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Mass spectrometry characterization of an *Aloe vera* mannan presenting immunostimulatory activity

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ABSTRACT

Aloe vera acemannan is a polysaccharide composed by a backbone of β-(1→4)-linked D-mannose residues interspersed by few glucose residues, acetylated in O-2, O-3, and O-6 containing side chains constituted by O-6-linked single α-D-galactose and α-L-arabinose residues. This structural features are rather similar to mannans from other sources, namely coffee and locust bean gum. However, *Aloe vera* acemannan and coffee mannans present immunostimulatory activity but locust bean gum does not. In order to know more about the structural features of a commercial preparation of *Aloe vera* presenting comparable immunostimulatory activity to that observed for coffee mannans, this preparation was submitted to sugar and methylation analysis. To gain further insight to the structural details of the mannans, focusing in the study of acetylation pattern, a specific hydrolysis with an *endo*-β-(1→4)-D-mannanase was performed and the resulting oligosaccharides (OS) were fractionated by size exclusion chromatography and characterized by ESI-MS, ESI-MS/MS and MALDI-MS. The majority of the OS obtained for acemannan had a ratio of two acetyl groups per sugar residue. The observation of OS highly acetylated as well as non-acetylated OS, allowed to infer a non-homogeneous distribution of the acetyl groups. Also, it was observed OS presenting fully acetylated arabinose residues. The occurrence of a high abundance of acetylated residues shows that this polysaccharide contains odd acetylation content. These unusual features are reinforced by the presence of acetylated side chains, only previously observed in chemically acetylated mannans with immunostimulatory activity prepared from coffee residue. The comparison with other galactomannans allowed to infer that lower branching, shorter chains, and higher acetylation seems to promote the immunostimulatory activity attributed to these polysaccharides.

1. Introduction

Aloe vera has been the object of several studies in the last two decades due to its reported health benefits. These studies, using *Aloe vera* extracts have shown the promotion of wound healing (Davis, DiDonato, Hartman, & Haas, 1994), skin hydration (Dal'Belo, Gaspar, & Maia Campos, 2006), anti-inflammatory (Davis et al., 1994), antifungal (Rodrıĭguez, Castillo, Garcıĭa, & Angulo-Sánchez, 2005), antidiabetic (Yongchaiyudha, Rungpitarangsi, Bunyapraphatsara, & Chokechaijaroenporn, 1996), anticancer (Steenkamp & Stewart, 2007), immunostimulatory (Simões et al., 2009; Talmadge et al., 2004), and gastro protective (Yusuf, Agunu, & Diana, 2004) properties. *Aloe vera* is a member of the *Liliaceae* plant family and their polysaccharides make up most of the dry matter of the *Aloe vera* protoplast of the parenchyma cells. *Aloe vera* polysaccharides are rich in acemannans (50%) and contain cellulose (25%), pectic polysaccharides (20%), and xylose-containing polysaccharides (5%) (Femenia, García-Pascual, Simal, & Rosselló, 2003; Femenia, Sánchez, Simal, & Rosselló, 1999). Some of the health benefit activities are attributed to the polysaccharides, namely the acemannans (Hamman, 2008).

Acemannan, also known by the commercial name of "carrysin", is the designation for the acetylated mannan from *Aloe vera* commercially available. The *Aloe vera* acemannans are composed by a backbone of acetylated β-(1→4)-mannose residues with α-(1→6)-galactose residues as single side chains (Reynolds, 1985). The acetylation occurs at the C-2, C-3 and C-6 of mannose residues with an acetyl/mannose ratio of approximately 1:1, and the mannan backbone contains interspersed β-(1→4)-linked glucose residues (Fogleman, Shellenberger, Balmer, Carpenter, & McAnalley, 1992; Hamman, 2008; Manna & McAnalley, 1993; McAnalley, Carpenter, & McDaniel, 1999; Talmadge et al., 2004). Chow, Williamson, Yates, and Goux (2005), using NMR and sugar and linkage analysis combined with selective enzymatic hydrolysis with *endo*-β-mannanase and acid hydrolysis, provide evidence of the glucomannan backbone, but raised some questions about the identity of the of *Aloe*

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vera acemanan side chains. Since deacetylation was performed prior to analysis, no data were added concerning the acetylation pattern of this mannan. Overall, little is known about the pattern of acetylation of these compounds, in particular the distribution profile of acetyl groups along the polymeric chain.

