Analysis of Pectin Structure by HPAEC-PAD 006286

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1 Introduction

Pectins are complex, branched, acidic polysaccharides that are located throughout the plant primary cell wall matrix. Pectin structure in cell walls has been described as block-like (Jarvis 1984) in which smooth and hairy regions exist (de Vries et al. 1982). The smooth pectin regions consist of homogalacturonan (McNeil et al. 1984). Variation in the degree of methyl-esterification exists within pectin, with calcium mediating the cooperative cross-linking of pectin chains containing seven adjacent nonesterified galacturonic acid residues (Powell et al. 1982). When pectin is treated with endo-polygalacturonase (endo-PG[1]), the homogalacturonan degrades to oligogalacturonic acids leaving the hairy, branched pectic regions unhydrolyzed (reviewed by O'Neill et al. 1990). The hairy regions of pectin consist of rhamnogalacturonan I (RG-I), rhamnogalacturonan II (RG-II), xylogalacturonan, and RG with arabinan side chains that can be separated based on size following endo-polygalacturonase (O'Neill et al. 1990) or rhamnogalacturonase (Voragen et al. 1993) hydrolysis. RG-I consists of alternating rhamnose and galacturonic acid residues in the backbone. Arabinose- and galactose-containing side chains are attached to the rhamnose residues. RG-II, from the cell walls of suspension-cultured sycamore cells, consists of a not fully characterized structure that contains unusual sugars such as apiose, 3-deoxy-D-manno-octulosonic acid (KDO), aceric acid, methyl-fucose, and methyl-xylose. Xylogalacturonan, present in the modified hairy regions (MHR, residue remaining after enzymatic liquefaction of pectin) of apple pectin, consists of a galacturonan backbone with each galacturonic acid residue substituted at the 3-position with a single β-linked xylose

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[1] Abbreviations: ASP, alkaline soluble pectin; CDTA, 1,2-cyclohexanediaminetetraacetic acid; CSP, chelator-soluble pectin; DEAE, diethylaminoethyl; DP, degree of polymerization; endo-PG, endo-polygalacturonase; FAB-MS, fast atom bombardment-mass spectrometry; GPC, gel-permeation chromatography; HPAEC-PAD, high-performance anion-exchange chromatography and pulsed amperometric detection; HPLC, high-performance liquid chromatography; HPSEC, high-performance size-exclusion chromatography; MHR, modified hairy regions; MHR-S, saponified modified hairy regions; MS, mass spectrometry; NMR, nuclear magnetic resonance; PAD, pulsed amperometric detection; PME, pectin methyl-esterase; QAE, diethyl[2-hydroxypropyl]aminoethyl; RG, rhamnogalacturonan; UV, ultraviolet; WAF, weeks after flowering.

residue (Voragen et al. 1993). After approximately ten galacturonic acid residues the xylogalacturonan backbone is interrupted by a rhamnose residue to which arabinose- and galactose-rich side chains may be attached. RG with arabinan side chains was also characterized in apple MHR as smaller in molecular weight compared to xylogalacturonan but larger than RG-I (Voragen et al. 1993). Curve-fitting of high-performance size-exclusion chromatograms indicated the presence of five macromolecular components for pectins from various sources (Fishman et al. 1989b, 1992). Electron microscopy of shadowed pectic components demonstrated a multimodal distribution of chain lengths (Fishman et al. 1992; McCann et al. 1992). Macromolecular pectic components are thought to represent different levels of noncovalent aggregation of these acidic polysaccharides. In this chapter, we demonstrate that enzymatic degradation of pectin subunits followed by high-performance anion-exchange chromatography and pulsed amperometric detection (HPAEC-PAD) analysis yields valuable information concerning the internal structure of these pectic subunits.

2 Pectin HPLC Separations

2.1 GPC

Gel-permeation (GPC) or size-exclusion chromatography has long been used to separate pectins based on size. Determination of pectin molecular weight is difficult with this type of chromatography due to aggregation. However, high-performance size exclusion chromatography (HPSEC) has been used to measure pectin molecular weight and size (Barth 1980; Fishman et al. 1984, 1987; Deckers et al. 1986), as well as to monitor pectin depolymerization by pectate lyase (Neidhart et al. 1994). These columns are calibrated with either pullulan, dextran, or pectin standards. HPSEC columns require long run times and possess relatively low oligosaccharide resolution compared to other forms of high-performance liquid chromatography (HPLC). Following the first reports of pectic oligosaccharide resolution with gel filtration chromatography (Rexova-Benkova 1970; Thibault 1980), Bio-Gel P-4 has recently been used to purify oligogalacturonic acids up to a degree of polymerization (DP) of 6 (MacDougall et al. 1992) and to monitor pectin molecular weight changes during fruit repening in avocado and tomato (Huber and O'Donoghue 1993). While the resolution of the P-4 column is better than other gel-filtration or size-exclusion columns, the run times are still rather long (8–11 h).

