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PRODUCTION OF NARROW-RANGE SIZE-CLASSES OF POLYGALACTURONIC ACID OLIGOMERS

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Abstract. **A structural component of citrus processing residues with significant functionality is the homogalacturonan region of pectin. Its functional properties include ion binding, gelation, water retention and elicitation of plant defense responses to pathogens. It is also the site of attack by commercial pectinolytic enzymes used as processing aids for viscosity reduction, enzymatic peeling and conversion of peel polysaccharides to monomeric sugars for subsequent fermentation. More recently endo-polygalacturonases (EPG) have been used as research tools to probe the mode of action of pectin methylesterases and to map pectin fine structure. Consequently, knowing how EPG acts on oligomers of polygalacturonic acid is needed. A major limitation in studying these oligomers has been the limitations on chromatographic detection. In the work presented here, three size-classes with a degree of polymerization ranging from 1-13, 8-24 and 22-46 were prepared by enzymatic digestion followed by a combination of differential pH and alcohol precipitation. The oligomers were characterized using improved chromatographic techniques that enabled us to estimate masses of individual oligomers. Strawberries were treated with each size-class to determine if the fragments elicited a biological response. Only the medium-DP size-class resulted in a delay in fruit decay not observed with the low-or high-DP size-classes.**

Pectin from citrus fruit peel is composed of 80-90% galacturonic acid (GA; Grohmann et al., 1995). The GA is found mainly in homogalacturonan (HG) regions of pectin. This region is a linear stretch of 100-200 GA residues in length in which a variable proportion may be methyl esterified at C6. The proportion of these residues that are demethylated, and their spatial patterning, are responsible for many of the functional properties inherent in the pectin molecule (Taylor, 1982; Willats et al., 2001). Mapping pectin fine structure (distribution of methylated and demethylated HG regions) has been the topic of numerous reports (Cameron et al., 2004; Catoire et al., 1998; Denes et al., 2000; Grasdalen et al., 1996). A common tool used in these studies is the enzyme endo-polygalacturonase (EPG, EC. 3.2.1.15) from *Aspergillus niger* (Limberg et al., 2000a, b). Multiple forms of EPG from *A. niger* have been isolated and characterized (Benen et al., 1999; Pařenicová et al., 1998). EPG mode of action has not been characterized on GA oligomers larger than a 7-mer (Benen et al., 1999; Pařenicová et al., 1998), although stretches of demethylated HG may be much longer than 7 GA residues (Catoire et al., 1998; Denes et al., 2000), depending on the percentage of galacturonic acid residues that are methyl esterified at the C-6 position (pectin degree of esterification; DE).

In order to fully utilize EPG as a tool to map the fine structure of HG regions in pectin and, subsequently, for discerning the mode of action of multiple forms of pectin methylesterase (PME) present in citrus fruit peel, we have made use of differential precipitation of polygalacturonic acid (PGA) oligomers based on their degree of polymerization (DP; Hotchkiss and Hicks, 1990) to produce and fully characterize narrow-range size-classes of PGA oligomers by high-performance anion exchange chromatography coupled to an evaporative light scattering detector (HPAEC-ELSD; Cameron and Grohmann, 2005; Cameron et al., 2004). In addition to preparing, characterizing and quantifying the DP size-classes for studying EPG action, we have tested their effect as surface treatments to inhibit fruit decay of harvested strawberries. Baldwin and Briggs (1988) previously showed that a mixture of PGA oligomers in the DP range of 10-15 was able to elicit the production of ethylene in citrus and Simpson et al. (1998) demonstrated that small DP fragments (DP 4-6) induced both ethylene production and the expression of the gene encoding ACC oxidase in tomato plants. Here we investigate the utility of the different DP size-classes to inhibit the growth of decay organisms on fresh strawberries.

Materials and Methods

Production of narrow-range size-classes. The free acid of polygalacturonic acid was made to a concentration of 2% (w/v) in 50 mM lithium acetate, pH 4.7 (LiPGA; unless specified all chemicals were purchased from Sigma-Aldrich, St. Louis, Mo., USA). A one liter volume of the 2% LiPGA was digested with 0.05 U/mL EPG (Lot 00801, Megazyme International Ireland Limited, Bray, Ireland) for 4.5 h at room temperature with constant stirring. The digested LiPGA was brought to pH 2.0 with concentrated HCl and then stored overnight at 4°C. The precipitated material was pelleted by centrifugation at 23,500 g for 30 min at 4°C. The pelleted precipitate, representing the High-DP size-class, was solubilized in 50 mM lithium acetate (LiOAc) and re-precipitated by adjusting to pH 2 with concentrated HCl and storing overnight at 4°C followed by centrifugation as described above. The pelleted High-DP (DP 22-46) material was solubilized in 50 mM LiOAc and stored at 4°C.

The supernatant from the initial centrifugation of the pH 2 precipitation, containing the Low- and Medium-DP frag-

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ments, was collected and brought to 50 mM sodium acetate (NaOAc) and 22.5% EtOH and then placed at 4°C overnight to precipitate the Medium-DP fragments (DP 8-24). Following centrifugation as described, above the supernatant (Low-DP fragments, DP 1-13) was pooled and stored at 4°C. The pelleted material was solubilized in 50 mM LiOAc and then re-precipitated by adjusting the solution to 50 mM NaOAc and 22.5% EtOH. This material, representing the Medium-DP fragments, was centrifuged again and the pellets were solubilized in 50 mM LiOAc and stored at 4°C.

