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Determination of pathogen-related enzyme action by mass spectrometry analysis of pectin breakdown products of plant cell walls

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Abstract

An analytical approach using matrix-assisted laser desorption/ionization mass spectrometry for the structural characterization and assessment of the degree of polymerization of cell wall pectin-derived oligosaccharides (PDOs) in three regions of *Botrytis cinerea*-infected tomato fruit tissue is described. The PDOs were isolated from lesion centers (extensively macerated tissue), the area just beyond visible lesion margins, and healthy and intact tissue of an inoculated fruit, sampled at a distance from developing lesions. PDO mixtures were directly analyzed by mass spectrometry without chromatographic separation, after minimum cleanup by membrane drop dialysis. The structures identified implied the action of three different pathogen pectin-modifying enzymes. Modifications such as methyl esterification were identified by determination of exact PDO molecular masses and tandem mass spectrometry via collision-induced dissociation. We have identified four PDO series that were generated through the breakdown of homogalacturonan pectins. The decayed and lesion edge areas had fewer and less diverse PDOs than healthy tissues, possibly due to metabolic by-products of the pathogen. This analytical technique provides a simple and rapid method to characterize the pectin-derived oligosaccharides produced by in vivo digestion during pathogen infection.

Pectins are a family of complex and highly heterogeneous polysaccharides found in plant primary cell walls that make important contributions to the texture of fruits, vegetables, and their processed products [1,2]. Pectin backbones mainly consist of α-1,4-linked–D-galacturonic acid residues, with various degrees of methyl esterification of the carboxyl groups on GalUA[1] residues.

Plant biochemists and food scientists are highly interested in methods for characterizing pectin polymers to understand their physiological and biochemical roles in plants. Plant pathogenic fungi produce extracellular enzymes which can degrade the cell wall components of plants. These fungi not only degrade cell wall polymers to use their sugars as an important nutrient source but also digest the cell wall to aid in penetrating cells and

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[1] *Abbreviations used:* GalUA, galacturonic acid; PDOs, pectin-derived oligosaccharides; MALDI, matrix-assisted laser desorption/ionization; TOF, time of flight; FTMS, Fourier transform mass spectrometry; DP, degree of polymerization; HPAEC, high-performance anion exchange chromatography; CE, capillary electrophoresis; PGC, porous graphitized carbon; SPE, solid-phase extraction; TFA, tri fluoroacetic acid; ACN, acetonitrile; CID, collision-induced dissociation; PG, polygalacturonase; PL, pectin lyase; PME, pectin methylesterase.

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spreading through the plant tissue. Cell wall pectin-derived oligosaccharides (PDOs) are generated as pectins are digested in fruit as they ripen [3] and as they are digested by pathogen enzymes during tissue colonization [4–6]. It is thought that these oligosaccharides play important roles in the regulation of fruit development such as ripening [3] and are important factors for regulating fruit responses to infection by pathogens [7–9]. Thus, the characterization of PDOs is important for (1) identifying the enzymes that act on cell wall pectins, (2) understanding pectolytic enzyme specificity as expressed in vivo, and (3) identifying structures of potentially important cellular signals.

Over the years, many techniques such as high-performance liquid chromatography (HPLC), high-performance anion exchange chromatography (HPAEC), capillary eletrophoresis (CE), and NMR spectroscopy have been used for the analysis of enzymatic or chemical degradation products of pectin polymers [10–16]. These techniques are very useful for the identification and structure characterization of degraded GalUA oligomers. However, chromatographic techniques are not straightforward, particularly for modified pectin oligomers, which may be partially methylated or acetylated, because oligomers having similar molecular weight and charge may give the same chromatographic properties. NMR is a useful technique and direct tool for analyzing PDOs but it requires relatively large amounts (mg scale) of material and often requires chromatographic separation [14,16–18].

Mass spectrometry has been shown to be highly effective in the analyses of GalUA oligomers [19–26]. Electrospray ionization with an ion trap mass analyzer was employed by Korner et al [27]. Tandem mass spectrometry (MS^n) was used to sequence partially methyl-esterified pectin or oligosaccharides produced by the in vitro digestion of pectin polymers [21,28,29]. The groups of Voragen and Roepstroff have used matrix-assisted laser desorption/ionization–time of flight (MALDI-TOF) to analyze GalUA oligomers [22,27,30–34].

In this paper, we describe an analytical method for the characterization of cell wall pectin-derived oligosaccharides extracted from tomato tissues infected by the gray mold pathogen, *Botrytis cinerea*. MALDI-Fourier transform mass spectrometry (FTMS) has not been used for these types of studies. However, FTMS has the advantages of mass accuracy, high sensitivity, and tandem MS (MS^n), even for ions produced by MALDI. There is considerable interest in the types of enzymes that the pathogen uses to infect fruit. This study illustrates the determination of pathogen-specific endoglycosidases based on the oligogalacturonide products. It differs from earlier studies of oligogalacturonides in that it deals with the complexity and heterogeneity of direct biological samples. Previous studies have focused on model compounds and their in vitro enzymatic reactions [33,35,36]. To facilitate the analysis various desalting techniques for acidic oligosaccharides were studied to increase the MS sensitivity and speed. After minimum clean up by drop dialysis, we were able to identify important structural domains of PDOs and screen their species using a combination of MALDI-FTMS and MALDI-TOF mass spectrometers. This technique provides a simple and rapid method to characterize the pectin oligomers from plant cell walls. To our best knowledge, this is the first report of the structure analysis of cell-wall-derived oligosaccharides produced in fruit tissues by in vivo digestion during pathogen infection.

Experimental

Materials

2,5-Dihydroxybenzoic acid and polygalacturonic acid (sodium salt) were obtained from Sigma (St. Louis, MO). Solvents were of HPLC grade. Evaporation of small amounts of solvent was done on a Centrivap Concentrator (Labconco, Kansas City, MO). Porous graphitized carbon (PGC) cartridges for desalting were purchased from Alltech Associates (Deerfield, IL). Membrane containing nitrocellulose for drop dialysis was obtained from Millipore (Bedford, MA).