2.2 Ion-Exchange and Ion-Pair RP

For over 40 years, ion-exchange chromatography has been used to separate pectins and their degradation products (Derungs and Deuel 1954). Separation of pectin

from neutral polysaccharides has typically involved ambient pressure ion-exchange chromatography on DEAE Sepharose or Cellulose (de Vries et al. 1981; Rouau and Thibault 1984), and QAE-Sephadex (Nothnagel et al. 1983). High-performance ion-exchange chromatography has been used to separate oligogalacturonic acids (Voragen et al. 1982) with a DP of up to 25 (Maness and Mort 1989), to isolate oligogalacturonic acids up to DP 7 in gram quantities (Hotchkiss et al. 1991), to determine the degree of esterification of pectin and monitor deesterification with pectin methyl esterase (Schols et al. 1989), and to monitor enzymatic depolymerization of polygalacturonic acid (Endress et al. 1991). While the run times are reasonable (<1 h), this method requires the use of a UV-absorbance detector which produces a significant baseline shift during the course of the buffer gradient. In order to resolve oligogalacturonic acids above approximately DP 10, it is necessary to first form 2-amino-pyridine derivatives (Maness and Mort 1989). Ion-pair reversed-phase chromatography has also been used to separate oligogalacturonic acids (Heyraud and Rochas 1982; Voragen et al. 1982) and monitor depolymerization of polygalacturonic acid by pectate lyase (Preston and Rice 1991). However, oligogalacturonic acids greater than DP 11 could not be resolved by this method. Additionally, the column effluent, monitored by UV absorbance, was subject to baseline shifts.

2.3 HPAEC-PAD

HPAEC-PAD is the most selective HPLC method available for the separation of monosaccharides, oligosaccharide positional isomers, and homopolymer series of oligosaccharides (Hardy and Townsend 1988; Koizumi et al. 1989; Lee 1990). This method allows for the separation of underivatized oligosaccharides up to DP 80 (Koizumi et al. 1989). The detector is as sensitive as the highest sensitivity refractive index HPLC detectors, while almost insensitive to changes in mobile phase buffer concentration. Hotchkiss and Hicks (1989, 1990) first applied HPAEC-PAD to the separation of pectic oligosaccharides. Subsequently, numerous publications have appeared demonstrating the value of HPAEC-PAD in structural analysis of pectins.

A series of oligogalacturonic acids terminated by a 4,5-unsaturated function at their nonreducing ends was observed to elute later from the CarboPac PA1 column used for HPAEC-PAD than the corresponding like-DP saturated counterparts (Hotchkiss et al. 1990; Hotchkiss and Hicks 1993; Lieker et al. 1993). This allowed for HPAEC-PAD analysis of pectate lyase (highly purified and "recombinant") action patterns demonstrating that both unsaturated and saturated oligogalacturonic acids were generated from polygalacturonic acid and plant cell walls during a depolymerization time course (Hotchkiss and Hicks 1993; Hotchkiss et al. 1995b). Oligogalacturonic acids were retained longer on the CarboPac PA1 column than the same DP oligosaccharide consisting of galacturonic acid residues terminated at the reducing end by a single galactosyl residue (Mort et al. 1993). In order to analyze the pattern of pectin methyl-esterification, the latter oligosaccharides

were formed by reduction of methyl-esterified galacturonic acid residues followed by HF solvolysis. HPAEC-PAD was also used to identify oligogalacturonic acids terminated at their reducing ends with galactaric acid isolated from commercial polygalacturonic acid (Pressey 1991). Uronic acid oxidase was found in squash and other plants (Pressey 1991), therefore, oxidized oligogalacturonic acids are likely cell wall components that are not generated by microbial attack. Since these oxidized oligogalacturonic acids stimulated auxin hormone oxidation (Pressey 1991), they may be plant signal compounds that have greater auxin antagonist activity than unoxidized oligogalacturonic acids (Fry et al. 1993).

HPAEC-PAD was used for the semipreparative (9 × 250 mm CarboPac PA1 column) isolation of oligogalacturonic acids between DP 10 and DP 15 (Spiro et al. 1993). The structure of DP 13 oligogalacturonic acid, a phytoalexin elicitor, was confirmed by ^{1}H-NMR and FAB-MS. Lo et al. (1994) used this semipreparative HPAEC-PAD method and reported ^{1}H- and ^{13}C-NMR chemical shift values and ^{1}H-^{1}H coupling constants for isolated oligogalacturonic acids up to DP 7 as well as a FAB-MS $[M-H]^{+}$ molecular ion for DP 14. Melotto et al. (1994) used a preparative-scale CarboPac PA1 column (23 × 280 mm) to isolate tomato (breaker stage of ripeness) rhamnogalacturonan oligosaccharides that elicited ethylene production in mature green tomato pericarp discs.

Schols et al. (1994b) used semipreparative HPAEC-PAD to isolate rhamnogalacturonan oligosaccharides up to DP 9 from rhamnogalacturonase-degraded, saponified, modified hairy regions (MHR-S) of apple pectin. ^{1}H-NMR spectroscopy of the isolated oligosaccharides confirmed the structure of the RG-I disaccharide repeating unit, α-(1 → 2)-L-Rha*p*-α-(1 → 4)-D-Gal*p*A (with approximately half of the rhamnose residues substituted at the 4-position with β-Gal*p*; Lau et al. 1985). This structure was reported earlier (Colquhoun et al. 1990) following NMR analysis of a mixture of rhamnogalacturonase-generated oligosaccharides in a low molecular weight fraction collected from a Sephadex G50 GPC column (Schols et al. 1990a). Molecular ions ($[M + Na]^{+}$) of these rhamnogalacturonan oligosaccharides were reported following HPAEC-thermospray MS (Schols et al. 1994a). Cell wall RG-I susceptible to the fungal rhamnogalacturonase (first purified by Schols et al. 1990a) appears to have wide taxonomic source range. A similar profile of rhamnogalacturonan oligosaccharides was generated from dicot (apple, potato, carrot, pear) and monocot (leek and onion) MHR-S pectin following rhamnogalacturonase treatment (Schols et al. 1994b). A different rhamnogalacturonase (RGase B) was reported by Kofod et al. (1994) that was a lyase (RG-lyase) based on HPAEC-PAD, UV (235 nm) detection and ^{1}H-NMR of the rhamnogalacturonan oligosaccharides released (Mutter et al. 1996).

