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Flexible chain conformation of (1 → 3)-β-D-glucan from *Poria cocos* sclerotium in NaOH/urea aqueous solution

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ABSTRACT

A water-insoluble polysaccharide (PCS3-II) extracted from sclerotium of *Poria cocos* was identified as a linear (1 → 3)-β-D-glucan by ^{13}C NMR and gas chromatography. Aqueous 0.5 M NaOH/0.2 M urea was a good solvent for PCS3-II and the dependence of intrinsic viscosity ($[\eta]$) on weight-average molecular weight (M_w) was established in the M_w range from 7.68×10^4 to 5.14×10^5 to be $[\eta] = 3.39 \times 10^{-2} M_w^{0.62}$ cm^{3}g^{-1} at 25 °C by using laser light scattering and viscometry. The chain conformation parameters of PCS3-II in the 0.5 M NaOH/0.2 M urea solution was 2.3 (± 0.3) nm for persistence length (q), 580 g mol^{-1} nm^{-1} for molar mass per unit contour length (M_L), 0.8 (± 0.2) nm for the diameter of the chain (d) and 3.63 for limited characteristic ratio (C_∞). The results revealed, for the first time, that PCS3-II existed as a flexible chain in 0.5 M NaOH/0.2 M urea aqueous solution.

1. Introduction

Polysaccharides have attracted much attention because of the increasing awareness of their biological activities and special solution properties (Chayed & Winnik, 2007; Fusco, Michon, Tai, & Blake, 1997; Hellerqvist, Lloyd, Wang, & Bardhan, 1996; Jeon, Katsuraya, Kaneko, Mimura, & Uryu, 1997; Koji et al., 2007; Liu & Zhang, 2007; Liu et al., 2007; Lu, Wang, Hu, Huang, & Wang, 2008; Nahm, Olander, & Magyarlaki, 1997; Saskia & Fred, 2007; Wang, Xu, & Chen, 2007; Wong, Wong, Chiu, & Cheung, 2007; Zaharoff, Rogers, Hance, Schlom, & Greiner, 2007). *Poria cocos* has been used as a traditional medicine in China and Japan for a long time. It is promising as a diuretic and anti-aging drug (Jin et al., 2003; Kanayama, Adachi, & Togami, 1983; Narui, Takakashi, Kobayashi, & Shibata, 1980; Yamada et al., 1992). *P. cocos* sclerotium grows on the roots of pine trees, and it contains many bioactive substances. Fractions extracted from *P. cocos* by 50% hot ethanol can effectively augment the secretion of interleukins IL-1 beta and IL-6 (Yu & Tseng, 1996). Pachymic acid (PA) extracted from *P. cocos* can significantly reduce cell proliferation (Gapter, Wang, Glinski, & Ng, 2005). Polysaccharides extracted from *P. cocos* can antiproliferate and antidifferentiate human leukemic cells (Chen & Cheng, 2004) and induce NF-kappaB/Rel activation and iNOS expression (Lee et al., 2004). Interestingly, a water-soluble heteropolysaccharide extracted from *P. cocos* having relatively high molecular weight has exhibited high inhibition ratio to tumor growth *in vivo* (Zhang, Chen, Xu, Zeng, & Cheung, 2005). The sulfated and carboxymethylated derivatives of polysaccharide extracted from *P. cocos* possess significant antitumor activities against S-180 and gastric carcinoma tumor cells (Wang, Zhang, Li, Hou, & Zeng, 2004).

The molecular weight and chain conformation of the polysaccharides are related to the antitumor bioactivities. It has been reported that triple-helical conformation is important to the immunomodulating properties and antitumor bioactivity of the (1 → 3)-β-D-glucan scleroglucan and schizophyllan (Falch, Espevik, Ryan, & Stokke, 2000; Kojima, Tabata, Itoh, & Yanaki, 1986). Lentinan having single flexible chains exhibited far lower *in vivo* antitumor activities than the triple-helix Lentinan samples (Zhang, Li, Xu, & Zeng, 2005). However, a single-helix conformation is a stimulant to host-mediated antitumor activity (Saito et al., 1991).