Preparation of pectin-derived oligosaccharides

Mature green tomatoes were harvested and held at 20 °C for 2 days. Their surfaces were then sterilized with 1% bleach, dried, and wounded in four places around the stem end to a depth of 2 mm with a sterile needle. Ten microliters of solution containing 10^5 *B. cinerea* spores were placed in each of three wounds. In the fourth wound 10 μL of sterile water was placed as a control. The spores had been frozen in sterile water at a concentration of 10^7/mL. They were thawed, diluted in 10 mM phosphate buffer (pH 7), 10 mM sucrose, and allowed to stand for 2 h at 20 °C before inoculation. Inoculated tomatoes were held for 5–7 days at 15 °C in a plastic container with moist paper towels to create high humidity, until the average lesion diameter was 20 mm. Then, with a scalpel, three areas of the tomato were excised: the macerated center of the lesion (designated "decay"), a 3-mm-wide ring of tissue just outside of the macerated area (designated "edge"), and an area of tissue at the blossom end of the fruit where there was no visible decay (designated "healthy-inoculated"). The excised tissue was frozen immediately in liquid nitrogen. In each experiment between 30 and 50 g of tissue of each type was collected. The frozen samples were cut into small sections with a scalpel and put into boiling 90% ethanol for 30 min. The solution was cooled and then ground in a Polytron (Beckman Instruments). The

slurry was vacuum filtered through glass fiber filters (GF/C, Whatman). The residue on the filters was suspended in water with 0.01% thimersol to prevent microbial growth and stirred overnight to solubilize some cell wall pectic polymers and wall digestion products. After centrifuging the slurry, the supernatant was collected, frozen, and lyophilized. The dried sample was dissolved in a small volume of 0.2 M $NH_4CO_2CH_3$ (pH 5.0) and assayed for uronic acid [37] and neutral sugar content [38]. A 1-mL fraction of this material was passed through a Bio-Gel P-4 column (25 cm length × 2 cm I.D, Bio-Rad Laboratories) and 1.1-mL fractions were collected. These fractions were assayed for uronic acid and neutral sugar content. Following the large peak of water-soluble uronic acid representing polymeric pectin, which appeared in the void fractions of the column, the fractions representing early and late-eluted uronic-acid-containing oligosaccharides in the fractionated volume of the column were pooled (generally pooled as early and late-eluting fractions, 22–30 and then 31–40, respectively). These two fractions of PDOs were frozen, lyophilized, and resuspended in water to give a concentration of uronic acid between 0.3 and 2 mg/mL. These mixtures were used for characterization of the PDOs by MALDI-MS. Aliquots of the P-4 fractionated and pooled fractions were also separated by HPLC using a Dionex instrument fitted with a Carbopac PA-1 column and PAD detection [3].

Desalting PDOs

Drop dialysis. Fractions (5 μL) of PDOs were treated with Bio-Gel P-4 separation and loaded on a nitrocellulose membrane (0.025 μm pore size) for drop dialysis against nanopure water (1–2 h). For MALDI analysis, a 1-μL sample desalted by drop dialysis was directly applied to the MALDI probe followed by a matrix.

On-probe cleanup by NH_4 resin. The cation exchange resin (ammonium form) was prepared by a previously published procedure [39]. An H^+-cation exchange resin (100–200 mesh; Bio-Rad) was stirred in a 1 M ammonium acetate solution for 12 h. The product was filtered and washed with 1 M ammonium acetate solution, deionized water, acetone, and hexane. The resin was dried and stored for future use. After loading the sample on the MALDI probe, a NH_4 resin was added to remove alkali metals.

Porous graphitized carbon cartridge. PDOs were purified by solid-phase extraction (SPE) using a porous graphitized carbon cartridge. A PGC cartridge was washed with H_2O followed by 0.05% (v/v) TFA in 80% ACN/H_2O (v/v). The solution of PDOs was applied to the PGC cartridge. Subsequently the cartridge was washed with nanopure water at a flow rate of about 1 mL/min to remove salts and buffer. PDOs were eluted with 10% ACN in H_2O and 40% ACN in 0.05% TFA in H_2O. Each fraction was collected and concentrated in vacuo prior to MALDI analysis.

Mass spectrometry

Mass spectra were recorded on an external source HiResMALDI (IonSpec, Irvine, CA) equipped with a 7.0-Tesla magnet. The HiResMALDI was equipped with a pulsed YAG laser (266 nm). MALDI-TOF mass spectra were recorded in linear mode using a Proflex III (Bruker Daltonics). 2,5-Dihydroxy-benzoic acid was used as a matrix (5 mg/100 μL in ethanol). For MALDI analysis, 1 μL of the desalted sample was applied first to the MALDI probe followed by the matrix (1 μL, 1 nmol). The sample was dried under a stream of air and subjected to mass spectrometric analysis.

Collision-induced dissociation (CID)

A desired ion was readily selected in the analyzer with the use of an arbitrary wave form generator and a frequency synthesizer. All CID experiments were performed at +1000 Hz off resonance to the cyclotron frequency of the isolated ion. The CID excitation time was 1000–2000 ms. Two pulses of argon were introduced into the analyzer chamber at 0 and 500 ms for collision gas. The excitation voltages ranged from 8 to 13 V depending on the desired level of fragmentation and the size of the oligosaccharide.

Results and discussion

Preseparation of PDOs on a Bio-Gel P-4 column

The PDO samples obtained from the *B. cinerea*-infected tomato fruit tissues were redissolved in a small volume of 0.2 M $NH_4CO_2CH_3$ (pH 5.0) and assayed for uronic acid and neutral sugar content. The PDOs were separated from a large amount of higher molecular weight uronide (water-soluble pectins) and roughly desalted by passage through the Bio-Gel P-4 column. The elution profiles of the three samples are shown in Fig. 1. These P-4 fractions were assayed for uronic acid. All fractions eluted after the void volume (V_0, approximately fraction 18) contained polymeric uronide. The extracts from the healthy-inoculated and the edge tissue samples produced similar profiles with a maximum at the void volume consistent with large, water-soluble uronide polymers in the healthy tissues. In contrast, the P-4 uronic acid profile for the extract from the decay tissues had a maximum at fraction 38, in the middle of the column's fractionation range, indicating the extent of pectin depolymerization in the decayed tissue.

