Carbohydrate Research 343 (2008) 2251–2257

Contents lists available at ScienceDirect

Carbohydrate Research

journal homepage: http://www.elsevier.com/locate/carres

Characterization of polysaccharide–protein complexes by size-exclusion chromatography combined with three detectors

Yongzhen Tao [a,b], Lina Zhang [a,*]

[a] Department of Chemistry, Wuhan University, Wuhan 430072, China
[b] Key Lab of Green Processing and Fictionalization of New Textile Materials, Ministry of Education, Wuhan University of Science and Engineering, Wuhan 430073, China

ARTICLE INFO

Article history:
Received 6 November 2007
Received in revised form 21 April 2008
Accepted 21 April 2008
Available online 27 April 2008

Keywords:
Polysaccharide–protein complex
Size-exclusion chromatography
Chain conformation

ABSTRACT

Two water-soluble samples (TM1 and TM2) were extracted from *Pleurotus tuber-regium* using 0.9% aqueous NaCl at 20 and 80 °C to obtain relatively low molecular mass fractions. The chemical structure of TM1 was analyzed to be a branched heteropolysaccharide–protein complex, and the sugar moiety was mainly β-(1→6)-, β-(1→4)-, and β-(1→3)-linked glucan containing galactose and mannose. TM2 was a branched polysaccharide–protein complex, and the sugar moiety was mainly β-(1→6)-, β-(1→4)-, and β-(1→3)-linked glucan. Preparative size-exclusion chromatography (SEC) and analytical SEC combined with three detectors were used to detect the TM1 and TM2 samples, confirming that the proteins were bonded to the polysaccharides. Furthermore, analytical SEC combined with online laser light scattering, differential refractive index detector, and UV to determine the components, weight-average molecular mass (M_w) and chain conformation of the samples. The relatively low exponent values (ν) of $\langle S^2\rangle_z^{1/2} = k_\nu M_w^\nu$ for the samples in 0.15 M aqueous NaCl at 25 °C suggested that TM1 and TM2 existed in compact sphere conformation in the aqueous solution. This work provided valuable information on the structure and chain conformation characterization of the polysaccharide–protein complex having relatively low M_w.

1. Introduction

Polysaccharide–protein complexes are important water-soluble biopolymers, and their biological activities have attracted much attention in the fields of biochemistry and pharmacology.[1–6] Antagonism to immunosuppressive factors in tumor-bearing hosts is the major action mechanism of the polysaccharide–protein complexes, which are recognized as biological response modifiers. In view of the cytokine gene expression, two antitumor polysaccharide–protein complexes increase the expression lever of macrophage colony-stimulating factor, suggesting that protein-bound polysaccharide activates some immunomodulating cytokines.[7] On the basis of the molecular level analysis, Matsunaga has investigated the action of the protein-bound polysaccharide on transforming growth factor-β, suggesting that the protein moiety of PSK plays an important role in the expression of the activity.[8] Moreover, carbohydrate-bound proteins residing in cell membranes are known to mediate the initial recognition of immunologically important events such as lymphocyte routing and neutrophil and monocyte recruitment to the injury site.[9] Though much information on the bioactivities of polysaccharide–protein complexes is available, their structure and chain conformation have not been fully investigated due to the small amount of yield and the difficulty in fractionating. However, studies of the physicochemical properties of the polysaccharide–protein complexes could increase understanding of their structure properties and conformational behavior in relationship to their immunomodulatory and antitumor activity.[10] So, it is critical to find a simple and feasible method to characterize the structure and chain conformation of the polysaccharide–protein complexes.

Size-exclusion chromatography (SEC) is a rapid method for separation and characterization of polymers.[11,12] The SEC column packed with polymeric gels such as methacrylate and vinyl types in the native forms is not suitable for separation of biopolymers because of their inappropriate surface properties.[13] In our laboratory, novel microporous beads have been prepared from the blending of cellulose and konjac glucomann (RC/KGM3) by an emulsification method. Then, the microporous beads were modified with silane to avoid the adsorption of polymers containing hydroxyl groups. The modified beads were coded as RC/KGM3-Si.[14] A prepared SEC column packed with RC/KGM3-Si has been used to successfully fractionate poly (ε-caprolactone) and polysaccharides, and good efficiency for fractionation was shown. In the present work, using the prepared SEC column, we attempt to clarify whether or not the protein is bound to the polysaccharide in the complex polysaccharide samples. Furthermore, analytical SEC combined with three detectors such as ultraviolet (UV), laser light scattering (LLS), and differential refractive index (RI) is used to further determine the structure, average molecular weight, and chain conformation of the complex biopolymers.

* Corresponding author. Tel.: +86 27 87219274; fax: +86 27 68754067.
E-mail address: lnzhang@public.wh.hb.cn (L. Zhang).

