Carbohydrate Polymers 195 (2018) 207–217

Contents lists available at ScienceDirect

Carbohydrate Polymers

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

Structural studies of water-extractable pectic polysaccharides and arabinogalactan proteins from *Picea abies* greenery

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ARTICLE INFO

Keywords:
Picea abies
Pectic polysaccharides
Arabinogalactan proteins
Coniferous tree greenery
Structure
NMR

ABSTRACT

Water-extractable arabinogalactan proteins (AGP) (the main constituent) and pectic polysaccharides were isolated from tree greenery of *Picea abies*. The carbohydrate part of AGP macromolecules consisted of AG-II, the side chains of which were represented by 1,6- and 1,3,6-β-D-Gal*p*, T-α-L-Ara*f*, 1,3- and 1,5-α-L-Ara*f*, T-β-D-Glc*p*A and 1,4-β-D-Glc*p*A, T-α-L-*Rha*p, T-α-L-Fuc*p* and 4-*O*-Me-α-L-Fuc*p* residues. It was established that the unusual 4-*O*-Me-α-L-Fuc*p* monosaccharide are located on the non-reducing ends of the side chains of carbohydrate part of AGP macromolecules, and are bound to 1,4-β-D-Glc*p*A residues by 1,4-bonds. The backbone of pectin macromolecules consisted mostly of 1,4-α-D-galactopyranosyluronan and RG-I, which side chains were represented by highly branched 1,5-α-L-arabinan. It was shown that RG-I is characterized mainly by short segments, which are alternated with the regions of the non-acetylated and non-methyl-esterified galacturonan. The study revealed that at least a part of the pectin is strongly associated with AGP. It was indicated that the RG-I segments are separated from the AGP-bound pectin by regions of 1,4-α-D-galactopyranosyluronane.

1. Introduction

The complexity of pectins as polysaccharide substances is manifested by a great variety of their major constituents, the main of which are galacturonan, rhamnogalacturonan-I, rhamnogalacturonan-II, arabinan, galactan and arabinogalactan. Pectins are present mainly in the intercellular space and primary cell wall, where they perform a lot of important biological functions.

Arabinogalactan proteins (AGPs) are a large class of highly glycosylated proteins characterized by complex structure. The bulk of AGP (up to 95%) comprises a carbohydrate part, which consists of arabinogalactan type II (AG-II), and 2–10% of a polypeptide core usually rich in hydroxyproline (Hyp), alanine (Ala), serine (Ser), or threonine (Thr) (Ellis, Egelund, Schultz, & Bacic, 2010; Gaspar, Johnson, McKenna, Bacic, & Schultz, 2001; Seifert & Roberts, 2007; Tryfona et al., 2010). AGPs are present in all plant tissues at all stages of growth. AGPs are involved in such processes as cell growth, cell proliferation, pattern formation and sexual reproduction (Seifert & Roberts, 2007).

The content of pectin and AGP, monosaccharide composition and structural elements can differ not only in different plant species, but also among individual tissues of a plant. These features are dependent on environmental conditions of growth, plant variety, a vegetation stage and age of a plant.

Intensive studies of these polymers are continuing in order to discover new structural features of these classes of complex natural compounds. It is expected that pectins can be bound with structural proteins via complex covalent bonds to form an interlaced network that is needed to maintain integrity and stability of the cell wall (Keegstra, Talmadge, Bauer, & Albersheim, 1973). However, in spite of the fact that significant progress has been achieved in the studies on structure of pectic polysaccharides and AGP, the structural features and interactions of these structural elements with each other and with other components of the cell wall are still being discussed.

Norway spruce (*P. abies*) is a coniferous tree, a species of the genus *Picea* in the family *Pinaceae*. It is known that spruce extracts exhibit a wide range of physiological activity, e.g. immunomodulating, immunostimulating, anti-inflammatory, antibacterial, antifungal and other activities (Fyhrquist et al., 2017; Le Normand et al., 2014).

In the previous work, arabinogalactan proteins and pectic polysaccharides were isolated from greenery of *P. abies* by water extraction. Major constituents of their structure were determined by ion exchange chromatography, partial acid and enzymatic hydrolysis and NMR spectroscopy (Shakhmatov, Belyy, & Makarova, 2017).

