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Synthesis of selenium-containing *Artemisia sphaerocephala* polysaccharides: Solution conformation and anti-tumor activities in vitro

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ABSTRACT

It has been reported in our previous work that selenized *Artemisia sphaerocephala* polysaccharides (SeASPs) with the Se content range of 168–1703 μg/g were synthesized by using $Na_2SeO_3/HNO_3/BaCl_2$ system. In the present work, the solution property of SeASP was studied by using size exclusion chromatography combined with multi angle laser light scattering (SEC-MALLS). A decrease in d_f values indicated that SeASPs with different conformational features that were highly dependent on M_W. SeASPs exhibited a more rigid conformation (d_f value of 1.29–1.52) in low molecular weight range (M_W of 1.026–1.426 × 10^4 g/mol) and compact spherical conformation in high molecular weight range (M_W of 2.268–4.363 × 10^4 g/mol). It could be due to the degradation of polysaccharide chains in HNO_3, which was supported in monosaccharide composition analysis. Congo red (CR) spectrophotometric method and atomic force microscopy (AFM) results also confirmed the conformational transition and the evidence on the shape of the rigid chains. In vitro anti-tumor assays, $SeASP_2$ displayed greater anti-proliferative effects against three tumor cell lines (hepatocellular carcinoma HepG-2 cells, lung adenocarcinom A549 cells and cervical squamous carcinoma Hela cells) in a dose-dependent manner. This suggested that selenylation could significantly enhance the anti-tumor activities of polysaccharide derivatives in vitro.

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1. Introduction

The biochemical role of selenium (Se) was clearly established almost subsequently with the discovery that the antioxidant enzyme glutathione peroxidase contains a selenocysteine residue at the active site. The upper limit of selenium intake no less than 400 μg/day is prescribed by the Institute of Medicine of The National Academy of Sciences (United States). Selenium deficiency leads to the development of many diseases, such as immune dysfunction and hypothyroidism (Thomas, 2012, Chapter 9). Inorganic and organic selenium are the main existence forms of Se in nature. It is believed that the decoration of inorganic selenium with polysaccharides showed higher bioavailability and provided safe and efficient delivery of microelements for humans (Yang et al., 2012; Bondarenko et al., 2016). Selenium-enriched food supplements are the major source of Se, which are generally considered as a dietary supplement (Wang et al., 2013). In organism, the biosynthesis of selenocysteine is very complex due to the inhibition of selenocysteine incorporation and a dramatic alteration in the enzymatic behavior (Thomas, 2012). Therefore, biotransformation and chemical synthesis of selenium containing proteins and polysaccharides have been found to be very useful and attractive.

Selenium-polysaccharides, including functionalized selenium with polysaccharides, natural Se polysaccharides extracted from plants, bio-enrichment method or their chemically synthesized derivatives, are drawing the attention of researchers recently. The bio-activities of Se-polysaccharides are involved in cancer-therapeutic benefit in vitro/vivo (Wang et al., 2013; Shang, Zhang, Wen, Li, Cui, 2009; Yang et al., 2012; Wu et al., 2013; Chen, Wong, Zheng, Bai, Huang, 2008), immunoregulation (Mao et al., 2016; Liu et al., 2015a; Gao et al., 2016), anti-fatigue (Chi et al., 2015),

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antibacterial (Lü, Gao, Shan & Lin, 2014), antioxidant and hypolipidemic activity (Mao et al., 2014; Liu et al., 2013). However, the selenium content of natural Se-polysaccharides is very low even in Se-enriched area. Therefore, there has been growing interests in artificial synthetic selenizing polysaccharides due to the efficient and high Se content. Several researchers have shown their results in chemically selenized polysaccharides with different Se contents, i.e. *Radix hedysari* polysaccharide of 3.29 mg/g (Wei et al., 2015), *Atractylodes macrocephala* polysaccharide of 11.27 mg/g (Liu et al., 2015a), *Capparis spionosa* polysaccharide of 5.547 mg/g (Ji et al., 2012), garlic polysaccharide of 29.4 mg/g (Gao et al., 2016) and pectic polysaccharide of 478.17 μg/g (Chen et al., 2014b). The selenylation of polysaccharide is one of the most intriguing scientific endeavors in carbohydrate chemistry.

In our previous study, selenized *Artemisia sphaerocephala* polysaccharide (SeASP) with the maximum Se content of 1703 μg/g was synthesized by employing H_2SeO_3 in HNO_3 solution and Ba^{2+} as catalyst. The effect of reaction conditions on the Se contents and yields of SeASPs was studied. The C-6 substituted pattern and a decrease in M_W were determined by using ^{13}C NMR spectroscopy and size-exclusion chromatograph combined with multi-angle laser light scattering photometer (SEC-MALLS). Compared to the native ASP, SeASP showed greater scavenge hydroxyl and superoxide radical activities in vitro (Wang, Zhao, Wang, Yao, Zhang, 2012). Moreover, some researchers have focused on the relations between conformation and biological activity of modified polysaccharides. The bio-activities and solution conformation are mainly influenced by molecular weight and electrostatic interactions of polysaccharide chains in modified polysaccharides (Tao, Zhang & Peter, 2006; Wang et al., 2016).

