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Extraction, fractionation and physicochemical characterization of water-soluble polysaccharides from *Artemisia sphaerocephala* Krasch seed

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ABSTRACT

The extraction and fractionation methods as well as the physicochemical properties of *Artemisia sphaerocephala* Krasch polysaccharide (ASKP) were studied. ASKP was extracted from the outer layer of the seeds with high ratio of hot water to seeds (400:1, v/w). The yield of water-soluble ASKP was 13.6% (w/w). High performance size exclusion chromatography (HPSEC) results indicated that ASKP had two components with different molecular weights. The high molecular weight (Mw, 551.3 KDa) fraction 60P was separated from the low molecular weight fraction 60S (Mw, 38.7 KDa) by precipitation in 60% (w/v) ammonium sulfate salt solution. Steady flow rheological tests revealed that the apparent viscosity of 1.5% (w/v) ASKP decreased logarithmically with the increasing temperature. Small strain oscillation tests suggested that 4% (w/v) 60P solution was a typical viscous fluid. ASKP was composed of 66.9% (w/w) neutral sugar and 15.8% (w/w) uronic acid; 60P comprised of 55.4% (w/w) neutral sugar and 25.8% (w/w) uronic acid, while in 60S, the percentage of neutral sugar and uronic acid were 87.1% and 10.4%, respectively. Relative monosaccharide composition analysis showed that 60S was composed of 38.3% glucose, 28.1% mannose, 24.2% galactose and 9.4% arabinose, while the 60P fraction contained 80.5% xylose, 10.9% arabinose, 5.0% glucose, 2.3% galactose and 1.2% rhamnose. 60S exhibited the highest surface activity compared to ASKP, 60P, ASKPE (ASKP with protein removed) and 60PE (60P with protein removed) although no protein was detected in this fraction.

1. Introduction

Artemisia sphaerocephala Krasch (ASK) is widely distributed in the desert and semi-desert regions of Shaanxi, Gansu and Inner Mongolia provinces in China. Seed of ASK, a traditional Chinese medicine and food additive, has been reported to have biofunctional properties such as detumescent (Bai, Yong, & Yun, 2000), antidiabetic and antioxidant effects (Hu et al., 2011; Xing, Zhang, Hu, Wu, & Xu, 2009; Zhang, Huang, Hou, & Wang, 2006). Artemisia seed polysaccharide (ASKP) is found in the outer layer of ASK seeds and is wildly used as thickener, stabilizer, water retention agent and film forming agent (Bai et al., 2000).

Most previous studies focused on the bioactivities and application of ASKP. Xing et al. (2009) found that ASK gum was able to alleviate hyperglycemia, hyperlipemia and insulin resistance in streptozotocin-induced type 2 diabetic rats. Hu et al. (2011) demonstrated the antioxidant effects of ASK gum on streptozotocin-induced type 2 diabetic rats; A study from Zhang et al. suggested that at a dose of 200 mg/kg body weight, ASKP could produce a significant decrease in blood glucose levels ($P < 0.01$) in alloxan-induced diabetic rats (Zhang et al., 2006). ASKP was also used as a food additive in food products to increase viscosity and improve mouth-feel (Huang, Liu, & Gu, 2007; Liu, Li, & Gu, 2006). However, to the best of our knowledge, only a couple of studies have reported on the physicochemical properties of ASKP and limited information available regarding its structural and conformational properties (Zhang et al., 2007) in food system.

The objectives of the present study were to develop extraction and fractionation methods, characterize the physicochemical properties in terms of monosaccharide composition, molecular weight, rheological properties and surface activities, and explore its potential applications in food industry.

2. Materials and methods

2.1. Materials

ASK seeds were purchased from a farm in Yulin, Shaanxi province of China. All chemicals were reagent grade.

