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Characterization of Enzymatic Pectin Digests by Matrix-assisted Laser Desorption/Ionization Mass Spectrometry

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The use of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry for the characterization of partially methyl-esterified enzymatic pectin digests is described. The sensitivities of several matrices, positive and negative ion modes and desalting techniques for these acidic oligosaccharides were compared. The most favorable results were obtained with a thin-layer preparation of a mixture of 2,4,6-trihydroxyacetophenone and nitrocellulose in the negative ion mode. Results are presented demonstrating the sensitive characterization of separated and unseparated high-ester pectin digests obtained after complete digestion using *Aspergillus niger* pectin lyase and the analysis of digests after chemical modification. In the case of unseparated digests, the analysis of methylation patterns is demonstrated. Oligomers with a degree of polymerization up to 40 were detected after enrichment of large oligomers by propan-2-ol precipitation. © **1998 John Wiley & Sons, Ltd.**

KEYWORDS: pectin; matrix-assisted laser desorption/ionization mass spectrometry; acidic oligosaccharides; pectin lyase; desalting

INTRODUCTION

Pectins are a family of complex, highly heterogeneous polysaccharides found in the primary cell walls and intercellular regions of higher plants. These biopolymers consist of chains of α-(1 → 4)-linked D-galacturonic acids (smooth region) interrupted by L-rhamnose-carrying branched neutral sugar side-chains (hairy regions).[1,2] In nature, the uronate residues are partially methyl esterified. Figure 1 shows a schematic overview of the pectin structure.

Oligogalacturonic acids with degrees of polymerization (DP) between 10 and 20 have been shown to have important regulatory activity for plant defense mechanisms,[3] plant development[4] and protein phosphorylation.[5] In all these cases, complete characterization of the active oligosaccharides which are formed by enzymatic pectin degradation is needed.

Pectins are of high commercial interest for the food industry owing to their gelling and stabilizing properties. However, in spite of progress in the structural characterization of pectins by NMR spectroscopy,[6,7] atomic force microscopy,[8] mass spectrometry[7,9] and anion-exchange chromatography (AEC),[10] the structure of pectin and most of its enzymatic formed fragments has not been fully determined. Evaluation of esterification patterns is fundamental to understand not only the structure–function relationships of commercial pectins but also the specificity of pectinases. Pectin lyase is an *endo*-acting hydrolytic enzyme specific for highly

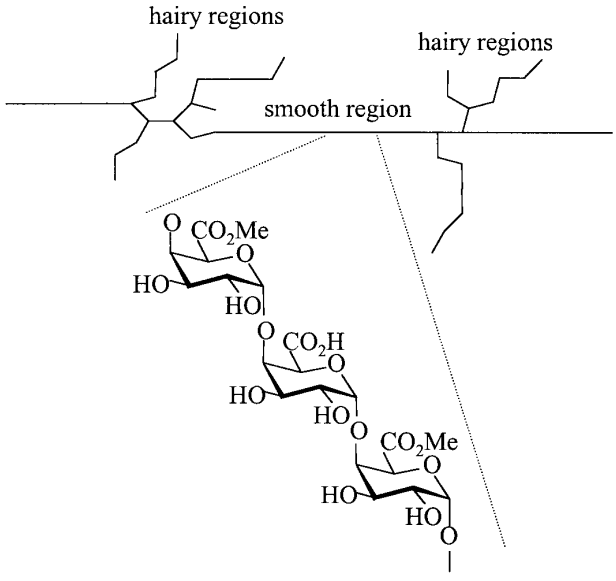

Figure 1. Schematic overview of the pectin structure. Pectin consists of a homogalacturonan backbone (smooth region) and branched neutral sugar side-chains (hairy region). The smooth region is composed of partially methylated α-(1 → 4)-linked D- -galacturonic acids as shown in the chemical structure.