The objective of the current study is to investigate the mechanisms governing the relations between chain conformation and structure features of SeASP. SEC-MALLS, monosaccharide components analysis (GC–MS method), Congo red (CR) spectrophotometric method and atomic force microscopy (AFM) were used to determine the chain conformation in solution and molecular topography. Moreover, the tumoricidal effects of ASP and SeASP were evaluated on human hepatocellular carcinoma HepG-2 cells, lung adenocarcinom A549 cells and cervical squamous carcinoma Hela cells in vitro.

2. Materials and methods

2.1. Materials

Trifluoroacetic acid (TFA), ammonium hydrochloride, pyridine, acetic anhydride and congo red (CR) were purchased from Jingchun Industry Co. Ltd. (Shanghai, China). The standard monosaccharides L-Rhamnose (L-Rha), L-Ribose (L-Rib), L-Arabinose (L-Ara), D-Xylose (D-Xyl), D-Lyxose (D-Lyx), D-Mannose (D-Man), D-Glucose (D-Glc) and D-Galactose (D-Gal) were purchased from Sigma Chemical Co. (imported from USA). In this study, all chemicals were of analytical reagent or better grade without further purification. The extraction and purification of ASP was shown in our earlier study (Wang et al., 2009). The protein (coomassie brilliant blue method), carbohydrate (phenol-sulfuric acid method) and uronic acid (sulfuric acid-carbazole method) contents of ASP was 2.4%, 90.2% and 16.3%, respectively.

2.2. Synthesis of selenized ASP

The preparation of selenized ASP (SeASP) and determination of selenium contents were reported in our previous report (Wang et al., 2012). Briefly, purified ASP was added in HNO_3 solutions (the concentration of 0.4–1.2%) with stirring for 10 h. Then, different amounts of H_2SeO_3 and $BaCl_2$ were added and reacted for 6 h. After the reaction, the mixture was neutralized (pH value was adjusted to 7–8 with 2 mol/L NaOH) and dialyzed (molecular weight cutoff 8–12 kDa) until the solution was colorless when ascorbic acid was added. SeASP was collected by filtration, washed with ethanol and lyophilized.

2.3. Molecular weight and solution conformation measurements

The weight average molecular weight (M_W) of ASP and SeASPs were determined by using size-exclusion chromatograph combined with multi-angle laser photometer and optilab refractometer (SEC-MALLS, wavelength of 690 nm, Wyatt Technology Co., USA). Ultrahydrogel™ column (7.8 × 300 mm, Waters, USA) was employed in SEC-LLS measurements. ASP and SeASPs were prepared in ultra-pure water at room temperature with desired concentration of 1 mg/mL and filtered through a cellulose filter (0.45 μm). The elution of the sample was performed using ultrapure water as mobile phase at a flow rate of 0.5 mL/min. The injection volume was 50 μL. The refractive index increment (dn/dc) value was 0.145 mL/g. The M_W and $\langle S^2\rangle_z$ were calculated using Zimm method. The equation was shown below

$$\frac{KC}{R_\theta} = \frac{1}{M_w}\left(1 + \frac{16\pi^2\langle S^2\rangle_z}{3\lambda^2}.\sin^2\left(\frac{\theta}{2}\right)\right) + 2A_2C \quad (1)$$

where C was the concentration of the samples (mg/mL); A_2, the second virial coefficient; K, an optical constant equal to $[4\pi^2 n_0^2(dn/dc)^2]/(\lambda^4 N_A)$; R_θ was the Rayleigh ratio; λ, the wavelength of incident light; n_0 was the refractive index of the solvent; $\langle S^2\rangle_z$, the mean square radius of gyration; N_A was the Avogadro' number; θ, the scattering angle. The Astra software (Wyatt Tech. Corp.) was employed for data analysis.

2.4. Monosaccharide components analysis

The monosaccharide composition was detected as previously described (Wang et al., 2014). ASP and SeASP (10 mg) were hydrolyzed with 4 M TFA(4 mL) in a test tube at 120 °C for 10 h under airtight condition. The residual TFA was removed by evaporation. The acetylation reaction of hydrolyzate was prepared by adding of ammonium hydrochloride (10 mg), pyridine (1 mL), acetic anhydride (1 mL) and reacting at 90 °C for 30 min. The standard monosaccharides including L-Rha, L-Rib, L-Ara, D-Xyl, D-Lyx, D-Man, D-Glc and D-Gal were prepared in the same way. The analysis of acetate derivatives was carried out by GC–MS (Thermo Polaris-Q) with a HP-5 capillary column. The chromatographic conditions were as follows: high-purity nitrogen gas was at a flow rate of 1 mL/min, temperature of injector and ion source was 250 °C. The initial column temperature was held at 120 °C and then increased gradually to 250 °C at a rate of 5 °C/min.

2.5. Congo red binding studies

Congo red (CR) spectrophotometric method has been commonly used to analyze the solution conformation of ionic biopolymer. The procedure of CR-polysaccharide binding experiment was performed according to the method of Pal et al. with some modifications (Pal, Maity, Sardar, Chakraborty, Halder, 2016). CR solution (concentration of 91 μmol/L) and SeASP solutions (concentrations of 0.2–2 mg/mL) were prepared in distilled water at room temperature. Then, 2 mL SeASP solutions were mixed with 2 mL CR solution to give polysaccharide-CR complex by stirring at room temperature for 1 h. UV/Vis wavelength sweeps were performed in the range of 400–600 nm at room temperature (UV1100, Labtech Inc., China). Furthermore, NaOH was added to observe the evolution of maxi-

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