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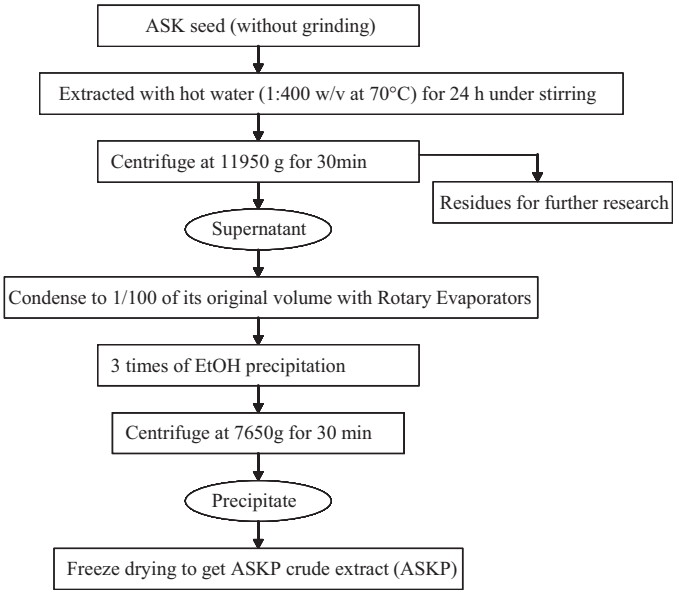

Fig. 1. Extraction of water-soluble polysaccharide from *Artemisia sphaerocephala* Krasch seeds.

2.2. Extraction and fractionation procedure for water-soluble ASKP

The extraction and fractionation procedures for water-soluble ASKP are demonstrated on Figs. 1 and 2 respectively. The 0.1 M NaOH adopted by the fractionation procedure was used to remove the ammonium sulfate residue (Wu, Cui, Eskin, & Goff, 2009). Three independent measurements were performed for both extractions and fractionation procedures.

2.3. Enzyme hydrolysis

In order to remove the protein from 60P and ASKP, protease (Megazyme, *Bacillus licheniformis*) was added (0.24 U/mL) to 0.2% (w/v) polysaccharide solution at 60 °C, pH 7.5 for 30 min with constant stirring, followed by dialysis (8000 Da Mw Cut-off) and freeze drying. The protein free samples were designated 60PE and ASKPE, accordingly.

2.4. Molecular weight determination

The molecular weight distribution profile was obtained by a high performance size-exclusion chromatograph (HPSEC) equipped with multiple detectors: a differential pressure viscometer (DP) for viscosity determination; a refractive index detector (RI) and a UV detector for concentration determination; a right angle laser light scattering detector (RALLS) and a low angle laser light scattering detector (LALLS) for direct molecular determination (Viscotek, tetra detector array from Malvern company).

2.5. Total uronic acid and total sugar determination

Total uronic acid was determined by the *m*-hydroxydiphenyl method (Blumenkrantz & Asboe-Hansen, 1973); total sugar was determined via the Dubois method using galactose, glucose and xylose (4:6:4 w/w) as standards (DuBois, Gilles, Hamilton, Rebers, & Smith, 1956). All the measurements were repeated three times.

2.6. Monosaccharide composition analysis

Monosaccharide compositions were determined by treating sample (20 mg) in 1 mL 12 M H_2SO_4 at 30 °C for 30 min, then diluting to 6 mL (2 M H_2SO_4) followed by hydrolysis at 100 °C for 2 h. Analysis was carried out using a high performance anion-exchange chromatograph (HPAEC) as per Wood, Weisz, and Blackwell, (1994). All the measurements were repeated three times.

