Carbohydrate Polymers 113 (2014) 420–429

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

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

Yuan-Feng Zou[a,*], Xing-Fu Chen[b], Karl Egil Malterud[a], Frode Rise[c], Hilde Barsett[a], Kari Tvete Inngjerdingen[a], Terje Einar Michaelsen[a], Berit Smestad Paulsen[a]

[a] Department of Pharmaceutical Chemistry, School of Pharmacy, University of Oslo, P.O. Box 1068, Blindern, 0316 Oslo, Norway
[b] Key Laboratory of Crop Ecophysiology and Farming System in Southwest China, Ministry of Agriculture, College of Agronomy, Sichuan Agricultural University, Wenjiang, 611130, PR China
[c] Department of Chemistry, University of Oslo, P.O. Box 1033, Blindern, 0315 Oslo, Norway

ARTICLE INFO

Article history:
Received 20 May 2014
Received in revised form 16 July 2014
Accepted 16 July 2014
Available online 24 July 2014

Keywords:
Codonopsis pilosula
Complement fixation activity
Polysaccharides
Rhamnogalacturonan I

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.

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.

* Corresponding author. Tel.: +47 22856549; fax: +47 22854402.
E-mail address: yuanfeng.zou@farmasi.uio.no (Y.-F. Zou).

http://dx.doi.org/10.1016/j.carbpol.2014.07.036
0144-8617/

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.

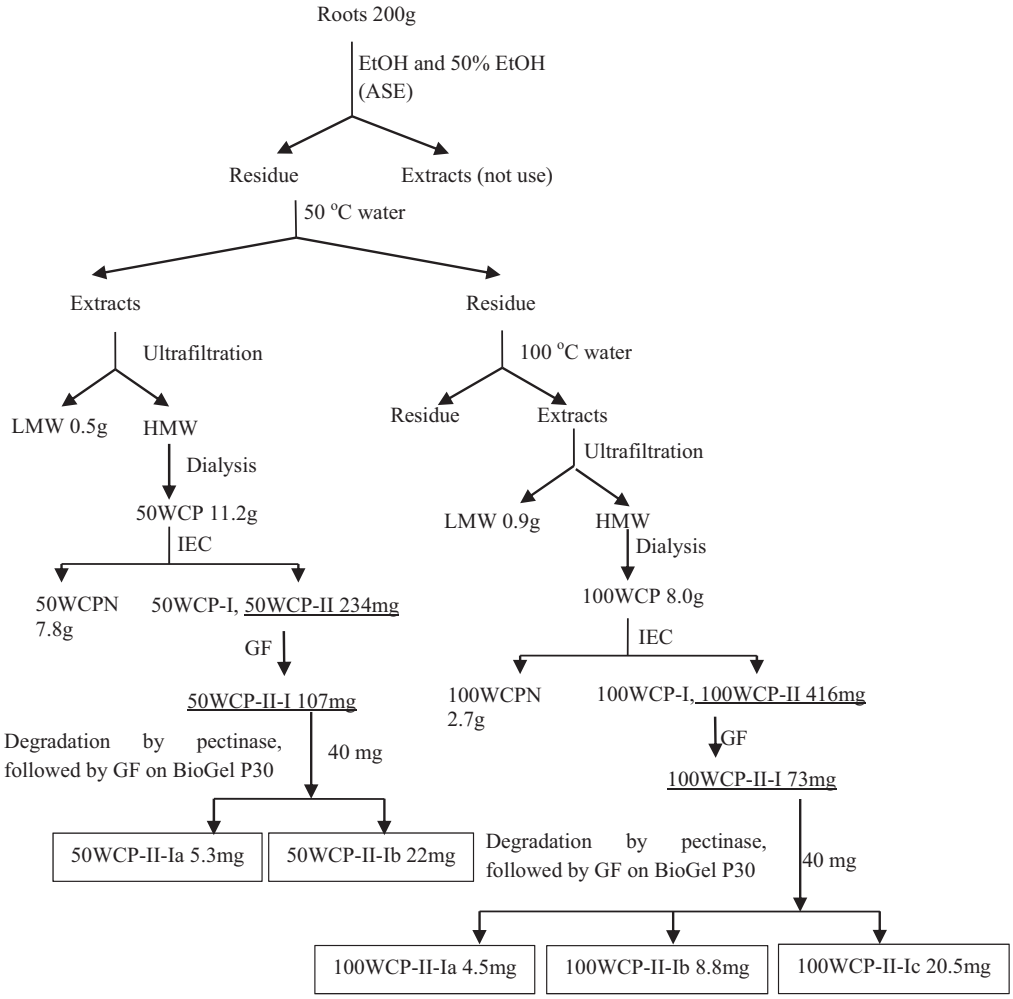

Fig. 1. Extraction and fractionation scheme of polysaccharides extracted by accelerated solvent extraction (ASE) from roots of *C. pilosula* (IEC, ion exchange chromatography; GF, gel filtration).

2.5. Molecular weight determination

The homogeneity and molecular weight of the native purified polysaccharide fractions were determined by size exclusion chromatography on a Hiload™ 16/60 Superdex™ 200 prep grade column (GE Healthcare) combined with the Äkta system (FPLC, Pharmacia Äkta) with RID-10 A detector (Shimadzu, Kyoto, Japan). Dextran polymers (Pharmacia) of 5.6, 19, 233 and 475 kDa were used as calibration standards. The phenol-sulphuric acid method was employed to determine the carbohydrate elution profiles of the polysaccharides fractions (Fig. 2G and H). The fractions obtained after enzymatic degradation of 50WCP-II-I and 100WCP-II-I followed by separation on BioGel P30 were estimated from the calibration curve of the elution volume of standard dextrans. Dextran polymers of 19.1, 11.7, 7.6, 5.6 and 2.3 kDa were used as calibration standards.

2.6. Precipitation with the Yariv β-glucosyl reagent

Precipitation with the Yariv β-glucosyl reagent was performed on the samples as described by van Holst and Clarke (1985). The Yariv β-glucosyl reagent forms a colored precipitate with compounds containing AG-II structures. A solution of Arabic gum in water (1 mg/mL) was used as a positive control.

