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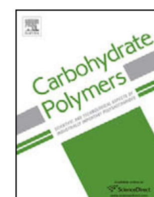

Structural features and complement fixing activity of polysaccharides from *Codonopsis pilosula* Nannf. *var. modesta* L.T.Shen roots

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ABSTRACT

Two pectic polysaccharides, 50WCP-II-I and 100WCP-II-I, were obtained from 50 and 100 °C water extracts of *Codonopsis pilosula* roots by ion exchange chromatography and gel filtration. The study of the sub-fractions obtained after pectinase degradation showed that the complement fixation activities of these pectins are expressed mainly by their ramified regions. The structure studies of native and sub-fractions showed the 50WCP-II-I is a pectic polysaccharide, with long homogalacturonan regions (some of the galacturonic acid units were methyl esterified), interrupted by one short rhamnogalacturonan I (RG-I) region. The side chains of the RG-I region are arabinogalactan type I (AG-I) and type II (AG-II) attached on position 4 of rhamnose. The 100WCP-II-I has two main ramified regions, one is galacturonan region with AG-I side chain on position 2 of GalA, and the other one is RG-I region with AG-II side chain on position 4 of Rha.

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1. Introduction

Radix Codonopsis is one of the most popular ingredients in traditional herbal medicines in China, Japan and Korea, and consists of the root of *Codonopsis pilosula* (Franch.) Nannf., *C. pilosula* Nannf. var. *modesta* L.T.Shen and *C. tangshen* Oliv. It was used in traditional medicine to lower blood pressure and increase white blood cell count, and is reported to cure appetite loss and boost immunity (China Pharmacopoeia Commission, 2010). Radix Codonopsis was utilized primarily as a substitution for ginseng (*Panax ginseng*), and was therefore called poor man's ginseng. Some reports indicate that the main components of *Radix Codonopsis* are sterols, triterpenes, lobetyolin (He, Zhu, Wang, Xu, & Hu, 2005), atractylenolide III (Wang, Xu, Namba, & Tsuneo, 1991), alkaloids and polysaccharides. Radix Codonopsis is an edible medicinal plant with abundant nutritive components, such as protein, essential amino acids and minerals (Bi, Zhang, Chen, & Wu, 2008).

Recently, several investigators reported that the polysaccharides extracted from Radix Codonopsis had several bioactivities such as significantly increased lymphocyte proliferation (Sun & Liu, 2008), stimulation of splenocytes mitogenic response (Wang, Ng, Yeung, & Xu, 1996), improvement of the compensatory hematopoiesis of spleen (Yang, Li, Liu, & Xian, 2005; Zhang, Zhu, Hu, Lai, & Mo, 2003), scavenging of oxygen free radicals (Li & Yang, 2001), and antitumor activities (Xin et al., 2012; Xu, Liu, Yuan, & Guan, 2012). Hot-water reflux extraction and ultrasound extraction techniques are the main extraction methods used to isolate polysaccharides from Radix Codonopsis in recent studies (Sun, Liu, & Kennedy, 2010; Zou, Chen, Yang, & Liu, 2011). Most of the current reports on *C. pilosula* polysaccharides focused on their isolation, pharmacological activity and therapeutic effects. Only a few of these compounds have been characterized, and most of them are neutral polysaccharides (Han, Cheng, & Chen, 2005; Ye et al., 2005; Zhang et al., 2005; Zhang, Zhang, Yang & Liang, 2010).

Pectins are generally known to be composed of linear homogalacturonan (HG) regions and branched rhamnogalacturonan (RG) I and II regions (Waldron & Faulds, 2007). The HG or "smooth

Abbreviations: AG-I, arabinogalactan type I; AG-II, arabinogalactan type II; Ara, arabinose; ASE, accelerated solvent extraction; EtOH, ethanol; Fuc, fucose; Fru, fructose; Gal, galactose; GalA, galacturonic acid; GF, gel filtration; Glc, glucose; GlcA, glucuronic acid; HG, homogalacturonan; HMW, high molecular weight; IEC, ion exchange chromatography; KDO, 3-deoxy-D-*manno*-2-octulosonic acid; LMW, low molecular weight; Man, mannose; *Mw*, molecular weight; PABR, phenol-acetone-boric acid reagent; RG-I, rhamnogalacturonan type I; RG-II, rhamnogalacturonan type II; Rha, rhamnose; Xyl, xylose.