0008-6215/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carres.2008.04.030

Pleurotus tuber-regium, an edible fungus, grows in tropic and subtropical regions of the world.[15] It is consumed not only for its flavor and nutritive value, but also for its medicinal effects including the treatment of asthma, smallpox, and high blood pressure.[16] The sclerotia are rich sources of proteins, fat, crude fiber, minerals as well as carbohydrates. This suggests the sclerotia have a wide range of food nutrients and balanced food values.[17] In our previous work,[18] polysaccharide–protein complexes having high molecular mass were extracted from the sclerotia of *Pleurotus tuber-regium* by using an aqueous solution of 0.5 M NaOH and 0.01 M $NaBH_4$ at 10 °C. In this work, low molecular mass fractions (coded as TM1 and TM2) were extracted from the sclerotia of *Pleurotus tuber-regium* using 0.9% aqueous NaCl at 20 and 80 °C. Their chemical structures were determined by using IR, and NMR spectroscopy, plus elemental analysis, and amino-acid analysis. Using preparative SEC and analytical SEC, the structure, molecular mass, and chain conformation of TM1 and TM2 were further investigated.

2. Experimental

2.1. Material

Sclerotia of *Pleurotus tuber-regium* were provided by the Department of Biology in the Chinese University of Hong Kong. The dried sclerotia powder of *Pleurotus tuber-regium* was defatted by Soxhlet extraction with EtOAc and acetone for over 6 h. The residue was immersed stepwise in 0.9% aq NaCl at 20 and 80 °C. In each step, the mixture was stirred overnight, and then was centrifuged at 9045*g* for 20 min to obtain supernatants. The supernatants were labeled as TM1 and TM2, respectively. The TM1 and TM2 supernatants were subjected to the Sevag method to remove free proteins, and then 30% H_2O_2 to decolorize. Subsequently, the supernatants were dialyzed using a regenerated cellulose tube (M_w cut-off 8000, Union Carbide USA) against tap water for 5 days and against distilled water for 3 days. The resultant solution was concentrated by rotary evaporation at reduced pressure below 45 °C, and finally lyophilized to give white powder of TM1 and TM2.

Individual TM1 and TM2 samples (100 mg) were dissolved in 10 mL of 0.2 M NaOH and 1 M $NaBH_4$, then these were heated to 45 °C with stirring, and standing for 24 h. The resulting solution was neutralized with 50% CH_3COOH, and then the protein was removed by the Sevag method for over five times. The supernatant was dialyzed with a regenerated cellulose tube (M_w cut-off 8000, Union Carbide, NJ, USA) against tap water for 5 days and distilled water for 4 days. The purified polysaccharide was finally lyophilized to dryness (Christ Alpha 1-2, Osterode am Harz, Germany) to obtain a white powder, coded as P-TM1 and P-TM2.

2.2. Analysis of chemical composition

Infrared spectra (FTIR) of TM1 and TM2 were recorded on a Nicolet 170SX FTIR (Spectrum One, Perkin Elmer Co., USA) spectrometer in the range 4000–400 cm^{-1} using the KBr-disk method.^{13}C NMR spectra of TM1, TM2, P-TM1, and P-TM2 were recorded on a Mercury 600 NMR spectrometer (Varian Inc., USA) at 30 °C. The samples were dissolved in D_2O to obtain a concentration of 50 mg/mL. Elemental compositions for C, H, and N of TM1 and TM2 were determined using an elemental analyzer (EA, Heraeus Co., Germany).

Total sugar content was determined by the phenol–sulfuric acid method with glucose as the standard.[19]Amino acids were analyzed with a Hitachi L-8880 amino-acid analyzer.

2.3. Preparation of cellulose beads and preparative SEC

Microporous beads (RC/KGM3) with a particle size of about 90 μm were prepared from cellulose and konjac glucomannan in aq 1.5 M NaOH and 0.65 M thiourea by an emulsification method.[14] The surfaces of the RC/KGM3 beads were modified with trimethylchlorosilane to get RC/KGM3-Si. Finally the RC/KGM3-Si beads were stored in a mixture of aq 20% 2-PrOH and 2% formaldehyde. When used, the RC/KGM3-Si beads were suspended in distilled water, and were then packed in a glass column (600 × 20 mm) to form a 500-mm bed for a preparative SEC column. The elution phase was 0.15 M aq NaCl, and the flow rate was adjusted to 0.5 mL/min during the runs to stabilize the preparative SEC column for a week at room temperature. TM1, TM2, and the mixture of dextran (D-1517) and bovine albumin (BSA, A-1900) samples were dissolved in 0.15 M aq NaCl to prepare a solution with a concentration of 12 mg/mL. Five milliliters of the solution was injected into the preparative SEC column. The eluate of TM1, TM2, and mixture of dextran and BSA was monitored using the UV detector at 200 nm for polysaccharide and 280 nm for protein, respectively.

2.4. SEC–LLS–RI–UV Measurements

Size-exclusion chromatography combined with laser light scattering measurements was performed on multi-angle laser photometer (DAWN®DSP, Wyatt Technology Co., Santa Barbara, CA, USA), combined with a P100 pump (Thermo Separation Products, San Jose, USA) equipped with TSK-GEL G6000 and G4000 PWXL column (7.8 mm × 300 mm) in series for aq solution at 25 °C. A differential refractive index detector (RI-150) and ultraviolet detector were simultaneously connected. The eluent was 0.15 M aq NaCl with a flow rate of 1.0 mL/min. All solutions with a concentration of 3.0–5.0 mg/mL were filtered first with a sand filter, followed by a 0.20-μm filter (Whatman, England), and then kept in sealed glass bottles before being injected onto the SEC column. ASTRA software (version 4.90.07) was utilized for the data acquisition and analysis. The refractive index increment (d*n*/d*c*) values of the samples in 0.15 M aq NaCl were determined using an Optilab refractometer (Dawn-DSP, Wyatt Technology Co., Santa Barbara, CA, USA) at 633 nm and 25 °C (shown in Table 1).