Although Ni, Yates, and Tizard (2004) reported that the acemannan found in *Aloe vera* has an unique structure among other well-known plant mannans, more recently, a similar structure has been also identified in coffee (Nunes, Domingues, & Coimbra, 2005; Simões, Nunes, Domingues, & Coimbra, 2010) and locust bean gum (Simões, Nunes, Domingues, & Coimbra, 2011). Interestingly, coffee mannans showed immunostimulatory activities similar to *Aloe vera* mannan, while locust bean mannan did not present immunostimulatory activity under the same experimental *in vitro* conditions (Simões et al., 2009). Although no reports are yet available concerning the relation between structure and biological activity of mannans, polysaccharides may be strongly affected by their structure (Yang & Zhang, 2009), namely the molecular weight and the degree branching. Also, the effect of the conformation and the polarity of the polysaccharides are reported to modulate their biological activity (Leung, Liu, Koon, & Fung, 2006). *Aloe vera* acemannan can interact with the mannose receptors present on several cells, including macrophages, stimulating the immune response (Leung et al., 2006). Simões et al. (2009) showed that a commercial sample of *Aloe vera* acemannan stimulated murine B- and T-lymphocytes, as evaluated by the *in vitro* expression of the surface lymphocyte activation marker CD69, more marked on B- than on T-lymphocytes.

The aim of the present study was to structurally characterize the acemannan obtained from a commercial sample of *Aloe vera* with known immunostimulatory activity, focusing in the identification of the acetylation pattern and the distribution profile of the acetyl groups along the mannan chain. Mass spectrometry has been shown to be a powerful tool for the characterization of the acetylation pattern of polysaccharides, allowing to infer the distribution and sometimes also the position of the acetyl groups (Nunes et al., 2005; Reis et al., 2005; Simões et al., 2010), making a valuable alternative to the NMR, especially when low amounts of material are available. The commercial preparation was characterized concerning its sugars and glycosidic-linkage composition. Since the commercial sample was a mixture, and in order to obtain information exclusively from the mannan present, it was selectively treated with an *endo*-β-D-mannanase. This enzyme allowed a selective degradation of the acemamman without interference of other polymers. The detailed structure of the mannosyl oligosaccharides present in selected fractions obtained by size exclusion chromatography was achieved by electrospray (ESI-MS and ESI-MS/MS) and matrix assisted laser ionization mass spectrometry (MALDI-MS). These results will be used for comparison with the structural features of mannans from other sources in order to relate their structures with their previously reported immunostimulatory activities (Simões et al., 2009).

2. Experimental

2.1. Aloe vera sample

A capsule from Molo-Cure® (now called AMP Floracel®), USA, containing 405.5 mg of powder Aloe mucilaginous polysaccharides (AMP) extracted from *Aloe vera* plant was used to obtain the *Aloe vera* mannan. The whole material present in the capsule was dissolved in water, dialyzed (12–14 kDa cut-off) and freeze-dried (Simões et al., 2009). This high molecular weight material (AV) was shown by Simões et al. (2009) to present *in vitro* immunostimulatory activity.

2.2. Sugar analysis

Neutral sugars were determined by gas chromatography (GC) as alditol acetates. The polysaccharides were treated with 72% (w/w) H_2SO_4 (10 mg/mL) during 3 h at room temperature with occasional stirring followed by hydrolysis for 2.5 h with 1 M sulfuric acid at 100 °C. Monosaccharides were reduced with $NaBH_4$ (15% in NH_3 3 M) during 1 h at 30 °C and subsequent acetylated with acetic anhydride (3 mL) in the presence of 1-methylimidazole (450 μL) during 30 min at 30 °C. Alditol acetate derivatives were separated with dichloromethane and analyzed by GC with a FID detector and equipped with a 30 m column DB-225 (J&W Scientific, Folsom, CA, USA) 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 until 220 °C, standing for 7 min, followed by a rate of 20 °C/min 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 (Nunes & Coimbra, 2001).

2.3. Linkage analysis

Linkage analysis was carried out by methylation as described by Ciucanu and Kerek (1984). The sample was dissolved in 1 mL of anhydrous dimethylsulfoxide (DMSO), then powdered NaOH (40 mg) were added and samples were methylated with CH_3I (80 μL) during 20 min. The material was remethylated to ensure complete methylation of the polysaccharides (Nunes & Coimbra, 2001). The methylated material was hydrolyzed with TFA 2 M at 121 °C for 1 h, and then reduced and acetylated as previously described for neutral sugar analysis. The partially methylated alditol acetates (PMAA) were separated and analyzed by gas chromatography–mass spectrometry (GC–MS) on an Agilent Technologies 6890N Network. The GC was equipped with a DB-1 (J&W Scientific, Folsom, CA, USA) capillary column (30 m length, 0.25 mm of internal diameter and 0.15 μm of film thickness). The samples were injected in splitless mode (time of splitless 5 min), with the injector operating at 220 °C, and using the following temperature program: 45 °C for 5 min with a linear increase of 10 °C/min up 140 °C, and standing for 5 min at this temperature, followed by linear increase of 0.5 °C/min up to 170 °C, and standing for 1 min at this temperature, followed by linear increase of 15 °C/min up to 280 °C, with further 5 min at 280 °C. The helium carrier gas had a flow rate of 1.7 mL/min and a column head pressure of 2.8 psi. The GC was connected to an Agilent 5973 mass quadrupole selective detector operating with an electron impact mode at 70 eV and scanning the range m/z 40–500 in a 1 s cycle in a full scan mode acquisition.

