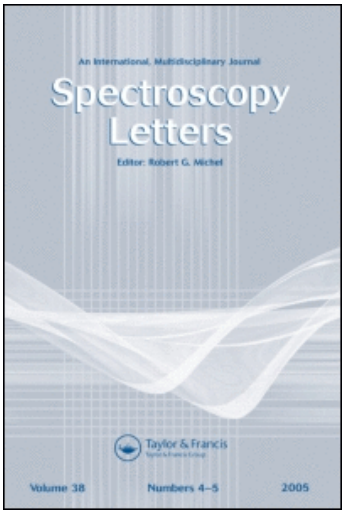

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Spectroscopic Characterization of Pachyman Sulfate and Its Binding Interaction with Azur A

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Spectroscopic Characterization of Pachyman Sulfate and Its Binding Interaction with Azur A

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Abstract: High-purity pachyman isolated from the sclerotium of *Poria cocos* was reacted with chlorosulfonic acid to obtain pachyman sulfate (PS). The FTIR spectrum indicated sulfation of glucoside hydroxyl. The NMR results indicated almost complete sulfation on C-6, C-2, and C-4 of glucoside. Interaction between azur A (AA) and PS with AA concentration from 2.745×10^{-5} to 6.863×10^{-5} M was investigated using spectrophotometry at 625 nm. It was found that AA was a promising spectroscopic probe for the detection of sulfated groups in the PS macromolecule. Self-interference of AA on AA–PS interaction caused an overestimation of the molar mass of PS. The molecular weight mass of 13,110 Da obtained with an improved method for PS used in the current work agreed well with the value of 10,233 Da calculated from HPLC results.

Keywords: Azur A, maximum binding number, molecular weight, pachyman sulfate, *Poria cocos*, spectroscopic probe

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INTRODUCTION

Poria cocos (Schw.Wolf) ("fu-ling" in Chinese) is a macroscopic fungus and is best known for its diuretic and sedative pharmaceutical activities.[1] The principal component (>93%) of *Poria cocos* sclerotium is a cell-wall polysaccharide named pachyman. Pachyman is a glucan of β-1,3-linked D-glucopyranose residues containing 9–10 branches of β-1,6-linked D-glucopyranosyl groups and an internal β-1,6-D-linkage.[2] Modifications of pachyman are required for its biotechnological and biomedical applications because pachyman is insoluble in water. The introduction of a charged group at hydroxyl groups on the glucan chain could improve its water solubility, change the polymer chain to adopt a certain conformation in aqueous solutions, and enhance the pharmaceutical activities.[3] Chihara et al.[4] prepared a linear β-1,3-D-glucan, named pachymaran, by severing β-1,6-glucoside linkages of pachyman with sodium metaperiodate oxidation and found that the pachymaran had antitumor activities. Hamuro et al.[5] found that the carboxymethylated pachyman was water soluble and had a marked antitumor activity. Pachyman sulfate (PS), one of the semisynthetic sulfated glucans, has good water solubility and a great potential for the antitumor bioactivity.[6]

The bioactivities of the derivatives of pachyman are related to its chemical structure, that is, the degree of sulfation (DS),[7] the molecular weight (MW)[8] and the glycosidic branchings.[9] Owing to the presence of the sulfate groups, the PS molecule has a high negative charge density. This allows PS to interact with cationic dyes such as azur A (AA). The mechanism of positively charged dyes interacting with negatively charged glycosaminoglycans was proposed by Jiao et al.[10,11] A mathematical model based on this mechanism agreed very well with experimental results.[12] Based on this mechanism, Chen et al.[12] proposed an improved method of estimating the molecular weight of the sulfated polysaccharides. However, interaction of pachyman sulfate with azur A was not studied. In the current work, pachyman sulfate was synthesized using an improved Wolfrom method,[13] and its binding interaction with azur A was spectroscopically examined.