3. Results and discussion

3.1. Chemical structure

The FTIR spectra of the samples are shown in Figure 1. The TM1 and TM2 samples exhibit an absorption peak at 890 cm^{-1}, which is characteristic of a β-D-glucan.[20] An IR absorption of the TM1 and

Table 1
^{13}C NMR chemical shifts (δ, ppm) of TM1, P-TM1, TM2, and P-TM2 in D_2O solution

Chemical shifts	C1	C2	C3	C4	C5	C6	C1′	C3′	C4′	C6′	C6s	–CONH–
TM1	102.6	73.5	76.6	70.2	76.6	61.1		84.3	79.3	70.2	66.8	168–176
	99.1	68.2	70.2		77.2	61.1						
			74.7	66.8	79.3	61.1						
P-TM1	102.9	73.2	75.6	69.7	75.6	60.8		84.3	79.3	69.7		
TM2	102.6	73.1	75.7	70.0	75.7	60.8		84.3	79.3	70.0	67.0	168–176
P-TM2	102.6	73.2	75.7	69.7	75.7	60.8		84.3	78.6	69.7		

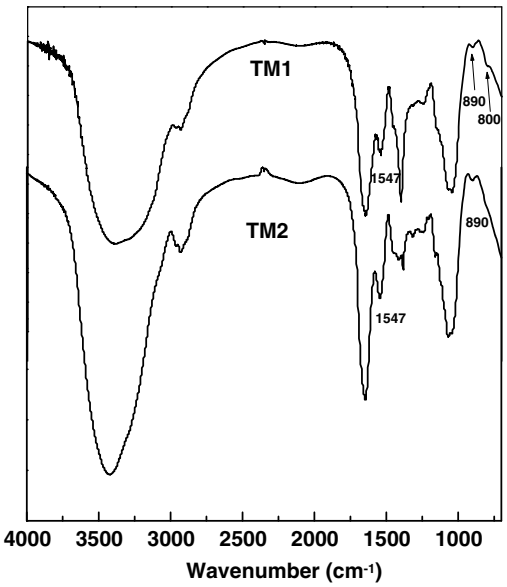

Figure 1. FTIR spectra of the polysaccharide–protein complexes.

TM2 samples at 1547 cm^{-1}, corresponding to the secondary –CONH– group of the protein, indicates the existence of proteins.[21] The proteins in the samples are mainly bonded to the polysaccharides because the procedure of Sevag was repeated for more than 10 times to remove free proteins. The IR absorption of TM1 at 800 cm^{-1} is the characteristic absorption of mannose. The ^{13}C NMR spectra of the TM1, P-TM1, TM2, and P-TM2 samples are shown in Figure 2. The resonance peaks at 168–176 ppm in the ^{13}C NMR spectra of the TM1 and TM2 samples are assigned to the –CONH– group of protein.[22] The main signals of TM1 are assigned to 102.6 ppm for C1, 76.6 ppm for C3 and C5, 73.5 ppm for C2, 70.2 ppm for C4, and 61.1 ppm for C6, similar to those of the carbons for β-D-glucopyranose.[23] Furthermore, the signals at 84.3 ppm, 79.8 ppm, and 70.2 ppm are assigned to some substituted residues at C3, C4, and C6, respectively. The results indicate that TM1 contains β-D-glucan with numerous terminal residues,[24] β-(1→6)-, β-(1→4)-, and β-(1→3)-linked residues. In addition, the peaks at 99.1, 70.2, 68.2, 77.2, and 61.1 ppm for TM1 could be attributed to chemical shifts of C1, C3, C2, C5, and C6 of α-D-galactose bonded to mannose, whereas those at 79.3, 74.7, 66.8 and 61.1 ppm are assigned to C5, C3, C4, and C6 of β-linked-D-mannose.[25] Therefore, TM1 is a branched heteropolysaccharide–protein complex, and the sugar moiety is mainly β-(1→6)-, β-(1→4)-, and β-(1→3)-linked glucan containing other sugar units such as galactose and mannose. In the same way, TM2 is a branched polysaccharide–protein complex, and the sugar moiety is mainly β-(1→6)-, β-(1→4)-, and β-(1→3)-linked glucan. The chemical shifts for the TM1, P-TM1, TM2, and P-TM2 samples are summarized in Table 1. Compared with the ^{13}C NMR spectra of the native TM samples, the peak at 168–176 ppm, which is the chemical shift of the –CONH– group of protein for TM1 and TM2, almost disappeared in the ^{13}C NMR spectra for P-TM1 and P-TM2. It suggests that the proteins have been largely removed from the TM samples by alkali treatment. Compared with the ^{13}C NMR spectra of the native TM samples, another resonance peak disappears in the spectra for the P-TM samples, namely, the signal at 66.8 ppm for TM1 and the one at 67.0 ppm for TM2. This indicates the proteins bonded with the polysaccharides at the position of O-6 in the TM1 and TM2 samples, and the C6 signal shifts downfield for 6 ppm or so. When the proteins were removed, the signal of substituted C6 (C6s) disappeared subsequently. Further, the signals at 99.1 ppm, 77.2 ppm, and 68.2 ppm, which are attributed to chemical shifts of α-D-galactose bonded to the mannose of TM1, disappear in the ^{13}C NMR spectra of P-TM1.

