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ARTICLE

Structural Characterization, Chain Conformation, and Morphology of a β-(1→3)-D-Glucan Isolated from the Fruiting Body of *Dictyophora indusiata*

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A water-soluble glucan, namely, PD3, was isolated from the fruiting body of *Dictyophora indusiata* as the method reported previously. Its chemical structure was characterized by GC, FTIR, and ^{13}C NMR. The results indicated that PD3 is a (1→3)-β-D-glucan, with (1→6)-β-glucopyranoside side branches. The chain conformation and morphology of PD3 in aqueous solution were investigated by viscometry, rheometer, and laser light scattering (LLS) measurements, atomic force microscopy (AFM), and transmission electron microscopy (TEM), respectively. The weight-average molecular mass (M_w), radius of gyration (R_g), hydrodynamic radius (R_h), and intrinsic viscosity ([η]) of PD3 in water were determined to be 5.1×10^5, 141 nm, 44 nm, and 1440 $cm^3\ g^{-1}$, respectively, by LLS and viscometry. The structural parameter ρ (Rg/Rh) of PD3 was calculated to be 3.4, and the [η] dependence of C_{NaOH} of PD3 is similar to that of triple helical polysaccharides Scleroglucan and Lentinan, suggesting that PD3 exists as a triple helical chain in water. This conclusion was further proved by rheological measurement and AFM observation. Interestingly, the [η] of PD3 dramatically decreased in a narrow range concentration of NaOH between 0.18 and 0.22 M, higher than that of Scleroglucan and Lentinan (both less than 0.1 M), indicating the helix−coil conformation transition of PD3 is more difficult than that of Scleroglucan and Lentinan. Moreover, with the increase of concentration, PD3 trends to self-assemble to fibrous aggregates in aqueous solution as measured by TEM.

KEYWORDS: structure characterization; chain conformation; morphology; (1→3)-β-D-glucan *Dictyophora indusiata*

INTRODUCTION

Numerous bioactive polysaccharides or polysaccharide−protein complexes from medicinal mushrooms, plants, and other resources are reported to enhance innate and cell-mediated immune responses (*1*−*3*) and exhibit antitumor activities in animals and humans (*4*−*6*). As an example, Lentinan, a (1→3)-β-D-glucan with (1→6)-glucosyl side groups from *lentinue edoes*, is clinically widely used in Japan, China, and other countries as an anticancer agent (*7*, *8*). Polysaccharides are also widely used in the food industry as functional ingredients (*9*). Many polysaccharides are exploited as thickeners in the food industry, including sodium alginate, carboxymethyl cellulose, the plant seed galactomannans, and λ-carrageenan (*9*). These polysaccahrides all exist as random coils in dilute solution, and their viscosity behaviors are nonspecific, i.e., when molecular weight is normalized, viscosity depends on shear rate and polymer concentration. Of great interest are those polysaccharides that confer novel rheological properties, such as rigid gel formation or weak gel properties. These polysaccharides, such as Xanthan (*10*), Schizophyllan (*11*), and Scleroglucan (*12*), are widely used as gelling, stabilizing, emulsifying, and water-binding agents. This relatively new class of polysaccharides usually exists as ordered chains (helices) in dilute solution and forms very viscous solutions at low concentration.

The reason that polysaccharides can exhibit large differences in solubility, aggregation, crystallization, viscosity, gel formation, digestibility, and biological activities is because their different primary chemical structures determine the shape that they can adopt in aqueous solutions (*9*). For example, (1→3)-β-D-glucans with some (1→6)-glucosyl side groups, including Lentinan (*13*), Schizophyllan (*11*), and Scleroglucan (*12*), all exist as triple helical chains, form very viscous solutions at low concentration, and have potential antitumor activity (*10*). Therefore, investigating the primary chemical structure, solution property, and chain conformation is very essential for the study of the polysaccharide

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bioactivity and the development of new polysaccharide based food additives.

