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ScienceDirect
Carbohydrate Polymers 65 (2006) 504–509
Carbohydrate Polymers
www.elsevier.com/locate/carbpol

Chain conformation of carboxymethylated derivatives of (1 → 3)-β-D-glucan from *Poria cocos* sclerotium

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Received 7 October 2005; received in revised form 29 January 2006; accepted 8 February 2006
Available online 31 March 2006

Abstract

A water-insoluble (1 → 3)-β-D-glucan (PCSG) isolated from the fresh sclerotium of *Poria cocos* was carboxymethylated to afford a water-soluble derivative coded as C-PCSG. The carboxymethylated (1 → 3)-β-D-glucan was fractionated to obtain eight fractions according to the nonsolvent addition method. The weight-average molecular mass (M_w), radius of gyration ($\langle s^2\rangle_z^{1/2}$) and intrinsic viscosity ([η]) of the fractions were determined by size-exclusion chromatography combined with laser light scattering (SEC-LLS) and viscometry in 0.2 M NaCl aqueous solution at 25 °C. The dependences of [η] and $\langle s^2\rangle_z^{1/2}$ on M_w for C-PCSG were found to be $[\eta] = 1.49 \times 10^{-2} M_w^{0.75}$(mL g^{-1}), and $\langle s^2\rangle_z^{1/2} = 3.65 \times 10^{-2} M_w^{0.56}$ (nm), respectively. Analysis of M_w and [η] in terms of the known theories for wormlike chain model yielded 633 nm^{-1} for molar mass per unit contour length (M_L), 5.5 nm for persistence length (q), and 20.2 for characteristic ratio (C_∞). These results indicated that C-PCSG exists as a relatively extended flexible chain in 0.2 M NaCl aqueous solution. Therefore, the introduction of the carboxymethyl groups into the β-glucan improved significantly the water solubility and enhanced the stiffness of the chains.

Keywords: (1 → 3)-β-D-Glucan; Carboxymethylated derivative; *Poria cocos* sclerotium; Solution properties; Chain conformation

1. Introduction

Recently, the fungal polysaccharides as a functional food and a source for the development of biomedical drugs have attracted much attention. It has been found that fungal polysaccharides exert their bioactivities mostly via activation of the host immune system rather than a direct cytotoxic action (Wasser, 2002). The chain conformation of fungal polysaccharides is already assumed to be an important contributing factor to their bioactivities. For instance, a triple-helical conformation of scleroglucan (1 → 3)-β-D-glucan is known to be important for their cytokine stimulating activity (Falch, Espevik, Ryan, & Stokke, 2000). It has also been confirmed that the (1 → 3)-β-D-glucan of schizophyllan with triple-helix conformation shows distinct antitumor activity (Kojima, Tabata, Itoh, & Yanaki, 1986). In contrast, Saito et al. (1991) have suggested that single-helix conformation is a stimulant to the host-mediated antitumor activity. In order to improve biological activities, some researches have been directed towards the modified polysaccharides (Ohno, Kurachi, & Yadomae, 1988; Yang & Du, 2003). Introduction of suitable ionic groups with appropriate degrees of substitution should not only enhance the water solubility of the polysaccharides, but also change the conformation of the polymer chain in solution (Zhang, Zhang, Chen, & Zeng, 2000). Therefore, a basic understanding of the conformations of the polysaccharides and their derivatives is essential for investigating structure–function relationships, especially the molecular mechanisms of the interactions with the target cells.

