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Note

The use of combined high-performance anion-exchange chromatography–thermospray mass spectrometry in the structural analysis of pectic oligosaccharides

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Pure and highly specific enzymes are used more and more as an important step in the elucidation of the fine structure of polysaccharides [1–3]. Chromatographic separation of the oligosaccharides formed is now generally performed using high-performance anion-exchange chromatography (HPAEC) in which a strong anion-exchange resin in combination with a pulsed electrochemical detector allows gradient elution for optimal resolution [2–4]. However, to reveal the exact structure of the oligosaccharides obtained, extensive purification steps such as preparative size-exclusion chromatography or HPAEC, followed by laborious desalting steps are required prior to submitting the isolated fragment to NMR or fast atom bombardment (FAB) mass spectrometry (MS). Recently, we demonstrated that the on-line combination of HPAEC–MS via a thermospray interface is a powerful tool in the characterization of sugar oligomers obtained by enzymic digestion of non-ionic plant polysaccharides [5–7].

In this communication, we report on the characterization of charged oligosaccharides, liberated by rhamnogalacturonase (RGase) during the incubation of pectic hairy regions of apple, by HPAEC–MS without sample pretreatment. The

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partial characterization of two unknown dimers, formed by the degradation of RGase oligomers by a mixture of rhamnosidases and galacturonosidases, is discussed.

HPAEC has been described as a versatile method for the analysis of homologous series of galacturonic acid oligomers [8,9] as well as for the separation of oligomers having a backbone of alternating rhamnose and galacturonic acid [3]. However, elution conditions for this type of charged oligosaccharide in this chromatography mode were not considered to be compatible with LC–MS interfaces, since sodium acetate gradients up to 500–1000 mM are used. To overcome this problem, two anion micromembrane suppressors (AMMS) were placed in series following the detector to exchange all sodium ions. During this step a rather high concentration of acetic acid is formed, but preceding experiments [10] showed that this does not result in negative effects on the molecular-mass determination of oligosaccharides under LC–MS conditions. Using this system, sodium acetate concentrations up to 400 mM sodium could be handled. At higher concentrations, significant salt deposits will occur in the ion source, which hampers further analysis. For this reason, the HPAEC system was disconnected from the MS when the gradient exceeded the concentration of 300 mM sodium acetate in 100 mM sodium hydroxide. The molecular mass of intact oligosaccharides can be studied by thermospray MS via ionization with alkali metal ions, e.g. sodium or lithium [10]. This was achieved by the addition of 0.5–1 mL/min of 0.1 mM sodium acetate to the AMMS effluent prior to the introduction of the mixture to the thermospray interface by a booster pump. Such a pump is needed since the operation of a thermospray requires a solvent pressure of ca. 4 MPa, while the AMMS is pressure-limited to ca. 0.7 MPa. The system is described in more detail elsewhere [5,6].

For neutral sugar oligomers it has been demonstrated that sodiated molecules $[M + Na]$ at m/z $M_r + 23$ can be detected, while no fragmentation is observed at all. For monomeric galacturonic acid, the sodiated sodium salt $[M - H + 2\,Na]^+$ as well as the sodiated acid can be recognized. For oligomers with higher dp values (dp > 4), doubly charged disodium ions are observed, i.e., $[M + 2Na]^{2+}$ at m/z $(M_r + 46)/2$. Triply charged ions are not observed.

To examine the developed HPAEC–MS method for the analysis of complex mixtures of uronic acid-containing oligosaccharides, a fraction containing the hairy (ramified) regions of apple pectin [11] was treated by RGase. The elution pattern of the digest on HPAEC and a characteristic chromatogram for the homologous series of galacturonic acid oligomers under the same conditions are shown in Fig. 1. Although the RGase oligomers were eluted at about the same acetate concentration as digalacturonic acid, it is known from previous studies [3] that the degree of polymerization (dp) ranges from 4 (I) to 9 (VI). To obtain information on the molecular mass without purifying the digest, analysis was performed by HPAEC–thermospray MS. Previously [5], we found that the total ion current chromatogram of HPAEC–MS analysis usually showed no peaks at all. Although this is a disadvantage, poor total-ion current traces should be considered as quite normal in LC–thermospray MS. For this reason, the full scan acquisition data were searched