The structure of RG-I (O'Neill et al. 1990; Schols et al. 1990b) was further characterized when HPAEC-PAD was used to separate a series of arabinose-containing oligosaccharides up to DP 20 following treatment of sycamore suspension-culture RG-I with endo-(1 → 5)-α-L-arabinase (Lerouge et al. 1993). A combination of endo- and exo-(1 → 4)-β-D-galactanases was required to remove most of the galactose-containing side chain region from de-arabinosylated RG-I.

Galacturonic acid (Lerouge et al. 1993), glucuronic acid, and 4-*O*-methyl-glucosyluronic acid (An et al. 1994a) were observed to be substituents on the galactose-containing side chain of RG-I. The latter two substituents were components of side chain oligosaccharides isolated by semipreparative HPAEC-PAD following acid hydrolysis of sycamore RG-I. An et al. (1994b) used semipreparative HPAEC-PAD to isolate rhamnogalacturonan oligosaccharides (up to DP 14) generated from sycamore RG-I after rhamnogalacturonase activity was observed in a mixture of commercial cell wall-degrading enzymes. Mutter et al. (1994) purified a fungal α-L-rhamnosidase specific for apple rhamnogalacturonan fragments and characterized its mode of action by HPAEC-PAD. This enzyme removes the nonreducing terminal rhamnose residue from rhamnogalacturonan oligosaccharides. The HPAEC-PAD peak area of the series of rhamnogalacturonan oligosaccharides with nonreducing ends terminated by galacturonic acid was greater than the peak area of those oligosaccharides with rhamnose residues at their nonreducing ends when both rhamnogalacturonase and rhamnosidase activities were present during sycamore RG-I hydrolysis (An et al. 1994b).

Most of the above methods for HPAEC-PAD analysis of pectin structure have utilized a high pH mobile phase (100 mM NaOH) and a sodium acetate gradient. The limit of DP size resolution using these conditions is a DP 20 oligogalacturonic acid (O'Neill et al. 1990). Using a potassium oxalate (pH 6) gradient mobile phase and post-column addition of hydroxide, it is possible to resolve oligogalacturonic acids up to approximately DP 50 (Hotchkiss and Hicks 1990). Since oligogalacturonic acids up to DP 50 were not isolated and characterized, it was debatable whether or not these HPAEC-PAD peaks represented 50 covalently connected galacturonosyl residues without rhamnose interruption. Koizumi et al. (1989) resolved malto-oligosaccharides up to DP 80 on a CarboPac PA1 column using a high-pH mobile phase, therefore, it is possible to resolve a DP 50 oligosaccharide with this column. Furthermore, Thibault et al. (1993) showed that apple, beet and citrus pectin, following partial acid hydrolysis, contained homogalacturonans (98 + % galacturonic acid content) with average molecular weights equivalent to DP 72–100 oligogalacturonic acids. The galacturonic acid-rhamnose molar ratio for the citrus (source of the polygalacturonic acid used by Hotchkiss and Hicks 1990) homogalacturonan was 247:1 (Thibault et al. 1993). Therefore, it is extremely likely that the oxalate gradient HPAEC-PAD peaks represent a homopolymer series of individually resolved oligogalacturonic acids. We correlated the average radius of gyration values calculated from GPC chromatograms with those values calculated from oxalate gradient HPAEC-PAD chromatograms of partially hydrolyzed polygalacturonic acid (Hotchkiss et al. 1995a). The smallest pectic macromolecular component was equivalent to the series of oligogalacturonic acids up to DP 40. Therefore, with the oxalate gradient mobile phase, it is posible to bridge the pectin structural information obtained from GPC with that obtained from high-pH HPAEC-PAD.

Quantitation of the carbohydrate composition by HPAEC-PAD is not a straightforward process. There has been some debate concerning the correlation