2.7. Complement fixation assay

The complement system is an important part of the innate immune system which also cooperates with the adaptive immune system in many ways. The complement system consists of over 20 serum proteins including nine complement components (C1–C9) and their regulators, and is normally present in blood serum in an inactive form. The component proteins can be activated through three separate pathways: the classical pathway, the alternative pathway, and the lectin pathway (Ricklin, Hajishengallis, Yang, & Lambris, 2010). The complement fixation assay method A (Michaelsen, Gilje, Samuelsen, Høgåsen, & Paulsen, 2000) was designed to detect the activities of substances through the classical pathway of complement system. The classical pathway is often referred to as "antibody-dependent" because it is strongly initiated by immune complexes containing IgM and/or IgG cluster with complement component 1 (C1) (Ricklin et al., 2010). The complement fixation test is based on inhibition of hemolysis of antibody sensitized sheep red blood cells (SRBC) by human sera as described by Michaelsen et al. (Method A) (2000). BPII, a highly active pectic polysaccharide from the aerial parts of *Biophytum petersianum* Klotzsch (syn. *B. umbraculum*) (Grønhaug et al., 2011; Inngjerdingen, Coulibaly, Diallo, Michaelsen, & Paulsen, 2006), was used as a positive control. Inhibition of lysis induced by the test samples was calculated by the formula $[(A_{control} - A_{test})/A_{control}] \times 100\%$. From these data, a

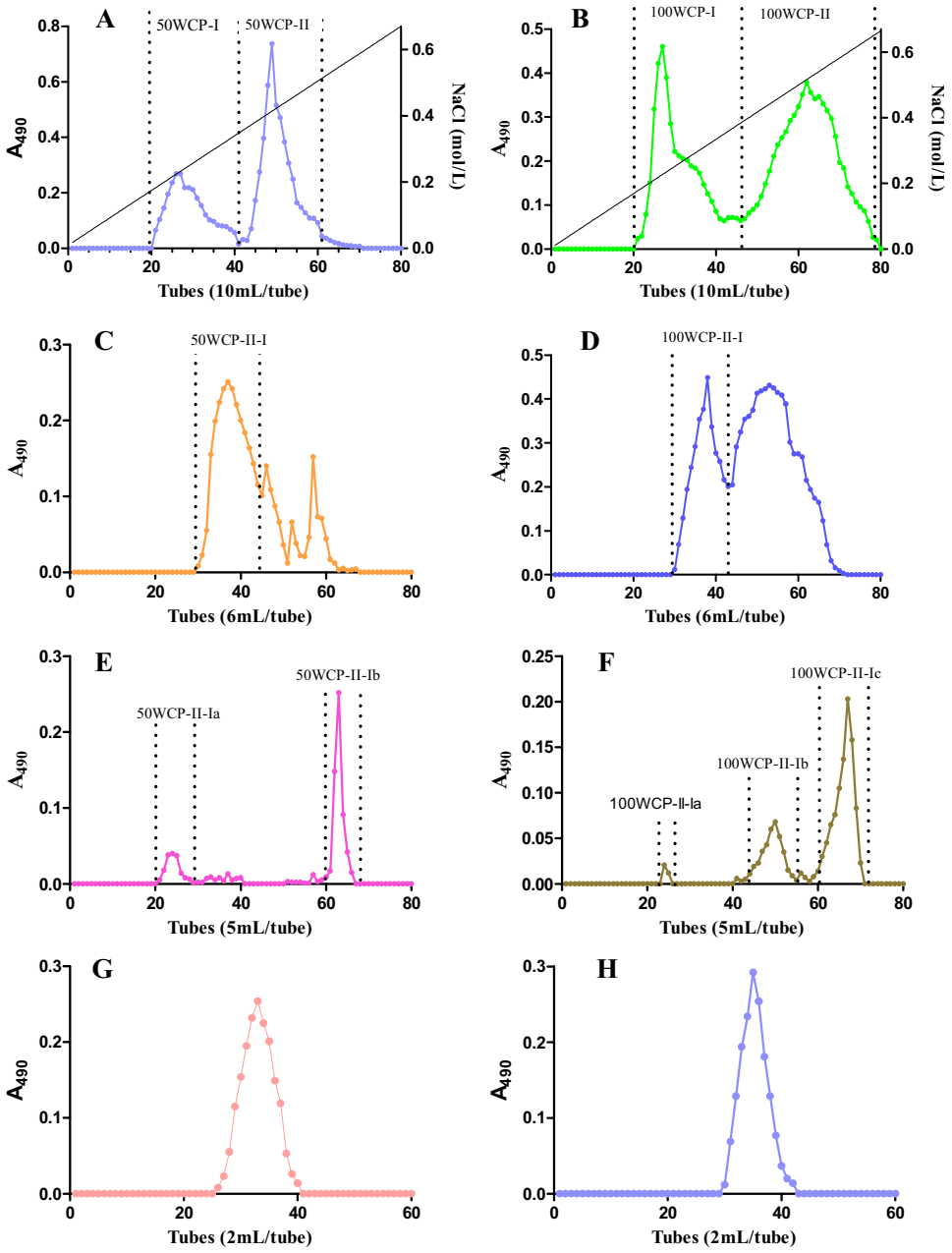

Fig. 2. Elution profiles. (A) IEC elution profile of fraction 50WCP. (B) IEC elution profile of fraction 100WCP. (C) GF elution profile of fraction 50WCP-II. (D) GF elution profile of fraction 100WCP-II. (E) GF elution profile of 50WCP-II-I after pectinase degradation. (F) GF elution profile of 100WCP-II-I after pectinase degradation. (G) Size exclusion chromatography elution profile of fraction 50WCP-II-I. (H) Size exclusion chromatography elution profile of fraction 100WCP-II-I. A_{490} stands for absorbance at 490 nm as described in phenol-sulphuric acid method.

dose-response curve was created to calculate the concentration of test sample giving 50% inhibition of lysis (ICH_{50}). A low ICH_{50} value means a high complement fixation activity. All samples were analyzed in triplicates.

2.8. Nuclear magnetic resonance (NMR) spectroscopy

50WCP-II-I and 100WCP-II-I were deuterium exchanged three times by freeze-drying in D_2O, after which one- and two-dimensional spectra were recorded in D_2O solution on a Bruker AV600 instrument (Bruker, Rheinstetten, Germany; ^1H NMR spectra at 600 MHz, ^{13}C spectra at 150 MHz) at a temperature of 335 K.

3. Results

3.1. Extraction and purification of polysaccharide fractions

The roots of *C. pilosula* were further extracted with 50 and 100 °C water after pre-extraction with 96% ethanol (EtOH) and 50% water–ethanol (50%EtOH). The crude water extracts 50WCP and 100WCP were subjected to anion exchange chromatography. Two active acidic fractions, 50WCP-II and 100WCP-II were obtained and applied to a gel filtration column. 50WCP-II-I and 100WCP-II-I were the most abundant and active fractions, isolated from 50WCP-II and 100WCP-II, respectively.

Table 1
Monosaccharide composition (mol%) and *Mw* of native fractions and fractions obtained after pectinase digestion of the major bioactive fractions from roots of *C. pilosula*.

	50WCP-II-I	50WCP-II-Ia	100WCP-II-I	100WCP-II-Ia	100WCP-II-Ib
Ara[a]	5.8	6.9	7.5	8.0	8.8
Rha[a]	5.7	2.9	6.4	3.5	20.8
Fuc[a]	1.0	1.7	Trace	1.6	2.8
Xyl[a]	1.0	1.2	0.8	2.1	0.4
Man[a]	0.4	5.7	0.3	6.6	0.4
Gal[a]	11.2	21.8	12.4	20.2	11.1
Glc[a]	2.6	6.3	2.3	6.0	2.8
GlcA[a]	1.3	3.0	1.2	2.8	4.8
GalA[a]	71.4	50.6	69.0	49.2	48.2
Mw (kDa)	71.6	17.3	53.2	17.3	1.3
The Yariv test[b]	+	+	+	–	+

[a] mol% related to total content of the monosaccharides Ara, Rha, Xyl, Man, Gal, Glc, GlcA, GalA.
[b] The presence of arabinogalactans type II (AG-II) was identified by precipitation with the β-glycosyl Yariv reagent.