Poria cocos is one of the most important traditional medicines in China and other Asian countries, and has many culinary and medical uses such as anti-inflammatory, antitumor, complement activating, and immune stimulating activities (Kanayama, Adachi, & Togami, 1983; Lee

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0144-8617/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carbpol.2006.02.014

& Jeon, 2003; Yasukawa et al., 1998; Yu & Tseng, 1996). Chihara, Hamuro, Maeda, Arai, and Fukuoka (1970) have indicated that the main component of sclerotium of *P. cocos* is a water-insoluble (1 → 3)-β-D-glucan, which hardly exhibits antitumor activity, but its carboxymethylated derivative has strongly inhibited against the growth of sarcoma 180 transplanted in mice (Hamuro, Yamashita, Ohsaka, Maeda, & Chihara, 1971). It has been illustrated that carboxylmethyl pachymaran has much better inductive effect to the production of tumor necrosis factor (TNF), interleukin-6 (IL-6) and interferon-γ (INF-γ) than conventional agent (Chen, 1998). In our previous work, carboxymethylated (1 → 3)-β-D-glucan from *P. cocos* sclerotium showed significant antitumor activity both in vivo and in vitro (Wang, Zhang, Li, Hou, & Zeng, 2004). Unfortunately, the solution properties of carboxymethylated (1 → 3)-β-D-glucan of *P. cocos* sclerotium have not been yet clarified. A basic understanding of molecular conformation of the polysaccharide is essential for the further insight on the correlation of structure to bioactivities and the application in the medicine fields. In this work, fractions of the carboxymethylated (1 → 3)-β-D-glucan having different molecular masses were prepared according to the nonsolvent addition method. Based on current theories for worm-like chain model, the molecular size and conformation of carboxymethylated (1 → 3)-β-D-glucan were studied by size-exclusion chromatography combined with laser light scattering (SEC-LLS) and viscometry.

2. Experimental

2.1. Materials

A water-insoluble (1 → 3)-β-D-glucan, termed as PCSG, extracted with 0.5 M NaOH aqueous solution from sclerotium of *P. cocos* was used. All the chemical reagents used were of analytical grade, and were obtained from commercial resources in China.

2.2. Preparation of carboxymethylated (1 → 3)-β-D-glucan

Six hundred milligram of PCSG was suspended in a solution of 10 mL of 20% NaOH and 25 mL isopropanol in an ice bath with stirring for 3 h. Then, a solution of 5.25 g chloroacetic acid, 10 mL of 20% NaOH and 25 mL isopropanol was slowly added with stirring. The reaction was continued at room temperature for 3 h and then at 60 °C for 1.5 h. After the solution was cooled to the room temperature, 0.5 M HCl was added to adjust pH to 7 and then dialyzed by a regenerated cellulose tube (M_w cut-off 8000) against tap water for 7 days and distilled water for 4 days, respectively. The resulting solution was concentrated by rotary evaporator at reduced pressure below 40 °C. Finally, the carboxymethylated (1 → 3)-β-D-glucan was lyophilized by using a lyophilizer (CHRIST Alpha1-2, Germany) to obtain a white powder, coded as C-PCSG.

2.3. Fractionation

C-PCSG was fractionated according to the nonsolvent addition method. Acetone as precipitant was slowly added to a 1% solution of C-PCSG in 0.2 M NaCl at 25 °C until the solution became turbid. The turbid solution was then heated to 50 °C and kept for 2 h, and its turbidity was slightly weakened. After cooled to 25 °C and kept for 12 h, the solution was centrifuged (8000 rpm, 20 min) to separate into liquid and gel phases. The gel was removed and the supernatant was subjected to the next fractionation. In this way, C-PCSG was divided into 12 fractions. Eight parts of these fractions that had sufficient quantity for molecular mass determination were chosen as tested sample, and coded as C-1, C-2, ..., and C-8. The fractions were reprecipitated individually from 0.2 M NaCl using acetone, washed with anhydrous acetone six times, and finally vacuum-dried for 7 days to obtain white powders.

2.4. Viscometry

Intrinsic viscosities ([η]) of the fractions in 0.2 M NaCl aqueous solution were measured at 25 °C using an Ubbelohde capillary viscometer. The kinetic energy correction was negligible. Huggins and Kraemer equations were used to estimate the [η] value by extrapolating concentration (c) to be zero as follows:

$$\eta_{sp}/c = [\eta] + k'[\eta]^2 c, \quad (1)$$

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

where k' and k'' are constants 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.