Dictyophora indusiata is an edible mushroom widely used in China and other Asian countries. It is called "queen of the mushrooms" in China due to its beautiful appearance and delicious taste. A few small compounds were isolated from the mushroom and reported to have neuroprotective and other bioactivities (*14*, *15*). Besides that, several water-soluble and water-insoluble polysaccharides were isolated and characterized by Hara and Ukai et al. (*16*−*18*). Among these polysaccharides, a branched (1→3)-β-D-glucan is of great importance due to its antitumor potential and possible triple helical conformation. However, the solution properties, chain conformation, and morphology of the triple helical polysaccharide still remain unknown.

The present work was aimed to investigate the chemical structure, chain conformation, and morphology of the possible triple helical polysaccharide in aqueous solution. We isolated the triple helical polysaccharide by the method reported by Hara et al. (*16*), characterized its monosaccharide composition by GC, and determined its chemical structure by FTIR and ^{13}C NMR. Viscometry and laser light scattering (LLS) experiments were carried out to measure its physico-chemical properties i.e., intrinsic viscosity [η], weight average molecular mass M_w, radius of gyration (R_g), hydrodynamic radius (R_h), and structural parameter ρ (R_g/R_h) in water. Rheological measurement and [η] dependence of C_{NaOH} experiments were used to investigate its triple helical conformation and conformation transition. Finally, the morphology of the polysaccharide was directly observed by atomic force microscopy (AFM) and transmission electron microscopy (TEM) methods.

MATERIALS AND METHODS

Polysaccharide Isolation and Purification. The polysaccharides were isolated and purified from *Dictyophora indusiata* according to the method by Hara et al. (*16*). Briefly, dried fruiting bodies of *Dictyophora indusiata* powder were defatted sequentially by using Soxhlet extraction with ethyl acetate and ethanol for over 6 h, respectively. The resultant residue was immersed stepwise in distilled water at 20, 40, 60, and 80 °C. In each step, the mixture was stirred overnight and then centrifuged (9000 rpm, 20 min) to get the supernatant, coded as PD1, PD2, PD3, and PD4, respectively. In this article, we focused on the fraction PD3, a polysaccharide forming, very viscous solution at low concentration (0.5 mg/mL). This polysaccharide was further purified by decolorization with 30% H_2O_2 and removal of free protein by the Sevag's method (*19*), followed by fractionation by actetone and dialysis against distilled water (M_w cutoff 8000). The polysaccharide was finally lyophilized to the purified PD3 sample (white powder).

Structure Characterization. *Monosaccharide Composition by GC*. PD3 was hydrolyzed to monosaccharides by 2 M sulfuric acid. The resulting monosaccharide was further reacted with acetic anhydride to prepare the monosaccharide alditol acetate derivatives according to the method of Blakaney et al. (*20*). The alditol acetate derivatives were then analyzed by gas chromatography (GC). GC was conducted on a Hewlett-Packard 6910 gas chromatograph system. The detailed experimental conditions were as follows: H_2 (30 mL/min); O_2 (200 mL/min); N_2 (20 mL/min); the initial column temperature of 140 °C was held for 3 min, and then the temperature was increased at 7 °C min^{-1} to 240 °C and held at 240 °C for 10 min. The temperature of the injector was 250 °C, and the temperature of the detector was 270 °C.

IR Spectrum by FTIR Spectrometer. The IR spectrum of PD3 was recorded with a Nicolet 170SX FTIR spectrometer (Spectrum One, Perkin−Elmer Corp., U.S.A.) in the range of 4000−400 cm^{-1} using the KBr-disk method.

^{13}C NMR by NMR Spectrometer. The ^{13}C NMR spectrum of PD3 was recorded on a 600 MHz NMR spectrometer (Varian Inova) by using a standard 5 mm probe and D_2O as the solvent at 60 °C.

Chain Conformation. *Weight-Average Molecular Mass (M_w) and Radius of Gyration (R_g) by Static Laser Light Scattering Measurement Using Multiangle Laser Light Scattering Instrument*. The light scattering intensities of PD3 sample in water were measured with a laser light scattering (LLS) instrument (ALV/CGS-8F, ALV, Germany) at 633 nm in an angular range from 30° to 150° with an interval of 15° at 25 °C. Pure benzene at 25 °C was used to calibrate the apparatus. The most concentrated solutions of the samples were prepared by continuous stirring at room temperature overnight, and the solutions of lower concentrations were obtained by sequential dilution. The weight concentrations of all test solutions were determined gravimetrically and converted to mass concentrations c by use of the densities of the solvent. These solutions were optically purified by filtration through Millipore filters of pore size 0.2 μm (PTFE, Puradisc 13 mm Syringe Filters, Whatman, England). The specific refractive index increment (dn/dc) at 633 nm and 25 °C for PD3 was determined to be 0.138 mL/g using an interferometric refractometer (Optilab/903, Wyatt Technology, U.S.A.).

