Ethylenediaminetetraacetic Acid (EDTA) as an Auxiliary Tool in the Electrospray Ionization Mass Spectrometry Analysis of Native and Derivatized β-Cyclodextrins, Maltoses, and Fructans Contaminated with Ca and/or Mg

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The effect of Ca^{2+} (and Mg^{2+}) and the disodium salt of ethylenediaminetetraacetic acid (EDTA), a well known Ca^{2+} (and Mg^{2+}) chelating agent, on the volatilization/ionization of carbohydrates by using electrospray ionization mass spectrometry has been studied. Model compounds such as maltoses (maltose to maltoheptaose), β-cyclodextrins (β-cyclodextrin, methyl-β-cyclodextrin, heptakis(2,6-di-O-methyl)-β-cyclodextrin, heptakis(2,3,6-tri-O-methyl)-β-cyclodextrin, and 2-hydroxypropyl-β-cyclodextrin) and fructans (sucrose, 1-ketose, nystose, and 1F-fructofuranosylnystose) were used. (J Am Soc Mass Spectrom 2010, 21, 1526–1529) © 2010 Published by Elsevier Inc. on behalf of American Society for Mass Spectrometry

Important advances in the direct analysis of soluble carbohydrates present in plant cells and tissues, such as probe electrospray (PESI) mass spectrometry (MS) [1], pressure probe and ultraviolet matrix assisted laser desorption/ionization (UV-MALDI) MS [2], and direct UV-MALDI-MS of tissues [3–6], have been recently reported. Studies of carbohydrate MS analysis generally deals with complex mixtures isolated from dry powdered plant tissues [7–10]. Carbohydrates soluble in cell sap are in an aqueous solution containing cations such as K^+ (50–100 mM), Na^+ (5.0–5.5 mM), as well as Mg^{2+} and Ca^{2+} (<2.5 mM) [11]. Thus, soluble carbohydrates are detected in MS positive ion mode mainly as potassiated species ($[M + K]^+$) [1–9] and in negative mode as deprotonated species ($[M - H]^-$) by using either sucked cell sap [2] or direct cell/tissue MS analysis [1, 3–6].

The situation is quite different for native carbohydrate samples obtained after extraction because cell walls are included in the plant material and its Ca^{2+} content is high [11]. The average natural content of cations in dried plant material is K^+ 1%, Ca^{2+} 0.5%, and Mg^{2+} 0.2% [11]. Furthermore, the content of Na^+ and the Na^+/K^+ ratio can change drastically due to use of different aqueous solutions and chemicals used during isolation, fractioning, and purification of carbohydrates. Thus, in these samples, the content of Na^+ is higher than K^+, which leads to the formation of mainly the sodiated species $[M + Na]^+$ together with variable amounts of the species $[M + K]^+$, $[M + Ca]^{2+}$, and $[M + Mg]^{2+}$ [7–10].

To improve the MS analysis of native extracted carbohydrates, which might contain a significant amount of Ca^{2+} (and/or Mg^{2+}), we studied the effect of Ca^{2+} (and Mg^{2+}) and the disodium salt of ethylenediaminetetraacetic acid (EDTA), a well known Ca^{2+} (and Mg^{2+}) chelating agent [12], on the volatilization/ionization of carbohydrates using as ionization method electrospray ionization (ESI).

Experimental

Materials and Methods

Carbohydrates (Glucose (Glc), maltose (Glc_2), maltotriose (Glc_3), maltotetraose (Glc_4), maltopentaose (Glc_5), maltohexaose (Glc_6), maltoheptaose (Glc_7), β-cyclodextrin (β-CD), methyl-β-cyclodextrin (M-β-CD), heptakis (2,6-di-O-methyl)-β-cyclodextrin (DM-β-CD), heptakis (2,3,6-tri-O-methyl)-β-cyclodextrin (TM-β-CD), and 2-hydroxypropyl-β-cyclodextrin (OHPM-β-CD) and inorganic salts (sodium, potassium, calcium and magnesium chloride) were obtained from Sigma Chemical Co. Ltd., Tokyo, Japan. Fructans [fructose (F),

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sucrose (F {2}), 1-ketose (F {3}), nystose (F {4}) and 1F-fructofuranosylnystose (F {5})] and the disodium salt dihydrate of ethylenediaminetetraacetic acid (EDTA) were obtained from Wako Pure Chemical Industries, Ltd, Japan. Acetonitrile (MeCN) and methanol (MeOH) (Sigma-Aldrich HPLC grade), were used as purchased without further purification. Water of very low conductivity (Milli-Q grade; 56–59 nS/cm with PURIC-S; ORUGANO Co., Ltd., Tokyo, Japan) was used.

