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Structural characterization of a glucuronoarabinoxylan from pineapple (*Ananas comosus* (L.) Merrill) gum exudate

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ABSTRACT

Native polysaccharide from pineapple gum (PANP) was obtained following alkaline extraction of gum and fractionation with cetylpyridinium chloride. It was characterized as a glucuronoarabinoxylan using NMR, methylation data, controlled Smith degradation, carboxy-reduction, and ESI-MS of oligosaccharides produced on mild acid hydrolysis of PANP. HSPEC-MALLS-RI of carboxy-reduced fraction showed homogeneous profile (M_w 1.943 × 10^5 g/mol). PANP was composed of Ara, Xyl, Gal, and Glc*p*A (40:23:7:30 molar ratio). Its main chain presented (1→4)-linked β-xylan, highly substituted at O-2 and O-3 by side chains of 3-*O*- and 3,5-di-*O*-linked α-Ara*f*, 2-*O*- and 4-*O*-linked α-Glc*p*A, and nonreducing end-units of α-Ara*f*, β-Ara*p*, β-Gal*p*, and α-Glc*p*A. ESI-MS of a mixture of oligosaccharides formed on the mild acid hydrolysis of PANP was consistent with repetitive structures of α-Glc*p*A O-3 linked at β-Xyl*p* units, whereas in others glucuronoarabinoxylan-type gum exudates, α-Glc*p*A units had been previously found to be linked at O-2.

1. Introduction

Gum exudates are composed mainly of polysaccharides, which have complex structures, with a great variety of monosaccharides and glycosidic linkages, most of them having highly branched structures (Aspinall, 1969). The most abundant polysaccharide structures found in gum exudates are arabinogalactans, such as those from *Acacia senegal* (gum arabic), which is composed of Ara (28%), Gal (39%), Glc*p*A (17.5%), 4-Me-Glc*p*A (1.5%), and Rha (14%). It has a linear main chain of (1→3)-linked β-Gal*p* residues, substituted at O-6 by complex side-chains of α-L-Ara*f*, β-D-Glc*p*A, α-L-Rha*p*, and β-D-Gal*p* (Anderson, Hirst, & Stoddart, 1966a; Anderson, Hirst, & Stoddart, 1966b; Tischer, Gorin, & Iacomini, 2002). Other polysaccharides have been described in gum exudates, although they are less common. Examples are glucuronoarabinoxylans (GAXs), which show structural relationship with those hemicellulosic glucurono(arabino)xylans from primary plant cell wall, especially those from species of family Poaceae such as sorghum (Verbruggen et al., 1998), maize (Allerdings, Ralph, Steinhart, & Bunzel, 2006), and wheat (Hromádková, Paulsen, Polovka, Kost'álová, & Ebringerová, in press; Sun, Cui, Gu, & Zhang, 2011). They consist of a backbone of β-(1→4)-Xyl*p* units substituted at O-3 prevalently by single α-L-Ara*f* units and at O-2 by α-Glc*p*A (or its 4-O-methyl ether) units (Ebringerová, Hromádková, & Heinze; Pastell, Tuomainen, Virkki, & Tenkanen, 2008; Smith & Harris, 1999). Short arabinans side-chains composed of 2-*O*- and 5-*O*-substituted Ara*f* units were described in arabinoxylans from wheat bran cell wall (Sun et al., 2011). Acetyl groups, ferulic acid and *p*-coumaric acid were also described as substituents of side-chains of GAXs from plant cell walls. Ferulic and coumaric acids may be ester-linked at O-5 of some of α-L-Ara*f* residues of GAX (Ishii, 1997). Glucuronoarabinoxylans from gum exudates were notably more highly branched than those of the hemicellulose type. Those from gum exudates of palm species, showed more than 40% of their Xyl*p* main chain units totally substituted (Maurer-Menestrina, Sassaki, Simas, Gorin, & Iacomini, 2003; Simas et al., 2004, 2006). Typically have Ara*f* units as nonreducing ends and (1→3)-substituted (Dutton & Kabir, 1973; Léon de Pinto, Martínez, & Rivas, 1994; Simas et al., 2004), although gum brea (*Cercidium australe*) showed (1→5)-substituted Ara*f* units (Cerezo, Stacey, & Webber, 1969). Nonreducing end-units were composed of Xyl*p*, Ara*p*, and Fuc*p*, being the last one found in gums from palm species (Dutton & Kabir, 1973; Léon de Pinto et al., 1994; Maurer-Menestrina et al., 2003; Simas et al., 2004, 2006).

Pineapple (*Ananas comosus* (L.) Merrill) belongs to the monocotyledon family Bromeliaceae and is the third most abundant

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tropical fruit cultivated in Brazil (Almeida, Santana, Rodriguez, & Costa, 2002). The major compound of pineapple cell wall is GAX, with a structure similar to those GAXs occurring in the primary cell walls of Poaceae species, although it was more substituted (Smith & Harris, 1995, 2001). It showed the ratio of branched (2,4-*O*- and 3,4-di-*O*-substituted) to unbranched (4-*O*-substituted) main chain Xyl*p* units of 1.6, in contrast with ratio of ~0.2 of GAXs of the Poaceae family (Smith & Harris, 1995).

Among the pineapple diseases "gummosis", which is caused mainly by *Fusarium subglutinans* contamination, is characterized by the exudation of gum from the rind of fruit. The gum can be found anywhere on the fruit, although it usually occurs on the lower half. The gum exudation occurs through the floral cavities and may start when the fruit is 4 or 5 inches and continues until it is ripe, affecting partially or totally the fruit quality (Pérez, 1957; Kimati, 1980). The chemical and structural properties of pineapple gum have not yet been described, and since these characteristics are important for industrial, technological, and biological gum applications, we have isolated and characterized the structure of a polysaccharide from this gum exudate.

2. Materials and methods

2.1. Collection of pineapple gum and isolation of its polysaccharides

The pineapple gum exudates were collected from the rind of various units of fruit of the variety "Pérola", in Sapé (State of Paraíba, Brazil), July–August, 2005.

