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Structural features of partially acetylated coffee galactomannans presenting immunostimulatory activity

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ABSTRACT

The galactomannans purified from coffee infusions have been shown to present *in vitro* immunostimulatory activity on murine B- and T-lymphocytes. These properties have also been shown characteristic of the galactomannans recovered from coffee residue by strong alkali solutions and rendered soluble in water by partial acetylation. In this study, purified fractions of galactomannans with immunostimulatory activity obtained from coffee infusion and from coffee residue were compared according to their average molecular weight determined by size-exclusion chromatography on Sephacryl S300, glycosidic-linkage composition by methylation analysis, selective hydrolysis by an *endo*-(1 → 4)-β-D-mannanase, analysis of the resultant oligosaccharide profile by Bio-Gel P2 separation, and determination of the pattern of acetylation by electrospray tandem mass spectrometry (ESI-MS/MS). It was found that the galactomannans have a comparable molecular weight (90–110 kDa), and similar glycosidic-linkage composition. However, the galactomannans from coffee residue were preferentially acetylated in the side chain residues whereas the galactomannans recovered from coffee infusions only had acetyl groups directly linked to the backbone residues. These results show that these polysaccharides that present comparable immunostimulatory properties have different acetylation patterns.

1. Introduction

Roasted coffee beans comprise 30–43% of polysaccharides (Redgwell, Trovato, Curti, & Fischer, 2002) and are composed mainly by galactomannans (48%), type II arabinogalactans (34%), and cellulose (18%). About 6–12% of them are soluble in water and can be extracted during the coffee beverage preparation (Nunes & Coimbra, 2002a; Redgwell et al., 2002). Coffee infusion polysaccharides are mainly composed by galactomannans (68%) and type II arabinogalactans (Nunes & Coimbra, 2001, 2002a, 2002b). A significant amount of coffee infusion galactomannans are acetylated (Nunes, Domingues, & Coimbra, 2005). Although several modifications can undergo on coffee galactomannans during the roasting process, such as depolymerization, debranching, and Maillard Reaction, caramelization, isomerisation, oxidation, and decarboxylation by their reducing end (Nunes, Reis, Domingues, & Coimbra, 2006a), several representative fractions of coffee galactomannans still maintain their characteristic structural features (Nunes and Coimbra, 2007), and present *in vitro* immunostimulatory activity (Simões et al., 2009).

In vitro immunostimulatory activity is also a characteristic of the galactomannans that have been recovered from coffee residue with strong alkali solutions and modified by acetylation with iodine and acetic anhydride in order to render them soluble in water (Simões et al., 2009). The chemically acetylated galactomannans had a higher degree of acetylation (0.84 mol acetyl groups per mol of sugar residue) than that presented by the naturally acetylated coffee infusion galactomannans (0.08). Nevertheless, the degree of acetylation of the chemically acetylated coffee residue galactomannans was of the same level of the bioactive *Aloe vera* acemannan (0.91) (McAnalley, 1988), which presented comparable immunostimulatory properties, contrasting with the galactomannans from locust bean gum, that do not present any stimulatory property (Simões et al., 2009). The purpose of this study was to evaluate and compare the structural features and acetylation pattern of both, coffee infusion and coffee residue acetylated galactomannans, which have been shown to present immunostimulatory activity (Simões et al., 2009).

2. Experimental

2.1. Coffee galactomannan samples

The coffee infusion used was prepared from Brazilian Arabica coffee beans from a roast with 5% matter loss on a dry weight basis (DR 5%), as described by Nunes and Coimbra (2001, 2002a). Briefly,

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the ground and defatted coffee was extracted with water (50 g/L) at 80 °C, 20 min, filtered, concentrated, dialysed, frozen, and freeze-dried, giving the high molecular weight material (HMWM). The galactomannan-rich fraction was obtained from the HMWM as described by Nunes et al. (2005, 2006a). Briefly, the HMWM was gradually precipitated in ethanol and the fraction insoluble in 50% ethanol (Et50) was recovered and further purified by anion exchange chromatography on Q-Sepharose. The material recovered on the nonretained fraction was further purified by phenylboronic acid (PBA) affinity chromatography. This fraction contains mannose (89 mol%), galactose (7%), arabinose (2%), and glucose (1%), and a total sugars of 76% (Simões et al., 2009). The amount of acetyl groups, on a molar basis in relation to the sugar residues, was 8%.