ESI-TOF-MS Experiments

Because clusters such as $[M + (MeCN)_n + Ca]^{2+}$ were detected when acetonitrile was used, water-acetonitrile (MeCN) mixtures was not used as solvents [13–15]. The ESI-TOF mass spectra were acquired using a Mariner Applied Biosystems (Foster City, CA, USA) ESI-TOF mass spectrometer, and 9:1 (vol/vol) MeOH-H_2O as solvent stream. A Harvard PHD 2000 syringe pump (Holliston, MA, USA) at a flow-rate of 5 L min^{-1} was used to introduce the carbohydrate solution. The spray tip potential was +3.69 kV, the nozzle potential was +100 V and the skimmer voltage was +15 V. The nozzle temperature was 137 °C. The carbohydrates were dissolved in 1 mL water (pH 6.20–6.80). Carbohydrate solution (100 μL) was mixed with the proper volume of salt solution to give solutions containing carbohydrate to salt molar ratio from 100:1 to 1:10 with a 0.1 μmol/mL carbohydrate concentration. Each solution and subsequent solutions made by a 1 in 10 dilution, was injected into the mass spectrometer. The ratios of carbohydrate to metal and to EDTA were varied to study the EDTA effect on the ESI were determined by using carbohydrate concentrations of 10 to 100 pmol/μL, and carbohydrate to $CaCl_2$ molar ratios 1:1, 1:5, and 1:10. The solutions compared in these experiments had carbohydrate to EDTA ratios of 100:1, 50:1, 20:1, 10:1, 5:1, 2:1, 1:1, 1:5, and 1:10 (mol/mol). Aqueous carbohydrate solutions with molar ratios of carbohydrate-$CaCl_2$ 1:1 (mol/mol) (pH 6.30–6.50) were used with enough EDTA to insure calcium to EDTA molar ratios 1:1 and 1:10. Experiments with $MgCl_2$ were conducted in a similar way.

Results and Discussion

Adding EDTA

Ethylenediaminetetraacetic acid (EDTA), as well as its different sodium salt forms, is a good chelator for Ca^{2+} and Mg^{2+} [12]. In connection with the ESI-MS analysis of carbohydrates, EDTA has only been used to show that the exchange reactions between the carrageenan molecules and the surrounding matrix, whereby protons furnished by the acidic matrix (ethylenediaminetetraacetic acid), take place with one or more Na^+ ions [16]. To the best of our knowledge, EDTA has never been used to "clean" native carbohydrate samples contaminated with Ca^{2+} and/or Mg^{2+} through chelation. Recently, the specific metal interactions (Na^+, Mg^{2+}) in nucleic acids have been studied using chelators in nanoESI-MS [17].

To check the efficiency of EDTA as a selective chelator in ESI-MS several experiments using the linear maltoses, β-CD and fructans mentioned above as models were conducted. To an aqueous solution containing either a 1:1 or 1:10 carbohydrate to $CaCl_2$ molar ratio, EDTA was added in 1:1 and 1:10 $CaCl_2$ to EDTA molar ratio. Aqueous solution of the corresponding carbohydrate and EDTA, in 1:1 and 1:10 molar ratio, were used as control solutions. The ESI-MS analysis of the samples was conducted before and after EDTA addition and showed that for linear maltoses Glc_2 to Glc_6, similar intensity for the $[M + Na]^+$ signals were observed. Similarly, for Glc_7 and β-CD the intensity for the pair of $[M + Na]^+$ and $[M + 2Na]^{2+}$ signals was similar. Maltoses and β-CD showed higher affinity for Ca^{2+} and/or higher stability of the gas species $[M + Ca]^{2+}$ than did fructans. This calcium-containing signal was completely eliminated when EDTA was added to the aqueous carbohydrate-$CaCl_2$ solution.