Crude gum (100 g) was partly dissolved in H_2O (2 L) (25 °C/24 h) and the remaining debris were removed by filtration, followed by centrifugation (12,430 × *g*/20 min/10 °C). The aqueous extract obtained was added to excess of ethanol (EtOH) (×3 vol.) to give a precipitate which was isolated by centrifugation (12,430 × *g*/20 min/10 °C). After dialysis (*cut-off* 12–14 kDa) and freeze-drying, it was called fraction NP (10.6 g). The remaining gum debris were treated with $NaBH_4$ to pH 10.0, and then dissolved in 0.5% aq. KOH (2.0 L). After complete solubilization, the alkaline extract was neutralized with 50% (v/v) aq. acetic acid and was added to excess of ethanol (EtOH) (×3 vol.) to give a precipitate which was dialyzed (*cut-off* 12–14 kDa) and freeze-dried, to give a fraction ANP (59.5 g).

2.2. Fractionation and purification of polysaccharides via cetylpyridinium chloride precipitation

As fraction ANP was obtained in higher yield than NP, it was chosen for examination of the pineapple gum polysaccharides. 20 g of ANP was dissolved in water (400 mL) and then 7.5% (w/v) aq. cetylpyridinium chloride (200 mL) was added. Since cetylpyridium chloride is a cationic quaternary ammonium salt, it reacted with uronic acids rich-fraction producing an insoluble complex, which was isolated by centrifugation (12,430 × *g*/20 min/10 °C) (Scott, 1965; Woranovicz-Barreira, Gorin, Sassaki, Marcelli, & Iacomini, 1999). Precipitate and supernatant fractions were both treated with 4 M NaCl to remove cetylpyridinium chloride, followed by precipitation with EtOH (×3 vol.). After dialysis (*cut-off* 12–14 kDa), the resulting polysaccharide fractions were named PANP (cetylpyridinium-precipitated fraction, 11.1 g) and SANP (cetylpyridinium-supernatant fraction, 4.21 g).

2.3. Controlled Smith degradation

In order to obtain more information about the structure of the main chain of polysaccharide from pineapple gum (PANP), it was dissolved in H_2O (400 mg in 50 mL) and 50 mL of 0.1 M $NaIO_4$ (oxidant agent) was added. The solution was maintained for 72 h in the dark, under magnetic stirring (Hay, Lewis, & Smith, 1965). After this, ethylene glycol (10 mL) was added to stop the reaction. The solution was dialyzed (*cut-off* 8 kDa/48 h) against tap water, treated with $NaBH_4$ (to pH 10.0) for 16 h, and then neutralized (HOAc) and dialyzed (*cut-off* 8 kDa/48 h) against tap water (Goldstein, Hay, Lewis, & Smith, 1965). The solution was concentrated to 40 mL using rotary evaporator (at 35 °C) and submitted to mild acid hydrolysis with TFA at pH 2.0, for 40 min at 100 °C (Gorin, Horitsu, & Spencer, 1965; Simas et al., 2004). It was then treated with 1 M NaOH to pH 5.0 and excess of ethanol was added (4:1, v/v) to give a precipitate, which was dialyzed (*cut-off* 2 kDa) for 24 h yielding polysaccharide fraction called PANP-S (112 mg; 28% yield).

2.4. Carboxy-reduction

Carboxy-reduction of native polysaccharide (PANP – 30 mg) was carried out using two successive cycles of the 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide method (Taylor & Conrad, 1972), $NaBD_4$ being used as reducing agent, to give carboxy-reduced polysaccharide (fraction PANP-CR; 73% yield).

2.5. Mild acid hydrolysis

Native polysaccharide (PANP – 1.0 g) was submitted to mild acid hydrolysis with 0.5 M TFA, at 100 °C for 3 h (Maurer-Menestrina et al., 2003; Simas et al., 2004, 2006). It was then adjusted to pH 5.0 with aq. NaOH and added to excess of EtOH (5:1, v/v) to give a precipitate, which was isolated and dialyzed (*cut-off* 2 kDa) for 24 h to give retained fraction PANP-PH (98 mg; 10% yield).

2.6. Analytical methods

2.6.1. Monosaccharide composition analysis

Each polysaccharide sample was hydrolyzed with 2 M TFA for 8 h at 100 °C, the product being reduced with $NaBH_4$ (Wolfrom & Thompson, 1963a) and acetylated with a mixture of acetic anhydride (Ac_2O) and pyridine (1:1; v/v) for 18 h at 25 °C (Wolfrom & Thompson, 1963b). The resulting alditol acetates were analyzed by GC–MS (Varian model 3800 gas chromatograph coupled to a Saturn 2000R mass spectrometer) using a DB-225 capillary column (25 m × 0.25 mm i.d.) at 50 °C during injection, then programmed at 40 °C/min to 220 °C with He as carrier gas. Uronic acid contents were determined by the colorimetric method of Filisetti-Cozzi and Carpita (1991).

2.6.2. Methylation analysis

Carboxy-reduced polysaccharide (PANP-CR), Smith degraded polysaccharide (PANP-S), and a fraction obtained after mild acid hydrolysis (PANP-PH) were methylated according to Ciucanu and Kerek (1984), by dissolution in dimethyl sulfoxide followed by addition of powdered NaOH and CH_3I. Each mixture was agitated strongly for 30 min and then left for 18 h. After dialysis, the products were freeze-dried and submitted to a second cycle of methylation, then being extracted from aqueous solutions with $CHCl_3$. The per-*O*-methylated products from fractions PANP-CR and PANP-S were hydrolyzed with 72% (w/w) H_2SO_4 (0.5 mL) at 0 °C for 1 h, followed by dilution to 8% (Saeman, Moore, Mitchell, & Millet, 1954), being maintained at 100 °C for 8 h, and then neutralized with $BaCO_3$. The per-*O*-methylated products from fraction PANP-HP were treated with methanolic HCl (1 M) for 3 h at 70 °C and neutralized with Ag_2CO_3. In order to detect partially *O*-methylated fragments arising from Glc*p*A of PANP-HP, its methanolysis product was carboxy-reduced with $NaBD_4$ in 0.1% NaOMe-MeOH at 70 °C for 2 h (Simas et al., 2004). The product was then hydrolyzed with 1 M H_2SO_4

at 100 °C for 8 h. After $NaBD_4$ reduction and acetylation with Ac_2O-pyridine, the resulting mixtures of partially *O*-methylated products were examined by GC–MS using a DB-225 capillary column (25 m × 0.25 mm i.d.), held at 50 °C during injection and then programmed at 40 °C/min to 215 °C (constant temp.). The partially *O*-methylated alditol acetates were identified by their typical electron impact breakdown profiles and retention times (Sassaki, Gorin, Souza, Czelusniak, & Iacomini, 2005).

