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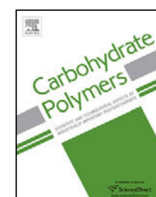

Structural characterization and antiviral effect of a novel polysaccharide PSP-2B from Prunellae Spica

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ABSTRACT

In the present study, a novel polysaccharide, PSP-2B, was isolated from aqueous extracts of Prunellae Spica by direct ultrafiltration membrane separation and gel chromatography purification. PSP-2B is a partially sulphated polysaccharide with a molecular weight of approximately 32 kDa. Its sulfate content is 10.59% by elemental analysis. The major sugars comprising PSP-2B are arabinose, galactose and mannose, in addition to small amounts of glucose and uronic acids. The framework of PSP-2B is speculated to be a branched arabinogalactomannan, and the side chains are terminated primarily by the Ara*f* residues. PSP-2B also contains 2.98% protein. PSP-2B exhibits activity against herpes simplex virus (HSV), with a half maximal inhibitory concentration (IC_{50}) of approximately 69 μg/mL for HSV-1 and 49 μg/mL for HSV-2. However, PSP-2B demonstrated no cytotoxicity even when its concentration was increased to 1600 μg/mL, suggesting that it has potential as an anti-HSV drug candidate.

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1. Introduction

Prunella vulgaris Linn is a perennial plant belongs to the Labiatae family and is widely distributed in Asia and Europe (Feng, Jia, Shi, & Chen, 2010; Şahin, Demir, & Malyer, 2011). The dried spikes of Prunellae Spica (PS) are used to treat wounds, reduce sore throat, and alleviate fever in traditional Chinese medicines (Committee, 2010). PS has also been utilized clinically to treat high blood pressure, breast hyperplasia, hyperthyrea, and herpetic keratitis (Cui & Miao, 2014; Gao & Dong, 2003; Yang, Guo, Wu, Ye, & Xia, 2007). Phytochemical studies have illustrated that PS is rich in triterpenoids (Kojima & Ogura, 1986; Ryu et al., 2000), polysaccharides (Dore et al., 2013; Tabba, Chang, & Smith, 1989; Xu, Lee, Lee, White, & Blay, 1999), phenolic acids (Liu, 2010), flavonoids (Cheung & Zhang, 2008; Ye, 2010) and steroids (Kojima, Sato, Hatano, & Ogura, 1990). Pharmacological investigations have also indicated that the crude aqueous extract of PS has immunostimulatory, antioxidant, anti-inflammatory, antitumor and antiviral activities (Choi et al., 2010; Han et al., 2009; Tabba et al., 1989; Xu et al., 1999).

Polysaccharides exist in a variety of organisms, including certain microorganisms, algae, plants and animals. Together with nucleotides and proteins, polysaccharides are essential biomacromolecules that regulate normal cellular activities (Yang & Zhang, 2009). Many polysaccharides obtained from natural sources exhibit promising biological effects (Deng, Zhou, & Chen, 2014; Diller, Mankowski, & Fisher, 1963; Hasenclever & Mitchell, 1964; Jong & Birmingham, 1993; Wasser, 2002). Furthermore, some polysaccharides have already been approved for clinical usage (Iyer, Ohtola, & Westerink, 2015; Tsiountsioura et al., 2014). Studies involving Prunellae Spica polysaccharides have demonstrated that these sugars have anti-virus capacities, including anti-herpes simplex virus (HSV) (Chiu, Zhu, & Ooi, 2004; Xu et al., 1999; Zhang et al., 2007), anti-human immunodeficiency virus (HIV) (Tabba et al., 1989; Yao, Wainberg, & Parniak, 1992) and anti-respiratory syncytial virus activities (Ma et al., 2002). Li et al. also found Prunellae Spica

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polysaccharides have antioxidant and immunomodulatory activities (Li et al., 2015).

The separation and purification process of polysaccharides are of great importance for both structure characterization and for understanding their biological activities (Mould & Synge, 1952). Current available methods for separation and purification of polysaccharides include solvent precipitation (Xu et al., 1999; Zhang et al., 2007), ion-exchange resin (Feng et al., 2010), gel chromatography (Zhang et al., 2007) and methods that combine these techniques. However, solvent precipitation only assists in the preliminary preparative stage of purification (Mould & Synge, 1952), and is unable to achieve fine separation. A major disadvantage of ion exchange is that it adds undesired ions to the polysaccharide during purification (Slokar & Le Marechal, 1998). Furthermore, ion exchange methods are not capable of separation when the available exchange sites are saturated (Robinson, McMullan, Marchant, & Nigam, 2001). Finally, gel chromatography relies on a molecular sieve effect and has become very valuable in the separation of molecules of various sizes (Andrews, 1965; Gelotte, 1960). However, the high price and low sample loading ability has made it difficult to purify polysaccharides on an industrial scale (Xie et al., 2014). Compared to traditional separation techniques, ultrafiltration membrane separation technology has emerged with several desired benefits, including the use of less organic solvents, its high-throughput nature, and its environmental friendliness (Hong & Choi, 2007; Ye, Wang, Zhou, Liu, & Zeng, 2008). More recently, it has been adopted for the separation and purification of several biological active biomolecules, including proteins, peptides, nucleic acids and polysaccharides (Cheang & Zydney, 2004; Cui & Wright, 1994; Luong, Male, & Nguyen, 1988; Susanto, Arafat, Janssen, & Ulbricht, 2008; Toba, Uemura, & Itoh, 1992; Turgeon & Gauthier, 1990; Wang, Yang, & Wei, 2012).