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methylated regions in the homogalacturonan chain.[11] The minimal length of these blocks and the preferred cleavage positions are still the subject of ongoing research. Complex mixtures of oligomers with variable DP and degree of methylation (DE) are produced by pectin lyase, making their characterization challenging. Since large, highly methyl-esterified oligomers co-elute with smaller, less methylated oligomers on anion-exchange columns, the analysis of methylation patterns by AEC alone is not possible (unpublished results). Rapid and sensitive analytical methods for mixtures of partially esterified oligogalacturonic acids are therefore needed. Matrix-assisted laser desorption/ionization mass spectrometry[12] (MALDI/MS) has been developed into a powerful tool for the analysis of oligosaccharides. Although sample preparation techniques for the analysis of neutral oligosaccharides by MALDI/MS are well established,[13–17] relatively little has been published about the analysis of acidic oligosaccharides.[18,19]

Oligogalacturonic acid oligomers with a DP up to 14 have been detected by fast atom bombardment MS in the negative mode[7], up to DP 7 by electrospray ionization (ESI) MS in the negative mode[20] and up to DP 12 by MALDI/MS in the positive mode.[21]

In this paper, we describe a study of sample preparation methods for the analysis of partially methylated oligogalacturonic acids by MALDI/MS, compare the sensitivity of positive and negative ion modes and describe the use of a sample desalting method based on ammonium-loaded cation-exchange resins developed for nucleic acid analysis.[22] Large oligomers can be enriched and suppression effects reduced by propan-2-ol precipitation of the initial oligomer mixture. Oligomers up to DP 40 could be detected in the negative linear MALDI/MS mode. Comparison of the MALDI/MS analysis of pectin lyase digests after de-methylation[23] with separations using MonoQ anion-exchange columns[24] shows that the results obtained are qualitatively in good agreement.

EXPERIMENTAL

Materials

Commercial ultra rapid set (URS) pectin produced from lime pectin with a DE of 81% and pectin lyase from *Aspergillus niger* were provided by Danisco (Danisco Biotechnology, Copenhagen, Denmark). Dihydroxybenzoic acid was obtained from Hewlett-Packard (Palo Alto, CA, USA), diammonium hydrogencitrate and α-cyano-4-hydroxycinnamic acid from Sigma (St Louis, MO, USA), 2,4,6-trihydroxyacetophenon and 3-hydroxypicolinic acid from Aldrich (Gillingham, Dorset, UK), analytical-grade acetone and propan-2-ol from BDH (Poole, Dorset, UK), sequencing grade methanol from Rathburn Chemicals (Walkerburn, UK) and Suprapur ammonia solution (25%) and analytical-grade ammonium acetate from Merck (Darmstadt, Germany). GELoader tips and plastic syringes (Combitips, 1.25 ml) were purchased from Eppendorf-Netheler-Hinz (Hamburg, Germany) and the nitrocellulose membrane (Trans-Blot Transfer Medium, pure nitrocellulose membrane, 0.45 μm) and the cation-exchange resin (50W-X8, 200–400 mesh, hydrogen form) from Bio-Rad (Richmond, CA, USA).

The arabinosazone matrix was synthesized as described by Chen *et al.*[25] Ultra-pure water from a Milli-Q purification system (Millipore, Bedford, MA, USA) was used in the preparation of all solutions.

Enzymatic digestion

A solution of 5 mg ml^{-1} URS pectin in 50 mM sodium acetate buffer (pH 5.0) was incubated with 0.1 U pectin lyase for 24 h at room temperature (1 U produces 1 μmol of double bonds per minute measured by the increase in the molar absorption at 235 nm using the described conditions). At that time the reaction was more than 98% complete. Less than 10% of the enzyme activity was lost during the incubation period, measured by adding another portion of URS pectin and determining the rate of digestion.

Chromatographic analysis of de-methylated pectin lyase digest

Hydrolysis of the methyl ester groups was achieved by treatment of 1 ml of crude digestion mixture with 0.1 ml of 0.5 M NaOH (resulting pH ≈ 12) at 3 °C for 16 h. The sample was neutralized by addition of 0.1 ml of 0.5 M HCl. For chromatographic analysis the samples were filtered through a 2 μm filter. Aliquots of 100 μl were loaded on to a 1 ml MonoQ HR 5/5 column (Pharmacia, Uppsala, Sweden) using the Äkta system (Pharmacia). Separation could be achieved by running a gradient using 0.05 M NH_4HCO_3 (eluent A) and 0.75 M NH_4HCO_3 (eluent B) in Milli-Q water. The gradient program was 8 column volumes 100% A, 10 column volumes 0–40% B and 30 column volumes 40–100% B.