The protein and carbohydrate content of TM1 and TM2 are summarized in Table 2. The protein content (37.5% for TM1, and 42.0 for TM2) that is obtained from amino acid analysis is in good agreement with the results from elemental analysis. The relative amino-acid composition content (mol %) of the protein moieties of the polysaccharide–protein complexes is summarized in Table 3.

3.2. Test of preparative SEC

Recent advances in biotechnology and biochemistry have been facilitated by efficient separation methods for biopolymers, such as proteins and nucleic acids.[26] It is difficult to characterize visually whether the protein is covalent to the sugar in a complex biopolymer. By using preparative SEC combined with a UV detector, we directly detect the protein-bound polysaccharide on the basis of the chromatogram. In our laboratory, the preparative SEC column has been used to fractionate a polysaccharide,[14] and a protein–polysaccharide complex[18] successfully. Its exclusion limit and fractionation range of the stationary phase are, respectively, M_w of 4.8×10^5 g/mol and $5.3 \times 10^3 - 4.8 \times 10^5$ g/mol.[14] Figure 3 illustrates the elution pattern of TM1 and TM2 in 0.15 M aqueous NaCl by using preparative SEC with a flow rate of 0.5 mL/min. The elution profile was detected using a differential UV detector at 280 nm and 200 nm, respectively. The signals detected by the UV detector at 280 nm and 200 nm reflect the concentration of protein and polysaccharide, respectively. In the SEC chromatogram of TM1 and TM2, the polysaccharide peak detected at 200 nm coincides with the protein peak detected at 280 nm on the preparative SEC column. The results indicate that proteins are bonded with polysaccharides to form a complex. In order to further test the preparative SEC, the mixture of the dextran and BSA was performed as the model (shown in Fig. 4). The SEC pattern of the mixture on the preparative SEC column shows that the polysaccharide peak does not coincide with the protein peak. It suggests that proteins and polysaccharides are not bonded to each other but are free in the mixture. In view of the results, the preparative SEC combined with UV detectors can be used to clarify whether the proteins are bonded to the polysaccharides in the complex biopolymers on the basis of the elution profile. It is feasible to characterize and separate polysaccharide–protein complexes using the preparative SEC column packed with RC/KGM3-Si microporous beads.

Furthermore, dextran, BSA, and a mixture of dextran and BSA were examined by analytical SEC using a combination of LLS, RI, and UV detectors. Figure 5 shows the SEC patterns of dextran, BSA, and mixture of dextran and BSA on the analytical SEC column in 0.15 M aqueous NaCl at 25 °C detected by the UV detector at 280 nm. Dextran alone did not give rise to any UV light absorption because of no chromophores absorbing in this spectral region. When BSA solution and the mixture solution were injected onto the analytical SEC column, both were detected by the UV detector at 280 nm. The UV absorption was detected in the elution volume 7.7–10.2 mL for BSA, and 7.5–10.5 mL for the mixture of dextran and BSA. In the experimental setup used, the UV absorption peak for the mixture appears within the same elution volume as that for BSA. Figure 6 shows the SEC patterns of dextran, BSA, and mixture of dextran and BSA on the analytical SEC column in 0.15 M aqueous NaCl at 25 °C detected by a differential refractive index detector. The chromatogram thus obtained describes the concentration of each fraction in the samples. Only one peak appears in the elution profile of dextran or BSA. In the experimental setup used, dextran elutes in the interval 6.0–10.0 mL, and BSA elutes in the interval 7.4–9.2 mL. Two peaks, labeled as peak 1 and peak 2, appear in the chromatogram of the mixture. Compared with