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, in accordance to the method developed by Nunes, Rocha, and Coimbra (2006). The samples (2–3 mg) were dispersed in water (2.4 mL) in vials with 1 mL capacity and sonicated for 10 min in a water bath at room temperature. The saponification of the polysaccharides occurred by the addition of 0.8 mL of 2 M NaOH, with a reaction time of 1 h at 25 °C. The reaction was finished by the addition of 0.8 mL of 2 M HCl, and the pH was adjusted to 2.0. The vials (10 mL) containing 4.0 mL of sample suspension (sample dispersed in 2.4 mL of water, 0.8 mL of 2 M NaOH and 0.8 mL of 2 M HCl) or standard solutions were thermostatized at 40 °C in a water bath, with continuous stirring. After 15 min, the SPME fiber coated with 50/30 μm divinylbenzene/carboxen on polydimethylsiloxane

(DVB/carboxen/PDM) was manually inserted through the Teflon septum into the headspace of the vial and exposed at 40 °C during 30 min. The SPME coating fiber containing the headspace volatile compounds was introduced into the GC injection port at 250 °C and kept for 10 min for the desorption. A Hewlett-Packard 5890 series II gas chromatograph (Hewlett-Packard, Wilmington, USA), equipped with a split/splitless injector and a flame ionization detector (FID) was used.

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

Samples (15 mg) were hydrolyzed with 1 U of a pure *endo*-β-(1→4)-D-mannanase preparation (Megazyme, EC 3.2.1.78) during 48 h at 37 °C with continuous stirring 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 (glucose), using a flow of 0.3 mL/min. Fractions (1 mL) were collected and assayed for sugars with evaporative light scattering detection. Fractions containing oligosaccharides were pooled and evaporated until all the eluent was removed. No lyophilization was performed since it has been shown that lyophilization promotes O-acetyl migration on galactomannans (Nunes et al., 2005).

2.6. Electrospray ionization mass spectrometry

The fractions obtained after the Biogel P2 column were dissolved in water and further diluted in methanol/water/formic acid (50:49.9:0.1, v/v/v). Positive ion ESI-MS and MS/MS spectra were acquired using a LXQ linear ion trap mass spectrometer (Finningan). Typical ESI conditions were as follows: nitrogen sheath gas 30 psi, spray voltage 5 kV, heated capillary temperature 275 °C, capillary voltage 1 V, and tube lens voltage 40 V. The flow rate was set to 8 μL/min and the voltage applied was 5 kV. Nitrogen was used as nebulizing and drying gas. Full scan mass spectra ranging from *m*/*z* 100 to 1500 were acquired in the positive mode. In the MS^2 experiments, collision energy varied between 15 and 25 of normalized collision. Data acquisition was carried out with Xcalibur data system.

2.7. Matrix-assisted laser desorption/ionization mass spectrometry

Sample preparation for MALDI analysis was performed by mixing 5 μL of sample dissolved in water to 20 μL of 2,5-dihydroxybenzoic acid (DHB) dissolved in a solvent mixture composed by acetonitrile:aqueous TFA (0.1%, v/v) (70:30, v/v). From this mixture, 0.3 μL were deposited on top of a layer of crystals of 2-cloromercaptobenzothiazole (CMBT) formed by deposition of 0.5 μL of CMBT solution in tetrahydrofuran:methanol:water (1:1:1, v/v/v) on the MALDI plate (Pfenninger, Karas, Finke, Stahl, & Sawatski, 1999). MALDI mass spectra were acquired using a MALDI-TOF/TOF Applied Biosystems 4800 Proteomics Analyser (Applied Biosystems, Framingham, MA) instrument equipped with a nitrogen laser emitting at 337 nm and operating in a reflectron mode. Full scan mass spectra ranging from *m*/*z* 500 to 4000 were acquired in the positive mode.

Table 1
Sugar and glycosidic-linkage composition of AV.

Linkage	%Area
T-Ara*f*	0.4
T-Ara*p*	1.4
Total Ara	1.8[a] (3)[b]
T-Man*p*	1.5
4-Man*p*	32.7
4,6-Man*p*	3.1
Total Man	37.3 (44)
T-Gal*p*	1.2 (6)
T-Glc*p*	3.5
4-Glc*p*	54.3
Total Glc	57.8 (47)

[a] Total molar percentage obtained by methylation analysis.
[b] Values in brackets are the molar percentage obtained by sugar analysis.

