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Note

Composition of pectic polysaccharides in a Portuguese apple (*Malus domestica* Borkh. cv Bravo de Esmolfe)

Ana S. Silva[1], Claudia Nunes[2], Manuel A. Coimbra[2], Luís F. Guido[1]*

[1]University of Porto/Faculty of Sciences/REQUIMTE – Dept. of Chemistry and Biochemistry, R. do Campo Alegre, 687 – 4169-007 – Porto – Portugal.
[2]University of Aveiro/QOPNA – Dept. of Chemistry, Campus de Santiago – 3810-193 – Aveiro – Portugal.
*Corresponding author <lfguido@fc.up.pt>

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ABSTRACT: *Malus domestica* Borkh. cv Bravo de Esmolfe is a typical Portuguese apple cultivar classified as *Protected Designation and Origin* (PDO). It is a traditional product produced under strict conditions and labelled with a specific law protected designation. This cultivar presents quite good sweetness and flavor. The monosaccharide composition of the pectic polysaccharides from this traditional apple is herein reported for the first time. Based on the molar ratios obtained from the sugar composition, the presumable pectin structure could be inferred. The cell-wall polysaccharides present in the alcohol-insoluble residue (AIR) of unpeeled *BE* apple were sequentially fractionated. In addition, pectic material was also extracted by citric acid treatment prior to heat extraction at acidic pH. The water soluble pectin, imidazole soluble pectin and sodium carbonate soluble pectin account for 44, 16 and 40 % of the AIR, respectively. The pectic polysaccharides extracted in the presence of citric acid had lower galacturonic acid content and higher neutral sugars content. The homogalacturonan (HG) and less-substituted rhamnogalacturonan (RG) domains are extracted first. Pectin treated with citric acid has been shown to contain more substituted polymers, especially RG-I. In addition, the relatively higher Xylose/Galacturonic acid ratio found in the citric acid extract demonstrates that the xylogalacturonan (XG) domain presumably is present in the pectic material of the unpeeled *BE* apple.

Keywords: pectin, alcohol-insoluble residue, galacturonic acid, uronic acids

Abbreviations: **AIR**, alcohol-insoluble residue; ***BE***, Bravo de Esmolfe; **CA**, citric acid; **CW**, cold water; **FID**, flame ionization detector; **GalA**, galacturonic acid; **HG**, homogalacturonan; **IMZ**, imidazole; **RG**, rhamnogalacturonan; **UA**, uronic acids; **Rha**, rahmnose; **XG**, xylogalacturonan

Introduction

Malus domestica Borkh. cv Bravo de Esmolfe (*BE*) is a typical Portuguese apple cultivar classified as *Protected Designation and Origin* (PDO) since 1994, which means that it corresponds to a traditional product produced under strict conditions and labeled with a specific law protected designation. This variety is produced in a restricted and small inland region in northern Portugal, corresponding to a production of ca. 200,000 kg year^{-1}, but commercial demand is now increasing thanks specifically to its appealing sensory properties, namely sweetness and flavor (Moldao-Martins et al., 2003). A number of reports have been published identifying the volatile profile of *BE* (Reis et al., 2009), as well as characterizing this apple variety in terms of chemical composition, polyphenols (Serra et al., 2012), protein, sugars, acids, fiber, vitamins and minerals (Feliciano et al., 2010).

Pectic polysaccharides comprise associated polysaccharides, such as homogalacturonans (HG), xylogalacturonans (XG), type I rhamnogalacturonans (RG-I), type II rhamnogalacturonans (RG-II), arabinans, and arabinogalactans (Caffall and Mohnen, 2009; Mohnen, 2008; Voragen et al., 2009; Harholt et al., 2010).

Pectic substances, such as water-soluble dietary fibers, are not metabolized by endogenous digestive enzymes during passage through the human digestive tract, but they are fermented by the microflora present in the colon (Gulfi et al., 2006; Khondkar et al., 2009). Daily consumption of this dietetic fiber has been linked to positive health effects such as cholesterol-lowering (Brown et al., 1999), cancer-preventing (Umar et al., 2003; Zong et al., 2012; Maxwell et al., 2012; Li et al., 2012), and plasma glucose-regulating (Jenkins and Jenkins, 1995). A pectic fraction isolated from guarana powder has recently exhibited antioxidant activity (Dalonso and Petkowicz, 2012), probably contributing to the positive biological effects of guarana.

