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Chemical characterization of *Artemisia* seed polysaccharide

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Abstract

Artemisia seed polysaccharide (ASP) has great potential to be used as stabilizer, water retention agent and filmogen due to its high viscosity. This study reports analyses of its composition and molecule weight using gas chromatography (GC) and light scattering techniques. The molecular structure of the ASP at different concentrations was observed with atomic force microscopy (AFM). The relationship between structural and physical characteristics of ASP was also described in details. Our results showed that the ASP was composed of L-arabinose (L-Ara), D-xylose (D-Xyl), D-lyxose (D-Lyx), D-mannose (D-Man), D-glucose (D-Glu) and D-galactose (D-Gal). Its molecular weight was about 1.42×10^5. The ASP molecule had a linear backbone with branches. The network structure of ASP was formed through entanglement between the molecules of the polysaccharide.

Keywords: *Artemisia* seed polysaccharide; Atomic force microscopy; Images; Monosaccharide constituents; Molecular weight

1. Introduction

Artemisia sphaerocephala Krasch (Asteraceae) is widely distributed in Gansu province and Inner Mongolia Autonomous Region, China (Chinese flora editorial committee, 1991). The surface of its seeds is covered by a layer of gum, *Artemisia* seed polysaccharide (ASP). ASP has been found 1800 times more viscous than gelatin. Its strong hydroscopicity can make it absorbing water as much as up to 60 times of its own weight. Due to these properties, ASP can be used as thickener, stabilizer, water retention agent, filmogen, etc. (Bai, Yong, & Yun, 2000). However, its monosaccharide components and molecular structure remains unknown.

Recently, the AFM has emerged to be a very powerful tool for single-molecule force spectroscopy experiments and for microcosmos observation in material analysis (Ludwing et al., 1999; Brant, 1999). It was used to characterize surface structures of non-conductive materials by directly scanning gaseous or aqueous samples (Brant, 1999; Round, Mac Dougall, Ring, & Morris, 1997; Capron, Alexdander, & Muller, 1998). For AFM observations, there are three classes of tip-sample interactions: tapping mode, contact mode and non-contact mode. Biological samples can be observed and imaged un-destructively by choosing a suitable mode (Morris, Gunning, & Kirby, 1997). There are also different sample carriers for AFM analysis such as mica, single-crystal silicon slice, graphitic materials and some bio-membranes. The AFM has been used to study morphology and reaction mechanism of biological macromolecules, and to determine molecular structure of polysaccharides.

In this study, the analyses of monosaccharide components of the ASP were conducted with gas chromatography (GC). The molecular weight was determined using light scattering method and the structure was imaged under atomic force microscopy (AFM).

2. Material and methods

2.1. Materials

Seeds of *A. sphaerocephala* Krasch were collected from the Inner Mongolia Autonomous Region of China. Pure

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monosaccharides standards of D-(+)-galactose, D-(+)-glucose, D-(+)-mannose, L-(−)-arabinose, D-(+)-xylose, D-(−)-arabinose and D-(−)-lyxose were obtained from China Sigma–Aldrich (Shanghai, China).

2.2. ASP extraction

The polysaccharide was extracted and purified following the reported procedure (Chen & Tian, 2003; Bao & Fang, 2001). Briefly, the *Artemisia* seed powder was kept in distilled water at 80 °C for 12 h under constant stirring. After removing the debris by centrifugation, the aqueous extracts were mixed with 95% ethanol (1:3, v/v), followed by centrifugation at 5000 rpm for 10 min. The pellets containing ASP were dissolved in double deionized water (dd H_2O) and the extraction procedure was repeated. The final ASP extracts were dissolved in dd H_2O and treated with H_2O_2 to obtain colorless and clear solutions. The associated proteins in the ASP solution were removed using the Sevag method (Wang, Li, Chen, & An, 2003).

2.3. Purification of ASP extracts and determination of its homogeneity

The extracted polysaccharide was further purified by gel permeation chromatography (GPC) (Zhang, 1999) on an Ultrahydrogel™ 500 column (Waters, USA), at a concentration of 1.000 g/l and flow rate of 0.08 ml/min. The purity of ASP collections was monitored under ultraviolet (UV) light at 280 nm. To determine the homogeneity of gel-purified ASP, the collection was resolved on a 1.0% agarose gel by electrophoresis and stained with toluidine blue.

