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Structural analysis of gellans produced by *Sphingomonas elodea* strains by electrospray tandem mass spectrometry

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ABSTRACT

A commercial gellan sample (Gelrite) and a gellan-like polymer (JB3) obtained by exposure of the producing strain to chemical mutagenesis were subjected to partial acid hydrolysis and the resultant oligosaccharides were identified by Electrospray Mass Spectrometry (ESI-MS) and Tandem Mass Spectrometry (ESI-MS/MS and MS^n). In both gellans, the main fragments were in accordance with the tetrasaccharide repeating unit, D-Glucose (Glc)-D-Glucuronic acid (GlcA)-D-Glucose (Glc)-L-Rhamose (Rha), described for the wild-type gellan, showing the higher acid lability of the Rha-(1 → 3)-Glc linkage when compared to the (1 → 4) of the other residues. Under the experimental conditions used in the study, as expected, no acyl substituents were observed in the commercial gellan but in JB3 oligosaccharides a glyceryl moiety was identified, substituted in the 3-linked Glc residue. Furthermore, the analysis of the MS/MS and MS^n spectra of both gellans allowed the identification of structural details, some of them not yet reported for these exopolysaccharides. The presence of oligosaccharides with single Glc and Rha residues substituent of the tetrasaccharide unit of gellan may represent novel side chains of the backbone unit that to our knowledge have never been reported previously for the gellan exopolysaccharide.

1. Introduction

Bacterial exopolysaccharides (EPS) are a diverse and versatile class of materials that have been described to have new and promising applications in the Biotechnology field (Crescenzi, 1995). Gellan, a commercial EPS member of the designated sphingan family, is an example of a multifunctional gelling agent, forming highly viscous aqueous solutions and thermo-reversible gels (Dlamini & Peiris, 1997), with a wide range of applications, particularly in the food, pharmaceutical and biomedical fields (Chandrasekaran & Radha, 1995; Fialho et al., 2008). Gellan is produced in high yield by the non-pathogenic bacterium *Sphingomonas elodea* ATCC 31461 (Mazen, Milas, & Rinaud, 1999), originally designated by *Pseudomonas elodea* and latter proposed to be a member of *Sphingomonas* genus by Pollock (1993). Gellan is a linear, anionic polysaccharide based on a tetrasaccharide repeating unit (Jannson, Lindeberg, & Sandford, 1983; Jay et al., 1998) composed by the backbone [→3)-β-D-Glc*p*-(1 → 4)-β-D-Glc*p*A-(1 → 4)-β-D-Glc*p*-(1 → 4)-α-L-Rha*p*-(1→] (Kuo, Mort, & Dell, 1986; Sá-Correia et al., 2002), as shown in Fig. 1. In this native form, the backbone can be partially esterified by acyl substituents; the (1 → 3)-D-Glc residue can have attached L-glycerate (OGl) at C-2 and acetate (OAc) at C-6, in a proportion of 1 mol of glycerate and 0.5 mol of acetate per repeating unit (Fialho et al., 1999, 2008; Kuo et al., 1986; Sá-Correia et al., 2002). Gellan has an average molecular mass of about 500 kDa and has been shown to adopt a double-helical structure in solution (Bemiller, 1996).

The potential for using gellan or gellan-like gums in industrial applications is determined by their physical properties (Sá-Correia et al., 2002). The side chains determine the rheological behaviours of the polysaccharide other than viscosity (Hashimoto et al., 1997). As demonstrated by Jay et al. (1998), the glycerate groups are responsible for significant changes in the rheological properties. So, the deacylation of the native gellan results in a change from soft, elastic thermo-reversible gels to harder, more brittle gels (Jay et al., 1998).

Gellan biosynthesis starts with the intracellular formation of the nucleotide-sugar precursors. Gellan repeat unit is formed by sequential transfer of the sugars to an activated lipid carrier, by committed glycosyltransferases (GT), followed by gellan polymerization and export. The gellan clusters includes genes coding for enzymes involved in dTDP-L-rhamnose-synthesis, GTs and proteins required for gellan polymerization and export (Fialho et al., 2008). In order to obtain gellan-like polymers with different properties than the gellan wild-type, the producing strains can be submitted to genetic engineering procedures or exposed to chemical muta-

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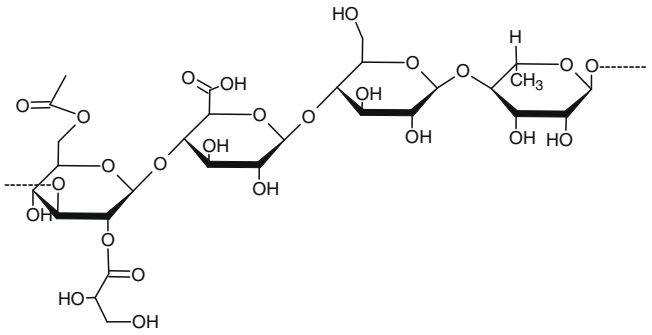

Fig. 1. Tetrasaccharide repeating unit characteristic of gellan exopolysaccharide produced by *Sphingomonas elodea* ATCC 31461.

genesis and/or to environmental stress (Jay et al., 1998; Richau, Choquenet, Fialho, & Sá-Correia, 1997).

Mass spectrometry (MS) has been used in the recent years for the structural characterisation of carbohydrates, allowing the identification of detailed structural features, namely sugars composition, sequence, and ramifications (Zaia, 2004). Structural information is obtained by the analysis of tandem mass spectra (MS/MS and MS^n) of ions formed by electrospray ionisation (ESI) and matrix assisted laser desorption ionisation (MALDI) (Harvey, 2006; Reis et al., 2005; Zaia, 2004). The analysis of polysaccharides in their native form is difficult due to their high molecular weight. In order to overcome this, polysaccharides are usually cleaved into oligosaccharides or polymers with lower degree of polymerization (DP). These can be achieved by different methodologies, namely enzymatic hydrolysis or partial acid hydrolysis. Both approaches have been used in order to obtain oligosaccharides suitable for structural characterisation using mass spectrometry (Liu, Jiang, Cui, & Guan, 2000; Reis, Coimbra, Domingues, Ferrer-Correia, & Domingues, 2002; Serrato et al., 2008; Reis et al., 2005), even when present in mixtures and with low abundance, without any manipulation/derivatisation being required (Zaia, 2004). Oligosaccharides under ESI conditions ionised preferentially as sodium adducts, $[M + Na]^+$, even without addition of any sodium salt (Cech & Enke, 2001; Reis et al., 2002).

In this work, a commercial (Gelrite) and a gellan-like polymer (JB3) were subjected to partial acid hydrolysis and the oligosaccharides obtained were partially separated by size exclusion chromatography and Electrospray Ionisation Mass Spectrometry (ESI-MS, ESI-MS/MS and $ESI-MS^n$) was carried out to obtain a closer look into their structural features.