between oligosaccharide molecular weight and PAD response. A better correlation does exist between carbohydrate molar concentration and PAD response than for carbohydrate mass concentration (Paskach et al. 1991). However, this correlation is not as linear as that obtained from refractive index or UV-absorbance HPLC detectors. Assuming the PAD detects on a molar basis when different carbohydrates with different chemical and physical properties are analyzed can lead to error. Clearly, when considering a homopolymer series of oligosaccharides, the PAD molar response is not linear with increasing molecular weight (Hotchkiss and Hicks 1990; Koizumi et al. 1991a; Mort et al. 1993). The change in relative PAD response with increasing DP of galacturonic acid oligosaccharides terminated by galactose at their reducing ends was linear up to DP 7 (Mort et al. 1993). However, PAD quantitation progressively underestimated the amounts of these oligosaccharides as the DP increased compared to UV detection following labeling with 2-aminopyridine. The latter labeling and detection method provides equimolar response for oligogalacturonic acids up to DP 20 (Maness and Mort 1989). With malto-oligosaccharides, where the increase in PAD molar response was reported to be proportional to the number of sugar ring hydroxyl groups as molecular weight increased (Koizumi et al. 1989), the molar response is nonlinear above DP 10 (Koizumi et al. 1991a). Therefore, HPAEC-PAD is only quantitative if exact standards for the analyte of interest are available. HPAEC-MS shows great potential for the identification of unknown HPEAC-PAD peaks when standards are not available. However, HPAEC-MS is currently limited (by the desalting method prior to the thermospray interface) to oligosaccharides not larger than a DP 3 oligogalacturonic acid or DP 9 rhamnogalacturonan oligosaccharide (Schols et al. 1994a). Detection based on mass was demonstrated for HPAEC-separated oligogalacturonic acids up to DP 15 using a continuous postcolumn permanganate reaction system and absorbance detection at 525 nm (Thomas and Mort 1994). Mass basis detection of oligogalacturonic acids avoids the loss of sensitivity from the PAD response, or from the molar response of reducing end labeled oligosaccharides that is associated with increasing DP length. However, it should be noted that the CarboPac HPAEC column still has unequaled selectivity for oligosaccharide separations, which makes up for disadvantages associated with HPAEC-PAD.

3 Pectin Analysis in Ripening Peach Fruit

3.1 Melting- and Nonmelting-Flesh Peaches

Many examples have been reported where endo-polygalacturonase (endo-PG) is associated with pectin depolymerization during fruit ripening (Brady 1987). Higher endo-PG activity was proposed to be responsible for the increased pectin solubilization and softening in melting-flesh (freestone) peaches relative to nonmelting-flesh (clingstone) peaches (Pressey and Avants 1978). Due to the rapid

decrease in tissue firmness (late during ripening) in melting-flesh peaches compared to nonmelting-flesh peaches, the former peaches are used for the fresh market and the latter used for canning (Lester et al. 1994). Melting-flesh peaches contain both exo- and endo-polygalacturonase enzymes, while nonmelting flesh peaches only have exo-polygalacturonase activity during ripening (Pressey 1986). Endo-PG gene expression, probed with a partial cDNA sequence from ripe melting-flesh peach fruit, occurred in low levels as the fruit began to ripen and high levels in ripe fruit (Lester et al. 1994). Low levels of a different RNA transcript were detected with this probe only in ripe nonmelting-flesh fruit (Lester et al. 1994). Three pectin methyl-esterase (PME) isozymes have been isolated from ripe melting-flesh peaches (Glover and Brady 1994). In tomato, the relationship between endo-PG activity and fruit ripening has become unclear because while expression of anti-sense RNA to this enzyme prevented pectin degradation, the tissue still softened (Sheehy et al. 1988). These observations led to an investigation of other carbohydrate-modifying enzyme activities that may play a role in tomato ripening (Maclachlan and Brady 1994).

Fishman et al. (1993b) demonstrated that pectin solubilization and softening were greatest in Redskin (melting-flesh) peaches between 21 weeks after flowering (WAF) and 22 WAF compared to Suncling (nonmelting flesh) peaches at the same stage of ripening. The size and intrinsic viscosity of the Redskin chelator-soluble pectin decreased and the molecular weight of both chelator-soluble and alkaline-soluble Redskin pectin increased between 21 and 22 WAF compared to Suncling pectin (Fishman et al. 1993b). Therefore, endo-PG is only active at the end of the melting-flesh peach ripening process when the most dramatic textural softening occurs. These authors suggested that other mechanisms may be involved in the initial stages of Redskin peach ripening and Suncling peach softening (Fishman et al. 1993b). Redskin peach pectin (20 WAF alkaline-soluble pectin) consists of four macromolecular components in which the larger components are aggregates of the smaller components (Fishman et al. 1992, 1993a). All but the smallest component could be visualized by electron microscopy as a network of segmented, kinked, and branched rods which had a discontinuous distribution of contour lengths.

3.2 HPAEC-PAD System

Conditions for HPAEC-PAD were similar to those reported previously (Hotchkiss and Hicks 1990). HPAEC-PAD was performed with a Dionex Bio-LC system, which included a PAD 2 (gold working electrode) pulsed amperometric detector (E_1 = 0.15 V, 480 ms; E_2 = 0.7 V, 120 ms; E_3 = −0.6 V, 360 ms), and a pressurized bottle post-column delivery system that added 500 mM potassium hydroxide prior to the detector. A CarboPac PA1 column (4 × 250 mm; Dionex Corp.) and a CarboPac PA Guard column (3 × 25 mm; Dionex Corp.) were utilized. The mobile phase consisted of a 25–500 mM potassium oxalate pH 6 nonlinear, 100-min mobile phase gradient (Table 1).