2.6.3. HPSEC-MALLS-RI analysis

HPSEC-MALLS-RI analysis of samples was carried out using a Waters high-performance size-exclusion chromatography (HPSEC) apparatus coupled to a differential refractometer (RI) (Waters 2410) and a Wyatt Technology Dawn-F Multi-Angle Laser Light Scattering detector (MALLS). Four columns of Waters Ultrahydrogel (2000, 500, 250, and 120) were connected in series and coupled to a multidetection system. 0.1 M $NaNO_2$ containing NaN_3 (0.5 g/L) was used as eluent. Fractions (1 mg/mL) were dissolved in this solvent and filtered (0.22 μm) before analysis. Data were analyzed using ASTRA 4.70.07 software.

2.6.4. Nuclear magnetic resonance (NMR) spectroscopy

^{13}C NMR, ^{13}C NMR-DEPT 135, ^{1}H NMR, ^{1}H (obs.), ^{13}C HMQC, COSY, and TOCSY spectra were obtained using a 400 MHz Bruker model DRX Avance spectrometer equipped with a 5 mm inverse probe. Analyses were performed at 50 °C or 70 °C in D_2O or Me_2SO-d_6 solutions. Chemical shifts of the samples are expressed in PPM (δ) relative to external standard of acetone (δ 30.2) or Me_2SO-d_6 (δ 39.51).

2.6.5. ESI-MS analysis

Fraction obtained after mild acid hydrolysis (PANP-PH; Section 2.5) was dissolved in H_2O (~300 μg/mL) and submitted to negative mass spectrometry, at atmospheric pressure ionization (API), recorded in a triple quadrupole, Quattro LC (Waters), with N_2 as nebulizer (75 L/H) and desolvation gas (350 L/H). The temperature of source was 120 °C and source block 300 °C. Offline as nebulizer and desolvation gas. Offline analyses were performed with an infusion pump at a flow rate of 10 μL/min. Employed were two different energies conditions to obtain mass spectra, one of them being 2.1 kV on the capillary and 30 V on the cone and other with 2.8 kV on the capillary and 90 V on the cone. Tandem-MS was obtained by collision induced dissociation-mass spectrometry (CID-MS), using argon as collision gas. The collision energy was 15 eV.

Table 1
Monosaccharide composition of polysaccharide fractions.

Fraction	Monosaccharide composition (%)[a]				
	Ara	Xyl	Gal	Glc	Uronic acids [b]
NP	29	20	7	tr	44
ANP	36	18	7	tr	39
PANP	40	23	7	tr	30
PANP-CR	38	29	7	26[c]	tr
PANP-S	25	63	2	–	10
PANP-PH	tr	60	tr	–	40

tr: traces (≤1%); NP: fraction obtained after aqueous extraction of gum; ANP: fraction obtained after alkaline extraction of gum; PANP: fraction obtained after cetylpyridinium chloride precipitation of ANP; PANP-CR: carboxy-reduced polysaccharide; PANP-S: Smith degraded polysaccharide; PANP-PH: oligosaccharide-rich fraction obtained after partial acid hydrolysis.

[a] Analyzed on a DB-225 column by GC–MS after total hydrolysis, reduction, and acetylation.

[b] Determined by the colorimetric method of Filisetti-Cozzi and Carpita (1991).

[c] The mass spectrum of glucitol hexaacetate showed key ions with *m/z* added of two mass units, arising from $NaBD_4$ carboxy-reduction of glucuronic acids.

3. Results and discussion

3.1. Homogeneity, molecular mass, and structural analysis of native polysaccharide from pineapple gum

Pineapple gum exudate was submitted to sequential aqueous and alkaline extractions obtaining native polysaccharide (NP) and alkali-obtained native polysaccharide (ANP) (Fig. 1). Fractions NP and ANP showed similar monosaccharide compositions (Table 1), being composed of Ara, Xyl, Gal, and uronic acids in a 29:20:7:44 and 36:18:7:39 molar ratio, respectively.

Since fraction ANP was obtained in higher yield than NP, it was chosen for further fractionation and isolation of polysaccharides. ANP gave rise to an heterogeneous profile on HPSEC-MALLS-RI (Fig. 1). As it contained a high uronic acid content, it was submitted to cetylpyridinium chloride treatment that fractionated acidic polysaccharides according to their uronic acid content (Scott,

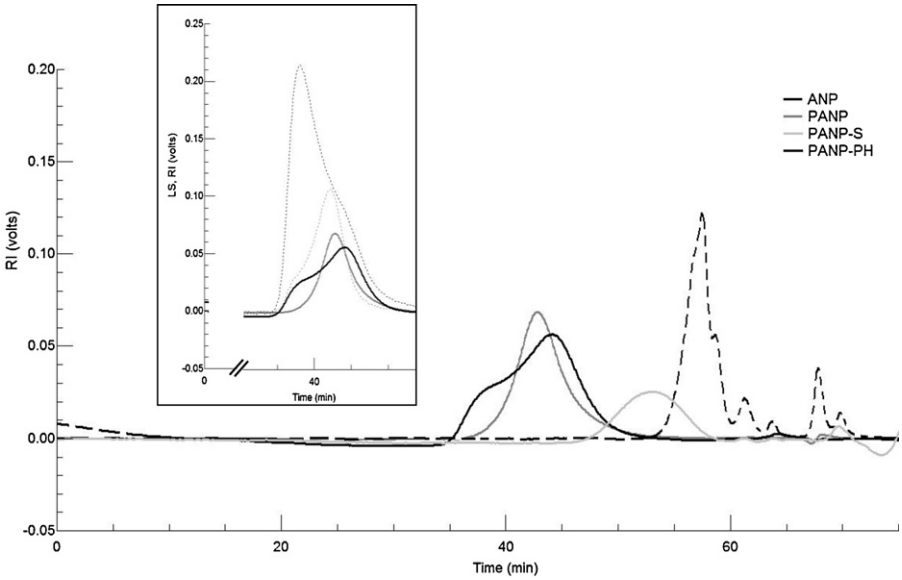

Fig. 1. Elution profiles of polysaccharides fractions using HPSEC with a refractive index (RI) detector. Inset: elution profiles of ANP and PANP fractions using RI (full lines) and LS (light scattering) (dotted lines) detectors.