Fractions collected from the Bio-Gel P4 column contained substantial buffer and salts, which interfered with

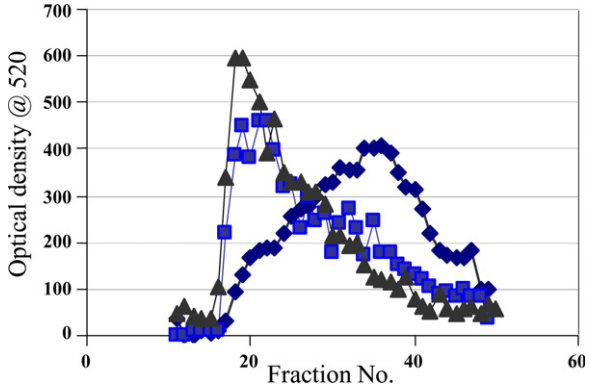

Fig. 1. Profile of uronic acid distributions in fractions from Bio-Gel P-4 separations of water-soluble pectin from three areas of tomato fruit infected with *Botrytis cinerea*: decay (◆), edge (■), and healthy-inoculated (▲).

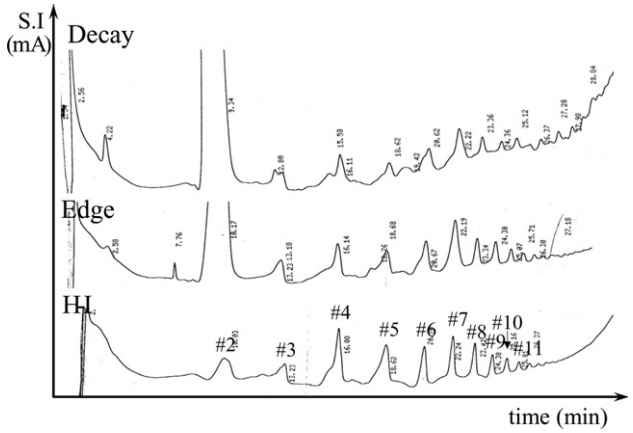

Fig. 2. HPLC separation of PDO mixture from three tomato tissues through a Bio-Gel P4 column.

the MS analyses. Various desalting methods for PDOs were studied to increase MS sensitivity. Solid phase extraction using a PGC cartridge, on-probe cleanup using NH_4 resin, and membrane drop dialysis were compared. The PGC-SPE effectively removes salts and buffer but requires relatively more time than the other methods. We obtained poor MS sensitivity with on-probe cleanup using NH_4 resin, although it is relatively fast. The use of cation-exchange resins in a resin-filled column with a 5% ammonia solution for desalting was reported by the Roepstorff group [22] for samples from commercial sources. In our hands, the best result for removing salts was obtained with drop dialysis against nanopure water through a 0.025-μm cut-off membrane. This cleanup step takes only 1–2 h per sample, although multiple samples can be treated easily and simultaneously. This procedure allows rapid analysis of complex mixtures of PDOs, even without preseparation by HPLC, CE, or gel electrophoresis before MS analysis. All MS spectra reported were obtained after minimal sample cleanup by employing drop dialysis, alone.

Separation of PDOs using HPLC

A number of attempts to separate the PDO components into pure fractions for further analyses were made however, the oligosaccharide heterogeneity of the mixtures made this difficult. Fractions 22–40 from the Bio-Gel P-4 column were combined and separated by HPAEC-PAD. Fig. 2 shows HPLC separation of the PDO samples from the three tissue regions—decay (D), edge (E), and inoculated-healthy (H-I). The three samples show very similar chromatograms with a number of overlapping peaks that were clearly not resolved in the HPLC. As a control, polygalacturonic acid from a commercial source was hydrolyzed with dilute HCl. The resulting PDOs were subjected to the same HPLC separation and yielded the chromatogram in Fig. 3A. The chromatogram shows a very regular pattern of peaks with the larger hexuronic acid oligomers eluting at later times. It was possible to distinguish peaks corresponding to degrees of polymerization (DP) up to 12 in the HPLC chromatogram. Mass spectrometry of individual HPLC fractions yielded a single product corresponding to the oligomer DP (data not shown). It was not possible to correlate the HPLC peaks in the HCl-digested polygalacturonic acid control with those from the tomato fruit tissues. The peaks from the three fruit samples were generally asymmetric and some had significant tailing. The lack of a simple correlation between the HPLC of the samples and the control suggested that the in vivo generated PDOs were not simply the result of the hydrolysis of unmethylated cell wall homogalacturonan. The MS of the samples supported this notion (see below). The MALDI-FT mass spectrum of the control mixture yielded a single oligomeric series consistent with the HPLC. Conversely, the samples from the fruit yielded a number of overlapping progressions corresponding to a number of oligomeric series. The MS analyses of the isolated HPLC peaks in the control produced single products, while the analyses of individual HPLC-separated peaks from the fruit produced complex spectra varying in relative abundances but generally showing the same oligomers in each, suggesting that only partial separation was accomplished by HPLC (see below). For this reason, the HPLC was abandoned and the sample mixtures from the pooled Bio-Gel P-4 column fractions were analyzed directly using MS. The individual components were isolated by tandem MS, and structure elucidations were accomplished by CID.

Characterization of PDOs by mass spectrometry

Pectin oligomers produce intense signals ($[M-H]^-$) in negative mode because of the negative charge from the deprotonated carboxyl group on galacturonic acid.