Coffee residue galactomannans were prepared from espresso coffee residue obtained by a commercial batch of Buondi coffee, a dark roasted Arabica coffee, produced in Portugal. The coffee residue (1 kg, 360 g dry weight) was sequentially extracted with: (a) 2 L distilled water at 90 °C during 1 h, then (b) with 2 L 0.5 M imidazole, pH 7.0, at 70 °C during 1 h, (c) 1 L 0.05 M NaOH, (d) 1 L 1 M NaOH, and (e) 1 L 4 M NaOH. All NaOH extractions were performed during 2 h at room temperature. To prevent peeling reactions and alkaline oxidation of the polysaccharides, the NaOH extractions were carried out under an inert atmosphere (N_2) with O_2 free solutions containing 0.02 M $NaBH_4$. The NaOH solutions were prepared using distilled water previously boiled for 20 min and cooled under a nitrogen atmosphere. After each extraction step the mixture was filtered, concentrated under reduced pressure, and dialysed for 3 days, with several changes of distilled water. The alkali extracts were previously acidified to pH 5.0 with glacial acetic acid. After dialysis, the extracts were centrifuged and the precipitates, if present, were recovered, frozen, and freeze-dried. The galactomannans present in the 4 M NaOH precipitate were then solubilised by acetylation according to the methodology proposed by Biswas, Shogren, and Willett (2006) for starch and cellulose. In a glass vial equipped with a magnetic stirrer, 1 g of sample was suspended in 2 mL of acetic anhydride containing 50 mg of iodine, sealed, and heated at 100 °C for 10 min. The reaction mixture was then cooled to room temperature and treated with a saturated solution of sodium thiosulfate (2 mL) with stirring. The mixture colour changed from dark brown to colourless, indicating the transformation of iodine to iodide. The mixture was poured into 50 mL of ethanol and stirred for 30 min. The dispersion was centrifuged (15,000 rpm, 10 min, 4 °C) and the precipitate was recovered, suspended in water and stirred overnight in order to solubilise the acetylated polysaccharides. This fraction contains mannose (69 mol%), galactose (23%), arabinose (6%), and glucose (2%), and a total sugars of 55% (Simões et al., 2009). The amount of acetyl groups, on a molar basis in relation to the sugar residues, was 84%.

2.2. Methylation analysis

The methylation procedure to obtain the partially methylated alditol acetates and the analysis by gas chromatography–mass spectrometry were performed as described by Nunes and Coimbra (2001).

2.3. Gel-filtration chromatography (Sephacryl S-300 HR)

Gel-filtration chromatography on Sephacryl S-300 HR (10–400 kDa), Pharmacia, was performed on a 100 × 1.6 cm (XK 100/16, Pharmacia) column at a flow rate of 0.5 mL/min. The samples were suspended in 1 mL of 0.1 M potassium phosphate buffer pH 6.5 with 3 M urea. The same phosphate-urea buffer was used as eluent. Fractions (2 mL) were collected and were assayed for polysaccharides (phenol–sulfuric acid method) (Nunes & Coimbra 2001). To calibrate the column, standard dextrans of 25, 150, and 410 kDa (Sigma) were used. The column internal volume was determined by elution of glucose.

2.4. Determination of the degree of acetylation

The degree of acetylation was determined by quantification of the acetic acid released by saponification of acetyl groups, acidification of solution, solid phase microextraction (SPME), and analysis by gas chromatography, as described by Nunes, Rocha, and Coimbra (2006b).

2.5. Enzymatic hydrolysis and size-exclusion chromatography (Bio-Gel P2)

Samples (15 mg) were hydrolysed with a pure *endo*-β-(1 → 4)-D-mannanase preparation (Megazyme, EC 3.2.1.78) during 48 h at 37 °C with continuous shacking in a 100 mM Na-acetate buffer, pH 5.5, containing 0.02% sodium azide. The freeze-dried material was dissolved in water, and loaded on a XK 1.6/100 column containing Bio-Gel P-2 (Bio-Rad) previously equilibrated with water, and calibrated with DP4 (stachyose), DP2 (cellobiose) and monosaccharide (Glc) using a flow of 0.3 mL/min. Fractions (1 mL) were collected and assayed for sugars with evaporative light scattering detection. The appropriated fractions were pooled and evaporated until all the eluent was removed. No lyophilisation was performed since it has been shown that lyophilisation promotes acetyl migration for non-reducing end of the oligosaccharides of galactomannans (Kabel, de Waard, Schols, & Voragen, 2003).