In static LLS, scattering light intensity known as Rayleigh ratio (R_θ) of a polymer solution at angle(θ) and concentration (c) is related to weight-average molecular mass (M_w) by (*21*)

$$Kc/R_\theta = 1/M_w(1 + 1/3(R_g^2q^2) + 2A_2c \quad (1)$$

where $K = 4\pi^2n_0^2(dn/dc)^2/(N_A\lambda_0^4)$ and $q = 4\pi n_0/\lambda \sin(\theta/2)$, with N_A, n_0, λ_0 as Avogadro's number, refractive index of the solvent, and wavelength of the light in vacuum, respectively. R_g is the z-average radius of gyration, and A_2 is the second virial coefficient. By measuring the R_θ at different c and q, we can obtain the M_w, R_g, and A_2 from the Zimm plot.

Hydrodynamic Radius Measurement by Dynamic Laser Scattering Instrument. Dynamic laser light scattering measurements were carried out to determine the hydrodynamic radius R_h for PD3 in water at 25 °C. In all measurements, we used an ALV/DLS/SLS-5000E light scattering goniometer (ALV/CGS-8F, ALV, Germany) with vertically polarized incident light of wavelength 632.8 nm from a He−Ne laser equipped with an ALV/LSE-5003 light scattering electronics and multiple tau digital correlator. Test solutions were prepared and optically purified in the same manner as in the case of static laser scattering measurements. The normalized autocorrelation function $g^{(2)}(t,q)$ of scattered light intensity, which related to the z-average translational diffusion coefficient D_z and q ($4\pi n_0/\lambda \sin(\theta/2)$) (*22*) was measured at five different concentrations and scattering angles ranging from 30° to 90°. The hydrodynamic radius R_h is calculated from the Stokes−Einstein relation as follows:

$$R_h = k_BT/(6\pi\eta_0D_z) \quad (2)$$

where k_B is the Boltzmann constant; T is the absolute temperature; and η_0 is the solvent viscosity.

Intrinsic Viscosity Measurement by Ubbelohde-Type Viscometer. The intrinsic viscosities of PD3 were determined by using an Ubbelohde-type viscometer in water at 25 °C. The kinetic energy correction was always negligible. Huggins and Kraemer

equations were used to estimate the intrinsic viscosity [η] by extrapolation to infinite dilution as follows:

$$\eta_{sp}/c = [\eta] + k'[\eta]^2 c \quad (3)$$

$$\ln \eta_r/c = [\eta] - (0.5 - k')[\eta]^2 c \quad (4)$$

where k' is constant for a given polymer at a given temperature in a given solvent; η_{sp}/c is the reduced specific viscosity; and $(\ln \eta_r)/c$ is the inherent viscosity. The C_{NaOH} dependence of [η] was obtained by measuring the intrinsic viscosity of PD3 at various NaOH concentrations between 0 and 0.3 M, using the same method as mentioned above.