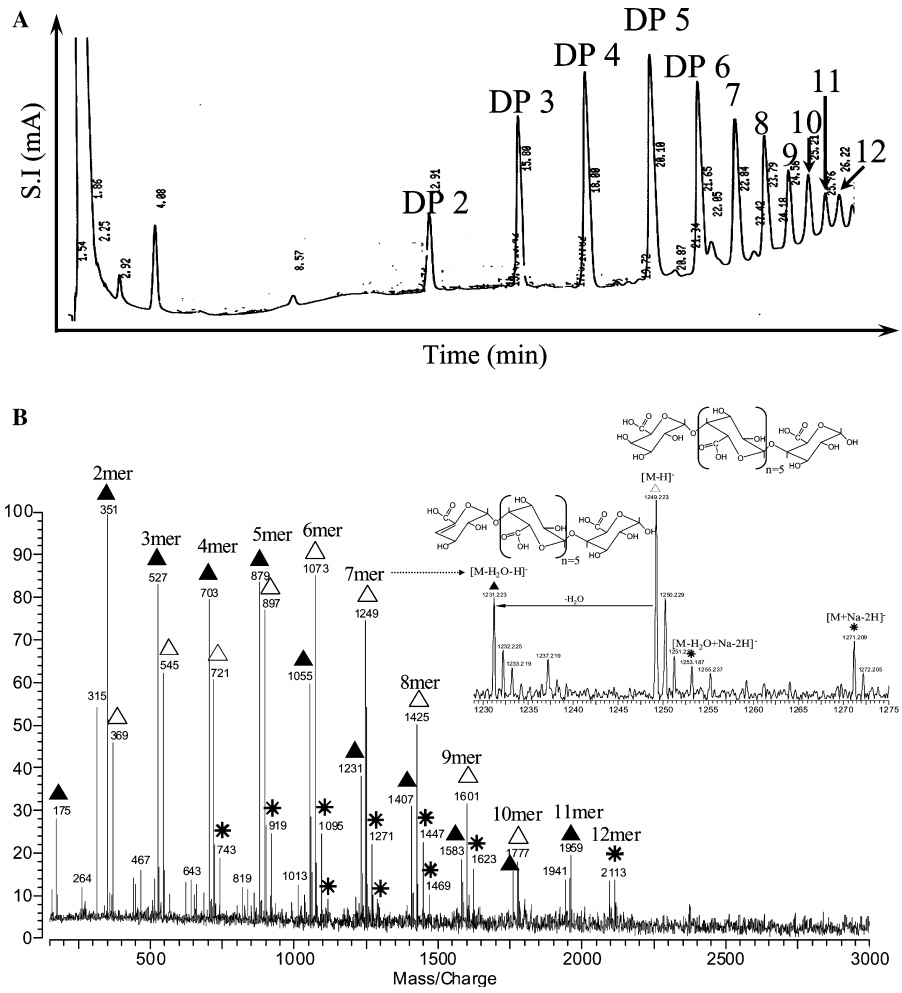

Fig. 3. (A) HPLC separation of PDOs prepared by an acid digestion of deesterified polygalacturonic acid. (B) Negative MALDI-FTMS spectrum of PDOs prepared by an acid digestion of deesterified polygalacturonic acid.

As the GalUA readily forms salts with alkali metals, especially Na($[M + Na]^+$), multiple peaks ($[M - nH^+ + (n+1)Na]^+$, n = No. of GalUA) are observed as the result of partial alkali–salt formation in the positive mode. Similar observations with regard to multiple sodium attachment have also been reported using electrospray ionization [40]. Fig. 3B shows the MALDI-FT mass spectrum of the acid-hydrolyzed pectin whose HPLC is shown in Fig. 3A. There are two major oligomeric series separated by one H_2O unit. Both series are expected products of acid hydrolysis of pectins. The analysis of each HPLC peak shows an oligomer corresponding to both acid hydrolysis products. Also the ion marked with asterisks corresponds to the replacement of one hydrogen with one sodium atom. In highly acidic samples, this type of substitution is often observed even in negative mode. Diligent removal of the salt is necessary to minimize this effect, and the drop dialysis technique is the best approach that we have found. Often mass spectra containing higher order substitution are produced when the samples have not been properly desalted. The additional peaks complicate the spectra; drop dialysis minimizes the problem. Attempts to analyze the individual fractions from the HPLC separations of the three fruit samples were made. The MALDI-FT mass spectrum of the HPLC peak labeled 3 (retention time 13.23 min; Fig. 2) is shown in Fig. 4A. A later-eluting fraction (11, retention time 25.81 min) is shown in Fig. 4B. The earlier fraction is richer in the oligomers of lower DP, while the later fraction is richer in larger oligomers. The fraction also shows a number of Na-exchanged peaks, suggesting that the HPLC separation did not sufficiently remove the salt contaminants. However, the MALDI-FTMS analysis indicated that both fractions contained oligomers of a range of DPs, suggesting that the HPLC had not accomplished effective size discrimination. Consequently, no further analyses were performed with the HPLC fractions.

Direct PDO structural analysis of fruit-derived samples was accomplished using drop dialysis of Bio-Gel P-4 purified samples. The samples were analyzed by MALDI-FTMS and MALDI-TOF MS. Fig. 5 shows the MALDI-FT mass spectrum of PDOs from healthy-inoculated tissue obtained from a *B. cinerea*-infected tomato.