This study aimed to characterize the sugar composition of the pectic polysaccharides of the *Bravo de Esmolfe* apple. The cell wall polysaccharides present in the alcohol-insoluble residue (AIR) of unpeeled apples were sequentially extracted and the chemical composition of each fraction was determined. The pectic material was further extracted by citric acid treatment prior to heat extraction at acidic pH. In the light of the molar ratios drawn from the sugar composition, the presumable pectin structure is discussed.

Materials and Methods

Chemicals and reagents

The monosaccharides, D-galactose (98 %), D-glucose (98 %), D-mannose (99 %), D,L-arabinose (99 %),

D-xylose (99 %), L-fucose (99 %) and L-rhamnose monohydrate (99 %), as well as D-galacturonic acid monohydrate (99 %), were used. Imidazole, methylimidazole, sodium carbonate, citric acid, trifluoracetic acid, phenol, 3-phenylphenol, xilenol, sulphuric acid, sodium borohydride, acetic anhydride, dichloromethane, diethyl ether were purchased. Ammonium acetate, glacial acetic acid and *o*-phosphoric acid were supplied. Sodium chloride, sodium hydroxide, hydrochloride acid, ammonia and ethanol (96 %) were used. Visking dialysis membranes were also used. The *BE* apples were purchased from a local market. High-purity water from a Millipore Simplicity 185 water purification system was used for all chemical analyses and glassware washing.

Alcohol-insoluble residue (AIR)

Unpeeled apple (100 g) was cut into small portions of approximately 1 cm^2 and dispersed in 750 mL of 65 % (v/v) ethanol and boiled for 10 min. The suspension was cooled to room temperature and filtered through a glass fiber filter under vacuum. The final residue was then washed with diethyl ether, filtered and allowed to dry overnight at 40 °C. The dried material obtained constituted the AIR.

Sequential extraction of AIR

The cell wall polysaccharides present in the AIR of unpeeled BE apples were fractionated in water (hot and cold) soluble, chelator (imidazole) soluble and sodium carbonate soluble polysaccharides. In addition, pectic material was also extracted from unpeeled BE apple by citric acid treatment prior to heat extraction at acidic pH, in accordance with the method developed by Kurita et al. (2008). A dialysis membrane was used to remove salts and low molar-mass carbohydrates.

Pectic polysaccharides were extracted from the AIR by sequential extraction, in accordance with the method described by Mafra et al. (2001), with some minor modifications. Initially, 5 g of AIR were sequentially extracted with: (*1*) water. The extract was dispersed and stirred in 750 mL of deionized water during 18h at 25 °C (*cold water*) or 1h at 95 °C (*hot water*). The suspension was centrifuged at 24515 × g for 20 min at 4 °C and the supernatant was collected, filtered through a glass fiber filter and further concentrated to 50 mL by dialysis against water (*cut-off* 12 to 14 kDa). The solid residue obtained from the cold water extraction was used for the subsequent extraction; (*2*) 0.5 M imidazole/HCl (pH 7.0). This extraction was performed with a chelating solution of imidazole. The solid residue was then recuperated and used for the last extraction; (*3*) 50 mM Na_2CO_3. All extraction procedures were carried out using the same volume of extraction agent as well as the same procedure reported above for the extraction with water. The Na_2CO_3 extract was neutralized to pH 5–6 with glacial acetic acid prior to dialysis. After dialysis, all extracts were concentrated under reduced pressure, frozen and freeze-dried.