3. Results and discussion

3.1. Chemical characterization of Aloe vera high molecular weight material (AV)

The sugar composition of the high molecular weight material recovered by dialysis from the commercial Aloe mucilaginous polysaccharides (AV) is shown in Table 1. The amount of carbohydrate material was estimated to be 33.5% by sugar analysis, and was mainly composed by glucose (47%) and mannose (44%). According to glycosidic linkage composition (Table 1), this glucose occurs mainly as (1→4)-linked residues, with 6% of terminally linked glucose residues in relation to total glucose. As this polysaccharide did not colored blue in the iodine test, it could be inferred that its origin should be an excipient with a cellulosic origin. AV mannose residues were mainly (1→4)-linked (32.7% of total glycosidic linkages observed), followed by (1→4,6)- (2.9%), and terminally linked mannose residues (1.5%). Galactose only occurs as terminally linked residues (1.2%). This glycosidic composition confirms the presence of a galactomannan.

Table 2 compares the glycosidic linkage composition of galactomannans from AV and coffee, presenting immunostimulatory activity, and also of locust bean gum (LBG) galactomannan, that do not present this activity, according to already published data (Simões et al., 2009). The ratio of total Man/(1→4,6)-Man was

Table 2
Comparison of galactomannan characteristics of *Aloe vera*, chemically acetylated coffee residue, coffee infusion, and locust bean gum.

Galactomannan features	AV	Chemically acetylated coffee residue[a]	Coffee infusion[b]	LBG[c]
Total Man*p*/4,6-Man*p*	13	13	28	4
Total Man*p*/T-Man*p*	26	23	20	68
Acetylation (mol acetic acid/mol sugar)	2.08	0.98	0.08	v[d]
Ara side chains	Yes	Yes	Yes	Yes
Acetylated Man backbone	Yes	Yes	Yes	Yes
Acetylated side chains	Yes	Yes	No	No

[a] Values from Simões et al. (2010).
[b] Values from Nunes et al. (2005).
[c] Values from Simões et al. (2011).
[d] Vestigial amounts.

13, which showed an average of 1 branched Man residue in each 13 Man residues (Table 2). This degree of branching was similar to that observed for the chemically acetylated coffee residue galactomannans (Simões et al., 2010), but was lower than that observed for coffee infusion galactomannans, which presented a total Man/(1→4,6)-Man ratio of 28 (Nunes et al., 2005). The AV polysaccharides were, however, much less branched than LBG galactomannan that presented a total Man/(1→4,6)-Man ratio of 4 (Simões et al., 2011). The ratio of total Man/T-Man provides information concerning the length of the polymers, although, as observed by Nunes and Coimbra (2001) and Simões et al. (2010), a possible degradation of the polysaccharides during the methylation procedure could result in underestimation of the degree of polymerization of these polysaccharides (Nunes & Coimbra, 2001). According to Table 2, the ratio of total Man/T-Man of 26 for AV was comparable to those observed for coffee infusion and chemically acetylated coffee residue galactomannans and was much smaller than the 68 observed for LBG.

An average of 2.08 acetyl groups was observed per sugar residue (Table 2). This value is higher than that observed for *Aloe vera* acemannans by Fogleman et al. (1992), and Hamman (2008), that determined approximately 1 acetyl group per mannose residue. The value observed of 2.08 acetyl groups is also higher than that observed for the mannans of coffee infusion (0.08, Simões et al., 2009) and LBG (below the limit of detection of the method used, Simões et al., 2011). Also, this acetylation content is higher than that observed in the chemically acetylated coffee mannan (0.84–0.94, Simões et al., 2009).

As observed for coffee and LBG mannans, according to Table 2, AV mannan is also composed arabinose by terminally linked arabinose residues (2%). However, in AV mannan, the arabinose residues occur mainly in the pyranose form (78%) instead of the furanose form found to occur in coffee and LBG.

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

In order to obtain exclusively mannan oligosaccharides that contain the structural details and retaining the sugar and acetyl substituents of the mannan backbone for further MS studies, the hydrolysis with the *Aspergillus niger endo*-β-(1→4)-D-mannanase was performed. This selective hydrolysis was used for the study of mannans from naturally acetylated mannans present in coffee infusion (Nunes et al., 2005), from chemically acetylated mannans from coffee residue (Simões et al., 2010), and from several galactomannans (Cerqueira et al., 2011; Simões et al., 2011). According to the known enzymatic mechanism of *Aspergillus niger endo*-β-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). This selective hydrolysis gives oligosaccharides with low molecular weight allowing their separation by size-exclusion chromatography on Biogel-P2 (Fig. 1) in fractions ready for MS analysis. This procedure also allowed discarding the contaminant glucan observed to be present in this preparation.

According to Fig. 1, the enzymatic cleavage of AV led to the formation of OS with a higher degree of polymerization than tetrasaccharides (DP4). This contrasted with the chromatographic profile observed for coffee infusion and LBG OS, where the more abundant compounds had a DP of 2 (Simões et al., 2010) and between 2 and 4 (Simões et al., 2011), respectively. However, this profile is similar to that observed for chemically acetylated coffee residue (Simões et al., 2010), except for the fact that the small amount of DP2 or DP3 observed in coffee residue was not observed in AV.