3.2. Chemical compositions and linkage analyses of parent fractions

After methanolysis, the monosaccharide compositions of the 50WCP-II-I and 100WCP-II-I were analyzed by GC as the TMS derivatives of the methyl-glycosides. The content of Fru was estimated using PABR method; the results indicated that neutral fractions 50WCPN and 100WCPN consisted of 96% Fru (data not shown). The monosaccharide compositions present in 50WCP-II-I and 100WCP-II-I, are similar and typical for pectins, consisting mainly of galacturonic acid (GalA), rhamnose (Rha), galactose (Gal) and arabinose (Ara). The Bio-Rad protein assay showed negligible amounts of protein to be present in 50WCP-II-I (0.06%) and 100WCP-II-I (0.46%). A higher amount of phenolic compounds was found in fraction 50WCP-II-I (1.43%) than fraction 100WCP-II-I (0.69%), determined by the Folin–Ciocalteu assay. Size exclusion chromatography using dextran standards was employed to determine the average *Mw* of the purified fractions, the results indicated that fraction 50WCP-II-I has a slightly higher *Mw* than 100WCP-II-I (Table 1).

In order to determine the nature of the glycosidic linkages of the different monosaccharides in the purified fractions 50WCP-II-I and 100WCP-II-I, permethylation of the reduced polymers was performed, partially *O*-methylated alditol acetates (PMAAs) were prepared and subjected to GC–MS. The results are given in Table 2. The main structural feature is the 1,4-linked galacturonan, with only a few units as branch points on position 2 or 3 of GalA. The Rha units are basically 1,2-linked, with branch points on position 4. As the RG-I backbone generally consists of alternating units of Rha and GalA, the low Rha to GalA ratio indicates that the backbone of these two polysaccharide fractions consist of shorter RG-I structures, and longer homogalacturonan regions. The Gal and Ara present have the normal type of linkages that are found in the AG-I/AG-II side chain. The presence of 1,3-linked, 1,6-linked and 1,3,6-linked Gal indicate the presence of AG-II structures in these two fractions, as the Yariv test gave a positive reaction. GlcA appearing in these two fractions are 1,4-linked units. These features have certain similarities with pectins that are composed of areas with hairy or ramified and smooth regions. The 1,2,4-linked Gal was only detected in fraction 100WCP-II-I.

Table 2
The linkages (mol%) of the monosaccharides present in native fractions and fractions obtained by gel filtration after pectinase treatment determined by GC–MS after methylation.

		50WCP-II-I	50WCP-II-Ia	100WCP-II-I	100WCP-II-Ia
Ara	T*f*	4.7	6.6	5.6	8.0
	1 → 5*f*	0.9	0.2	1.2	n.d.
	1 → 3,5*f*	0.2	0.1	0.7	n.d.
Rha	1 → 2*p*	4.0	0.7	4.3	3.5
	1 → 2,4*p*	1.7	2.2	2.1	n.d.
Gal	T*p*	3.5	4.8	1.9	4.9
	1 → 3*p*	0.9	0.4	1.2	n.d.
	1 → 4*p*	3.3	14.9	5.8	12.6
	1 → 6*p*	1.1	0.7	0.6	n.d.
	1 → 2,4*p*	n.d.	n.d.	1.0	2.7
	1 → 3,6*p*	2.3	0.9	1.8	n.d.
Glc	T*p*	1.3	3.0	1.9	0.5
	1 → 4*p*	1.3	3.3	0.4	5.5
GlcA	1 → 4*p*	1.3	3.0	1.2	2.8
GalA	T*p*	0.8	1.6	0.8	n.d.
	1 → 4*p*	67.7	38.6	66.4	38.0
	1 → 3,4*p*	1.0	n.d.	0.3	n.d.
	1 → 2,4*p*	2.0	10.3	1.4	11.2

n.d., Not detected.

3.3. Enzymatic hydrolysis

The purified fractions 50WCP-II-I from 50 °C crude water extracts and 100WCP-II-I from 100 °C crude water extracts were subjected to enzymatic degradation by pectinase after de-esterification by 50 mM NaOH to isolate the hairy regions of the pectins. Pectinase hydrolyzes the smooth regions of de-esterified 1,4-linked GalA residues, leaving the hairy regions from the polymer intact, which could be isolated by gel filtration (Inngjerdingen et al., 2006). Treating high molecular pectin with the enzyme usually generates RG-I, RG-II and oligogalacturonides with a degree of polymerization between 1 and 5 (O'Neill & York, 2003). The pectinase hydrolysate was applied to a Bio-Gel P30 column to obtain sub-fractions. The highest molecular weight fraction, 50WCP-II-Ia (17.3 kDa), was eluted first from the column, followed by the elution of 50WCP-II-Ib (0.3 kDa). Three pectinase-resistant sub-fractions, 100WCP-II-Ia (17.3 kDa), 100WCP-II-Ib (1.3 kDa) and 100WCP-II-Ic (0.2 kDa) were obtained from 100WCP-II-I.

The monosaccharide composition of the different fractions is given in Table 1. The *Mw*'s of the fractions 50WCP-II-Ib and 100WCP-II-Ic are very low, and they are most probably disaccharides or monosaccharides of GalA. 50WCP-II-Ia, a high *Mw* fraction from 50WCP-II-I, consists of GalA (50.6 mol%), Gal, Glc, Ara, Man, Rha and GlcA. Sub-fractions 100WCP-II-Ia and 100WCP-II-Ib contain the same types of monosaccharides as 50WCP-II-Ia, albeit in different amounts. Compared to 100WCP-II-Ia, 100WCP-II-Ib contains higher amounts of Ara, Rha and GlcA. Fractions 50WCP-II-I, 50WCP-II-Ia, 100WCP-II-I, 100WCP-II-Ia and 100WCP-II-Ib all gave a negative reaction in the thiobarbituric acid assay, indicating that the monosaccharide 3-deoxy-D-*manno*-2-octulosonic acid (KDO) and thereby RG-II region, are not present in these fractions.

3.4. Linkage analysis of the fractions after enzymatic degradation

The most active fractions after enzymatic degradation from each native fraction which also had the highest *Mw*, 50WCP-II-Ia and

Table 3
Complement fixation activity (ICH_{50}; Concentration of the samples at 50% inhibition of hemolysis) of native fractions and fractions obtained by gel filtration after pectinase treatment.

Fractions	ICH_{50} (μg/mL)
50WCP-II-I	31.7
50WCP-II-Ia	6.5
50WCP-II-Ib	>500
100WCP-II-I	32.0
100WCP-II-Ia	12.2
100WCP-II-Ib	>500
100WCP-II-Ic	>500
BPII[a]	11.9

[a] Positive control, a high active pectic polysaccharide from *B. petersianum*.