Single step extraction in the presence of citric acid

The procedure employed for the extraction with citric acid was performed according to Kurita et al. (2008), with minor adjustments. Five grams of unpeeled apple were cut and suspended in distilled water and the pH was adjusted to 7.0 with 4 M NaOH. Following the adjustment of the citric acid concentration to 1 M, the suspension was heated to 50 °C for 2 h and then cooled to room temperature. The suspension pH was then adjusted to 2.2 with HCl and heated for 15 min in a boiling water bath. The crude extracts were cooled and centrifuged at 24515 × g for 15 min at 4 °C. The supernatant was adjusted to pH 7.0 with 4 M NaOH. For pectin precipitation, ethanol was added to the solution at final concentrations of 60 % (v/v) and the precipitate was collected by centrifugation, washed twice with 80 % ethanol, and air-dried at 50 °C for 14 h. The dried precipitate was dissolved in distilled water and dialyzed against water (*cut-off* 12 to 14 kDa). The dialyzed solution was frozen and freeze-dried.

Pre-hydrolysis and saponification of AIR

The following three conditions had been previously chosen for determining the composition of pectic polysaccharides from the AIR. *(1)* non-hydrolyzed: 2 mg of the dried AIR were dispersed in 2 mL of deionized water. The suspension was maintained in an ultrasonic bath for 10 min at 25 °C; *(2)* pre-hydrolyzed: 2 mg of the dried AIR were hydrolyzed in 200 µL of 11 M H_2SO_4 for 3 h at 25 °C. The final volume was adjusted to 2 mL and hydrolysis conducted for 2.5 h at 100 °C; *(3)* saponified: 2 mg of dried AIR were saponified by the addition of 0.1 M NaHO (2 mL). The reaction time was 1 h at 4 °C and then overnight at room temperature and light-protected.

Determination of total neutral sugars and uronic acids content

The content of total neutral sugar (galactose equivalent) was determined by the phenol-sulfuric acid method (Dubois et al., 1956) after correction for interference from uronic acids, according to Yamazaki and Kurita (2007). Uronic acid content was determined by two methods, the xylenol (Walter et al., 1993) and *m*-hydroxydiphenyl (Blumenkr and Asboehan, 1973) colorimetric methods. With regard to the *m*-hydroxydiphenyl method, samples were prepared by pre-hydrolysis in 0.2 mL of 11 M H_2SO_4 for 3 h at room temperature followed by 1 h hydrolysis in 1 M H_2SO_4 at 100 °C. D-Galacturonic acid solutions (0-80 µg mL^{-1}) were used to construct the calibration curves for both methods. The data are reported as the mean values of three independent experiments.

Determination of neutral sugar composition

Neutral sugars were determined by gas chromatography (GC) as alditol acetates (Coimbra et al., 1996). The hydrolysis was performed with 2 M trifluoroacetic acid at 100 °C for 1 h. Monosaccharides were reduced

with $NaBH_4$ (15 % in NH_3 3 M) for 1 h at 30 °C and subsequently acetylated with acetic anhydride (3 mL) in the presence of 1 methylimidazole (450 µL) for 30 min at 30 °C. Alditol acetate derivatives were separated with dichloromethane and analyzed by GC with an FID detector and equipped with a 30 m column DB-225 (J&W Scientific, Folsom, CA) with i.d. and film thickness of 0.25 mm and 0.15 µm, respectively. The oven temperature program used was: initial temperature 200 °C, a rise in temperature at a rate of 40 °C min^{-1} until 220 °C, standing for 7 min, followed by a rate of 20 °C min^{-1} until 230 °C and maintain this temperature 1 min. The injector and detector temperatures were, respectively, 220 and 230 °C. The flow rate of the carrier gas (H_2) was set at 1.7 mL min^{-1}.

Statistical treatment

All data are reported as the mean and standard deviation (SD) values of three independent experiments. In order to test whether the differences between the means were significant the t-test of the mean (two-tailed) and the F-test of variances were applied.

Results and Discussion

Characterization of the pectic polysaccharides extracted from BE apple

Both the neutral sugar content (from 80 to 310 µg mg^{-1} AIR) and the uronic acid content (from 80 to 178 µg mg^{-1} AIR) have increased ($p < 0.01$) with the pre-hydrolysis with acid (Figure 1). The galacturonic acid content of AIR of 'Golden Delicious' and 'Fuji' apples has been found to be between 252 and 292 mg g^{-1} AIR while the total neutral sugar content has been found to be between 567 and 716 mg g^{-1} AIR (Billy et al., 2008). The ratio between acidic and neutral sugars (approximately 1:2) found for the AIR by these authors is approximately the same observed herein for the *BE* apple after the hydrolysis step, confirming that pre-hydrolysis of AIR is required prior to the application of the colorimetric methods.