Table 1. HPAEC-PAD mobile phase gradient

Time (min)	Eluant A 500 mM K^+ oxalate (%)	Elunat B H_2O (%)	Oxalate (mM)
0–1	5	95	25
9	20	80	100
40	40	60	200
65	50	50	250
80	53	47	265
100	100	0	500
110	100	0	500
111	5	95	25

3.3 Sample Preparation

Peach cell walls were isolated and extracted sequentially with a chelating agent (50 mM CDTA) and then under alkaline conditions (50 mM Na_2CO_3, 2 mM CDTA) as reported previously (Fishman et al. 1993b), producing chelator-soluble pectin (CSP) and alkaline-soluble pectin (ASP), respectively. CSP and ASP were hydrolyzed by incubating 10 mg of pectin in 0.1 M NaCl, pH 4, 30 °C to which 1 unit of purified endo-polygalacturonase (Doner et al. 1988) was added (1 ml total volume). Hydrolysis was terminated by raising the pH to 6 with KOH and heating the sample in a boiling water bath for 10 min. A 200-μl aliquot was removed, filtered (0.45 μm), and injected for HPAEC-PAD analysis. The pH of the remaining sample was adjusted to 4 with CH_3COOH and hydrolysis continued (30 °C) after adding an additional unit of endo-polygalacturonase. Due to a limited amount of sample available for analysis, this process was repeated for each time point during the hydrolysis time course.

3.4 HPAEC-PAD of Redskin and Suncling Peach Pectin

HPAEC-PAD analysis of Redskin and Suncling peach pectins revealed only trace amounts (if any) of oligogalacturonic acids and a broad, occasionally bimodal, peak at approximately 90 min that we have described as a "polymer" peak (Hotchkiss and Fishman 1992). The polymer peak represents the beginning of the size range at which the CarboPac PA1 column no longer resolves individual oligogalacturonic acids and/or the size range at which the forces that aggregate the larger pectic subunits are stronger than the dissociating properties of the potassium oxalate buffer. The earlier-eluting components of the bimodal polymer peak appears to consist of homogalacturonan since it is depolymerized fastest by endo-PG (Figs. 1a, 2a,b, 3a). However, endo-PG hydrolysis of the polymer peak proceeds by a two-stage reaction. The later-eluting second components of the polymer peak, that which is more slowly depolymerized, is more difficult to characterize. The second polymer peak component probably does not result solely from a higher degree of branching or degree of esterification, which are characteristics of pectins

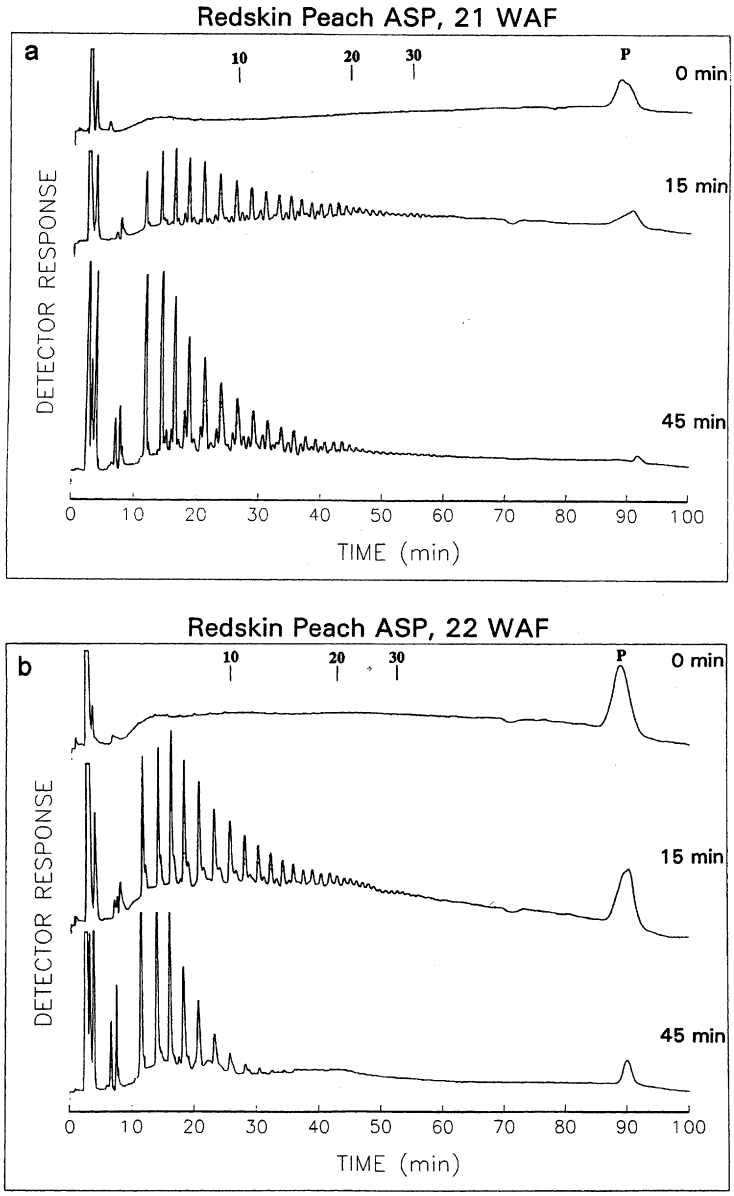

Fig. 1a,b. HPAEC-PAD chromatograms of endo-PG depolymerization of Redskin peach ASP at **a** 21 WAF and **b** 22 WAF. The time after addition of the enzyme is indicated at the end of each chromatogram. The *numbers* over each chromatogram refer to the oligogalacturonic acid DP determined by comparison of retention times with standards (Doner et al. 1988) and then extrapolation; *P* polymer peak