2.4. Determination of the molecular weight of ASP

The molecular weight of the ASP was determined by following the method of Zhang (1999). The purified ASP was diluted with dd H_2O to the final concentrations of 0.400 mg/ml, 0.200 mg/ml, 0.133 mg/ml and 0.100 mg/ml. After filtration (0.2 μm) and de-dusting, the molecular weight was determined at 488 nm on a molecular weight analyzer (BI-200SM/9000AT, goniometer/autocorrelator and BI-MwA; Brookhaven Instruments Corporation).

2.5. Composition analysis

The composition was analyzed by following the procedure of Zhang (1999). Briefly, 10 ml of ASP extract was dissolved in 4 ml of 4 M Trifluoro acetic acid acetate (TFA, CF_3COOH) in a test tube and then hydrolyzed at 120 °C for 10 h under airtight condition. TFA was then evaporated through decompression and distillation. When the tube was dry, 10 mg of ammonium hydrochloride and 0.5 mg pyridine were added and allowed to react in a 90 °C water bath for 30 min. Then 0.5 ml cold (kept at 4 °C in a refrigerator) acetic anhydride was added to the test tube and the mixture was incubated in the 90 °C water bath for another 30 min to allow the acetylation reaction to complete. The end product was decompressed and distilled to dryness. The acetate derivatives were analyzed by GC with a HP-5 capillary column (HP6820, Hewlett – Packark). The temperature program was set to increase from 120 to 300 °C at an increment of 5 °C/min and N_2 was the carrier gas. The standard monosaccharides were measured following the same procedure.

2.6. ASP imaging with atomic force microscopy

ASP stock solution (1 mg/ml) was prepared by adding some purified ASP into dd H_2O. The aqueous solution was stirred for about 1 h at 80 °C in a sealed bottle under N_2 stream so that the ASP was dissolved completely. After cooled to room temperature, the solution was diluted to the final concentration of 1, 2 and 10 μg/ml. About 10 μl of diluted ASP solution was dropped on the surface of mica sample carrier, allowed to dry (Kirby, Gunning, & Morris, 1995), and then imaged in air at room temperature. The atomic force microscopy used in this study was a SPI3800N instrument and was operated in the tapping-mode. The resulting imaging force was estimated to be 0.05–1.6 nN and the resonant frequency was about 2 KHz (Gunning et al., 2003).

3. Results and discussion

3.1. Purity and homogeneity of ASP preparation

The UV-spectroscopic quantification of proteins is dependent upon the presence of aromatic amino acids in the proteins. Tryptophan and tyrosine absorb ultraviolet light strongly at 280 nm. The tryptophan and tyrosine content of many proteins remains fairly constant, and so the absorbance of a protein solution at 280 nm can be used to determine its protein concentration. In this study, when the ASP gel filtration collection was measured under UV light, the absorbance at 280 nm was zero, indicating that protein contamination in the ASP solution was undetectable. The purity of the extracts was good for further characterization. When the polysaccharide was resolved on an agarose gel, only one spot was observed (Fig. 1), confirming that the ASP collection contained only one kind of polysaccharide molecule and it was homogeneous.

3.2. ASP monosaccharide composition

Fig. 2b showed all the peaks of eluents when the ASP acetate derivatives were analyzed on GC. Compared with the monosaccharide standards (Fig. 2a), the peaks of the ASP acetate derivatives were identified as L-Ara, D-Xyl, D-Lyx, L-Man, L-Glc and L-Gal, respectively. It is thus concluded that the ASP was composed of these six monosaccharides and their molar ratio is 1:5:2:28:4:14 (Table 1).