Table 2
Partially *O*-methylalditol acetates formed on methylation analysis of carboxy-reduced polysaccharide (PANP-CR), Smith-degraded polysaccharide (PANP-S), oligosaccharide-fraction obtained after partial acid hydrolysis (PANP-PH).

Partially *O*-methylated alditol acetates	R_t (min)	Linkage type	Polysaccharide (%)		
			PANP-CR	PANP-S	PANP-PH
2,3,5-Me_3-Ara	6.9	Ara*f*-(1→	17	8	–
2,3,4-Me_3-Ara	7.3	Ara*p*-(1→	tr	2	–
2,3,4-Me_3-Xyl	7.4	Xyl*p*-(1→	4	–	–
2,5-Me_2-Ara	8.4	→3)-Ara*f*-(1→	8	12	–
2,3,4,6-Me_4-Glc[b]	8.7	Glc*p*A-(1→	17	–	–
2,4-Me_2-Xyl	9.1	→3)-Xyl*p*-(1→	–	–	18
2,3,4,6-Me_4-Gal	9.2	Gal*p*-(1→	5	2	–
2,3-Me_2-Xyl	9.4	→4)-Xyl*p*-(1→	tr	62	10
3,4,6-Me_3-Glc[b]	11.0	→2)-Glc*p*A-(1→	9	–	–
2,3,4-Me_3-Glc[c]	11.1	Glc*p*A-(1→	–	–	33
2-Me-Ara	11.3	→3,4)-Ara*p*-(1→, →3,5)-Ara*f*-(1→	7	–	–
2-Me-Xyl[a]	11.9	→3,4)-Xyl*p*-(1→	tr	9	39
3-Me-Xyl[a]	11.9	→2,4)-Xyl*p*-(1→	tr	5	–
2,3,6-Me_3-Glc[b]	12.1	→4)-Glc*p*A-(1→	6	–	tr
Xyl	15.4	→2,3,4)-Xyl*p*-(1→	27	tr	–

tr: traces (≤1%).
[a] These are relative values obtained by comparison of the intensities of ions with *m/z* 118 (2-isomer) with that with *m/z* 129 (3-isomer).
[b] These derivatives arose from carboxy-reduced glucuronic acid units.
[c] This derivative correspond to 2,3,4-Me_3-glucitol-1-D,6-D_2 produced after a $NaBD_4$ reduction of carboxyl methyl ester groups from glucuronic acid units.

1965). Fraction PANP, precipitated on cetylpyridinium chloride treatment, showed an homogeneous profile using an RI detector (Fig. 1) when analyzed by HPSEC-MALLS-RI. However there was a shoulder on MALLS detector profile (Fig. 1 – inset) that could be explained by molecular aggregates, characteristic of polyelectrolyte rich-fractions. PANP showed M_w 1.943 (±0.160) × 10^5 g/mol (*dn/dc* = 0.168) and it was composed of Ara, Xyl, Gal, and uronic acids in a 40:23:7:30 molar ratio (Table 1), suggesting an acidic arabinoxylan structure. Fraction PANP was carboxy-reduced to characterize the uronic acid present in its structure. Carboxy-reduced fraction (PANP-CR) contained glucose (26%; Table 1), arising from carboxy-reduction of glucuronic acid units. This result was confirmed by the mass spectrum of glucitol hexaacetate obtained in $NaBD_4$ carboxy-reduced fraction (PANP-CR), which gave rise to ions with *m/z* 117, 141, 155, 189, and 219, corresponding to glucitol hexaacetate key ions with 2 mass units added to *m/z* (Supplementary material).

Methylation analysis of fraction PANP-CR (Table 2) showed a highly branched structure, with nonreducing end-units of Ara*f* (17%) (2,3,5-Me_3-Ara), Xyl*p* (4%) (2,3,4-Me_3-Xyl), and Gal*p* (5%) (2,3,4,6-Me_4-Gal). The presence of 2,3,4,6-Me_4-Glc (17%), 3,4,6-Me_3-Glc (9%), and 2,3,6-Me_3-Glc (6%) derivatives from PANP-CR indicated that Glc*p*A units were present as nonreducing end-, 2-*O*- and 4-*O*-substituted, respectively, in the original fraction (PANP). The 4-*O*-substituted Glc*p*A units have been described in arabinogalactan-type gum exudates, as gum arabic (Nie et al., 2013; Tischer, Gorin, et al., 2002), gum ghatti (Tischer, Iacomini, Wagner, & Gorin, 2002), *Anadenanthera colubrina* gum (Delgobo, Gorin, Tischer, & Iacomini, 1999), and *Anacardium occidentale* gum (Menestrina, Iacomini, Jones, & Gorin, 1998). In contrast with GAX from pineapple gum (PANP), GAXs from plant cell walls of monocotyledons (Hromádková et al., in press; Smith & Harris, 1999; Verbruggen et al., 1998) and other GAX-type gum exudates showed Glc*p*A units as nonreducing end-units and/or 2-*O*-substituted (Cerezo et al., 1969; Dutton & Kabir, 1973; Léon de Pinto et al., 1994; Maurer-Menestrina et al., 2003; Simas et al., 2004, 2006). Ara*f* side-chains units in PANP-CR were 3-*O*- (8%) and 3,5-di-*O*-substituted (7%) and Xyl*p* were 2,3,4-tri-*O*-substituted (27%) (Table 2). GAXs from gum exudates of palms species (Maurer-Menestrina et al., 2003; Simas et al., 2004, 2006) and *Cercidium praecox* (Léon de Pinto et al., 1994) commonly present Ara*f* units as nonreducing end- and 3-*O*-substituted. The di-*O*-substituted Ara*f* units, as observed in PANP structure, have not been described previously. Moreover, the polysaccharide from pineapple gum (PANP) was more branched than those other GAX-type gum exudates (Cerezo et al., 1969; Léon de Pinto et al., 1994; Maurer-Menestrina et al., 2003; Simas et al., 2004, 2006), since almost all of its main chain xylose units were 2,3-di-*O*-substituted (Table 2).