2.6. Mass spectrometry

The fractions obtained after the Bio-Gel P2 column were dissolved in 40 μL of water and than 2 μL of the solution obtained was diluted in 200 μL 1:1 MeOH–water containing 1% (v/v) formic acid. Samples were introduced into the mass spectrometer using a flow rate of 10 μL/min. Positive ion ESI-MS and MS/MS spectra were acquired using a Q-TOF 2 instrument (Micromass, Manchester, UK), setting the needle voltage at 3000 V with the ion source at 80 °C and cone voltage at 35 V. Each spectrum was produced by accumulating data during approximately 1–2 min. MS/MS spectra of $[M + Na]^+$ ions were obtained by collision induced dissociation (CID), using argon as the collision gas and varying collision energy between 40–50 eV. Data acquisition was carried out with a MassLynx 4 data system.

3. Results and discussion

3.1. Chemical characterisation of galactomannans

The results of methylation analysis of the galactomannans recovered from coffee residue and presenting immunostimulatory activity are shown in Table 1. These polysaccharides have a ratio of (1 → 4,6)-linked/Total Man of 0.078, which is indicative of a degree of branching (DB) of 8%, which is higher than the 4% observed for the galactomannans purified from coffee infusion. However, the ratio of total Man/terminally linked Man, diagnostic of the degree of polymerisation of the galactomannans, is comparable (23 for coffee residue and 20 for coffee infusion). These ratios are in the range of those found for roasted coffee infusion galactomannans (DB 4–5 and 17–24, respectively) (Nunes & Coimbra 2001) and coffee residue galactomannans (DB 4%) (Redgwell et al., 2002). Both samples contained a contamination of type II arabinogalactans, as inferred by the presence of (1 → 3)- and (1 → 3,6)-linked galactose residues, as well as (1 → 5)-linked arabinose residues (3%). Also present were terminally-linked galactose and arabinose residues and

Table 1
Methylation analysis of the coffee infusion and residue galactomannans presenting immunostimulatory activity.

Linkage	Coffee infusion[a] (mol%)		Coffee residue (mol %)	
T-Ara*f*	0.4		4.8	
5-Ara*f*	0.2		2.9	
Total	0.6[b]	2[c]	7.6	6
T-Man*p*	4.6		3.0	
4-Man*p*	83.9		60.8	
4,6-Man*p*	3.3		5.4	
Total	91.8	89	69.1	69
T-Gal*p*	4.0		6.4	
6-Gal*p*	0.3		0.9	
3-Gal*p*	1.4		8.5	
3,6-Gal*p*	0.4		6.2	
Total	6.1	7	22.0	23
4-Glc*p*	1.8		1.2	
Total	1.8	2	1.2	2
4,6-Man*p*/Total Man*p*	0.036		0.078	
Total Man*p*/T-Man*p*	20		23	

[a] Values from Nunes et al. (2006a, 2006b).
[b] Total molar percentage obtained by methylation analysis.
[c] Values are the molar percentage obtained by sugar analysis.

(1 → 4)-linked glucose residues, which are known to be structural features of coffee galactomannans (Nunes et al., 2005). Nevertheless, the amount of type II arabinogalactans is higher in the coffee residue sample (24%) than in the coffee infusion used (3%).

In order to obtain an estimation of the molecular weight of the polysaccharides present in coffee residue, a size-exclusion chromatography on Sephacryl-300HR was performed (Fig. 1). The average molecular weight was estimated as 109 kDa, which is a value similar to that obtained for the coffee infusion sample (140–90 kDa). The difference found for the molecular weight of coffee galactomannans determined by size-exclusion chromatography and end-group analysis by methylation has been previously observed (Nunes & Coimbra, 2001), attributed to a possible degradation of the polysaccharides during methylation (Nunes & Coimbra 2001). From these results, the primary glycan structure for the acetylated coffee residue galactomannans does not appear to differ significantly from the coffee infusion ones.

3.2. Selective hydrolysis of coffee galactomannans by an endo-β-(1 → 4)-D-mannanase

In order to evaluate and compare the acetylation pattern of these immunostimulatory galactomannans obtained from a roasted coffee infusion and from the residue, they were selectively hydrolysed with an *endo*-β-(1 → 4)-D-mannanase. This selective degradation procedure allows cleaving the galactomannan backbone between adjacent β-(1 → 4)-linked mannose residues, allowing to obtain mannan oligosaccharides that contain structural details on the substituents of the mannan backbone (Dhawan & Kaur, 2007; Moreira & Filho, 2008).