Storage Modulus (G′) and Loss Modulus (G″) Dependence of Temperature by Dynamic Temperature Sweep Measurement Using Strain-Controlled Rheometer. The dynamic properties of PD3 were carried out on ARES-RFS III strain-controlled rheometer (TA Instruments, U.S.A.). The double-concentric cylinder geometry consisting of a rotating outer cylinder (cup) and an inner cylinder (bob) was used. The diameters of the cup and bob were 34 and 32 mm, respectively, and the bob length was 33 mm. The rheometer was equipped with two force transducers allowing the torque in the range from 0.004 to 1000 g·cm. The shear rate ranges from 0.02 to 1000 s^{-1}. Temperature control in the range of 0−15 °C was achieved by using a circulating fluid bath (a julabo FS18 cooling/heating bath) kept within ±0.4 °C over an extended time. In dynamic measurements, both strain and frequency are very important parameters, as only at lower strain limits will shear storage modulus (G') and loss modulus (G'') be independent of strain. Therefore, dynamic strain sweep measurements were carried out at 1 rad/s to determine the linear viscoelastic regime with a strain range from 0.3 to 900%. For each measurement, a fresh sample solution was initially transferred into the cup, and then the bob was suspended from an air bearing and torque was measured using transducers in the frequency range of 0.1−100 rad/s. The dynamic time sweep at a given frequency was also performed to monitor and/or store the transient stress and strain signals graphically in real time to provide additional information on materials during testing by Waveform and fast data sampling option. The dynamic temperature ramp test was conducted from 1 to 20 °C at an angular frequency of 1 rad/s and strain of 1% with heating rates of 1 °C/min and soak times of 5 min at each elevated temperature for PD3 at a concentration of 0.1%. All sample solutions were loaded at room temperature and allowed to be equilibrated for about 20 min in order to make temperature equilibration and stress relaxation. The solution was covered with a thin layer of low-viscosity silicone oil in order to prevent water evaporation during rheological measurements. All the dynamic measurements are performed in the linear visocoelastic region.

Morphology. *Atomic Force Microscopy*. AFM imaging was conducted with a PicoScan atomic force microscope (Molecular Imaging, U.S.A.). Freshly prepared samples were mounted on AFM stage and imaged under MAC Mode in air (relative humidity = 40−50%, T = ~25 °C) using MAClever type II probes (spring constant = 2.8 N/m, resonant frequency = ~85 kHz, Molecular Imaging, U.S.A.). Scan rates were about 1.5 line/s. The images were rastered at 256 × 256 pixels, unfiltered and flattened when needed. A drop of 10 μL of sample solution (1 μg/mL) was dropped onto freshly cleaved ruby muscovite mica substrate (Digital Instruments, U.S.A.) and allowed to dry overnight.

Transmission Electron Microscopy (TEM) and Image Analysis. The PD3 polysaccharide aqueous solution (10 μg/mL) is dropped on copper grids and stained by a 0.2% (w/v) solution of phosphotungstic acid before observation; after that the sample was dried overnight at room temperature for viewing. A Tecnai G2 20 electron microscope was used to obtain the electron micrographs of PD3. The magnification of the micrographs was described in the picture. TEM image of PD3 was obtained at an accelerating voltage of 120 kV.

RESULTS AND DISCUSSION

Structural Characterization. The chemical structure of PD3 was characterized by GC, Fourier transform infrared (FTIR), and ^{13}C NMR, respectively. **Figure 1** showed the GC trace of the alditol acetate derivatives of the hydrolyzed PD3 product. Compared with standard monosaccharide derivates, we found that PD3 consists mainly of glucose (~96.7%), which indicated that PD3 is almost a pure glucan.

The FTIR spectrum of PD3 is shown in **Figure 2**. The band in the region of 3350 cm^{-1} is due to the hydroxyl stretching vibration of polysaccharide. The bands in the region of 2932 cm^{-1} are due to C−H stretching vibration, and the bands in the region of 1640 cm^{-1} are due to associated water. Absorptions at 891 cm^{-1} are typical for β-D-glucose in pyranose form. The absorptions at 1034 cm^{-1} could be attributed to the C−O stretching

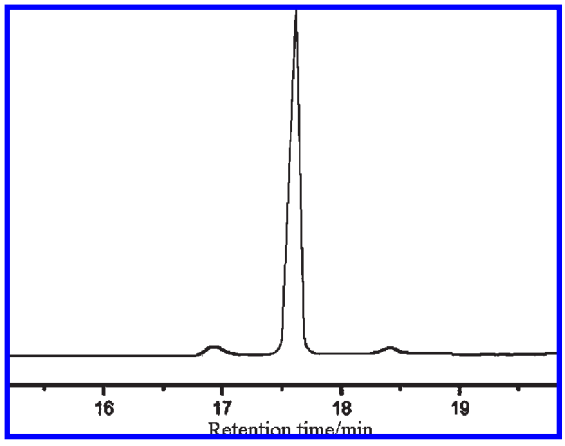

Figure 1. GC trace of glucan PD3 monosaccharides alditol acetate derivatives.

