Coverletter

Microwave-assisted synthesis, structure and anti-tumor activity of selenized *Artemisia sphaerocephala* polysaccharide

Corresponding author: Junlong Wang

College of Life Science, Northwest Normal University, Lanzhou 730070,
People's Republic of China

Bioactive Products Engineering Research Center For Gansu Distinctive Plants,
Northwest Normal University, Lanzhou 730070, People's Republic of China

E-mail: nuno-vai@163.com Fax: +86-0931-7971414

Telephone number: +86-0931-7971999

Co-authors:

Xiaopin Yang

College of Life Science, Northwest Normal University, Lanzhou 730070,
People's Republic of China

E-mail: 13060729@qq.com

Aijuan Bao

College of Life Science, Northwest Normal University, Lanzhou 730070,
People's Republic of China

E-mail: wyq127127@163.com

Xinlong Liu

College of Life Science, Northwest Normal University, Lanzhou 730070,
People's Republic of China
E-mail: xlliu@163.com

Junyuan Zeng

College of Life Science, Northwest Normal University, Lanzhou 730070,
People's Republic of China
E-mail: 1622081261@qq.com

Xiurong Liu

College of Life Science, Northwest Normal University, Lanzhou 730070,
People's Republic of China
E-mail: xrliu@sina.com

Jian Yao

College of Life Science, Northwest Normal University, Lanzhou 730070,
People's Republic of China
Bioactive Products Engineering Research Center For Gansu Distinctive Plants,
Northwest Normal University, Lanzhou 730070, People's Republic of China
E-mail: yaojiani@nwnu.edu.cn

Ji Zhang

College of Life Science, Northwest Normal University, Lanzhou 730070,
People's Republic of China
Bioactive Products Engineering Research Center For Gansu Distinctive Plants,
Northwest Normal University, Lanzhou 730070, People's Republic of China

Key Laboratory of Eco-Environment-Related Polymer Materials Ministry of
Education, College of Chemistry and Chemical Engineering, Northwest Normal
University, Lanzhou 730070, People's Republic of China

E-mail: nuno-vai@hotmail.com.cn

Ziqiang Lei

Key Laboratory of Eco-Environment-Related Polymer Materials Ministry of
Education, College of Chemistry and Chemical Engineering, Northwest Normal
University, Lanzhou 730070, People's Republic of China

Tel: +86-0931-7971414 Fax: +86-0931-7970686

E-mail: leiziq@nwnu.edu.cn

Microwave-assisted synthesis, structure and anti-tumor activity of selenized *Artemisia sphaerocephala* polysaccharide

Junlong Wang^{a,b*}, Xiaopin Yang^a, Aijuan Bao^a, Xinlong Liu^a, Junyuan Zeng^a, Xiurong Liu^a,

Jian Yao^{a,b}, Ji Zhang^{a,b,c}, Ziqiang Lei^c

^a College of Life Science, Northwest Normal University, Lanzhou 730070, People's Republic of China

^b Bioactive Products Engineering Research Center For Gansu Distinctive Plants, Northwest Normal University,

Lanzhou 730070, People's Republic of China

^c Key Laboratory of Eco-Environment-Related Polymer Materials Ministry of Education, College of Chemistry

and Chemical Engineering, Northwest Normal University, Lanzhou 730070, People's Republic of China

* Corresponding author: Junlong Wang Address: College of Life Science, Northwest Normal University, Anning eastroad 967 Lanzhou China

Fax: +86-0931-7971414 e-mail: nuno-vai@163.com

Abstract:

In this work, selenylation of *Artemisia sphaerocephala* polysaccharide (SeASP_{MW}) was studied by using H₂SeO₃/HNO₃/BaCl₂ reaction system in microwave field. SeASP_{MW} exhibited the Se content range of 111-264 µg/g with high yields (72.1-94.9%). ¹³C NMR results indicated that the weak C-6 substitution was occurred. The decrease (from 7.348×10⁴ g/mol to 1.736-4.667×10⁴ g/mol) in weight average molecular mass (*M*_w) of SeASP_{MW} was observed in size exclusion chromatography combined with multi angle laser light scattering (SEC-MALLS) analysis. SeASP_{MW} exhibited a more rigid solution conformation which might be due to the degradation of polysaccharide chains in acidic reaction reagent. This was also supported by atomic force microscopy (AFM) result that SeASP_{MW} showed short chains and island-like topography. In anti-tumor activity assays, SeASP_{MW6} exhibited the inhibition rates of 32.381% and 39.776% against human non-small cell lung cancer cell line (H1650) at the concentration of 100 and 200 µg/mL, respectively. The relatively weak inhibition effect of SeASP_{MW} was not related to cell apoptosis and cell cycle arrest, suggesting Se content might be a key factor to influence the anti-tumor activities of selenized polysaccharides *in vitro*.

Keywords: Selenylation; microwave assisted synthesis; anti-tumor activity

1. Introduction

Selenium (Se) is an essential trace element which can be introduced as inorganic selenium or organic selenium. It is well known that organic selenium compounds exhibit low toxicity and high bioavailability. Reports suggest that selenium as a potential countermeasure against toxicity is mediated via sequestration of toxic elements into biologically inert conjugates and selenium-dependent antioxidant enzymes [1]. Recently, organic selenium compounds such as seleno-proteins and seleno-polysaccharides have emerged as important selenium supplements because selenium is a cofactor of many selenium-dependent enzymes [2]. The metabolism of inorganic selenium or organic selenium is fundamentally different in organism. Organic selenium supplement is reported as reducing the levels of mRNA encoding proteins known to be up-regulated during oxidative stress and cancerous states in animals [3].

Seleno-polysaccharides are organic selenium species with various biological activities. The chemical structure and anti-tumor activities of purified Se-polysaccharides from Se-enriched Ziyang green tea (Se-ZYTP) and *Ganoderma lucidum* (SeGLP-2B-1) are published. The cell viabilities in human osteosarcoma U-2 OS cells are 31.4% and 21.2% for 100 µg/mL and 200 µg/mL in Se-ZYTP treated groups, respectively [4]. SeGLP-2B-1 exhibits an approximately 10-fold stronger anti-proliferative activity against six human cancer cell lines [5]. Mao et al. also report the anti-tumor activity and immunologic function of Se-polysaccharide purified from Se-enriched *Grifola frondosathe* (Se-GP11). Se-GP11 inhibits the growth of Heps

tumor in vivo. The increases in thymus index, tumor necrosis factor α (TNF- α) and interleukin-2 (IL-2) levels in serum are observed. The results reveal that Se-GP11 exhibits the anti-tumor activity through improving immunomodulation activities of the tumor bearing mice [6]. Moreover, the biological activities of seleno-polysaccharides are related to antibacterial [7], antioxidant and hypolipidemic activity [8-9], anti-fatigue [10] and immuno-enhancement [11-12]. The structures and biological activities of selenized polysaccharides are one of the most intriguing scientific endeavors in carbohydrate chemistry.