(1 → 3)-β-D-glucan extracted from *P. cocos* by 0.5 M NaOH is water-insoluble and forms aggregates in aqueous NaOH solution, leading to difficulty in studying the application of the Mark–Houwink equation and determining the molecular conformation (Ding, Jiang, Zhang, & Wu, 1998). Recently, a linear (1 → 3)-β-D-glucan PCS3-II has been isolated from *P. cocos*, and it is also water-insoluble but the molecular parameters are hitherto unknown (Wang et al., 2004). To examine the solution properties of PCS3-II in aqueous system, it is necessary to first develop an aqueous solution to dissolve PCS3-II. A new solvent, NaOH/urea aqueous solution, has been developed, which can break strong intermolecular and intra-

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molecular hydrogen bonds of cellulose, leading to the good dissolution of cellulose (Cai & Zhang, 2006; Cai et al., 2007; Zhou, Zhang, & Cai, 2004). Therefore this solution was seen as a route to finding of a solvent system for PCS3-II to study its chain conformation and molecular size.

2. Experimental

2.1. Preparation of sample

The sclerotium of the fungus *P. cocos* was cultivated in Luotian (Hubei, China) and its sclerotium dried (Fig. 1). The sclerotium powder was defatted sequentially by using Soxhlet extraction with ethyl acetate and acetone for 6 h. The resultant residues were immersed in 0.15 M NaCl at 25 °C and stirred overnight. Then the mixture was centrifuged and the residues were immersed in distilled water at 120 °C for 40 min and then centrifuged to yield the residues again, which were further extracted with 0.5 M NaOH solution for 4 h at 25 °C and then centrifuged. The supernatant was neutralized with 50% acetic acid solution, and then precipitate was appeared. The mixture was centrifuged to yield a precipitate, coded as PCS3-II.

PCS3-II was dissolved in dry dimethyl sulfoxide (DMSO) and then fractionated according to a non-solvent addition method. A mixture of acetone and DMSO (4:1) as non-solvent was slowly added to PCS3-II solution at 25 °C until the solution turned turbid. The turbid solution resulted in the precipitate of polysaccharide. The turbid liquid was heated to 50 °C to become transparent again to eliminate the low molecular mass species of the precipitation (Young, 1981). After being cooled to 25 °C and allowed to stand for 12 h at 25 °C, the solution turned turbid again. The turbid solution was centrifuged for separation of liquid (supernatant) and gel phases. The gel was collected as fraction 1, and the supernatant was further fractionated. A mixture of acetone and DMSO (4:1) was slowly added to the supernatant at 25 °C until the solution turned turbid, and the turbid liquid was heated to 50 °C to become transparent again. After being cooled to 25 °C and allowed to stand for 12 h at 25 °C, the solution turned turbid again. The turbid solution was centrifuged again for separation of liquid and gel phases. The gel was collected as fraction 2, and the supernatant was subjected in next step to obtain other fractions. This step was repeated until no further gel phase appeared. In this way, the PCS3-II sample was divided into 9 fractions. The 9 fractions were code as F1 for the first fraction to F9 for the last fraction as well as F2–F8 in order of molecular weight. To further purify, 9 fractions were dissolved in DMSO and precipitated by the addition of acetone and then the precipitates were washed with anhydrous acetone three times, and finally vacuum-dried to yield white powder.

Fig. 1. The photogram of sclerotium of *Poria cocos*.

2.2. Structure characterization

Infrared spectra (IR) of the PCS3-II samples were recorded with a Nicolet 170SX FT-IR spectrometer (Spectrum One, Perkin Elmer Co., USA) using the KBr-disk method. Acetylated derivatives of PCS3-II were made according to the literature (Tezuka, Imai, Cshima, & Chiba, 1987). Gas chromatography (GC) of the acetylated monosaccharide derivates of the polysaccharide was performed with an HP-5 capillary column (30 m × 0.32 mm) programmed from 180 to 220 °C at 4 °C/min and held at 220 °C for 30 min. The injection sample volume was l μL, the carrier gas was high-purity nitrogen, and detection was made by flame ionization. ^{13}C NMR measurement of PCS3-II was analyzed on a Mercury 600 NMR spectrometer (Varian Inc., USA) at 20 °C. PCS3-II was dissolved, respectively, in DMSO–d_6 and 0.5 M NaOH/0.2 M urea/D_2O to obtain polysaccharide solution with a concentration of 5×10^{-2} g cm^{-3}.

