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Investigation of molecular masses and aggregation of β-D-glucan from *Poria cocos* sclerotium by size-exclusion chromatography

Lina Zhang[a,*], Qiong Ding[a], Diya Meng[b], Liwei Ren[b], Guang Yang[a], Yonggang Liu[a]

[a]*Department of Chemistry, Wuhan University, Wuhan 430072, China*

[b]*Agricultural Testing Center of Hubei Academy of Agriculture Science, Wuhan 430064, China*

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Abstract

β-D-Glucan PC3 (weight-average molecular mass=$9.15 \cdot 10^4$, polydispersity=1.6), a linear glucan from *Poria cocos* sclerotium, was fractionated by a preparative size-exclusion chromatography (SEC) column packed with regenerated cellulose gels using dimethyl sulfoxide (DMSO) as mobile phase. The molecular masses and aggregation of the fractions were investigated by using analytical SEC, viscometry and membrane osmometry under different conditions. The results of SEC analysis proved that glucan PC3 forms partial aggregates in 0.25 *M* LiCl/DMSO (namely, 0.25 *M* LiCl in DMSO) at 40°C, showing a new shoulder peak of a higher-molecular-mass component in the chromatogram, and dissolves as a single-stranded chain at 80°C, giving a sharper peak. The mean apparent aggregation number (N_{ap}) of the fractions in 0.25 *M* LiCl/DMSO at 40°C was 1.9, and much smaller than that of glucan PC3 in 20% cadoxen. It is indicated that the analytical SEC is more sensitive and direct in detection of the aggregates than other methods, and aggregation can be avoided by carrying out SEC measurement at 80°C. The Mark-Houwink equation for glucan PC3 in DMSO at 25°C was established as $[\eta]=0.107M_w^{0.50}$ in the M_w range of $7–11 \cdot 10^4$, suggesting that it behaves as a flexible coil.

Keywords: *Poria cocos*; Carbohydrates; Preparative chromatography; Glucan

1. Introduction

In the carbohydrate field, size-exclusion chromatography (SEC), a rapid method for separation and characterization, has been widely used in the fractionation and molecular mass distribution analysis of polysaccharides of industrial or biochemical importance [1]. It is worth noting that the calculation of molecular mass and its distribution in polymers from SEC data has been greatly facilitated by the availability of computer software. Functional properties of the polysaccharides in many applications are large due to their ability to control the rheology of aqueous media, thus they have wide applications as thickening and gelling agents in the food and oil industries [2]. Understanding of solution properties of the polysaccharides is important for these application. However, the polysaccharides have generally a tendency to aggregate in solution because of the abundance of interchain hydrogen bonds, thereby con-

*Corresponding author. Tel.: +86-27-87682322/3016; fax: +86-27-8788-2661.

E-mail address: lnzhang@elm.pdexpress.com (L. Zhang)

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siderably complicating their fractionation and analysis of their molecular mass. Moreover, the aggregation behavior of the polysaccharide in solution leads to uncertainty in molecular masses. It is essential that a sensitive technique in detecting aggregates and effective method in avoiding aggregation should be sought. The use of SEC may be advantageous [3,4].

Poria cocos has been long been used as traditional Chinese herb with diuretic and sedative activities, and polysaccharides from it have an antitumor effect [6]. In the previous papers [5,7], we proved that β-D-glucan (PC3) from a sclerotium of *Poria cocos*, a linear β-(1→3)-D-glucan, forms aggregates in 20% cadoxen (a saturated aqueous solution of CdO in $H_2NCH_2CH_2NH_2$ complex [5]) or 0.5 *M* NaOH aqueous solution, and dissociated to single chains in cadoxen or in dimethyl sulfoxide (DMSO). Because of the aggregation of the glucan PC3 in aqueous solution, no satisfactory fractions were obtained by addition of precipitant. In our laboratory, a preparative SEC column packed with regenerated cellulose gel particles has recently been successfully used for the fractionation of a dextran in water [8]. It was indicated that preparative SEC is simple, fast and suitable for large-scale fractionation of various polysaccharides in aqueous solution or organic solvent. In addition, we have demonstrated that DMSO with lithium chloride has better capability of dissolving varied polysaccharides than DMSO, but more easily absorbs moisture, resulting in aggregation of glucan PC3. The purpose of this work was to resolve the complication from aggregation of the glucan in 0.25 *M* LiCl/DMSO, to develop a fractionation method for the glucan PC3 in DMSO by preparative SEC and to study their weight-average molecular mass M_w, number-average molecular mass M_n and polydispersity index $d(M_w/M_n)$. From the experimental results, the Mark-Houwink equation was established, and chain flexibility was discussed.