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regions" are consisting of 1,4-linked galacturonic acid (GalA) that may be interrupted by ramified rhamnogalacturonan ("hairy") regions with backbone of alternating of 1,2-linked rhamnose (Rha) and 1,4-linked GalA residues (RG-I). The side chains of RG-I consist usually of arabinogalactans (AG) type I and II, attached on position 4 of Rha. The branched regions of the pectins are thought to be related to their immunomodulating activities (Yamada & Kiyohara, 2007). RG-II contains various rare sugars and complex oligosaccharide chains, and has an extremely complex structure (Perez, Rodriguez-Carvajal, & Doco, 2003). The chemical characteristics and biological activities of pectic polysaccharides, especially those from plants used in the treatment of wounds, ulcer and cancer have been reported (Austarheim, Mahamane, et al., 2012; Lin et al., 2013; Samuelsen et al., 1996; Yamada & Kiyohara, 1999; Zong, Cao, & Wang, 2012). Only one antitumor pectic polysaccharide, CPP1b, was isolated from *C. pilosula* (Franch.) Nannf, and this has been well characterized (Yang et al., 2013); no study on the pectins from *C. pilosula* Nannf.var.*modesta* L.T.Shen has been published until now.

In this paper, we describe the isolation, structure elucidation, and biological activity of polysaccharides isolated from roots of *C. pilosula* Nannf.var.*modesta* L.T.Shen. The aim of the study was also to determine what part of the two major polysaccharides was the most important for the effect on the complement system. Enzymatic treatments of the pectins to isolate their different structural elements have been performed and the sub-fractions compared regarding both structure and complement fixation activity.

2. Materials and methods

2.1. Plant material

The roots of *C. pilosula* Nannf. var. *modesta* L.T.Shen were collected from Jiuzhaigou County (Sichuan Province, P.R. China), and identified by Prof. Xing-Fu Chen, College of Agronomy, Sichuan Agricultural University. The roots were washed, dried and pulverized to a fine powder in a mechanical grinder.

2.2. Extraction of polysaccharides

Accelerated solvent extraction (ASE) was performed on a Dionex ASE350 Accelerated Solvent Extractor (Dionex, Sunnyvale, CA, USA). Two hundred g of powdered roots were weighed, mixed with 50 g of diatomaceous earth and packed in eight 100 mL stainless steel cells. The extractions were performed at 1500 psi, with 5 min heating, 5 min static time, and a 60 s purge for a total of three cycles. In order to remove lipophilic and low molecular weight compounds, the samples were pre-extracted twice with 96% ethanol and 50% ethanol–water at 70 °C. The samples were further extracted twice with distilled water of 50 and 100 °C. After extraction, the water extracts were subjected to ultrafiltration (cut off 5 kDa), and the high molecular weight (HMW) fraction was concentrated, dialyzed at cut-off 3500 Da and lyophilized.

The HMW fractions were filtered through 0.45 μm filters and applied to an anion exchange column packed with ANX Sepharose™ 4 Fast Flow (high sub) (GE Healthcare Bio-Sciences, Uppsala, Sweden). The neutral fractions were eluted with distilled water (at 2 mL/min), while the acidic fractions were eluted with a linear NaCl gradient in water (0–1.5 M) at 2 mL/min. The carbohydrate elution profiles were monitored using the phenol-sulfuric acid assay (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). The related fractions were pooled (Fig. 2 A and B), dialyzed at cut-off 3500 Da against distilled water for removal of NaCl, and lyophilized.