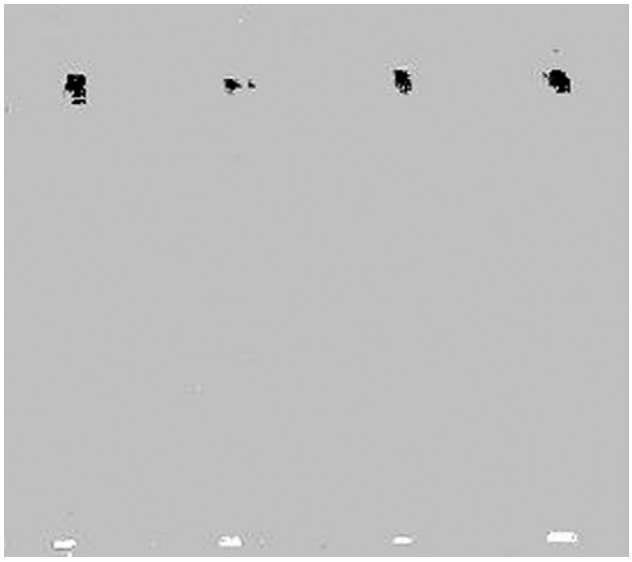

Fig. 1. Agarose gel electrophoresis and toluidine blue staining of *Artemisia* seed polysaccharide (ASP) from *A. sphaerocephala* Krasch. Seed polysaccharides of *A. sphaerocephala* Krasch in 1.0% agarose gel in Tris–HCl buffer (pH 9.1), (Zhang, 1999) and stained with 0.1% toluidine blue in acetic acid/ethanol/water (0.1:5:5, v/v/v).

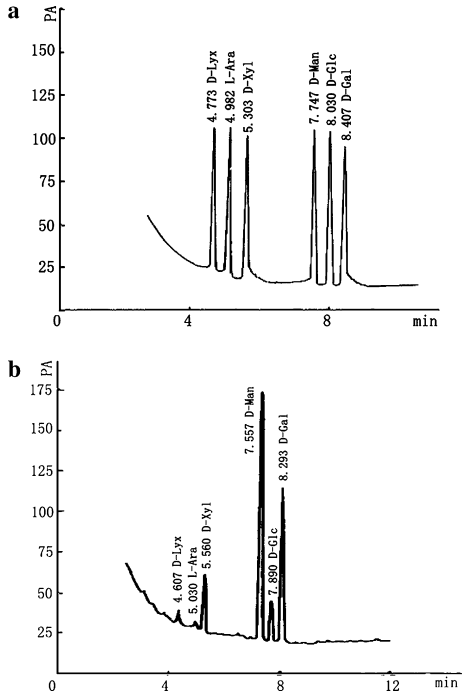

Fig. 2. (a) Relative retention times on GC of acetate derivative of standard monosaccharides. (b) Relative retention times on GC of acetate derivative of *Artemisia* seed polysaccharide (ASP) from *A. sphaerocephala* Krasch. GC analysis of the stand monosaccharides (SMs) and ASP. *Artemisia* seed polysaccharide (ASP) and pure standard monosaccharide (SMs) (Sigma) were treated with the standard acetate derivative method and injected into the GC HP-5 capillary column (HP6820, *manufacturer's name*). The temperature was programmed to increase from 120 to 300 °C at the rate of 5 °C/min and N_2 was the carrier gas.

Table 1
Peak area of standard monosaccharide and ASP hydrolysates by GC method

SM	D-Lyx	L-Ara	D-Xyl	D-Man	D-Glc	D-Gal
BIANK	4.773	4.982	5.303	7.747	8.03	8.407
ASP	5.03	5.127	5.56	7.557	7.89	8.293
ASP + D-Lyx	4.807	5.092	5.362	7.682	7.893	8.350
ASP monosaccharide composite molar ration	1.74:1:4.64:27.6:3.90:14.4					

3.3. ASP molecular weight (Mw) and structure

Based on the analysis of light scattering at 488 nm (Fig. 3), ASP molecular weight was about 1.42×10^5 and the second viral coefficient was 3.64×10^{-5} cm^3 mol/g^2. The Radius of Gyration was about 102.5 nm, and the mean square end to end distance was $102.5 \times 6 = 615$ nm. The size of one five-member ring of the ASP was 0.24 nm and six-member ring was 2.878 nm. The size of one glycosidic bond was 3.609 nm. In ASP, the molar ratio of L-Ara, D-Xyl, D-Lyx, D-Man, D-Glc and D-Gal was 1:5:2:28:4:14, so the molecular weight of one structural motive was 8534, and the size of one structural motive was 32.95 nm. The contour length of the ASP with molecular weight of 1.42×10^5 was approximately 548 nm. Because 548 nm/615 nm < 1, so the molecular shape of ASP should be highly branching.