MATERIALS AND METHODS

Apparatus

A Hitachi U-3000 Spectrophotometer (Tokyo, Japan) was used to record absorption spectra and to measure the absorbance at a given wavelength, using a 1 cm pathlength. A BP221S electronic balance (Sartorius, Goettingen, Germany) was used for weight measurements. A FlashEA-1112 elemental analyzer (Thermo, Waltham, MA, USA) was used to measure the content of

element S in PS. A NEXUS870 spectrometer (Nicolet, Madison, WI, USA) was used to record Fourier-transform infrared spectra. A DRX-500 spectrometer (Bruker, Karlsruhe, Germany) was used to record ^{13}C NMR spectra.

Reagents

Dried fruit body of fu-ling (*Poria cocos*) from Zhejiang province, China, was purchased from a local herbal drugstore in Nanjing. Pyridine (purity >99.5%) was obtained from Shanghai Chemical Reagent Ltd. (Shanghai, China). Chlorosulfonic acid (purity >99.5%) was obtained from Chemical Industry Reagent Factory of Shanghai (Shanghai, China). Heparin (average MW, 15,000 Da), low-molecular-weight heparin (average MW, 8,000 Da), and chondroitin sulfate (average MW, 20,000 Da) were obtained from Chemical Reagent Distribute Factory of Shanghai (Shanghai, China). The AA was purchased from Shanghai Xinzhong Chemical Co. Ltd. (Shanghai, China).

Reagents of anhydrous alcohol, acetone, ethyl ether, acetic acid (10% v/v), and NaOH (0.5 M) were of analytical or guaranteed reagent grades.

Isolation and Sulfation of Pachyman

Fu-ling powder of 100 g was soaked in 3 L of 0.5 M NaOH overnight at 4°C; acetic acid (10%, v/v) was added into the isolated supernatant to adjust the pH to 6–7 so that the jellylike material precipitated; the precipitated material was washed with distilled water three times, and then with anhydrous alcohol, acetone, and ethyl ether subsequently to remove water from the white pachyman. The yield of the dried white pachyman was 77.8%.

Pachyman sulfate was prepared by an improved Wolfrom method.[13] Pyridine 100 mL was placed in a three-necked flask previously cooled in an ice bath. To the cooled pyridine was added slowly, through a dropping funnel, 10 mL of chlorosulfonic acid over a period of 30–40 min. To this mixture, 6 g of the pachyman powder was added and the whole was heated on a boiling water-bath for 1 hr under stirring. About 50 mL of pyridine was retrieved by concentrating the mixture under the reduced pressure at 80°C. After cooling to the room temperature, the reaction mixture was poured into 100 mL of water to give a clear brown solution, and 2.5 N sodium hydroxide was added to adjust pH to 7–8. The sodium salt of crude sulfated pachyman was then precipitated with 400 mL of ethanol. The precipitate was redissolved in 100 mL of water and subjected to dialysis (MW cutoff, 3500 Da) for 2 days against tap water and 1 additional day for distilled water to remove pyridine, salts, and the degradation products. The solution, which

turned slightly acidic during dialysis, was neutralized with NaOH; this makes the final product a sodium salt. After the solution was concentrated to 100 mL under the reduced pressure, the product was precipitated as its sodium salt with 200 mL of ethanol. The final dry PS product of 4.5 g was obtained with the yield of 75%.

Characterization of Pachyman Sulfate

Sulfur content was obtained by elemental analysis (EA); the degree of sulfation (DS), which expresses the number of sulfation groups linked to hydroxyls on monose residue of sugar chain, was calculated using the following equation:[14]

$$DS = 1.62 \times S\% \div (32 - 1.02S\%) \quad (1)$$

Theoretically, the maximum DS is 3 for the pachyman sulfate because sulfation can only occur at the C-2, C-4, and C-6 positions. Average molecular weight was measured by HPLC analysis with reference to the standards of low molecular-weight heparin, heparin, and chondroitin sulfate.