2. Experimental

2.1. Preparation of fractions

The previously investigated sample of β-(1→3)-D-glucan from a fresh sclerotium of *Poria cocos*, PC3 with weight-average molecular mass of $8.93\cdot10^4$ in cadoxen [5], was used in this work. The glucan PC3 was fractionated by using the preparative SEC column (550 mm·20 mm) packed with regenerated cellulose gel particles. The exclusion limit and fractionation range of the stationary phase were molecular masses $7\cdot10^5$ and $3\cdot10^3$–$7\cdot10^5$, respectively [8]. The glucan PC3 was dissolved in DMSO to prepare 0.05 g ml^{-1} concentration. For each run 5 ml glucan solution was injected into the column, and DMSO was used as eluent at 25°C. The flow-rate was adjusted to 1.0 ml min^{-1} by using a peristaltic pump during the run. The column effluent and fractions were monitored by using a UV–Vis spectrophotometer (UV-160, Shimadzu, Japan) at 350 nm according to turbidimetry with DMSO–acetone (2:1, v/v). Fig. 1 shows the elution pattern of the preparative SEC for glucan PC3. The slicing indicates that 10 fractions collected from 10 injections were got to be 0.15–0.25 g of each of the fractions (PC3-1–PC3-10). The fractions PC3-2, PC3-4, PC3-6, PC3-8 and PC3-10 were used in this work.

2.2. Analytical SEC

A HPLC instrument (Waters) equipped with TSK GMH6 column (300 mm×7.5 mm) packed with cross-linked polystyene–divinylbenzene particles of 8–10 μm, 717 plus autosampler, 600 pump, 410 differential refractometer and 2010 Millennium Workstation was used for analytical SEC experiments. The pullulan standards (P-10, P-50, P-100, P-400 and P-800) were used for the calibration curve, and two samples of glucan ($14.4\cdot10^4$ and $56.3\cdot10^4$ in M_w, $9.6\cdot10^4$ and $37.1\cdot10^4$ in M_n), which were a gift from National Research Center for Certified Reference Materials in Beijing, were used to determine *G* factors for band broadening correction. The eluent was 0.25 *M* LiCl/DMSO (namely, 0.25 *M* LiCl in DMSO) and the flow-rate was 1.0 ml min^{-1} at 40 and 80°C. The calibration curves obtained from pullulan standards were shown in Fig. 2 and represented as follows:

$$\text{Log } M = 11.96 - 0.886\, V_e \text{ (at 40°C)} \quad (1)$$

$$\text{Log } M = 11.94 - 0.888\, V_e \text{ (at 80°C)} \quad (2)$$

Where V_e is elution volume. Pullulan is a linear

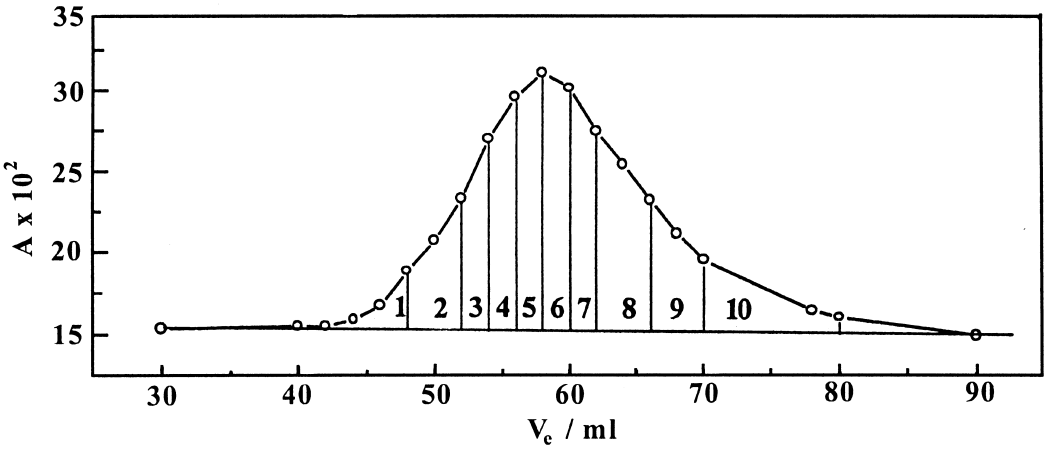

Fig. 1. Elution pattern and fractionation of glucan PC3 in DMSO at 25°C by using the preparative SEC (550 mm×20 mm) with flow-rate of 1ml min^{-1}

glucan [9] similar to glucan PC3, so that the SEC universal calibration was not used here. The viscosity of DMSO is very high, thus the column with large pore size gels was chosen here to reduce flow resistance. Therefore, the analytical SEC underwent a band broadening correction. The factor G of the band broadening correction was calculated by determining the SEC values of M_w and M_n for two samples of glucan with known molecular masses, namely true values, from the following relation:

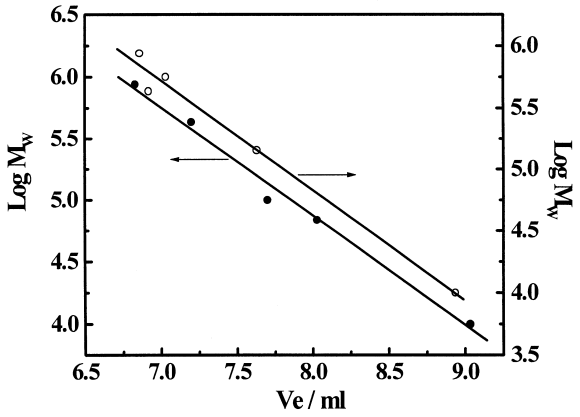

Fig. 2. Calibration curves for pullulan standards in 0.25 M LiCl/DMSO at 40°C (●) and 80°C (○) by using HPLC instrument equipped with TSK GMH6 column (300 mm×7.5 mm) with differential refractometer at a flow-rate of 1.0 ml min^{-1}.

$$G = \left[\frac{(M_w/M_n)_{SEC}}{(M_w/M_n)_{true}} \right]^{1/2} \quad (3)$$

The G values were found to be 1.54 at 40°C and 1.31 at 80°C, respectively. The average molecular masses M_w and M_n were calculated as follows:

$$M_w = M_{w,\,SEC}/G \quad (4)$$

$$M_n = M_{n,\,SEC}\,G \quad (5)$$

The GPC software was used in data treatment.

The five fractions and unfractionated glucan PC3 were dissolved in 0.25 M LiCl/DMSO to prepare 0.005–0.01 g ml^{-1} concentration, and stored in the refrigerator for 3 days before measurement. The injected volume was 50 μl.

2.3. Viscometry

Viscosity of the fractions and unfractionated glucan PC3 in DMSO were measured at 25±0.1°C by using a modified capillary viscometer, which was a gift from the Institute of Industrial Science in Tokyo University. The kinetic energy correction was always negligible. Huggins and Kraemer plots were used to estimate the intrinsic viscosity $[\eta]$ and the Huggins constant k'.

2.4. Osmometry

Osmotic pressure (π) of the fractions of glucan PC3 in 0.25 *M* LiCl/DMSO were measured with an improved Bruss membrane osmometer equipped with a regenerated cellulose semi-permeable membrane having a pore size of 8 nm prepared in our laboratory. Dynamic osmometry was employed to determine osmotic pressure [10]. Number-average molecular mass M_n and second viral coefficient A_2 were evaluated from five solution concentration c using the relation:

$$(\pi/c)^{1/2} = (RT/M_n)^{1/2}(1 + 0.5A_2M_nc) \quad (6)$$

3. Results and discussion

Figs. 3 and 4 show SEC chromatograms of the fractions PC3-4 and PC3-6 with 0.25 *M* LiCl/DMSO as mobile phase at 40°C and 80°C. When the elution was performed at 80°C, the samples all gave a sharper peak than at 40°C. A new shoulder, corresponding to a component of higher molecular mass, appears in the chromatograms of each fraction at 40°C. Its appearance implies that aggregation of glucan PC3 occurs in 0.25 *M* LiCl/DMSO at 40°C. The values of M_w of each fraction obtained by SEC analysis at 40 and 80°C are summarized in Table 1. The M_w values obtained at 40°C were obviously higher than those at 80°C, where the aggregates break up to form single chains. It can be proved that the M_w value of glucan PC3 at 80° was close to that as a single chain in cadoxen and in DMSO, as determined by light scattering [5,7]. The apparent aggregation number (N_{ap}) can be deduced from the mass of the aggregates, namely $N_{ap} = M_w$(at 40°C)/M_w(at 80°). The mean N_{ap} value of 1.9 (Table 1) for the fractions in 0.25M LiCl/DMSO at 40° were much smaller than that in 20% cadoxen ($N_{ap} = 4–6$) [5], indicating appearances of partial aggregation. Usually, when a few aggregates exist in polymer solution, the sensitivity in detecting aggregates by light scattering and osmometry is low. It is considered that the aggregates were more readily, directly and sensitively detected by SEC than by other methods, and that SEC at 80° was effective in avoiding aggregation of the glucan PC3.

Fig. 5 illustrates concentration dependence of π/c for the five fractions in 0.25 *M* LiCl/DMSO at 30°C. The values of M_n and A_2 are summarized in Table 1. The M_n results obtained by membrane osmometry are generally in agreement with the values given by SEC at 40°C. However, when the molecular mass was lowest, the M_n obtained by SEC at 40°C was markedly smaller than that by membrane osmometry

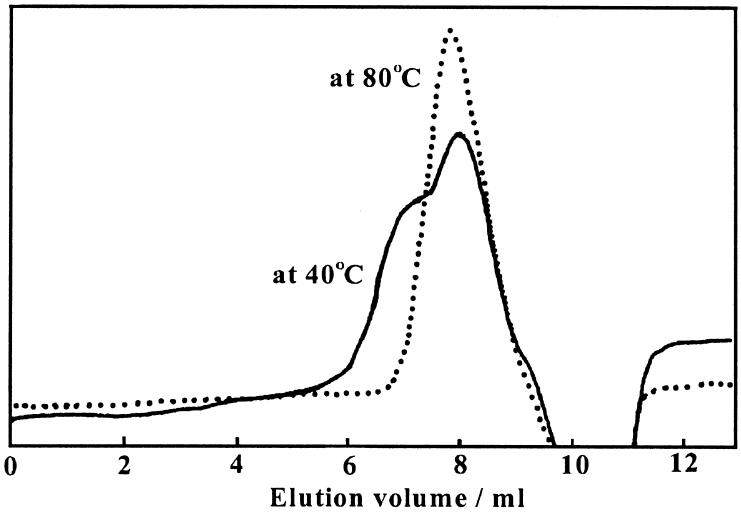

Fig. 3. SEC chromatogram of the fraction PC3-4 on TSK GMH6 column with 0.25 *M* LiCl/DMSO as mobile phase at 40°C (—) and 80°C (. . . . .)