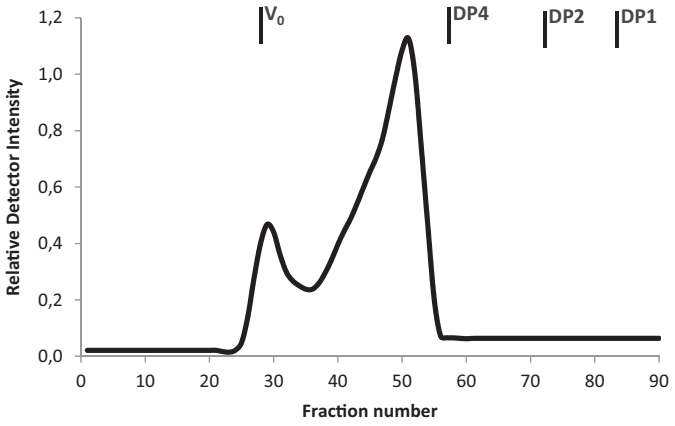

Fig. 1. Size-exclusion chromatography on Bio-Gel P2 of the OS obtained after enzymatic hydrolysis with an *endo*-β-D-mannanase of galactomannan from AV. V_0 – void volume, DP2 and DP3 correspond to the elution volume of DP2 and DP4 standard oligosaccharides, and DP1 corresponds to the elution volume of monomers.

3.3. Characterization of oligosaccharides from AV by ESI and MALDI mass spectrometry

To study the structural features of acemannan, OS obtained after selective hydrolysis with an *endo*-β-D-mannanase treatment and further fractionated by size exclusion chromatography, were analyzed by mass spectrometry (MS). The ions observed in the MS spectra was identified as $[M+Na]^+$ ions. Because Man, Gal, and Glc cannot be distinguished due to their isomeric nature, these hexoses are represented generically by "Hex". "Pent" represents a pentose, probably Ara, as this was the only pentose detected by sugar and methylation analysis, and "Ac" represents the acetyl groups. Fig. 2a shows the ESI-MS spectrum of the ions in the range of *m*/*z* 680 and 865, where it is possible to observe a series of $[M+Na]^+$ ions of OS composed by 4 hexose residues with acetyl groups ranging from 0 to 4 ($Ac_{0-4}Hex_4$). Also, in the range of *m*/*z* 800 and 1090, it is possible to observe a series of $[M+Na]^+$ ions of OS composed by 5 hexose residues with acetyl groups ranging from 0 to 5 ($Ac_{0-5}Hex_5$, Fig. 2b). The AV OS identified using electrospray ionization (ESI) are summarized in Table 3 where it is possible to observe acetylated OS in the range $Ac_{0-8}Hex_{3-6}$, including OS presenting a ratio of acetyl groups higher than one, as for example, Ac_6Hex_3, Ac_7Hex_4, and Ac_8Hex_5. This acetylation pattern shows much more acetyl groups than those observed in coffee (Nunes et al., 2005; Simões et al., 2010) and LBG mannan OS (Simões et al., 2011). The sample that seems to approach a similar acetylation pattern is the chemically acetylated mannans from coffee residue, where it was possible to observe an $Ac_{0-3}Hex_4$ series (Simões et al., 2010).

Fig. 2c shows the occurrence, although with a small relative abundance, of $[M+Na]^+$ ions corresponding to acetylated OS with hexoses and a pentose residue, namely, $Ac_{1-4}PentHex_{1-4}$. Table 3 shows that it is possible of find highly acetylated OS containing a pentose, namely, $Ac_5PentHex_2$ and $Ac_6PentHex_3$. The occurrence of mannan OS containing arabinose has also been observed in coffee and LBG, although in these samples it was possible to detect non-acetylated structures, which were not found in these AV samples.

To evaluate the presence of OS with higher DP than those observed by ESI-MS, a MALDI-MS analysis was performed (Fig. 3 and Table 4). MALDI-MS analysis allowed to identify the $[M+Na]^+$ ions in the range of $Ac_{0-18}Pent_{0-2}Hex_{4-14}$, which included the highly acetylated OS $Ac_{15}Hex_9$, $Ac_{16}Hex_{10}$, $Ac_{15}Hex_{11}$, and $Ac_{18}Hex_{12}$. Also, MALDI-MS allowed to identify highly acetylated OS containing pentoses, as for example, $Ac_8PentHex_{11}$, and $Ac_{11}Pent_2Hex_9$. This observation confirms the high degree of acetylation even in OS of higher DP.