Fourier-transform infrared spectra were recorded with the scanned wave number ranging from 4000 to 400 cm^{-1} using the KBr-disk method. ^{13}C NMR spectra were recorded at 30°C in Me_2SO-d_6 for pachyman and in D_2O for PS.

AA-PS Binding Interaction

A PS stock solution of 0.5 g/L was prepared by dissolving 0.05 g of PS in 100 mL of distilled water. An operating solution of PS of 0.01 g/L was prepared by diluting 1 mL of the stock solution with distilled water in a 50 mL volumetric flask. An AA stock solution (3.43×10^{-3} M) was prepared by dissolving 0.1 g of dye in 100 mL of distilled water. An AA operating solution (5.49×10^{-4} M) was prepared by diluting 8 mL of the AA stock solution with distilled water to 50 mL. The PS and AA stock solutions were stored in the dark.

The PS operating solution was transferred into a series of 12 × 100 mm test tubes; AA operating solution was then added to each test tube. The mixtures were diluted to a desired volume with distilled water, and the mixing was achieved by either inversion or vortexing. After the mixture had been in the test tube for 5 min, the absorption spectrum from 400 to 800 nm or the absorbances at 625 nm and 522 nm were measured for the solutions, with reference to water. All of the measurements were performed at pH 7 in a thermostated room of 20°C within 2 hr after the AA–PS mixtures were made. Each sample was measured in triplicate, with the relative standard deviation $<3\%$. Thus, an average of the three measurements was reported.

RESULTS AND DISCUSSION

The total carbohydrate content of isolated pachyman was 98.5% quantified by the phenol–sulfuric acid method.[15] No nitrogen was detected in the isolated pachyman from elemental analysis. The $[\alpha]_D^{25}$ of PS was -17.55°; the intrinsic viscosity, $[\eta]$, was 20.1 mL/g; sulfur content was 18.52%, that is, DS was 2.3 according Eq. (1). This DS value is very high compared with the literature results.[3] The average molecular-weight of PS was 10,233 Da calculated from the results of HPLC.

Spectral Characterization of Pachyman Sulfate Molecular Structure

The UV-Vis spectrum of PS obtained in this work is shown in Fig. 1 with a new broad absorption band at 260 nm, which is due to $n \rightarrow \pi^*$ transition of sulfate.[16] The IR spectra of pachyman and PS are shown in Fig. 2. The characteristic absorption at 890 cm^{-1} for pachyman was assigned to the β-configuration.[17] Compared with the IR spectrum of pachyman, two new absorption bands appeared in the IR spectrum of PS (Fig. 2), one at 1240 cm^{-1} describing an asymmetrical S═O stretching vibration and the other at 804 cm^{-1} representing a symmetrical C-O-S vibration associated with a C-O-SO_3 group,[17] indicating incorporation of the sulfating group. The -OH stretching vibration bands at 3700–3000 cm^{-1} for the sulfated derivatives were narrowed.[17] The signal of absorption at 1650 cm^{-1} in the

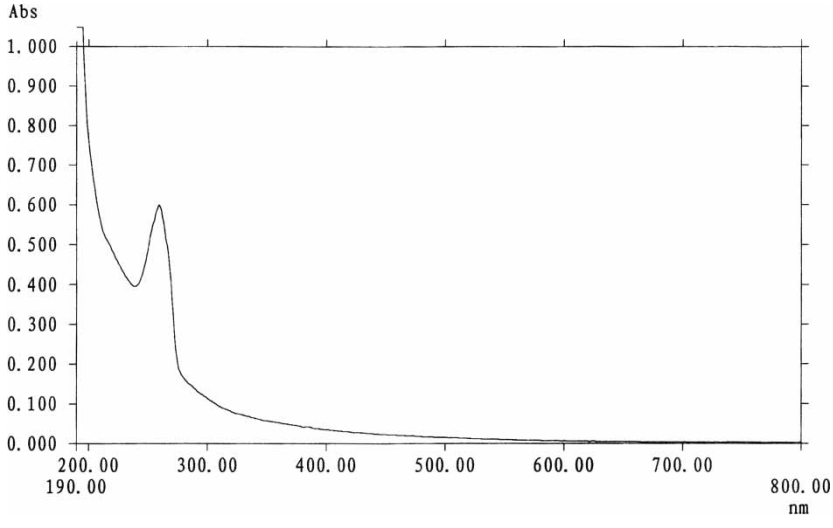

Figure 1. UV-Vis spectrum of PS.