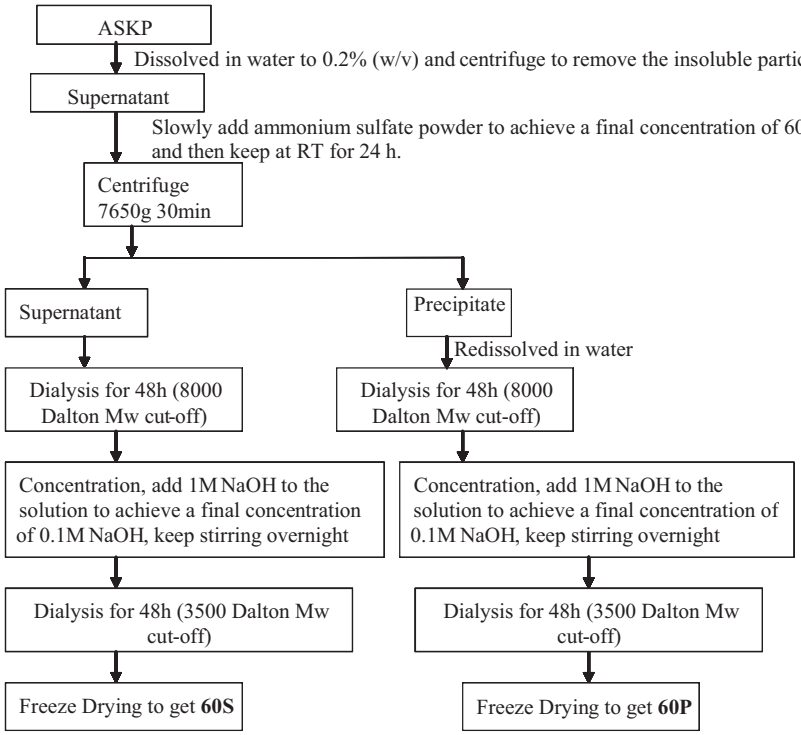

Fig. 2. Fractionation procedure of *Artemisia sphaerocephala* Krasch polysaccharide.

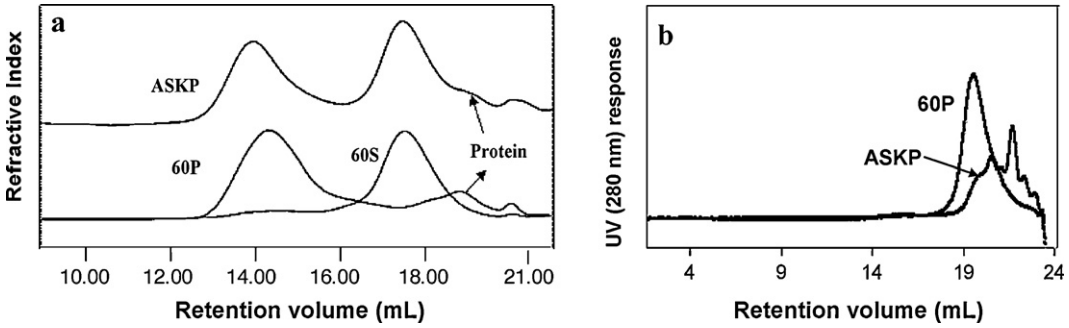

Fig. 3. The elution profiles of *Artemisia sphaerocephala* Krasch polysaccharide and its fractions. (a) monitored by RI detector; (b) monitored by UV detector.

2.7. Protein analysis

Nitrogen content was determined using a NA2100 Nitrogen and Protein analyzer (ThermoQuest, Milan, Italy); a conversion factor of 6.25 was used to calculate protein content. Each sample was measured in duplicate.

2.8. FT-IR analysis

FT-IR spectra of ASKP and its fractions were carried out on a Golden-gate Diamond single reflectance ATR in an FTS 7000 FT-IR spectrometer equipped with a DTGS detector (DIGILAB, Randolph, MA). The spectrum for each sample was recorded at absorbance mode from 1800 to 800 cm^{-1} at a resolution of 4 cm^{-1} with 128 co-added scans.

2.9. Rheological properties determination

All rheological measurements were performed on an ARES rheometer (TA instruments, New Castle, USA). All samples were prepared by dissolving the solute into 70 °C water with constant stirring for 2 h, followed by cooling at room temperature for 1 h. Cone and plate geometry (4°/50 mm) was employed for the steady flow and dynamic rheological tests with the gap size of 0.047 mm. Each sample was measured in duplicate.

2.10. Surface tension analysis

Surface tension was measured using a semiautomatic Surface Tensiomat model 21 (Fisher Scientific, Toronto, Canada) at 23.0 °C. The various concentrations of solutions were placed in 50 mL beakers (PYREX®). All samples were prepared by dissolving solute into 70 °C water with constant stirring for 2 h followed by cooling down at room temperature for 1 h. Samples were kept steady for another hour to reach equilibrium before tests. Each measurement was repeated six times.