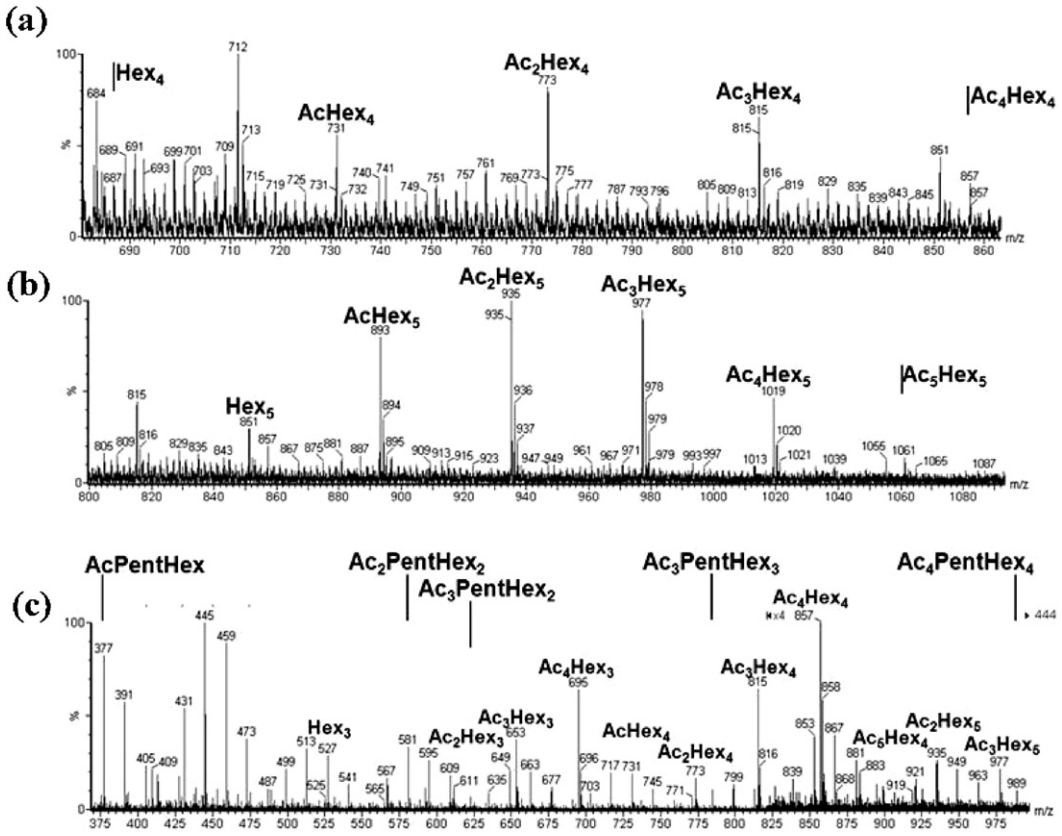

Fig. 2. ESI-MS spectra of AV OS (a) fraction nr. 41: *m*/*z* 680–865, (b) fraction nr. 41: *m*/*z* 800–1090 and (c) fraction nr. 47: *m*/*z* 350–1500.

3.4. Identification of structural features in OS by tandem mass spectrometry

In order to confirm the structural features of the OS seen in the MS spectra and the presence of pentose residues and acetyl groups as structural features of this mannan, all the OS were also studied by ESI-MS/MS. With this tool, it is possible to observe product ions formed by glycosidic cleavages allowing to confirm the presence of sugar residues. Also, the product ions formed by cross-ring cleavages can give information about the type of linkages (cleavage of two bonds within the sugar ring), and presence of substituting groups (Simões et al., 2010, 2011). To demonstrate this outcome, it will be described in detailed the product ion spectra (ESI-MS/MS) obtained for the ions $[M+Na]^+$ of an highly acetylated hexose OS ($[Ac_7Hex_4+Na]^+$ (*m*/*z* 983), and a highly acetylated OS with a pentosyl residue $[Ac_5PentHex_3+Na]^+$ (*m*/*z* 869).

The typical fragmentations observed in the MS/MS spectra of the $[M+Na]^+$ ions of β-(1→4)-mannosyl oligosaccharides under ESI-MS/MS conditions are the loss of a hexose residue (Hex_{res}, −162 Da) due to glycosidic bond cleavage, the loss of water (−18 Da), and the formation of fragment ions resultant from cross-ring cleavages, $^{0,2}A_3$, with loss of 60 Da (Simões et al., 2010, 2011).

3.4.1. MS/MS of Ac_7Hex_4

The ESI-MS/MS spectrum of $[Ac_7Hex_4+Na]^+$ (*m*/*z* 983) ions (Fig. 4) showed the presence of ions at *m*/*z* 965 ($[Ac_7Hex_3+Na]^+$), formed due to glycosidic bond cleavage with loss of one Hex_{res} (−162 Da). The product ions at *m*/*z* 965, due to loss of water (−18 Da), and at *m*/*z* 923, resulting from cross-ring cleavage (−60 Da) were also observed, in accordance to the characteristic fragmentation of β-(1→4)-linked hexose oligosaccharides. This ion at *m*/*z* 923 could be resulting also due to loss of an acetyl group (CH_3COOH) (Reis et al., 2005; Simões et al., 2010). This spectrum shows also the product ions at *m*/*z* 761, 719, and 677, due to loss of AcHex, Ac_2Hex, Ac_3Hex, respectively, and also the product ions at *m*/*z* 557, 515, 473, and 431, resultant from loss of $Ac_{2-5}Hex_2$. These fragmentation pathways confirm the presence of acetyl groups linked to Hex in the OS structures, reaching