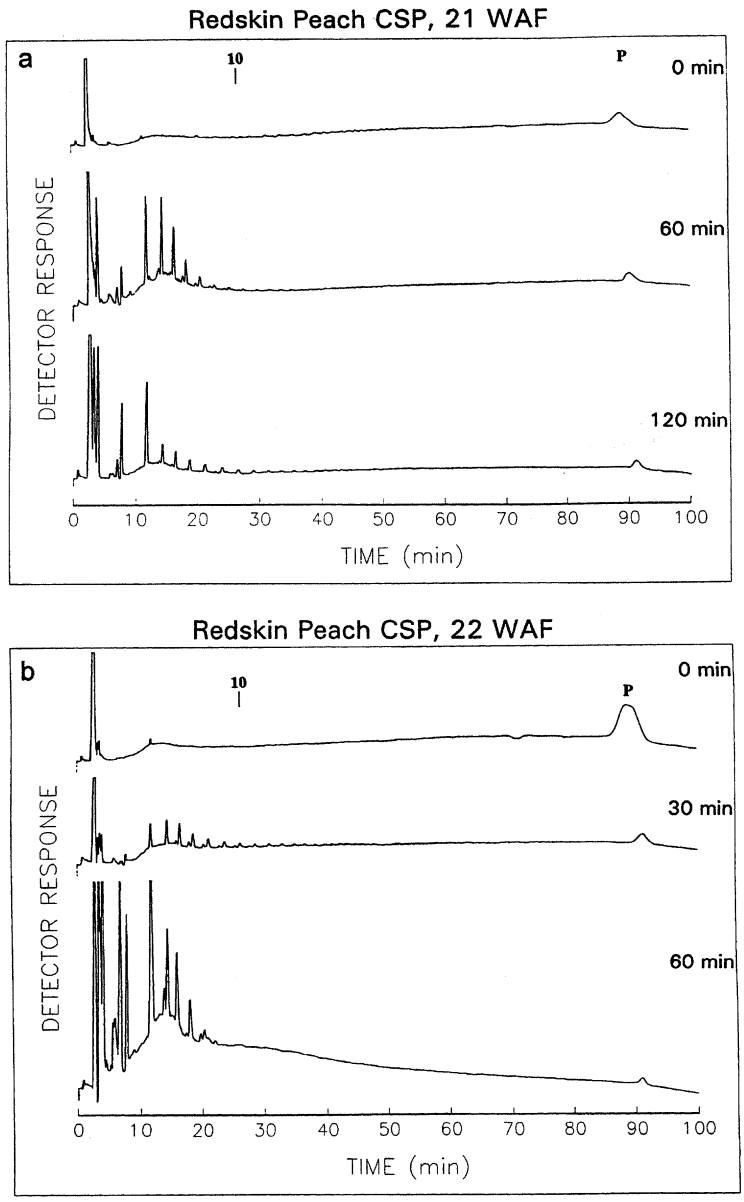

Fig. 2a,b. Endo-PG depolymerization of Redskin CSP at **a** 21 WAF and **b** 22 WAF. See Fig. 1 for description of labels

extracted under alkaline conditions. Increases in both of these structural properties in oligosaccharides leads to earlier elution from the CarboPac PA1 column (Mutter et al. 1994; Schols et al. 1994b). Possibly, a combination of branching, methyl-esterification, and pectin component aggregation may sequester endo-PG susceptible linkages in this later-eluting polymer peak component.