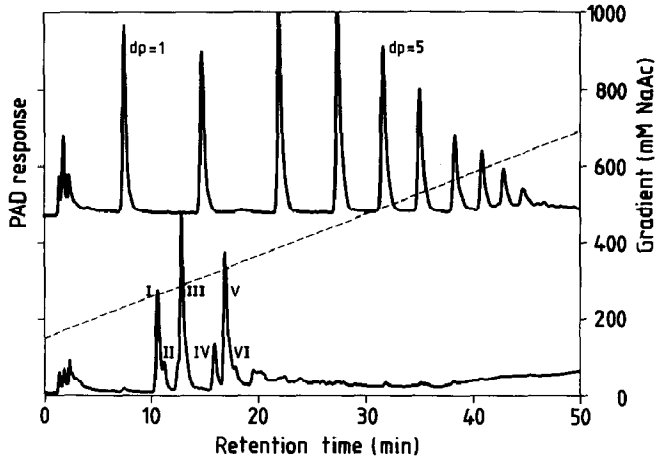

Fig. 1. Elution profile on HPAEC of apple MHR (saponified) after treatment with RGase (lower trace) and polygalacturonic acid after treatment with polygalacturonase (upper trace). The degree of polymerization (dp) of the galacturonic acid oligomers is given in the figure.

for specific m/z values expected in the digest under investigation. Since RGase is reported [12] to liberate only oligomers consisting of rhamnose, galactose, and galacturonic acid, a search was carried out for all m/z values calculated for combinations of hexoses, deoxyhexoses, and uronic acid up to the m/z value of 1500. This resulted in the assignment of the major peaks present in the digest. The poor peak shape observed when operated in full-scan mode can be significantly improved by a sequential run in the multiple-ion detection (MID) mode. The mass chromatograms of the rhamnogalacturonan digest are shown in Fig. 2. All peaks observed in the HPAEC elution pattern have been assigned. It is also confirmed that peak III (Fig. 1) consists of two different oligomers having m/z values of 847 and 1009, respectively, as described before [3]. Although peaks V and VI were separated under normal HPAEC conditions (Fig 1), they were hardly separated under HPAEC–MS conditions but could be recognised rather easily by the difference in m/z value. The m/z value of the peaks and the sugar composition derived therefrom are given in Table 1. The information is in full agreement with the exact structure of these oligomers as derived by ^{1}H NMR after laborious sample preparation [3], also presented in the same Table.

The above findings illustrate that the elution behaviour of oligosaccharides is influenced not only by size but also by the sugar residue and type of linkage present. It can be calculated that ca. 40 μg of oligomers were injected, indicating that under HPAEC–MS conditions the sensitivity is much lower (20 times) than in cases when pulsed amperometric detection (HPAEC–PAD) is used. Since important information on the size and sugar composition of the oligosaccharides can be obtained, it can be concluded that HPAEC–MS is a powerful technique in the characterization of charged, uronic-acid containing oligosaccharides up to dp 9. Combination of the knowledge of the polysaccharide under investigation (sugar