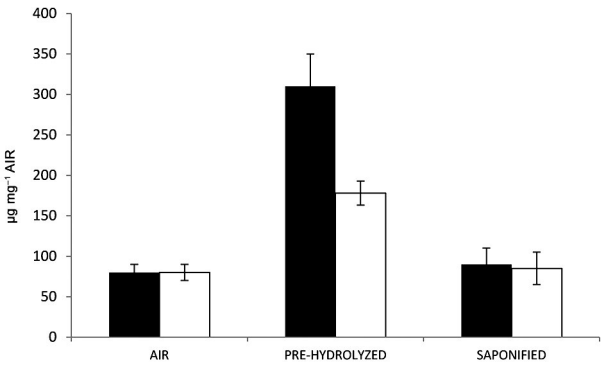

Figure 1 – Total neutral sugar (■) content and uronic acid (□) content of the alcohol-insoluble residue (AIR) of unpeeled *BE* (Bravo de Esmolfe) apple (AIR, pre-hydrolysed AIR and saponified AIR). Data represents means with standard deviation bars.

Cellulose represents the bulk of cell wall material and corresponds to more than 20 % of the AIR. The presence of pectic substances in the cellulosic residue of ripe apples, supported by the neutral sugar distribution and the uronic acid content, confirmed the association of the pectic polysaccharide with cellulose (Oechslin et al., 2003). *BE* apple pectins may bind cellulose, according to the uronic acid/neutral sugars ratios observed for non-hydrolyzed AIR and pre-hydrolised AIR (Figure 1).

Koch and Pein (1985) have defended the proposition that the esterification of galacturonic acid could inhibit the formation of the chromophore furfural derivative detected at 450 nm in the xylenol method and thus could sub-estimate galacturonic acid. In order to cleave such possible ester-linkages, saponification of the AIR was carried out prior to the colorimetric assays. A 6 % increase only was observed for the uronic acid content after saponification (from 80 µg mg^{-1} to 85 µg mg^{-1}) which is within the coefficient of variation linked to the measurements (Figure 1). Either the xylenol method is not influenced by the esterified galacturonic acid residues or these residues are not considerably methylated or esterified with other sugars. Alkali treatment with NaOH at high concentrations (1 M and 4 M) was used to yield the fractions rich in hemicelluloses and the cellulosic residue (Oechslin et al., 2003). The saponification with 0.1 M NaOH used herein was not enough to cause degradation of hemicelluloses and, thus, the content of total neutral sugars after saponification (90 µg mg^{-1}) roughly equals the non-hydrolysed sample (80 µg mg^{-1}).

The water soluble pectin, imidazole soluble pectin and sodium carbonate soluble pectin were respectively 44 %, 16 % and 40 % of the AIR (based on the concentrations of µg GalA per mg freeze-dried extract) (Figure 2B). Water extracts the pectin polysaccharides loosely attached to the cell wall material, since this condition preserves covalent bonds, and β-elimination does not occur (Basanta et al., 2012). Extraction with hot water was shown to be a more efficient procedure for removing these pectic polysaccharides than cold water (670 mg g^{-1} AIR *vs* 505 mg g^{-1} AIR) (Figure 2A).

The use of imidazole, a strong chelating agent, meant a further 15 % of pectic material could be obtained, presumably originating from dimers of homogalacturonan chains mediated by calcium ions. The GalA residues can bind to calcium ions allowing the pectic polysaccharide chains to assemble by calcium bridges (Ferreira et al., 2006). Thus, isolated homogalacturonans were found cross-linked with calcium ions in the cell junctions (M'sakni et al., 2006). Carbonate soluble pectin, assumed to contain pectic polysaccharides that are covalently bound, via ester-bond, to other cell wall polysaccharides, represents 40 % of total pectic polysaccharides (Figure 2B). This evidence may suggest the presence of alkali-labile ester bonds between pectic substances and other polysaccharides from the AIR. The