Application to Substituted β-CD

For this study, several commercial β-cyclodextrin derivatives (M-β-CD, DM-β-CD, TM-β-CD, and OHP-β-CD) were used. The corresponding ESI mass spectra showed that these derivatives consisted of a mixture of β-CD with different degrees of substitution. DM-β-CD showed the expected molecular ion $[(CH_3)_{14}$-β-CD] together with the species with more methyl groups than predicted $[(CH_3)_{15}$-β-CD] and $[(CH_3)_{16}$-β-CD] as three monosodiated ions ($[(CH_3)_{14}$-β-CD + $Na]^+$, $[(CH_3)_{15}$-β-CD + $Na]^+$, and $[(CH_3)_{16}$-β-CD + $Na]^+$), and the corresponding disodiated species $[(CH_3)_{14}$-β-CD + $2Na]^{2+}$, $[(CH_3)_{15}$-β-CD + $2Na]^{2+}$, and $[(CH_3)_{16}$-β-CD + $2Na]^{2+}$. The $[M + 2Na]^{2+}$ signal intensities were similar to the $[M + Na]^+$ signals. DM-β-CD showed not only the signals corresponding to the expected disodiated species but also additional high intensity satellite signals (Figure 1a), which were completely eliminated after adding EDTA to the aqueous DM-β-CD solution [Figure 1b; DM-β-CD:EDTA 1:1 (mol/mol)]. To prove that these additional signals were $[M + Ca]^{2+}$ and/or $[M + Mg]^{2+}$ species, several experiments were conducted. To a DM-β-CD:$CaCl_2$ 1:1 (mol/mol) aqueous solution, an increasing amount of EDTA was successively added [Figure 1c and d, $CaCl_2$:EDTA 1:1 and 1:10 (mol/mol)]. Similar experiments were performed using $MgCl_2$ instead of $CaCl_2$, mixtures of $CaCl_2$ + $MgCl_2$ (1:1) and treatment of the carbohydrate-$MgCl_2$ (or $CaCl_2$ + $MgCl_2$) mixtures with EDTA. The results obtained are interesting because they show that EDTA can be a useful tool in the ESI-MS analysis of carbohydrates naturally contaminated with Ca^{2+} and/or Mg^{2+}.

It should be noted that signals of the species $[M - H + Ca]^+$ and $[M - H + Mg]^+$ were not detected for linear