The oligosaccharides obtained by selective enzymatic hydrolysis of the mannan backbone were fractioned by size-exclusion chromatography, giving hydrolysis product profiles different for the two galactomannan samples (Fig. 2). The major oligosaccharides released from coffee infusion galactomannans were the disaccharides (DP2) and, in lower amount, the trisaccharides (DP3), although a small amount of monosaccharides, in the inclusion limit of the gel, and a small amount of oligosaccharides of higher degree of polymerisation, in the void volume of the gel (>2 kDa), were also present. Contrarily to what was observed for coffee infusion galactomannans hydrolysis products, the major oligosaccharides released for the coffee residue galactomannans were higher than DP3 and presented a heterogeneous degree of polymerisation (Fig. 2). For these galactomannans, DP3 and DP2 oligosaccharides accounted for a small proportion of the total oligosaccharides released and monosaccharides were absent. Also, the carbohydrate material present in the void volume of the gel was higher than that observed for the coffee infusion galactomannans. This high molecular weight material contains the contaminating Type II arabinogalactans present in this polysaccharide fraction. Also, because the galactomannans of coffee residue presented a high degree of acetylation, it is possible that the higher molecular weight carbohydrate fraction found in coffee residue galactomannans after enzymatic hydrolysis contains undegradable mannan portions for *endo*-β-(1 → 4)-D-mannanase, as the substitution pattern of mannans influences the range of oligosaccharides released after enzymatic hydrolysis (Daas, Schols, & Jongh, 2000).

3.3. ESI-MS analysis

To evaluate and compare the acetylation pattern of the naturally acetylated galactomannans of coffee infusion and the chemically acetylated galactomannans obtained from coffee residue (CR), the oligosaccharides obtained after mannanase treatment were analysed by mass spectrometry (MS) with electrospray ionization (ESI). The oligosaccharides identified as $[M + Na]^+$ ions in ESI-MS spectra from coffee infusion and coffee residue samples are shown in Table 2. "Hex" represents a hexose, as the *m/z* of Man, Gal or Glc is the same; "Pent" represents a pentose, probably Ara, as this was

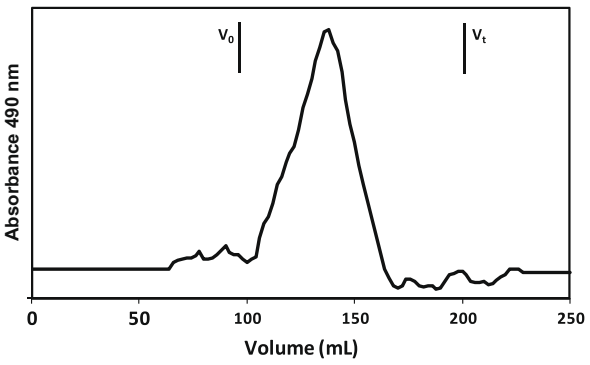


Fig. 1. Size-exclusion chromatographic profile on Sephacryl-300 HR of galactomannans from coffee residue. V_0, void volume and V_t, total volume.

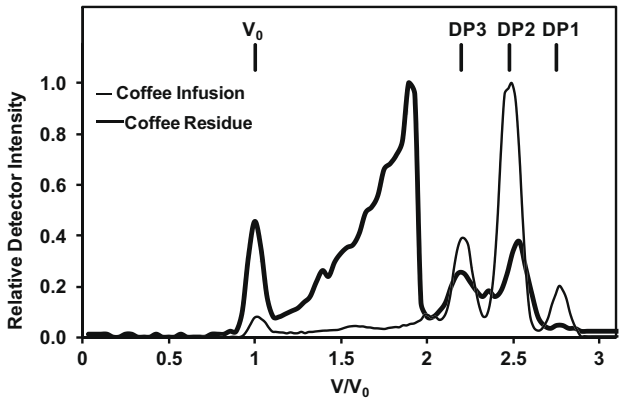


Fig. 2. Size-exclusion chromatography on Bio-Gel P2 of the oligosaccharides obtained after enzymatic hydrolysis with an *endo*-β-D-mannanase of acetylated galactomannans from coffee infusion and coffee residue. V_0- void volume, DP 2 and DP 3 correspond to the elution volume of DP 2 and DP 3 standard oligosaccharides, and DP 1 corresponds to the elution volume of monomers.