2.5. SEC-LLS measurements

Size-exclusion chromatography combined with laser light scattering (SEC-LLS) measurements were performed on a multi-angle laser photometer ($\lambda = 633$ nm; DAWN®DSP, Wyatt Technology Co., Santa Barbara, CA, USA) combined with a P100 pump (Thermo Separation Products, San Jose, USA) equipped with TSK-GEL G5000 and G3000 PWXL columns (7.8 mm × 300 mm) in 0.2 M NaCl aqueous solution at 25 °C. A differential refractive index detector (RI-150) was simultaneously connected. 0.2 M NaCl aqueous solution was used as the eluent with a flow rate of 1.0 mL min^{-1}. All solutions, having a sample concentration of 2.0–3.0 × 10^{-3} g mL^{-1}, were filtered by a 0.20 μm pore-size filter (Whatman, England), and then kept in sealed glass bottles before injection onto the SEC column. The refractive index increments (dn/dc) were determined by using an Optilab refractometer (DAWN®DSP, Wyatt Technology Co., Santa Barbara, CA, USA) at 633 nm and 25 °C. The dn/dc value of samples in 0.2 M NaCl aqueous solution was determined to

be 0.140 mL g^{-1}. Astra software (Version 4.70.07) was utilized for the data acquisition and analysis.

3. Results and discussion

3.1. Mark-Houwink equation

The chemical structure of carboxymethylated (1 → 3)-β-D-glucan has been shown in our previous work (Wang, Zhang, Ruan, 2004), indicating that carboxymethylated substitution of PCSG was nonselective and occurred mainly at C-6 position and secondly at C-4 and C-2 position of the (1 → 3)-β-D-glucan. The degree of substitution (DS) of carboxymethylated glucan determined by NMR spectra was 1.27. The native (1 → 3)-β-D-glucan is water insoluble, and has a tendency to aggregate in aqueous solution. However, the carboxymethylated (1 → 3)-β-D-glucan and its fractions can dissolve well in water as a result of introduction of suitable ionic group.

SEC-LLS has been applied conveniently for determination of the molecular mass, molecular mass distribution and chain size of polymers without the aid of standard samples. In this research, SEC-LLS as an absolute method is applicable for the characterization of the fractions in the M_w range tested. SEC-LLS chromatograms for the fractions in 0.2 M NaCl aqueous solution are shown in Fig. 1. "LS, AUX (volts)" represents an arbitrary unit of scattering intensity. Therefore, the radius of gyration ($\langle s^2\rangle_z^{1/2}$) and weight-average molecular mass (M_w) were determined with Zimm fit from the light scattering signals. The values of M_w, $\langle s^2\rangle_z^{1/2}$, polydispersity index ($M_w/M_n$), and [η] of the C-PCSG fractions are summarized in Table 1. The M_w of unfractionated sample was determined to be 25.1×10^4, and M_w of the fractions range from 6.1 to 45.4×10^4. The molecular mass distributions of the fractions are narrower than unfractionated sample, indicating that the fractionation of the C-PCSG sample in 0.2 M NaCl aqueous solution is successful. Therefore, these fractions can be used for investigation of their solution properties.

Table 1
Experimental results from viscosity and SEC-LLS for the C-PCSG fractions in 0.2 M NaCl aqueous solution at 25 °C

Sample	[η] ($cm^3\ g^{-1}$)	SEC-LLS		
		$M_w \times 10^{-4}$ (g mol^{-1})	$\langle s^2\rangle^{1/2}$ (nm)	M_w/M_n
C-PCSG[a]	167.1	25.1	45.0	1.7
C-1	263.1	45.4	50.2	1.4
C-2	251.2	41.7	48.8	1.3
C-3	240.9	38.8	48.6	1.3
C-4	229.9	36.1	46.8	1.4
C-5	199.5	27.4	41.2	1.3
C-6	153.8	18.2	31.2	1.4
C-7	124.3	16.1	29.6	1.4
C-8	57.5	6.1	[b]	1.3

[a] Previous results quoted for comparison (Wang, Zhang, Li, et al., 2004).
[b] Not determined.