Seleno-polysaccharides can be obtained by extracting from plants, biotransformation and chemical modification of natural polysaccharides. The most commonly employed method is chemical synthesis using H_2SeO_3 (or Na_2SeO_3 in HNO_3 solution) and $\text{Ba}^{2+}/\text{Ca}^{2+}$ as catalyst. However, $\text{H}_2\text{SeO}_3/\text{HNO}_3$ method is time-consuming and not efficient enough to synthesize seleno-polysaccharides with high Se content due to the weak reactivity of H_2SeO_3 . The most obvious one is its long heating period that may result in degradation of polysaccharide chains in HNO_3 . For instance, Ji et al. synthesize seleno-*Capparis spionosa* polysaccharides (Se content of 5.547 mg/g) in $\text{H}_2\text{SeO}_3/\text{HNO}_3$ system for 7.5 h at 71 °C, and Wei et al. (Se content from 1.04 to 3.29 mg/g) prefer a long reaction time of 8 h at 65 °C [13-14]. Similar results of selenized *Atractylodes macrocephala* polysaccharides, pectic polysaccharide and garlic polysaccharide with the Se content range of 0.478-29.4 mg/g are obtained in the reaction time range of 5-10 h [11-12, 15].

In recent years, the use of microwave heating in chemical reactions has attracted

a considerable amount of attention, owing to its advantages such as dramatically reduce processing times, increase product yields and enhance product purity or material properties compared to conventionally processed experiments [16-17]. A large number of publications have been reported the application of microwaves in drug synthesis/development [18], organic synthesis [16], polymer chemistry [19], material sciences [20] and carbohydrates chemistry [21]. Several reports have proved that microwaves to a significant extent improve the efficiency in the synthesis of polysaccharides derivatives and polysaccharide-based co-polymers [22-23].

In our previous work, selenized *Artemisia sphaerocephala* polysaccharides (SeASP) were prepared by using $\text{H}_2\text{SeO}_3/\text{HNO}_3$ system and Ba^{2+} as catalyst. The Se content of SeASP varied from 168 to 1703 $\mu\text{g/g}$ depended on the reaction conditions. The substitution position, molecular weight and solution conformation were characterized by employing ^{13}C NMR spectroscopy, size-exclusion chromatograph combined with multi-angle laser light scattering photometer (SEC-MALLS), congo red (CR) spectrophotometric analysis and atomic force microscopy (AFM) observation. After selenylation, SeASP exhibited better scavenge hydroxyl and superoxide radical activities *in vitro* [24]. In anti-tumor assays, SeASP displayed greater anti-proliferative effects against HepG-2 cells, A549 cells and Hela cells in a dose-dependent manner [25].

In the current study, selenized ASP (SeASP_{MW}) was synthesized in microwave field with the irradiation time controlled and all other parameters remain unaltered.

Fourier transform infrared spectroscopy (FT-IR), ^{13}C NMR spectroscopy,

SEC-MALLS were used to study the chemical structure and conformation parameter of SeASP_{MW}. AFM was employed to observe the shape and size of the polysaccharide molecules. The tumoricidal effects of ASP and SeASP_{MW} were evaluated on human non-small cell lung cancer cell line (H1650) *in vitro*. Furthermore, the apoptotic effect and cell cycle distribution was analyzed by flow cytometry.

2. Materials and methods

2.1 Materials

Selenious acid (H₂SeO₃), HNO₃, BaCl₂, NaOH, ascorbic acid and 5-fluorouracil (5-Fu) were purchased from Jingchun Industry Co. Ltd. (Shanghai, China). Dulbecco's modified Eagle medium (DMEM) was obtained from HyClone (GE Healthcare, USA). Fetal bovine serum was supplied by Clark (Australia). Cell counting kit-8 was purchased from Dojindo Laboratories Co. Ltd. (Shanghai, China). RNaseA, TritonX-100, penicillin and streptomycin were obtained from Solarbio Co. Ltd. (Beijing, China). FITC Annexin V apoptosis detection kit containing propidium iodides (PI) staining solution was purchased from Pharmingen (BD Biosciences, USA). All reagents and chemicals were of analytical grade without further purification in this study.

ASP was extracted and purified according to our earlier study. The carbohydrate, protein, and uronic acid contents of ASP were determined to be 90.2%, 2.4% and 16.3%, respectively. The monosaccharide composition of ASP was L-arabinose, D-xylose, D-mannose, D-glucose and D-galactose (the molar ratio of

1:4.2:45.9:9.7:11.4) with the weight average molecular mass (M_w) of 7.348×10^4 g/mol [26].

2.2 Microwave assisted synthesis of selenized ASP ($SeASP_{MW}$)

ASP (500 mg) was suspended in 50 mL HNO_3 solution (0.8%). The polysaccharide solution was stirred for 10 h at room temperature. Then, H_2SeO_3 (1.0 g) and $BaCl_2$ (1.65 g) were added. Microwave synthesis was carried out in a microwave reaction system (MG08S-2B, Huiyan, Nanjing, China) under open-vessel reflux conditions with a frequency of 2450 ± 50 MHz. To achieve a precise controlling of reaction temperature, microwave irradiation at low microwave power of 300W was performed for desired amount of reaction time ranging from 15 to 480 min at $60^\circ C$. The microwave exposure was paused periodically during the reaction. When reaction was complete, sodium sulfate was added to remove Ba^{2+} and the pH value of mixture was adjusted to 7-8 with NaOH solution (1 mol/L). After that, the mixture was dialyzed (molecular weight cutoff 8-12 kDa) against tap water and distilled water until the solution was colorless when ascorbic acid was added. Finally, the mixture was evaporated, precipitated with EtOH (95%) and lyophilized to give $SeASP_{MW}$. For comparison, selenylation reaction without $BaCl_2$ was also studied.

Polysaccharide samples were digested in a microwave digestion system (MARS-6, CEM, USA). Selenium content determination was performed by a high-resolution continuum source atomic absorption spectrometer (ContrAA700, Analytical Jena, Germany) equipped with a hydride generator.

2.3 FT-IR spectra

FT-IR analysis was performed on a fourier transform infrared spectrometer (iS10, Thermo Nicolet, USA) with KBr pellets. Sixteen scans at a resolution of 4 cm^{-1} were recorded in the wavenumber range of $4000\text{--}400\text{ cm}^{-1}$ and referenced against air.

2.4 ^{13}C NMR spectra

^{13}C NMR spectroscopy was performed on a Bruker Avance DPX-400 spectrometer, operating at the frequency of 100.593 MHz. Samples were deuterium-exchanged several times by freeze drying from D_2O , and then examined in D_2O with a 5-mm probe at 25°C . Chemical shifts were reported in ppm using Me_4Si as the internal reference.

2.5 Molecular weight and conformation analysis

Size-exclusion chromatograph combined with multi-angle laser photometer (SEC-MALLS, Wyatt Technology Co., USA) and optilab refractometer (wavelength of 690 nm) were employed to determine the weight average molecular mass (M_w) and solution conformation of the samples. Polysaccharide solutions were obtained in ultra-pure water with the concentration of 1 mg/mL at room temperature and filtered through a $0.45\text{ }\mu\text{m}$ cellulose filter. The elution of the sample was performed using a UltrahydrogelTM column ($7.8\times 300\text{ mm}$, Waters, USA) and ultra-pure water as mobile phase at a flow rate of 0.5 mL/min with the injection volume of 50 μL . The refractive

index increment (dn/dc) value was determined to be 0.145 mL/g. The M_w and $\langle S^2 \rangle_z$ were calculated using Zimm method. The Astra software (Wyatt Tech. Corp.) was used for data analysis.