Table 2
Oligosaccharide *m/z* ([M + Na]$^+$) ions observed by ESI-MS after enzymatic hydrolysis with an *endo*-β-(1 → 4)-D-mannanase of the mannans obtained from coffee infusion and coffee residue after chemical acetylation.

n	Coffee infusion		Coffee residue			
	2	3	2	3	4	5
Hex_n	365	527	365	527	689	851
$PentHex_n$	497		497	659	821	
$AcHex_n$	407	569	407	569	731	893
$AcPentHex_n$		701	539	701	863	1025
Ac_2Hex_n	449	611		611	773	935
$Ac_2PentHex_n$		743	581	743	905	1067
Ac_3Hex_n					815	977
$Ac_3PentHex_n$			623	785	1079	1109

the only pentose detected by sugar analysis and also identified in coffee infusion galactomannans, and "Ac" represents the acetyl groups.

The number of oligosaccharide products observed for coffee residue galactomannan enzymatic hydrolysis was much higher than that observed for coffee infusion galactomannans, which is in accordance with the higher variability observed by the size-exclusion chromatographic profile. In coffee residue galactomannans it was identified di- to pentasaccharides constituted only by hexoses (Hex_{2-5}) whereas in coffee infusion galactomannans only di- and trisaccharides (Hex_{2-3}) were identified. As previously shown (Nunes et al., 2005), the mannanase hydrolysis of coffee infusion galactomannans gives origin to oligosaccharides containing pentoses. These oligosaccharides are also present in coffee residue galactomannans, allowing to infer the occurrence of galactomannans in coffee residue that present this structural feature previously observed in coffee infusion galactomannans (Nunes et al., 2005). From sugar analysis, this pentose should be arabinose.

Oligosaccharides containing acetyl groups were identified in both coffee galactomannan samples. It was possible to identify monoacetylated oligosaccharides and diacetylated oligosaccharides. Only for coffee residue galactomannans tri-acetylated hexose oligosaccharides have been observed (Table 2). This feature seems to be related with the higher degree of acetylation of the coffee residue galactomannan.

3.4. ESI-MS/MS analysis

In order to elucidate the structure of coffee oligosaccharides released from galactomannans, namely, the location of the acetyl groups on the original galactomannan polysaccharide backbone, they were submitted to a fragmentation under ESI-MS/MS conditions. ESI-MS/MS fragmentations are the result of glycosidic cleavages between two sugar residues and of cross-ring cleavages (cleavage of two bonds within the sugar ring) (Domon & Costello, 1988; Zaia, 2004). Analysing the ESI-MS/MS spectra of the [M + Na]$^+$ ions of acetylated oligosaccharides with the same molecular weight, and with the same sugars composition, but originated from different samples (coffee residue or coffee infusion galactomannan enzymatic hydrolysis products), it was possible to observe different fragmentations. The observed differences will allow to infer the presence of acetyl groups in different monosaccharide residues and thus to identify the acetylation pattern of each oligosaccharide (Nunes et al. 2006a; Reis et al., 2004).

To demonstrate this outcome, the ESI-MS/MS spectra of two pairs of isomeric oligosaccharides, $AcPentHex_3$, *m/z* 701, and $Ac_2PentHex_3$, *m/z* 743, obtained from the galactomannans from coffee infusion and coffee residue, are presented in Figs. 3 and 4, respectively. Comparing the MS/MS spectra of these pairs of isomers it is possible to see that the MS/MS spectra are distinct, revealing differences in the fragmentation pattern of these ions

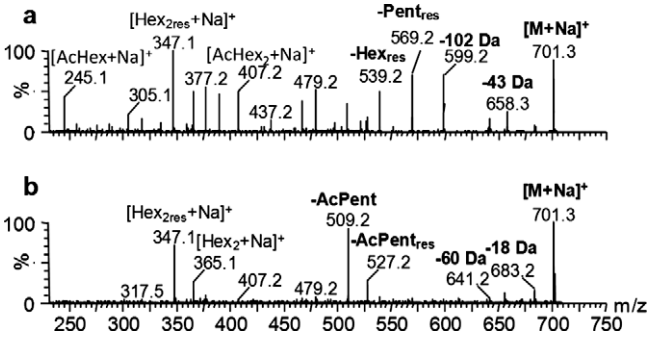


Fig. 3. ESI-MS/MS spectra of the [M + Na]$^+$ adducts of $AcPentHex_3$ (*m/z* 701) obtained respectively from (a) coffee infusion and (b) coffee residue.