Fig. 2 shows the double-logarithmic plot of [η] against M_w for the fractions in 0.2 M NaCl aqueous solution at 25 °C. The Mark-Houwink equation for the carboxymethylated (1 → 3)-β-D-glucan with M_w ranging from 6.1 to 45.4×10^4 (the solid line in Fig. 2) is represented by

$$[\eta] = 1.49 \times 10^{-2} M_w^{0.75}\ (\text{mL g}^{-1}). \quad (3)$$

The exponent (α) value is related to the shape of the macromolecule and nature of the solvent. The α value of polymers, including linear and branched, ranges widely from 0.5 to 0.8. For flexible linear polymers in a good solvent, the α value is usually in the range from 0.7 to 0.8. Therefore, the α value of 0.75 for the carboxymethylated (1 → 3)-β-D-glucan in 0.2 M NaCl aqueous solution lies in the range of flexible polymer chains. Some published

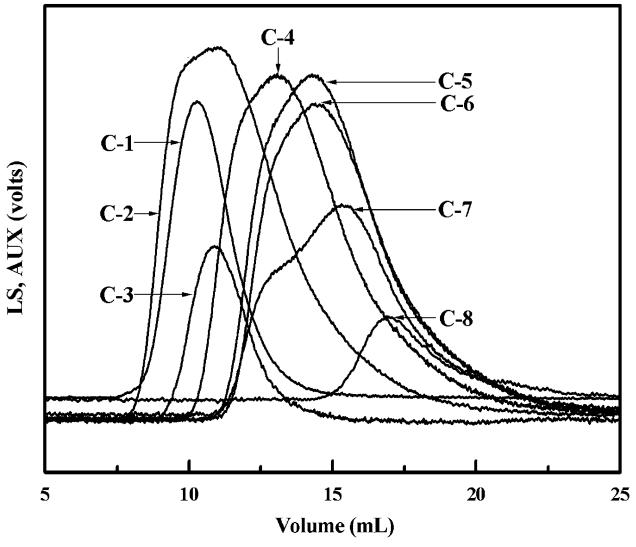

Fig. 1. Size-exclusion chromatograms of the C-PCSG fractions from C-1 to C-8 determined by multi-angle laser light scattering photometry in 0.2 M NaCl aqueous solution at 25 °C.

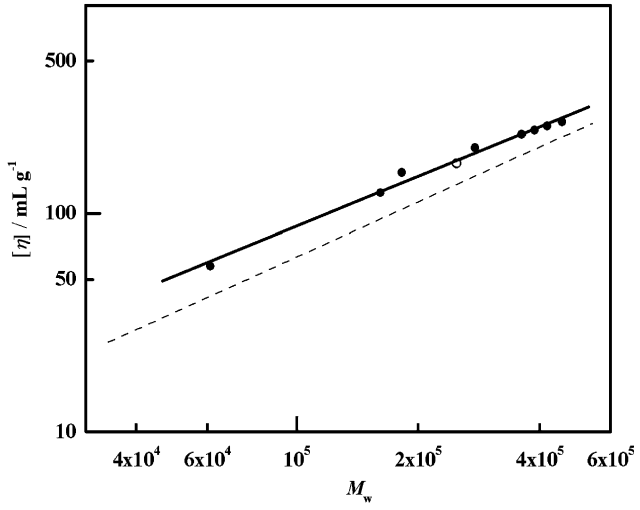

Fig. 2. The double-logarithmic plot of [η] against M_w for the C-PCSG (●) fractions in 0.2 M NaCl aqueous solution at 25 °C (the solid line) and the unfractionated sample C-PCSG (○), in comparison with the carboxymethylated glucan from sclerotia of *Pleurotus tuber-regium* in PBS aqueous solution at 37 °C (the dash line; Zhang et al., 2003).

data of M_w and $[\eta]$ for the carboxymethylated glucan from sclerotia of *Pleurotus tuber-regium* in PBS aqueous solution at 37 °C (the dash line in Fig. 2) (Zhang, Zhang, & Cheung, 2003) is included for comparison. The slope of the line for the C-PCSG fractions was similar to that of carboxymethylated glucan from sclerotia of *P. tuber-regium* ($\alpha = 0.78$), suggesting that the two kinds of carboxymethylated glucans exist as relatively extended flexible chains in aqueous solution.