Propan-2-ol precipitation

One part of aqueous sample solution was added dropwise to two parts of propan-2-ol. The mixture was shaken and the precipitate was isolated by centrifugation at 5000*g* for 10 min. The precipitate was washed three times with 60% aqueous propan-2-ol and finally freeze-dried.

Sample desalting

Cation-exchange resins were converted from the hydrogen form to the ammonium form by washing a resin-filled column with 5% ammonia solution followed by extensive washing with 3 M ammonium acetate solution. Finally, water was passed over the column. Aliquots of 50 μl of resins and the same volume of water were prepared in plastic tubes and stored in a cold-room for further use.

Miniaturized cation-exchange columns were prepared similarly to the procedure described by Kussmann *et al.*[26] for reversed-phase columns by flattening the outlet

of a GELoader pipette tip near the outlet to ensure that the packing medium did not leak out. A 10 µl volume of a suspension of packing medium was dispensed in the column body from the wide end, followed by the same volume of water. The column body was fitted with a plastic syringe filled with air and the suspension was pressed down to form a column. A 10 µl volume of sample was applied to the top of the column using another GELoader pipette tip. The sample was slowly pressed through the column and the effluent was collected in a small plastic tube. Finally, 0.5 µl of ammonium-loaded cation-exchange suspension was added to the sample solution.

MALDI/MS sample preparation

Dihydroxybenzoic acid (DHB). DHB was dissolved in 0.1% trifluoroacetic acid (TFA)–acetonitrile (70:30, v/v) to a concentration of 20 µg μl^{-1}. A 0.5 µl volume of the matrix was mixed on the MALDI target with 0.5 µl of 20 mM dibasic ammonium citrate and 0.5 µl of sample solution. The sample was allowed to dry in air.

2,4,6-Trihydroxyacetophenone (THAP)–nitrocellulose. THAP was dissolved in methanol to a concentration of 200 µg μl^{-1}. Nitrocellulose was dissolved in acetone to a concentration of 30 µg μl^{-1} and diluted with propan-2-ol to a final concentration of 15 µg μl^{-1}. THAP and nitrocellulose solutions were mixed in the ratio 4:1. A 0.2 µl volume of matrix solution was placed on the target. The solution spread out fast, forming a thin layer of homogeneous, very fine crystals. This crystal layer can be washed with water to remove salt contaminants. A 0.2 µl volume of 20 mM dibasic ammonium citrate solution and 0.2 µl of sample solution were placed on top of the matrix layer and were allowed to dry in air.

Alternatively, THAP was used without addition of nitrocellulose in dried droplet preparation: THAP was dissolved at a concentration of 20 µg μl^{-1} in methanol-water (1:1, v/v). A 0.5 µl volume of the matrix was mixed on the MALDI target with 0.5 µl of 20 mM dibasic ammonium citrate and 0.5 µl of sample solution. The sample was allowed to dry in air.

Arabinosazone. Arabinosazone was dissolved in methanol to a concentration of 10 µg μl^{-1}. A 0.5 µl aliquot of matrix solution was mixed on the target with 0.5 µl of 20 mM dibasic ammonium citrate and 0.5 µl sample solution. The sample was allowed to dry in air.

3-Hydroxypicolinic acid (3-HPA). 3-Hydroxypicolinic acid was dissolved in 30% (v/v) aqueous acetonitrile to a concentration of 30 µg μl^{-1}. A 0.5 µl volume of the matrix was mixed on the MALDI target with 0.5 µl of 20 mM dibasic ammonium citrate and 0.5 µl of sample solution. The sample was allowed to dry on air.

Mass spectrometry

MALDI time-of-flight (TOF) mass spectra were acquired on a Bruker Reflex II spectrometer (Bruker-Franzen Analytik, Bremen, Germany) and on a Voyager Elite spectrometer (Perseptive Biosystems, Framingham, MA, USA). Both instruments were equipped with delayed extraction technology and delay times of 400 ns (Reflex II) and 150 ns (Voyager Elite) were used. In order to avoid saturation of the detector by matrix ions, low-mass detector gating (cut-off between 400 and 1400 Da, depending on the mass range studied) was used on both instruments. The instruments were calibrated externally with a mixture of galacturonic acid oligomers.

