Carbohydrate Polymers 101 (2014) 699–704

Contents lists available at ScienceDirect

Carbohydrate Polymers

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

Hot-water extracts from the inner bark of Norway spruce with immunomodulating activities

Myriam Le Normand[a], Hugo Mélida[b], Bjarne Holmbom[c], Terje E. Michaelsen[d], Marit Inngjerdingen[e], Vincent Bulone[b], Berit Smestad Paulsen[d], Monica Ek[a,*]

[a] Division of Wood Chemistry and Pulp Technology, School of Chemical Science and Engineering, KTH Royal Institute of Technology, Teknikringen 56, SE-10044 Stockholm, Sweden
[b] Division of Glycoscience, School of Biotechnology, KTH Royal Institute of Technology, AlbaNova University Centre, SE-10691 Stockholm, Sweden
[c] Chemistry Centre, Åbo Akademi University, Porthansgatan 3, FI-20500 Turku, Finland
[d] Department of Pharmaceutical Chemistry, School of Pharmacy, University of Oslo, P.O. Box 1068, Blindern, N-0316 Oslo, Norway
[e] Department of Anatomy, Institute of Basic Medical Sciences, University of Oslo, P.O. Box 1105, Blindern, N-0317 Oslo, Norway

ARTICLE INFO

Article history:
Received 8 May 2013
Received in revised form
16 September 2013
Accepted 18 September 2013
Available online 27 September 2013

Keywords:
Bark
Biorefinery
Immunomodulating activities
Compositional analysis
Pectins

ABSTRACT

The inner bark of Norway spruce (*Picea abies*) was sequentially extracted with hot water at 100 °C, 140 °C and 160 °C. The hot-water extracts (IB 100 °C, IB 140 °C and IB 160 °C) contained pectic polysaccharides and showed immunostimulating activities. Structural analyses of their carbohydrate content, including glycosidic linkage analyses, revealed the presence of pectins with a large rhamnogalacturonan RG-I domain ramified with highly-branched arabinans. IB 100 °C also contained a large amount of terminal glucosyl residues, indicating the presence of highly substituted polymers. IB 160 °C was mainly composed of starch. The hot-water extracts were tested for two biological activities, namely complement fixation and macrophage stimulation. IB 100 °C exhibited the highest complement fixation activity, with a 1.7-times higher IC_H50 than the control pectin, while IB 140 °C and IB 160 °C gave similar IC_H50 values as the control. Macrophages were stimulated by IB 100 °C and IB 140 °C in a dose-dependent manner, but not by IB 160 °C. IB 100 °C presented the highest activity toward macrophages, comparable to the control pectin.

1. Introduction

In Scandinavia, Norway spruce (*Picea abies*) and Scots pine (*Pinus sylvestris*) are the most important raw materials used by the forest industry. Manufacturing processes for pulp, paper and timber production from Norway spruce produce large quantities of complex lignocellulosic leftovers, such as bark, which could be recovered and converted into higher-value bio-based products. The quantity of bark from Norway spruce is estimated to represent around 1.5 million tons/year in Sweden alone.

The most studied components of Norway spruce bark are hydrophilic extractives such as tannins (Matthews, Mila, Scalbert, & Donnelly, 1997; Zhang & Gellerstedt, 2009) and stilbenes (Mannila & Talvitie, 1992), and lipophilic compounds (Norin & Winell, 1972). In previous works, we determined the carbohydrate composition of fractions that were sequentially extracted from the bark of Norway spruce (Krogell, Holmbom, Pranovich, Hemming, & Willför, 2012; Le Normand, Edlund, Holmbom, & Ek, 2012). High concentrations of galacturonic acid, arabinose, galactose and rhamnose, indicative of the presence of pectic polysaccharides, were measured in hot-water extracts. The amount of pectins was estimated to represent 9.0% of the outer bark, 12.6% of the inner bark and 10.5% of the bark collected in a paper mill immediately after the debarking process. These values are significantly higher than the concentration values of pectins (~3.5%) in the wood of Norway spruce (Bertaud & Holmbom, 2004; Willför, Sundberg, Hemming, & Holmbom, 2005). Pectins have also been identified in the bark of various conifer species such as *Picea mariana* (Anderson & Pigman, 1947), *Picea glauca* (Painter & Purves, 1960), *Abies amabilis* (Bhattacharjee & Timell, 1965) and, more recently, in the bark of *Pinus pinaster* (Fradinho et al., 2002) and *P. sylvestris* (Valentín et al., 2010). The fine structure and properties of the pectins from the bark of *P. abies* is however scarce, despite the potential economic importance of this raw material.