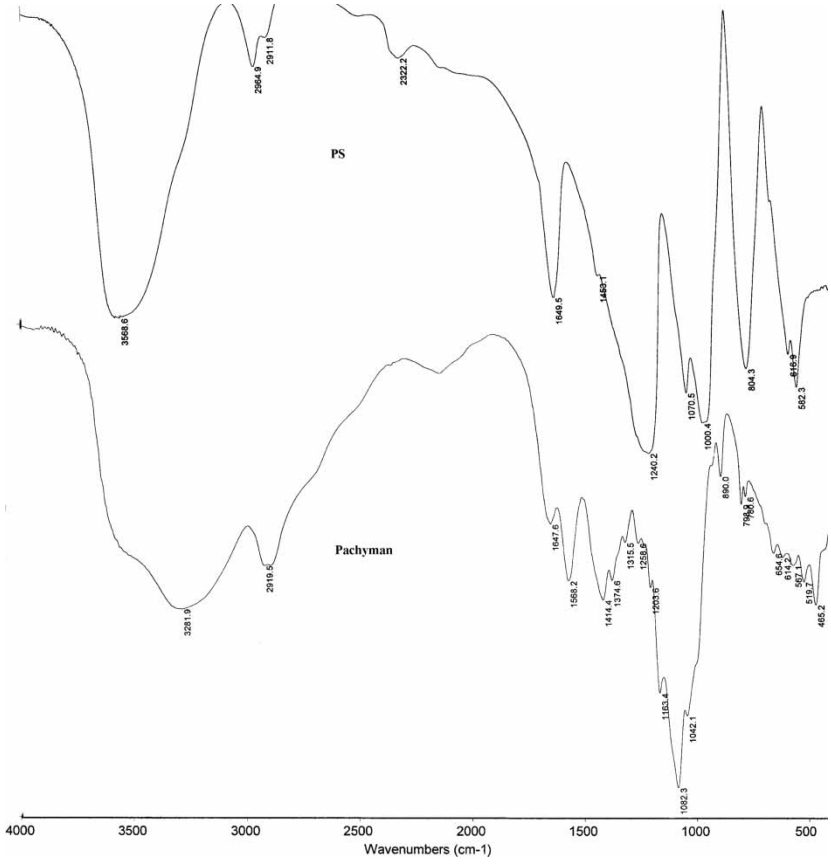

Figure 2. FTIR spectra of pachyman (lower) and PS (upper).

spectrum of PS is much stronger than that in the spectrum of pachyman, indicating C═C conjugate vibration associated with the oxidization of the pyranoid ring of the sugar unit by chlorosulfonic acid;[17] this may have caused the formation of the dark-brown color of PS when the PS was prepared at the harsher reaction conditions.

^{13}C NMR spectra of Pachyman and PS are shown in Fig. 3. Compared with the chemical shifts of the native pachyman, there are six new peaks in the PS spectrum while the peaks of the original pachyman disappeared resulting from sulfation of the hydroxyl groups at positions 6, 4, and 2. Alban et al.[7] found that the OH groups bound to C-6 atom had about 10-fold higher accessibility to sulfation compared with those bound to C-2 or C-4 atoms. The accessibility of the different OH groups for the sulfation was in the following order: C-6 > C-2 > C-3 > C-4;[7] this order of the accessibility was independent of the original starting