2.6 Molecular morphology observation

Atomic force microscopy (AFM) measurements were performed using a Nanoscope IIIa microscopy (Veeco Instruments, USA) in tapping mode. Commercial RTESP tips were purchased from Bruker (Antimony doped Si) with a nominal spring constant of 40 N/m and probe length of 125 μm and width of 35 μm . The bulk solution of sample was dissolved in HPLC grade water and filtered through a 0.45 μm cellulose filter. Then, the solution was diluted to 10 and 1 $\mu\text{g/mL}$ with vigorous stirring for 24 h and placed on a freshly cleaved mica disk and dried in ambient air for 4-8 h at room temperature. All images were obtained with the resolution of 256 $\times$ 256 point.

2.7 Cancer cell proliferation assay

CCK-8 assay was employed to evaluate the anti-tumor activities of the samples against human non-small cell lung cancer cell line (H1650) *in vitro*. H1650 cell line was purchased from Gansu Academy of Medical Sciences (Gansu, China). Cells were incubated in 90% DMEM supplemented with 10% fetal bovine serum and penicillin-streptomycin (10 units/mL) at 37 $^{\circ}\text{C}$ in a humidified atmosphere of 5% CO_2 .

H1650 cells were incubated in 96-well plates (the density of 8×10^3 cells per well).

After cell attachment, the wells were washed and incubated in DMEM for 24 h. Then, cells were treated with serial concentrations of 5-Fu and polysaccharide samples. After 48 h, 10 μ L of CCK-8 was added and cultured for another 1.5 h. The absorbance was read at 450 nm on a micro-plate enzyme linked immunosorbent assay (ELISA) reader (xMark, BioRad, USA) according to CCK-8 colorimetric procedure. The anti-proliferative activity of samples was calculated as the formula:

$$\text{Inhibitory rate (\%)} = (A_{\text{control}} - A_{\text{treated}}) / A_{\text{control}} \times 100\% \quad (1)$$

where A_{control} and A_{treated} were the average absorbance of the control and treated group, respectively.

2.8 Analysis of cell apoptosis and cell cycle distribution

The apoptosis rate was quantitatively measured by Annexin V-FITC and propidium iodides (PI) double staining. The cell suspension was prepared with a sterile filter screen and washed by cold phosphate buffer saline (PBS) twice and adjusted to the density of 1×10^6 cells/mL. Then, Annexin V-FITC and PI were added and incubated away from light for 15 min at room temperature. A total of 10,000 cells were collected for each sample. Depending on fluorescence intensity of Annexin V-FITC and PI, the populations could be distinguished into double negative (healthy) cells, Annexin-V positive (early apoptotic cells) and double positive (late apoptotic and necroptotic) cells. Stained cells were analyzed by flow cytometry (FACSVerse, USA).

For analyzing cell cycle-phase distribution, H1650 cells was labeled with PI and

measured by flow cytometry. Briefly, cells were harvested and suspended in PBS, and then fixed overnight at 4°C in cold ethanol (70%). After that, RNaseA (1 mg/mL) was added and incubated for 30 min at 37°C. Subsequently, TritonX-100 (2%) and PI (50 µg/mL) were added and incubated away from light for 15 min at 4°C. A total of 50,000 cells from each sample were acquired and analyzed. The percentage of cells in each cell cycle phase was estimated by measuring the percentage of remaining cells at G₀/G₁, S and G₂/M phase cells by side scatter analysis.

2.9 Statistical Analysis

Results were listed as means±standard deviations (S.D.) of three replicates. One way ANOVA was used to analyze the differences between control and treated group by SPSS version 18.0 software. Differences were considered to be statistically significant at $p < 0.05$ or $p < 0.01$.

3. Results and discussion

3.1 Synthesis of SeASP_{MW}

In many cases, H₂SeO₃/HNO₃ system is selected for the selenylation of polysaccharides in aqueous. In this study, the effect of reaction time on microwave assisted selenylation of ASP was evaluated in aqueous H₂SeO₃/HNO₃ solution with or without BaCl₂. For a better controlling of reaction temperature, a low microwave power of 300 W was selected in preparation of SeASP_{MW}.

Table 1 summarized the data of reaction conditions, yields, Se and carbohydrate

contents of SeASP_{MW}. Six selenized derivatives (SeASP_{MW1-6}) with the Se contents of 111-264 µg/g were obtained in the reaction time range of 15-480 min by using BaCl₂ as catalyst in microwave field. It was obvious that a maximum Se content of 264 µg/g in SeASP_{MW4} was obtained with the increasing of reaction time up to 120 min. Further increasing in the reaction time from 120 to 480 min did not lead to substantial increments in Se contents of SeASP_{MW5} and SeASP_{MW6}, which showed the similar values of 235 and 179 µg/g, respectively. Reaction times required for microwave heating of SeASP_{MW} were decreased to about 2 h compared to the references [11-15]. According to the results from Table 1, the proposed reaction conditions of SeASP_{MW} in microwave field were reaction time 2h, reaction temperature 60°C and HNO₃ concentration of 0.8%. Moreover, we compared the Se contents with our previous result of selenized ASP in the presence of BaCl₂ (SeASP_{cata}) using conventional heating method. Under the similar reaction conditions, the Se content of SeASP_{cata} (969 µg/g) was significantly higher than that of SeASP_{MW4} (Table 1). Selenized ASP using conventional heating method exhibited an approximately 10-fold higher Se contents (the maximum Se content of 1703 µg/g) compared to SeASP_{MW} [24].

It was reported that divalent cation such as Ba²⁺ or Ca²⁺ was one of the important parameters in selenylation reaction using H₂SeO₃/HNO₃ as selenized reagents [14-15]. The mechanism of Ba²⁺ as catalyst involved the formation of coordinate barium complexes and enhanced the interaction between hydroxyl groups and H₂SeO₃ [24]. In order to assess the Se content of microwave assisted selenylation in a more comprehensive way, SeASP_{MW7} was synthesized without BaCl₂ as compared with

SeASP_{MW4} in the same reaction conditions. Unexpectedly, the decrease in Se content of SeASP_{MW7} was not observed. SeASP_{MW7} exhibited the Se content of 257 µg/g, which was similar to SeASP_{MW} obtained in microwave field (Table 1). This result indicated that Ba²⁺ could not improve the Se content of SeASP_{MW} under microwave irradiation. Such an interpretation was further confirmed by a significant decrease in the Se content of SeASP (79 µg/g), which was prepared without BaCl₂ using conventional heating method.

In contrast, the yields (72.1-94.9%) and carbohydrate contents (77.3-85.4%) of SeASP_{MW} were much higher than the references, such as selenized *Schisandra chinensis* polysaccharides with the yields of 32.6-54.8% and carbohydrate contents of 47.2-56.8% [27], selenized *Atractylodes macrocephala* polysaccharides with the yields of 19.7-31.7% and carbohydrate contents of 36.1-63.0% [12] and selenized garlic polysaccharides with the yields of 14.8-36.1% and carbohydrate contents of 19.4-50.4% [11]. The yields of SeASP_{MW} were further comparable to that obtained from selenylation of *Radix hedysari* polysaccharides (Se-RHP), which showed the yield range of 86.2-94.5% [14]. Although the Se content was relatively low, selenylation reaction of SeASP_{MW} in microwave field was characterized by time saving and high yields. However, the effect of microwave irradiation in chemical reactions was a combination of many factors such as the thermal and non-thermal effects. The issue of non-thermal effect was still a controversial matter [28]. Thus, further investigation along those directions is necessary to clarify more details in the effect of microwave irradiation on synthesis of selenized polysaccharides under

different microwave reaction conditions.