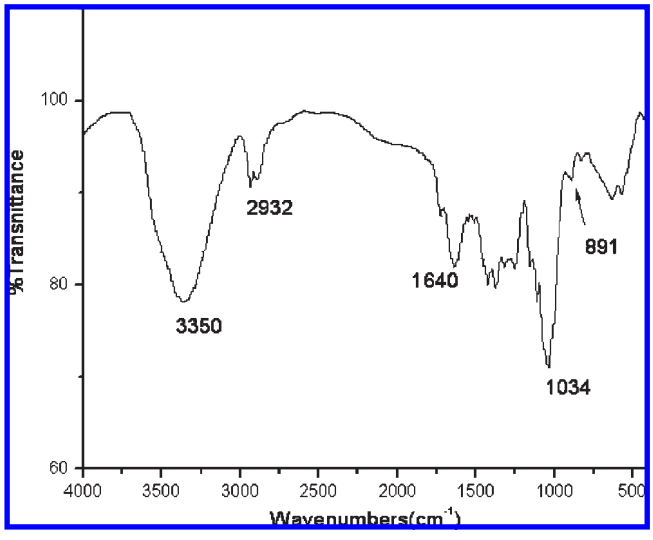

Figure 2. FTIR spectrum of the glucan PD3.

of polysaccharide. These results are well consistent with the conclusion of Hara et al. (*16*).

The ^{13}C NMR spectrum of PD3 in D_2O is shown in **Figure 3**. The chemical shifts of PD3 were very similar to those of β-(1→3)-D-glucans with side branches of β-(1→6) glucosyl units such as Grifolan, Glucan TM8, Scleroglucan, and Lentinan (*23*−*26*). The assignments of ^{13}C NMR chemical shifts of PD3, Grifolan, and Glucan TM8 are listed in **Table 1**. Peaks at 103.8(C1), 73.9(C2), 86.5(C3), 68.7(C4), 76.8(C5), and 61.3(C6) ppm are signals of the carbon atoms of the β-(1→3)-D-glucan backbone, while peaks at 69.6(C4′), 74.8(C5′), and 70.7(C6′) ppm resulted from branching effect. Our results indicated that PD3 has a similar structure to those of Grifolan and Glucan TM8, i.e., β-(1→3)-D-glucan backbone with β-(1→6) glucosyl side chain.

Chain Conformation. Figure 4 illustrated measurement of the intrinsic viscosity of PD3 in water. The intrinsic viscosity [η] of PD3 was determined to be 1440 $cm^3\ g^{-1}$, which is much higher than that of flexible polysaccharides in good solvents (usually $< 200\ cm^3\ g^{-1}$). Moreover, this data is comparable with that of Schizophyllan (500 $cm^3\ g^{-1}$, M_w 5.0×10^5) (*27*) and Lentinan (903.3 $cm^3\ g^{-1}$, unfractionated sample) (*28*) in water, suggesting they have a similar chain conformation in aqueous solution. Since Lentinan and Schizophyllan exist as helical conformation in aqueous solution, PD3 thus possibly exists as a helix in aqueous solution as well.

Figure 5 showed the Zimm plot of PD3 in aqueous solution. The weight-average molecular weight (M_w) and radius of gyration (R_g) were calculated to be 5.1×10^5 and 141 nm, respectively. The hydrodynamic radius (R_h) of PD3 was also measured to be 44 nm according to eq 2 by dynamic light scattering method. It is well-known that the parameter ρ defined as R_g/R_h could be employed to describe the shape of linear chains to some extent as follows (*29*, *30*). When ρ is 0.775, the polymer chains exist as spheres, and for a long rod chain, $\rho = \infty$, indicating more rigid chains have higher ρ values. For example, random coils have

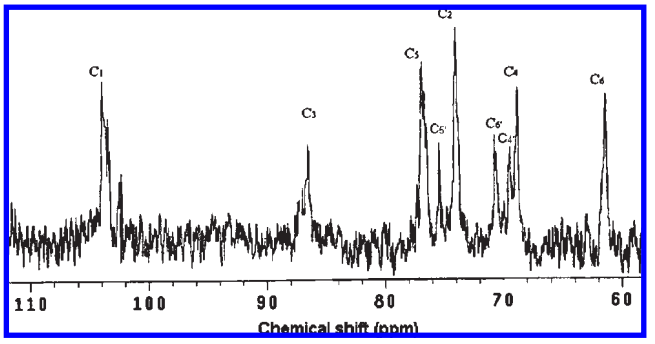

Figure 3. ^{13}C NMR spectrum of Glucan PD3 in D_2O at 60 °C.