RESULTS AND DISCUSSION

Sample preparation

The matrices dihydroxybenzoic acid (DHB), 2,4,6-trihydroxyacetophenone (THAP)–nitrocellulose, arabinosazone, 3-hydroxypicolinic acid (3-HPA) and α-cyano-4-hydroxycinnamic acid (4-HCCA)[26] were compared for the analysis of enzymatic pectin digests. Only weak signals were obtained using 4-HCCA and 3-HPA. DHB, THAP and arabinosazone gave intense signals in both the positive and negative modes and can be considered as good matrices for the analysis of pectin digests. The sample preparations were optimized for these three matrices. The best results for the detection of oligomers with a high DP were obtained, in our hands, with the THAP–nitrocellulose matrix in a thin-layer preparation.

Owing to the rapid crystallization of matrix material, an even and homogeneous layer of very small crystals was formed, very little spot-to-spot variation of the ion signal was observed and significantly more homogeneously composed spots could be obtained compared with a dried droplet preparation. The thin-layer preparation method was first introduced by Vorm *et al.*[27] for 4-HCCA and by Mørtz *et al.*[28] for THAP. We found that the further addition of nitrocellulose was advantageous for the formation of a mechanically stable and homogeneous matrix layer of very fine crystals. The inclusion of nitrocellulose in addition to ammonium citrate slightly improved the sensitivity for oligomers with high DP. Our results are in agreement with the work of Papac *et al.*[18] who compared several matrices for the analysis of acidic oligosaccharides and achieved the highest sensitivity and minimal prompt fragmentation using THAP with a fast vacuum drying process.

Owing to the acidic character of the studied oligosaccharides and the sodium acetate buffer used in the enzymatic digestion, intense metal–cation adducts were observed when no sample desalting was used. Most of the metal–cations were removed by passing the sample over miniaturized, ammonium-loaded cation-exchange columns. Further reduction of metal–cation adduct formation was achieved by mixing the sample with diammonium hydrogencitrate (10–50 mM) on the target. The ammonium ions replace the metal–cations on the acidic binding sites and, in contrast to metal–cations, are effectively released from the analyte via proton transfer upon ionization.[22] The extensive desalting procedures were found to be essential for the acquisition of spectra in both positive and negative ion modes.

Comparison of positive and negative ion modes

Intense ion signals were obtained in both the positive and negative ion modes. The sensitivity for larger oligomers was higher in the negative mode [compare Fig. 2(a) and (b)]. Whereas mainly deprotonated ions were formed in the negative mode, sodiated and potassiated ions and multiple alkali–metal adducts were observed in the positive mode [see insets in Fig. 2(a) and (b)]. Heterogeneous alkali metal adduct ion formation is undesirable for several reasons. First, the peaks of sodiated and potassiated ions overlap with the methylation pattern. As an example, the potassiated 8-mer with five methyl groups has a 2 Da higher mass than the sodiated 8-mer with six methyl groups [see inset in Fig. 2(b)]. Such a small mass difference is difficult to resolve when spectra are recorded in the linear mode as for higher oligomers, resulting in overlapping peaks and therefore decreased accuracy in mass determination. Second, the total available charge is distributed among a larger number of ions, thereby decreasing the overall sensitivity. This can be seen by comparing the signal intensities of, for example, the 11-mer in the negative [Fig. 2(a)] and positive [Fig. 2(b)] ion modes. Completely methylated oligomers could not be detected in the negative ion mode since there are no free acidic groups available for deprotonation.

However, only short fully methylated oligomers up to DP 3 are present in high abundance, because pectin lyase cleaves in methylated regions of the homogalacturonan backbone, and because the digestion with pectin lyase was almost complete. These short oligomers were analyzed in the positive ion mode by ESI/MS (unpublished results) instead of the positive MALDI ion mode because of interference of the analyte signals with matrix ions in the latter.