2. Material and methods

2.1. Gellan samples

The commercial gellan gum was purchased from Fluka (Sigma-Aldrich). The JB3 gellan polymer was produced as was described previously by Jay et al. (1998). Sugars analysis as alditol acetates (Blakeney, Harris, Henry, & Stone, 1983) and m-phenylphenol colorimetric assay showed that JB3 gellan was composed mainly by rhamnose (26.3 mol %), uronic acid (29.6 mol %) and glucose (44.1 mol %).

2.2. Partial acid hydrolysis and size exclusion chromatography

The gellan samples (commercial and JB3) were treated with trifluoroacetic acid (50 mM) at 100 °C for 45 min. The resultant oligosaccharides were then separated by size exclusion chromatography using a Biogel P2, and water as eluent. Elution profile was monitored using an evaporative light scattering detector (ELSD).

2.3. Methylation analysis and GC–MS

Oligosaccharides, obtained after partial acid hydrolysis of the gellan samples, were treated with powdered NaOH and methylated with CH_3I, as described by Coimbra, Delgadillo, Waldron, and Selvendran (1996). The methylated material the was dissolved in 3 mL of water and extracted with 4 mL of chloroform. The organic layer was washed three times with 3 mL of water and evaporated to dryness. The methylated oligosaccharides were hydrolyzed with 2 M TFA at 121 °C for 1 h, reduced by $NaBD_4$ and acetylated in the presence of acetic anhydride and 1-methylimidazole. The partially methylated alditol acetates (PMAA) were analysed by gas chromatography–mass spectrometry (GC–MS) in an OV-1 capillary column (30 m length, 0.32 mm of internal diameter and 0.25 μm of film thickness). The samples were injected in splitless mode (time of splitless 4 min), with the injector and detector operating at 220 °C and 250 °C, respectively, 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 flow of the carrier gas (H_2) was set at 1.7 mL/min. GC–MS analysis was performed in a HP series 2 gas chromatograph and Trio-1 S VG mass-lab with scans between 499 and 33 m/z/s with a 70 eV ionisation energy.

2.4. Mass spectrometry

Positive-ion ESI-MS and ESI-MS/MS were carried out on a Micromass (Manchester, UK) Q-TOF2 hybrid tandem mass spectrometer. For ESI analysis, gellan oligosaccharides fractions were diluted in methanol/water/formic acid (50:50:0.1, v/v/v). Samples were introduced at a flow rate of 10 μL/min into the ESI source. In the MS and MS/MS experiments, the time-of-flight (TOF) mass resolution was set to approximately 9000. The cone voltage was 35 V, and the capillary voltage was 3 kV. The source temperature was 80 °C and the desolvation temperature was 150 °C. MS/MS spectra were obtained using argon as the collision gas with the collision energy set between 20 and 45 eV. The data was processed using MassLynx software (version 4.0).

A LXQ linear ion trap mass spectrometer (Finningan) was used for sequence analysis. The heated capillary was kept at 350 °C. In each experiment, the ion transmission parameters were optimised automatically in order to improve the detection of the analytes of interest. Gellan fractions were diluted in methanol/water/formic acid (50:50:0.1, v/v/v). The flow rate was set to 8 μL /min and the voltage applied was 5.5 kV. Nitrogen was used as nebulising and drying gas. Full scan mass spectra ranging from m/z 100 to 1500 were acquired in the positive mode. In the MS^n experiments, collision energy varied between 15 and 25 of normalised collision energy.

3. Results and discussion

The commercial and the JB3 gellan samples were subjected to partial acid hydrolysis and the resultant oligosaccharides were fractionated by size exclusion chromatography. The ESI-MS spectra obtained for the main fractions are shown in Fig. 2 and all oligosaccharides identified in these and other fractions are summarized in Table 1, with the indication of the m/z value of the $[M + Na]^+$ ion.

3.1. Characterisation of tetrasaccharide units

In the ESI-MS spectra of the main fraction of commercial gellan (Fig. 2a) it is possible to observe that the major ion occurs at m/z

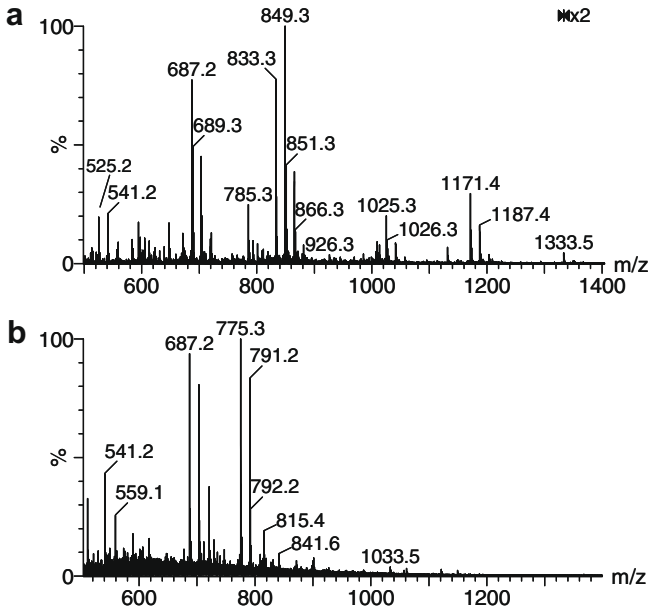

Fig. 2. ESI-MS spectra of the: (a) Commercial gellan and (b) JB3 gellan.

Table 1
Oligosaccharides identified in the ESI-MS spectra from commercial gellan and from JB3 gellan, with the indication of the *m*/*z* value of the $[M+Na]^+$.