The ^{13}C NMR spectrum of PANP confirmed its highly branched structure (Fig. 2A). On its anomeric region, signals at δ 108.7–107.6 arose from α-L-Ara*f* units (Gorin & Mazurek, 1975). Signals at δ 99.1–97.9 could be assigned to Glc*p*A in an α-configuration (Cavagna, Deger, & Puls, 1984; Simas et al., 2004) and those at δ 103.1 and δ 101.6 were assigned to β-Gal*p* and β-Xyl*p* units, respectively (Simas et al., 2004; Tischer, Iacomini, et al., 2002). The downfield signals at δ 176.3 and δ 175.7 were from carboxyl (–$\underline{C}O_2H$) groups from α-Glc*p*A units (Gorin & Mazurek, 1975). Those at δ 65.2, δ 63.0, and δ 61.1 were assigned to C-5 of nonreducing end-units of β-Xyl*p* (Gorin & Mazurek, 1975), 4-*O*-linked β-Xyl*p* units (Simas et al., 2004), and α-Ara*f* units (Delgobo et al., 1999), respectively.

3.2. Structural analysis of Smith degraded polysaccharide (PANP-S): elucidation of main chain of PANP polysaccharide

Since the polysaccharide of pineapple gum (PANP) showed a highly branched structure, controlled Smith degradation was used to obtain a less complex polysaccharide which gave information about the main-chain structure of PANP. The first step of the procedure was periodic acid oxidation, which cleave *vic*-glycols linkages with the formation of two aldehydic groups, consuming one molecular proportion of periodate (Hay et al., 1965). Units that did not possess adjacent hydroxyl groups such as 2,3,4-tri-*O*-substituted Xyl*p* units, 3,5-di-*O*-substituted Ara*f* units, and 3-*O*-substituted Ara*f* units were not oxidized by periodate. At the second step, the aldehydic groups were reduced with $NaBH_4$ to give the corresponding alcohol. At the last step of the procedure, the formed alcohols, sensitive to acid, were cleaved by a mild acid hydrolysis (Goldstein et al., 1965). The monosaccharides units that were not oxidized by periodate were more stable to mild acid hydrolysis.

The Smith degraded polysaccharide (PANP-S) was homogeneous on HPSEC-RI (Fig. 1) and it was composed of Ara, Xyl, Gal, and uronic acids in a 25:63:2:10 molar ratio (Table 1). Methylation analysis of PANP-S (Table 2) showed mainly Xyl*p* units 4-*O*-substituted (62%), characterizing the main chain of the original polysaccharide (PANP) as (1→4)-linked xylan and suggesting that high amount