2.3. Viscosity measurement

Intrinsic viscosities ($[\eta]$) of the PCS3-II fractions in 0.5 M NaOH/0.2 M urea and DMSO, respectively, were measured at 25.0 ± 0.1 °C by using an Ubbelohde capillary viscometer. The kinetic energy correction was assumed to be negligible. Huggins and Kraemer equations were used to estimate the $[\eta]$ value by extrapolating to an infinite dilute concentration formulated as:

$$\eta_{sp}/c = [\eta] + k'[\eta]^2 c \tag{1}$$

$$(\ln \eta_r)/c = [\eta] - \beta[\eta]^2 c \tag{2}$$

Both k' and β are constants for a given polymer at a given temperature in a given solvent; and the value of η_{sp}/c represents a reduced specific viscosity; $(\ln \eta_r)/c$, inherent viscosity. The measurement of the viscosity for the PCS3-II in 0.5 M NaOH/0.2 M urea aqueous solution on the storage time was carried out immediately once the sample was dissolved in the solvent.

2.4. Laser light scattering

The weight-average molecular weight (M_w) and radius of gyration ($<S^2>^{1/2}$) of the PCS3-II sample and its fractions in 0.5 M NaOH/0.2 M urea and in DMSO were measured, respectively, with a multiple angle laser light scattering instrument equipped with an He-Ne laser (MALLS, λ = 633 nm; DAWN®DSP, Wyatt Technology Co., Santa Barbara, CA, USA) at 25 °C. The basic light scattering equation is as follows:

$$\frac{Kc}{R_\theta} = \frac{1}{M_w}\left[1 + \frac{16\pi^2 n^2 \langle S^2\rangle_z}{3\lambda^2} \cdot \sin^2\left(\frac{\theta}{2}\right)\right] + 2A_2 c + \cdots \tag{3}$$

Where K is an optical constant, $K = [4\pi^2 n^2(dn/dc)^2]/(\lambda^4 N_A)$; c is the polymer concentration in g cm^{-3}, R_θ is the Rayleigh ratio; λ is the wavelength; n is the refractive index of the solvent; dn/dc is the refractive index increment; N_A is the Avogadro number; A_2 is the second virial coefficient. The polysaccharide solution with desired concentrations was prepared, and optical clarification of the solution was achieved by filtration through a 0.2 μm pore size filter (PTFE, Puradisc 13-mm Syringe Filters, Whatman, England) into a scattering cell. The refractive index increment (*dn/dc*) was measured with a double-beam differential refractometer (DRM-1020, Otsuka Electronics Co. Osaka, Japan) at a wavelength of 633 nm. The *dn/dc* values of PCS3-II in 0.5 M NaOH/0.2 M urea and DMSO, respectively, were 0.119 cm^3 g^{-1} and 0.060 cm^3 g^{-1}. The scattering cells used were K5 type for samples in DMSO and standard vials in 0.5 M NaOH/0.2 M urea, respectively. Astra software v4.90.07 was utilized for data acquisition and analysis.

3. Results and discussion

3.1. Chemical Structure

The IR spectrum (not shown) of the PCS3-II exhibits an absorption peak at 890 cm^{-1}, which is characteristic of the β-configuration of glucan (Kiho, Sakushima, Wang, Nagai, & Ukai, 1991). The GC chromatogram of PCS3-II has showed only one peak, corresponding to acetylated glucose, suggesting that PCS3-II is a homopolysaccharide. In the ^{13}C NMR spectra of the PCS3-II polysaccharide in DMSO-d$_6$ (Fig. 2b) the major signals are assigned to 103.7 ppm for C1, 86.9 ppm for C3, 77.0 ppm for C5, 73.5 ppm for C2, 69.1 ppm for C4, and 61.6 ppm for C6. These peaks positions are almost the same as those of carbons for linear (1 → 3)-β-D-glucans (Satio et al., 1987). Moreover, there is no peak in the ^{13}C NMR corresponding to other polysaccharides or branching, indicating existence of only a linear glucan. In view of the IR, GC, NMR results, PCS3-II is proved to be a linear (1 → 3)-β-D-glucan. The ^{13}C chemical shifts of PCS3-II in 0.5 M NaOH/0.2 M urea solution (Fig. 2a) are almost the same as those of PCS3-II in DMSO. A new peak (162.8 ppm) appeared in the ^{13}C NMR spectrum of PCS3-II in the NaOH/urea system compared to the peaks in Fig. 2b, which was assigned to the chemical shift of the carbonyl carbon for urea (Carbon-13 NMR. Sadtler Research Laboratories, 1979). This suggests the absence of any derivatives of PCS3-II. Therefore, the 0.5 M NaOH/0.2 M urea aqueous solution is a good solvent of the polysaccharide. Thus, the PCS3-II solution in this system could be used to study the solution properties and molecular parameters of the polysaccharide.