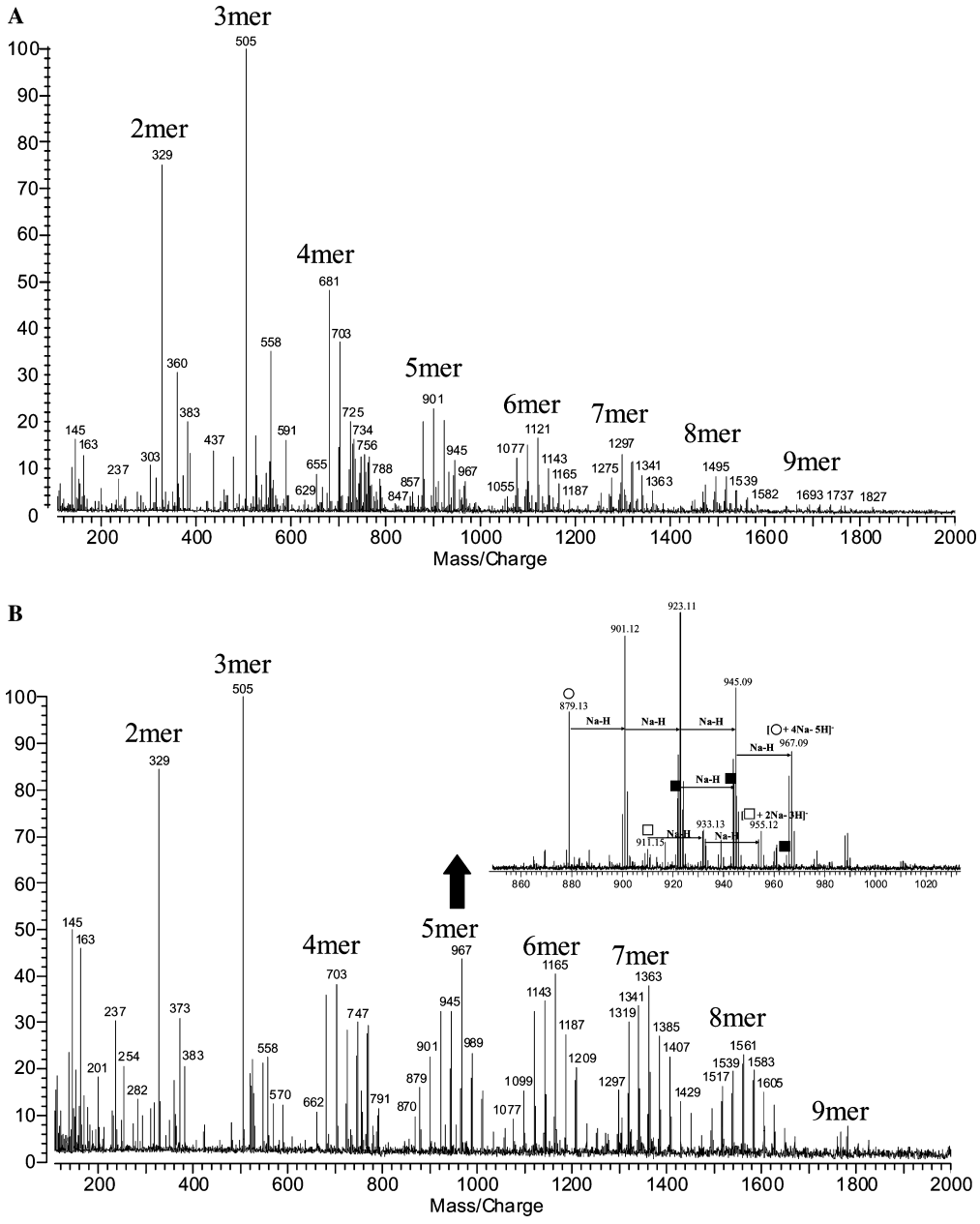

Fig. 4. Negative MALDI-FTMS spectrum of HPLC fraction of PDOs from healthyinoculated tissue with retention time at (A) 12.23 min (fraction 3) and (B) 25.51 min (fraction 11).

Several PDO series were observed with signal separation of 176 Da, corresponding to the GalUA residue. The following series of peaks were identified: series A (filled circles) with *m*/*z* 329.03, 505.06, 681.09, 857.12, 1033.15, and 1209.18; series B (open circles) with *m*/*z* 351.05, 527.08, 703.11, 879.14, 1055.17, 1253.18 (an H^+ replaced by Na^+), and 1451.20 ($2H^+$ replaced by $2Na^+$); series C (open squares) with *m*/*z* 383.06, 559.09, 735.12, 911.15, and 1087.18; and series D (filled squares) with *m*/*z* 569.08, 745.12, 921.15, 1097.17, and 1273.21. All major signals correspond to a deprotonated parent ion ($[M-H]^-$). The GalUA readily forms salts with sodium as the number of GalUA in an oligomer is increased. Although the PDOs were not doped with Na, partial sodium adduct formation was observed. PDOs, including Na-exchanged peaks, are summarized in Table 1. The DP range of these PDOs is 2–8. The DP was analyzed more efficiently with MALDI-TOF as metastable decomposition sometimes affected MALDI-FT mass spectra. Fig. 6 shows the MALDI-TOF-MS spectrum of GalUA oligomers from healthy-inoculated fruit tissue with increasing DP (2–16 mer). Several series of PDO peaks were observed. The labeled symbols correspond to the same series of PDOs as those deduced from the FTMS spectrum (Fig. 5). The negative mode MALDI-FTMS spectra of PDOs of edge and decay fruit tissue obtained from infected tomato are

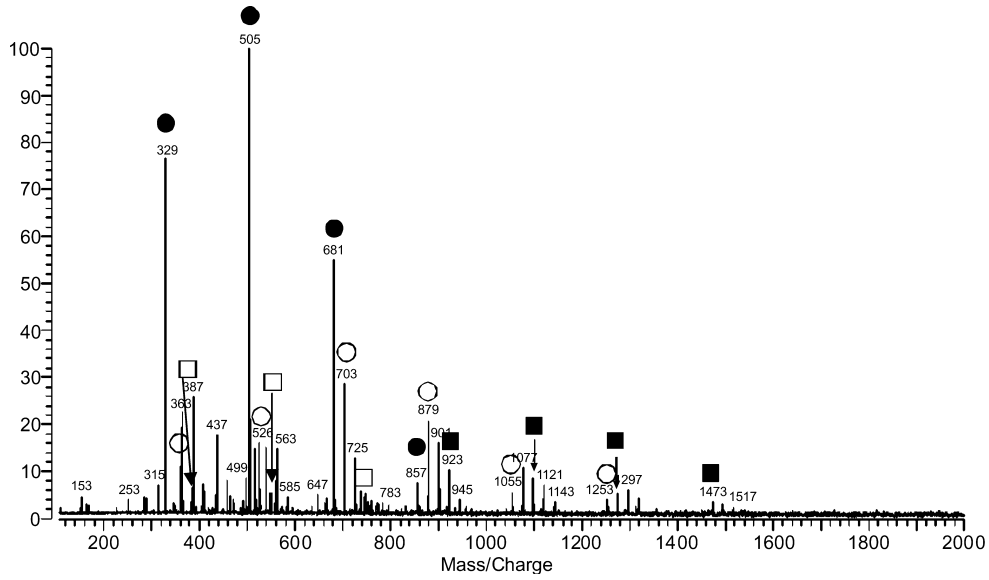

Fig. 5. MALDI-FTMS spectrum of PDOs from healthy-inoculated tissue obtained from a *B. cinerea*-infected tomato in negative mode. See Fig. 9 for key.