Table 1. Assignment of ^{13}C NMR Chemical Shifts of the PD3, Glucan TM8, and Grifolan A

		chemical shift (ppm)						
sample	solvent	C-1	C-2	C-3	C-4	C-5	C-6	source
PD3	D_2O	103.8	73.9	86.5	68.7 (69.6)	76.8 (74.8)	61.3 (70.7)	this work
Glucan TM8	D_2O	103.0	73.7 (72.5)	86.3	68.5	76.1 (74.6)	60.9 (70.0)	ref (*23*)
Grifolan A	D_2O	102.8	73.5 (72.4)	87.0	68.3	76.8 (74.5)	60.7 (70.0)	ref (*24*)

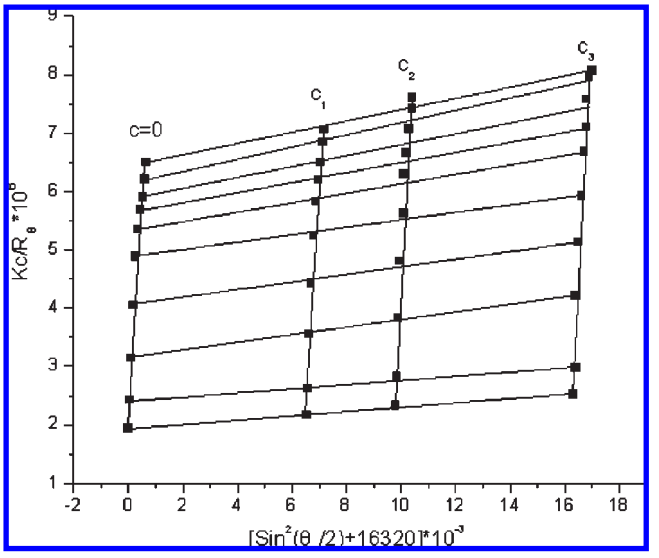

Figure 5. Zimm plots for PD3 in aqueous solution (C_1 = 0.4 mg/mL, C_2 = 0.6 mg/mL, C_3 = 1.0 mg/mL).

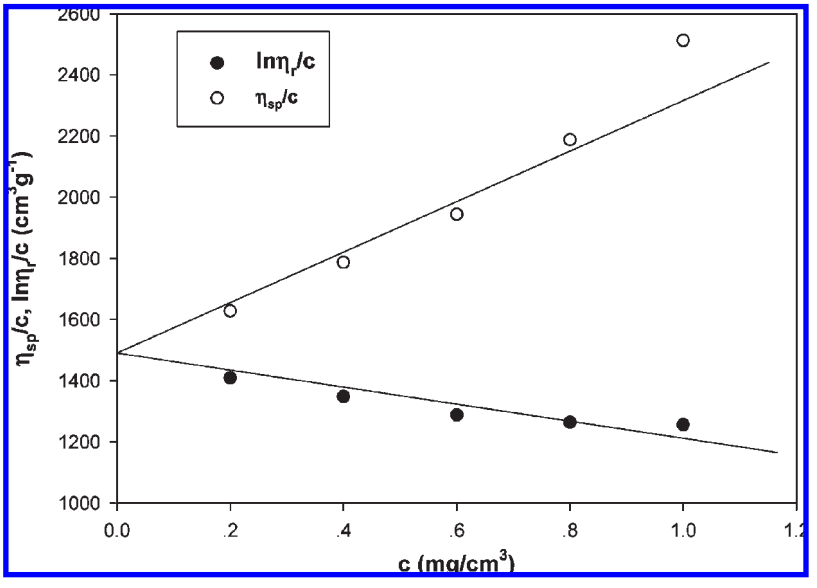

Figure 4. η_{sp}/c and ln η_r/c dependence of c in water at 25 °C.

