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Cell wall polysaccharides from Ponkan mandarin (*Citrus reticulata* Blanco *cv. Ponkan*) peel

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ABSTRACT

Cell wall polysaccharides from ponkan peel were investigated with the aim of gain knowledge about their potential for different applications and the use of ponkan peel as raw material for pectin extraction. The plant material was defatted using MeOH:$CHCl_3$, pretreated with DMSO and then subjected to sequential extractions with cold and hot water, ammonium oxalate, HCl, Na_2CO_3, 2 M and 4 M NaOH in order to obtain polysaccharides. The polysaccharide fractions were analyzed by chemical, chromatographic and spectroscopic methods Cold and hot water-soluble pectins contained higher amounts of GalA and higher degrees of methyl-esterification (DM) than ammonium oxalate and HCl fractions. Na_2CO_3 extraction provided non-esterified arabinose-rich pectins which formed gel in a dialysis step. NaOH solubilized hemicelluloses, composed mainly of xyloglucans, galactomannans and galactoglucomannans. The water-soluble fraction (WSP) was purified using α-amylase and amyloglucosidase and gave rise to the subfraction named α-WSP. The α-WSP was a pectin composed of HG and RG-I domains containing side chains of arabinans and short-chains of galactans, with low DM (39.4%) and M_w of 1.615×10^5 g/mol.

1. Introduction

Cell wall polysaccharides are important and promising raw materials for biomedical (Liu, Willför, & Xu, 2015) and food applications (Chapple & Carpita, 1998; Harris & Smith, 2006). Since the composition of the polysaccharides differs depending on the plant source (Nevins, English, & Albersheim, 1967) and even the cell type (Keegstra, 2010), it is important to increase the knowledge about the polysaccharides present in different species. Cellulose is the most characteristic plant cell wall polysaccharide and forms the structure of the cell wall by association with other two groups of polysaccharides: pectins and hemicelluloses (Keegstra, 2010).

Pectins are acidic heteropolysaccharides which are classified into three main groups: homogalacturonans (HG), rhamnogalacturonan I (RG-I) and rhamnogalacturonan II (RG-II) (Willats, Knox, & Mikkelsen, 2006). HG consists of a linear chain of covalently linked →4)-α-D-GalA*p*-(1→ units, which can be partially methyl-esterified at C-6 and acetylated at *O*-2 and/or *O*-3 (Voragen, Coenen, Verhoef, & Schols, 2009; Yapo, 2011). In RG-I, the backbone is composed of [→4)-α-D-GalA*p*-(1 → 2)-α-L-Rha*p*(1 →] repeated disaccharide and side chains of arabinans, galactans and/or arabinogalactans are attached to the backbone at *O*-4 of some rhamnosyl residues (Voragen et al., 2009; Yapo, 2011). In its turn, RG-II consists of HG main chain containing quite complex side chains. (Mohnen, 2008; Voragen et al., 2009). Pectins are widely used due to their gelling properties (Willats et al., 2006), which are strongly affected by their structure, especially on the degree of methyl-esterification (Thakur, Singh, & Handa, 1997).

Hemicelluloses are described as the polysaccharides that are solubilized from the cell wall by aqueous alkaline solutions and have β-(1 → 4)-linked pyranosyl residues with the O-4 in the equatorial position. They comprise the groups of xylans, mannans and xyloglucans (Caffall & Mohnen, 2009).

Citrus peel are the major sources of commercial pectins (Ciriminna, Chavarría-Hernández, Hernández, & Pagliaro, 2015; Willats et al., 2006). Often, the botanical source is not precisely identified in citrus commercial pectins, but orange, lime, grapefruit and lemon are commonly used (Yapo, 2009).