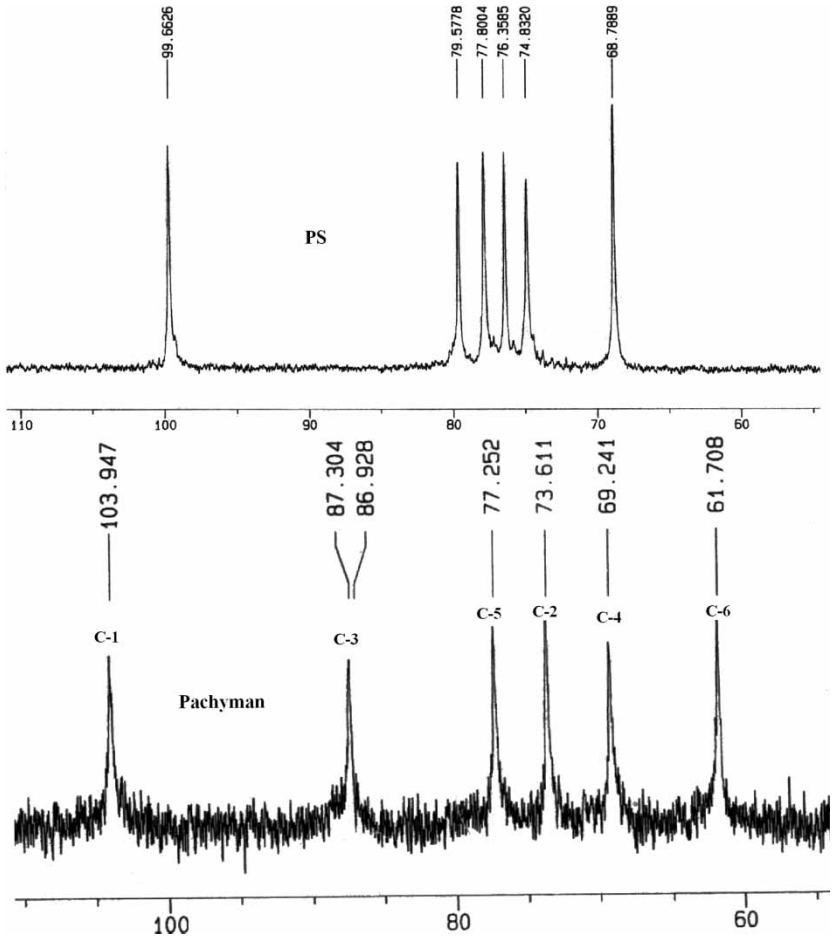

Figure 3. ^{13}C NMR spectra of pachyman (lower) and PS (upper).

polysaccharide, probably owing to the steric hindrance.[7] From the results of ^{13}C NMR spectra of pachyman and PS in Fig. 3, a complete sulfation of the hydroxyl groups at position C-6 is confirmed by the 7 ppm downfield shift of C-6 atom.[17] The 4.2 ppm upfield shift of C-1 is caused by adjacent C-2 being substituted.[18] The new peaks at 79.6 ppm for the PS may be assigned to fully substituted C-2,[18] which is a 6 ppm downfield shift compared with that of pachyman. The chemical shifts in 74–80 ppm are designated as the signals of C-3, C-4, and C-5 because C-4 directly attached to electronegative sulfate ester groups would shift to lower field position, whereas C-3 and C-5 indirectly attached to sulfate ester groups would shift to higher field position.[19]

It is noted that the DS of PS was 2.3, whereas the results of ^{13}C NMR of PS denote that C-2, C-4, and C-6 were substituted completely, that is, DS should be 3. This may be due to the weak or overlapped signals of unsubstituted C (C-2 or C-4).