Commercial	JB3	Oligosaccharide composition
525	–	$[HexA{\cdot}Hex{\cdot}dHex + Na]^+$
541	541	$[HexA{\cdot}Hex_2 + Na]^+$
–	629	$[HexOGl{\cdot}HexA{\cdot}Hex + Na]^+$
687	687	$[HexA{\cdot}Hex_2{\cdot}dHex + Na]^+$
–	775	$[HexOGl{\cdot}HexA{\cdot}Hex{\cdot}dHex + Na]^+$
833	833	$[HexA{\cdot}Hex_2{\cdot}dHex_2 + Na]^+$
849	849	$[HexA{\cdot}Hex_3{\cdot}dHex + Na]^+$
–	921	$[HexOGl{\cdot}HexA{\cdot}Hex{\cdot}dHex_2 + Na]^+$
–	937	$[HexOGl{\cdot}HexA{\cdot}Hex_2{\cdot}dHex + Na]^+$
1171	–	$[HexA_2{\cdot}Hex_3{\cdot}dHex_2 + Na]^+$
1333	–	$[HexA_2{\cdot}Hex_4{\cdot}dHex_2 + Na]^+$

687. Attending to sugars analysis and considering that all of the identified ions in both ESI-MS spectra were attributed to the respective sodium adducts, this ion can be attributed to a tetrasaccharide composed by two hexoses, one deoxyhexose, and one uronic acid. In order to confirm the sugars composition and sequence of the ESI-MS identifications, the product ion spectrum of the ion at *m*/*z* 687 was obtained on a Q-TOF2 instrument (Fig. 3a). The occurrence of the ion at *m*/*z* 525, due to the loss of a hexose residue (Hex_{res}, −162 Da) instead of a hexose (Hex, −180 Da), can be attributed to a loss from the non-reducing end of the polysaccharide (Reinhold, Reinhold, & Chan, 1996; Reis, Coimbra, Domingues, Ferrer-Correia, & Domingues, 2004). This loss was followed by loss of a uronic acid residue ($HexA_{res}$, −176 Da), to give the ion at *m*/*z* 349, and a further loss of another Hex_{res}, with formation of the ion at *m*/*z* 187, corresponding to a deoxyhexose (dHex) that, according to Reinhold et al. (1996) and Reis et al. (2004), should be at the reducing end of the oligosaccharide. This fragmentation pathway confirmed the structure previously described for the tetrasaccharidic repeating unit, Glc-GlcA-Glc-Rha (Kuo et al., 1986). Attending to the linear ion trap mass spectrometer potentialities, which comes from the ion trap capacity to perform multiple stages of mass spectrometry (MS^n) (Fernández, 2007), this monosaccharide sequence was confirmed by an ESI-MS^n experiment carried out for the ion at *m*/*z* 687 (Fig. 3b).

For the JB3 gellan, together with the ion at *m*/*z* 687, another ion with similar relative abundance, at *m*/*z* 775, was also present in the ESI-MS spectrum (Fig. 2b). The ESI-MS/MS and MS^n spectra obtained for the ion of *m*/*z* 687, observed in the ESI-MS spectrum of JB3 gellan presented the same fragmentation pathway as reported above (data not shown) indicating that it corresponded to the same tetrasaccharidic repeating unit composed by Glc-GlcA-Glc-Rha. The ion at *m*/*z* 775 was identified as a tetrasaccharide composed by two hexoses, one deoxyhexose, one uronic acid and a glyceryl group. In the product ion spectrum, obtained in a Q-TOF2 instrument, (Fig. 3c), it is possible to observe an initial loss of a neutral residue with 250 Da. This elimination was attributed to the loss of an OGl substituent linked to a Hex_{res} ($HexOGl_{res}$). It was also observed further loss of $HexA_{res}$ (−176 Da) with formation of the ion of *m*/*z* 349, which corresponded to the sodium adduct of one Hex and one dHex from the reducing end, $[Glc - Rha + Na]^+$. Loss of dHex from reducing end (−164 Da, to the ion of *m*/*z* 611) followed by, or combined with loss of Hex_{res} (−162 Da, to the ion of *m*/*z* 449) was also observed. This fragmentation suggests the presence of a tetrasaccharide with a glyceryl group as substituent, with the following structure GlcOGl-GlcA-Glc-Rha. The monosaccharide sequence was confirmed by the analysis of the ESI-MS^n spectra obtained in a linear ion trap mass spectrometer (Fig. 3d). The ion of *m*/*z* 775 corresponds, in fact, to the linear tetrasaccharidic repeating unit, typical of gellan, substituted with a glyceryl group in the (1 → 3)-Glc residue.

By the analysis of the ESI-MS spectra obtained from both gellans, it was possible to observe that, after partial acid hydrolysis, the resultant oligosaccharides are, in a great proportion, constituted by the characteristic tetrasaccharidic repeating unit (with and without glyceryl groups). Contrarily to what was observed for glyceryl groups, no acetyl groups, also linked by ester bonds, were observed. This should be due to the major acid lability of acetyl than glyceryl groups. The repeating unit resultant from partial acid hydrolysis, contained Rha in the reducing end, allowing to infer the acid lability linkage along the gellan backbone is the [-α-L-Rha*p*-(1 → 3)-β-D-Glc*p*], which is in accordance with the higher acid lability of 6-deoxyhexose pyranosyl residues in relation to other residues (Fry, 1988).

3.2. Characterisation of trisaccharide units

Oligosaccharides correspondent to ions with lower *m*/*z* value than the tetrasaccharidic repeating unit were also identified in the ESI-MS spectra of both gellans. The ions of *m*/*z* 525 and 541, identified in the commercial gellan, and the ions of *m*/*z* 541 and 629, identified in the JB3 gellan, can be attributed to the $[M + Na]^+$ ions of oligosaccharides with different combinations of Glc, Rha and GlcA, as result of the random cleavage of glycosidic linkages during partial acid hydrolysis. In order to identify their composition, the product ion spectra (MS/MS and MS^n) of the observed ions were obtained in the two different mass spectrometers, ESI-Q-TOF2 (MS/MS) (Fig. 4) and ESI-Linear ion trap (MS^n) (data not shown). The ion of *m*/*z* 525 fragments by loss of $HexA_{res}$ (−176 Da) with formation of the ion of *m*/*z* 349, which corresponds to $[Glc - Rha + Na]^+$ (Fig. 4a), allows to infer for a GlcA-Glc-Rha structure. Following the same methodology, by analysis of the ESI-MS/MS spectrum, obtained in a Q-TOF2, for the ion of *m*/*z* 541 of commercial gellan (Fig. 4b), it is possible to see that this ion fragments by loss of Hex_{res} (−162 Da), following by loss of 176 Da (−$HexA_{res}$) to the ion of *m*/*z* 203, which corresponds to the sodium adduct of a Hex unit from the reducing end. This allows to infer that the ion of *m*/*z* 541 has a Glc-GlcA-Glc structure. The ESI-MS/MS and MS^n spectra of the ion of *m*/*z* 541, observed in the ESI-MS spectrum of JB3 gellan (data not shown), presented the same fragmentation pathway as reported above indicating that