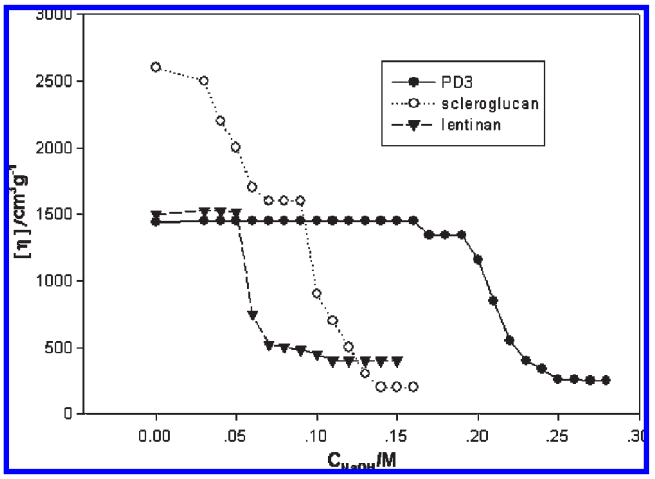

Figure 6. Dependence of $[\eta]$ on C_{NaOH} for PD3 in aqueous NaOH solution at 25 °C (solid dot). The open dot indicates the data of *Scleroglucan* F-1 by Sato et al. (*31*). The line with solid triangles represents the data of *Lentinan* by Zhang et al. (*30*). The concentrations of PD3 at various C_{NaOH} are fixed as 0.5 mg/mL.

ρ values of 1.3−1.5, while stiff chains have ρ values larger than 2. Ding et al. have reported that a very extended chain, i.e., Pachyman in dimethyl sulfoxide (DMSO), has a ρ value around 2.5 (*31*). By combining the results of R_g from static light scattering with those of R_h from dynamic light scattering, the ρ value for PD3 is calculated to be 3.4. This data is higher than that of Pachyman in DMSO, suggesting the rigidity of PD3 in water is higher and possibly adopts a more ordered chain conformation.

The C_{NaOH} dependence of $[\eta]$ for PD3 at 25 °C is shown in **Figure 6**. For comparison, the literature data for triple helical polysaccharides such as Lentinan and Scleroglucan was also summarized in this figure. It is very interesting that PD3 also has the similar conformation changes from the triple helix to random coil as Lentinan and Scleroglucan. The main differences are the range of NaOH concentrations in which the sharp decrease of $[\eta]$ occurred. For PD3, $[\eta]$ sharply decreased in a range of NaOH concentration at 0.18−0.25 M, while Lentinan and Scleroglucan decreased in a range at 0.05−0.08 M (*32*) and 0.08−0.15 M(*33*), respectively. The differences of the C_{NaOH} dependence of $[\eta]$ among different types of triple helical polysaccharides may relate with its sample-preparation procedure. For Lentinan and Scleroglucan, it was isolated by NaOH aqueous solution extraction. In this procedure, part of the hydrogen bonds was broken; thus, their hydrogen bond strength was relatively weaker than that of PD3, which was isolated by water extraction. Nevertheless, the chain conformation transitions of these triple helical polysaccharides are very similar, as their $[\eta]$ sharply decreased to 100−200 $cm^3\ g^{-1}$ from about 2000 $cm^3\ g^{-1}$ at certain NaOH concentration.

Several investigators have found that the intramolecular conformation transition from ordered triple helix I to disordered triple helix II for Schizophyllan in water occurred at temperature of ~7 °C (*34*−*36*), but no such specific phenomena were observed for random coil Pullulan and simple saccharides such as sucrose (*34*). This low-temperature intramolecular conformational transition involves formation of hydrogen bonding among the side group and water molecules, and behaves as a drop decrease of shear modulus G' in dynamic temperature sweep experiment (*37*), which was also found in Lentinan (*38*). **Figure 7** depicts the changes of G' with temperature for sample PD3 in water with concentration of 0.1%. Similar to the evolution of G' for the original triple helical Lentinan and Schizophyllan in water, G' for

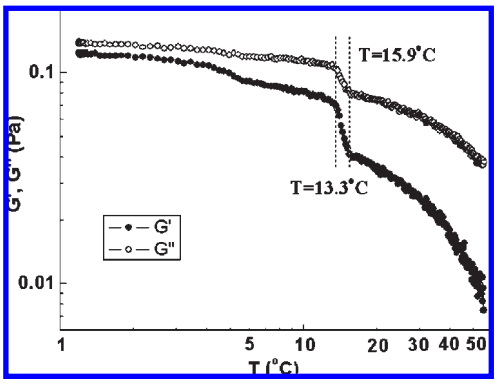

Figure 7. Storage modulus G' (solid symbols) and loss modulus G'' (open symbols) dependence of temperature in aqueous solutions.