Ponkan *(Citrus reticulata* Blanco *cv. Ponkan*) is a non-climateric citrus fruit originated from Asia which belongs to a cultivar of mandarin (Bao, Yuan, Zhao, Liu, & Gao, 2015; Lee, Zhong, & Chang, 2015). It is one of the most appreciate mandarins for consumption, being the main mandarin cultivated in Brazil (Mendonça, Ramos, Rufini, Araújo Neto, & Rossi, 2006; Ramos et al., 2009). The fruits are flattened, with few seeds and the peel is rough and loose (Coelho, 1996), which makes it easy for consumption *in natura*. The fruits are also processed for the production of concentrated juice (Pavan, Mazzocato, Jacques, & Dias,

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2008).

Previous studies on the ponkan peel composition investigated mainly secondary metabolites, such as flavonoids, carotenoids (Wang, Chuang & Hsu, 2008), polymethoxyflavones (Du & Chen, 2010), and the essential oil (Lota, Serra, Tomi & Casanova, 2000). Ascorbic acid and mineral contents were also evaluated (Barros, Ferreira & Genovese, 2012). Concerning the polysaccharides, the pectin content in ponkan peel was reported to be around 37% (Wang et al., 2008). However, only the amount of pectin was evaluated, and no studies were found in the literature concerning the structure and properties. Thus, the present work describes the fractionation and characterization of polysaccharides from cell wall of ponkan peel.

2. Materials and methods

2.1. Preparation of plant material

Fresh ripe fruits were collected in Ponta Grossa, Paraná, in southern Brazil (25°04′18.7″S; 50°07′44.3″W). Fruits were washed and peeled, and the peels were freeze-dried and ground into powder using an analytical mill IKA A-11 basic. The peel powder was defatted in a Soxhlet apparatus using chloroform/methanol, in a ratio of 2:1. Then, a pretreatment with DMSO 90% for 12 h was carried out for removal of starch.

2.2. Sequential extraction of cell wall polysaccharides from ponkan peel

After starch removal, sequential extractions of ponkan peel (PP) were performed using water, chelating agents, dilute acid and alkali in order to obtain pectins (Voragen, Pilnik, Thibault, Axelos, & Renard, 1995) and increasing concentrations of aqueous NaOH for extraction of hemicelluloses (Vriesmann & Petkowicz, 2009), as shown in Fig. 1. All the extractions were carried out using a solid/liquid ratio of 1:25 under mechanical stirring. NaOH extractions were performed in the presence of 20 mM $NaBH_4$ to avoid alkaline degradation. After each extraction, the material was centrifuged at 5000 rpm for 20 min at 10 °C and the supernatant was filtered with a synthetic fabric. For salt removal, ammonium oxalate and sodium carbonate extracts were dialyzed (6–8 KDa membrane) against tap water for three days. The extracts obtained using water (at 25 °C and 100 °C), ammonium oxalate and citric acid were precipitated with 2 vols of ethanol, kept at 4 °C overnight, and the polysaccharides were recovered by filtration, washed three times with ethanol and dried under vacuum, resulting in WSP (water-soluble pectin), HWSP (hot water-soluble pectin), CSP (chelator-soluble pectin) and HSP (diluted acid-soluble pectin) fractions, respectively. The sodium carbonate extract formed a gel within the membrane during dialysis which was then separated in soluble and insoluble parts, giving rise to fractions ASPS (soluble part from diluted alkali-soluble pectin after dialysis) and ASPI (insoluble part from diluted alkali-soluble pectin after dialysis). For NaOH extractions, the extracts were neutralized with 50% acetic acid and the precipitate formed in 2 M NaOH extract resulted in the polysaccharide fraction HA2 (hemicellulose A soluble in 2 M NaOH), whereas no precipitate was found for 4 M extract after neutralization. After the neutralization and removal of the precipitate by centrifugation, the supernatants were dialyzed for three days, precipitated with 2 vols of ethanol and the polysaccharides were recovered and dried as previously described, giving rise to fractions HB2 (hemicellulose B soluble in 2 M NaOH) and HB4 (hemicellulose B soluble in 4 M NaOH).