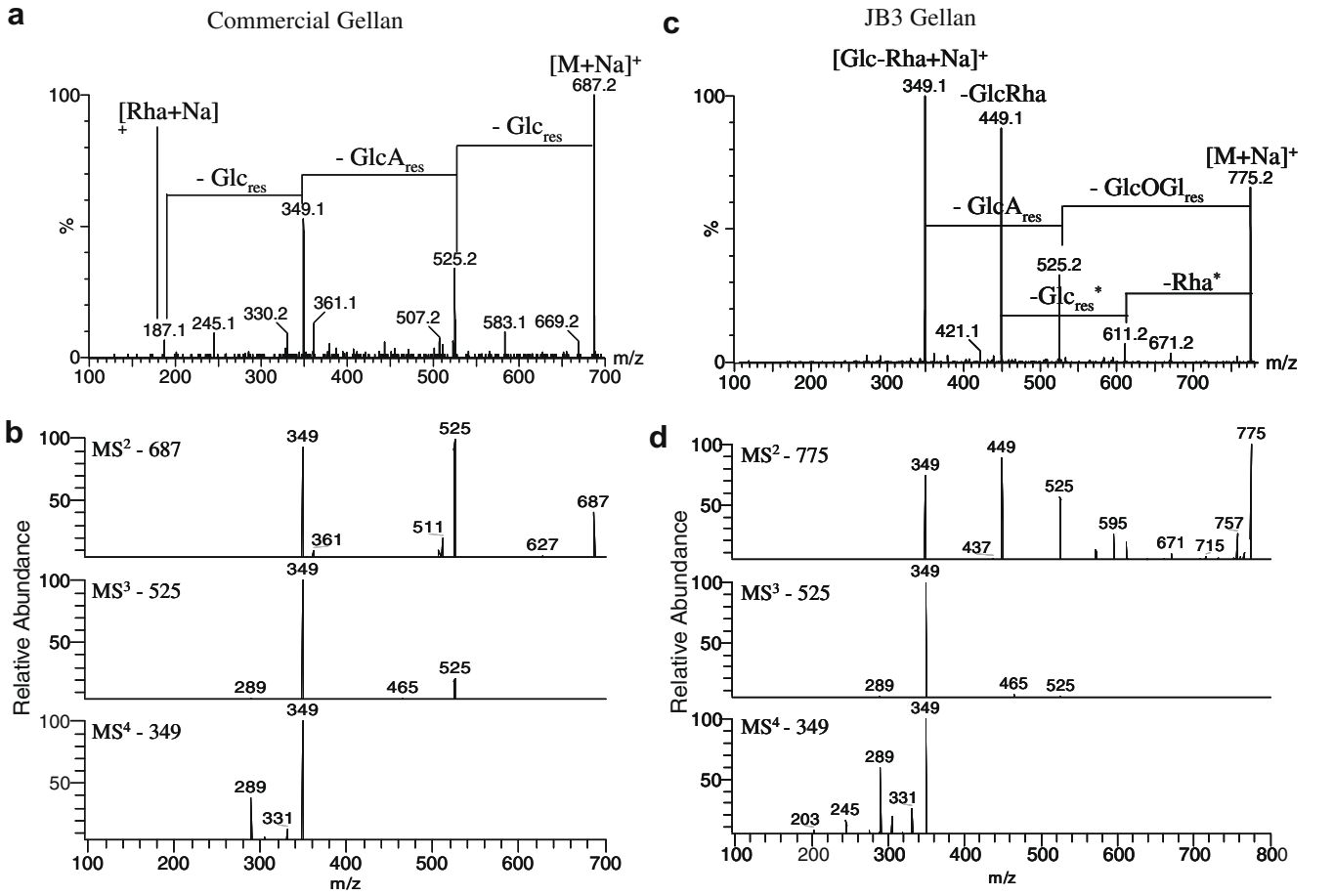

Fig. 3. Product ion spectra of the ion of *m*/*z* 687 from commercial gellan obtained (a) in a Q-TOF2 instrument, (b) in a linear ion trap instrument. Product ion spectra of the ion of *m*/*z* 775 from JB3 gellan obtained, (c) in a Q-TOF2 instrument and (d) in a linear ion trap instrument.

it corresponds to the same trisaccharide unit. From the product ion spectrum of the ion of *m*/*z* 629 (Fig. 4c), it is possible to see that this ion fragments mainly by loss of $HexOGl_{res}$ from the non-reducing end (−250 Da, to the ion of *m*/*z* 379) followed by loss of $HexA_{res}$ (−176 Da) with formation of $[Hex + Na]^+$ of *m*/*z* 203. The presence of a non-substituted Hex on the reducing end is confirmed by the presence of the ion of *m*/*z* 203 and a major loss of 180 Da from the precursor ion (to the ion of *m*/*z* 449), which corresponds to loss of a Hex unit. Thus, the oligosaccharide corresponding to this ion is a trisaccharide with the structure GlcOGl-GlcA-Glc. This structure confirms the occurrence of the OGl group as a substituent of the β-(1 → 3)-Glc residue. The backbone of this trisaccharide corresponds to the one identified for the ion of *m*/*z* 541 but, in this case, with an additional glyceryl group as substituent. The structure of these oligosaccharides, obtained by partial acid hydrolysis, revealed that they have in common a GlcA-Glc disaccharide unit, linked with either additional Rha or Glc residues. This fact suggests that the linkage between GlcA and Glc residues forming an aldobiuronic acid, is resistant to cleavage during partial acid hydrolysis (Fry, 1988).

3.3. Characterisation of pentasaccharide and higher oligosaccharides

Oligosaccharides with higher *m*/*z* values than the tetrasaccharide units were also found in the ESI-MS spectra of both gellan samples, namely the ions of *m*/*z* 833, 849, 1171, and 1333 in commercial gellan and the ions of *m*/*z* 833, 849, and 937 in JB3 gellan. It was found that some of the ions identified were present in both samples, as the ions of *m*/*z* 833 and 849. Ions with the same *m*/*z* value obtained from both samples showed similar product ion spectra, revealing that they have the same structure. The ion of *m*/*z* 833 was identified as a pentasaccharide composed by two Glc, two Rha and one GlcA. In the product ion spectrum obtained in a Q-TOF2 (Fig. 5a), it is shown the loss of a $dHex_{res}$ (−146 Da), followed by loss of a Hex_{res} (−162 Da) and finally loss of a $HexA_{res}$ (−176 Da) with formation of the ion of *m*/*z* 349, which corresponds to the sodium adduct of the disaccharide Glc-Rha in the reducing end. The results allow to infer that this pentasaccharide had the following structure: Rha-Glc-GlcA-Glc-Rha, which corresponds to the gellan gum characteristic tetrasaccharidic repeating unit with an additional Rha in the non-reducing end.