3.4. AFM images of ASP

Fig. 4 presents ASP images obtained when 1 μg/ml of ASP solution was applied onto the atomic force microscopy. As shown in Fig. 4a, both A and B had the same molecule diameter, which was measured to be 2.53 nm. The wider strand C (5.74 nm) was formed by entanglement between A and B. Moreover, C and D were further entangled with each other to form a much wider chain.

The images in Fig. 4a–d and were very similar. The most obvious difference is that the strands became wider as more molecules were entangled. In Fig. 4b, the width of E and F was about 5.46 nm. G was the stand entangled between E and F and its width became 13.80 nm. Similarly, in Fig. 4c, the width of H and I was 42.84 nm and J was about 142.8 nm. In Fig. 4d, the width of K and I was 83.9 nm and M was 298.4 nm.

When 1 μg/ml of ASP was applied to the mica carrier, a single ASP molecule entangled to form wider strands and these strands could cross-link (Fig. 4a). As the entanglement process goes on, the ASP molecules would appear more and more enlarged. In addition, only parts of the ASP molecules, not the whole structure, entangle together because of the steric hindrance. Thus the ASP should have branches on the linear molecules. This conclusion supports the result of light scattering analysis. At the concentration of 2 and 10 μg/ml (Fig. 5), more ASP molecules were deposited on the mica. In this case, ASP chains are associated with each other to form loops (Gunning, Kirby, & Morris, 1996).

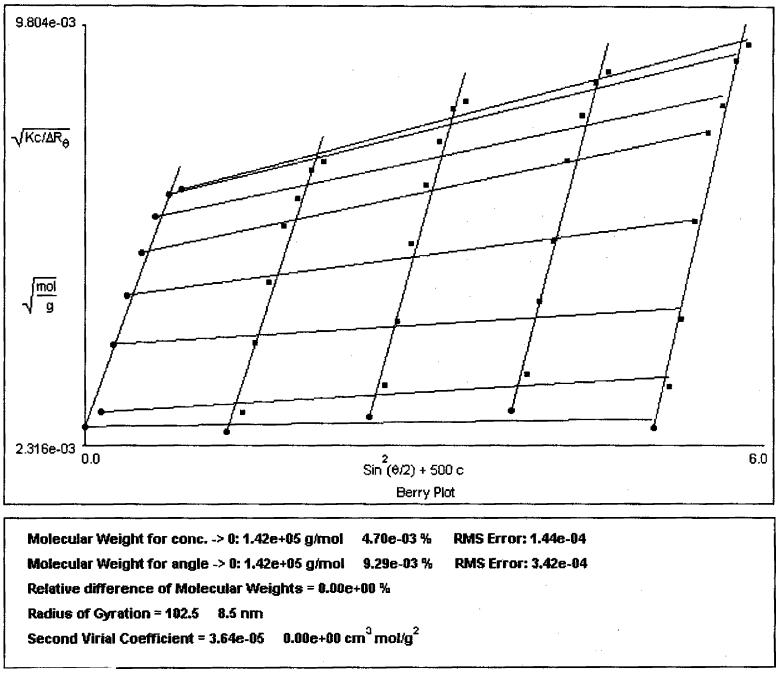

Fig. 3. Berry plot of ASP. Berry Plot for determination of molecular weight and molecular size of *Artemisia* seed polysaccharide (ASP) from *A. sphaerocephala* Krasch using light scaterring method. The instrument was BI-200SM/9000AT, goniometer/autocorrelator and BI-MwA; Brookhaven Instruments Corporation. The measurement was taken at 488 nm wavelength.