100WCP-II-Ia, were selected for linkage analysis. The results are given in Table 2.

The fraction 50WCP-II-Ia consists of a 1,4-linked galacturonan with a high degree of branching on position 2. The Rha is present as 1,2-linked units with branching on position 4. Gal and Ara are present as typical linkage units for AG-II, which also was shown by a positive Yariv reaction (Table 1). The presence of 1,4-linked Gal may indicate the presence of AG-I. These features indicate the 50WCP-II-Ia consists of an RG-I backbone with AG-I and AG-II side chains.

The linkage units of 100WCP-II-Ia are different from 50WCP-II-Ia. The Rha is present as 1,2-linked only. 1,3-linked Gal, 1,6-linked Gal and 1,3,6-linked Gal were not detected in 100WCP-II-Ia indicating the absence of AG-II, this fraction gave also a negative reaction in the Yariv test (Table 1). The Gal is present mainly as terminal units and 1,4-linked Gal and also with some branching points on position 2. The proposed feature of 100WCP-II-Ia is a 1,4-linked galacturonan, interrupted by 1,2-linked Rha, with side chains substituted on position 2 of GalA. The side chains consist of 1,4-linked galactans with branch points on position 2.

3.5. Complement fixation activity

The results of complement fixation assay of the Fru rich neutral fractions 50WCPN and 100WCPN showed no activity (data not shown). As shown in Table 3, the purified fractions 50WCP-II-I and 100WCP-II-I exhibited similar potent human complement fixation activities *in vitro*. These two fractions were less active than BPII, but still quite active compared with other polysaccharides previously studied (Samuelsen et al., 1996). The activity of the fractions after pectinase degradation and separation by gel filtration showed that the highest *Mw* sub-fractions, 50WCP-II-Ia and 100WCP-II-Ia, had a higher complement fixation activity than their parent fractions. The activity of fraction 50WCP-II-Ia is significantly higher than fraction100WCP-II-Ia. The low *Mw* fractions 50CP-II-Ib, 100WCP-II-Ib and 100WCP-II-Ic exhibited much lower activity compared to their parent fractions.

3.6. Structural features

Fractions 50WCP-II-I and 100WCP-II-I were characterized by 1D and 2D NMR spectroscopy and comparison with chemical shift values from the literature (Hromádková, Košťálová, Vrchotová, & Ebringerová, 2014; Košťálová, Hromádková, & Ebringerová, 2013; Yang et al., 2013), and by monosaccharide composition and linkage types. The monosaccharide composition and linkage analysis indicate that these two fractions are fairly similar. Fraction 100WCP-II-I had somewhat better solubility (25 mg/mL) than fraction 50WCP-II-I (15 mg/mL). From the ^{1}H and ^{13}C one-dimensional spectra and the HSQC two-dimensional spectrum, most of the signals could be assigned as shown in Table 4. Some signals from minor sugars could not be observed.

The ^{1}H NMR spectrum of fraction 50WCP-II-I contains four main peaks in the anomeric region, which were labeled as A, B, C and D (Fig. 3A-a). The anomeric proton signals at δ 5.12 and 4.98 ppm correspond to the non-methyl esterified and methyl esterified α-D-Gal*p*A, while signals at δ 5.08 and 5.29 ppm correspond to H1 of α-L-Ara*f* and α-L-Rha*p*, respectively. The intense ^{1}H signal at δ 3.82 ppm, the ^{13}C signal at δ 55.7 ppm and cross peak δ 55.7/3.82 (—OCH_3 in Fig. 3A) and δ 74.2/4.70 ppm (B5 in Fig. 3A) in the HSQC spectrum and cross peaks δ 173.6/3.82 and 173.6/5.11 ppm in the HMBC spectrum (data not shown) indicate that α-D-Gal*p*A is partly methyl esterified. In the downfield region of ^{13}C NMR spectrum, signals at δ 173.6 and 177.7 ppm (Fig. 3A-b) were assigned to the carboxyl groups of methyl esterified and non-methyl esterified α-D-Gal*p*A, respectively (Yang et al., 2013). Integration of signals at δ 4.70 ppm (H5 of non-esterified α-D-Gal*p*A), δ 4.98 ppm (H1 of esterified α-D-Gal*p*A) and δ 5.12 ppm (H1 of non-esterified α-D-Gal*p*A and H5 of esterified α-D-Gal*p*A) indicates a ratio between non-esterified and esterified α-D-Gal*p*A of about 2:1. The weak cross peak at δ 19.5/1.22 ppm in the HSQC spectrum (C6 in Fig. 3A-c) corresponds to C6/H6 (methyl group) of α-L-Rha*p*, and the weak cross peak δ 110.4/5.08 ppm (D1in Fig. 3A) corresponds to C1/H1 of terminal α-L-Ara*f* (Hromádková et al., 2014). The location of other residues of fraction 50WCP-II-I in Table 2 could not be determined due to their very low concentration.

The ^{1}H NMR spectrum of fraction 100WCP-II-I contains five main peaks in the anomeric region, which were labeled J, K, L, M and N (Fig. 3B-a). The anomeric proton signals at δ 5.12 and 4.98 ppm corresponded to the non-methyl esterified α-D-Gal*p*A and methyl esterified α-D-Gal*p*A, while signals at δ 5.10, 5.29 and 4.64 ppm corresponded to the H1 of α-L-Ara*f*, α-L-Rha*p* and β-D-Gal*p*, respectively. The intense ^{1}H signal at δ 3.82 ppm, the ^{13}C

Table 4
^{13}C and ^{1}H Chemical shifts (δ in ppm) of the polysaccharide fractions 50WCP-II-I and 100CP-II-I.

Residues		C1/H1	C2/H2	C3/H3	C4/H4	C5/H5	C6/H6
50WCP-II-I							
A	-4-α-D-GalA*p*A6Me[a]-(1-	103.1/4.98	71.0/3.82	71.8/3.98	81.2/4.47	73.4/5.12	173.6[b]
B	-4-α-D-GalA*p*-(1-	102.2/5.12	70.8/3.75	71.0/4.01	81.6/4.45	74.2/4.70	177.7
C	-2-α-L-Rha*p*-(1-	n.d./5.29	n.d./n.d.	n.d./n.d.	n.d./n.d.	n.d./n.d.	19.5/1.22, n.d.
D	t-α-L-Ara*f*-(1-	110.4/5.08	n.d./n.d.	79.6/3.94	n.d./n.d.	63.9/3.82	
100WCP-II-I							
J	-4-α-D-GalA*p*A6Me-(1-	103.1/4.98	71.0/3.82	71.9/4.00	81.7/4.47	73.4/5.12	173.6[b]
K	-4-α-D-GalA*p*-(1-	102.2/5.12	70.8/3.75	71.2/4.01	81.2/4.45	74.2/4.70	177.7
L	-2-α-L-Rha*p*-(1-	101.8/5.29	79.2/4.12	72.6/3.98	n.d./n.d.	n.d./n.d.	19.5/1.26,1.30
M	t-α-L-Ara*f*-(1-	110.4/5.10	84.2/4.14	79.5/3.92	86.8/4.06	63.9/3.78	
N	-4-β-D-Gal*p*-(1-	107.2/4.64	74.8/3.68	76.2/3.78	80.9/4.16	77.4/3.70	64.0/3.80

n.d., Not detected.
[a] GalA*p*A6Me stands for methyl esterified galacturonic acid.
[b] —OCH_3 at δ 55.7/3.82.