3.2. Chain conformation

The solution properties of polysaccharides can be investigated according to the theory of polymer solution. On the basis of the data of M_w, $\langle s^2\rangle_z^{1/2}$ and $[\eta]$ for the fractions, a wormlike cylinder model can be used for conformational characterization of polysaccharides. Bushin and Bohdanecky have independently indicated that the Yamakawa–Fujii–Yoshizaki (Y–F–Y) theory (Yamakawa & Fujii, 1974; Yamakawa & Yoshizaki, 1980) for $[\eta]$ of an unperturbed wormlike cylinder may be expressed in a good approximation by:

$$(M^2/[\eta])^{1/3} = A_\eta + B_\eta M^{1/2}, \tag{4}$$

$$A_\eta = \phi_{0,\infty}^{-1/3} A_0 M_L \ (\mathrm{g}^{1/3}\,\mathrm{cm}^{-1}), \tag{5}$$

$$B_\eta = \phi_{0,\infty}^{-1/3} B_0 (2q/M_L)^{-1/2} \ (\mathrm{g}^{1/3}\,\mathrm{cm}^{-1}), \tag{6}$$

where M is molecular mass; q and M_L are the persistence length and the molar mass per unit contour length, respectively. The value of A_0 and B_0 had been tabulated in Bohdanecky's paper (Bohdanecky, 1983), and $\phi_{0,\infty}$ was found to be 2.87×10^{23}. The plot of $(M_w^2/[\eta])^{1/3}$ against $M_w^{1/2}$ for the C-PCSG fractions is shown in Fig. 3. Substituting the intercept and slope of this plot into Eqs.(4)–(6) yielded 633 nm^{-1} for M_L and 5.5 nm for q, indicating a flexible chain characteristic of C-PCSG. The double-logarithmic plot of $\langle s^2\rangle_z^{1/2}$ against M_w for the C-PCSG fractions is shown in Fig. 4. For the C-PCSG fractions with M_w ranging from 16.1 to 45.4×10^4, the plot can be represented by the following equation:

$$\langle s^2\rangle_z^{1/2} = 3.65 \times 10^{-2} M_w^{0.56} \ (\mathrm{nm}), \tag{7}$$

The value of the exponent 0.56 is in the range of a normal flexible polymer (0.5–0.6).

Based on Kratky–Porod wormlike chain model (Kratky & Porad, 1949), an equation, which should be suitable for semi-flexible polymers, may be represented by

$$(M_w/\langle s^2\rangle_z)^{1/2} = (3M_L/q)^{1/2} + 3M_L(3qM_L)^{1/2}/2M_w. \tag{8}$$

Fig. 5 shows the plot of $(M_w/\langle s^2\rangle_z)^{1/2}$ against $1/M_w$ for the C-PCSG fractions in 0.2 M NaCl aqueous solution at 25 °C. From the plot, the values of M_L and q have been