3.2. Stability of polysaccharide solution

In the time dependence of the inherent viscosity ($(ln\,\eta_r)/c$) for PCS3-II in 0.5 M NaOH/0.2 M urea (Fig. 3), the ($(ln\,\eta_r)/c$) values decreased slowly. Leveling off of the viscosity values was only reached at a storage time of greater than 50 h. However, after 3 h, ($(ln\,\eta_r)/c$) was decreased only 0.45%. It is, therefore, believed that the 0.5 M NaOH/0.2 M urea aqueous solution of the polysaccharide was stable in the period of the measurement.

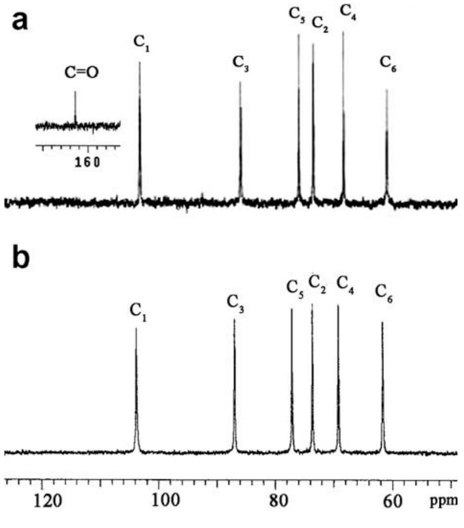

Fig. 2. Liquid ^{13}C NMR spectrum of water-insoluble polysaccharide PCS3-II in 0.5 M NaOH/0.2 M urea/D$_2$O solution (a) and PCS3-II in DMSO (b).

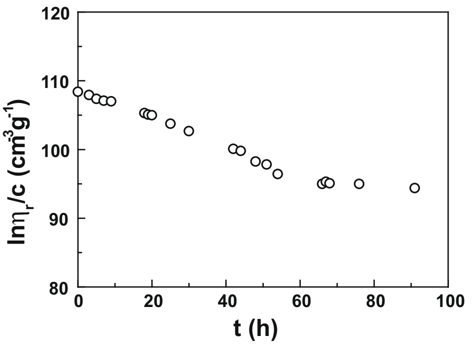

Fig. 3. Time dependence of the inherent viscosity for water-insoluble polysaccharide PCS3-II in 0.5 M NaOH/0.2 M urea solution at 25 °C.

3.3. Mark–Houwink equation

From the Zimm plots of PCS3-II in DMSO (Fig. 4a) and in 0.5 M NaOH/0.2 M urea aqueous solution at 25 °C (Fig. 4b) the M_w, A_2 and $<S^2>^{1/2}$ values of the PCS3-II fractions were obtained (Table 1). It is noted that there is not much difference between the M_w values of PCS3-II in 0.5 M NaOH/0.2 M urea and that in DMSO, indicating that PCS3-II exists as individual chains in the two solvents. It can be believed that there are no aggregates of the PCS3-II glucan in the 0.5 M NaOH/0.2 M urea aqueous solution. The A_2 value of PCS3-II in 0.5 M NaOH/0.2 M urea is the order of 10^{-4} mol g^{-2} cm^3, further confirming it is a good solvent for PCS3-II.

Dependence of $[\eta]$ on M_w for PCS3-II fractions in 0.5 M NaOH/0.2 M urea and DMSO is plotted in Fig. 5. Mark–Houwink equation in the M_w range from 7.68×10^4 to 5.14×10^5 in 0.5 M NaOH/0.2 M urea aqueous solution was established as:

$$[\eta] = 3.39 \times 10^{-2} M_w^{0.62} \quad (\mathrm{cm^3\ g^{-1}}) \tag{4}$$

The exponent value α is related to the shape of the macromolecules and the nature of the solvent. It is well known that the α value of a flexible polymer in good solvent lies in the range from 0.6 to 0.8 (David, Picout, & Ross, 2002; Peng, Zhang, Zhang, Xu, & Kennedy, 2005; Tao, Zhang, Yan, & Wu, 2007). The α value for PCS3-II in the new solvent is 0.62, suggesting a flexible chain. For instance, pullulan, a polysaccharide produced by a fungus, *Aureobasidium pullulans,* is a linear (1 → 6)-β-D-glucan which exists as a flexible chain in aqueous solution with an α value of 0.67 in water (Kato, Okamoto, Tokuya, & Takahashi, 1982). Moreover, the Mark–Houwink equation for PCS3-II in DMSO in the M_w range from 6.73×10^4 to 4.10×10^5 was established as:

$$[\eta] = 1.38 \times 10^{-1} M_w^{0.54} \quad (\mathrm{cm^3\ g^{-1}}) \tag{5}$$

The exponent value is 0.54, indicating that the PCS3-II molecules also exist as flexible chains in DMSO, close to that in 0.5 M NaOH/0.2 M urea aqueous solution.