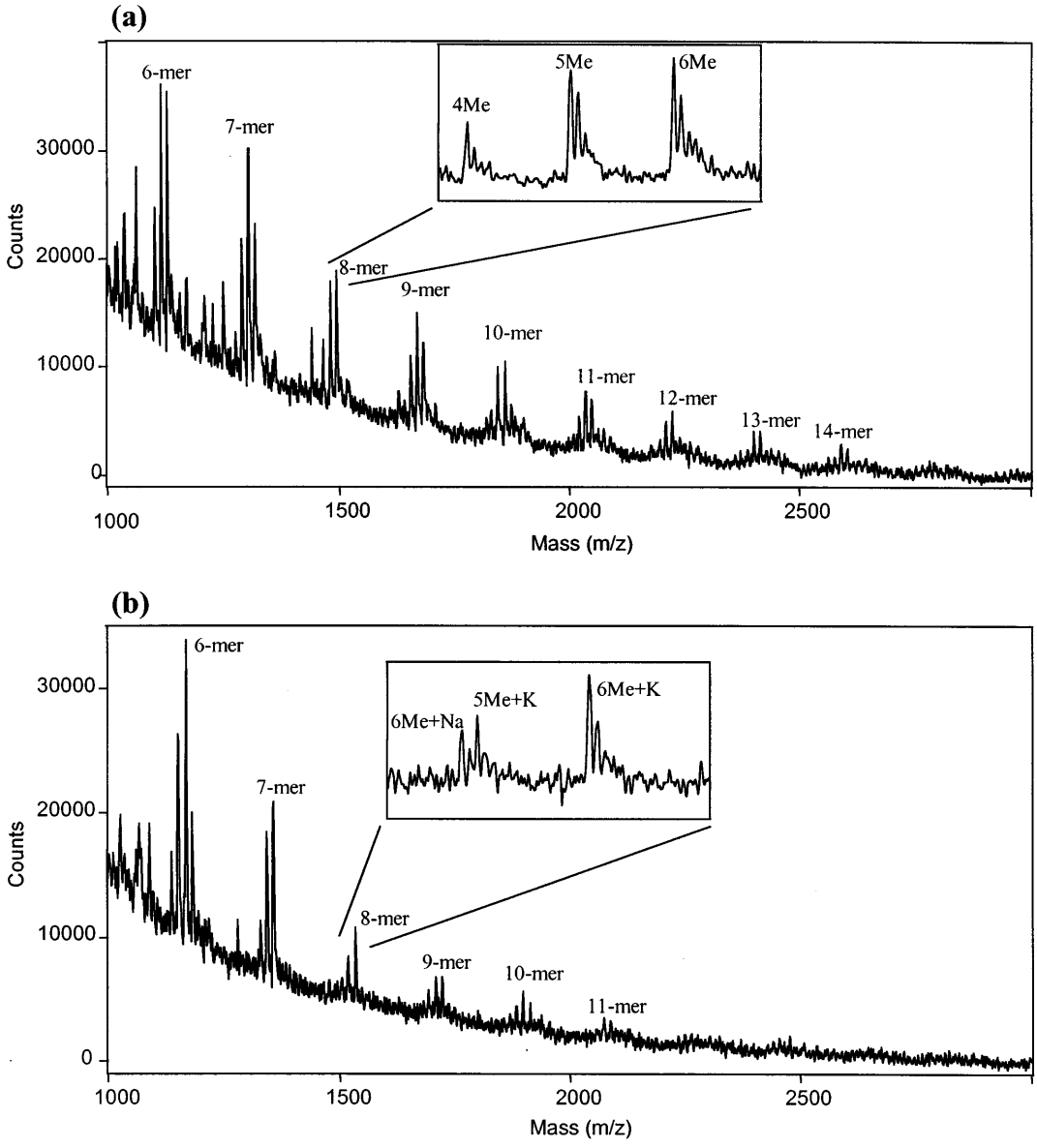

Figure 2. MALDI mass spectra of a pectin lyase digest of highly methylated pectin (81% DE) in (a) negative ion reflector mode and (b) positive ion reflector mode. The insets show (a) deprotonated 8-mer with 4–6 methyl groups and (b) sodiated and potassiated 8-mer with 5–6 methyl groups. A THAP–nitrocellulose matrix in a thin-layer preparation was used as the matrix for both experiments.