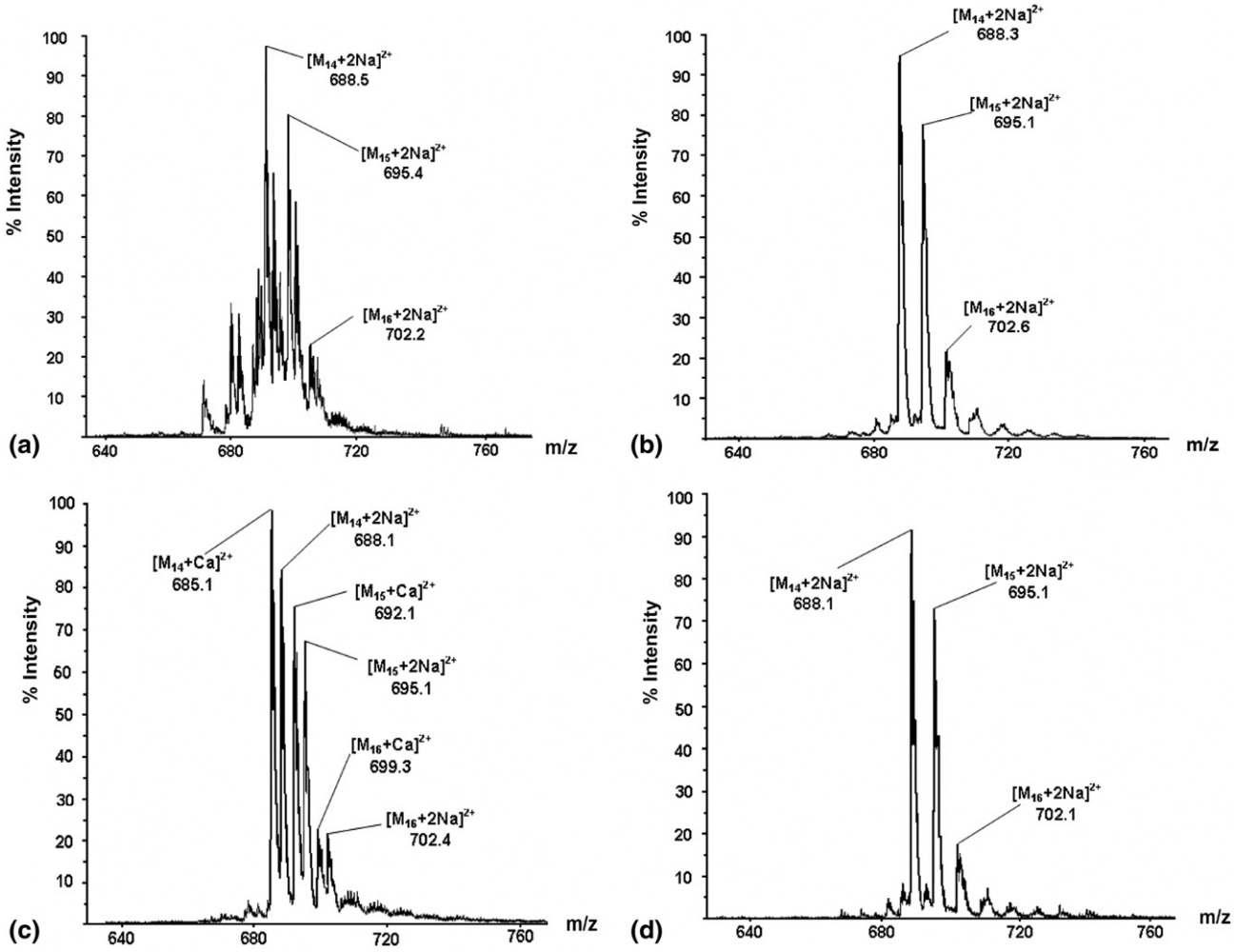

Figure 1. Effect of $CaCl_2$ and EDTA on DM-β-CD ESI-MS analysis: (**a**) DM-β-CD; (**b**) DM-β-CD + EDTA 1:1 (mol/mol); (**c**) DM-β-CD + $CaCl_2$ + EDTA 1:1:1 (mol/mol/mol); (**d**) DM-β-CD+$CaCl_2$ + EDTA 1:1:10 (mol/mol/mol); solvent: water; *m/z* region: 640–760 Da; $[M_n + 2Na]^{2+}$ = $[(CH_3)_n$-β-CD $+2Na]^{2+}$, $[M_n + Ca]^{2+}$ = $[(CH_3)_n$-β-CD + $Ca]^{2+}$, n = 14–16.

maltoses, fructans or β-CD. These monocharged satellite signals of the peaks $[M + Na]^+$ and $[M + K]^+$ were observed in the ESI-MS of the β-CD derivatives studied. As an example, a complex pattern of signals in the $[M + Na]^+$ and $[M + 2Na]^{2+}$ regions were observed for M-β-CD. The family of eight species from $[(CH_3)_9$-β-CD] to $[(CH_3)_{16}$-β-CD] was observed in both *m/z* regions. The *m/z* region between 1250 and 1450 Da, the $[M + Na]^+$ region, was also "cleaned" after EDTA addition [Figure 2, M-β-CD:EDTA 1:1 and 1:10 (mol/mol)]. Addition of $CaCl_2$ to the M-β-CD solution clearly increased the intensity of these satellite signals, which were completely eliminated after EDTA addition.