Table 3
Oligosaccharide $[M+Na]^+$ ions from AV observed in the ESI-MS, with the identification of the *m*/*z* value and the most probable composition.

	n	Number of acetyl groups								
		0	1	2	3	4	5	6	7	8
		m/*z*								
Hex_n	3	527	569	611	653	695	737	779		
	4	689	731	773	815	857	899	941	983	
	5	851	893	935	977	1019	1061	1103	1145	1187
	6	1013	1055	1097	1139	1181				
$PentHex_n$	2			581	623		707			
	3		701		785		869	911		
	4				947	989				

Table 4
Oligosaccharide $[M+Na]^+$ ions observed in the MALDI-MS spectra of the several OS fraction obtained from AV with the identification of the *m*/*z* value and the most probable composition.

$Pent_mHex_n$		Number of acetyl groups																	
m	*n*	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
		m/*z*																		
0	4				815	857														
0	5	851	893	935	977		1061													
0	6	1013	1055	1097		1181														
1	6									1481										
0	7	1175	1217	1259				1427	1469	1511	1553									
1	7									1643										
2	7												1901							
0	8							1589	1631	1673	1715	1757								
1	8									1805										
2	8											2021	2063	2105						
0	9										1877	1919	1961				2129			
1	9									1967										
2	9												2225							
0	10										2039	2081	2123			2249		2495		
1	10									2129										
0	11										2201	2243	2285	2327	2369	2411	2453			
1	11									2291										
0	12											2405	2447	2489	2531	2573	2615	2657	2699	2741
0	13												2609	2651	2693	2735	2777	2819	2861	2903
0	14															2897	2939	2981	3023	3065

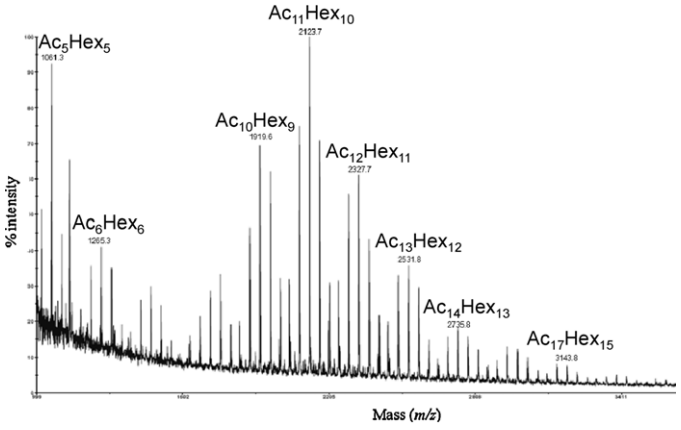

Fig. 3. MALDI-MS spectrum of fraction nr. 31 of AV (*m*/*z* 1000–3600).

the number of three acetyl groups per hexose. Assuming the β-(1→4)-linkage, the occurrence of Ac_3Hex and Ac_5Hex_2 shows that all hexose carbons, C2, C3, and C6, should be acetylated (Manna & McAnalley, 1993; Talmadge et al., 2004).

The loss of 162 Da allows to infer that this OS does not contain any acetyl group at the non-reducing end residue. This confirms that this OS is resultant from the *endo*-β-(1→4)-D-mannanase hydrolysis, as for the enzymatic hydrolysis to occur a non-substituted mannose residue should be present forming the glycosidic linkage to be cleaved, thus leading to the formation of OS with a non-substituted mannose residue at the new formed non-reducing end.

3.4.2. MS/MS of $Ac_5PentHex_3$

The ESI-MS/MS spectrum of the $[Ac_5PentHex_3+Na]^+$ (*m*/*z* 869) ion (Fig. 5) shows the product ions at *m*/*z* 719 due to loss of Pent (−150 Da), correspondent to $[Ac_5Hex_{3res}+Na]^+$, confirming the presence of a pentosyl group in this OS. It was also observed the characteristic loss of a hexose residue (−Hex_{res}; −162 Da), corresponding to the ion observed at *m*/*z* 707, $[Ac_5PentHex_2+Na]^+$. The ions at *m*/*z* 695 and 653 correspond to $[Ac_4Hex_3+Na]^+$ and $[Ac_3Hex_3+Na]^+$, respectively, formed due to the loss of $Ac_{1-2}Pent_{res}$. The ions at *m*/*z* 677, 635, and 593 correspond to the ions $[Ac_4Hex_{3res}+Na]^+$, $[Ac_3Hex_{3res}+Na]^+$, and $[Ac_2Hex_{3res}+Na]^+$, respectively, formed due to loss of $Ac_{1-3}Pent$, showing that pentosyl groups of the mannan understudy were also acetylated. The loss of Ac_3Pent shows that all pentose carbons are acetylated. This is a surprising result, not expect to be obtained in acemannan according to the available literature (Talmadge et al., 2004). However, a similar pattern has been observed when the mannans from coffee residue were chemically acetylated, showing the occurrence of a higher extent of acetylation than that observed in the natural polymer, as well as acetylated pentoses (Simões et al., 2010). Also, the absence in all spectra of the loss of 102 Da, attributed to a combined loss of cross ring fragmentation $^{0,2}A_3$ with an acetyl group linked, is comparable with the product obtained by the chemical