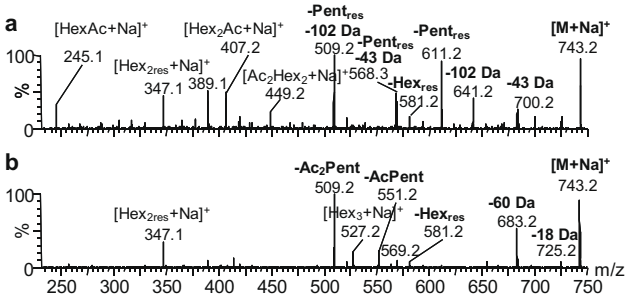
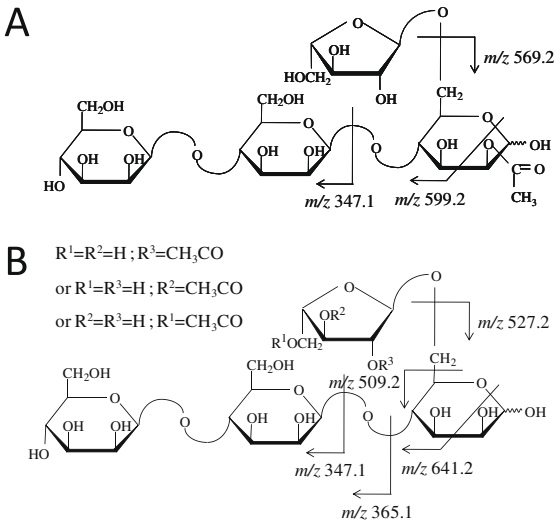


Fig. 4. ESI-MS/MS spectra of the [M + Na]$^+$ adducts of $Ac_2PentHex_3$ (*m/z* 743) obtained respectively from (a) coffee infusion and (b) coffee residue.

with the same *m/z* value, indicating different structures for the oligosaccharides obtained from the chemically and naturally acetylated galactomannans.

3.4.1. ESI-MS/MS analysis of monoacetylated tetrasaccharides containing a pentose

The ESI-MS/MS spectrum of [$AcPentHex_3$ + Na]$^+$ (*m/z* 701) ions from coffee infusion sample showed the presence of ions at *m/z* 569, 407, and 245, identified as [$AcHex_3$ + Na]$^+$, [$AcHex_2$ + Na]$^+$, and [AcHex + Na]$^+$, resultant of loss of one $Pent_{res}$ (132 Da), one $Pent_{res}$ plus Hex_{res} (294 Da), and one $Pent_{res}$ plus two Hex_{res} (294 Da), respectively (Fig. 3a and Scheme 1). Also observed with



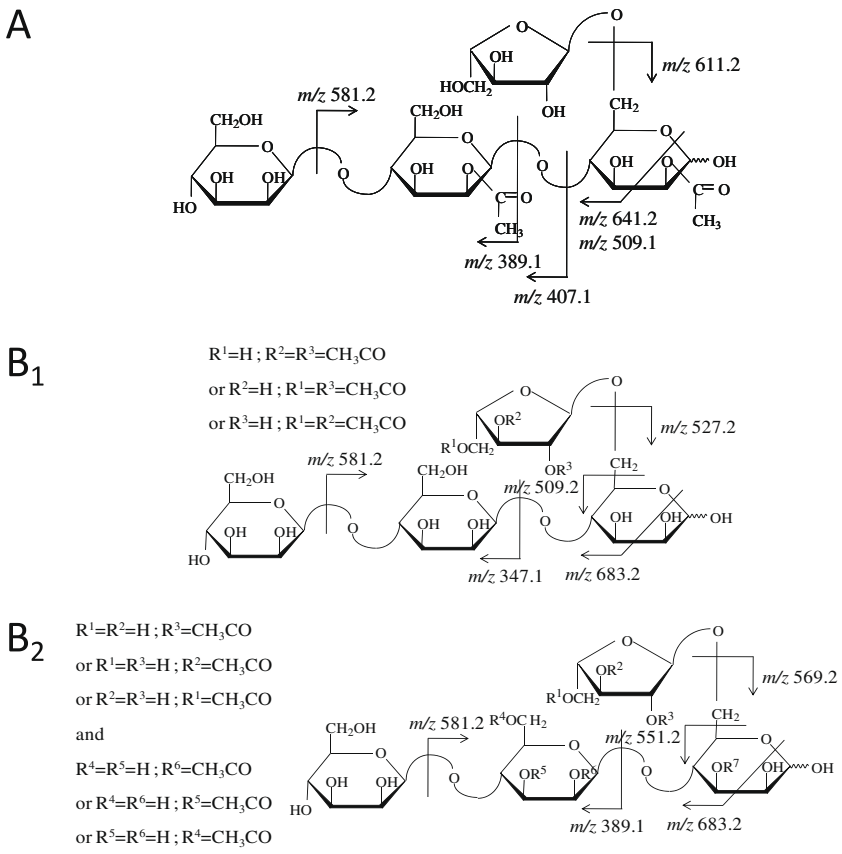
Scheme 1. Fragmentation pattern observed in the ESI-MS/MS spectra of the [M + Na]$^+$ adducts of $AcPentHex_3$ (*m/z* 701) obtained respectively from (A) coffee infusions and (B) coffee residue.