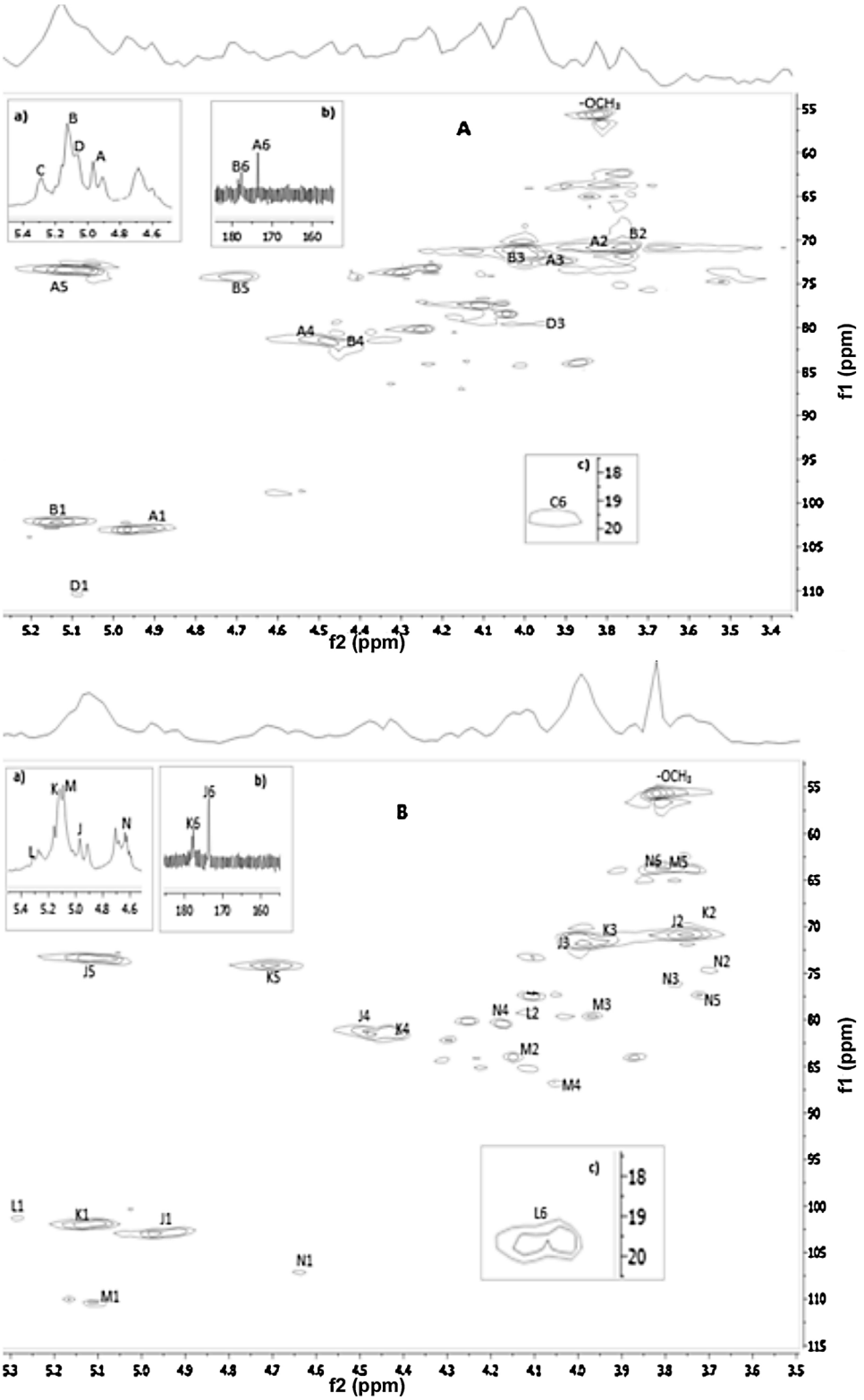

Fig. 3. $^{13}C/^{1}H$ HSQC NMR spectrum of 50WCP-II-I (A) and 100WCP-II-I (B). The insets represent the enlargement of (a) anomeric region (δ 4.5–5.5 ppm), (b) downfield region of ^{13}C NMR spectrum (160–180 ppm), (c) methyl group of rhamnose.

signal at δ 55.7 ppm, cross peaks at δ 55.7/3.82 (—OCH_3 in Fig. 3B) and δ 74.2/4.70 ppm (K5 in Fig. 3B) in the HSQC spectrum, and cross peaks at δ 173.6/3.82 and 173.6/5.11 ppm in the HMBC spectrum (data not shown) indicate methyl esterified α-D-Gal*p*A. In the downfield region of ^{13}C NMR spectrum, signals at δ 173.6 and 177.7 ppm (Fig. 3B-b) were assigned to the carboxyl groups of methyl esterified and non-methyl esterified α-D-Gal*p*A, respectively (Yang et al., 2013). Integration of signals at δ 4.70 ppm (H5 of non-esterified α-D-Gal*p*A), δ 4.98 ppm (H1 of esterified α-D-Gal*p*A) and δ 5.12 ppm (H1 of non-esterified α-D-Gal*p*A and H5 of esterified α-D-Gal*p*A) indicates a ratio between non-esterified α-D-Gal*p*A and esterified α-D-Gal*p*A of about 1.25:1. Thus, there is a higher

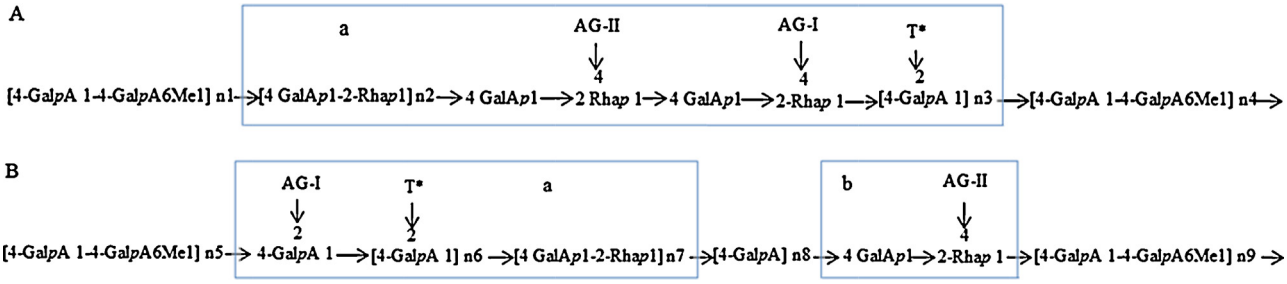

Fig. 4. Proposed structure of fraction 50WCP-II-I (A) and fraction 100WCP-II-I (B). The letters n1–n9 stand for number of residues. (* Terminal linked units of GlcA or Glc or Gal or Ara).