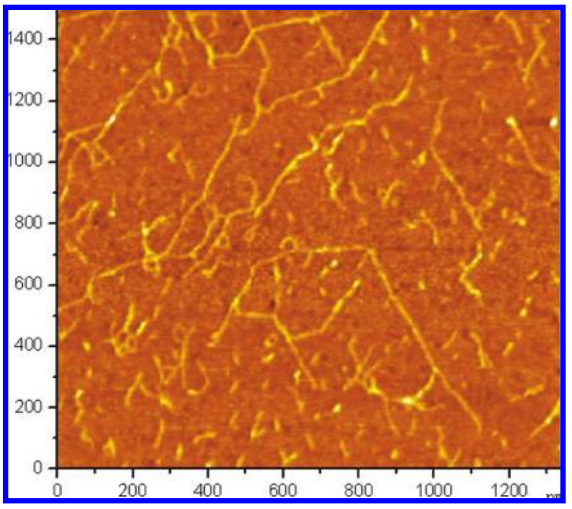

Figure 8. AFM topographic image of the PD3 deposited on mica as a 1 μg/mL solution in pure water.

PD3 first hardly changed and then decreased sharply at temperature from 13.3 to 15.9 °C with increasing temperature, which was ascribed to the formation of triple helical structures.

On the basis of the analysis of results from viscometry, light scattering, and rheology, it can be concluded that PD3 exists as a triple helical conformation in water.

Morphology of PD3 in Aqueous Solution. Figure 8 shows an AFM image of the PD3 sample in pure water. The AFM topographic image of sample PD3 reveals almost rodlike structures. The measured mean thickness of the PD3 is 1.2 ± 0.3 nm calculated by computer averaged over hundreds of molecules. The chain thickness of PD3 is consistent with that of the native triple helix chain of Lentinan, Scleroglucan, and Schizophyllan (~1.0 nm) (*39*, *40*), giving further evidence of PD3 existing as triple helical chains in dilute solution.

Figure 9 showed the TEM picture PD3. Clearly, the triple helical polysaccharide chains self-assembled to rigid fibrous network. It is indicated that PD3 trends to aggregates at relative higher concentration and forms fiber supermolecular structure. Nature routinely chooses the fiber as its structural material of choice. Examples include cellulose, chitin, collagen, keratin, and silk. Hydrogen bond, hydrophobic interaction, and electrostatic interaction are the source of power to maitain the supermolecular structure. In our case, hydrogen bonds are the only possible strength to maintain the fibrous network.

In summary, a novel triple helical polysaccahride, PD3, was isolated and purified from the fruiting body of *Dictyophora indusiata*.

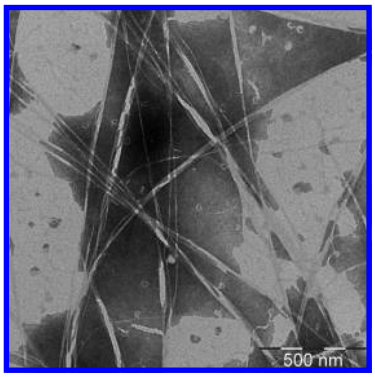

Figure 9. TEM topographic image of the PD3 deposited on mica as a 10 μg/mL solution in pure water.

PD3 has a very similar structure with Glucan TM8 and Grifolan A. PD3 formed a very viscous solution at low concentration and exhibited similar conformation and conformation transition behavior with triple helical glucans, i.e., Lentinan and Scleroglucan in aqueous solution, suggesting its potential application in the food industry and medicine. It is interesting to note that TEM results showed PD3 self-assembled to a fibrous network structure. To exploit its application in the food industry, more detailed studies on the rheological properties and aggregation and self-assembly behavior are needed.

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