a high abundance was the ion at *m/z* 347, identified as [Hex-Hex_{res} + Na]$^+$, resultant from loss of AcPentHex (354 Da). These results show that this oligosaccharide is acetylated in one of the hexoses, excluding the non-reducing mannose. The presence of one acetyl group is also noticed by the ions at *m/z* 658 and 479, resultant of loss of –$CH_3COO^{\cdot}$ (43 Da) and AcHex (222 Da), respectively (Fig. 3a). The loss of AcHex (222 Da) suggests the presence of an isomeric structure where the Pent is linked to the inner mannose residue and the acetyl group is linked to the mannose at the reducing end. The occurrence of the ion at *m/z* 599, resultant from loss of 102 Da, attributed to a $^{0,2}A_3$ cross ring fragment containing the acetyl group at *O*-2 position (Nunes et al., 2005), shows that the acetyl group is linked to the *O*-2 of the hexose residue of the reducing end terminal (Fig. 3a and Scheme 1).

The ESI-MS/MS spectrum of the [$AcPentHex_3$ + Na]$^+$ (*m/z* 701) ion from coffee residue sample (CR, Fig. 3b) shows a different fragmentation pattern when compared with the one reported above for the isomeric oligosaccharide from coffee infusion. In this MS/MS spectrum, the major ions are observed at *m/z* 509 [Hex_{3res} + Na]$^+$ and 527 [Hex_3 + Na]$^+$, resultant from the loss of an acetylated pentose, AcPent (192 Da), and loss of an acetylated pentose residue, $AcPent_{res}$ (−174 Da) (Scheme 1B). Also, the ion resultant of the loss of $Pent_{res}$ (132 Da) at *m/z* 569, correspondent to [$AcHex_3$ + Na]$^+$, which is the major product ion of the MS/MS spectra of coffee infusion oligosaccharide, is absent in this spectrum of coffee residue. In addition, the ion at *m/z* 245, correspondent to [AcHex + Na]$^+$, is also absent. These results allow to conclude that the chemically acetylated galactomannan from coffee residue is acetylated in the pentose residue, a structural feature not observed for the naturally acetylated coffee infusion galactomannan. The information obtained from the MS/MS spectra does not allow to exactly locating the esterified position on the pentose residue. The ions [$HexHex_{res}$ + Na]$^+$, at *m/z* 347, and [Hex_2 + Na]$^+$, at *m/z* 365, formed by the combined loss of AcPent plus Hex_{res}, and $AcPent_{res}$ plus Hex_{res}, respectively, are also observed with high abundance. Ions due to loss of $Pent_{res}$ are absent in this spectrum. The ion at *m/z* 407, correspondent to [$AcHex_2$ + Na]$^+$ was observed, but in very low relative abundance, indicating that acetylation also occurs at the hexose residues, although in a low abundance. The absence of ions resultant of loss of 102 Da ($^{0,2}A_3$ cross ring fragment containing the acetyl group at *O*-2 position) and of the ion at *m/z* 658, resultant of a loss of 43 Da (–$CH_3COO^{\cdot}$) show that the terminal mannose residue of this oligosaccharide is not acetylated. These features are also observed for the other oligosaccharides with higher degrees of polymerisation and related structure ($AcHex_nPent$, results not shown).