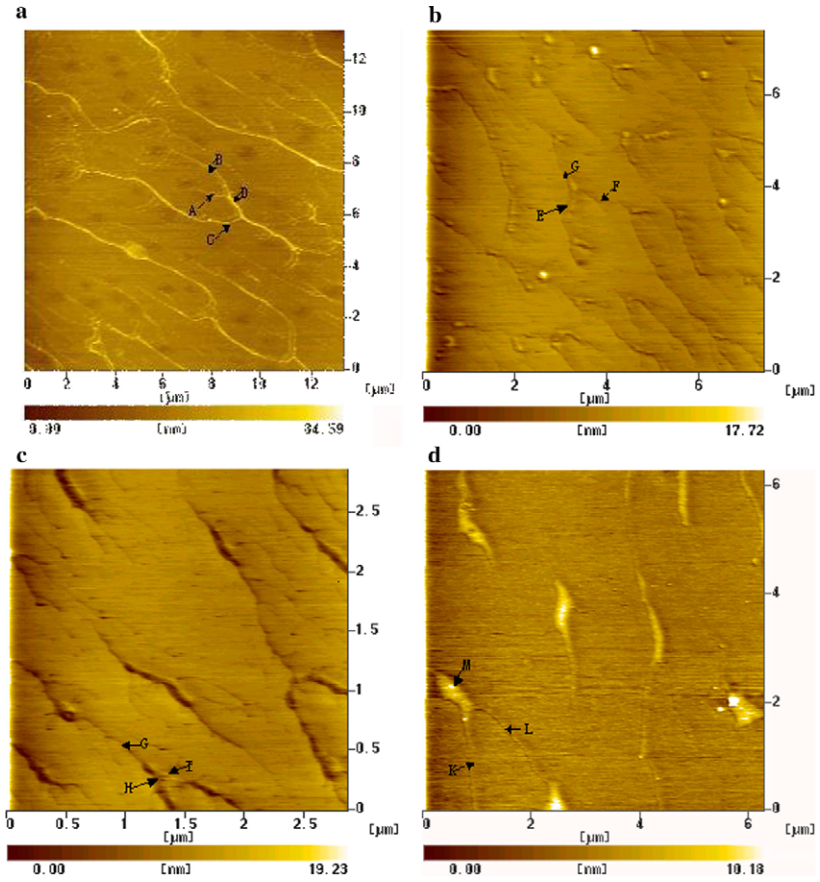

Fig. 4. Atomic force microscopy (AFM) images of linear molecules of *Artemisia* seed polysaccharide (ASP) from *A. sphaerocephala* Krasch. The ASP concentration was 1 μg/ml. The atomic force microscopy was an SPI3800N instrument and was operated in the tapping-mode. The resulting imaging force was estimated to be 0.05–1.6 nN and the resonant frequency was about 2 KHz (Kirby et al., 1995).