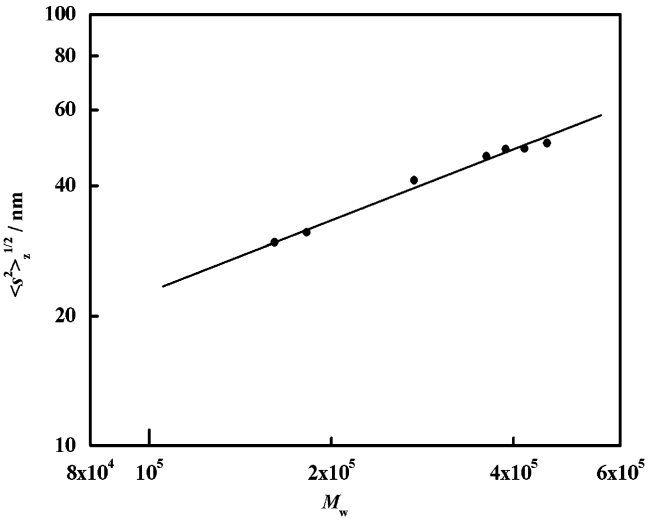

Fig. 4. The double-logarithmic plots of $\langle s^2\rangle_z^{1/2}$ against M_w for the C-PCSG fractions in 0.2 M NaCl aqueous solution at 25 °C.

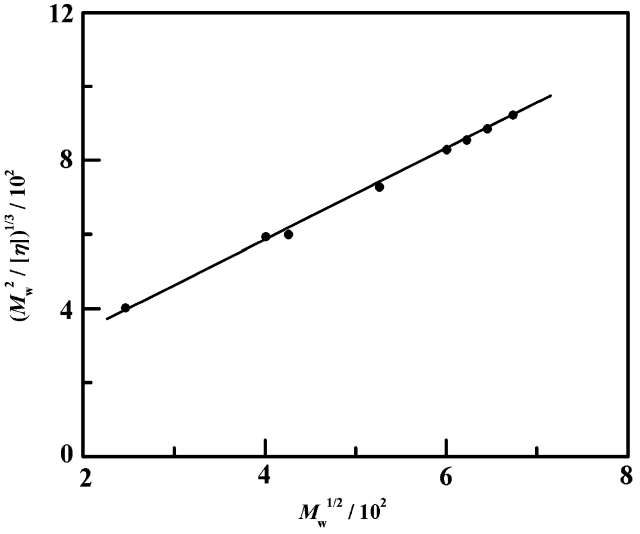

Fig. 3. Plot of $(M_w^2/[\eta])^{1/3}$ vs. $M_w^{1/2}$ for the C-PCSG fractions in 0.2 M NaCl aqueous solution at 25 °C.

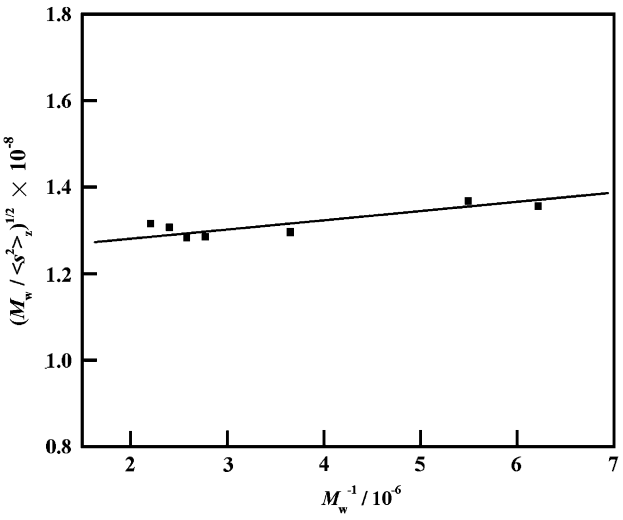

Fig. 5. Plot of $(M_w/\langle s^2\rangle_z)^{1/2}$ vs. M_w^{-1} for the C-PCSG fractions in 0.2 M NaCl aqueous solution at 25 °C.

estimated roughly to be 614 nm^{-1} and 9.4 nm, respectively. The M_L value is consistent with the calculated value of 663 nm^{-1} obtained from $[\eta]$. In addition, the difference between the q values obtained from $[\eta]$ and that from $\langle s^2\rangle_z^{1/2}$ may be related to the scatter of the points obtained from $\langle s^2\rangle_z^{1/2}$. The characteristic ratio (C_∞) represents how much the chain is extended by steric hindrance. The C_∞ is defined as the following (Zoberi, 1973):