3.4.2. ESI-MS/MS analysis of diacetylated tetrasaccharides containing a pentose

The ESI-MS/MS spectrum of the isomeric diacetylated oligosaccharide [$Ac_2PentHex_3$ + Na]$^+$ (*m/z* 743) ions obtained from coffee infusion and coffee residue galactomannans are shown in Fig. 4. The oligosaccharides obtained from coffee infusion (Fig. 4a) presented the fragmentations due to the loss of $Pent_{res}$ (ion at *m/z* 611), loss of Hex_{res} (ion at *m/z* 581), and combined loss of $Pent_{res}$ plus Hex_{res} (ion at *m/z* 449), thus indicating that the pentose residue is not acetylated neither the hexose from the non-reducing end. Abundant ions due to loss of 102 and 43 Da from the [M + Na]$^+$ (ions at *m/z* 641 and 700) and from [(M-$Pent_{res}$ + Na)]$^+$ (ions at *m/z* 509 and 568), resultant from cross ring and acetyl group cleavages, and the ion at *m/z* 245, attributed to [AcHex + Na]$^+$, were also observed, indicating that one acetyl group is at



Scheme 2. Fragmentation pattern observed in the ESI-MS/MS spectra of the [M + Na]$^+$ adducts of $Ac_2PentHex_3$ (*m/z* 743) obtained respectively from (A) coffee infusion and (B1 and B2) coffee residue.

the C-2 of the reducing end mannose residue (Fig. 4a, Scheme 2a). All these fragmentations confirmed the presence of the acetyl groups linked to galactomannan backbone.

The ESI-MS/MS spectrum of the $[Ac_2PentHex_3 + Na]^+$ ion, at *m/z* 743, obtained from coffee residue (Fig. 4b, Scheme 2b) shows fragments due to the loss of Ac_2Pent and Ac_2Pent_{res}, with formation of the ions at *m/z* 509, correspondent to $[Hex_{3res} + Na]^+$, and 527, correspondent to $[Hex_3 + Na]^+$, respectively, as resumed in Scheme 2B1. These fragmentations indicate that both acetyl groups are linked to the pentosyl residue that is linked to the mannan backbone. From the MS/MS data no evidence of the exact location of the acetyl groups can be achieved. The combined loss of Ac_2Pent plus Hex_{res} (ion at *m/z* 347) is also observed (Scheme 2B1). Another fragmentation pathway correspondent to the loss of AcPent is observed by the ion at *m/z* 551, with formation of the ion $[AcHex_2Hex_{res} + Na]^+$ and loss of $AcPent_{res}$, by formation of the ion at *m/z* 569, correspondent to $[AcHex_3 + Na]^+$. These ions show the presence of an isomeric oligosaccharide structure bearing one acetyl group linked to the pentosyl residue and another linked to a hexose residue (Scheme 2B2). As observed previously for the tetrasaccharides, the product ion spectra of coffee residue oligosaccharides do not show loss of $Pent_{res}$ (−132 Da), loss of 102 Da neither loss of 43 Da. The absence of the cross ring fragmentation ($^{0,2}A_3$ cross ring fragment containing the acetyl group at *O*-2 position) and the absence of the ion formed by the loss of 43 Da ($-CH_3COO^{\cdot}$) show that the terminal mannose residue of this oligosaccharide is not acetylated. These fragmentation pathways were also observed for the other ions of the series $Ac_2PentHex_n$. Thus, it is possible to conclude that the acetylation in these oligosaccharides from chemically acetylated mannans occurred also preferentially in the pentose ramification.

4. Concluding remarks

The chemically acetylated galactomannans isolated from coffee residue have a degree of branching and a molecular weight similar to those found for coffee infusion galactomannans. Nevertheless, the acetylation pattern and the degree of acetylation of these galactomannans were significantly different from the natural acetylation pattern and degree of acetylation occurring in coffee infusion galactomannnans. Whereas the acetylation of coffee infusion galactomannans occurs in the mannose residues of the backbone, preferentially at the *O*-2 position, the acetylation of the chemically acetylated coffee residue galactomannans occurs preferentially at the side chains, here demonstrated for the pentose residues present as substituents of the coffee mannan backbone. Nevertheless, the galactose residues present in coffee galactomannans as side chains should also be acetylated. However, since they have the same molecular weight as the mannose residues of the backbone, it is not possible to differentiate their presence from the acetylated mannose residues. These results show that these polysaccharides that present comparable immunostimulatory properties have different acetylation patterns, suggesting that further structure–activity studies need to be performed in order to understand their action.

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