3.2 FT-IR analysis

Fig. 1A showed the FT-IR spectra of ASP and its selenized derivatives. For ASP, the absorption peaks at 3411.3 and 2928.1 cm^{-1} were emerged from the stretching vibration of O-H and -CH₂- (mainly existed in the backbone of polysaccharide). The peak at 1052.6 cm^{-1} came from the stretching vibration of C-O in hydroxyl groups. These peaks indicated the existence of carbohydrate in ASP. After selenylation, the intensity of the peaks in selenized samples near 2928 cm^{-1} was decreased. It might be due to the substitution of hydroxyl groups in SeASP_{MW4} and SeASP_{MW5}. Compared to ASP, new peak in selenized sample appeared at 1118.3 cm^{-1} describing Se=O stretching vibration. Moreover, peak at 735.1 cm^{-1} described C-O-Se stretching vibration [11, 27]. These absorptions indicated that the selenylation had actually occurred.

3.3 ¹³C NMR analysis

The ¹³C NMR spectra of ASP and its selenized derivatives were shown in Fig. 1B-1D. The assignment of the chemical shifts of ASP was shown in our previous study [29]. As shown in Fig. 1B, the resonances in the region near 90-110 ppm of ASP were attributed to the anomeric carbon atoms of arabinofranosyl (Araf, 111.1 ppm), glucopyranosyl (Glc_p, 103.5 ppm), galactopyranosyl (Gal_p, 100.9 ppm) and mannopyranosyl (Man_p, 99.3 ppm), respectively. In the region of high field near 60

ppm, the signals at 64.5, 62.8 and 61.5 ppm were assigned to the unlinked C-6 chemical shifts of Galp, Glcp and Manp, respectively. Meanwhile, the signals of linked C-6 were shown at 69.8 (Glcp) and 68.1 (Manp) ppm. It was also important to point out that the signal at 178.9 ppm was due to the carboxylate group.

After selenylation, it was found that the ^{13}C NMR spectra of SeASP_{MW1} and SeASP_{MW4} were very similar to the native ASP. No significant displacements in the signals range of 65-200 ppm were observed in selenized samples. It could be due to the low Se contents and weak substitution in SeASP_{MW}. The signals at 62.3, 60.7 and 59.3 ppm (Fig. 1C-1D) were assigned to the unlinked C-6 chemical shifts of Galp, Glcp and Manp in SeASP_{MW1} and SeASP_{MW4}, respectively. New peak at 64.5 ppm of SeASP_{MW1} and SeASP_{MW4} was attributed to the C-6 substituted carbon signal of Manp. It was attributed to the downfield shift of primary carbon (C-6) directly attached to electron-withdrawing $-\text{SeO}_3^-$ groups [30]. However, this C-6 substituted carbon signal in SeASP_{MW1} was very weak owing to the lower Se content compared to SeASP_{MW4}. Moreover, a significant decrease in the intensity of the C-6 peak for Manp at 59.3 ppm was observed. Wei et al. also reported that selenized *Radix hedysari* polysaccharide (Se-RHP3) showed a new peak at 62.8 ppm which was assigned to the O-6 substituted carbons. Meanwhile, C-6 peaks still remained at 60.5 ppm and no obvious decrease in the intensity of C-6 peak was observed [14]. The ^{13}C NMR result confirmed the fact that the weak C-6 substitution was shown in SeASP_{MW1} and SeASP_{MW4}.

3.4 Molecular weight and solution conformation analysis

The SEC-MALLS chromatograms of ASP and SeASP_{MW1-6} were shown in Fig. 2. The values of M_w , polydispersity (PD, M_w/M_n) and relation equations of the samples were summarized in Table 2. As shown in our previous article, the M_w and polydispersity of ASP was found to be 7.348×10^4 g/mol and 3.184, respectively [26]. More information about the solution conformation of soluble biopolymer could be determined by the power law describing the M_w dependence of $\langle S^2 \rangle_z^{1/2}$. It was reported by Zhang et al. that a fractal dimension (d_f) value of 1-1.5 for a rigid rod chain, 1.5-2 for flexible polymer in good solvent, 2-3 for polymers with high degree of branching of compact chain conformation and higher than 3 for globular shape [31-32]. Fig. 2C illustrated the plot of M_w versus $\langle S^2 \rangle_z^{1/2}$ in double logarithmic coordinates. The d_f value of ASP was 2.86 suggesting a conformation between hard sphere and fully swollen branched polymer in aqueous solution.

The SEC-MALLS chromatography and molecular mass distribution of SeASP_{MW1-6} were shown in Fig. 2A and Fig. 2B, respectively. Compared to the native ASP, the decrease in M_w (1.736 - 4.667×10^4 g/mol) with narrow molecular distribution (1.073-2.241) was shown in all selenized samples. No relations between microwave radiation time and M_w were found. The reason for the decreasing in M_w could be explained by the extensive degradation of polysaccharide chains in acid environment. This was in accordance with our earlier report that selenized ASP exhibited a lower M_w range of 1.026 - 5.407×10^4 g/mol compared to ASP using conventional heating method. The narrow molecular distribution might be due to the loss of small

molecular mass fractions during dialysis. It could be concluded that the combination of microwave radiation and $\text{H}_2\text{SeO}_3/\text{HNO}_3/\text{BaCl}_2$ reaction system also led to the degradation of polysaccharide chains in the reaction time range of 15-480 min, which was similar to the references. It had been shown by Wei et al. that chemically selenized *Radix hedysari* polysaccharide (Se-RHP) showed lower M_w (27.7-62.7 kDa) compared to RHP (84.7 kDa) using $\text{HNO}_3\text{-Na}_2\text{SeO}_3$ method [14]. A sharp decrease in M_w ($1.77\text{-}10.9\times 10^4$ g/mol) was also reported in selenized *Potentilla anserina* polysaccharide compared to the native one (M_w of 22.9×10^4 g/mol) by employing $\text{HNO}_3\text{-H}_2\text{SeO}_3$ as reaction reagent [33].

After selenylation, SeASP_{MW} showed a decrease in the d_f value according to the power law of M_w versus $\langle S^2 \rangle_z^{1/2}$ (Fig. 2C). As shown in Table 2, the d_f value of $\text{SeASP}_{\text{MW}5}$ was calculated to be 2.27, which was consistent with the behavior of a hard sphere and branched macromolecule in aqueous solution. The d_f values of $\text{SeASP}_{\text{MW}3}$ and $\text{SeASP}_{\text{MW}4}$ were 1.30 and 1.47, which exhibited the solution conformation between rigid rod and random coil. It had also been documented that selenized *Codonopsis pilosula* polysaccharide showed a linear random coil conformation in 0.9% NaCl/0.02% NaN_3 aqueous solution [15]. In our earlier report using conventional heating method, selenized ASP showed a compact globular conformation in high M_w range ($2.268\text{-}4.363\times 10^4$ g/mol) and a more rigid conformation in low M_w range ($1.026\text{-}1.426\times 10^4$ g/mol). Anionic groups ($-\text{SeO}_2\text{H}$) had very little influence on d_f values due to the low Se contents (168-1703 $\mu\text{g/g}$) of selenized samples [25]. Similarly, considering the very low Se contents in SeASP_{MW} ,

it could also indicated that the more rigid solution conformation of SeASP_{MW} was solely governed by low M_w . It had been proved that chemically modified polysaccharides (i.e. sulfated polysaccharides) exhibited less flexible conformation, which might be due to the degradation of polysaccharide chains in acidic reaction reagent such as sulfuric acid and chlorosulfonic acid [32, 34]. Polysaccharides having lower molecular weight could readily exhibited a more extended chain conformation. Zhang et al. also reported the formation of ordered aggregates associated with short triple helical chains of lentinan with a low M_w of 5.0×10^5 g/mol [35].