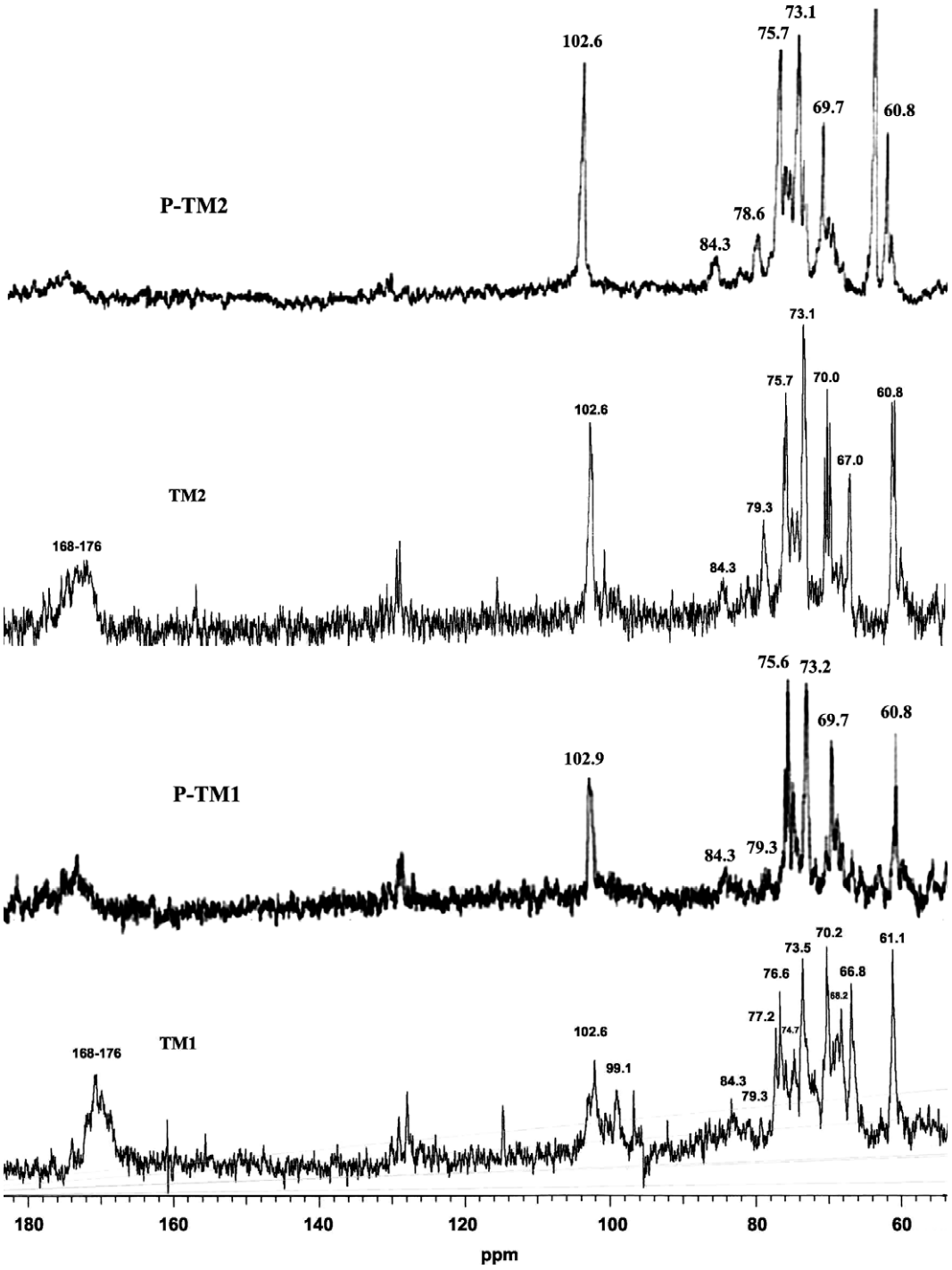

Figure 2. ^{13}C NMR spectra of TM1, P-TM1, TM2, and P-TM2 in D_2O solution.

Table 2
Total carbohydrate content, protein content, and molecular parameters of the TM1 and TM2 polysaccharide–protein complexes in 0.15 M aqueous NaCl at 25 °C

Samples	Total carbohydrate content (%)	Protein content (%)		d*n*/d*c*	$\langle S^2\rangle_z^{1/2}$ (nm)	$M_w \times 10^{-4}$	M_w/M_n	ν[a]
		From elemental analyzer	From amino-acid analyzer					
TM1	44.2	37.5	36.1	0.084	67.0	6.0	1.2	0.10
TM2	47.6	42.0	42.4	0.104	27.6	14.1	3.4	0.05

[a] ν is the exponent of equation $\langle S^2\rangle_z^{1/2} = k_\nu M_w^\nu$.

the elution profiles of dextran and BSA, peak 1 and peak 2 are, respectively, assigned to the signals for dextran and BSA in the mixture. Peak 2 in the chromatogram of the mixture detected by the differential refractive index detector corresponds to the absorption peak of the mixture obtained by the UV detector. The proteins and polysaccharides are free in the solution of the mix-

Table 3
Amino acid compositions (mol %) of the polysaccharide–protein complexes

Amino acid	TM1	TM2
Asp	11.6	13.5
Thr	7.1	6.8
Ser	11.6	11.3
Glu	8.9	9.4
Pro	8.6	8.0
Gly	10.9	9.9
Ala	10.1	8.9
Cys	2.4	2.09
Val	5.5	6.29
Met	2.0	1.7
Ile	5.1	5.6
Leu	4.6	4.8
Tyr	1.9	1.9
Phe	4.2	3.9
Lys	1.2	0.6
His	1.3	1.8
Arg	3.0	3.6