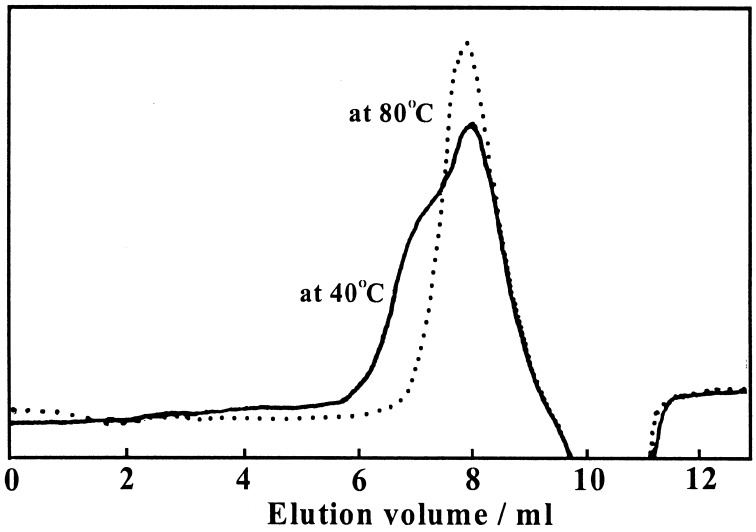

Fig. 4. SEC chromatogram of the fraction PC3-6 on TSK GMH6 column with 0.25 *M* LiCl/DMSO as mobile phase at 40°C (—) and 80°C (. . . . .)

at 30°C. It can be explained that under different conditions the aggregation of low size macromolecules in the solution takes place more easily and is broken than high size macromolecules [11], so that an enhancement in aggregation of PC3-10 with changing temperature from 40 to 30°C was higher than that of other fractions. The values of M_n for PC3-4 at 30°C was higher than that of PC3-2, as were the values of M_w and M_n from SEC, owing to more aggregation, which exhibited a more pronounced shoulder peak of high molecular mass components in the SEC chromatogram of PC3-4 at 40°C (Fig. 3). In addition, the values of A_2 are very positive, suggesting that 0.25 *M* LiCl/DMSO is a good solvent for glucan PC3 at 30°C.

It was demonstrated that glucan PC3 dissociates to

Table 1
Experimental results of M_w, M_n, *d* and [η] for the fractions of glucan PC3 in 0.25 *M* LiCl/DMSO by SEC, viscometry and membrane osmometry

Fraction	Temperature	SEC			$\frac{M_{w,40°C}}{M_{w,80°C}}$	Osmometry[a]		Viscometry[b]	
	(°C)	$M_w\times10^{-4}$	$M_n\times10^{-4}$	d		$M_n\times10^{-4}$	$A_2\times10^4$ (mol ml g^{-2}	[η] (ml g^{-1})	k'
PC3-2	40	19.5	10.0	2.0	1.8	10.2	5.40	35.3	0.46
PC3-4	40	20.6	10.3	2.0	2.1	10.7	4.76	33.4	0.48
PC3-6	40	17.1	8.51	2.0	1.9	8.89	5.95	32.1	0.36
PC3-8	40	14.8	7.22	2.0	1.9	8.16	6.49	30.7	0.38
PC3-10	40	8.63	4.03	2.1	1.2	5.12	13.9	28.9	0.47
PC3-2	80	10.9	6.81	1.6					
PC3-4	80	9.73	6.29	1.5					
PC3-6	80	8.98	5.99	1.5					
PC3-8	80	7.97	5.33	1.5					
PC3-10	80	7.50	3.86	1.9					
PC3	80	9.15	5.63	1.6					

[a] At 30°C.
[b] In DMSO at 25°C.

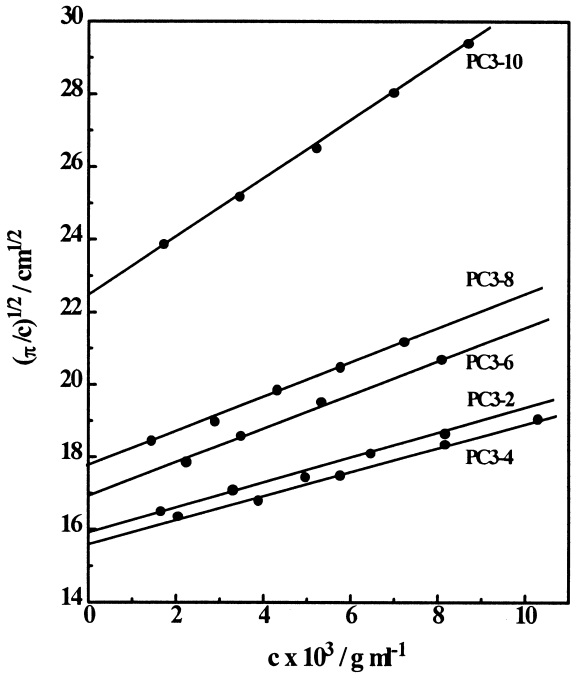

Fig. 5. Concentration dependence of π/c for the fractions in 0.25 *M* LiCl/DMSO at 30°C