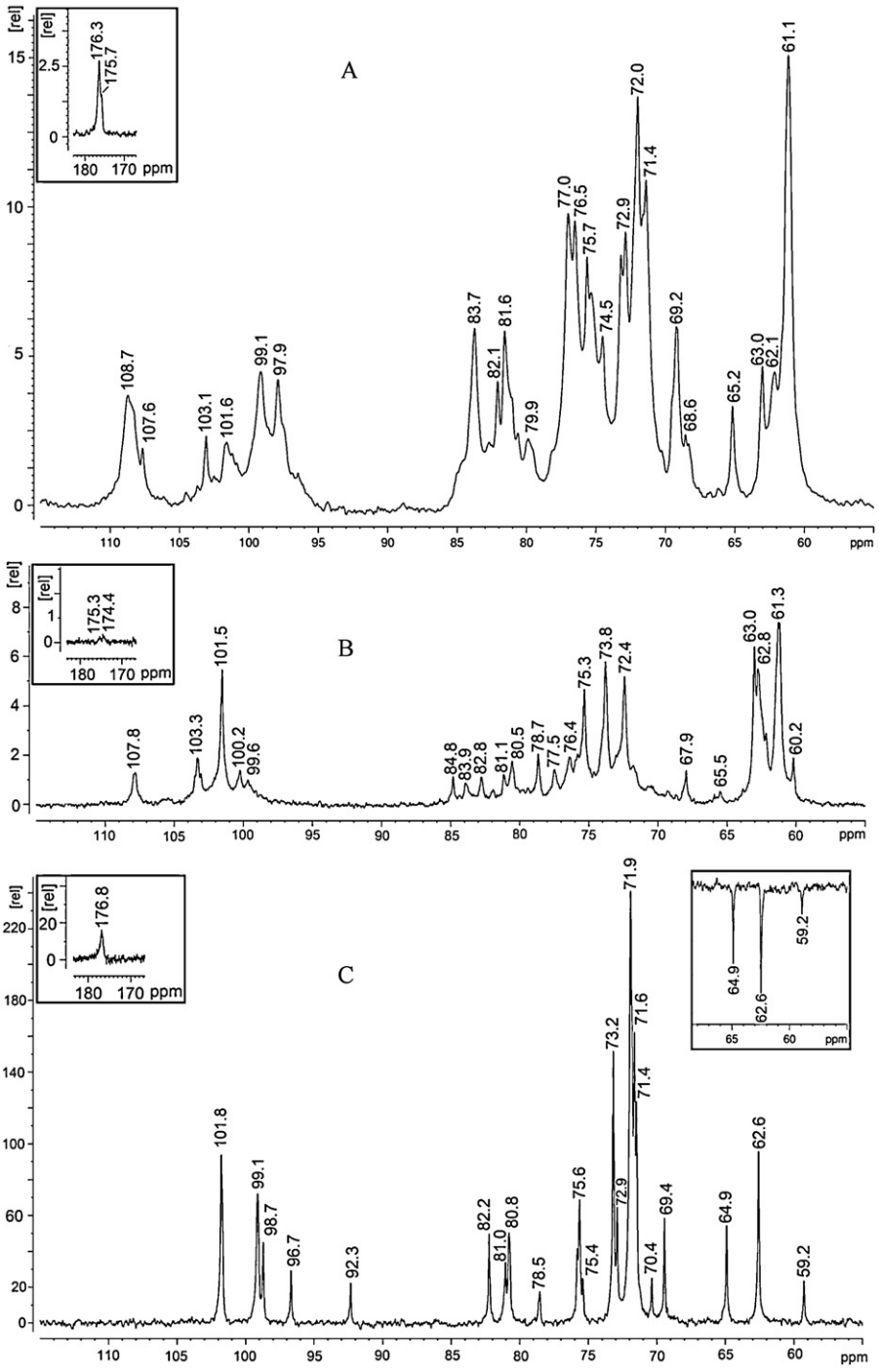

Fig. 2. NMR spectra of native polysaccharide (PANP) (A), Smith degraded polysaccharide (PANP-S) (B), and fraction obtained after mild acid hydrolysis (PANP-PH) (C). Solvent: D_2O (PANP and PANP-PH) and Me_2SO-d_6 (PANP-S) at 50 °C (PANP) and 70 °C (PANP-PH and PANP-S) with numerical values in δ (PPM). Insets: CO_2H region (left side); inverted signals in a ^{13}C DEPT 135 spectrum (right side).

of these units were substituted at O-3 and O-2 by periodate sensitive side-chains, which were degraded. According to analysis of oligosaccharides produced by mild acid hydrolysis (described in Section 3.3), some of the side-chains linked at O-3 of Xyl*p* were composed of Glc*p*A units. Some units of the xylan-main chain of Smith degraded polysaccharide (PANP-S) were 3-*O*- (9%) and 2-*O*-substituted (5%) by Ara*f* units 3-*O*-linked (11%) and nonreducing end-units of Ara*f* (8%), Ara*p* (2%), and Gal*p* (2%) (Table 2). The pattern of substitution of Ara*f* units in PANP and PANP-S structures differ of those classical GAXs from monocotyledons of family Poaceae. In Poaceae, Ara*f* residues were typically nonreducing end-units or 2-*O*-substituted units attached mainly at O-3 (and at a lower amount at O-3 and O-2) to Xyl*p* units of the (1→4)-β-xylan backbone (Gruppen et al., 1992; Hromádková et al., in press; Smith & Harris, 1999; Verbruggen et al., 1998). This structural characteristic was also observed in GAX of primary cell walls of pineapple fruit (Smith & Harris, 1995).

The ^{13}C NMR spectrum of PANP-S (Fig. 2B) contained 5 main signals at δ 101.5, δ 75.3, δ 73.8, δ 72.4, and δ 63.0 arising from C-1, C-4, C-3, C-2 and C-5 of (1→4)-linked β-Xyl*p*- main chain units (Gast, Atalla, & McKelvey, 1980; Simas et al., 2004). Signals at δ 107.8 and δ 61.3 were from C-1 and C-5 of residual α-L-Ara*f* units (Gorin &

Mazurek, 1975). Other C-1 signals at δ 103.3, δ 100.2, and δ 99.6 suggested the presence of β-Gal*p*, β-L-Ara*p*, and α-Glc*p*A (Delgobo et al., 1999; Gorin & Mazurek, 1975; Léon de Pinto et al., 1994; Tischer, Gorin, et al., 2002), since the monosaccharide composition (Table 1) and methylation analysis (Table 2) showed low amounts of these units in the PANP-S structure.

3.3. Analysis of PANP-PH oligosaccharide rich-fraction: information on PANP acidic side-chains

According to Adams (1965a, 1965b), sugar furanoside linkage is more acid labile than the pyranoside one, being readily hydrolyzed by mild acid treatment. Thus, Ara*f* residues attached to the original polysaccharide of pineapple gum (PANP) may be preferentially cleaved by mild acid hydrolysis, while Glc*p*A glycosyl linkages were resistant to this treatment. Thus, this procedure yielded an acidic oligosaccharide rich-fraction (PANP-PH).

Fraction PANP-PH showed an heterogeneous profile on HPSEC-RI (Fig. 1), suggesting a mixture of oligosaccharides with different molecular weight. PANP-PH was cmposed of Xyl (60%) and uronic acid (40%) (Table 1).

Methylation analysis of PANP-PH (Table 2) involving a $NaBD_4$ reduction of carboxyl methyl ester groups gave rise to the acetate of 2,3,4-Me_3-glucitol-1-D,6-D_2 (33%) (Supplementary material), indicating the presence of glucuronic acids as nonreducing end-units. The Xyl*p* units were mainly 3,4-di-*O*-substituted (39%), with some 3-*O*- (18%) and 4-*O*-substituted (10%) units. The 3-*O*-linked Xyl*p* units present in PANP-PH, not detected in other fractions, correspond to 2,3,4-linked Xyl*p* units present in original gum polysaccharide (PANP) structure. After mild acid hydrolysis, O-4 and O-2 substitutions were cleaved, since they were neutral residues. The fragments Glc*p*A-(1→3)-Xyl*p* were not cleaved, considering that linkages between uronic acids and neutral residues were acid-resistant (Adams, 1965a, 1965b).

The ^{13}C NMR spectrum of PANP-PH (Fig. 2C) contained C-1 signals at δ 101.8, δ 99.1/98.7, δ 96.7, and δ 92.3 which were attributed to internal β-Xyl*p*, nonreducing end-units of α-Glc*p*A, reducing end-unit of Xyl*p* in β- and α-configurations, respectively (Simas et al., 2004; Utille, Kovác, Sauriol, & Perlin, 1986). The downfield signal at δ 176.8 arose from $-CO_2H$ of α-Glc*p*A units and upfield ones at δ 64.9, δ 62.6, and δ 59.2, which were inverted in a ^{13}C DEPT 135 spectrum (Fig. 2C – inset at right side), were attributed to C-5 of internal residues of β-Xyl*p* and reducing end-units of Xyl*p* in β- and α-glycosidic configurations, respectively (Gast et al., 1980; Simas et al., 2004). The other ^{13}C NMR assignments and C/H correlations were made according to COSY, TOCSY (Supplementary material) and HMQC (Fig. 3) spectra and are shown in Table 3. Those at δ 82.2–75.4 were characteristic of *O*-substituted carbons. Signals at δ 82.2/3.675 and δ 80.8/3.767 corresponded to C-3/H-3 substituted of β-Xyl*p* units and β-Xyl*p* 4-*O*-substituted units (which produced a β-effect on the C-3 signal), respectively. Signals at δ 81.0/3.767 were from C-3/H-3 substituted of β-Xyl*p* 4-*O*-substituted reducing end-units. Signals at δ 75.6/4.030, δ 75.4/4.017, and δ 78.5/3.979 arose from C-4/H-4 substituted of internal β-Xyl*p* residues and Xyl*p* reducing end-units at β- and α-anomeric configurations, respectively.