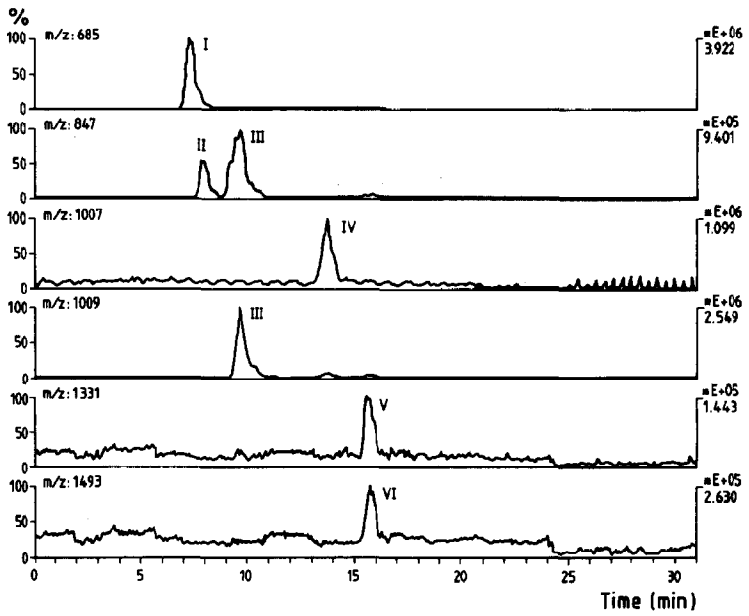

Fig. 2. HPAEC–MS of the RGase digest as shown in Fig 1. Mass chromatograms obtained by multiple ion detection. Right-hand abscissa is ion intensity in arbitrary units.

composition and sugar linkage composition), the known mode of action of the enzyme used, and the molecular-mass information of the oligomers as obtained by HPAEC–MS enables us to predict to a great extent the exact structure of the oligomers. However, the exact location of the galactose residues has still to be established by NMR spectroscopy which requires complete purification of the oligomers.

The HPAEC–MS method described was also used to reveal the molecular mass of some unknown oligosaccharides formed from the rhamnogalacturonan oligomers as described above. In a study carried out in our department dealing with the isolation and characterization of enzymes active on MHR fragments generated by RGase [13], special attention was paid to the action of galacturonosidases and rhamnosidases. A typical elution pattern of a reaction mixture of RGase oligomers after treatment with a partially purified enzyme-fraction is shown in Fig. 3. Next to the monosaccharides rhamnose (peak a), galactose (peak b), and galacturonic acid (peak d), as identified by their retention times, one major, unknown compound (peak c) is observed. Analysis by HPAEC–MS resulted in the mass chromatograms (MID detection) shown in Fig. 4. The peaks of rhamnose (m/z 187), galactose (m/z 203), and galacturonic acid (m/z 217) can be recognized very easily. The relative intensities of rhamnose and galactose are probably not correct, since some loss occurs in the AMMS.

Next to monomers, one major and one minor peak are found at m/z 363, representing a dimer of a deoxyhexose and a hexuronic acid residue. From the

Table 1
Sodiated oligosaccharides: m/z values, sugar composition deduced therefrom, and the structure as published before [3]

m/z	Identity	Structure
685	$(Rha)_2$-$(GalA)_2$	α-Rha *p*-(1 → 4)-α-GalA *p*-(1 → 2)-α-Rha *p*-(1 → 4)-GalA *p*
847/435	$(Rha)_2$-$(Gal)_1$-$(GalA)_2$	α-Rha *p*-(1 → 4)-α-GalA *p*-(1 → 2) α-Rha *p*-(1 → 4)-GalA *p* β-Gal *p*-(1 → 4)
847/435	$(Rha)_2$-$(Gal)_1$-$(GalA)_2$	β-Gal *p*-(1 → 4)-α-Rha *p*-(1 → 4)-α-GalA *p*-(1 → 2)-α-Rha *p*-(1 → 4)-GalA *p*
1009/516	$(Rha)_2$-$(Gal)_2$-$(GalA)_2$	β-Gal *p*-(1 → 4)-α-Rha *p*-(1 → 4)-α-GalA *p*-(1 → 2) α-Rha *p*-(1 → 4)-GalA *p* β-Gal *p*-(1 → 4)
1007/515	$(Rha)_3$-$(GalA)_3$	α-Rha *p*-(1 → 4)-α-GalA *p*-(1 → 2)-α-Rha *p*-(1 → 4)-α-GalA *p*-(1 → 2)-α-Rha *p*-(1 → 4)-GalA *p*
1331/677	$(Rha)_3$-$(Gal)_2$-$(GalA)_3$	β-Gal *p*-(1 → 4)-α-Rha *p*-(1 → 4)-α-GalA *p*-(1 → 2) α-Rha *p*-(1 → 4)-α-GalA *p*-(1 → 2)-α-Rha *p*-(1 → 4)-GalA *p* β-Gal *p*-(1 → 4)
	and	β-Gal *p*-(1 → 4)-α-Rha *p*-(1 → 4)-α-GalA *p*-(1 → 2)-α-Rha *p*-(1 → 4)-α-GalA *p*-(1 → 2) α-Rha *p*-(1 → 4)-GalA *p* β-Gal *p*-(1 → 4)
1493/758	$(Rha)_3$-$(Gal)_3$-$(GalA)_3$	β-Gal *p*-(1 → 4)-α-Rha *p*-(1 → 4)-α-GalA *p*-(1 → 2) α-Rha *p*-(1 → 4)-α-GalA *p*-(1 → 2) β-Gal *p*-(1 → 4) α-Rha *p*-(1 → 4)-GalA *p* β-Gal *p*-(1 → 4)