degree of methyl-esterification (DM) in the fraction 100WCP-II-I (*ca.* 44%) than in the fraction 50WCP-II-I (*ca.* 33%). A cross peak at δ 101.8/5.29 ppm (L1 in Fig. 3B) indicates the C1/H1 of α-L-Rha*p*, and cross peaks δ 19.5/1.22 and 19.5/1.30 ppm (L6 in Fig. 3B-c) of the HSQC spectrum correspond to C6/H6 (methyl group) of α-L-Rha*p* (Hromádková et al., 2014; Košťálová et al., 2013). The cross peaks M1–M5 in the HSQC spectrum (Fig. 3B) indicate that the α-L-Ara*f* is mainly terminal linked, which was also shown by the methylation results (Table 2). The $^{13}C/^{1}H$ signals (N1–N6 in Fig. 3B) of 1,4-linked β-D-Gal residues (Table 4) confirmed the presence of β-(1,4)-galactan, which forms AG-I side chains of the RG-I region (Hromádková et al., 2014; Košťálová et al., 2013). Due to the very low content of other residues in Table 2, their location in the spectra could not be determined.

4. Discussion

Two purified polysaccharide fractions, 50WCP-II-I and 100WCP-II-I, from the 50 and 100 °C water extracts of the roots of *C. pilosula*, were isolated after ion exchange chromatography and gel filtration. 50WCP-II-I had complement fixation activity similar to 100WCP-II-I; both being lower than BPII, but still quite active compared with activities of other active anti-complementary polysaccharides previously studied, such as PMII from *Plantago major* (Samuelsen et al., 1996), GOA2 from *Glinus oppositifolius* (Inngjerdingen et al., 2007), BR-2-IIb from *Bupleurum falcatum* (Yamada, Ra, Kiyohara, Cyong, & Otsuka, 1989), and polysaccharides from *Lessertia frutescens* (Zhang et al., 2014). These two fractions were degraded by pectinase after de-esterification. As shown in Table 3, the hairy regions (the highest *Mw* compounds after pectinase treatment of the native fractions) are more active than their parent polymers; this has been observed for other pectic polymers as well (Paulsen & Barsett, 2005).

The feature of the fraction 50WCP-II-Ia is RG-I backbones, with branching AG-I and AG-II side chains on position 4 of Rha. This is consistent with a highly ramified pectic polysaccharide Z100W-II-I with AG-I and AG-II side chains, isolated from *L. frutescens* leaves, with strong complement fixation activity (Zhang et al., 2014). Based on monosaccharide composition, linkage analyses (both before and after pectinase treatment) and 1D and 2D NMR spectra, the structure of 50WCP-II-I is proposed as shown in Fig. 4A. The proposed structural feature of fraction 50WCP-II-I is 1,4-linked galacturonan (some of the residues are methyl esterified), interrupted by ramified regions. The ramified region (Fig. 4A, region a) consists of alternating 1,4-linked α-D-Gal*p*A and 1,2-linked α-L-Rha*p*, with branching AG-I/AG-II side chains on position 4 of α-L-Rha*p*. The ramified region also contains 1,2,4-linked α-D-Gal*p*A, branched by terminal linked monosaccharides on position 2. This proposed structure is based on normal feature of pectin, but we cannot verify the exactly branching position, which means the AG-I/AG-II side chains could be branched on position 2 of α-D-Gal*p*A as well. While these structures cannot be regarded as fully proven, they appear to be the most probable ones based on the available data. The high *Mw* sub-fraction 50WCP-II-Ia (Fig. 4A, region a) is responsible for expression of the main part of the complement fixation activity of 50WCP-II-I.

After pectinase treatment, three main fractions were obtained from the parent fraction 100WCP-II-I. The highest *Mw* sub-fraction 100WCP-II-Ia consists of a ramified region with AG-I side chains attached on position 2 of GalA, the second sub-fraction 100WCP-II-Ib contains AG-II side chains. Compared to fraction 100WCP-II-Ia (only containing AG-I side chains), the fraction 50WCP-II-Ia contains AG-I and AG-II side chains, which may explain the higher activity detected in fraction 50WCP-II-Ia. The pectic polysaccharide Oc50A2 from *Opilia celtidifolia* with AG-I and AG-II side chains showed higher complement fixation activity than Oc50A2.II.pur with only AG-II side chains (Grønhaug et al., 2010; Togola et al., 2008). These findings indicate that AG-I side chains contribute to the complement fixation activity in the present study. Although 100WCP-II-Ib contains AG-II side chain, the low activity might be due to the low molecular weight (1.3 kDa).

The structure of 100WCP-II-I was proposed as shown in Fig. 4B, using the same methods as for fraction 50WCP-II-I. The proposed structure of fraction 100WCP-II-I is 1,4-linked galacturonan (some of the residues are methyl esterified), interrupted by ramified regions. The ramified region (Fig. 4B, region a) consists of alternating 1,4-linked α-D-Gal*p*A (with/without methyl esterification) and 1,2-linked α-L-Rha*p*, with branching AG-I side chain and terminal linked monosaccharides on position 2 of 1,4-linked α-D-Gal*p*A. The ramified region (Fig. 4B, region b) contains alternating 1,4-linked α-D-Gal*p*A and 1,2-linked α-L-Rha*p*, with side chain AG-II branched on position 4 of α-L-Rha*p*. The complement fixation assay results suggest that the activity of fraction 100WCP-II-I mainly is expressed by the ramified region 100WCP-II-1a (Fig. 4B, region a).

A pectic polysaccharide, CPP1b, with antitumor activity was isolated from the root of *Codonopsis pilosula* (Franch.) Nannf. The structural features of the polysaccharides 50WCP-II-I and 100WCP-II-I are more complex than that of CPP1b (Yang et al., 2013). The latter has a higher *Mw* (145 kDa) and only four types of monosaccharides (Rha, Ara, Gal and GalA). CCP1b and our samples all contain GalA units that are 1,4-linked, and all are partly methyl esterified. The main structural difference lies in the branches where CCP1b has a low degree of branched Ara, while our samples are highly branched with both AG-I and AG-II side chains.