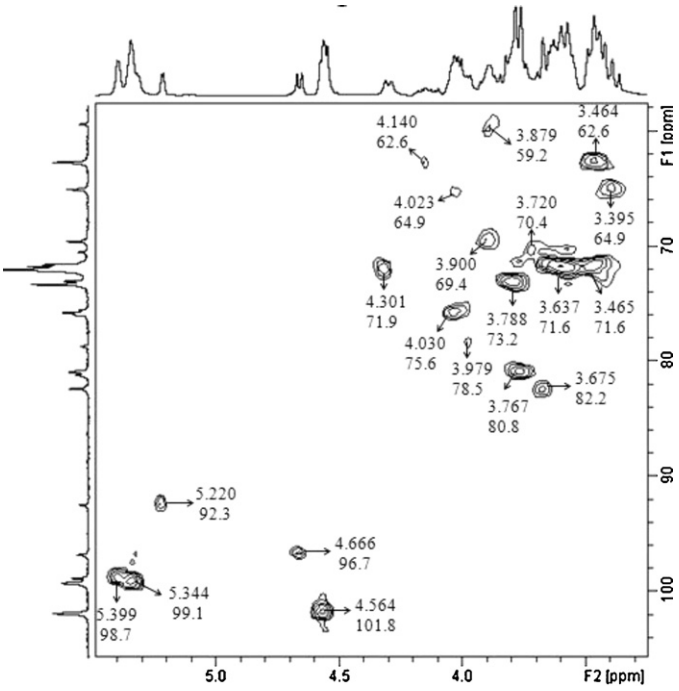

Fig. 3. ^{1}H (obs.), ^{13}C HMQC spectrum of fraction obtained after mild acid hydrolysis (PANP-PH). Solvent: D_2O at 70 °C with numerical values in δ (PPM).

Considering the negative net charge of oligosaccharides from PANP-PH, ESI-MS in the negative ionization mode was chosen for their characterization. In a first approach, a low energy was applied to the cone and capillary (30 V and 2.1 kV, respectively), in order to prevent *in-source* fragmentation. Under this lower energy condition the oligosaccharides appeared as multi-charged ions, which was useful in determining the number of Glc*p*A units present in each oligosaccharide in the mixture. Thus, using isotopologue rules, we found main ions at *m/z* 311 corresponding to Xyl_5-$GlcA_5$ $[M\text{-}5H]^{5-}$, *m/z* 312 to Xyl_4-$GlcA_4$ $[M\text{-}4H]^{4-}$, *m/z* 313 to Xyl_3-$GlcA_3$ $[M\text{-}3H]^{3-}$, and *m/z* 316 to Xyl_2-$GlcA_2$ $[M\text{-}2H]^{2-}$ (Fig. 4A and Table 4). Minor ions at *m/z* 357, 382, and 416 corresponded to *m/z* of Xyl_4-$GlcA_3$ $[M\text{-}3H]^{3-}$, Xyl_3-$GlcA_2$ $[M\text{-}2H]^{2-}$, and Xyl_4-$GlcA_4$ $[M\text{-}4H]^{3-}$, respectively (Fig. 4A). In tandem-MS, the precursor ion at *m/z* 311 (Xyl_5-$GlcA_5$) gave rise to a MS^2 spectrum with fragments at *m/z* 312 (Xyl_4-$GlcA_4$), *m/z* 313 (Xyl_3-$GlcA_3$), *m/z* 316 (Xyl_2-$GlcA_2$),

Table 3
$^{1}H/^{13}C$ assignments of fraction PANP-PH according to HSQC, COSY, and TOCSY spectra.

Fraction PANP-PH	Nonreducing end-units of α-Glc*p*A	Internal β-Xyl*p* units	Xyl*p* reducing end-units	
			β	α
H-1/C-1	5.344/99.1[b]	4.564/101.8	4.666/96.7	5.220/92.3
	5.399/98.7[c]			
H-2/C-2	3.637/71.6	3.465/71.6	3.437/71.5	3.720/70.4
H-3/C-3	3.788/73.2[b]	3.675/82.2[a]	3.767/81.0[a]	n.a.
	3.810/72.9[c]	3.767/80.8[a]		
H-4/C-4	3.597/71.9	4.030/75.6[a]	4.017/75.4[a]	3.979/78.5[a]
H-5/C-5	4.301/71.9	3.395, 4.023/64.9	3.464, 4.140/62.6	3.879/59.2

n.a.: not assigned.
[a] Downfield signals characterizing *O*-substitution.
[b] It was from α-Glc*p*A linked to Xyl*p* reducing end-units at β-anomeric configurations.
[c] It was from α-Glc*p*A linked to Xyl*p* reducing end-units at α-anomeric configurations.

Table 4
ESI-MS and tandem-MS profile of main multi-charged ions from fraction PANP-PH.