The present work is devoted to further elucidation of structural features of the water-extractable pectic polysaccharides and arabinogalactan proteins from Norway spruce greenery using enzyme digestion and Smith degradation followed by 1D and 2D NMR analysis of the degradation products.

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https://doi.org/10.1016/j.carbpol.2018.04.074
Received 14 March 2018; Received in revised form 17 April 2018; Accepted 18 April 2018
Available online 24 April 2018

The results obtained in this work not only confirmed, but also substantially supplemented the conclusions obtained earlier. General regularities and features of structure of carbohydrate chains of pectic polysaccharides and carbohydrate part of AGP from *Picea abies* greenery were specified and supplemented.

2. Materials and methods

2.1. Preparation of plant raw material and isolation of polysaccharides

Coniferous greenery of *P. abies* was collected near Syktyvkar (Komi Republic, Russia) in the period of January 2016. The samples were taken from 10 to 20 growing trees in the middle of the month. For the study of coniferous greenery, thin branches (less than 8 mm in diameter) with needles, from the top, middle and bottom sections of a tree crown were cut in four different geodesic directions. Fresh plant material was homogenized using an RM-120 knife mill (particle size 10–15 mm, Russia).

The isolation was performed according to the procedure described earlier (Makarova, Shakhmatov, & Belyy, 2017). The residual raw material (100 g) was extracted with distilled water (1 L) under continuous stirring for 2 h at 70 °C. The fivefold extraction was applied. The combined extract was filtered, concentrated and centrifuged. The supernatant was collected and precipitated with four volumes of 96% ethanol. The precipitate was separated by centrifugation and redissolved in water. The resulting polysaccharide PA_W was dialyzed (12–14 kDa membranes) and freeze-dried.

2.2. General methods

The glycuronic acid (UA) content was determined by the reaction with 3,5-dimethylphenol in the presence of concentrated sulphuric acid. A calibration plot was constructed for D-galacturonic acid (Sigma–Aldrich), and photocolorimetry was carried out at 400 and 450 nm (Usov, Bilan, & Klochkova, 1995). Protein concentration was determined using the Bradford procedure with bovine serum albumin (BSA) as a standard (Bradford, 1976). The degree of methyl esterification was calculated as the molar ratio between methanol (determined by the method of Wood and Siddiqui (1971)) and uronic acids; the photocolorimetry was conducted at 412 nm. Absorption spectra of the analyzed solutions were measured on a Shimadzu UV-1700 PharmaSpec (Shimadzu, Japan) spectrophotometer. Each experiment was run in triplicate.

The solutions were concentrated on a rotary evaporator (Heidolph, Germany) under reduced pressure at 40–45 °C. The samples were centrifuged at 5000–10,000*g* for 10–20 min and then lyophilized from the frozen state using a Christ Alpha 2–4 LD lyophilizer.

The solutions were dialyzed using 3.5 or 12–14 kDa molecular weight cut-off membranes for 3 days, with periodic change of water every 4 h (10 L each time).

2.2.1. Size exclusion chromatography

Gel filtration chromatography was carried out on a Sephacryl S-300 (Sigma-Aldrich) column (1.3 cm × 37 cm, a void volume of 12 mL, Sigma, USA). Distilled water was used as an eluent at a flow rate of 0.3 mL/min, the fractions volume was 3 mL (a fraction collector was used). The carbohydrate content in the fractions was determined using the phenol–sulphuric acid procedure with photocolorimetry carried out at 480 nm (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). Fractions corresponding to separate peaks were combined, concentrated, dialysed and lyophilized.

2.2.2. Molecular weight determination of carbohydrates

Molecular weights and polydispersities of the polysaccharide fractions were determined by the high performance liquid chromatography. The chromatographic system (Shimadzu, Japan) used for the analysis consisted of an LC-20AD pump, a DGU-20A3 degasser, a CTO-10AS thermostat, an RID-10A refractometer as the detector, and a Shodex OH-pak SB-804 HQ column (Shimadzu, Japan). Pullulans from Fluka, Germany (1.3, 6, 12, 22, 50, 110, 200, 400, 800 kDa), were used as standards.