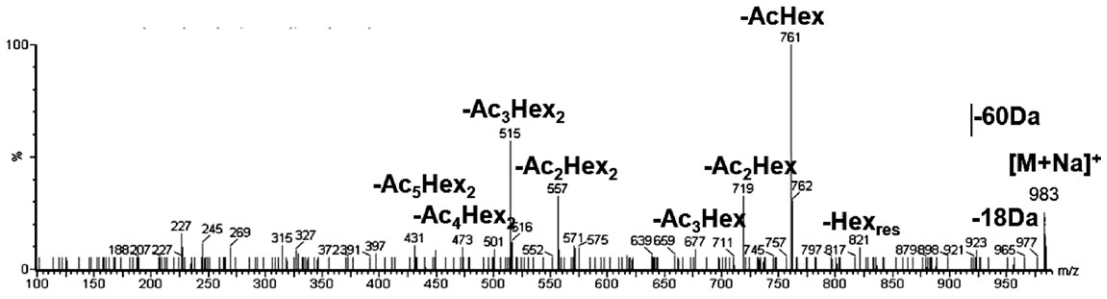

Fig. 4. ESI-MS/MS spectrum of the $[M+Na]^+$ ion of Ac_7Hex_4 (*m*/*z* 983) obtained from AV.

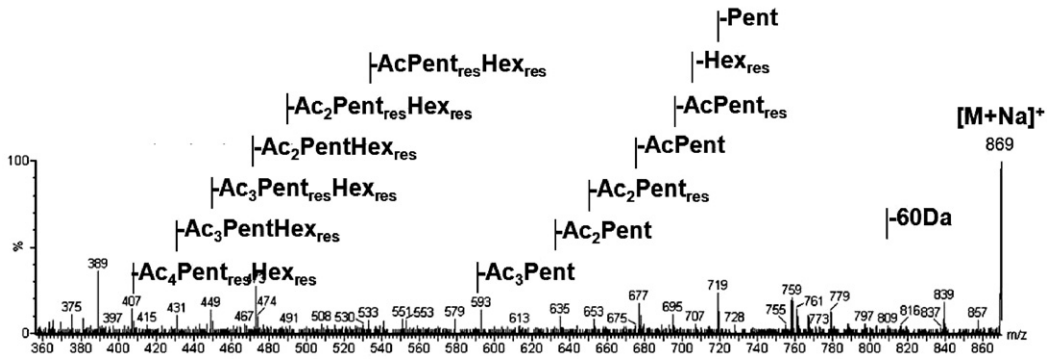

Fig. 5. ESI-MS/MS spectrum of the $[M+Na]^+$ ion of $Ac_5PentHex_3$ (m/z 869) obtained from AV.

acetylation of coffee residue mannan. However, the OS from coffee infusion mannans showed this loss of 102 Da, confirming the location of the acetyl group at the C2 of the hexose of the naturally acetylated product (Simões et al., 2010).

4. Conclusions

This is the first report on mass spectrometry of *Aloe vera* acemannan structural features, allowing the estimation of the acetylation profile of a commercially bioactive acemannan and to observe for the first time the presence of arabinose residues in this structure. Analysis of electrospray mass spectra and tandem mass spectra of oligosaccharides obtained from this bioactive *Aloe vera* acemannan, which was used as positive control for the *in vitro* evaluation of the immunostimulatory activity of mannans, allowed to conclude that it was highly acetylated. This mannan contained, on average, 2 acetyl groups per sugar unit, which is the double of that reported in bibliography for this polysaccharide. Also, it is more acetylated than the coffee mannans that showed similar immunostimulatory activity. Furthermore, in this polysaccharide, acetyl groups were non-homogeneously distributed, as it was possible to observe oligosaccharides with three acetyl groups per hexose unit, as well as non-acetylated hexose OS presenting DPs that varied between 3 and 7. In addition, pentosyl side chains were also identified as well as acetylated pentosyl residues. These pentosyl side chains can be attributed to arabinose residues, as identified in sugar and methylation analyses. The results obtained and the comparison with other galactomannans allow to infer that the lower branching, shorter chains, and higher acetylation seems to promote the immunostimulatory activity of these polysaccharides.

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