3.4. Conformation parameters

The unperturbed chain dimension ($\langle R^2\rangle_0/M_w$) can be calculated using Stockmayer–Fixman equation (Stockmayer & Fixman, 1963).

$$[\eta]/M_w^{1/2} = K_\theta + C'BM_w^{1/2} \tag{6}$$

$$K_\theta = \phi_0\left(\frac{\langle R^2\rangle_0}{M_w}\right)^{3/2} \tag{7}$$

Where $\phi_0 = 2.87 \times 10^{23}$ mol^{-1}. By plotting $[\eta]/M_w{}^{1/2}$ against $M_w{}^{1/2}$ as shown in Fig. 6, K_θ was evaluated as 0.11 cm^3 g^{-1}. So, ($<R^2>_0/M_w$) was calculated as 5.27×10^{-3} nm^2. The limited characteristic ratio (C_∞) can represent how much the chain is extended by steric

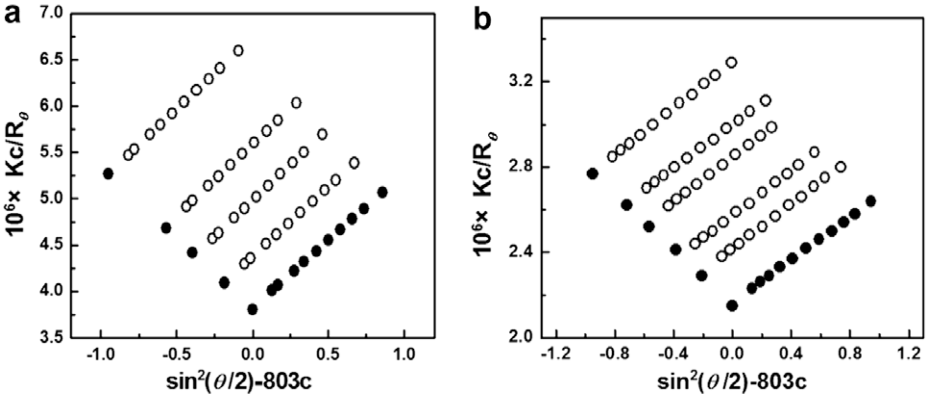

Fig. 4. Zimm plot of water-insoluble polysaccharide PCS3-II in DMSO(a) and PCS3-II in 0.5 M NaOH/0.2 M urea aqueous solution(b) at 25 °C.

Table 1
Experimental results of M_w, A_2, $[\eta]$ of the water-insoluble polysaccharide PCS3-II fractions in 0.5 M NaOH/0.2 M urea aqueous solution and in DMSO at 25 °C

Fraction	In NaOH/urea			In DMSO		
	$M_w \times 10^{-5}$ (g mol^{-1})	$[\eta]$ (cm^3 g^{-1})	$A_2 \times 10^4$ mol g^{-2} cm^3	$M_w \times 10^{-5}$ (g mol^{-1})	$[\eta]$ (cm^3 g^{-1})	$A_2 \times 10^4$ mol g^{-2} cm^3
F1	5.14	134.0	3.14	–	–	–
F2	4.50	124.0	3.40	4.10	165.0	5.77
F3	4.32	105.0	3.86	3.90	146.5	3.11
F4	3.81	100.0	3.46	3.69	138.9	2.49
F5	3.68	90.6	3.56	3.10	137.1	5.88
F6	3.34	76.7	3.25	–	–	–
F7	1.71	72.2	2.61	1.90	91.9	1.56
F8	1.10	46.9	1.73	–	–	–
F9	0.77	35.7	3.34	0.67	59.4	13.50