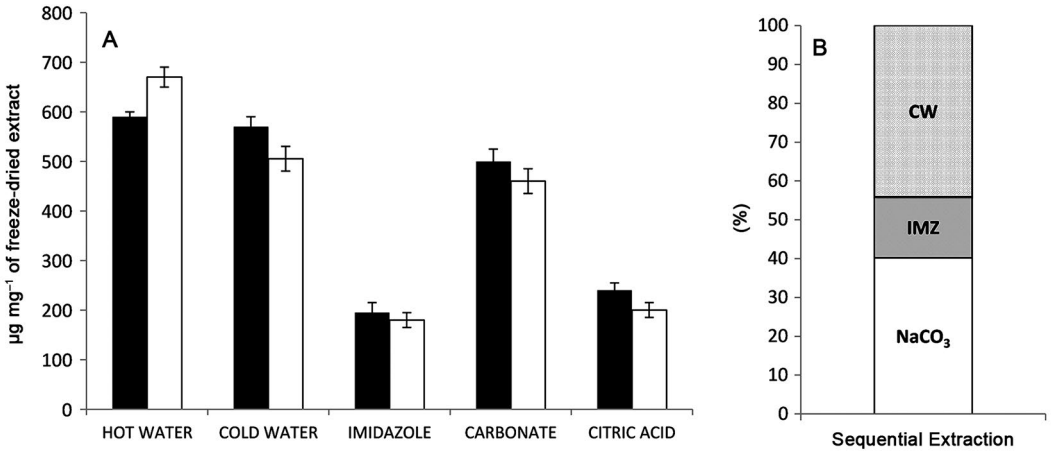

Figure 2 – Total neutral sugar (■) content and uronic acid (□) content of the extracts from unpeeled *BE* (Bravo de Esmolfe) apple (A) and relative amount of galacturonic acid in the sequential extraction fractions (B). Data represents means with standard deviation bars.

binding of the pectic polysaccharide to cellulose was observed in the cellulosic residue of ripe apples, supported by the neutral sugar distribution and the uronic acid content (Oechslin et al., 2003).

Treatment of pectin with citric acid leads to lower content of uronic acids (200 mg g^{-1} AIR, Figure 2A), which is in accordance with other reports showing that the pectin with citric acid treatment contained lower galacturonic acid and higher neutral sugars (Kurita et al., 2008).

Uronic acids, which are attributed to galacturonic acid (GalA), were the major sugars in all extracts of the *BE* apple, representing more than 90 mol % for the extracts obtained with sequential extraction (water, imidazole, and sodium carbonate) (Table 1). Although there are no studies showing that glucose (Glc) is part of the pectin structure, Glc was found in the pectin extracted in the presence of citric acid (8 mol %). This Glc content might be from non-pectic polysaccharides that were also extracted. Zhang et al. (2013) have reported Glc as the second most abundant component of apple pectin (28.7 mol %), which was ascribed to remnant soluble sugar that was not completely removed during the processing of pectin. However, Nunes et al. (2012) have shown the occurrence of GalA substituted by Glc, and Glc-β-(1-4)–Glc as structural features occurring in pectic polysaccharides, which could account for the Glc found in the composition of pectic material.

The presence of Fuc (4.3 mol %) in apple pectin has been reported (Zhang et al., 2013); however this monosaccharide was not detected in the *BE* apple extracts analyzed herein. Vestigial amount of Fuc and Xyl were reported for the pectin extracted from Yuza pomace (*Citrus junos*) by using combined physical and enzymatic treatment (Lim et al., 2012).

The unpeeled *BE* apple extract obtained with cold water contained 94 mol % of GalA. This extract contained minor amounts of arabinose (Ara, 2 mol %) and galactose (Gal, 2 mol %). The relative higher proportion of Ara was found for the imidazole (4 mol %) and sodium carbonate (5 mol %) fractions (Table 1). The lower relative content of GalA (71 mol %) and the corresponding higher relative content of neutral sugars, mainly Gal (11 mol %) and Ara (6 mol %), were found for the citric acid fraction. This evidence corroborates the suggestion that citric acid induces the liberation of Gal, in particular from hairy region of pectin, as previously reported by Kurita et al. (2008).