Enrichment of large oligomers by propan-2-ol precipitation

As mentioned above, pectin lyase digests of high-ester pectin consist predominantly of relatively short oligomers of DP < 10 (> 90% for pectin with DE 81%). Therefore, detection of large oligomers is difficult owing to the high abundance of short oligomers and the low absolute concentration of larger oligomers. In addition, Harvey[29] concluded from a study of oligosaccharide mixtures that the MALDI sensitivity for each ion species in the mixture decreases with increasing heterogeneity of the mixture and increasing molecular mass of the oligosaccharides. In order to enrich the content of larger oligomers, propan-2-ol precipitation of a pectin lyase digest was performed. Figure 3(a) shows that considerably larger (up to DP 26) oligomers could be detected compared with the analysis before propan-2-ol precipitation [up to DP 14, see Fig. 2(a)] under similar conditions, i.e. reflector mode. The linear mode further enhances the detection limit [see Fig. 3(b)] up to DP 40, but in this mode the resolution was not sufficient to resolve methylation patterns clearly. The spectrum of the 13-mer [Fig. 3(a), inset] shows oligomers having 2–10 methyl ester groups. Despite of the high DE, pectin lyase did not degrade these oligomers, indicating that blocks of at least 3–4 methylated galacturonic acid residues are needed for the degradation by this enzyme.

Comparison of the analysis of a de-methylated pectin lyase digest using either MonoQ AEC or MALDI/TOF-MS

High-ester pectin lyase digests can only be partially separated by anion-exchange chromatography. Therefore, the samples were de-methylated by alkaline treatment prior to AEC analysis. Pectin lyase introduces a double bond between C-4 and C-5 in the galacturonic