3.5 Atomic force microscopy (AFM) observation

In this study, AFM was used to characterize the molecular morphology of selenized samples. The surface morphologies of SeASP_{MW3}, SeASP_{MW4} and SeASP_{MW6} with different M_w were observed. AFM images at the concentrations of 10 and 1 $\mu\text{g/mL}$ were shown in Fig. 3 and Fig. 4, respectively. To avoid tip broadening effect, molecular height in section analysis (for linear and tangled molecular morphologies) and particle analysis (for spherical molecular morphology) were employed to measure the size of SeASP_{MW}.

As presented in Fig. 3A, the molecules of SeASP_{MW6} entangled with each other to form cross-linked pearl-necklace structures. According to the result of section analysis, the molecule height with the average value of approximately 1 nm was shown. Similarly, SeASP_{MW3} showed a morphology of single bead-like chain with the molecule height near 1.5-2 nm (Fig. 3B). This results demonstrated that SeASP_{MW}

with higher M_w exhibited entangled network and aggregation structures in aqueous solution. The morphology of SeASP_{MW4} was less regular in shape as visualized in Fig. 3C, moreover, enhanced aggregation was also observed. When comparing with the molecule size of SeASP_{MW6} and SeASP_{MW3}, SeASP_{MW4} with lower M_w adopted spherical morphologies linked with worm-like chains in aqueous solution. The heights of the spherical particles and the short chains and were estimated to be 3 and 1 nm, respectively. As shown in Fig. 3, the formation of aggregates was observed in all of the samples at the concentration of 10 $\mu\text{g/mL}$.

The molecular morphologies (Fig. 4) of the samples were also obtained at a lower concentration of 1 $\mu\text{g/mL}$. As for SeASP_{MW6}, the transformation from cross-linked pearl-necklace structures into the spherical morphology and bead-like chains was observed in concentration-dependent AFM images (Fig. 4A). By counting 26 particles, the mean height of the spherical molecules was calculated to be 1.82 nm in particle analysis. The height profile of the bead-like chains had a height distribution ranging between approximately 0.5 and 0.7 nm in section analysis. In this case, single molecular morphology of SeASP_{MW6} was obtained and some aggregations could also be observed. As shown in Fig. 4B, mono-dispersed spherical morphology with a mean height of 1.85 nm (by counting 37 particles in particle analysis) was observed for SeASP_{MW3}, which was similar to the image of SeASP_{MW6}. The topographical AFM image of SeASP_{MW4} appeared an island-like structure and slightly disassociated rod-shaped lumps (Fig. 4C). The molecular height with a mean value of 2.7 nm was determined in particle analysis. AFM observation was similar to the results obtained

in Section 3.4 by SEC-MALLS analysis and our previous study in selenized ASP that short chains of SeASP_{MW} might be attributed to the lower M_W caused by acid degradation [25]. Moreover, as observed in the molecular morphology of SeASP_{MW}, it was deduced that the molecular aggregation was strong at all concentrations. Similar result was reported by Liu et al. that mycelia selenium-polysaccharide (extracted from *Catathelasma ventricosum*) showed branched chains with the height of 8.5 nm at the concentration of 1 µg/mL, which was consistent with the thickness of the aggregated molecule chains [9]. It had been documented that the aggregation of polysaccharide molecules in AFM analysis was probably due to the hydroxyl groups providing strong inter-molecular and intra-molecular interactions [36].

3.6 Evaluation of anti-tumor activities in vitro

To investigate the effects of ASP and its selenized derivatives on the proliferation of cancer cell, H1650 cells were cultivated with different sample concentrations. H1650 cells proliferation in the 5-Fu treated group (positive control) was significantly decreased when compared with control group. The IC₅₀ value of 5-Fu was calculated to be 61.379 µg/mL in the concentrations range of 6.25-100 µg/mL (shown in *Supporting file*). After incubated with ASP and SeASP_{MW1-6} for 48 h at the concentration range of 25-400 µg/mL, the inhibition effects against H1650 cells were observed and compared with the control group. Fig. 5A showed the growth of H1650 cells in the presence of various samples. However, there was no significant suppressive effect of ASP on the proliferation of H1650 cells at all concentrations. No

dose-dependency relationship between the inhibition rates and concentrations was observed.

As for selenized samples, CCK-8 assays showed that SeASP_{MW2}, SeASP_{MW3}, SeASP_{MW4} and SeASP_{MW5} exhibited no proliferation inhibitory activity on H1650 cells at the concentration range of 25-400 µg/mL (Fig. 5A). It was deserved to note that SeASP_{MW1} exhibited relatively high inhibition rates (37.965% and 48.446%) than ASP ($p < 0.01$) at high concentrations of 300 and 400 µg/mL. Compared with ASP and SeASP_{MW1}, the anti-proliferation effect was higher in the groups treated with SeASP_{MW6} at all concentrations. The inhibition rates were 32.381% and 39.776% at the concentrations of 100 and 200 µg/mL, respectively. In our previous work, selenized ASP (Se content of 1703 µg/g) exhibited enhanced anti-proliferative activity against HepG-2 cells, A549 cells and Hela cells *in vitro*. The IC₅₀ value was calculated to be 161.6 µg/mL on A549 cells [25]. However, the IC₅₀ values of SeASP_{MW1} and SeASP_{MW6} could not be determined due to the low inhibition rates (< 50%) in the tested concentration range. This results suggested that SeASP_{MW} presented relatively low anti-tumor activities against H1650 cells.

It had been reported that some polysaccharides and its derivatives showed anti-tumor activity by inducing cell apoptosis and arresting cell cycle distributions [37-38]. Accordingly, SeASP_{MW1} and SeASP_{MW6} were selected to determine the cell apoptosis and cell cycle distribution by flow cytometry (Table 3). Compared to control group, apoptotic cells were not significantly increased in the tumor cells administered with ASP, SeASP_{MW1} and SeASP_{MW6} at the concentrations of 100 and

200 µg/mL (Fig. 5B). Furthermore, the proportion of G₀/G₁ phase in ASP treated group increased from 52.75% to 63.88% ($p<0.01$) and 67.18% ($p<0.05$) at the concentration of 100 and 200 µg/mL, respectively (Fig. 5C). Meanwhile, the DNA content of H1650 cells in G₂/M phase was decreased ($p<0.01$ or $p<0.05$) when compared with the control group. ASP significantly caused H1650 cells arrest at G₀/G₁ phase and decreased the cell population of G₂/M phase, which indicated that DNA synthesis was blocked after 48 h treatment. However, the cell cycle proportion of SeASP_{MW1} and SeASP_{MW6} was the same as ASP. The concentration dependent manner was not observed in selenized samples (Fig. 5C). Therefore, the relatively weak inhibition effect of SeASP_{MW1} and SeASP_{MW6} was not related to cell apoptosis and cell cycle arrest in H1650 cells.