Table 1 – Sugar composition of the pectic polysaccharide extracts from unpeeled *BE* (Bravo de Esmolfe) apple (Rha, rhamnose; Fuc, fucose; Ara, arabinose; Xyl, xylose; Man, mannose; Gal, galactose; Glc, glucose; GalA, galacturonic acid).

	Sugars (mol %)								Total (mg g^{-1})
	Rha	Fuc	Ara	Xyl	Man	Gal	Glc	GalA	
Cold water	1	0	2	1	0	2	0	94	862
Imidazole	1	0	4	1	0	2	1	91	568
Carbonate	1	0	5	1	0	2	1	90	820
Citric acid	1	0	6	3	0	11	8	71	706

Molar ratios from the sugar composition of the BE apple pectic polysaccharides

Table 2 shows the molar ratios obtained for each extract, in order to give further insights into the structure of *BE* apple pectin. This heteropolysaccharide is divided into three or four principal domains, the HG, the RG-I, the RG-II and/or XG. HG is a linear homopolymer of α-1,4-linked-D-GalA. This homopolymer is covalently linked to RG-I and RG-II. The RG-I backbone is characterized by the presence of alternate unities of GalA and Rha. The RG-II is a homogalacturonic acid backbone with numerous complex side chains containing Rha and types of glycosyl unities (Caffall

Table 2 – Molar ratios for the pectic polysaccharide extracts from unpeeled *BE* (Bravo de Esmolfe) apple (Rha, rhamnose; Ara, arabinose; Xyl, xylose; Gal, galactose; GalA, galacturonic acid).

	Molar Ratio		
	Xyl/GalA	GalA/Rha	(Ara + Gal)/ Rha
Cold water	1/94	94	4
Imidazole	1/91	91	6
Carbonate	1/90	90	7
Citric acid	1/24	71	17

and Mohnen, 2009; Harholt et al., 2010). The proportions among HG, XG, RG-I, and RG-II are variable, but typically HG is the most abundant polysaccharide, constituting about 65 % of the pectin, while RG-I constitutes 20 to 35 %. XG and RG-II are minor components, each constituting less than 10 % (Harholt et al., 2010). In fact, the very high GalA/Rha molar ratios (Table 2) suggest that HG is by far the most abundant polysaccharide.

As the molar ratio of Rha to GalA is indicative of the degree of branching, it is predictable that pectin treated with citric acid, showing the lowest GalA/Rha ratio (71), is more branched as was already inferred by the higher content of Gal and Ara observed in this extract, and is thus in accordance with Kurita et al. (2008). Neutral sugars such as Ara and Gal are found in side chains attached to Rha residues forming hairy blocks of RG-I while Fuc may be present in the side chains of RG-II. From the molar ratio (Ara + Gal) / Rha the presence of hairy regions of pectin, in particular RG-I, can be inferred. Table 2 reveals that the (Ara + Gal)/Rha ratio of pectic polymers increased from 4 to 7 for the sequentially extracted AIR. This result indicates that the less-substituted RG are extracted first, in agreement with previous reports, which have suggested that a higher substitution in RG-I domains contributes to pectin anchoring in the cell wall matrix (Pena and Carpita, 2004; Vincken et al., 2003).

Pectic polysaccharides extracted in the presence of citric acid showed the highest (Ara + Gal)/Rha ratio (17), confirming that the pectin treated with this acid contains more substituted polymers, especially RG-I, as previously reported (Kurita et al., 2008). XG is the fourth structural unit of pectin, which was recently presumed to be present in modified hairy regions of apple pectin (Arnous and Meyer, 2008). The relatively higher Xyl/GalA ratio found for the citric acid extract (1:24) demonstrates that the XG domain presumably occurs in the pectic material of the unpeeled *BE* apple.

Conclusion

A ratio of approximately 1: 2 was found between acidic and neutral sugars for the AIR after the additional hydrolysis step, confirming that pre-hydrolysis of AIR is required prior to the application of colorimetric methods. Extraction with hot water was more efficient in removing the pectin polysaccharides loosely attached to the cell wall material than cold water.

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