5. Conclusions

The water extract of roots of *C. pilosula* Nannf. var. *modesta* L.T.Shen contains polysaccharides with potent complement fixation activity. Two of the most abundant purified polysaccharide fractions, 50WCP-II-I and 100WCP-II-I, were obtained by ion exchange chromatography and gel filtration from 50 and 100 °C water crude extracts, respectively. The structural features of these two fractions were investigated based on monosaccharide compositions, linkage analyses (both before and after pectinase

treatment) and 1D and 2D NMR analyses. The main features of fraction 50WCP-II-I are long HG region (some of the residues are methyl esterified) and a main RG-I region with AG-I and AG-II side chains. The AG-I and AG-II side chains attach on position 4 of Rha of backbone. The high *Mw* sub-fraction 50WCP-II-Ia is responsible for expression of the main part of the complement fixation activity of 50WCP-II-I. The main features of 100WCP-II-I are long HG regions (some of the residues are methyl esterified) and 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. The results from complement fixation assay suggest that the activity of fraction 100WCP-II-I is expressed mainly by the ramified region 100WCP-II-Ia. The complement system is essential for the operation of the innate as well as the adaptive immune defenses, and the complement fixation assay was used for a long time as a screening system for interactions with the human immune system. Samples showing inhibition in the assay are thus having a direct effect on the human immune system (Michaelsen et al., 2000). The polysaccharides from *C. pilosula* showed high activity in complement fixation assay, and may therefore have effect on the human immune system. This might be seen as a rationale for the traditional use of *C. pilosula* to boost immunity. However, clinical studies are needed to elucidate the activity *in vivo* of *C. pilosula* preparations.

Acknowledgments

This project is part of the FP7EU project MUTHI, project number 266005. The first author is also grateful for receiving funding from the China Scholarship Council. The authors are indebted to Hoai Thi Nguyen Aas, Department of Pharmaceutical Chemistry, University of Oslo, for recording of the GC–MS experiments in the determination of glycosidic linkages.

References

Austarheim, I., Christensen, B. E., Hegna, I. K., Petersen, B. O., Duus, J. O., Bye, R., et al. (2012). Chemical and biological characterization of pectin-like polysaccharides from the bark of the Malian medicinal tree *Cola cordifolia*. *Carbohydrate Polymers*, *89*, 259–268.

Austarheim, I., Mahamane, H., Sanogo, R., Togola, A., Khaledabadi, M., Vestrheim, A. C., et al. (2012). Anti-ulcer polysaccharides from *Cola cordifolia* bark and leaves. *Journal of Ethnopharmacology*, *143*, 221–227.

Barsett, H., Paulsen, B. S., & Habte, Y. (1992). Further characterization of polysaccharides in seeds from *Ulmus glabra* Huds. *Carbohydrate Polymers*, *18*, 25–130.

Bi, H. Y., Zhang, L. P., Chen, Z., & Wu, B. (2008). An overview of research on germplasm resources of Radix Codonopsis and their utilization. *China Journal of Chinese Materia Medica*, *33*, 590–594.

Boratynski, J. (1984). Colorimetric method for the determination of ketoses using phenol-acetone-boric acid reagent (PABR). *Analytical Biochemistry*, *137*, 528–532.

Bradford, M. M. (1976). A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, *72*, 248–254.

Chambers, R. E., & Clamp, J. R. (1971). Assessment of methanolysis and other factors used in the analysis of carbohydrate-containing materials. *Biochemical Journal*, *125*, 1009–1018.

Chaplin, M. F. (1994). Monosaccharides. In M. F. Chaplin, & J. F. Kennedy (Eds.), *Carbohydrate analysis: A practical approach* (pp. 1–42). Oxford: Oxford University Press.

China Pharmacopoeia Commission. (2010). . *Pharmacopoeia of People's Republic of China* (Vol. I) Beijing: Chemical Industry Press.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., & Smith, F. (1956). Colorimetric method for determination of sugars and related substances. *Analytical Chemistry*, *28*, 350–356.

Grønhaug, T. E., Ghildyal, P., Barsett, H., Michaelsen, T. E., Morris, G., Diallo, D., et al. (2010). Bioactive arabinogalactans from the leaves of *Opilia celtidifolia* Endl. ex Walp. (Opiliaceae). *Glycobiology*, *20*, 1654–1664.

Grønhaug, T. E., Kiyohara, H., Sveaass, A. M., Diallo, D., Yamada, H., & Paulsen, B. S. (2011). Beta-D-(1(4)-galactan-containing side chains in RG-I regions of pectic polysaccharides from *Biophytum petersianum* Klotzsch contribute to expression of immunomodulating activity against intestinal Peyer's patch cells and macrophages. *Phytochemistry*, *72*, 2139–2147.

Han, F., Cheng, L., & Chen, Y. (2005). Study on isolation and composition of *Codonopsis tangshen* polysaccharide. *Chinese Pharmaceutical Journal*, *40*, 1381–1383.

He, Q., Zhu, E. Y., Wang, Z. T., Xu, L. S., & Hu, Z. B. (2005). HPLC analysis for lobetyolin in Radix Codonopsis. *Chinese Pharmaceutical Journal*, *40*, 56–58.

Hromádková, Z., Košťálová, Z., Vrchotová, N., & Ebringerová, A. (2014). Non-cellulosic polysaccharides from the leaves of small balsam (*Impatiens parviflora* DC.). *Carbohydrate Research*, *389*, 147–153.

Inngjerdingen, K. T., Coulibaly, A., Diallo, D., Michaelsen, T. E., & Paulsen, B. S. (2006). A complement fixing polysaccharide from *Biophytum petersianum* Klotzsch, a medicinal plant from Mali, West Africa. *Biomacromolecules*, *7*, 48–53.

Inngjerdingen, K. T., Patel, T., Chen, X., Kenne, L., Allen, S., Morris, G. A., et al. (2007). Immunological and structural properties of a pectic polymer from *Glinus oppositifolius*. *Glycobiology*, *17*, 1299–1310.

Kim, J. B., & Carpita, N. C. (1992). Changes in esterification of the uronic-acid groups of cell-wall polysaccharides during elongation of maize coleoptiles. *Plant Physiology*, *98*, 646–653.

Knutsen, S. H. (1991). Carrageenase production in a culture of *Pseudomonas carrageenovora* growing on κ-carrageenan. In G. Garcia-Reina, & M. Pedersen (Eds.), *Proceedings of a COST-48 workshop at Grand Canaria, Spain*.

Košťálová, Z., Hromádková, Z., & Ebringerová, A. (2013). Structural diversity of pectins isolated from the Styrian oil-pumpkin (*Cucurbita pepo* var. styriaca) fruit. *Carbohydrate Polymers*, *93*, 163–171.

Li, G., & Yang, S. (2001). Extraction of *Codonopsis pilosula* polysaccharide and its effects of anti-active oxygen free radicals. *Chemistry World*, *8*, 421–423.

Lin, M., Xia, B., Yang, M., Gao, S., Hou, Y., & Lou, G. (2013). Characterization and antitumor activities of a polysaccharide from the rhizoma of *Menispermum dauricum*. *Carbohydrate Polymers*, *92*, 2212–2217.

Michaelsen, T. E., Gilje, A., Samuelsen, A. B., Høgåsen, K., & Paulsen, B. S. (2000). Interaction between human complement and a pectin type polysaccharide fraction, PMII, from the leaves of *Plantago major* L. *Scandinavian Journal of Immunology*, *52*, 483–490.