The ever increasing improvements of biological activities in modified polysaccharides had been often obtained by means of selenylation. It was reported that chemically selenized *Capparis spionosa* polysaccharide (Se-CSPS) could inhibit the proliferation of human gastric cancer SGC-7901 cells in a dose-dependent manner. The Se content of Se-CSPS was 5547 µg/g with the IC₅₀ value of 111.90 µg/mL [13]. Compared to our previous report, SeASP_{MW} with lower Se contents (111-264 µg/g) presented weaker anti-proliferative activities than selenized ASP with high Se content of 1703 µg/g [25]. Similar result was shown by Mao et al. that Se-enriched *Grifola frondosa* polysaccharide with a low Se content of 0.5981 µg/g exhibited no anti-tumor activity against HepG-2 cells *in vitro* [6]. It could be postulate that Se content is a key factor to influence the anti-tumor activities of selenized polysaccharides. However,

Shang and colleagues showed that Se-containing *Ganoderma lucidum* polysaccharide (with a similar Se content of 186.7 $\mu\text{g/g}$ as SeASP_{MW}) exhibited significantly higher anti-proliferative activities against human leukemia K562 cells and human breast cancer MCF-7 cells by inducing apoptosis and G₁/S phase arrest [5, 39]. Wang et al. also offered the evidence that Se-enriched Ziyang green tea polysaccharide with lower Se content of 2.14 $\mu\text{g/g}$ possessed anti-cancer activity in human osteosarcoma U-2 OS cells *in vitro* and *vivo* [4]. The possible reason was that the anti-tumor activities of selenized polysaccharides were not a function of a one factor but a multiple action of many factors, such as Se content, substitution patterns and average molecular mass. Further studies on the preparation of selenized polysaccharides with different Se contents and structure features are necessary to expose the structure-bioactivity relations.

4. Conclusion

In this work, selenylation of ASP was finished in a short reaction period (~ 2 h) with high yields (72.1-94.9%). SeASP_{MW} showed the Se content range of 111-264 $\mu\text{g/g}$. According to the result of ^{13}C NMR analysis, C-6 substitution was shown in SeASP_{MW}. In SEC-MALLS analysis, a decrease in M_w ($1.736\text{-}4.667 \times 10^4$ g/mol) with narrow molecular distribution was observed. It could be explained by the extensive degradation of polysaccharide chains in acid environment. Considering the low Se content, a more rigid solution conformation of SeASP_{MW} was solely governed by low M_w . This was further confirmed by AFM observation that SeASP_{MW} presented short

chains and island-like topography after selenylation. In anti-proliferative activity assays, the relatively weak inhibition effect of SeASP_{MW} was not related to cell apoptosis and cell cycle arrest in H1650 cells. Se content was one of the key factors to influence the anti-tumor activities of selenized polysaccharides.

Acknowledgments

Financial support of this research from National Science Foundation of China (51463022) and Natural Science Foundation of Gansu Province (1308RJZA200) are gratefully acknowledged. We would also like to thank Prof. Fengyiny Jing (Chang Chun Institute of Applied Chemistry, Chinese Academy of Science) for the measurement of NMR and Prof. Ruibin Guo (The Key Laboratory of Ecological Environment Related Polymer Materials of Ministry of Education, Northwest Normal University) for the use of SEC-MALLS.

List of abbreviations

Artemisia sphaerocephala polysaccharide, ASP

Selenized *Artemisia sphaerocephala* polysaccharide, SeASP

Size-exclusion chromatograph combined with multi-angle laser light scattering photometer, SEC-MALLS

Atomic force microscopy, AFM

Selenized ASP synthesized in microwave field, SeASP_{MW}

Fourier transform infrared spectroscopy, FT-IR

Selenious acid, H_2SeO_3

Weight average molecular mass, M_w

Cell counting kit-8, CCK-8

Propidium iodides, PI

Phosphate buffer saline, PBS

Fractal dimension, d_f

References

- [1] I. Zwolak, H. Zaporowska. Selenium interactions and toxicity: a review. Selenium interactions and toxicity. Cell Biol. Toxicol. 28 (2012) 31-46.
- [2] W. Thomas. Organoselenium Chemistry Synthesis and Reactions, Wiley-VCH, Weinheim, 2012, pp. 361-363.
- [3] S.F. Liao, K.R. Brown, A.J. Stromberg, W.R. Burris, J.A. Boling, J.C. Matthews. Dietary supplementation of selenium in inorganic and organic forms differentially and commonly alters blood and liver selenium concentrations and liver gene expression profiles of growing beef heifers. Biol. Trace Elem. Res. 140 (2011) 151-169.
- [4] Y.C. Wang, J. Chen, D.Z. Zhang, Y.F. Zhang, Y.H. Wen, L.H. Li, L.H. Zheng. Tumoricidal effects of a selenium (Se)-polysaccharide from Ziyang green tea on human osteosarcoma U-2 OS cells. Carbohydr. Polym. 98 (2013) 1186-1190.
- [5] D.J. Shang, J.N. Zhang, L. Wen, Y. Li, Q. Cui. Preparation, characterization, and antiproliferative activities of the Se-containing polysaccharide SeGLP-2B-1 from Se-enriched *Ganoderma lucidum*. J. Agr. Food Chem. 57 (2009) 7737-7742.

- [6] G.H. Mao, Y. Ren, Q. Li, H.Y. Wu, D. Jin, T. Zhao, C.Q. Xue, D.H. Zhang, Q.D. Jia, Y.P. Bai, L.Q. Yang, X.Y. Wu. Anti-tumor and immunomodulatory activity of selenium (Se)-polysaccharide from Se-enriched *Grifola frondosa*. Int. J. Biol. Macromol. 82 (2016) 607-613.
- [7] H.T. Lü, Y.J. Gao, H. Shan, Y.T. Lin. Preparation and antibacterial activity studies of degraded polysaccharide selenide from *Enteromorpha prolifera*. Carbohydr. Polym. 107 (2014) 98-102.
- [8] G.H. Mao, Y. Zou, W.W. Feng, W. Wang, T. Zhao, C.W. Ye, Y. Zhu, X.H. Wu, L.Q. Yang, X.Y. Wu. Extraction, preliminary characterization and antioxidant activity of Se-enriched Maitake polysaccharide. Carbohydr. Polym. 101 (2014) 213-219.
- [9] Y.T. Liu, J. Sun, S.Q. Rao, Y.J. Su, J.H. Li, C.M. Li, S.D. Xue, Y.J. Yang. Antidiabetic activity of mycelia selenium-polysaccharide from *Catathelasma ventricosum* in STZ-induced diabetic mice. Food Chem. Toxicol. 62 (2013) 285-291.
- [10] A.P. Chi, H. Li, C.Z. Kang, H.H. Guo, Y.M. Wang, F. Guo, L. Tang. Anti-fatigue activity of a novel polysaccharide conjugates from Ziyang green tea. Int. J. Biol. Macromol. 80 (2015) 566-572.
- [11] Z.Z. Gao, J. Chen, S.L. Qiu, Y.Y. Li, D.Y. Wang, C. Liu, X.P. Li, R.R. Hou, C.J. Yue, J. Liu, H.Q. Li, Y.L. Hu. Optimization of selenylation modification for garlic polysaccharide based on immune-enhancing activity. Carbohydr. Polym. 136 (2016) 560-569.
- [12] J. Liu, X. Chen, C.J. Yue, R.R. Hou, Y. Chen, Y. Lu, X.P. Li, R.J. Li, C. Liu, Z.Z. Gao, E.T. Li, Y.Y. Li, H. Wang, Y. Yan, H.Q. Li, Y.L. Hu. Effect of selenylation modification on immune-enhancing activity of *Atractylodes macrocephala* polysaccharide. Int. J. Biol.