Table 1
Mass list of PDOs observed in MALDI-FTMS spectrum (see Figs. 5–7)

Symbol	Molecular ion	Observed mass						
		DP 2	DP 3	DP 4	DP 5	DP 6	DP 7	DP 8
●	$[M-H]^-$	329.03	505.06	681.09	857.12	1033.15	1209.18	1385.21
● + Na	$[M+Na-2H]^-$	351.01	527.04	703.07	879.10	1055.13	1231.16	1407.19
● + 2Na	$[M+2Na-3H]^-$		549.03	725.06	901.09	1077.12	1253.15	1429.18
○	$[M-H]^-$	351.05	527.08	703.11	879.14	1055.17	1231.20	1407.23
○ + Na	$[M+Na-2H]^-$	373.03	549.06	725.09	901.12	1077.15	1253.18	1429.21
○ + 2Na	$[M+2Na-3H]^-$		571.05	747.08	923.11	1099.14	1275.17	1451.20
□	$[M-H]^-$	383.06	559.09	735.12	911.15	1087.18	1263.21	1439.24
□ + Na	$[M+Na-2H]^-$		581.07	757.10	933.13	1109.16	1285.19	1461.22
□ + 2Na	$[M+2Na-3H]^-$			779.09	955.12	1131.15	1307.18	1483.21
■	$[M-H]^-$			745.12	921.15	1097.18	1273.21	1449.24
■ + Na	$[M+Na-2H]^-$				943.13	1119.16	1295.19	1471.22
■ + 2Na	$[M+2Na-3H]^-$				965.12	1141.15	1317.18	1493.21

shown in Fig. 7. These tissues had less diverse PDOs of generally smaller DP (edge: 2–8 mer; decay: 2–6 mer) than those identified in the inoculated-healthy fruit tissue. This likely indicates that the oligomers in infected tissues are generated and then modified by *B. cinerea* pectin-digesting enzymes [41,42]. Studies of pathogen pectolytic enzyme activities in and beyond macerated tissue areas of infected tomatoes have indicated that different enzymes are present in lesion centers and less heavily disrupted areas beyond lesion margins [42]. *B. cinerea* is known to express multiple genes encoding the pectin hydrolase, polygalacturonase (PG). Particularly intriguing is the presence of the PDOs in the healthy tissue at a distance from all visible indications of tissue rot. The PDOs may have been generated by pathogen enzymes. However, it may be more reasonable to propose that the localized but expanding zone of infection has generated signals that have induced the healthy tissue to activate endogenous pectolytic enzymes and these then acted to generate the PDOs found in healthy tissues. Bergey et al. [43] have reported the activation of tomato PG in leaves at a distance from a wound site. A similar pathway may operate in pathogen-infected fruit. We (Yang et al., unpublished) have shown the induction of the tomato fruit PG-encoding gene in healthy tissues of infected fruits.

Although PDO masses and the mass differences are sufficient to determine the oligomeric families, tandem MS using CID was also used to confirm the assignment. For example, the ion with *m*/*z* 681.086 shown in Fig. 5 was isolated using standard FT-ICR MS procedures (arbitrary waveform). Subsequent MS^2 of this ion yielded the spectrum in Fig. 8A. The fragment ion observed at *m*/*z* 505.060 corresponds to the loss of a

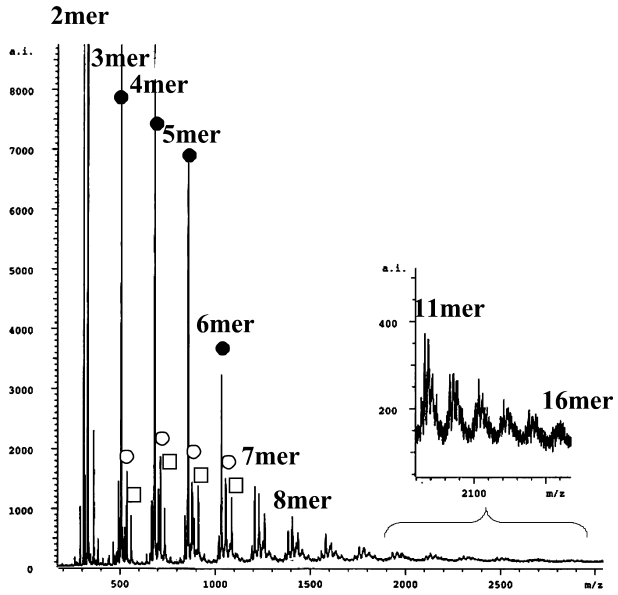

Fig. 6. MALDI-TOF-MS spectrum of PDOs from healthy-inoculated tissue obtained from a *B. cinerea*-infected tomato in negative mode. See Fig. 9 for key.

galacturonic acid residue (−176 mass units). A further fragmentation of this ion in an MS^3 experiment yielded the ions with *m*/*z* 329.032, corresponding to the loss of another galaturonic acid residue (Fig. 8B). Using CID, the oligomer unit and the mass of the oligomer head group could be identified.

The PDO structures deduced from the combined MS analysis are summarized in Fig. 9. The PDO head group of series A (filled circles) is still unknown. MS^4 of the ion with *m*/*z* 329.032 was performed using various collision energies to identify the molecular ion but no fragmentation was observed even at very high energy. As a control, the PDOs prepared by acid digestion of polygalacturonic acid were analyzed by MALDI-MS (Fig. 3B). However, we did not observe the ion with *m*/*z* 329.032 or PDOs of the class A series (filled circles). As shown in Fig. 2, the intense peak at 9–10 min in the HPLC fractionations was observed only in three tomato tissues extracts and not in the control. We found that this peak did not contain hexuronic acid. This suggests that this peak may be related to the unknown head group of series A. Elemental composition based on accurate mass (329.032 ± 0.002) and restriction to C, H, N, and O gave 10 hits with only one candidate, $C_7H_{11}N_3O_{12}$, resembling a carbohydrate formula. We believe that this residue may represent a modified or unknown sugar which is bound to a pectin polymer backbone and is digested during pathogen development.