3. Results and discussion

3.1. Extraction

Intact ASK seeds, instead of ground seeds (Zhang et al., 2007), were selected for extraction (Fig. 1) for two reasons. (1) As ASKP is present in the outer layer of the seed, it is unnecessary to grind the seed. (2) Contamination (such as protein and oil) could result from the interior of the seeds during the extraction process with ground seeds. For example, the protein content in the sample would be much lower when we use intact seed (around 16.6%) instead of ground one (around 25%) for the extraction. The water to material ratio of 400:1 was adopted in this procedure mainly due to the strong hygroscopicity of ASKP. If the ratio is too low, the water-soluble ASKP could be trapped in the mucilage and resulted in a relative low yield of ASKP. HPSEC results revealed that ASKP has two components with different molecular weights as shown in Fig. 3. The latter result is in disagreement with the report of Zhang et al. (2007), in which only one component was detected. This was probably due to the difference in the extraction method and the different source material.

3.2. Fractionation and molecular weight distribution

In the current study, 60% (w/v) ammonium sulfate was used to further separate the two components in ASKP as shown in Fig. 2.

Ammonium sulfate was mainly used to precipitate protein, especially enzyme, in the past (Braswell, Knox, & Frere, 1986; Seow, Jinap, Lee, & Lee, 1994). Recently, many researchers have used this technique to precipitate polysaccharides (Li, Cui, & Kakuda, 2006; Wang, Wood, Huang, & Cui, 2003). Based on the previous research, two mechanisms have been proposed: (1) NH_4^+ is able to suppress the negative charges on the outer layer of polysaccharides which can lead to polymer–polymer aggregation (Jullien, 1987; Wu et al., 2009); (2) ammonium sulfate is able to reduce and/or change the solvent properties of water for the polysaccharides which could lead to the precipitation polysaccharides with different molecular weight (Izydorczyk & Biliaderis, 1996)

In this study, the use of $(NH_4)_2SO_4$ (60%, w/v) allowed to fractionate ASKP into two main parts (Fig. 3a). Using multiple detectors, the molecular parameters were extracted and summarized in Table 1. The number average molecular weight (Mn) and weight average molecular weight (Mw) of 60P were 348.9 KDa and 551.3 KDa, respectively, which were much higher than those of 60S (Mn: 21.4 KDa and Mw: 38.7 KDa). The intrinsic viscosity of 60P (6.3 dL/g) and 60S (0.5 dL/g) showed the same trend as molecular weight. The polydispersity index values of 60P (1.6) and 60S (1.8) revealed that both fractions had wide distribution in molecular size (Table 1). The peak at retention volume of 18.83 mL was recognized as protein signal by UV detector at λ_0 = 280 nm, the different retention volume between protein and polysaccharide revealed that the protein was not conjugated with the polysaccharides (Fig. 3b). The molecular weight obtained for either fraction was different with that obtained by Zhang et al. (2007), in which the molecular weight was 142 KDa for the crude polysaccharide.

Table 1
Molecular characterization[a] of ASKP, 60P and 60S.

Fractions	Mn (KDa)	Mw (KDa)	[η] (dL/g)	Mw/Mn
ASKP	113.8	325.8	3.6	2.9
60P	348.9	551.3	6.3	1.6
60S	21.4	38.7	0.5	1.8

[a] Data was obtained using OmniSEC 4.6.1 software.

Table 2
Yield, total sugar, uronic acid and protein content for ASKP and its fractions (dry base).

Fraction	Yield (%)	Total sugar (%)	Uronic acids(%)	Protein (%)
ASKP	13.6 ± 1.2[a]	66.9 ± 5.9	15.8 ± 0.2	16.6 ± 0.1
60P	48.7 ± 1.0[b]	55.4 ± 3.6	25.8 ± 0.3	24.1 ± 0.4
60S	51.3 ± 1.0[b]	87.1 ± 1.1	10.4 ± 0.1	ND[c]

[a] Based on the total weight of seeds.
[b] Based on the weight of ASKP.
[c] Not detected.