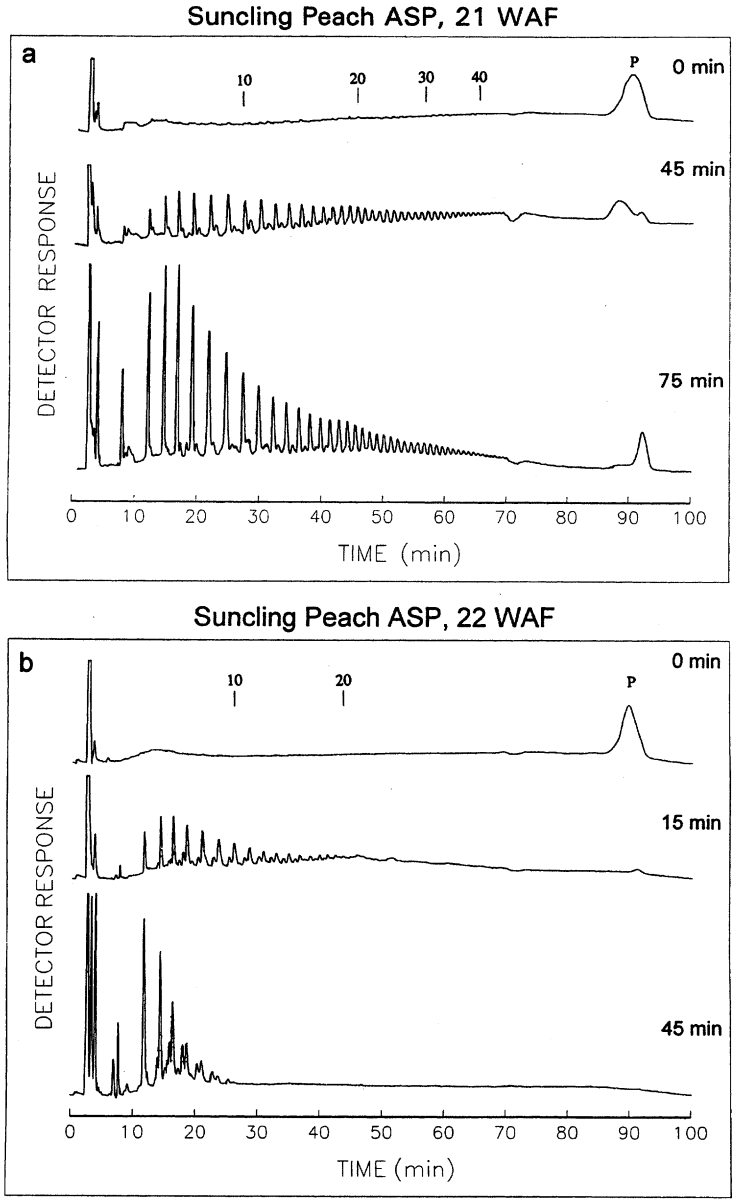

Fig. 3a,b. Endo-PG depolymerization of Suncling peach ASP at **a** 21 WAF and **b** WAF. See Fig. 1 for description of labels

We previously demonstrated that a series of oligogalacturonic acids were generated from 20 WAF Redskin peach ASP following a 30-min treatment with endo-PG (Fishman et al. 1993a). Endo-PG treatment of Redskin peach pectin at 21 and 22 WAF also generated a series of oligogalacturonic acids. However, ASP was generally more susceptible to endo-PG hydrolysis than CSP based on the time

required to hydrolyze oligogalacturonic acids. The alkaline conditions, used to extract ASP, saponified methyl-ester groups, rendering more pectin available for hydrolysis. Methyl-esterification of pectin (Thibault and Mercier 1978) and acetyl-esterification of galacturonic acid at C-2 and C-3 (Rexova-Benkova et al. 1977) decreases the extent of pectin degradation by endo-PG. The difference between the susceptibility of ASP and CSP to endo-PG is much more noticeable at 21 WAF than at 22 WAF, which correlates with changes in radius of gyration and intrinsic viscosity observed at this stage of ripening. The radius of gyration and intrinsic viscosity of CSP is much higher than that of ASP at 21 WAF (Fishman et al. 1993b). However, by 22 WAF these parameters for CSP and ASP are almost identical (Fishman et al. 1993b). Additionally, larger oligogalacturonic acids were released from ASP by endo-PG hydrolysis relative to CSP. While de-esterification with alkali occurs in a random pattern (Rexova-Benkova and Markovic 1976), larger blocks of homogalacturonan may have been produced by the alkaline extraction conditions in the ASP compared to that present in the CSP.

An increase in susceptibility of the Redskin CSP to endo-PG hydrolysis between 21 and 22 WAF is coincident with the significant decreases in radius of gyration and intrinsic viscosity, and increase in molecular weight reported earlier (Fishman et al. 1993b). Endo-PG-mediated hydrolysis of long, thin CSP aggregates (smooth regions) during this stage of ripening leaving short thick pectin aggregates (hairy regions) was proposed to be the major pectin structural difference between melting-flesh and nonmelting-flesh peach softening (Fishman et al. 1993b). Our results reflect the action of PME or acetyl-esterase during this stage of Redskin peach ripening. These enzymes deesterify CSP such that the 22 WAF CSP is more susceptible to endo-PG hydrolysis than it was at 21 WAF (Fig. 2). A higher CSP degree of esterification at 21 WAF could also be responsible for the higher radius of gyration and intrinsic viscosity values observed since it would be less able to aggregate due to calcium cross-linking. Chilling injury of peach fruit during storage that leads to a mealy texture called "woolliness", has been explained by an imbalance between PME and endo-PG activities such that high molecular weight, low-methoxy pectins accumulate (Ben-Arie and Sonego 1980). The enrichment in hairy regions in the 22 WAF Redskin CSP compared to 21 WAF is illustrated by the increased number of secondary peaks adjacent to the oligogalacturonic acid peaks (more minor peaks and shoulders in Fig. 2b, 60 min compared to Fig. 2a, 60 min). These secondary peaks are produced by oligogalacturonic acids containing one or more neutral sugars such as rhamnose. These secondary peaks are also present in Redskin ASP hydrolysates (Fig. 1; see also Fig. 10 in Fishman et al. 1993a).

HPAEC-PAD analysis of Suncling peach pectin (Figs. 3 and 4) demonstrated trends similar to those observed with Redskin peach pectin. ASP was more susceptible to endo-PG hydrolysis than CSP, and 22 WAF pectin (ASP and CSP) was more susceptible than 21 WAF pectin. Therefore, while endo-PG does not play a role in the normal ripening of this nonmelting-flesh peach, the Suncling pectin is just as susceptible to endo-PG hydrolysis at 21 WAF as Redskin pectin. The increase in endo-PG susceptibility between 21 and 22 WAF suggests that *in muro* esterase activity may play a role in Suncling peach softening. Oligogalacturonic acids re-