Characterization of the Interaction of AA Binding to PS

Azar A was very responsive to carboxyl and sulfation-rich macromolecules.[20] Typical absorption spectra of AA binding to PS are illustrated in Fig. 4, which shows the intensity of the absorption peaks at 625 nm decreased while a new series of absorption peaks at 522 nm emerged with increasing PS concentration. This new peak series and two isobestic points formed at 552 and 697 nm were attributed to the formation of AA–PS complexes in aqueous solution and the molar absorptivities at 552 and 697 nm of AA and AA–PS complex being equal to each other.[21] The decrease in absorbance at 625 nm was about four times as sensitive as the increase in absorbance at 522 nm in the linear range. Thus, the absorbance at 625 nm was used thereafter in order to obtain the maximum sensitivity. The interaction between AA and PS led to a change in dye conformation; this conformation change resulted in hydrophobic interactions between dye molecules binding to PS, which produced a color change in the mixture.[10–12]

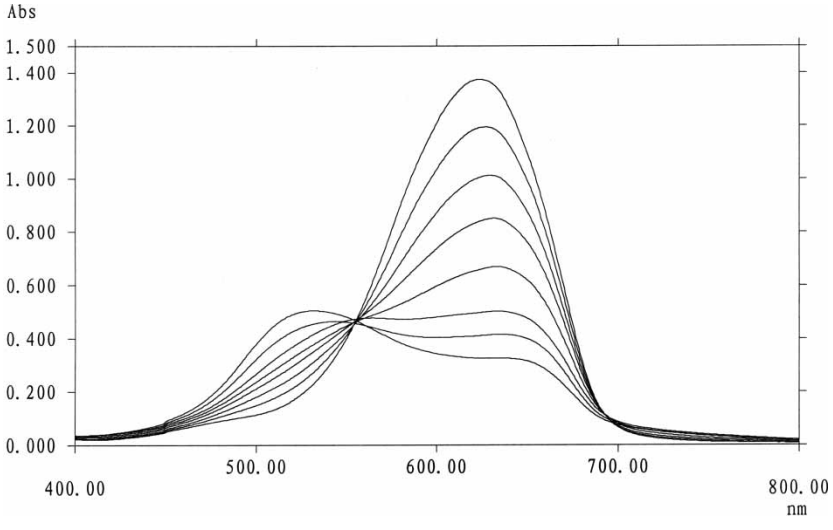

Figure 4. Absorption spectra of PS–AA mixtures. In order of decreasing and increasing absorbance peaks at 625 and 522 nm, respectively, the PS concentrations were 0, 1×10^{-3}, 2×10^{-3}, 3×10^{-3}, 4×10^{-3}, 5×10^{-3}, 6×10^{-3}, and 7×10^{-3} g/L in total assay volume. The AA concentration was constant at 5.49×10^{-5} mol/L, and pH = 7.00.

Model for AA–PS Interaction

Based on the dye–polysaccharide interaction mechanism proposed by Jiao et al.,[10,11] the following equation was obtained:

$$\Delta A = \Delta\varepsilon(1 + KD_t)/K - \Delta\varepsilon N(D_t\Delta\varepsilon/\Delta A - 1)C_p \quad (2)$$

where $\Delta A = A_0 - A$ and $\Delta\varepsilon = \varepsilon_b - \varepsilon_f$, in which A and A_0 are the absorbances of the pure AA and AA–PS solutions, respectively, with the same AA concentration; ε_b and ε_f are the molar absorptivities of the free and bound dye, respectively; ε_b and ε_f are constant and are determined from the absorbance of the pure AA solution and the AA–PS solution with an excess amount of PS, respectively. The value of $\Delta\varepsilon$ represents the sensitivity of the dye–polysaccharide binding assay at a constant dye concentration. $K = D_b/(D_f \times C_p)$, in which D_b and D_f are the binding dye and free dye concentrations, respectively, and C_p is the total polysaccharide concentration in the solution expressed in mol/L. D_t is the total dye concentration. N is the maximum binding number when C_p is expressed in mol/L; this is the maximum number of dye molecules that interact with one polysaccharide macromolecule. The maximum-binding number is a useful parameter because it can be used to calculate the molecular weight of polysaccharide.