$$C_\infty = M_0/(\lambda M_L l^2), \quad (9)$$

where M_0 is the average molar mass of a glucose residue repeating unit, λ^{-1} is the Kuhn's segment length ($\lambda^{-1} = 2q$), and l is the virtual bond length which equals to the distance between two successive glycosidic oxygens O(3) and O(3′) in the present case. If we assumed that the value of l for C-PCSG may be 0.443 nm, similar to that of (1 → 3)-β-D-glucan (Zhang et al., 2001), together with $M_0 = 240$ (the effects of substitution group on the average molar mass of a glucose residue repeating unit to be considered), $\lambda^{-1} = 11.0$ nm and $M_L = 663\ nm^{-1}$, C_∞ of C-PCSG in 0.2 M NaCl aqueous solution was calculated to be 20.2. Usually, the C_∞ of polymers is affected by bond angle and steric hindrance. In this case, relatively large C_∞ of C-PCSG is possibly attributable to induction of ionic groups that increase the steric hindrance of backbone and strengthen the effect of electrostatic repulsion. Therefore, the resulting parameters ($q = 5.5$ nm, $M_L = 663\ nm^{-1}$, and $C_\infty = 20.2$) indicate that C-PCSG exists as a relatively extended flexible chain in the 0.2 M NaCl aqueous solution. It has been demonstrated that the molecules of (1 → 3)-β-D-glucan (PC3) from *P. cocos* sclerotium exist as a random coil in dimethyl sulfoxide (Zhang et al., 1999). Therefore, The C-PCSG sample possesses more stiff chain than its native glucan.

It is well known that fungal polysaccharides in aqueous solution exhibit different chain conformation such as random coil, single helix, double helix, triple helix, and aggregate, even if they are (1 → 3)-β-D-glucan. Interestingly, the most common antitumor fungal polysaccharides, such as lentinan and schizophyllan, have a triple-helix conformation with stiff chains. In our laboratory, it has been confirmed that the relatively extended chain conformation of carboxymethylated β-glucan from sclerotia of *P. tuber-regium* (Zhang et al., 2003), and extracellular polysaccharides from *Ganoderma tsugae* mycelium (Peng, Zhang, Zeng, & Xu, 2003) are beneficial to the antitumor activity. The information of molecular parameters and the extended chain conformation in aqueous solution of the carboxymethylated (1 → 3)-β-D-glucan from the *P. cocos* sclerotium, for the first time have been reported in this research. Our previous work has confirmed that antitumor activities of the carboxymethylated (1 → 3)-β-D-glucan are significantly higher than its native one. Therefore, the important of antitumor activity is related to the enhancement of the molecular chain stiffness and water-solubility.

4. Conclusions

Water insoluble (1 → 3)-β-D-glucan isolated from the sclerotium of *P. cocos* was carboxymethylated to afford a water-soluble derivative. The carboxymethylated (1 → 3)-β-D-glucan in aqueous solution was fractionated successfully by using acetone as precipitant according to a nonsolvent addition method. The Mark-Houwink equation for the C-PCSG fractions in the range of M_w from 6.1 to 45.4×10^4 in 0.2 M NaCl aqueous solution at 25 °C was established to be $[\eta] = 1.49 \times 10^{-2} M_w^{0.75}$ (mL g^{-1}). The dependence of radius of gyration ($\langle s^2\rangle_z^{1/2}$) on M_w was found to be $\langle s^2\rangle_z^{1/2} = 3.65 \times 10^{-2} M_w^{0.56}$ (nm) in the M_w range tested. The conformational parameters of the carboxymethylated (1 → 3)-β-D-glucan were 633 nm^{-1} for M_L, 5.5 nm for q and 20.2 for C_∞, indicating a relatively extended flexible chain in the aqueous solution. The introduction of the carboxymethyl groups into the β-glucan improved significantly the water solubility and enhanced the stiffness of the chains.

Acknowledgments

This work was supported by major grant of the National Natural Science Foundation of China (30350850) (20074025), and the Area of Excellence on Plant and Fungal Biotechnology Project of the Hong Kong SAR Government.

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