- Macromol. 72 (2015) 1435-1440.
- [13] Y.B. Ji, F. Dong, L. Lang, L.W. Zhang, J. Miao, Z.F. Liu, L.N. Jin, Y. Hao. Optimization of synthesis, characterization and cytotoxic activity of seleno-*Capparis spionosa* L. polysaccharide. Int. J. Mol. Sci. 13 (2012) 17275-17289.
- [14] D.F. Wei, T. Chen, M.F. Yan, W.H. Zhao, F. Li, W.D. Cheng, L.X. Yuan. Synthesis, characterization, antioxidant activity and neuroprotective effects of selenium polysaccharide from *Radix hedysari*. Carbohydr. Polym. 125 (2015) 161-168.
- [15] W.X. Chen, J.Y. Chen, H.M. Wu, Y.Q. Gou, F.D. Hua, L.J. Liu, X. Gao, P. Zhang. Optimization of selenylation conditions for a pectic polysaccharide and its structural characteristic. Int. J. Biol. Macromol. 69 (2014) 244-251.
- [16] C.O. Kappe. Controlled microwave heating in modern organic synthesis. Angew. Chem. 43 (2004) 6250-6284.
- [17] D. Dallinger, C. O. Kappe. Microwave-assisted synthesis in water as solvent. Chem. Rev. 107 (2007) 2563-2591.
- [18] V. Santagada, F. Frecentese, E. Perissutti, F. Fiorino, B. Severino, G. Caliendo. Microwave assisted synthesis: a new technology in drug discovery. Mini-Rev. Med. Chem. 9 (2009) 340-358.
- [19] S.L. Pedersen, A.P. Tofteng, L. Malik, K.J. Jensen. Microwave heating in solid-phase peptide synthesis. Chem. Soc. Rev. 41 (2012) 1826-1844.
- [20] G.A. Tompsett, W.C. Conner, K. S. Yngvesson. Microwave synthesis of nanoporous materials. Chemphyschem. 7 (2006) 296-319.
- [21] H. Wang, Y. Shi, G. Le. Rapid microwave-assisted synthesis of polydextrose and

- identification of structure and function. *Carbohydr. Polym.* 113 (2014) 225-230.
- [22] Meenkashi, M. Ahuja, P. Verma. MW-assisted synthesis of carboxymethyl tamarind kernel polysaccharide-g-polyacrylonitrile: optimization and characterization. *Carbohydr. Polym.* 113 (2014) 532-538.
- [23] M.U. Chhatbar, R. Meena, K. Prasad, D.R. Chejara, A.K. Siddhanta. Microwave-induced facile synthesis of water-soluble fluorogenic alginic acid derivatives. *Carbohydr. Res.* 346 (2011) 527-33.
- [24] J.L. Wang, B.T. Zhao, X.F. Wang, J. Yao, J. Zhang. Synthesis of selenium-containing polysaccharides and evaluation of antioxidant activity in vitro. *Int. J. Biol. Macromol.* 51 (2012) 987-991.
- [25] J.L. Wang, Q.Y. Li, A.J. Bao, X.R. Liu, J.Y. Zeng, X.P. Yang, J. Yao, J. Zhang, Z.Q. Lei. Synthesis of selenium-containing *Artemisia sphaerocephala* polysaccharides: Solution conformation and anti-tumor activities in vitro. *Carbohydr. Polym.* 152 (2016) 70-78.
- [26] J.L. Wang, J. Zhang, X.F. Wang, B.T. Zhao, Y.Q. Wu, J. Yao. A comparison study on microwave-assisted extraction of *Artemisia sphaerocephala* polysaccharides with conventional method: Molecule structure and antioxidant activities evaluation. *Int. J. Biol. Macromol.* 45 (2009) 483-492.
- [27] C. Yue, J. Chen, R. Hou, J. Liu, X. Li, Z. Gao, C. Liu, D. Wang, Y. Lu, H. Li, Y. Hu. Effects of selenylation modification on antioxidative activities of *Schisandra chinensis* polysaccharide. *PloS One* 10 (2015) e0134363.
- [28] A. Hoz, A. Diaz-Ortiz, A. Moreno. Microwaves in organic synthesis. Thermal and non-thermal microwave effects. *Chem. Soc. Rev.* 34 (2005) 164-178.

- [29] J.L. Wang, W. Yang, J.C. Wang, X. Wang, F. Wu, J. Yao, J. Zhang, Z.Q. Lei. Regioselective sulfation of *Artemisia sphaerocephala* polysaccharide: Characterization of chemical structure. Carbohydr. Polym. 133 (2015) 320-327.
- [30] X.B. Yang, X.D. Gao, F. Han, R.X. Tan. Sulfation of a polysaccharide produced by a marine filamentous fungus *Phoma herbarum* YS4108 alters its antioxidant properties in vitro. BBA-Gen. Subjects 1725 (2005) 120-127.
- [31] Y. Peng, L.N. Zhang. Characterization of a polysaccharide-protein complex from *Ganoderma tsugae* mycelium by size-exclusion chromatography combined with laser light scattering. J. Biochem. Bioph. Meth. 56 (2002) 243-252.
- [32] Y.Z. Tao, L.N. Zhang, C.K. Peter. Physicochemical properties and antitumor activities of water-soluble native and sulfated hyperbranched mushroom polysaccharides. Carbohydr. Res. 341 (2006) 2261-2269.
- [33] B.T. Zhao, J. Zhang, J. Yao, S. Song, Z.X. Yin, Q.Y. Gao. Selenylation modification can enhance antioxidant activity of *Potentilla anserina* L. polysaccharide. Int. J. Biol. Macromol. 58 (2013) 320-328.
- [34] J.L. Wang, S.F. Niu, B.T. Zhao, T. Luo, D. Liu, J. Zhang. Catalytic synthesis of sulfated polysaccharides. II: comparative studies of solution conformation and antioxidant activities. Carbohydr. Polym. 107 (2014) 221-231.
- [35] Y.Y. Zhang, S. Li, L.N. Zhang. Aggregation behavior of triple helical polysaccharide with low molecular weight in diluted aqueous solution. J. Phys. Chem. B 114 (2010) 4945-4954.
- [36] L. Kong, L. Yu, T. Feng, X. Yin, T. Liu, L. Dong. Physicochemical characterization of the polysaccharide from *Bletilla striata*: Effect of drying method. Carbohydr. Polym. 125 (2015)

1-8.