2.2.3. Analysis of monosaccharide composition

Samples (3–5 mg) were hydrolyzed with 2 M TFA (1 mL) containing myo-inositol as an internal standard (1 mg/mL) at 100 °C for 5 h. The mixture of neutral monosaccharides was converted to alditol acetates (York, Darvill, McNeil, Stevenson, & Albersheim, 1986) and identified by gas chromatography (GC) on the Shimadzu GC-2010AF chromatograph equipped with a flame ionisation detector, using an HP-1 capillary column (Agilent, 30 m × 0.25 mm × 0.25 μm). Helium was used as the carrier gas. GC of alditol acetates was carried out at temperature programmed from 175 °C (1 min) to 250 °C (2 min) with a rate of 3 °C/min. The content of monosaccharides as percentage of the total mass was calculated from the peak areas using coefficients of detector response.

2.2.4. NMR spectroscopy

^1H and ^{13}C NMR spectra were recorded on a Bruker Avance II 300 spectrometer at operating frequencies of 300.17 and 75.48 MHz, respectively; using 3-5% solutions of polysaccharides in D_2O (99.9% D) containing DSS (sodium 4,4-dimethyl-4-silapentan-sulfonate) (Sigma–Aldrich), at 303 K and 328 K as described earlier (Shakhmatov et al., 2016). Samples were freeze-dried from 95% D_2O and subsequently dissolved in 99.9% D_2O. Chemical shifts were referenced to internal DSS (^1H and ^{13}C at 0.00 ppm). Two-dimensional spectra were recorded using standard Bruker procedures. Complete description of the signals in the ^1H and ^{13}C NMR spectra of the obtained polysaccharides was carried out using two-dimensional homonuclear (COSY, TOCSY, ROESY) and heteronuclear (HSQC, HSQC-TOCSY, HSQC-NOESY and HMBC) experiments.

2.3. Structural analyses of the polysaccharide PA_W

2.3.1. Enzymatic hydrolysis of the polysaccharide PA_W

The mixture of PA_W (320 mg in 52 mL of H_2O) with 0.2 mL of aqueous solution of *endo*-1,4-α-D-polygalacturonase (12 mg, activity > 1 U/mg, EC 3.2.1.15; Fluka, Germany) was incubated at 37 °C for 4 h. The polygalacturonases were inactivated by boiling at 100 °C and removed by centrifugation. The supernatant was concentrated and precipitated with four volumes of 96% ethanol to obtain the polysaccharide fragment PA_W-F (218.8 mg).

The water-alcohol supernatant was concentrated and dialyzed in water (3.5 kDa membranes) and freeze-dried to give a carbohydrate fraction that was further purified using gel-filtration chromatography on a Sephacryl S-300 column. The fragment PA_W-F_1 (Fig. 1) was eventually obtained that corresponded to the major peak in the output elution curve; the yield was 18.5 mg.

2.3.2. Anion-exchange chromatography of the polysaccharide PA_W-F on DEAE-cellulose

The sample of PA_W-F (190 mg in 3 mL of 0.01 M NaCl) was fractionated on a DEAE-cellulose OH^--form column (34.5 cm × 2.2 cm). The fractions were eluted consecutively with 0.01, 0.1, 0.2, 0.3, and 0.4 M NaCl at a flow rate of 1 mL/min, then collected and analyzed using the phenol–sulphuric acid procedure with photocolorimetry carried out at 480 nm (Dubois et al., 1956). The fractions corresponding to separate peaks were combined, concentrated, dialyzed (12–14 kDa membranes), and freeze-dried.

Five fractions were obtained: eluted with 0.01 M NaCl, 69.8 mg, eluted with 0.1 M NaCl, 52.0 mg, eluted with 0.2 M NaCl, 7.7 mg, eluted with 0.3 M NaCl, 3.7 mg, and eluted with 0.4 M NaCl, 4.0 mg. The fraction eluted with 0.01 M NaCl (PA_W-F-I_1) was dissolved in distilled