The values of K and N can be calculated from the intercept and slope, respectively, from the linear plot of ΔA and $(\Delta A_{\max}/\Delta A - 1)C_p$ according to Eq. (2). In the following section, application of Eq. (2) to the effect of the concentration of AA is presented in order to further understand the AA–PS interaction.

Model Prediction of the Effect of Azur A Concentration

The effect of dye concentration on color yield for PS concentrations from 0 to 0.007 g/L as a function of AA concentration from 2.745×10^{-5} to 6.863×10^{-5} mol/L is shown in Fig. 5. It can be seen from Fig. 5 that decreases in absorbance at 625 nm were not linear to the increase in the PS concentration for the AA concentrations from 2.745×10^{-5} to 5.490×10^{-5} mol/L; the deviation from the linearity is more significant at lower AA concentrations. This is because the amount of AA molecules at low AA concentrations is less than that of the binding sites on PS molecules so that there is not enough AA binding to the PS molecules as the PS concentration increases. However, when the AA concentration is greater than 6.863×10^{-5} mol/L, it was found that the dye AA was easily deposited on the wall of a tube or cuvette in this work.

Application of Eq. (2) to the above data at all the AA concentrations from 2.745×10^{-5} to 6.863×10^{-5} mol/L yielded a series of highly correlated linear lines with correlation coefficients greater than 0.999, as shown in

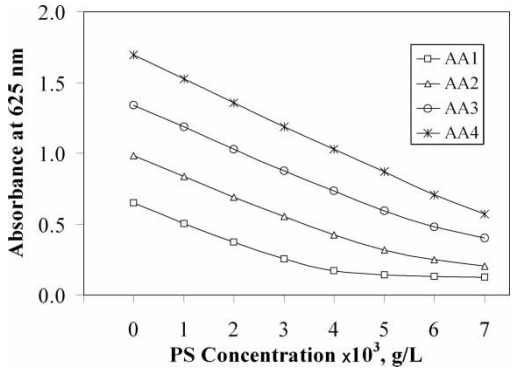

Figure 5. Absorbances of PS–AA complex at different concentrations of AA solution at 625 nm, pH 7.0. AA concentrations are (AA1) 2.745×10^{-5}, (AA2) 4.118×10^{-5}, (AA3) 5.49×10^{-5}, and (AA4) 6.863×10^{-5} mol/L.

Fig. 6. This indicates that the dye/PS concentration ratios did not affect the linear line of ΔA versus $(D_t \Delta\varepsilon/\Delta A - 1)C_p$ in Eq. (2) and confirms that the mechanism proposed by Jiao et al.[10,11] is also applicable to the AA–PS interaction. Thus, the experiment can be carried out at the lower AA concentrations to avoid the AA deposition on the wall of a tube or cuvette.

The effect of AA concentration on $\Delta\varepsilon$ and K values is shown in Fig. 7. The variation of $\Delta\varepsilon$ values seen in Fig. 7 indicates that the sensitivity of the AA–PS binding assay was essentially constant with decreasing AA concentration in the range studied. The K value decreases with increasing AA concentration, and the decrease in K values is more significant at the lower AA concentration.

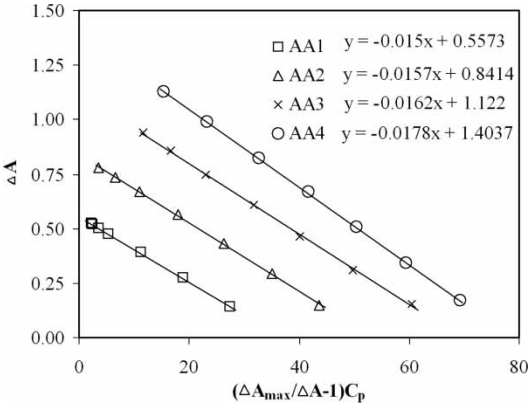

Figure 6. Typical linear regression line for $(\Delta A_{max}/\Delta A - 1)C_p$ versus ΔA according to Eq. (2) pH = 7.0 in all solutions.