MS^1 (*m/z*)	Type of MS^1 ion	MS^2 fragments (*m/z*)	Calculated molecular weight	Corresponding oligosaccharide
311	$[M-5H]^{5-}$	312, 313, 316, 325	1558	Xyl_5-$GlcA_5$
312	$[M-4H]^{4-}$	313, 316, 325	1250	Xyl_4-$GlcA_4$
313	$[M-3H]^{3-}$	316, 325	942	Xyl_3- $GlcA_3$
316	$[M-2H]^{2-}$	325, 457	634	Xyl_2-$GlcA_2$

and *m/z* 325 (Xyl-GlcA). The precursor ion at *m/z* 312 (Xyl_4-$GlcA_4$) gave fragment-ions at *m/z* 313 (Xyl_3-$GlcA_3$), *m/z* 316 (Xyl_2-$GlcA_2$), and *m/z* 325 (Xyl-GlcA). The precursor ion at *m/z* 313 (Xyl_3-$GlcA_3$) provided fragments at *m/z* 316 (Xyl_2-$GlcA_2$) and *m/z* 325 (Xyl-GlcA). From the precursor ion having *m/z* 316 (Xyl_2-$GlcA_2$), fragment ions appeared at *m/z* 325 (Xyl-GlcA) and 457 (Xyl_2-GlcA). These MS^2 results of main ions are summarized in Table 4.

In a second approach, higher cone (90 V) and capillary (2.8 kV) energies were used. Interestingly only singly charged ions were formed (Fig. 4B), confirming structures observed in the lower energy approach. Thus, the main ions that appeared at *m/z* 325, 633, 941, and 1249 correspond to singly charged Xyl-GlcA, Xyl_2-$GlcA_2$, Xyl_3-$GlcA_3$, and Xyl_4-$GlcA_4$, respectively. Minor ions were at *m/z* 457.5 (Xyl_2-GlcA), 765.5 (Xyl_3-$GlcA_2$), 1073.4 (Xyl_4-$GlcA_3$), 1381.0 (Xyl_5-$GlcA_4$), and 1557 (Xyl_5-$GlcA_5$) (Fig. 4B). Thus, ESI-MS analysis confirmed that fraction PANP-PH was composed of repetitive structures of Xyl*p* and Glc*p*A, appearing mainly in a 1:1 molar ratio, indicating via tandem-MS that each Xyl*p* is linked to a Glc*p*A unit.

The repetitive oligosaccharide structures identified by ESI-MS were composed of α-Glc*p*A units linked at O-3 to Xyl*p* units of the (1→4)-β-xylan backbone, according to NMR and methylation analysis (described above). This is in contrast with typical GAXs from Poaceae cell wall (Smith & Harris, 1999), GAXs from flesh of the pineapple fruit (Smith & Harris, 1995; 2001) and other GAX-type gum exudates (Cerezo et al., 1969; Dutton & Kabir, 1973; Lambert, Dickey, & Thompson, 1968; Léon de Pinto et al., 1994; Maurer-Menestrina et al., 2003; Simas et al., 2004, 2006) where single or 2-*O*-susbstituted Glc*p*A (and/or 4-Me-Glc*p*A) units were linked at O-2 to Xyl*p* units of the (1→4)-β-xylan backbone. A recent study of a GAX from the seeds of *Plantago asiatica* L., a dicotyledonous species, described nonreducing end-units of Glc*p*A linked at O-3 to side-chains Xyl*p* units and to those units of the (1→4)-β-xylan backbone (Yin, Lin, Nie, Cui, & Xiea, 2012), as we have found in GAX from pineapple gum exudate.

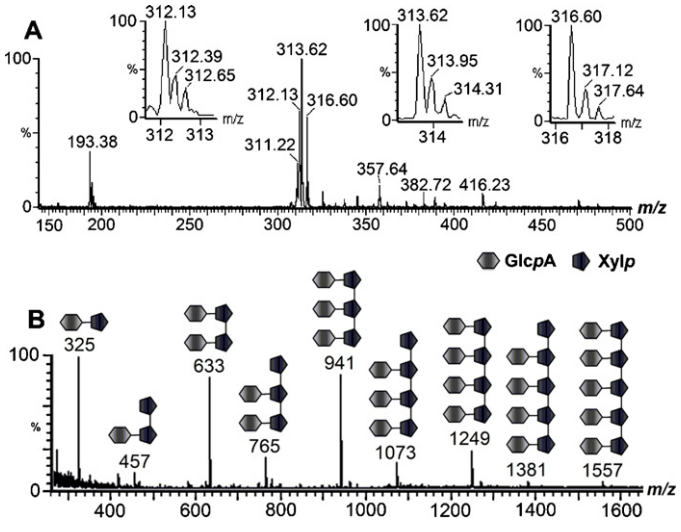

Fig. 4. (A) ESI-MS of multi-charged oligosaccharides from fraction PANP-PH. Insets: Zoom of main ions showing isotopologous distribution indicating the presence of 4, 3, and 2 charges at ions with *m/z* 312.13, 313.62, and 316.60, respectively. (B) ESI-MS of singly charged oligosaccharides from fraction PANP-PH. The corresponding Glc/Xyl ratios are schematically depicted above each ion.

4. Conclusions

Pineapple gum polysaccharide (PANP) was characterized using NMR, methylation data, HPSEC-MALLS-RI, controlled Smith degradation, carboxy-reduction, and ESI-MS of oligosaccharides formed on mild acid hydrolysis. This resulted in characterization of a glucuronoarabinoxylan structure with a main chain composed of (1→4)-linked β-Xyl*p* units highly substituted at O-2 and O-3. Their side chains were composed of 3-*O*- and 3,5-di-*O*-substituted α-Ara*f*, and 2-*O*- and 4-*O*-substitued α-Glc*p*A units, and nonreducing end-units of α-Ara*f*, β-Ara*p*, β-Xyl*p*, β-Gal*p*, and α-Glc*p*A. ESI-MS (negative mode) of fraction PANP-H (mixture of oligosaccharides produced via mild acid hydrolysis) using variable cone and capillary energies produced multi-charged or singly charged ions. These results, together with NMR and methylation data, identified the presence of repetitive structures where α-Glc*p*A units were linked to O-3 of β-Xyl*p* units, although in other glucuronoarabinoxylan-type gum exudates Glc*p*A units were previously found to be linked to O-2 (Cerezo et al., 1969; Dutton & Kabir, 1973; Lambert et al., 1968; Léon de Pinto et al., 1994; Maurer-Menestrina et al., 2003; Simas et al., 2004, 2006).

In future investigations, the knowledge about chemical structure of pineapple gum exudate will be important for understanding its solution properties, its applicability as hydrocolloid in foods and other industrial products, and its biological activity.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2012.12.059.

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