Miller, G. L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical Chemistry*, *31*, 426–428.

O'Neill, M. A., & York, W. S. (2003). The composition and structure of plant primary cell walls. In J. K. C. Rose (Ed.), *The plant cell wall* (pp. 1–54). Oxford: Blackwell Publishing.

Paulsen, B. S., & Barsett, H. (2005). Bioactive pectic polysaccharides. *Advances in Polymer Science*, *186*, 69–101.

Perez, S., Rodriguez-Carvajal, M. A., & Doco, T. (2003). A complex plant cell wall polysaccharide: Rhamnogalacturonan II. A structure in quest of a function. *Biochimie*, *338*, 651–671.

Ricklin, D., Hajishengallis, G., Yang, K., & Lambris, J. D. (2010). Complement: A key system for immune surveillance and homeostasis. *Nature Immunology*, *11*, 785–797.

Samuelsen, A. B., Paulsen, B. S., Wold, J. K., Otsuka, H., Kiyohara, H., Yamada, H., et al. (1996). Characterization of a biologically active pectin from *Plantago major* L. *Carbohydrate Polymers*, *30*, 37–44.

Singleton, V. L., & Rossi, J. A. (1965). Colorimetry of total phenolics with phosphomolybdic–phosphotungstic acid reagents. *American Journal of Enology and Viticulture*, *37*, 144–158.

Sun, Y. X., & Liu, J. C. (2008). Structural characterization of a water-soluble polysaccharide from the roots of *Codonopsis pilosula* and its immunity activity. *International Journal of Biological Macromolecules*, *43*, 279–282.

Sun, Y., Liu, J., & Kennedy, J. F. (2010). Application of response surface methodology for optimization of polysaccharides production parameters from the roots of *Codonopsis pilosula* by a central composite design. *Carbohydrate Polymers*, *80*, 949–953.

Togola, A., Inngjerdingen, M., Diallo, D., Barsett, H., Rolstad, B., Michaelsen, T. E., et al. (2008). Polysaccharides with complement fixing and macrophage stimulation activity from Opilia celtidifolia, isolation and partial characterization. *Journal of Ethnopharmacology*, *115*, 423–431.

van Holst, G. J., & Clarke, A. E. (1985). Quantification of arabinogalactan-protein in plant extracts by single radial gel diffusion. *Analytical Biochemistry*, *148*, 446–450.

Waldron, K. W., & Faulds, C. B. (2007). Cell wall polysaccharides: Composition and structure. In J. P. Kamerling, G. J. Boons, Y. C. Lee, A. Suzuki, N. Taniguchi, & A. G. J. Voragen (Eds.), *Comprehensive glycoscience – From chemistry to systems biology* (1) (pp. 181–201). Amsterdam: Elsevier.

Wang, Z. T., Ng, T. B., Yeung, H. W., & Xu, G. J. (1996). Immunomodulatory effect of a polysaccharide-enriched preparation of *Codonopsis pilosula* roots. *General Pharmacology*, *27*, 1347–1350.

Wang, Z. T., Xu, G. J., Namba, T., & Tsuneo, N. (1991). HPLC analysis of atractylenolide III in roots of *Codonopsis* plants. *Journal of China Pharmaceutical University*, *23*, 48–50.

Xin, T., Zhang, F., Jiang, Q., Chen, C., Huang, D., Li, Y., et al. (2012). The inhibitory effect of a polysaccharide from *Codonopsis pilosula* on tumor growth and metastasis in vitro. *International Journal of Biological Macromolecules*, *51*, 788–793.

Xu, C., Liu, Y., Yuan, G., & Guan, M. (2012). The contribution of side chains to antitumor activity of a polysaccharide from *Codonopsis pilosula*. *International Journal of Biological Macromolecules*, *50*, 891–894.

Yamada, H., & Kiyohara, H. (1999). *Complement-activating polysaccharides from medicinal herbs*. In H. Wagner (Ed.), *Immunomodulatory agents from plants* Basel: Birkhäuser Verlag.

Yamada, H., & Kiyohara, H. (2007). Immunomodulating activity of plant polysaccharide structures. In J. P. Kamerling, G. J. Boons, Y. C. Lee, A. Suzuki, N. Taniguchi, & A. G. J. Voragen (Eds.), *Comprehensive glycoscience – From chemistry to systems biology* (Vol. 4) (pp. 663–694). Amsterdam: Elsevier.

Yamada, H., Ra, K. S., Kiyohara, H., Cyong, J. C., & Otsuka, Y. (1989). Structural characterization of an anti-complementary pectic polysaccharide from the roots of *Bupleurum falcatum* L. *Carbohydrate Research*, *189*, 209–226.

Yang, C., Guo, Y., Chen, J., An, J., Chen, W., & Hu, F. (2013). Structural characterization and antitumor activity of a pectic polysaccharide from *Codonopsis pilosula*. *Carbohydrate Polymers*, *98*, 886–895.

Yang, G., Li, F., Liu, H., & Xian, F. (2005). The effect of Codonopsis polysaccharide on immune function of mice. *Pharmacology & Clinics Chinese Materia Medica*, *21*, 39–40.

Ye, G., Li, C., Huang, C., Li, Z., Wang, X., & Chen, Y. (2005). Chemical structure of fructosan from *Codonopsis pilosula*. *China Journal of Chinese Materia Medica*, *30*, 1338–1339.

Zhang, B., Leung, W. K., Zou, Y., Mabusela, W., Johnson, Q., Michaelsen, T. E., et al. (2014). Immunomodulating polysaccharides from *Lessertia frutescens* leaves: Isolation, characterization and structure activity relationship. *Journal of Ethnopharmacology*, *152*, 340–348.

Zhang, X., Zhu, C., Hu, L., Lai, X., & Mo, J. (2003). Pharmacological action of polysaccharides from Radix Codonopsis on immune function and hematopoiesis in mice. *Traditional Chinese Drug Research & Clinical Pharmacology*, *14*, 174–176.

Zhang, Y., Liang, Z., Zhao, W., Huo, X., Zhang, L., & Zhang, X. (2005). Separation, purification and compositional analysis of water soluble polysaccharide $CPPS_3$ from *Codonopsis pilosula*. *Chinese Pharmaceutical Journal*, *40*, 1107–1109.

Zhang, Y., Zhang, L., Yang, J., & Liang, Z. (2010). Structure analysis of water-soluble polysaccharide $CPPS_3$ isolated from *Codonopsis pilosula*. *Fitoterapia*, *81*, 157–161.

Zong, A., Cao, H., & Wang, F. (2012). Anticancer polysaccharides from natural resources: A review of recent research. *Carbohydrate Polymers*, *90*, 1395–1410.

Zou, Y. F., Chen, X. F., Yang, W. Y., & Liu, S. (2011). Response surface methodology for optimization of the ultrasonic extraction of polysaccharides from *Codonopsis pilosula* Nannf. var. *modesta* L.T.Shen. *Carbohydrate Polymers*, *84*, 503–508.