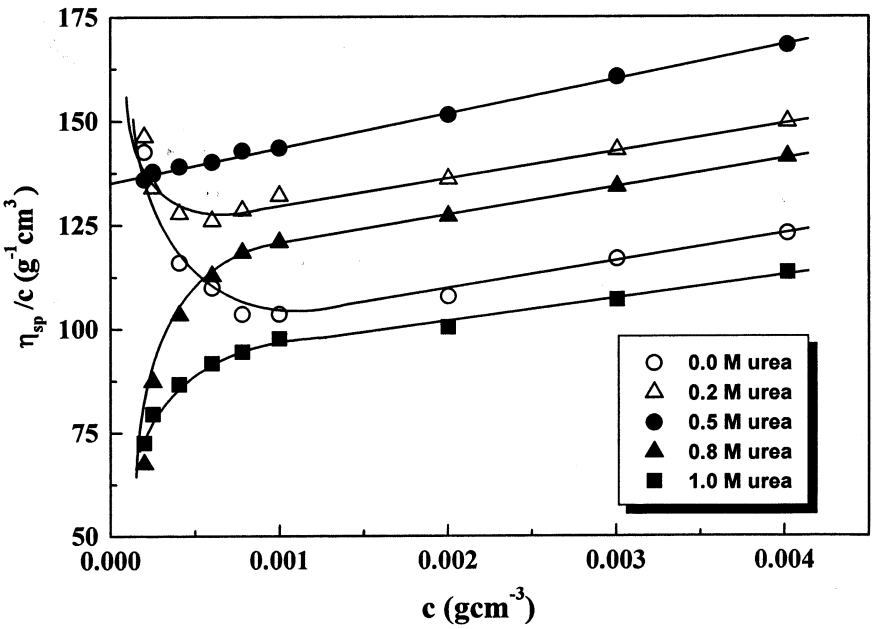

Fig. 6. $\eta_{sp}/c \sim c$ curves of the glucan in aqueous urea–0.5 M NaOH obtained from diluting urea–1.0 M NaOH to one-half concentration at 25 ± 0.1 °C (cited from the literature [16]).

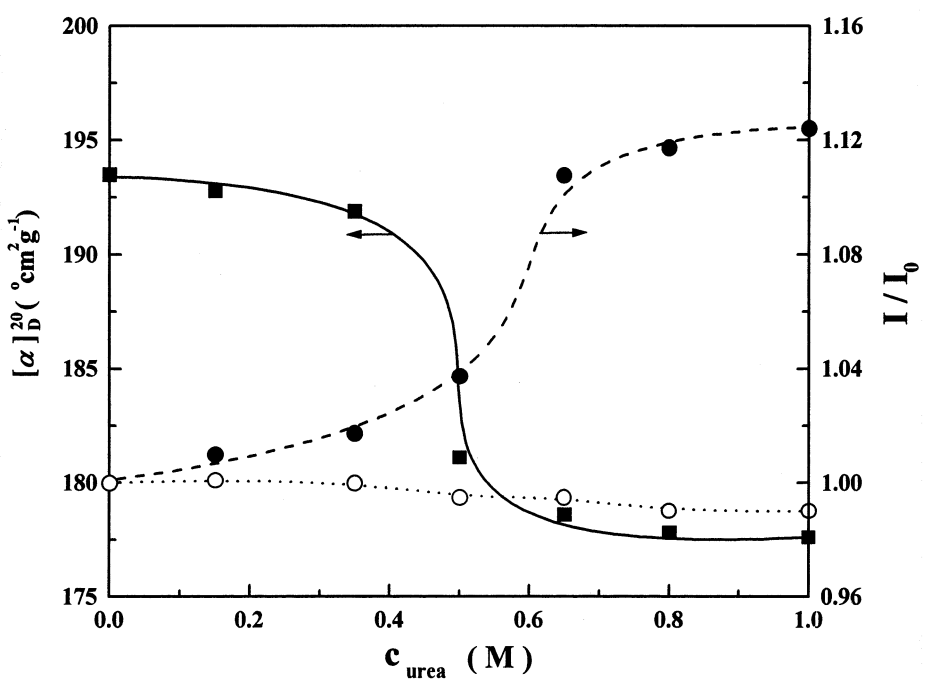

Fig. 7. Urea concentration dependences of $[\alpha]_D^{20}$ (■) and fluorescence emission ratio (I/I_0) of phenanthrene (● for the glucan) in 0.5 M NaOH aqueous solution containing urea from 0 to 1.0 M (○ for blank experiment).

I/I_0 of the glucan is shown in Fig. 7. The I_0 and I represent fluorescence–emission intensity in the absence and presence of glucan, respectively. The blank experimental result in Fig. 7 (○) showed that the urea concentration in 0.5 M NaOH containing urea almost did not affect the I/I_0. Interestingly the I/I_0 of the glucan in aqueous 0.5 M NaOH solution containing urea increased sharply with increasing c_{urea} from 0.4 to 0.6 M, similar to the change of the $[\alpha]_D^{20}$. These results suggested that a change in the molecular conformation of the glucan in aqueous 0.5 M NaOH containing urea occurred in the range 0.4–0.6 M urea.

4. Conclusions

There is a strong intermolecular hydrogen bonding in the α-(1→3)-D-glucan (L-FV-II), resulting in its water-insolubility. Degradation of the glucan was obvious after storage for 15 months, or in 1.0 M NaOH aqueous containing urea for 1 day. A mixture of 1.0 M urea and 1.0 M NaOH broke the hydrogen- and partial covalent-bonding of the glucan in aqueous solution, leading to increased water-solubility and decreased molecular weight. Urea decomposed the intermolecular hydrogen bonding of the glucan in aqueous solution more easily than NaOH, and plays an important role in enhancement of the water-solubility for the polysaccharide. Urea and NaOH concentration in the aqueous solution, storage time of the solution, and preparative mode of the polysaccharide solution affected considerably the determination of molecular weight and viscosity for the glucan. The obtained molecular weight of the glucan in 0.5 M NaOH aqueous solution deviated to a large value because of the presence of microgel, due to the poor solvent. The dimension of the molecular chains of the glucan in aqueous 0.5 M NaOH containing urea increased with urea concentration, and a change in the molecular conformation occurred in the range 0.4–0.6 M urea.

Acknowledgements

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