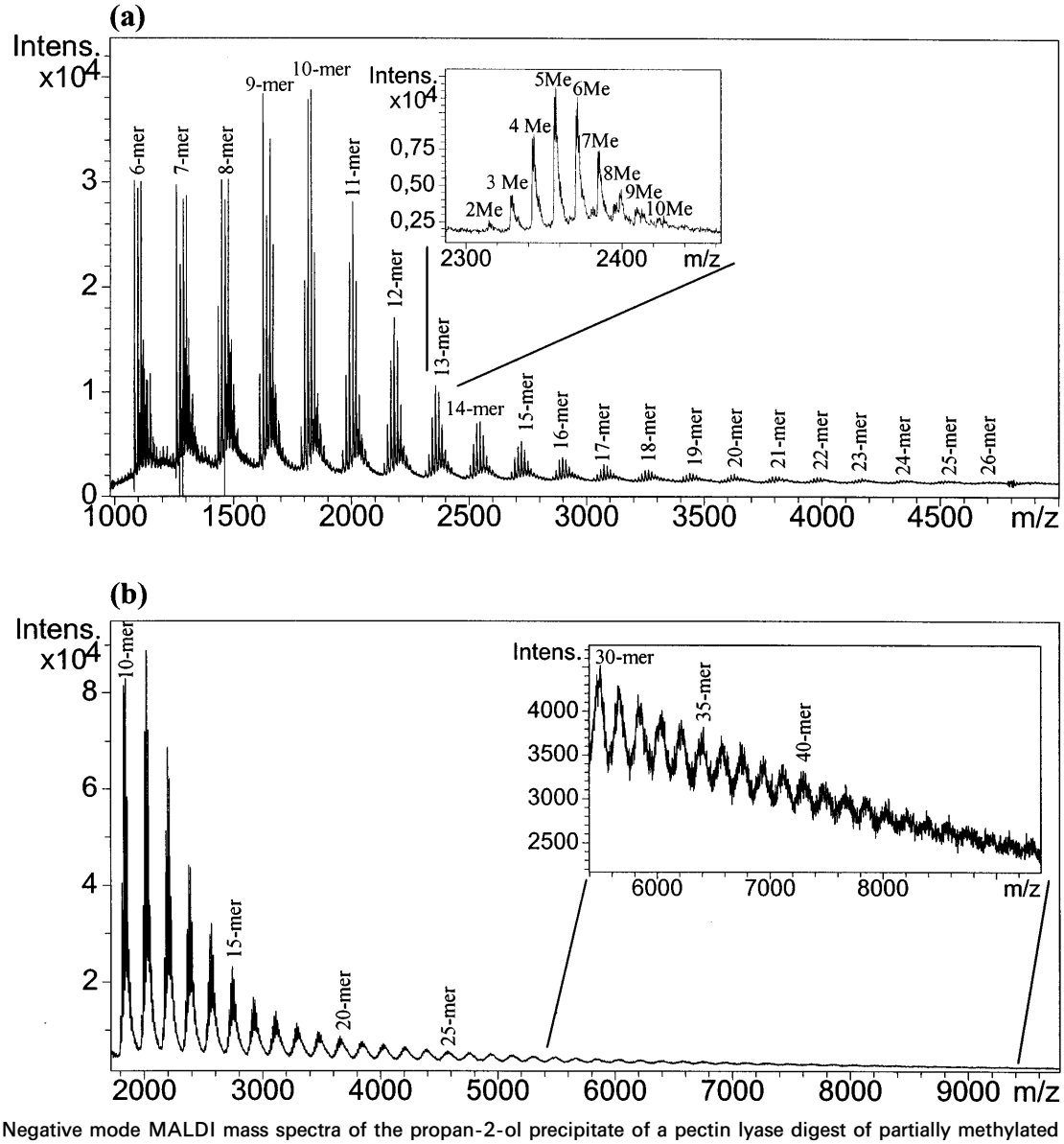

Figure 3. Negative mode MALDI mass spectra of the propan-2-ol precipitate of a pectin lyase digest of partially methylated pectin (81% DE). The spectra were recorded in (a) reflector and (b) linear mode. The insets show (a) 13-mer with 2–10 methyl groups and (b) an enlargement of the higher mass range. A THAP–nitrocellulose matrix in a thin-layer preparation was used as the matrix.

acid unit at the non-reducing end, allowing the detection of the cleavage products by measuring the UV absorption at 235 nm. AEC fractions were analyzed by ESI/MS and MALDI/MS to determine the DP (data not shown). The chromatogram [Fig. 4(a)] shows oligomers between DP 2 and 18 with maximum peak intensity at the 3-mer.

The same digest was analyzed by MALDI/MS [Fig. 4(b)] and consumed 500 times less sample. The observed size distribution (DP 3–17) and relative signal intensities show discrimination effects in both the high- and low-mass region. The sensitivity for short oligomers is reduced even when no detector gating is used, shifting the maximum peak intensity from DP 3 [Fig. 4(a)] to DP 5 [Fig. 4(b)]. A possible explanation could be that larger ions have stronger interactions with the matrix upon crystallization, making their ionization more favorable. Another reason for the observed discrimination of short galacturonic acid oligomers could be a lower ionization probability due to the lower number of acidic groups available for deprotonation. By comparing the relative signal intensities for 5-mers with 1–4 methyl ester groups upon analysis by MALDI and nano-ESI methods,[30] we found that oligomers with more acidic groups seem to be favored for ionization in MALDI. Thus, in nano-ESI the highest peak intensity was found for the 5-mer with four methyl groups, whereas in MALDI the maximum peak was shifted to the 5-mer with three methyl groups (data not shown). The ionization efficiency in nano-ESI is higher than in conventional ESI[31] and in MALDI. Consequently, the probability in MALDI of ionizing a molecule might be dependent on the number of acidic groups due to competition for the total available amount of charge.

The MALDI sensitivity also decreases for larger oligomers,[29] leading to a slight under-representation of these compounds in the mass spectrum. This can be explained by the reduced sensitivity of channel-plate