3.3. Monosaccharide analysis of ASKP and its fractions

Yield, total sugar, and total uronic acid contents of ASKP, 60P and 60S are summarized in Table 2. The yield of ASKP was 13.6%. It contained 66.9% (w/w) neutral sugar and 15.8% (w/w) uronic acids. The yield ratio of the two fractions was close to 1:1 after precipitation with 60% (w/v) $(NH_4)_2SO_4$. 60P comprised 55.4% (w/w) neutral sugar and 25.8% (w/w) uronic acids, while in 60S, the percentage of neutral sugar and uronic acid were 87.1% and 10.4%, respectively. The protein contents of ASKP (16.6%, w/w), 60P (24.1%, w/w) and 60S (trace) revealed that after fractionation, all the protein was co-precipitated with 60P. It is worth noting that the total sugar content of the three fractions, especially 60P, could be overestimated, because uronic acids could also contribute to the colorimetric reaction in the phenol-sulfuric acid assay. This explains why the total percentage of total sugar, uronic acid and protein in 60P fraction was above 100% (Table 2).

Monosaccharide composition analysis (Table 3) showed that six types of monosaccharides were detected in ASKP although the amount of rhamnose was very small. The relative monosaccharide composition of 60S was 38.3% glucose, 28.1% mannose, 24.2% galactose and 9.4% arabinose, while the 60P fraction mainly contained 80.5% xylose and 10.9% arabinose. It also contained 5.0% glucose, 2.3% galactose and 1.2% rhamnose. It is worthwhile noting that the total percentage of neutral sugar for 60P (30.12%, w/w) and 60S (71.28%, w/w) determined by HPAEC was less than the values derived from colorimetric analysis (Table 2). This was probably caused by the incomplete hydrolysis in HPAEC test and/or the contribution of uronic acid in colorimetric analysis as aforementioned.

3.4. FT-IR analysis

The FT-IR spectra of ASKP, 60P, 60S, ASKPE (protein free ASKP) and 60PE (protein free 60P) are presented in Fig. 4.

Peaks in 800–1200 cm^{-1} (finger-print region) for ASKP, 60S and 60P fractions were different indicating these three fractions had different structure features, which was in agreement with monosaccharide composition analysis (Table 2). ASKP and ASKPE, 60P and 60PE, had no difference in this region indicating that protease treatment did not affect the structure of the polysaccharides, which further confirmed that the protein was not conjugated with the polysaccharide in ASKP and 60P. The signals at 1652.75 and 1529.12 cm^{-1} (Fig. 4) were likely derived from protein, as these

Table 3
Relative monosaccharide composition for ASKP and its fractions.

Fractions	ASKP	60P	60S
Monosaccharides (% w/w)			
Rhamnose	0.7	1.2	Trace amount
Arabinose	9.1	10.9	9.4
Galactose	18.1	2.3	24.2
Glucose	30.2	5.0	38.3
Xylose	23.8	80.5	ND[a]
Mannose	18.1	ND	28.1

[a] Not detected.

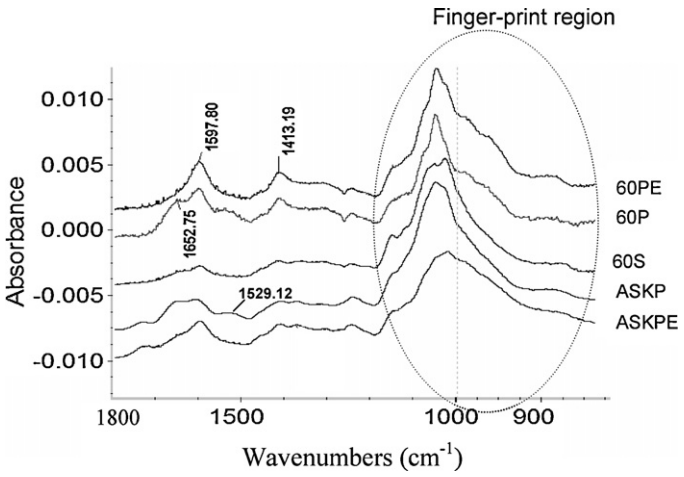

Fig. 4. FT-IR spectra of *Artemisia sphaerocephala* Krasch polysaccharide and its fractions (60PE: 60P with protein removed; ASKPE: ASKP with protein removed).