2.3. Purification of WSP

WSP was solubilized (1 mg/ml) and kept at 55 °C in a water bath. 30 µl α-amylase (Sigma, Germany) and 30 µl amyloglucosidase (Sigma, Germany) were added and the system was incubated for 1 h, followed by 15 min boiling for inactivation and precipitation of the enzymes. The solution was filtered, dialyzed for 2 days against deionized water in 6–8 kDa membrane and then lyophilized, giving rise to the fraction α-WSP which was then characterized.

2.4. Monosaccharide composition

For determination of neutral monosaccharides, fractions were hydrolyzed with 2 M TFA at 120 °C for 2 h. The hydrolyzate was evaporated to dryness, solubilized in water, reduced with $NaBH_4$ (Wolfrom & Thompson, 1963a) and acetylated with pyridine–acetic anhydride (1:1 v/v, 16 h, 25°) (Wolfrom & Thompson, 1963b). The resulting alditol acetates from fractions obtained by sequential extractions were extracted with $CHCl_3$ and examined by a Varian Saturn 2000R − 3800 gas chromatograph coupled to a Varian Ion-Trap 2000R mass spectrometer using a DB-225-MS column (0.32 mm internal diameter × 30 m × film thickness 0.25 µm) programmed from 50 to 220 °C at a heating rate of 40 °C/min, using He as carrier gas at 1 ml/min.

The purified polysaccharide was hydrolyzed and derivatized as described above and analyzed by a Thermo Scientific Trace GC Ultra gas chromatograph using a DB-225-MS column (0.32 mm internal diameter × 30 m × film thickness 0.25 µm) programmed from 60 °C to 200 °C at a heating rate of 40 °C/min, using a mixture of He, N_2 and compressed air as carrier gas at 1 ml/min.

Uronic acids were quantified according to Blumenkrantz and Asboe-Hansen (1973) method, using galacturonic acid as standard, and were identified by anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD). After hydrolysis with 2 M TFA (8 h, 100 °C), drying and repeated washing until total removal of the acid, the sample (1 mg/ml) was filtered through a membrane of 0.22 µm, injected in a Thermo Scientific Dionex ICS-5000 chromatograph (Thermo Fisher Scientific, USA) with CarboPac PA20 column (3 × 150 mm) using gradient of 1 M NaOH and 1 M NaOAc as eluent (Nagel, Sirisakulwat, Carle, & Neidhart, 2014) in N_2 atmosphere in a flow of 0.2 ml/min at 30 °C. Analyses were carried out in triplicate. Data were collected and analyzed using the ChromeleonTM 7.2 Chromatography Data System software.

2.5. Colorimetric methods

The acetyl content was determined by Hestrin (1949) method, using erythritol tetraacetate as standard, and the degree of acetylation (DA) was calculated as described by Colodel, Bagatin, Tavares, and Petkowicz (2017). Protein was measured by the Bradford (1976) method using BSA as standard. Total phenolics were estimated by the Singleton and Rossi (1965) method, using galic acid as standard.

2.6. High pressure size exclusion chromatography (HPSEC)

HPSEC analyses were performed using a Waters unit coupled to a refractive index (RI) and a Wyatt Technology Dawn-F multi-angle laser light scattering (MALLS). Four Waters Ultrahydrogel columns (2000; 500; 250; 120) were connected in series and coupled to the multi-detection instrument. A solution of 0.1 M $NaNO_2$ and 0.02% NaN_3 was used as eluent at a flux of 0.6 ml/min. Prior to the analyses, the samples (1.0 mg/ml) were filtered through a 0.22 µm cellulose acetate membrane. All the analyses were carried out at 25 °C. The refractive index increment of the solvent-solute solution with respect to a change in solute concentration (dn/dc) was determined using concentrations 0.2–1.0 mg/ml, and the average molar mass (Mw) was calculated using a Wyatt Technology ASTRA software.

2.7. Spectroscopic methods

The heteronuclear single quantum coherence nuclear magnetic resonance (HSQC NMR) spectrum was obtained for α-WSP in D_2O