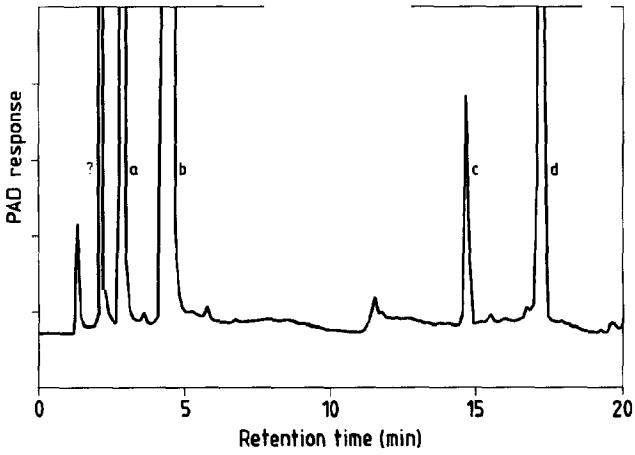

Fig. 3. Elution profile on HPAEC of rhamnogalacturonan oligomers after degradation by a mixture of rhamnosidases and galacturonosidases: a, rhamnose; b, galactose; c, unknown dimer; d, galacturonic acid.

sugar composition of the starting material used, it can be deduced that the dimer is composed of rhamnose and galacturonic acid. From the mass chromatogram, it can also be observed that a second (minor) dimer with the same sugar composition is eluted between the first dimer and galacturonic acid. Considering the structure of

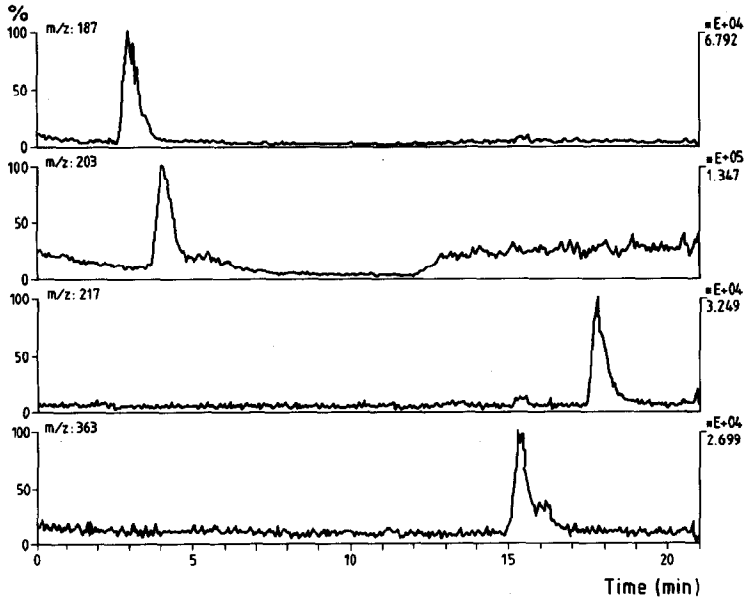

Fig. 4. HPAEC–MS of RGase oligomers after further degradation by a mixture of enzymes. Mass chromatograms obtained by multiple ion detection.