The product ion spectrum obtained in a Q-TOF2 (Fig. 5b) of the ion of *m*/*z* 849, composed by three Glc, one Rha and one GlcA, allowed to infer the following structure: Glc-GlcA-Glc-Rha-Glc. Furthermore, the oligosaccharide of *m*/*z* 937, identified in the ESI-MS spectrum of JB3 gellan, was characterised by tandem mass spectrometry as GlcOGl-GlcA-Glc-Rha-Glc (Fig. 5c), corresponding to the tetrasaccharidic repeating unit plus a Glc linked to Rha and an OGl linked to one Glc residue. This ion corresponds to the one of *m*/*z* 849 with an additional OGl substituent, which was identified by the initial loss of $HexOGl_{res}$ (−250 Da) followed by loss of $HexA_{res}$ (−176 Da) and loss of Hex_{res} (−162 Da), leading to a low abundant ion of *m*/*z* 349, correspondent to $[Hex - dHex + Na]^+$. The structure of these ions at *m*/*z* 849 and 937 showed the presence of a trisaccharide, constituted by Glc-GlcA-Glc, in all of them, with additional residues of Rha and/or Glc. This fact suggests that

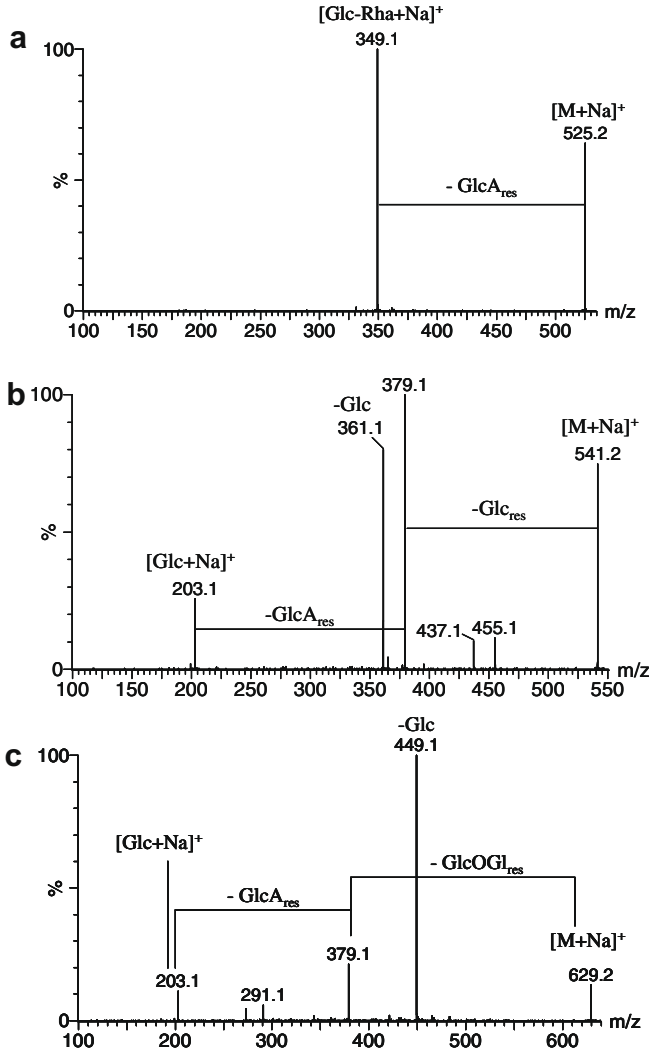

Fig. 4. Product ion spectra, obtained in a Q-TOF2 instrument, for the trisaccharides identified of (a) *m*/*z* 525 (from commercial gellan), (b) *m*/*z* 541 (from commercial gellan) and (c) *m*/*z* 629 (from JB3 gellan).

the linkage between Glc-GlcA, although not so resistant as GlcA-Glc, is not so straightforward to cleave during partial acid hydrolysis.

Following the same methodology, the ions of *m*/*z* 1171 and 1333, identified in commercial gellan (Figs. 5d and e) were characterised, allowing to propose for the ion of *m*/*z* 1171 the following heptasaccharide structure: GlcA-Glc-Rha-Glc-GlcA-Glc-Rha. The ion of *m*/*z* 1333 was characterised as the following octasaccharide structure: Glc-GlcA-Glc-Rha-Glc-GlcA-Glc-Rha, thus composed by two adjacent tetrasaccharide repeating units.

3.4. Characterisation of oligosaccharides not related to the tetrasaccharide repeating structure

All ions yet reported lead us to a typical structure of gellan gum, which correspond to its tetrasaccharidic repeating unit. However, surprisingly, the analysis of the MS/MS and MS^n data obtained for some of the oligosaccharides allows the assignment of fragmentations suggesting the presence of new oligosaccharide configurations that had never been described before for gellan. This is the case of the ions of *m*/*z* 833 and 849 for both gellans and the ions of *m*/*z* 921 and 937 from JB3 gellan (Fig. 5).

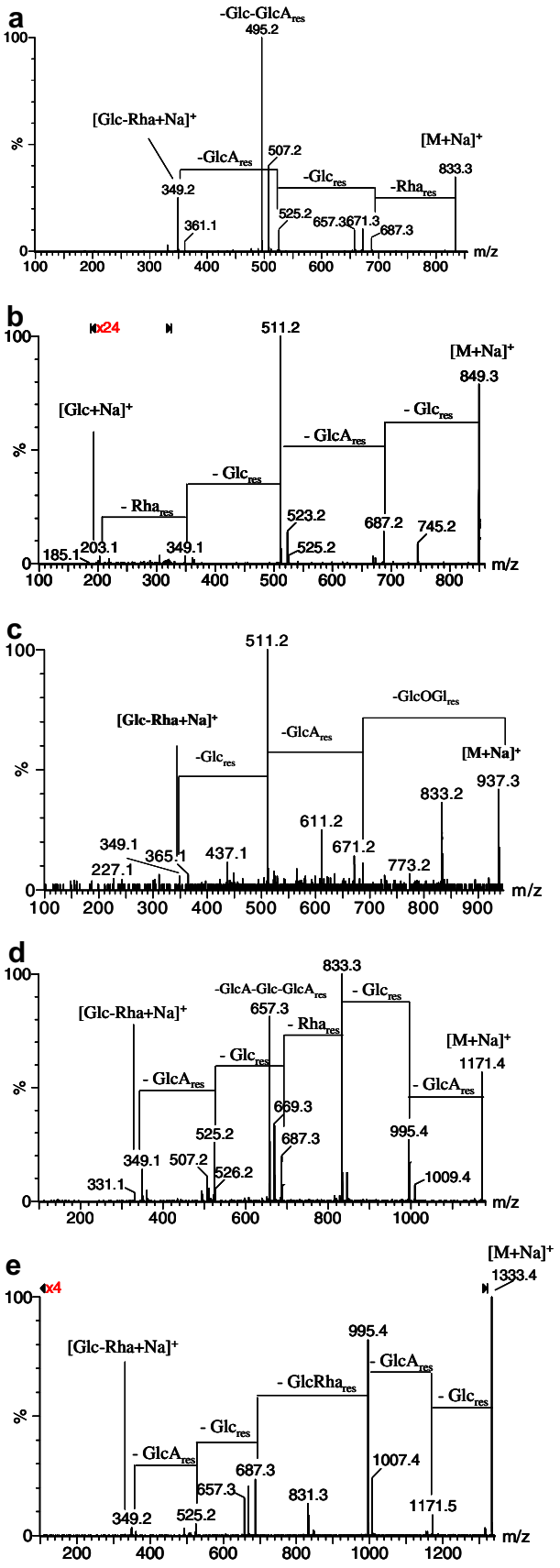

Fig. 5. Product ion spectra, obtained in a Q-TOF2 instrument, for the oligosaccharides identified of (a) *m*/*z* 833 (from commercial gellan), (b) *m*/*z* 849 (from commercial gellan), (c) *m*/*z* 937 (from JB3 gellan), (d) *m*/*z* 1171 (from commercial gellan) and (e) *m*/*z* 1333 (from commercial gellan).