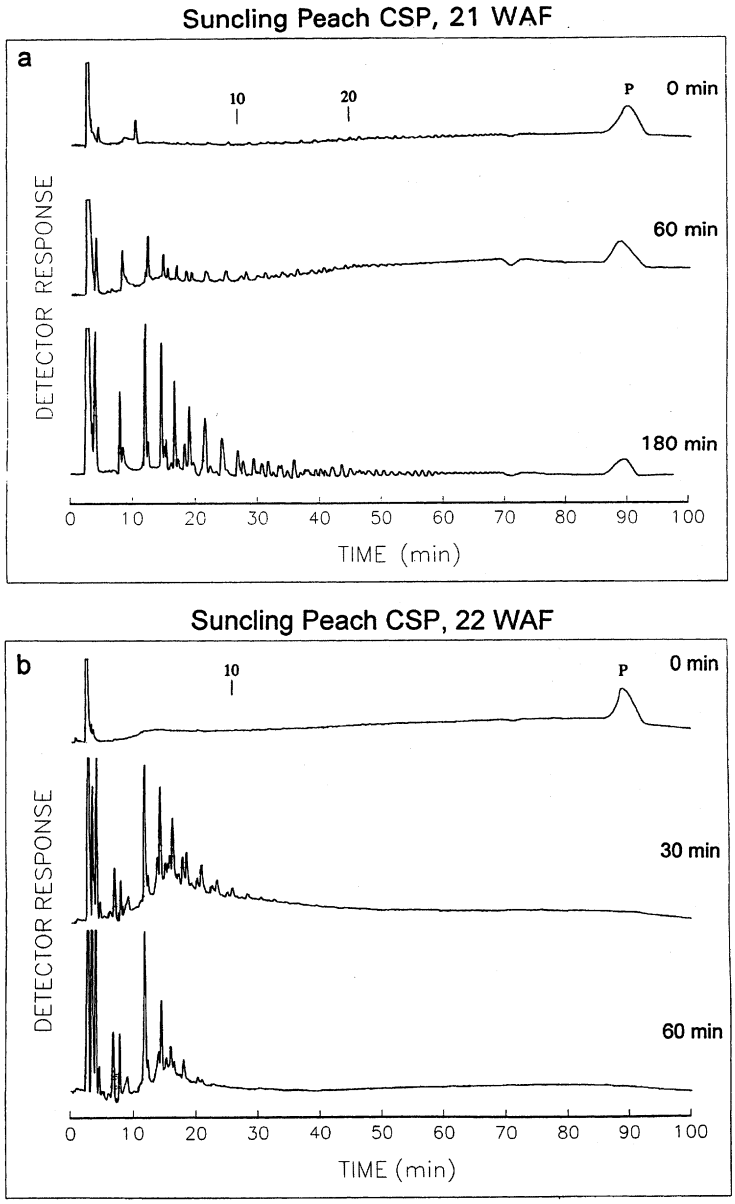

Fig. 4a,b. Endo-PG depolymerization of Suncling peach CSP at **a** 21 WAF and **b** 22 WAF. See Fig. 1 for description of labels

leased from Suncling pectin were consistently larger than those released from Redskin pectin during endo-PG hydrolysis. This suggests that the homogalacturonan DP size is larger in Suncling pectin, which probably reflects the relatively low levels of endo-PG activity during ripening.

4 Conclusions and Future Directions

HPAEC-PAD analysis of peach pectin and the susceptibility of these polysaccharides to endo-polygalacturonase hydrolysis provided valuable insights concerning the ripening process of these fruits. The degree of pectin esterification was implicated as the molecular control of peach softening since ASP as more susceptible to endo-PG hydrolysis than CSP and susceptibility increased between 21 and 22 WAF. Other carbohydrate-modifying enzymes, such as PME, may play a significant role in the initial stages of melting-flesh peach ripening. PME also appears to play a role in nonmelting-flesh peach ripening. The larger size range of oligogalacturonic acids released from nonmelting flesh peach pectin compared to melting-flesh peach pectin confirmed the previously reported role of endo-PG in the softening of the latter peaches. Through comparison with the HPSEC data reported for these pectins, meaningful interpretations of the HPAEC-PAD data could be made. Homogalacturonan and an aggregation of larger pectic components were the two parts of the polymer peak represented in HPAEC-PAD chromatograms. The complete picture of changes in pectin structure is only drawn when both levels of analytical resolution are applied.

The development of CarboPac PA columns advanced dramatically the field of oligosaccharide separations. New stationary phases consisting of a highly cross-linked polystyrene-divinylbenzene surface quaternized with amino functions (Corradini et al. 1994), amine surface-functionalized nonporous microspherical zirconia (Yu and Elrassi 1994), DEAE-functionalized polystyrene-divinylbenzene (301 VHP, Vydac; GlycoSep C, Oxford Glycosystems), porous graphitized carbon (Koizumi et al. 1991b; Hypercarb, Shandon; GlycoSep H, Oxford Glycosystems), aminopropylsilica gel covalently coated with polysuccinimide (Alpert 1990; Alpert et al. 1994; PolyHydroxyethyl A and PolyGLYCOPLEX, PolyLC), and β-cyclodextrin bonded silica gel (Simms et al. 1995; Cyclobond I, Advanced Separations Technologies) may expand oligosaccharide separation selectivity further in the future. Additionally, capillary zone electrophoresis has great potential for pectic oligosaccharide separations (Liu et al. 1992).

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