the oligomers from which these dimers are formed, the two possibilities are α-Rha*p*-(1 → 4)-GalA*p* (**1**) and α-GalA*p*-(1 → 2)-Rha*p* (**2**). Starting from the principle that almost all exo-enzymes and/or glycosidases have been reported to act from the nonreducing end of their substrate, we speculate that the major unknown dimer in the digest has a rhamnose residue at the nonreducing end (**1**). The formation of such a dimer from the RGase oligomers implies the presence of at least three glycosidases acting from the nonreducing end: ß-galactosidase, α-rhamnosidase, and α-galacturonosidase. The presence of very small amounts of compound **2** suggests the existence of an additional α-galacturonosidase, acting from the reducing end of the oligomer. The presence of these glycosidases has been confirmed by other experiments and the isolation and characterization of the pure enzymes will be described and discussed elsewhere. It can be concluded that molecular-mass information as obtained by on-line HPAEC–MS gives valuable knowledge of unknown oligosaccharides in complex mixtures, especially since the elution order in HPAEC is rather unpredictable (Fig. 1). Even when oligomers having a different molecular mass are not separated under the chromatographic conditions used, this can be recognised by HPAEC–MS. Although no information concerning the position of the various residues in the oligomer or types of glycosidic linkages can yet be obtained by HPAEC–MS, detailed knowledge of the polysaccharide and the specificity of the enzyme used enables the translation of the molecular mass information into the sugar composition of the various oligomers in the enzymic digest. Typically this will result in only two or three possible structures. Elucidation of the exact structure still requires NMR-analysis where the data interpretation can be simplified by the molecular-mass information and the sugar composition derived therefrom.

When series of analogous oligomers having linkages at different positions, different length, or different degree of branching have been identified and become available for comparison of the elution behaviour on HPAEC columns, molecular-mass information probably will be even more straightforward in suggesting the corresponding structure.

In addition, experiments dealing with HPAEC–MS/MS are in progress. Preliminary results on neutral oligosaccharides indicate that differentiation between isomeric oligomers based on the type of fragments and/or relative peak intensity is possible.

1. Experimental

Enzymic degradation.—Modified hairy regions (MHR) from apple [11] were degraded by RGase as described [3]. The digest was concentrated 10 times by lyophilization prior to injection on the LC–MS system. Rhamnogalacturonan oligomers were further degraded by enzymes, fractionated from the technical enzyme preparation Ultra Sp (*Aspergillus aculeatus*) which was kindly provided by Novo Nordisk Ferment AG (Dittingen, Switzerland)

HPAEC–MS.—A Finnigan MAT TSQ 70 tandem mass spectrometer, equipped with a 20-kV conversion dynode detection system, was used. A thermospray interface, operated at a block temperature of 400°C, a vaporizer temperature of 100°C and filament off, was applied in combination with HPAEC [performed on a Dionex system (Sunnyvale, CA, USA) equipped with a CarboPac PA100 column and a PED electrochemical detector] as described [3]. After an equilibration step of at least 15 min with 150 mM NaOAc in 100 mM NaOH, 25 μL of the sample was injected and a linear gradient to 400 mM NaOAc in 100 mM NaOH within 40 min was started. After the detector, two AMMS-II micromembrane suppressors were connected in series, regenerated with 10–15 mL/min of 0.15 mM H_2SO_4 delivered by a Chem/Tech Iwaki (Lowell, MA, USA) Model EP B15 pump. The effluent (1 mL/min) from the AMMS was mixed with 1 mL/min of 0.1 mM aq NaOAc and repressurized using a booster pump (Kratos, Manchester, UK) Spectroflow 400 LC pump. The digest of RGase oligomers after treatment with a mixture of rhamnosidases and galacturonosidases was eluted with a slightly modified gradient starting at 100 mM NaOH.

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