Analysis of enzyme specificity

Determination of the DP and structures of PDOs is important for understanding the structure–function relationship of pectin oligomers and the specificities of pathogen and plant pectolytic enzymes. Enzymatic cleavage of pectins by bacteria or fungi has been reported in the literature and is considered to be an early important aspect of pathogen colonization of plant tissues [4,6]. Both pectin hydrolases (e.g., PG) and pectin lyases (PL) have been reported to be important pathogen enzymes involved in host plant pectin polymer degradation [44]. PDOs that would be produced by both of these classes of pectolytic enzymes are found in *B. cinerea*-infected tomato fruits (Fig. 9). In classes B and D (open circles and filled squares in Figs. 4 and 5), PDOs with a 4,5-unsaturated nonreducing end are shown. These PDOs will be generated by PL action on homogalacturonan polymers. The PDOs with a saturated nonreducing end in class C (open squares in Figs. 4 and 6) would have been generated by PG action on homogalacturonans, with -*endo*-, rather than *exo*-, PG being more likely. In fact, PDO "synthesis" could involve PG action on a homogalacturonan substrate and either PL action on the PG products that are still sufficiently polymeric to be substrates for the lyase or PL action followed by PG action. The PDO structure of the head group in class A (filled circles in Figs. 4 and 6) is not yet completely characterized. It is likely that other enzymes are also involved in the generation of infection-related PDOs. Some structures (classes C and D) contain methylester groups, suggesting that pectin methylesterase (PME) might have acted on other residues in the backbone to open up sites for PL or PG action. A recent report [45] indicates that tomato fruit express genes encoding PL when they ripen. Thus, the unsaturated PDOs that have been identified in the healthy tissues of infected fruits may also be generated by the action of endogenous, rather than pathogen, enzymes.

The FTMS spectra of PDOs obtained from the three tomato tissues were very similar, although the edge and decay samples had fewer and less diverse PDOs. However, a distinction between these and PDOs from healthy tissues was identified by MALDI-TOF. The DP of PDOs from healthy tissues (~16 mer) is greater than that in edge and decay samples (~8 mer). This suggests that pectolytic enzyme activities, including PG, PL, and PME, are more diverse and/or active in infected areas. This can be explained by the presence of pathogen enzymes acting, perhaps in addition to fruit pectolytic enzymes, while only fruit enzymes are responsible for the PDOs in healthy tissues from infected fruits. It also may reflect the more intense conversion of pectin polymers into metabilizable substrate to be used by the pathogen biomass in lesion centers. We believe that PDOs are signals regulating plant responses to pathogens, so structural analysis will add to our understanding of factors affecting the expression of plant defenses and fruit susceptibility. This will include the testing of the ability of the different classes of infected fruit PDOs to activate defense gene expression in infected tomato fruit tissues.

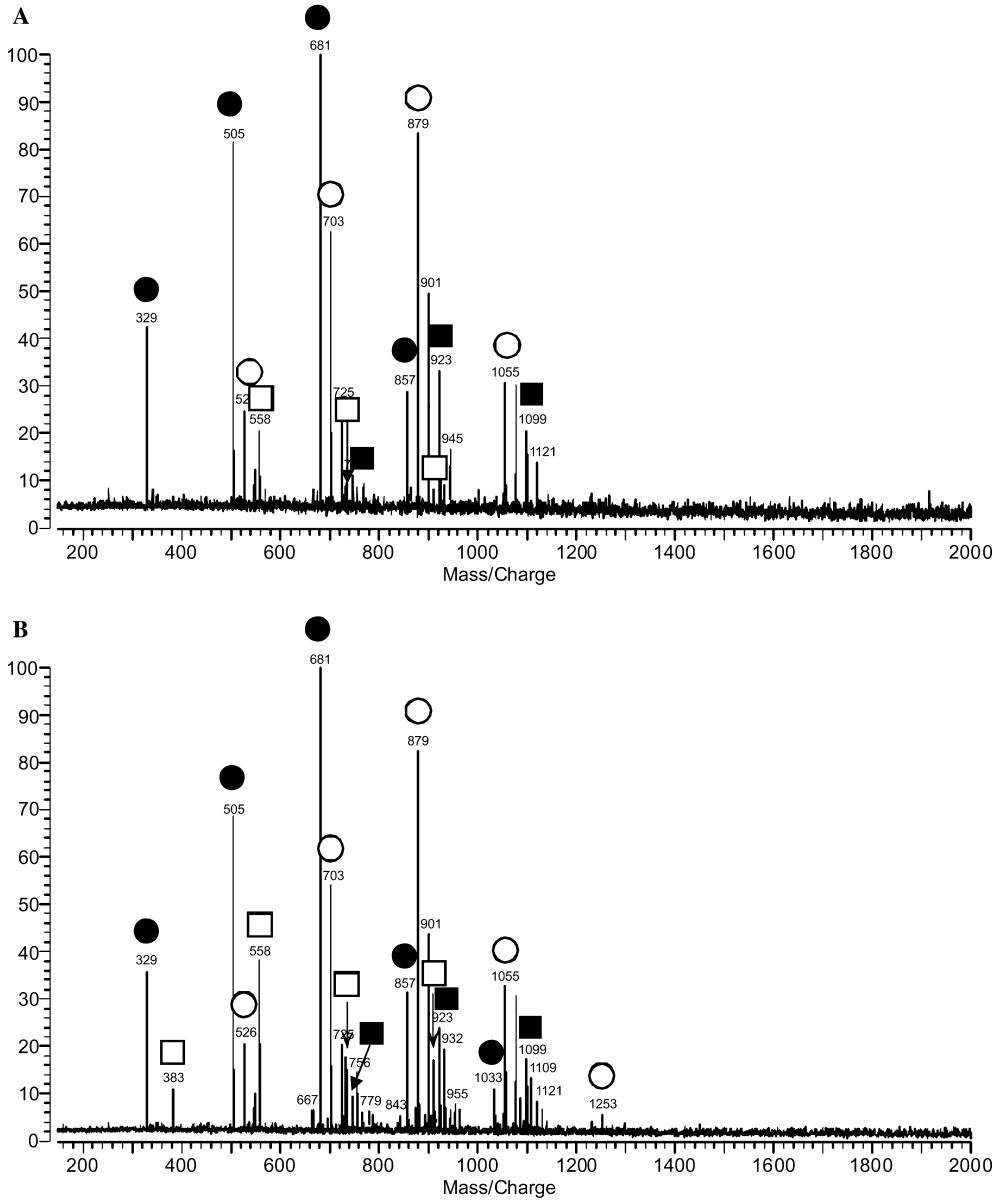

Fig. 7. MALDI-FTMS spectrum of PDOs from (A) decay and (B) edge tissue obtained from a *B. cinerea*-infected tomato in negative mode. See Fig. 9 for key.