This study aimed to utilize an ultrafiltration membrane process combined with gel chromatography to separate anti-HSV PSPs from aqueous Prunellae Spica extracts. From the Prunellae Spica extracts, a novel sulphated polysaccharide (PSP-2B) was obtained and structurally characterized using both chemical and modern spectroscopic methods. The anti-HSV activity of PSP-2B was also investigated using different evaluation methodologies.

2. Materials and methods

2.1. Materials and reagents

Prunellae Spica (*P. vulgaris* Linn) was collected in Zhejiang Province and identified by Professor Songlin Li, Jiangsu Province Academy of Traditional Chinese Medicine. Voucher specimens were deposited at the Department of Pharmacology at the Shanghai University of Traditional Chinese Medicine, with voucher number 130730. A series of reference compounds, including D-glucose (11833–201205), D-mannose (140651–200602), galactose (10026–201105), rhamnose (11683–200401), arabinose (111506–200001), D-glucuronic acid (140648–200602), D-galacturonic acid (111646–200301), and bovine serum albumin (BSA, 140619–201421) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (NICPBP, Beijing, China). A series of standard pullulans, including D-1, D-2, D-3, D-4, D-5, D-6, D-7, and D-8 pullulans, were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden).

Vero cells (African green monkey kidney cells) and herpes simplex virus type 1 (HSV-1) (strain KOS) and type 2 (HSV-2) (strain G) were purchased from American Type Culture Collection (ATCC, Virginia, USA). All other regents were of analytical grade and commercially available.

2.2. Extraction of polysaccharides from Prunellae Spica

Prunellae Spica samples (500 g) were powdered and extracted with boiling water (4 L) for 1.5 h. The extraction process was repeated twice and the extracts were combined. The supernatant was filtered through a 0.45-μm microfiltration membrane. The filtrate was concentrated and proteins were removed using Sevag reagent (chloroform: n-butyl alcohol = 4: 1, v/v) for seven times. Crude Prunellae Spica polysaccharide (PSP) was obtained by filtration using a dialysis bag with a cut-off of 3 kDa and thereafter lyophilizing *in vacuo* (FD-1C-50, Beijing Bo Kang laboratory instruments Medical Co., Ltd., Beijing, China). (Scheme of extraction was shown in Fig. 1A)

2.3. Fractionation of PSP by ultrafiltration

The crude PSP was re-dissolved in distilled water and pretreated by passing the solution through a membrane with a pore size of 0.45 μm to avoid damaging the ultrafiltration membranes (Ye et al., 2008). Ultrafiltration was performed with a Millipore system (Massachusetts, USA) equipped with multiple membranes. The feed solution was pumped to the membrane surface (tangential flow) and the filtrate was collected while the retained portion was directed back to the recycling tank. The filtrate obtained after each ultrafiltration process became the feed solution for the next round of ultrafiltration using a different membrane. Membranes with different nominal molecular weight cut-offs (1.0×10^{-4} mm, 100 kDa, 30 kDa) were chosen to fractionate the crude PSP. Finally, crude polysaccharide PSP-1 (>100 kDa), PSP-2 (30–100 kDa) and PSP-3 (3 kDa–30 kDa) were obtained. Based on our initial sugar content and antiviral test, PSP-2 showed encouraging results and was selected for further study.

2.4. Isolation and purification of PSP-2

PSP-2 was further purified through a Sephadex G-200 gel permeation column (1.6 × 100 cm, GE Healthcare, Pennsylvania, USA) using water as the eluent at a flow rate of 0.4 mL/min using an ÄKTA Purifier 100 system (GE Healthcare, Pennsylvania, USA). Eluate was collected automatically (5 mL/tube) using a Fraction Collector Frac-900. Finally, 80 tubes were collected for each run (Gan, Ma, Jiang, Xu, & Zeng, 2011). Every other of the test tubes were monitored for polysaccharide content using the phenol-sulphuric acid method, with glucose as the standard on a Perkin Elmer Lambda 35 UV/Visible spectrophotometer (Perkin Elmer Inc., Massachusetts, USA) (Deng et al., 2014). One major fraction and one minor fraction were collected, corresponding to PSP-2B and PSP-2A, respectively. (Supplementary data Fig. S1). Extensive structural characterization study of PSP-2B was carried out then.

2.5. Characterization of PSP-2B

2.5.1. Homogeneity and molecular weight determination

The homogeneity and molecular weight of PSP-2B were determined according to methods previously described (Xu et al., 1999; Zhang et al., 2007). After filtration through a 0.45-μm filter, PSP-2B and D-series pullulan standards (M_w: 708, 344, 200, 107, 47, 21 and 0.96 kDa) were subjected to high-performance gel permeation chromatography (HPGPC) analysis system using an Agilent 1100 HPLC (California, USA) equipped with Shodex KS 804 and 802 columns (8.0 × 300 mm, Shanghai, China) coupled to an Agilent refractive index detector (RID, California, USA). Detailed chromatographic conditions were as follows: injection volume, 20 μL; mobile phase, 0.2 M NaCl solution; flow rate, 0.8 mL/min; run time, 25 min. The log molecular weights of the D-series pullulan standards and

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