Pectins are major components of primary cell walls and middle lamellae of all plants (Willats, McCartney, Mackie, & Knox, 2001). They have multiple roles in plant growth (Ridley, O'Neill, & Mohnen, 2001) and are widely used in the food industry as gelling and stabilizing agents (Thakur, Singh, Handa, & Rao, 1997). Although most plant tissues contain pectins, the commercial

* Corresponding author. Tel.: +46 8 790 8104; fax: +46 8 790 6166.
E-mail address: monicaek@kth.se (M. Ek).

0144-8617/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.carbpol.2013.09.067

production of this family of polysaccharides is based almost entirely on citrus peel and apple pomace, which fulfill the properties required by the food industry (Willats, Knox, & Mikkelsen, 2006). Pectins are heterogeneous polysaccharides and their structure can vary considerably between plants, tissues and even within a single cell wall. Pectins consist of three main domains: a linear homogalacturonic backbone (HG) alternating with two types of highly branched rhamnogalacturonans regions designated as RG-I and RG-II (Albersheim, Darvill, O'Neill, Schols, & Voragen, 1996). These three domains are typically considered to be covalently linked and form a complex pectic network (Scheller, Jensen, Sørensen, Harholt, & Geshi, 2007). RG-I are substituted with side chains of arabinose and galactose units. Their chain length and sugar composition can be extremely heterogeneous between plants. On the contrary, RG-II has a highly conserved structure, consisting of a HG backbone branched with eleven different monosaccharides, including some rare sugars such as 2-O-methylxylose, 2-O-methylfucose, apiose, aceric acid, 2-keto-3-deoxy-D-manno-octulosonic acid and 3-deoxy-D-lyxo-2-heptulosaric acid (O'Neill et al., 1996). In our previous articles, we reported that pectins from the bark of Norway spruce are rich in arabinose and may have a rather large and branched RG-I region (Le Normand et al., 2012). These structural characteristics often have a positive impact on the pectins' health-promoting capacities (Yamada, 1996; Yamada & Kiyohara, 2007).

Several recent papers have described the immunomodulating activity of hemicelluloses and pectins extracted from a variety of medicinal plants, with effects ranging from complement fixation to macrophage stimulation (Austarheim et al., 2012; Inngjerdingen et al., 2008; Michaelsen, Gilje, Samuelsen, Høgåsen, & Paulsen, 2000; Nergard et al., 2005). Pectins extracted from plants are abundant and relatively nontoxic, thus providing ideal candidates for the development of therapeutics with immunomodulatory action. Although the mechanism of action of plant pectins on the immune system is not fully understood, the therapeutic effects of these polymers are thought to occur *via* modulation of part of the innate immune system such as the complement system and by stimulating macrophages. The complement system consists of more than 30 serum and cellular proteins, linked in two biochemical cascades, that play an important role in the primary defense against bacterial and viral infections. The activation of complement encompasses a series of initiation, amplification and lytic steps (Makrides, 1998). Macrophages play an important role in host defense and inflammation. They have different functions, including surveillance, phagocytosis, destruction of targeted foreign micro-organisms as well as antigen presentation to T lymphocytes (Schepetkin & Quinn, 2006).

In the present study, three hot water extracts of the inner bark of Norway spruce were compared with respect to their carbohydrate composition, structural patterns, complement fixation activity and macrophage activation.