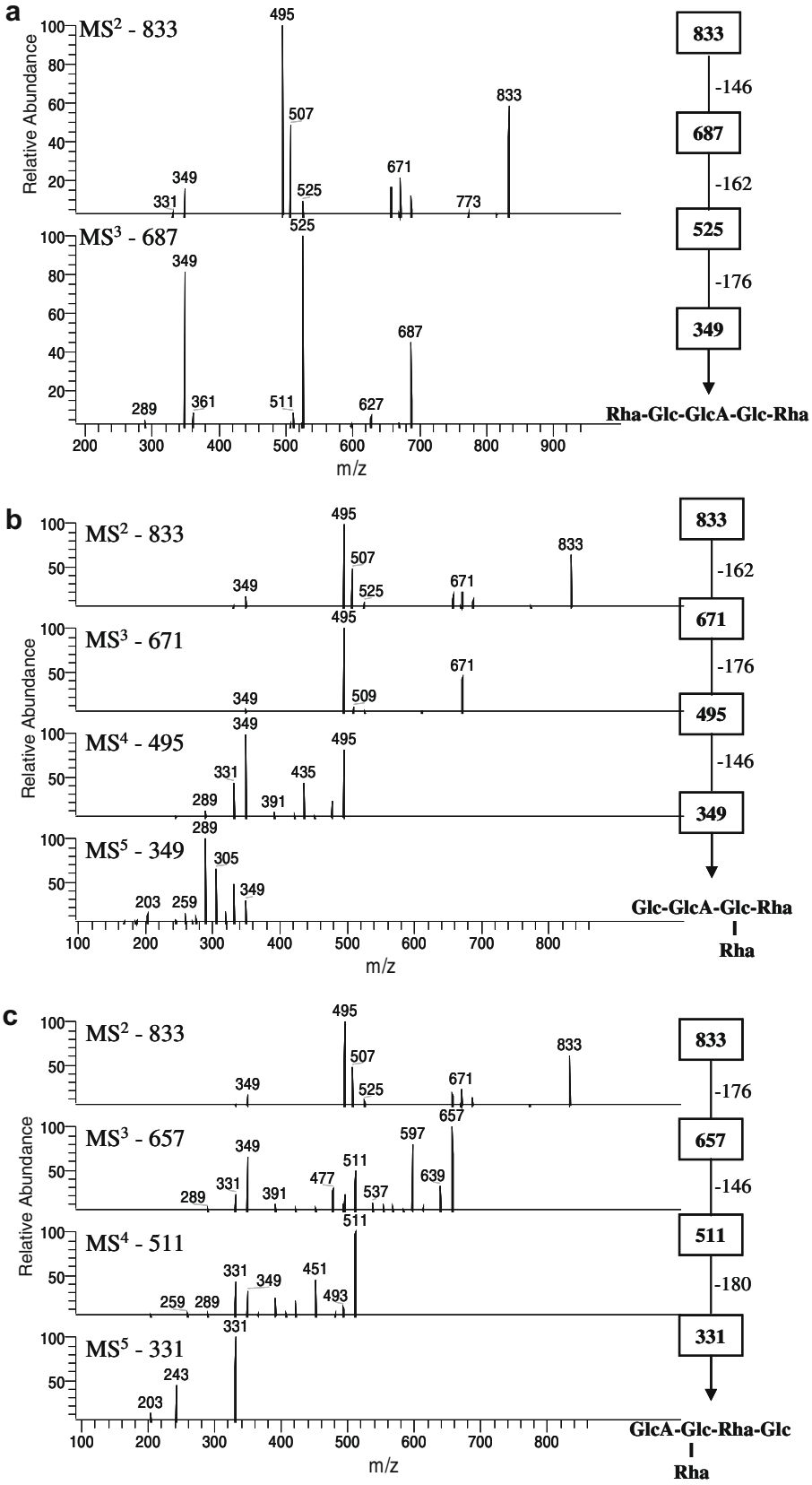

Fig. 6. Different possible fragmentation pathways proposed for the ion of *m*/*z* 833 from commercial gellan, based on the product ion spectra obtained in a linear ion trap instrument: (a) Rha-Glc-GlcA-Glc-Rha, (b) Glc-GlcA-(Rha-)-Glc-Rha and (c) GlcA-(Rha-)-Glc-Rha-Glc.

By the analysis of the product ion spectra obtained from the linear ion trap mass spectrometer of the ion of *m*/*z* 833 it was possible to identify three possible fragmentation pathways, which could correspond to different structures. The structure shown in Fig. 6a corresponds to the linear pentasaccharide Rha-Glc-GlcA-Glc-Rha, which contains the tetrasaccharide repeating unit, as described in

Fig. 5a. The initial loss of a Hex_{res} (Fig. 6b) suggests the presence of another oligosaccharide containing the same sugars composition, only explained if considering one of the Rha residues occurring as a side chain of the tetrasaccharide unit: Glc-GlcA-(Rha-)-Glc-Rha. Also, the initial loss of a $GlcA_{res}$ (Fig. 6c) suggests the presence of a third isomeric oligosaccharide, also only explained if considering the occurrence of one Rha residue as a side chain of the tetrasaccharide unit: GlcA-(Rha-)-Glc-Rha-Glc.

The MS^n spectra of the ion of *m*/*z* 921, identified in the JB3 gellan, showed two different fragmentation pathways, suggesting the presence of two isomeric pentasaccharides. One was identified as GlcOGl-GlcA-(Rha-)-Glc-Rha, fragmenting as described in Fig. 7a. This fragmentation pathway was also observed in the ESI-MS/MS spectrum (data not shown), through the loss of $HexOGl_{res}$, followed by loss of $HexA_{res}$ and loss of $dHex_{res}$ leading to the ion of *m*/*z* 349 $[Hex - dHex + Na]^+$. This fragmentation cannot be explained by the tetrasaccharide repeating unit characteristic of the gellan gum, however, this structure is similar to the previously identified oligosaccharide of *m*/*z* 833 (Fig. 6b), but having, in this case, an additional OGl. The other fragmentation pathway leads to the structure Rha-GlcOGl-GlcA-Glc-Rha (Fig. 7b), which is explained by the tetrasaccharidic repeating unit.