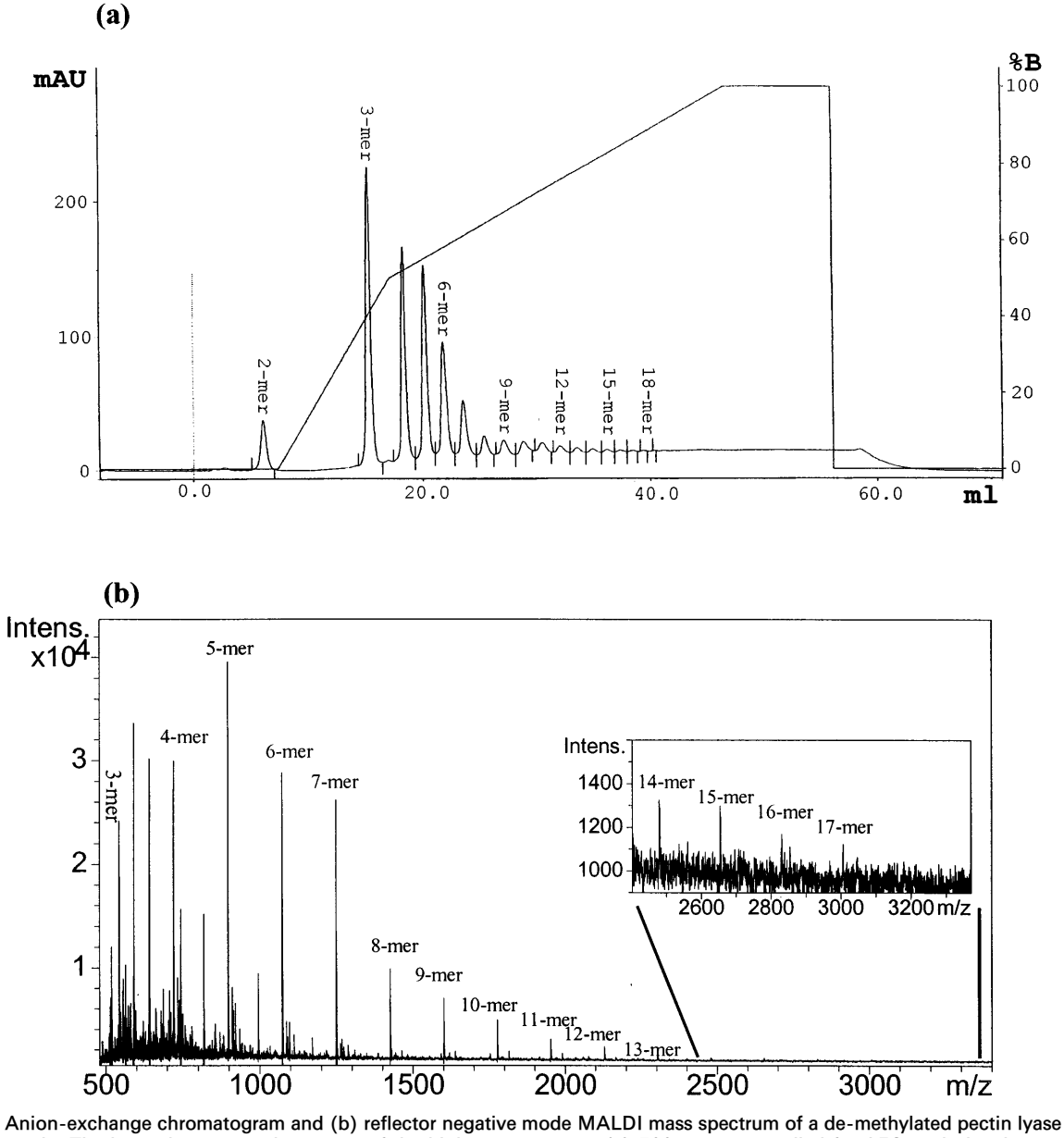

Figure 4. Anion-exchange chromatogram and (b) reflector negative mode MALDI mass spectrum of a de-methylated pectin lyase digest of high-ester pectin. The inset shows an enlargement of the higher mass range. (a) 500 μg were applied for AEC analysis whereas (b) 1 μg was consumed for MALDI sample preparation. A THAP–nitrocellulose matrix in a thin-layer preparation was used as the matrix.

detectors for larger ions with lower velocities in TOF-MS. In addition, metastable fragmentation increases with increasing oligomer length, also leading to an under-representation of larger molecules in reflector mode MALDI mass spectra. However, despite the observed discrimination effects, MALDI/MS allows a fast and sensitive qualitative analysis of enzymatic pectin digests and is, in contrast to detection based on UV absorption, applicable to enzymes that do not introduce a chromophoric group.

CONCLUSION

MALDI/MS was found to be a rapid, specific, sensitive and reproducible technique for the analysis of pectin digests. Methylation patterns of these digests can be analyzed to give important information on the structure of the studied pectins and the specificity of enzymes such as pectin lyase. Furthermore, typical modifications such as de-methylation can be verified by determining the correct molecular masses. Limitations in the analysis by MALDI are discrimination effects in the high- and low-mass ranges, and therefore calibration standards will be needed for quantitative analysis. As a consequence of the presence of acid groups, pectins tend to form adducts with sodium and potassium ions. Therefore, sample preparation including careful desalting is crucial for the analysis of complex mixtures. The best results were obtained upon desalting on miniaturized columns containing ammonium-loaded cation beads followed by the addition of diammonium hydrogencitrate and the use of a thin-layer preparation of THAP–nitrocellulose as matrix. Higher sensitivity and considerably less salt adducts were observed in the negative than in the positive ion mode.

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