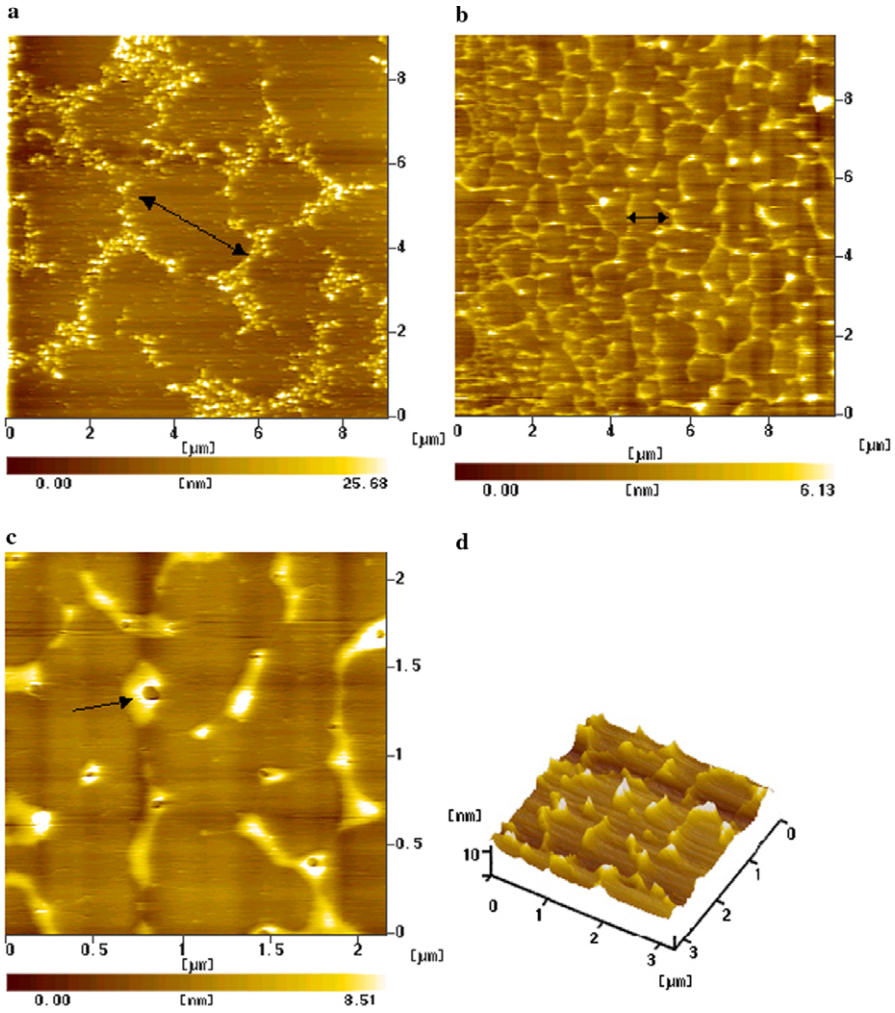

Fig. 5. Atomic force microscopy (AFM) images of network structure of *Artemisia* seed polysaccharide (ASP) from *A. sphaerocephala* Krasch. The ASP concentrations were 2 μg/ml (a) and 10 μg/ml (b–d). The atomic force microscopy was an SPI3800N instrument and was operated in the tapping-mode. The resulting imaging force was estimated to be 0.05–1.6 nN and the resonant frequency was about 2 KHz (Kirby et al., 1995).

Entanglement of these loops can form network structure, i.e., gel.

In Fig. 5a, the arrow-pointed aperture was about 2860 nm whereas the average aperture was about 2000 nm. Fig. 5b–d show the ASP images when a higher concentration of ASP (10 μg/ml) was applied to the mica. A strong 3-dimensional network structure appeared on the image. The aperture was about 100–1200 nm, indicating that the degree of molecular cross-linking and the acting force increased with ASP concentration (Fig. 5b–d). When ASP concentration was low, the molecules formed the structure with bigger apertures (Fig. 5a). On the contrary, when the ASP concentration was high, the molecules formed more but smaller loops as shown in Fig. 5b.

The feature of the images was in agreement with the strong hydroscopicity of ASP. Because of this three-dimensional network structure, just like sponge, the ASP can absorb large amount of water in the aqueous solution. On the other hand, due to the strong interaction between water molecules and the hydroxyl groups (–OH) of polysaccharides in the solution, ASP releases water slowly. Therefore, ASP can be used as water retention material. In addition, the fluidity of ASP solution could be very low because of strong network structure. This may explain the high viscosity of ASP.

Fig. 5c is an enlarged image of Fig. 5b. It clearly shows the network structure and branches of ASP molecules. We observed that one strand has crossed over another strand in light spot (arrow). The concave in the three-dimensional images in Fig. 5d correspond to loops in 2D images of Fig. 5b. This characteristic of the ASP molecules explains the mechanism of its strong hydroscopicity because excessive water would be need to fill the concaves.

4. Conclusion

The molecule of ASP was 1.42×10^5 g/mol. The polysaccharide was composed of L-Ara, D-Xyl, D-Lyx, D-Man, D-Glu and D-Gal. From light scattering data, it can be concluded that ASP structure is highly branching. Data on the AFM images showed that ASP is a linear molecule with branches. In the solution, the linear molecules could

entangle with each other to form strong network structure, i.e., gel. However, the way for the cross-linking of ASP molecules remains unknown. Further research on ASP is necessary to study this important ecological material.

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