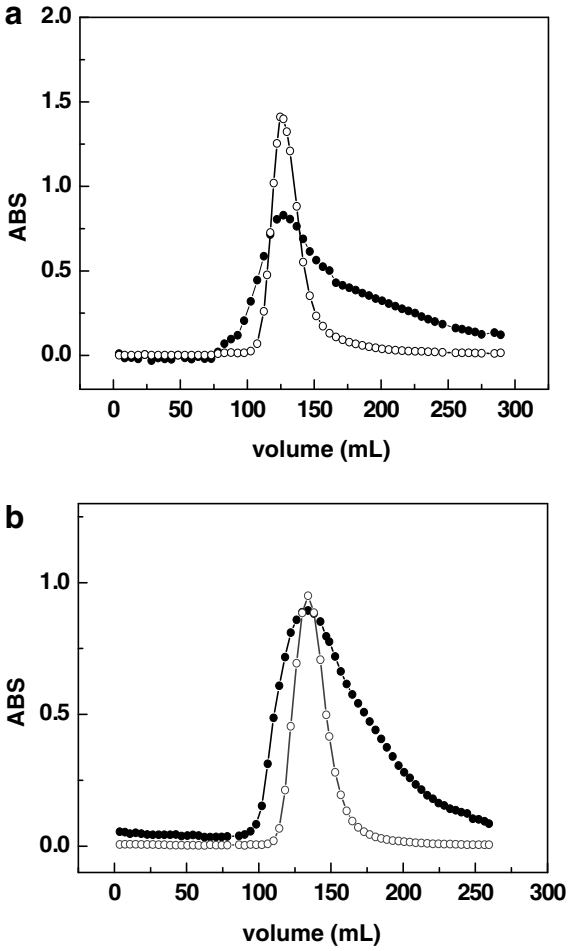

Figure 3. Elution pattern of TM1 (a) and TM2 (b) in 0.15 M aq NaCl at 25 °C using a preparative SEC column (500 mm × 20 mm) packed with RC/KGM3-Si and a flow rate of 0.5 mL/min. The absorbance of the effluent was monitored by a UV detector at 200 (•) nm and 280 (○) nm, respectively.

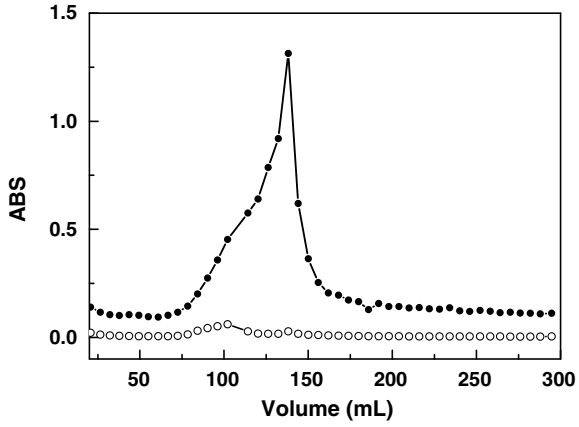

Figure 4. Elution pattern of mixture of dextran and BSA in 0.15 M aq NaCl at 25 °C by using a preparative SEC column (500 mm × 20 mm) packed with RC/KGM3-Si with a flow rate of 0.5 mL/min. Absorbance of the effluent was monitored by a UV detector at 280 (•) nm and 200 (○) nm, respectively.

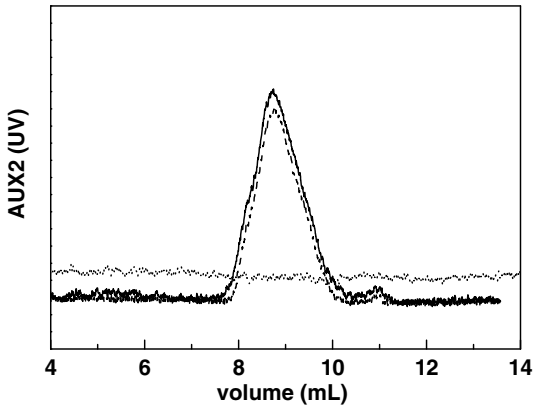

Figure 5. SEC chromatograms for dextran (···), BSA (---), and mixture of dextran and BSA (—) in 0.15 M aq NaCl at 25 °C detected by a UV detector. The detector's AUX2 value is from a signal from the UV detector.

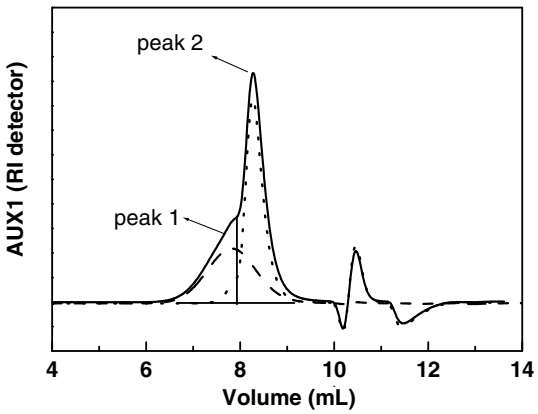

Figure 6. SEC chromatograms for dextran (---), BSA (···), and mixture of dextran and BSA (—) in 0.15 M aq NaCl at 25 °C detected by a differential refractometer. The detector's AUX1 value is from a signal from the refractive index detector.

ture, resulting in two peaks that appear in the chromatogram detected by the differential refractive index detector, but only one peak by UV detector.