- [37] S. Zhang, S. Nie, D. Huang, Y. Feng, M. Xie. A novel polysaccharide from *Ganoderma atrum* exerts antitumor activity by activating mitochondria-mediated apoptotic pathway and boosting the immune system. J. Agr. Food Chem. 62 (2014) 1581-1589.
- [38] P. Shao, X. Chen, P. Sun. Chemical characterization, antioxidant and antitumor activity of sulfated polysaccharide from *Sargassum horneri*. Carbohydr. Polym. 105 (2014) 260-269.
- [39] D. Shang, Y. Li, C. Wang, X. Wang, Z. Yu, X. Fu. A novel polysaccharide from Se-enriched *Ganoderma lucidum* induces apoptosis of human breast cancer cells. Oncol. Rep. 25 (2011) 267-272.

Tables

Table 1 Microwave assisted selenylation of ASP with the HNO₃ concentration of 0.8% at 60°C

Sample	Reaction time (min)	Microwave power (W)	BaCl ₂ (g)	Yield (%)	Se content (μg/g)	Carbohydrate (%)
ASP	-	-	-	-	- ^a	90.3
SeASP _{MW1}	15	300	1.654	93.1	115	80.7
SeASP _{MW2}	30	300	1.650	86.4	141	79.6
SeASP _{MW3}	60	300	1.652	89.3	111	84.4
SeASP _{MW4}	120	300	1.651	72.1	264	85.4
SeASP _{MW5}	300	300	1.650	94.9	235	83.2
SeASP _{MW6}	480	300	1.613	91.3	179	77.3
SeASP _{MW7}	120	300	-	66.9	257	80.6
SeASP _{cata} ^b	360	-	1.652	69.9	969	69.8
SeASP	120	-	-	79.8	79	86.8

^a Not detected.

^b Data from our previous study [24]. The HNO₃ concentration used in the synthesis of SeASP_{cata} was 0.7%.

Table 2 M_w , polydispersity (PD) and d_f values of ASP and SeASP_{MW}

Samples	$M_w \times 10^4$ (g/mol) ^b	M_w/M_n	Relation equation	d_f	Solution Conformation
					between hard sphere
ASP	7.348	3.184	$\langle S^2 \rangle_z^{1/2} = 0.203 M_w^{0.35 \pm 0.027}$	2.86	and random coil (fully swollen)
SeASP _{MW1}	2.772	1.175	$\langle S^2 \rangle_z^{1/2} = 1.11 M_w^{0.22 \pm 0.16}$	4.54	hard sphere
SeASP _{MW2}	2.633	1.188	$\langle S^2 \rangle_z^{1/2} = 3.85 M_w^{-0.61 \pm 0.40}$	- ^a	-
SeASP _{MW3}	3.486	1.249	$\langle S^2 \rangle_z^{1/2} = -1.65 M_w^{0.77 \pm 0.33}$	1.30	between rigid rod and random coil
SeASP _{MW4}	1.736	1.073	$\langle S^2 \rangle_z^{1/2} = -2.68 M_w^{0.68 \pm 0.39}$	1.47	between rigid rod and random coil
SeASP _{MW5}	2.752	1.100	$\langle S^2 \rangle_z^{1/2} = 0.835 M_w^{0.44 \pm 0.15}$	2.27	between hard sphere and random coil (not swollen)
SeASP _{MW6}	4.667	2.241	$\langle S^2 \rangle_z^{1/2} = 2.44 M_w^{-0.13 \pm 0.01}$	-	-

^a Not detected

Table 3. Effects of ASP and its selenized derivatives on cell apoptosis and cell cycle-phase distribution of H1650 cells after 48 hours treatment

Samples	Concentration ($\mu\text{g/mL}$)	Apoptosis (%)	Cell cycle (%)		
			G ₀ /G ₁	S	G ₂ /M
Control	0	1.31	52.75 $\pm$ 0.55	35.97 $\pm$ 0.23	11.28 $\pm$ 0.34
ASP	100	2.29	63.88 $\pm$ 0.10 ^{**}	27.06 $\pm$ 0.13 ^{**}	9.06 $\pm$ 0.06 [*]
	200	1.62	67.18 $\pm$ 4.40 [*]	25.90 $\pm$ 4.17	6.92 $\pm$ 0.23 ^{**}
SeASP _{MW1}	100	5.41	65.50 $\pm$ 1.21 ^{**}	25.42 $\pm$ 0.93 ^{**}	9.08 $\pm$ 0.27 [*]
	200	3.04	58.90 $\pm$ 8.17	33.88 $\pm$ 8.76	7.22 $\pm$ 0.60 [*]
SeASP _{MW6}	100	2.70	63.05 $\pm$ 0.93 ^{**}	26.72 $\pm$ 1.53 [*]	10.24 $\pm$ 0.60
	200	2.65	63.37 $\pm$ 8.70	29.69 $\pm$ 9.96	6.94 $\pm$ 1.17 [*]

* $p < 0.05$ and ** $p < 0.01$ compared with the control

Figure Captions:

Fig. 1 FT-IR spectra (A) and ^{13}C NMR spectra of ASP (B), SeASP_{MW1} (C) and SeASP_{MW4} (D).

Fig. 2 SEC-MALLS chromatograms of ASP and SeASP_{MW} (A) detected by laser light scattering photometry (B) Molar mass distribution analysis and (C) Log-log plot of M_w versus $\langle S^2 \rangle_z^{1/2}$.

Fig. 3 AFM images and section analysis of SeASP_{MW} at the concentration of 10 $\mu\text{g/mL}$ (A) SeASP_{MW6} (B) SeASP_{MW3} and (C) SeASP_{MW4}.

Fig. 4 AFM images and particle analysis of SeASP_{MW} at the concentration of 1 $\mu\text{g/mL}$ (A) SeASP_{MW6} and section analysis (B) SeASP_{MW3} and (C) SeASP_{MW4}.

Fig. 5 Inhibition rates (A), cell apoptosis (B) and cell cycle proportions (C) of ASP, SeASP_{MW1} and SeASP_{MW6}, against H1650 cells after incubated for 48 h in vitro. The inhibition rates were presented as mean values (n=3). * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared with the control. # $p < 0.05$, ## $p < 0.01$ and ### $p < 0.001$ compared with ASP.

Figures

Fig. 1 FT-IR spectra (A) and ^{13}C NMR spectra of ASP (B), SeASP_{MW1} (C) and SeASP_{MW4} (D).

Fig. 2 SEC-MALLS chromatograms of ASP and SeASP_{MW} (A) detected by laser light scattering photometry (B) Molar mass distribution analysis and (C) Log-log plot of M_w versus $\langle S^2 \rangle_z^{1/2}$.

Fig. 3 AFM images and section analysis of SeASP_{MW} at the concentration of 10 µg/mL (A)

SeASP_{MW6} (B) SeASP_{MW3} and (C) SeASP_{MW4}.

Fig. 4 AFM images and particle analysis of SeASP_{MW} at the concentration of 1 µg/mL (A)

SeASP_{MW6} and section analysis (B) SeASP_{MW3} and (C) SeASP_{MW4}.

Fig. 5 Inhibition rates (A), cell apoptosis (B) and cell cycle proportions (C) of ASP, SeASP_{MW1} and SeASP_{MW6}, against H1650 cells after incubated for 48 h in vitro. The inhibition rates were presented as mean values (n=3). * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared with the control. # $p < 0.05$, ## $p < 0.01$ and ### $p < 0.001$ compared with ASP.