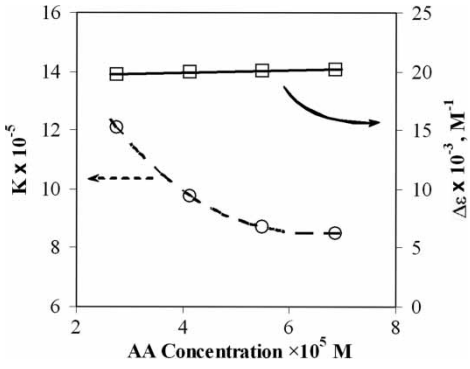

Figure 7. Effect of AA concentration on values of K and $\Delta\varepsilon$ according to Eq. (2). pH = 7.0 in all solutions.

The effect of AA concentration on N is shown in Fig. 8. It can be seen that the N value decreases with decreasing AA concentration, toward an asymptotic value, 76.03, when an infinitely dilute AA solution is approached. It is very likely that AA molecules that are close to a PS macromolecule will block other molecules that are far from the PS macromolecule from approaching the PS macromolecule at high AA concentrations. This self-interference of AA on AA–PS interaction, which was also reported for the AA binding interaction to hyaluronic acid,[12] suggests that the molecular weights of polysaccharides such as PS in this work can easily be overestimated if the N value obtained at high AA concentrations is used to calculate the molecular weight.

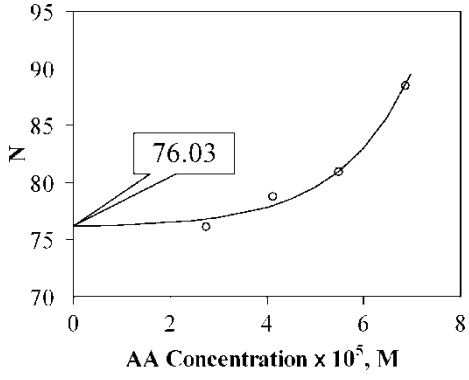

Figure 8. Effect of AA concentration on values of N according to Eq. (2). pH = 7.0 in all solutions.

Using the asymptotic N value of 76.03 and the value of DS (i.e., 2.3), the molar mass of PS used in this work, MW_{PS}, can be calculated as:

$$MW_{PS} = 76.03 \div 2.3 \times (162 + 102 \times 2.3) = 13{,}110\,\text{Da}$$

where 162 is the molecular weight of glucoside; 102 is equal to the molecular weight of -SO_4 Na (i.e., 119) subtracting that of -OH (i.e., 17). The MW_{PS} value of 13,110 Da agreed well with that of 10,233 Da calculated from HPLC results.

CONCLUSIONS

High-purity polysaccharide of pachyman isolated from the powder of *Poria cocos* sclerotium was reacted with chlorosulfonic acid to obtain pachyman sulfate. The yield of the pachyman and PS were 77.8% and 75%, respectively. The DS and molecular mass were important biochemical parameters for the PS. The PS spectra results of FTIR and NMR showed typical sulfation substitute of glucoside hydroxyl and almost complete sulfation on C-6, C-2, and C-4 of glucoside. The $[\alpha]_D^{25}$ of PS was -17.55°; the intrinsic viscosity, $[\eta]$, was 20.1 mL/g; sulfur content was 18.52%.

The binding of AA on PS was due to electrostatic force. The effect of AA concentration on AA–PS interaction indicated that there existed a self-interference of AA with the AA–PS interaction. Molecular weight of PS was estimated using an asymptotic maximum binding number when AA solution approached dilution. The molecular weight of 13,110 Da obtained with this improved method was in close agreement with 10,233 Da calculated from HPLC results.

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