TM1 and TM2 were performed on analytical SEC combined with LLS, RI, and UV detectors. The SEC patterns of TM1 and TM2 obtained with the SEC column in 0.15 M aqueous NaCl at 25 °C are shown in Figure 7. The samples exhibit a single peak, respectively, detected by laser light scattering photometry, a differential refractive index detector, and a UV detector. The results from ^{13}C NMR spectroscopy, elemental analysis, and amino-acid analysis have indicated the presence of protein (37–42%) in the polysaccharides. These findings further confirm that TM1 and TM2 are polysaccha-

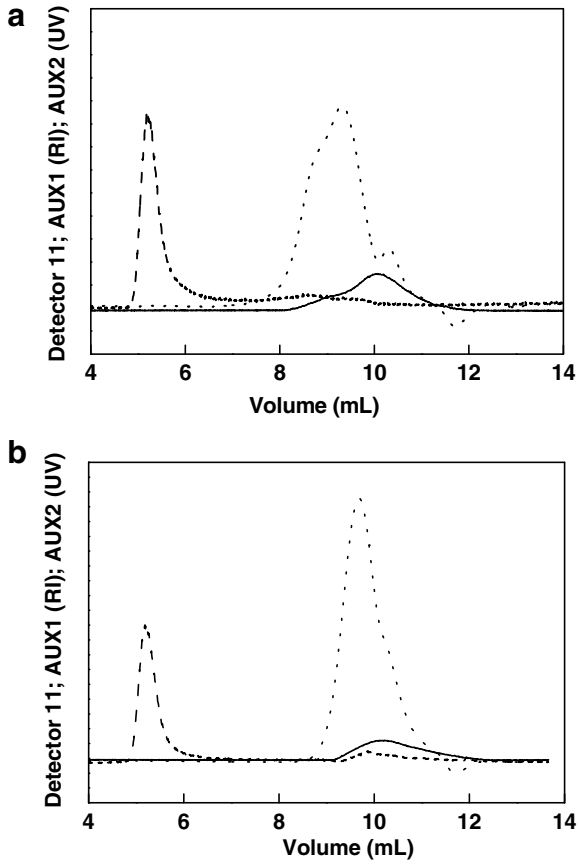

Figure 7. SEC, LLS, and UV chromatograms for TM1 (a) and TM2 (b) in 0.15 M aq NaCl at 25 °C detected by laser light scattering photometry (---), differential refractometer (···), and UV detector (—). The detector 11, AUX1, and AUX2 values are from signals from the LLS at 90°, the refractive index detector, and UV detector, respectively.

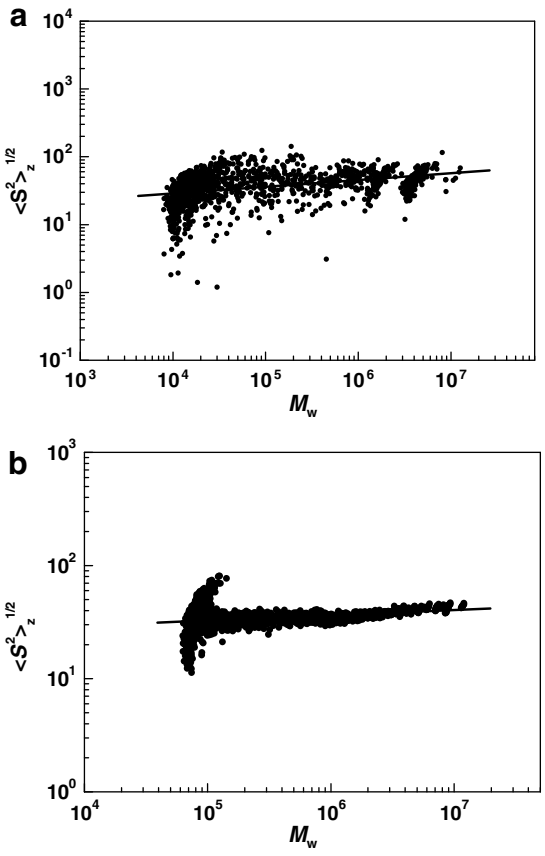

Figure 8. Plots of $\langle S^2\rangle_z^{1/2}$ versus M_w for the polysaccharide–protein complexes TM1 (a) and TM2 (b) in 0.15 M aq NaCl at 25 °C.

ride–protein complexes. The SEC mass recovery rate corresponds to the proportion (%) of injected material to recovered one in the column effluent. In this case, the dn/dc values of the samples and AUX calibration constant are known, so the SEC recovery rate was calculated using the product c_i=Δn/(dn/dc) for each sampling time over the entire profile. Using this method, the recovery rates were calculated to be 63.8% for TM1 and 82.5% for TM2, respectively.

3.3. Chain conformation

The values of weight-average molecular weight (M_w), radius of gyration ($\langle S^2\rangle_z^{1/2}$), and polydispersity ($M_w/M_n$) are obtained from analytical SEC combined with LLS, RI, and UV. The experimental data of the samples in 0.15 M aqueous NaCl are also summarized in Table 1. More information on chain confirmation could be obtained from SEC chromatograms. The power law of $\langle S^2\rangle_z^{1/2} = f(M_w)$ can be estimated from many experimental points in the SEC chromatogram. Figure 8 shows log–log plots of $\langle S^2\rangle_z^{1/2}$ versus M_w for TM1and TM2 in 0.15 M aqueous NaCl at 25 °C. The straight line fitting the experimental points from the SEC chromatogram is represented by

$$\langle S^2\rangle_z^{1/2} = k_v M_w^v$$

The exponent (v) may provide additional insights into polymer solution conformation. The v values for TM1and TM2 are shown in Table 1. Usually, the exponents of 0.33, 0.50–0.60, and 1.0 reflect the chain shape in adapting for a sphere, random coil, and rigid rod, respectively. Both of the samples exhibit very low v values, suggesting a spherically branched polymer.[27] On basis of a self-consistent field model, de Gennes and Hervet predict $r \approx M^{0.2}$ for dendrimers at low values of dendrimer generation (g).[28] We speculate that the very low v values result from the existence of protein and the high branching structure of the polysaccharide.