signals were observed in ASKP and 60P profiles but were absent in 60PE and ASKPE profiles. This result was also consistent with the previous research (Venyaminov & Kalnin, 1990), in which the two signals were attributed to amide band I (stretching vibrations of the C═O and C–N group) and amide band II (mainly from N–H bending), respectively. In addition, the peak observed at 1597.80 cm^{-1} (COO^- asymmetric stretching) and 1413.19 cm^{-1} (COO^- symmetric stretching) of the six fractions confirmed the presence of uronic acid (Table 1) (Cui, Phillips, Blackwell, & Nikiforuk, 2007).

3.5. Rheological properties

3.5.1. Effects of temperature on viscosity

The apparent viscosities of 1.5% (w/v) ASKP at different temperatures were investigated (Fig. 5). The apparent viscosity decreased with increasing temperature. For ideal Newtonian fluid, the relation of apparent viscosity is in accordance with Arrhenius equation (1).

$$\log\ \eta = \log A + \frac{E}{R}\frac{1}{T} \quad (1)$$

where A is a constant (Pa s), T is the absolute temperature (K), R is the gas constant (8.3144 J mol^{-1} K^{-1}), and E is the activation energy (kJ mol^{-1}).

Based on this equation, it is obvious that the apparent viscosity decreases logarithmically with increasing temperature. The

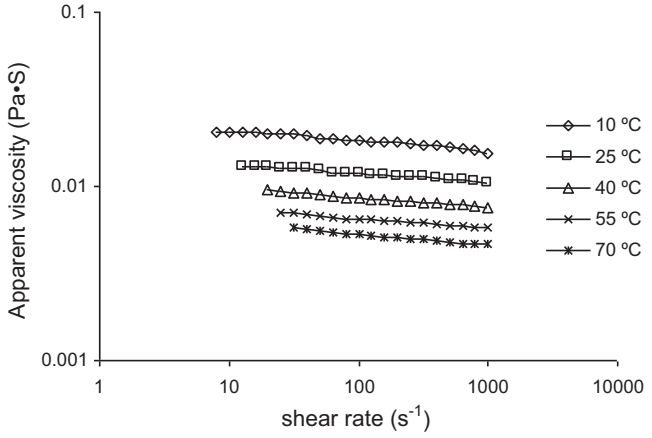

Fig. 5. Apparent viscosity of *Artemisia sphaerocephala* Krasch polysaccharide at various temperatures with the concentration of 1.5% (w/v).

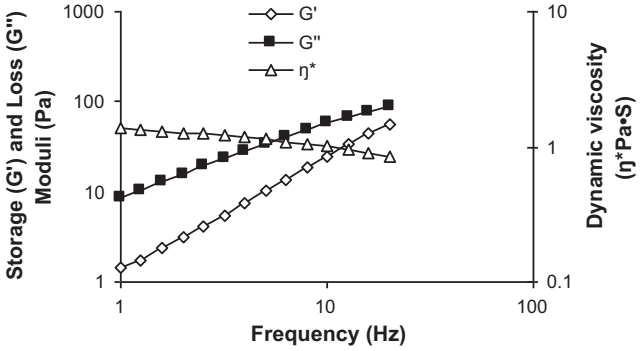

Fig. 6. Small strain oscillation of the high molecular weight fraction (60P). Temperature: 25 °C; concentration: 4% (w/v).

activation energy at shear rate 100 s^{-1} is 8.96 kJ mol^{-1}. The activation energy is related to the chain flexibility in solution. The relative low activation energy for ASKP indicated less inter- and intra-molecular interactions between polysaccharide chains in the investigated concentration, which could be due to the electrostatistic effect imposed by uronic acid (Balaghi, Mohammadifar, & Zargaraan, 2010).