For the ion of *m*/*z* 849, two pentasaccharides were characterised by MS^n, corresponding to Glc-GlcA-Glc-Rha-Glc (according to the tetrasaccharide repeating unit) and other with two linked Glc residues (Fig. 8a), only explained if considering the occurrence of one Glc residue as a side chain of the tetrasaccharide unit: GlcA-(Glc-)-Glc-Rha-Glc.

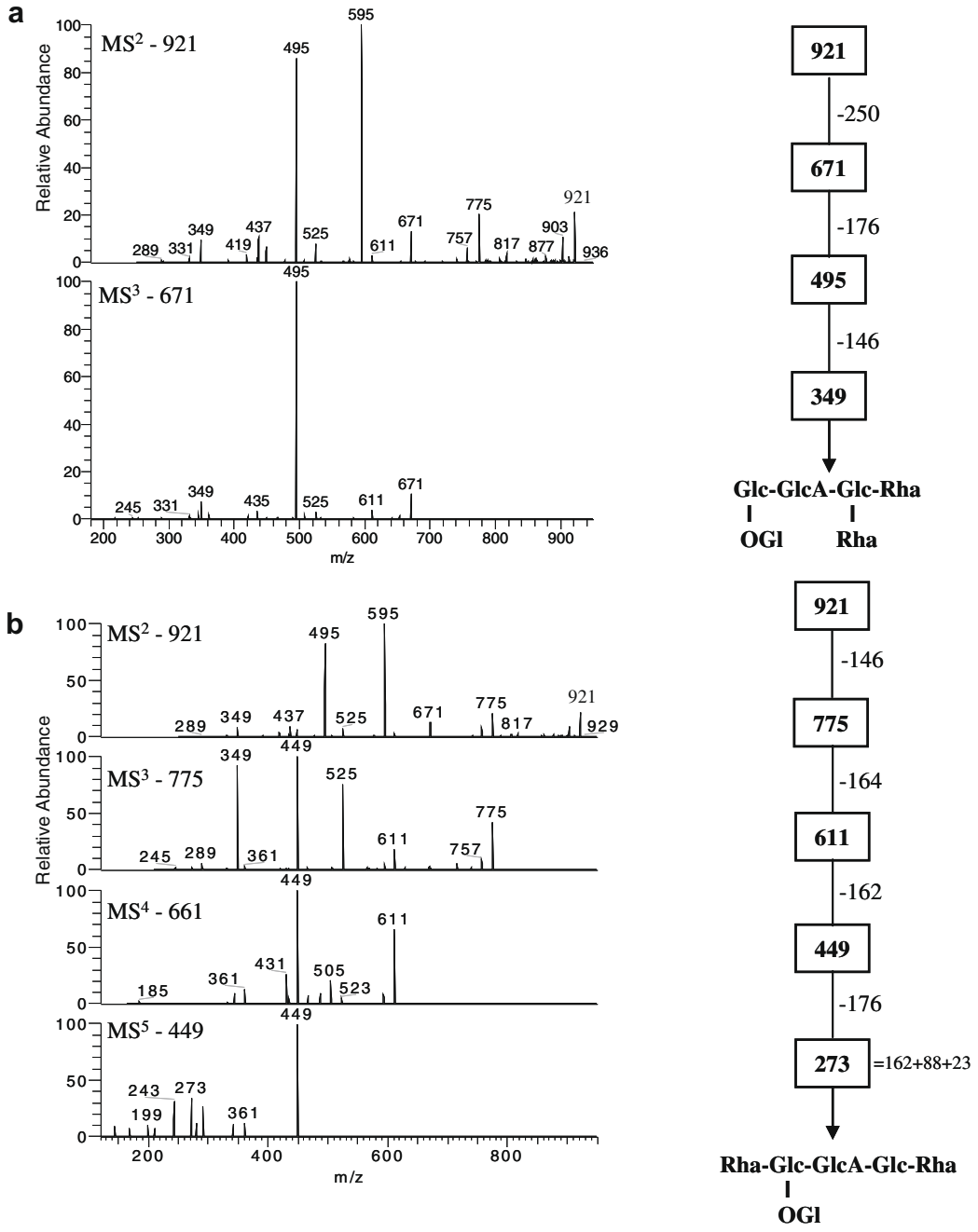

Fig. 7. Fragmentation pathways proposed for the product ion spectra obtained in a linear ion trap instrument for the ion of *m*/*z* 921 from JB3 gellan: (a) GlcOGl-GlcA-(Rha)-Glc-Rha and (b) Rha-GlcOGl-GlcA-Glc-Rha.