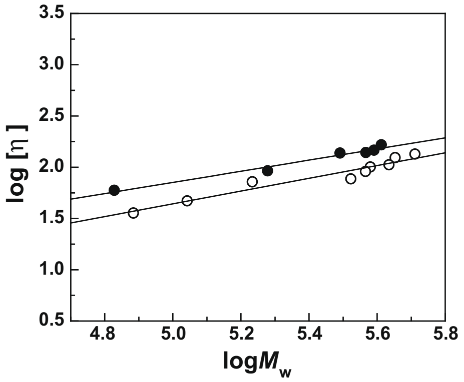

Fig. 5. M_w dependence of $[\eta]$ for the water-insoluble polysaccharide PCS3-II fractions (F1–F9) in 0.5 M NaOH/0.2 M urea aqueous solution (○) and PCS3-II fractions (F2–F9) in DMSO (●).

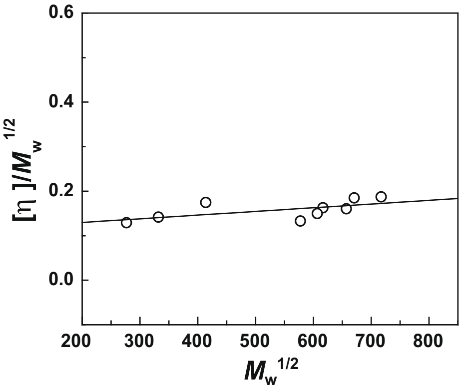

Fig. 6. Stockmayer-Fixman plot for water-insoluble polysaccharide PCS3-II fractions (F1–F9) in 0.5 M NaOH/0.2 M urea aqueous solution at 25 °C.

hindrance compared with the freely jointed chain. C_∞ can be defined as (Kato et al., 1982)

$$C_\infty = \lim_{n\to\infty}\frac{\langle R^2\rangle_0}{nl^2} \cong \left(\frac{\langle R^2\rangle_0}{M_w}\right)\left(\frac{M_0}{l^2}\right) \quad (8)$$

Where M_0 is the average molar mass of anhydroglucose in the repeating unit and l is the bond length of the anhydro glucose unit in the glucan. With the l value of 0.485 nm for glucopyranose (Nakata, Kawaguchi, Kodama, & Konno, 1998), and $M_0 = 162$, C_∞ of the PCS3-II in 0.5 M NaOH/0.2 M urea aqueous solution has been calculated as 3.63. The small C_∞ value reflects that the chain of PCS3-II exists as a flexible random coil in 0.5 M NaOH/0.2 M urea. PCS3-II has exhibited more flexible chain in the aqueous solution than pullulan having a value of 4.3 of C_∞ (Kato et al., 1982) .

Based on the analysis mentioned above, a worm-like touched-bead model can be used for characterization of the chain conformation of PCS3-II (Yamakawa, 1997). The value of $[\eta]$ can be calculated according to the following equation:

$$\begin{aligned}[\eta]_{KP}^{YNY} &= 6^{3/2}\phi_\infty\frac{\langle S^2\rangle_{KP^{3/2}}}{M}\Big[1+e^{-5L/2q}\sum_{i=0}^{3}C_i(L/2q)^{i/2} \\ &\quad + e^{-q/2L}\sum_{i=0}^{7}C_i(L/2q)^{-(i-3)/2}\Big]^{-1}+\frac{5}{12}\pi N_A\frac{Nd_b^3}{M_0}C_i \\ &= \sum_{j=0}^{2}a_{ij}d_b^j+\sum_{j=0}^{1}\beta_{ij}d_b^{2j}\ln d_b \end{aligned} \quad (9)$$

Where L is contour length, q is the persistence length and M_L is the molar mass per unit contour length. L equals the ratio of M (the molecular weight) to M_L (the molar mass per unit contour length).ϕ_∞ is the Flory viscosity factor for unperturbed random coils with values of 2.87×10^{23}; α_{ij}, β_{ij} are constants which are listed in the literature (Yamakawa, 1997). The value of d_b is the diameter of the bead in a touched-bead model. M_0 is the average molar of anhydroglucose repeating unit. A trial-and-error method was used to search for a set of q, L and chain diameter (d_b), which leads to the closest agreement between the values of M_w and $[\eta]$. Fig. 7 shows comparison of $[\eta]$ for PCS3-II with the theoretical data. The solid curve represents the theoretical values computed with the parameters of PCS3-II, $q = 2.3(\pm 0.3)$ nm, $M_L = 580$ ($\pm$ 40) g mol^{-1} nm^{-1}, and $d_b = 0.8$ ($\pm$ 0.2) nm, indicating a flexible chain conformation.