The decrease of viscosity with the increasing temperature is caused by the disentanglement of polysaccharide chains; in some situations, increase of temperature could also cause conformational changes in solution; both could cause the decrease in viscosity of the solution (Nielsen, 1977).

3.5.2. Dynamic rheological properties

The dynamic rheological properties of 60P fraction at concentration of 4% (w/v) were investigated in the present study using small-strain oscillatory testing (Fig. 6). A typical G' (storage modulus), G'' (loss modulus)-frequency profile is demonstrated in Fig. 6. Both G' and G'' increased with the increase of frequency. At low frequency, G' was much lower than G''; with the increase of frequency, they became closer, but even at the frequency of 20 Hz, there was still no crossover; this observation indicated that 4% (w/v) 60P water solution is a viscous system. This phenomenon suggested that less inter-molecular association or entanglement was exist in the 60P water solution even the concentration was up to 4%.

3.6. Surface tension determination

The surface tension of six samples at different concentration was investigated (Fig. 7). Compared with the surface tension at the air/water interface (72.6 dynes/cm), ASKP and its various fractions demonstrated the ability to reduce the surface tension of pure water. In the concentration range of 0.01–1.50% (w/v), the trend of surface activity decreased in the order of 60S, 60P, ASKP, 60PE and ASKPE. The presence of protein played an important role in surface activity as seen by comparing the surface activity of ASKP and 60P with that of ASKPE and 60PE (without protein). However, 60S exhibited the strongest surface activity even no protein was detected. The surface activity was comparable to gum Arabic (Huang, Kakuda, & Cui, 2001). This result was probably caused by the following reasons: (1) the presence of some hydrophobic groups could be the main cause of the high surface activity as per Dickinson (2003). Some hydrophobic groups such methoxy and acetyl groups have been evidenced the presence by the NMR Data which will be shown in the future paper. (2) Compared with 60P, the smaller molecular weight of 60S could also make contribution on the surface activity (Garti & Leser, 2001). (3) Although no protein was detected in the test, small amount of protein could still be existed, which might carry weight on the surface activity of the system. To better understand its corresponding mechanisms, the emulsification properties of 60S and its structure information will be further investigated in future work.

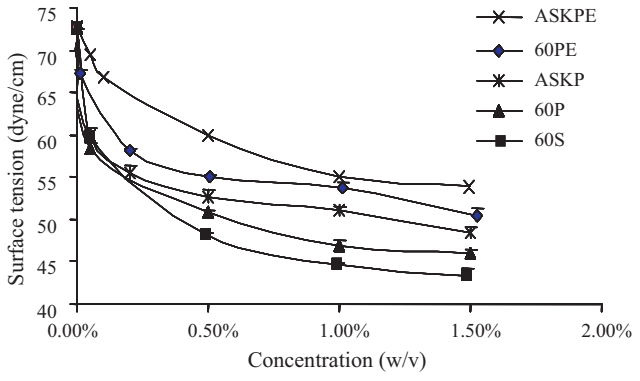

Fig. 7. Surface activity of *Artemisia sphaerocephala* Krasch polysaccharide and its fractions (60PE: 60P with protein removed; ASKPE: ASKP with protein removed).

4. Conclusion

In the current study, polysaccharide (ASKP) and its two fractions (60S and 60P) were obtained by 400:1 (v/w) hot water extraction and precipitation with 60% (w/v) ammonium sulfate solution. The Mw of 60P and 60S were 551.3 KDa and 38.7 KDa, respectively. ASKP was composed of 66.9% (w/w) neutral sugar and 15.8% (w/w) uronic acid; 60P comprised 55.4% (w/w) neutral sugar and 25.8% (w/w) uronic acid, while in 60S, the percentage of neutral sugar and uronic acid were 87.1% (w/w) and 10.4% (w/w) respectively. The apparent viscosity of 1.5% (w/v) ASKP decreased logarithmically with the increasing temperature and 60P formed typical viscous fluids in 4% (w/v) aqueous solutions. 60S exhibited substantial surface activity even no protein was detected.

Based on the aforementioned data, the physicochemical properties of 60P and 60S fractions are substantially different. Further structural and conformational research need to be conducted on 60P and 60S.

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