single chains in DMSO [7]. Hence, the fractionation in DMSO by preparative SEC with DMSO as mobile phase was effective in avoiding aggregation of glucan PC3. The values of M_w and M_n for the fractions in 0.25 *M* LiCl/DMSO at 80°C decreased with the progress of fractionation, and *d* values (Table 1) were lower (1.6 of unfractionated glucan PC3 and 1.5–1.6 for most of the fractions) except for PC3-10,which was collected from broad slicing. It is well known that it is very difficult to obtained narrow-distribution polysaccharide fractions by the nonsolvent addition method. The present work has indicated that fractions with different molecular masses can be obtained from narrow-distribution glucan PC3 by preparative SEC. Thus, it is possible that the solution properties of the glucan PC3 can be investigated by examination of such fractions.

The results of $[\eta]$ for five fractions in DMSO are listed in Table 1. The double-logarithmic plot of $[\eta]$ against M_w (from SEC at 80°C) for the fractions of glucan PC3 is shown in Fig. 6. The Mark-Houwink equation was established as follows:

$$[\eta] = 0.107M_w^{0.50} \quad (7)$$

The exponent (α) is 0.50, suggesting that the molecules of glucan PC3 exist as random coils in DMSO. Interestingly, the Mark-Houwink equation is almost the same as that of pullulan in the lower-molecular mass range ($1–9\times10^4$) in water at 25°C: $[\eta]=(0.133\pm0.005)M^{0.5\pm0.1}$, which has led to the conclusion that the pullulan chain has significant flexibility and behaves as a flexible coil in water [9].

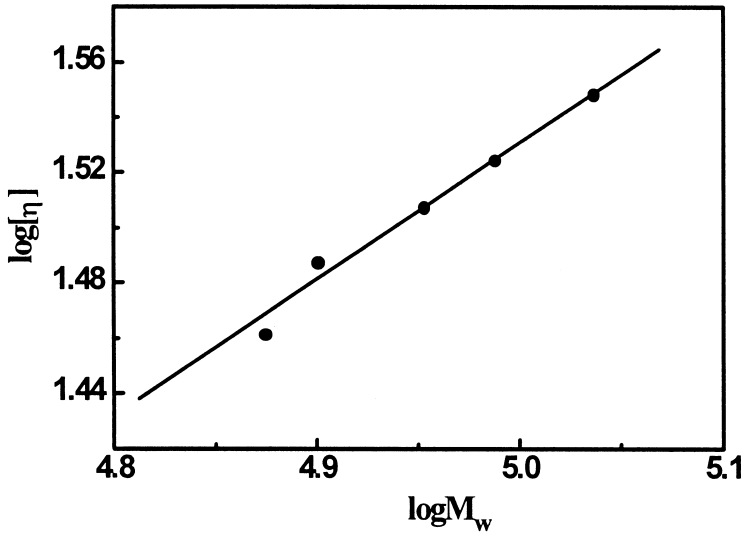

Fig. 6. Double-logarithmic plot of $[\eta]$ versus M_w for the fractions of glucan PC3 in DMSO at 25°C

4. Conclusion

The narrow-distribution glucan PC3 was fractionated by the preparative SEC with DMSO as mobile phase, avoiding aggregation. The results of SEC analysis proved that glucan PC3 forms partial aggregates in 0.25 *M* LiCl/DMSO at 40°C and dissolves as a single-stranded chain at 80°C. The analytical SEC method used is more sensitive and direct in detection of the aggregates than other methods, and the effects of aggregation are avoided by carrying out the measurement at 80°C. The Mark-Houwink equation in M_w range of $7–11\times10^4$ for glucan PC3 in DMSO at 25°C was established as $[\eta]=0.107M_w^{0.50}$. The data analysis of M_w and $[\eta]$ suggests that the molecules of glucan PC3 in DMSO exist as a random coil.

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