2. Materials and methods

2.1. Extraction of polysaccharides

2.1.1. Bark material

The bark of Norway spruce was sampled from a fresh 30-year-old tree cut in Gävleborg County (Sweden) in July 2009. It was stored in the dark at −20 °C. The inner and outer barks were manually separated using a scalpel. The inner bark was ground with a hand blender to particle sizes not exceeding 5 mm × 2 mm.

2.1.2. Extraction

The ground inner bark was sequentially extracted with acetone at 100 °C, followed by hot water at 100 °C, 140 °C and 160 °C, using an Accelerated Solvent Extractor (ASE) (Dionex, CA). Each extraction step included three cycles of 20 min. In total, 90 g of wet inner bark was extracted with ASE with a load of approximately 10 g in 34 ml-cells. The total dissolved solid (TDS) of all hot-water extracts was determined gravimetrically after freeze-drying 10 ml of each extract.

The fractions were filtered and dialyzed against distilled water in a Spectra/Por dialysis tube with a molecular mass cut-off of 3.5 kDa prior to analyses and bioactivity tests.

2.2. Structural analyses of the bark fractions

2.2.1. Carbohydrate composition and Klason lignin content

The freeze-dried fractions were analyzed for carbohydrate content and sugar unit composition in duplicate experiments. Total amounts of carbohydrates in the ground bark, residues and extracts were determined by acid methanolysis, followed by silylation with hexamethyldisilazane and trimethylchlorosilane, and analysis by gas chromatography (Sundberg, Sundberg, Lillandt, & Holmbom, 1996).

Klason lignin was determined after acid hydrolysis following the TAPPI test method T222 om-06 (TAPPI, 2006).

2.2.2. Molecular weight determination of carbohydrates

Size-exclusion chromatography (SEC) in alkaline solvent (NaOH) was used to estimate the molecular weight and polydispersity of the polysaccharides obtained by hot-water extractions. The SEC system consisted of three TSK gel columns (Tosoh Bioscience, Tokyo, Japan) coupled in series (G3000PW, 7.5 mm × 300 mm, 10 μm particle size; GP4000PW, 7.5 mm × 300 mm, 17 μm particle size; G3000PW) and connected to a refractive index detector (Waters 2410). The mobile phase was 10 mM NaOH and the flow rate was of 1 ml min^{-1}. The system was calibrated using pullulan standards of known molecular masses ranging from 320 to 400,000 Da.

2.2.3. Glycosidic linkage analyses

Polysaccharides in the freeze-dried fractions (5 mg) were carboxyl reduced with sodium borodeuteride ($NaBD_4$) (Kim & Carpita, 1992) and methylated (1 mg, four technical replicates) in the presence of NaOH/CH_3I (Ciucanu & Kerek, 1984). The methylation step was repeated five times on each sample to avoid undermethylation. The samples were then hydrolyzed with 2 M TFA at 121 °C for 2 h, reduced and acetylated (Albersheim, Nevins, English, & Karr, 1967). The permethylated alditol acetates were separated and analyzed by GC/EI-MS on a SP-2380 capillary column (30 m × 0.25 mm i.d.; Sigma–Aldrich) with a temperature program increasing from 160 °C to 210 °C at a rate of 1 °C min^{-1}. The mass spectra of the fragments obtained from the permethylated alditol acetates (EI-MS) were compared with those of reference polysaccharide derivatives and to available data (Carpita & Shea, 1989). The quantification was based on the carbohydrate composition and effective carbon response of each compound as detected by GC-MS.

2.2.4. Amylase treatment

Starch was removed from the filtered and dialyzed extracts by treatments with α-amylase from porcine pancreas (Type VI-B, 23 units/mg, Sigma–Aldrich). 50 mg of each extract was dissolved in 30 ml of 0.01 M phosphate buffer (pH 7) and stirred in a water bath at 37 °C. 7 mg of the enzyme was added every 24 h during three days. The solution was then filtered, dialyzed and freeze-dried.