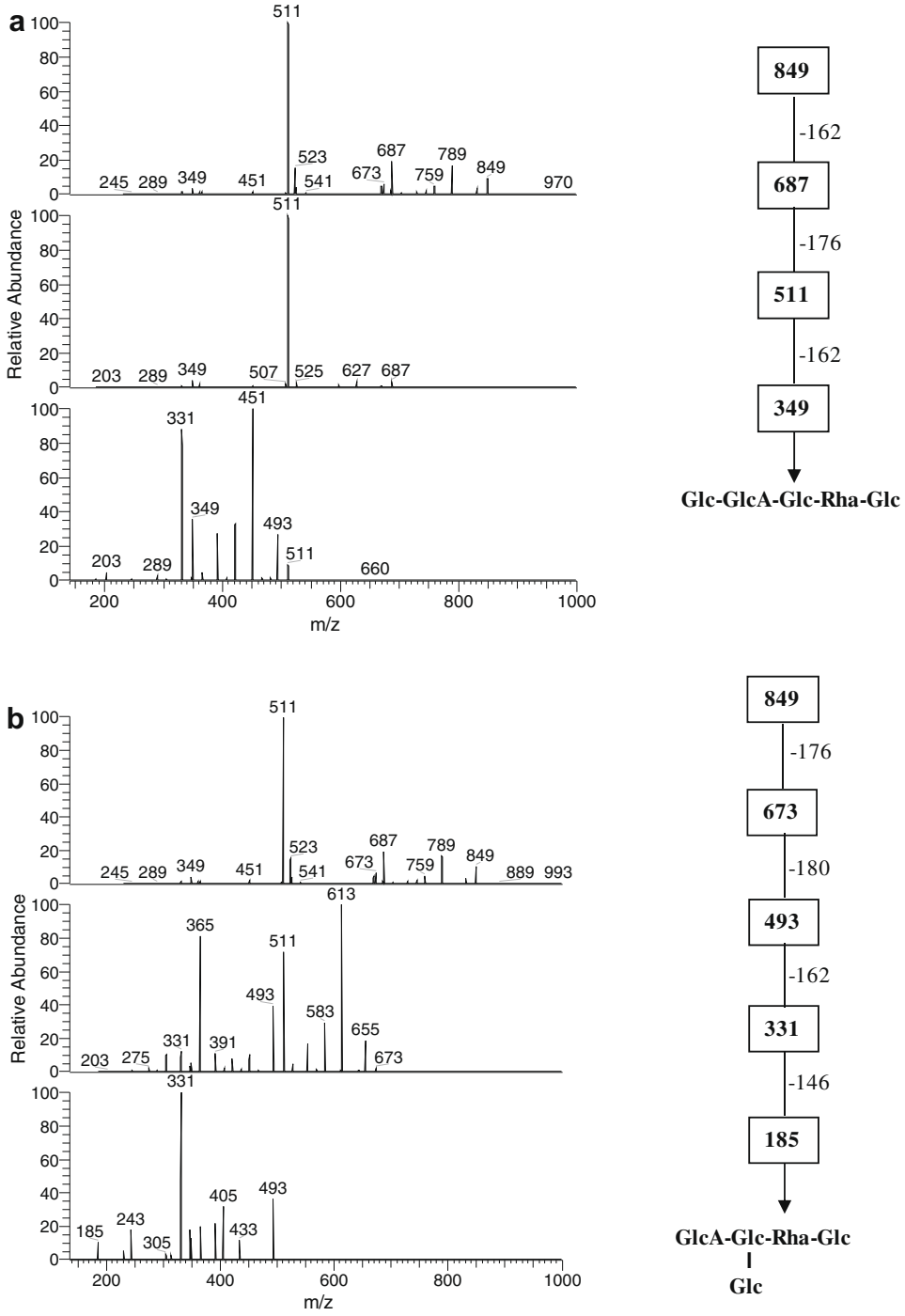

Fig. 8. Fragmentation pathways proposed for the product ion spectra obtained in a linear ion trap instrument for the ion of *m*/*z* 849 of gellan: (a) Glc-GlcA-Glc-Rha-Glc and (b) GlcA-(Glc)-Glc-Rha-Glc.

For the ion of *m*/*z* 937 it was also possible to observe a fragmentation pathway suggesting the presence of Glc-Glc residues by the loss of $dHex_{res}$ from the ion of *m*/*z* 511, leading to the ion of *m*/*z* 365 attributed to $[Hex_2 + Na]^+$ (Fig. 5c).

3.5. Methylation analysis

The possibility of the presence of unexpected structures suggests ramifications in the gellan tetrasaccharide backbone, namely with one Rha residue and/or one Glc residue. In order to confirm their occurrence, the oligosaccharides were submitted to a methylation analysis. Fig. 9a shows the chromatogram of the partially methylated alditol acetates (PMAA) of gellan oligosaccharides obtained by partial acid hydrolysis. As the carboxyl-reduction of uronic acid was not performed and the different acid lability of the different glycosidic linkages, the analysis is not quantitative. Beyond the glycosidic linkages of the neutral sugar residues component of the gellan backbone, (1 → 3)-Glc*p*, (1 → 4)-Glc*p* and (1 → 4)-Rha*p*, because the analysis was performed on oligosaccharides, terminally-linked Rha*p* and Glc*p* have been detected. Also detected were (1 → 2,4)-Glc*p*, at retention time 38.79 min, and (1 → 2)-Glc*p*. Fig. 9b shows the mass spectrum of the peak corre-

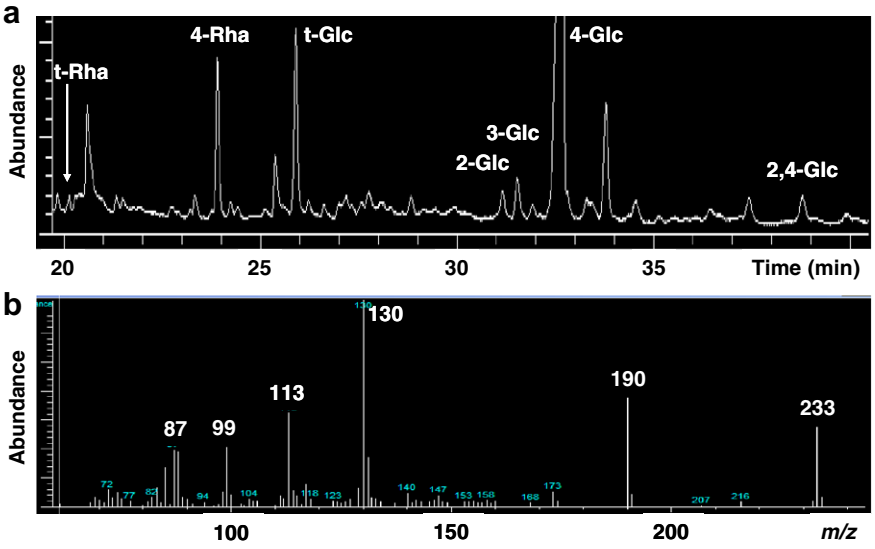

Fig. 9. (a) Chromatogram of the partially methylated alditol acetates obtained from gellan oligosaccharides obtained by partial acid hydrolysis and (b) Mass spectrum of (1 → 2,4)-Glc*p* peak, at retention time 38.79 min, corresponding to 1-*d*-1,2,4,5-tretraacetyl-3,6-dimethyl-glucitol.

sponding to 1-*d*-1,2,4,5-tretraacetyl-3,6-dimethyl-glucitol, which corresponds to a branched glucose residue in C-2 and C-4. No 3-linked branched Glc residues have been observed. These results allow confirming the presence of branched residues and to infer that the ramification occurs only at the *O*-2 position of the (1 → 4)-linked Glc directly linked to the Rha residue, as observed by tandem mass spectrometry.

4. Conclusions

Electrospray tandem mass spectrometry revealed to be suitable for the structural characterisation of gellan polysaccharides. It allowed the confirmation of the presence and sequence of the tetrasaccharide repeating unit without acetyl or glyceryl substituents in the commercial gellan. On the other hand, it allowed ascertain the presence of glyceryl substituent at the (1 → 3)-Glc residue of tetrasaccharide unit and the absence of the acetyl substituent in the JB3 gellan. Both gellans showed fragmentation pathways under MS/MS and MS^n that suggest the occurrence of Glc and Rha residues as ramifications in the backbone, linked to the (1 → 4)-Glc residue.

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