Conclusions

The $[M + 2Na]^{2+}$ species is detected as a peak with a similar or higher intensity than the $[M + Na]^+$ signal in the ESI-MS analysis of Glc_7, β-CD, and substituted β-CD. The Ca^{2+} and/or Mg^{2+} adducts formed as determined by the observed satellite signals near by the $[M + 2Na]^{2+}$ peak. These satellites are detrimental when a mixture of carbohydrates is analyzed, as demonstrated for the β-CD derivatives (DM-β-CD, M-β-CD, and OHP-β-CD). To make the matter worse, these β-CD derivatives also showed satellite signals with the structure $[M - H + Ca]^+$ and $[M - H + Mg]^+$ in the $[M + Na]^+$ and $[M + K]^+$ *m/z* region (Figure 2). In the case of fructans, although the presence of Ca^{2+} diminishes the intensity of the $[M + Na]^+$ signal, the $[M + Ca]^{2+}$ signal is not detected until the fructan:$CaCl_2$ molar ratio was higher than 1:1. This complication can cause serious mistakes in the quantification of fructans.

As a conclusion, the behavior of carbohydrates in the presence of Ca^{2+} (and Mg^{2+}) is not uniform, but rather depends on the carbohydrate structure. The addition of EDTA to a carbohydrate solution quenches the Ca^{2+} cationizing effect in the ESI spectrum by chelating the divalent cation. Because EDTA does not covalently bind to the carbohydrate, the intensity of the signals

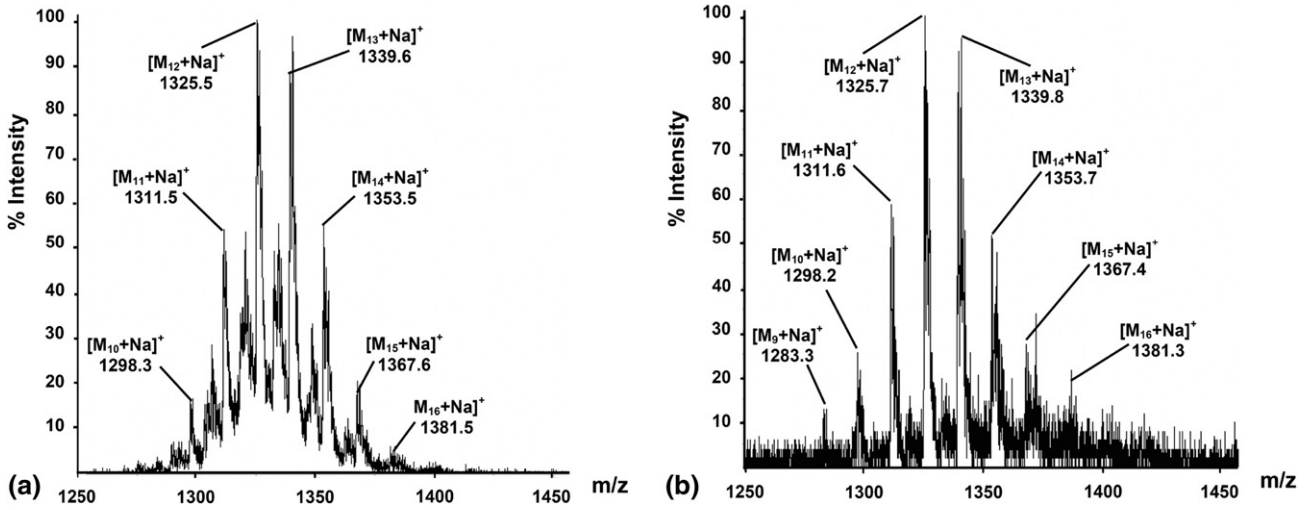

Figure 2. Effect of EDTA on M-β-CD ESI-MS analysis: (**a**) M-β-CD; (**b**) M-β-CD + EDTA 1:10 (mol/mol); solvent: water; *m/z* region: 1250–1450 Da; $[M_n + Na]^+ = [(CH_3)_n$-β-CD $+Na]^+$, $n = 9–16$.

$[M + Na]^+$ and$[M + 2Na]^{2+}$ are restored. The presence of Ca^{2+} and Mg^{2+} must be taken into account for qualitative and quantitative ESI-MS analyses of native extracted carbohydrates and carbohydrates in general. In this context, EDTA is a convenient auxiliary reagent that can be used in the ESI- MS analysis of carbohydrates.

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