The acidic fractions marked in Fig. 1 were dissolved in elution buffer (10 mM NaCl), filtered through a Millipore filter (0.45 μm), and subjected to gel filtration after application on a Hiload™ 26/60 Superdex™ 200 prep grade column (GE Healthcare) combined with the Äkta system (FPLC, Pharmacia Äkta, Amersham Pharmacia Biotech, Uppsala, Sweden), and eluted with 10 mM NaCl at 1.0 mL/min. Fractions were pooled based on the elution profile (Fig. 2C and D), as determined by the phenol-sulfuric acid assay, dialyzed and lyophilized.

2.3. Enzymatic degradation of the polymers

The main fractions 50WCP-II-I and 100WCP-II-I were subjected to enzymatic degradation. *Endo*-α-D-(1–4)-polygalacturonase was used for production of "hairy" or ramified regions of the polymers. The sample (40 mg) was dissolved and de-esterified in 4 ml 50 mM NaOH for 24 h at 0 °C. The reaction solution was neutralized by adding a few drops of glacial acetic acid. The de-esterified sample was diluted with 50 mM acetate buffer (pH 4.0) to a concentration of 5 mg/ml and 2 μl of enzyme was added (Pectinase, 3800 U/ml, Megazyme, Wicklow, Ireland). The enzymatic degradation proceeded at 37 °C until the increase in reducing end groups stopped (26 h), determined with dinitrosalicylic acid (Knutsen, 1991; Miller, 1959). The reaction was terminated by heating at 100 °C. The partially enzymatic degraded fractions were further fractionated by size exclusion chromatography on a BioGel P30 (Bio-Rad, Hercules, CA, USA) column (2.5 cm × 75 cm) with distilled water as eluent at 0.5 mL/min. The carbohydrate elution profile was determined with phenol-sulphuric acid method and the relevant acidic fractions were pooled (Fig. 2E and F) and lyophilized directly.

2.4. Chemical compositions and linkage determination

The monosaccharide compositions of fractions were determined by gas chromatography (GC) of the trimethylsilylated (TMS) derivatives of the methyl-glycosides obtained after methanolysis with 3 M hydrochloric acid in anhydrous methanol for 24 h at 80 °C (Austarheim, Christensen, et al., 2012; Barsett, Paulsen, & Habte, 1992; Chambers & Clamp, 1971). Mannitol was used as an internal standard. The TMS derivatives were analyzed by capillary gas chromatography on a Focus GC (Thermo Scientific, Milan, Italy). The quantitative determination of fructose (Fru) was performed using the phenol-acetone-boric acid reagent (PABR). The PABR assay was carried out as described by Boratynski (1984) and modified by Chaplin (1994). The total amount of phenolic compounds in the purified polysaccharide fractions were quantitatively determined using the Folin–Ciocalteu assay (Singleton & Rossi, 1965). The protein content of the polysaccharide fractions was determined by the Bio-Rad protein assay, based on the method of Bradford (Bradford, 1976).

Glycosidic linkage elucidation was performed by methylation studies. Prior to methylation, the free uronic acids were reduced with $NaBD_4$ to their corresponding neutral sugars. After reduction of the polymers, methylation, hydrolysis, reduction and acetylation (Kim & Carpita, 1992) were carried out. The derivatives were analyzed by GC–MS using a GCMS-QP2010 (Shimadzu, Kyoto, Japan) attached to a Restek Rxi-5MS column (30 m; 0.25 mm i.d.; 0.25 μm film). The injector temperature was 280 °C, the ion source temperature 200 °C and the interface temperature 300 °C. The column temperature was 80 °C when sample was injected, then increased with 10 °C/min to 140 °C, followed by 4 °C/min to 210 °C and then 20 °C/min to 300 °C. Helium was the carrier gas (pressure control: 80 kPa). The compound at each peak was characterized by an interpretation of the retention times and the characteristic mass spectra. The estimation of the relative amounts of each linkage type was related to the total amount of each monosaccharide type as determined by methanolysis.

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