For further evidence of the flexible chain conformation of PCS3-II, another models such as BSF, Hearst, Bohdanecky (Bohdanecky, 1983; Picout, Ross-Murphy, Errington, & Harding, 2001) were used to calculate q to be 0.88, 1.55 and 2.40 nm, respectively. All these values indicate a flexible chain conformation, similar to q value calculated from worm-like touched-bead model. The calculated results also prove that PCS3-II exists as flexible chain conformation. The difference of q was maybe connected with the different modes. The Hearst and Bohdanecky models are commonly used for stiff chain, and worm-like touched-bead model is used for flexible chain.

On the basis of the molecular weight and size of the PCS3-II, a schematic model describing the chain conformation is presented in Fig. 8. The molecular weight and size have been obtained by laser light scattering. ($M_w = 3.34 \times 10^5$, $\langle S^2 \rangle^{1/2} = 30$ nm). The straight chain length of the polysaccharide is calculated as $L = (M_w/M_0)\, l = 1000$ nm, and the bead in the chain denotes the chain segment of macromolecules. Thus, the sketch map visually shows the chain shape of PCS3-II molecule in 0.5 M NaOH/0.2 M urea aqueous solution.

4. Conclusions

A 0.5 M NaOH/0.2 M urea aqueous solution was demonstrated to be a good solvent for the water-insoluble PCS3-II glucan. PCS3-II is relatively stable in this solvent. The molecular chain conformation of PCS3-II in the NaOH/urea system was successfully studied by viscometry and light scattering. The Mark–Houwink equation for PCS3-II in the M_w range from 7.68×10^4 to 5.14×10^5 was established to be $[\eta] = 3.39 \times 10^{-2} M_w^{0.62}$ for PCS3-II in 0.5 M NaOH/0.2 M urea aqueous solution at 25 °C. The conformation parameters such as persistence length (q), molar mass per unit contour length (M_L), and limited characteristic ratio (C_∞) of PCS3-II in the dilute solution were 2.3 ($\pm$ 0.3) nm, 580 g mol^{-1} nm^{-1}, and 3.63, respectively, The results indicated that PCS3-II existed as a flexible chain in 0.5 M NaOH/0.2 M urea aqueous solution.

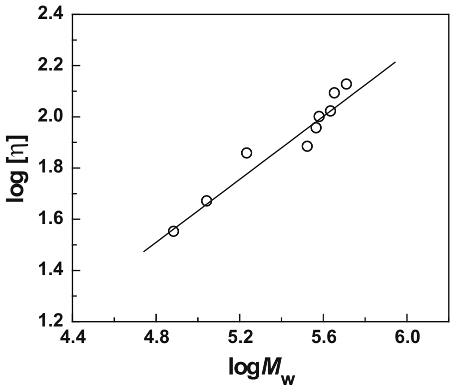

Fig. 7. M_w dependence of $[\eta]$ for water-insoluble polysaccharide PCS3-II fractions (F1–F9) in 0.5 M NaOH/0.2 M urea aqueous solution at 25 °C (○), compared with the theoretical curve for the touched-bead chain (—) with $\lambda^{-1} = 4.5$ nm, $M_L = 580$ nm^{-1}, and $d = 0.8$ nm.

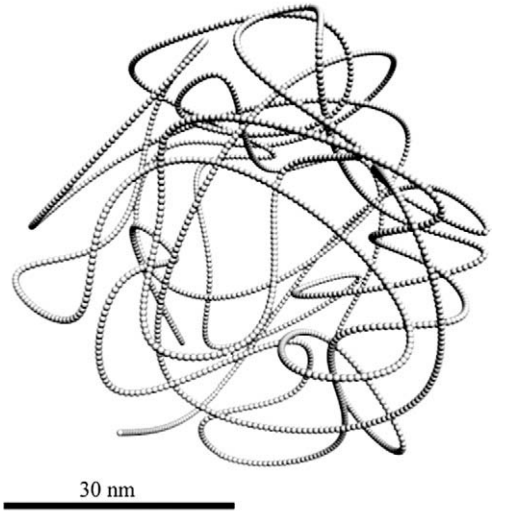

Fig. 8. Touch-bead model sketch map of water-insoluble polysaccharide fraction F6 in 0.5 M NaOH/0.2 M urea aqueous solution.

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