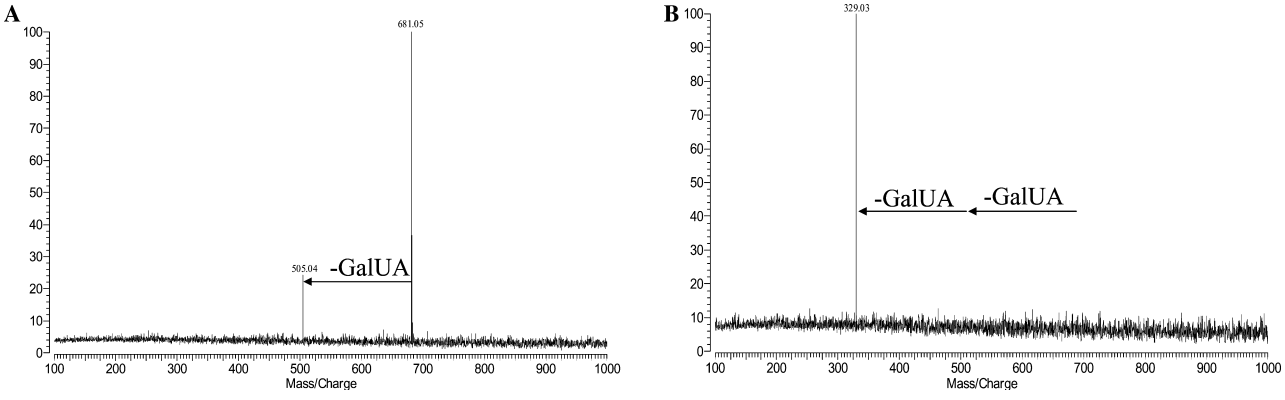

Fig. 8. CID spectrum of *m/z* 681 obtained from PDO oligomer mixture (A) MS/MS (MS^2, 681/505) and (B) MS/MS/MS (MS^3, 681/505/329).

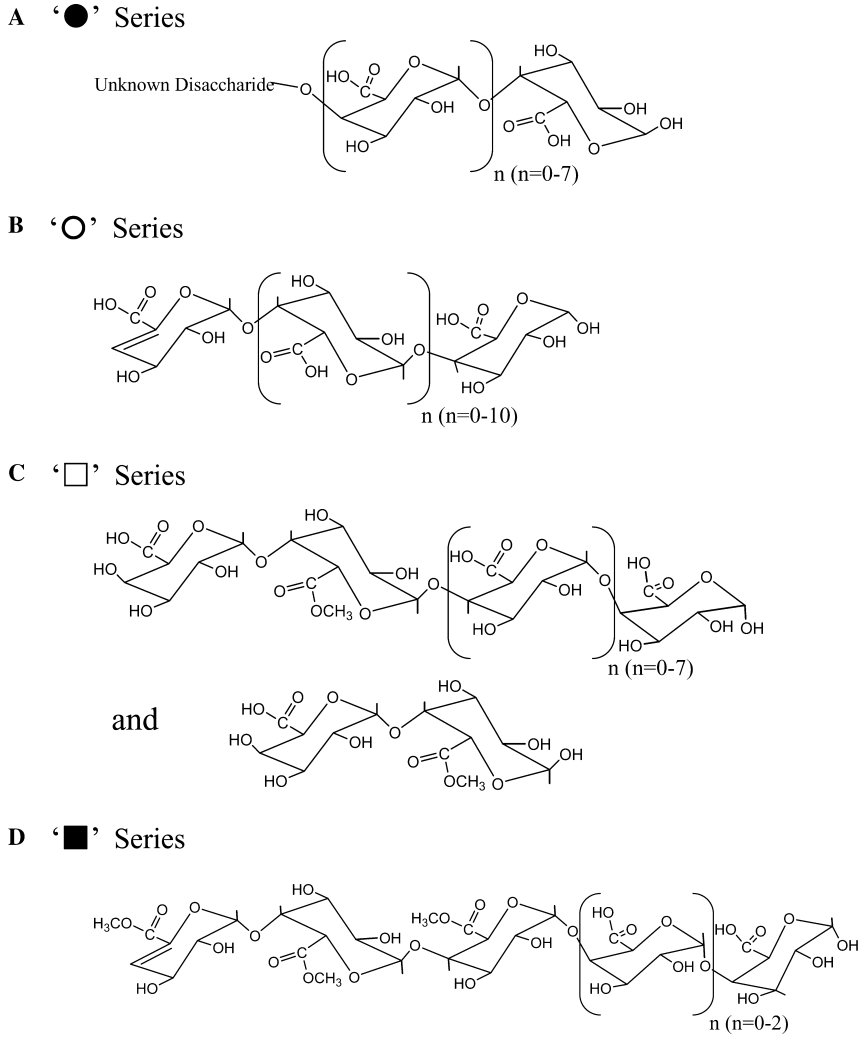

Fig. 9. The four classes of PDOs deduced from FTMS analysis.

Conclusions

Pectin-derived oligosaccharides in *B. cinerea*-infected tomato tissues were isolated and characterized by MALDI-MS after minimal sample cleanup by drop dialysis. The highest sensitivity in MS was obtained in the negative mode. We were able to obtain more exact information about PDO degree of polymerization and a detailed structure analysis of PDOs using two types of mass spectrometry, MALDI-FTMS and MALDI-TOF. This combination is a rapid, specific, sensitive, and reproducible technique for the characterization of PDOs without time-consuming sample preparation. The PDOs obtained from three different portions of tissue from infected fruits were apparently generated through the breakdown of homogalacturonan pectins. The PDO structures suggest that *endo*-polygalacturonase, pectin lyase, methylesterase activities were involved in the pectin backbone scission. The PDOs from edge and decay areas of infected fruits, tissues with which the gray mold pathogen interacted directly, had fewer and less diverse PDOs than those from the healthy tissues remote from infection sites. We believe that the characterization of these endogenous pectin breakdown products will add to our understanding of the regulation of processes that determine the outcome of plant interactions with pathogens.

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