4. Conclusions

Proteins and polysaccharides co-existed in the water-soluble TM1 and TM2 samples, which were isolated from the sclerotia of *Pleurotus tuber-regium*. Their chemical structures and chain conformations were successfully characterized using preparative SEC with a UV detector, and analytical SEC with LLS, RI, and UV detectors. The results confirmed that the proteins were bonded to the polysaccharides in TM1 and TM2, and that both the polysaccharide–protein complexes possessed a sphere–chain conformation in 0.15 M aqueous NaCl at 25 °C. The preparative SEC column packed with RC/KGM3-Si is effective in estimating whether the proteins were bond to the polysaccharides or not. Analytical SEC with different detectors had the advantages of being able to study the structure and chain conformation of complex biopolymers having low molecular mass on line.

Acknowledgments

This work was supported by major grant of the National Natural Science Foundation of China (30530850), and the High-Technology Research and Development Program of China (2006AA02Z102).

References

1. Lee, C. L.; Yang, X. T.; Wan, J. M. F. *Enzyme Microb. Technol.* **2006**, *38*, 14–21.
2. Ng, T. B. *Gen. Pharmacol* **1992**, *30*, 1–4.
3. Chen, T.; Embree, H. D.; Wu, L.; Payne, G. F. *Biopolymers* **2002**, *64*, 292–302.
4. Mi, F. *Biomacromolecules* **2005**, *6*, 975–987.
5. Ogasawara, H.; kusui, K.; Takasaki, S. *Immunol. Lett.* **1995**, *48*, 35–38.
6. Cui, J.; Chisti, Y. *Biotechnol. Adv.* **2003**, *21*, 109–122.
7. Liu, F.; Fung, M. C.; Ooi, V. E. C.; Chang, S. T. *Life Sci.* **1996**, *58*, 1795–1803.
8. Matsunaga, K. *Immunopharmacology* **1998**, *40*, 219–230.
9. Lasky, L. A. *Science* **1992**, *258*, 964–969.
10. Kulicke, W. M.; Lettau, A. I.; Thielking, H. *Carbohydr. Res.* **1997**, *297*, 135–143.
11. Goetz, H.; Kuschel, M.; Wulff, T.; Sauber, C.; Miller, C.; Fisher, S.; Woodward, C. *J. Biochem. Biophys. Methods* **2004**, *60*, 281–293.
12. Tarazona, M. P.; Saiz, E. *J. Biochem. Biophys. Methods* **2003**, *56*, 95–116.
13. Vianna-Soares, C. D.; Kim, C. J.; Borestein, M. R. *J. Porous Mater.* **2002**, *9*, 67–75.
14. Xiong, X.; Zhang, L.; Wang, Y. *J. Chromatogr., A* **2005**, *1063*, 71–77.
15. Zoberi, M. H. *Nigerian Field* **1973**, *38*, 81–90.
16. Oso, B. A. *Mycologia* **1971**, *69*, 271–279.
17. Ezenwugo, A. E. N.; Agu, R. C. *J. Food Sci. Technol.* **2001**, *38*, 612–614.
18. Tao, Y.; Yan, F.; Zhang, L. *J. Polym. Sci. Part B: Polym. Phys.* **2007**, *45*, 2546–2554.
19. Dubois, M.; Gilles, K. A.; Hamilton, J. K.; Rebers, P. A.; Smith, F. *Anal. Chem.* **1956**, *28*, 350–356.
20. Kiho, T.; Sakushima, M.; Wang, S.; Nagai, K.; Ukai, S. *Chem. Pharm. Bull.* **1991**, *39*, 798–800.
21. Williams, D. H.; Fleming, I. In *The Spectroscopic Methods in Organic Chemistry*; McGraw-Hill: London, 1980; p 58.
22. Temeriusz, A.; Anulewicz, R.; Wawer, I.; Krygowski, T. M.; Bartoszewicz, B. P.; Rowińska, M. *Carbohydr. Res.* **2001**, *334*, 71–79.
23. Bock, K.; Pedersen, C. *Adv. Carbohydr. Chem. Biochem.* **1983**, *41*, 27–66.
24. Satoh, T.; Imai, T.; Ishihara, H.; Maeda, T.; Kitajyo, Y.; Sakai, Y.; Kaga, H.; Kaneko, N.; Ishii, F.; Kakuchi, T. *Macromolecules* **2005**, *38*, 4202–4210.
25. Chaubey, M.; Kapoor, V. P. *Carbohydr. Res.* **2001**, *332*, 439–444.
26. Yang, F.; Ito, Y. *Anal. Chem.* **2002**, *74*, 440–445.
27. Beri, R. G.; Walker, J.; Reese, E. T.; Rollings, J. E. *Carbohydr. Res.* **1993**, *238*, 11–26.
28. de Gennes, P. G.; Hervet, H. *J. Phys. Lett.* **1983**, *44*, 351–360.