The concentration of galacturonic acid in each size-class was determined with a spectrophotometric microtiter plate assay using 3,5 dimethylphenol to produce the chromophore (Luzio, 2004).

Characterization of narrow-range size-classes. The population of the constitutive DP oligomers present in each size-class, as well as the parent LiPGA, was characterized by high-performance anion exchange chromatography coupled to an evaporative light scattering detector (Cameron and Grohmann, 2005). The chromatography system was composed of a Perkin Elmer Series 200 Pump (Shelton, Conn.) and a Perkin Elmer Series 200 Autosampler connected to a CarboPac PA1 (4 × 250 mm; Dionex Corporation, Sunnyvale, Calif.) anion exchange column. Detection of analytes was accomplished with a Sedex 75S Evaporative Light Scattering Detector (Lawrenceville, N.J.). Data collection was accomplished with an A/D converter connected to a Dell (Round Rock, Texas) personal computer using EZ Chrome Elite software (Scientific Software, Pleasanton, Calif.).

Treatment of strawberries with narrow-range size-classes of GA oligomers. Strawberry fruit were harvested on two different dates from the University of Florida Experiment Station in Dover, Fla. (cv. Festival) and a commercial farm near Plant City, Fla. (cv. Camino Real), in April 2005. The spore load present on the fruit surface was determined by placing the fruit in a sterile sampling bag, weighing and then washing the fruit with a sterile phosphate buffer. Samples of the phosphate buffer were plated onto agar plates, incubated and colonies counted. The main pathogens on these fruits were *Rhizopus stolonifer* and *Botrytis cinerea*, some yeast and a few bacteria that were secondary invaders. The numbers of colony forming units per gram weight of fruit were about 1×10^5. To determine the affect of the three GA oligomer size-classes on fruit decay, the fruit were sorted, dipped individually for 10-20 s in chilled solutions containing 0.2% (w/v, on the basis of GA concentration) of the different oligomer size-classes, drained and individual replicates were stored in commercial clam-shell containers at 15 or 19°C. Percent decay (number of fruit with lesions) was monitored during storage for 5-6 d by visual inspection and a visible lesion was scored as a decayed fruit. There were 10 fruit per replicate and three replicates per treatment for each harvest. Treatments included 1) Low-DP oligomers in 50 mM LiOAc, 50 mM NaOAc, 22.5% EtOH, 2) Medium-DP oligomers in 50 mM LiOAc, 3) High-DP oligomers in 50 mM LiOAc, and the two buffers without GA oligomers as control treatments 4) 50 mM LiOAc, 50 mM NaOAc, 22.5% EtOH and 5) 50 mM LiOAc.

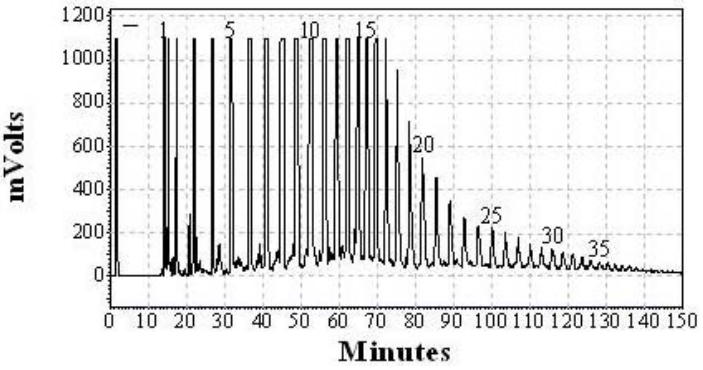

Fig. 2. HPAEC-ELSD chromatogram of 2% LiPGA after a 4.5 h digestion with EPG. Numbers indicate the DP of designated peaks.

Results and Discussion

The undigested LiPGA consisted of a population of GA oligomers with a size distribution heavily skewed towards fragments with a DP greater than 35 as indicated by the area under the unresolved hump (Fig. 1). After a 4.5 h digestion with EPG the population consists of much smaller fragments (Fig. 2) with a DP of 46 being the largest fragment observed. Adjusting the EPG digest to pH 2 precipitated the larger DP oligomers, which was designated as the High-DP size-class. HPAEC-ELSD of the re-solubilized High-DP size-class (Fig. 3) demonstrated the presence of oligomers in the DP range of 13-46. Adjusting the pH 2 supernatant to 50 mM NaOAc and 22.5% EtOH precipitated the 8-20 medium-DP size-class (Fig. 4, Table 1) while the Low-DP size-class (DP ≤ 12, Fig. 5) remained in solution.

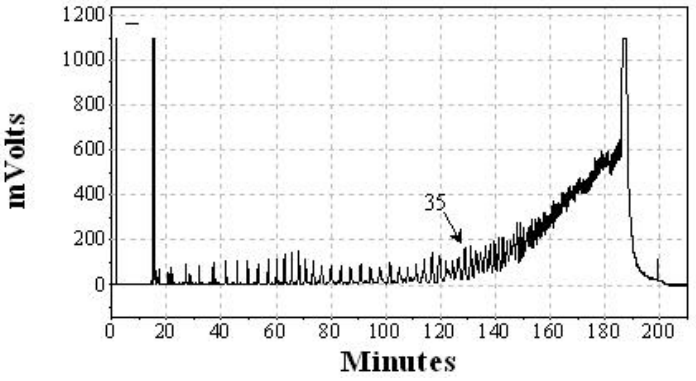

Fig. 1. HPAEC-ELSD chromatogram of undigested 2% LiPGA. Numbers indicate the DP of designated peaks.

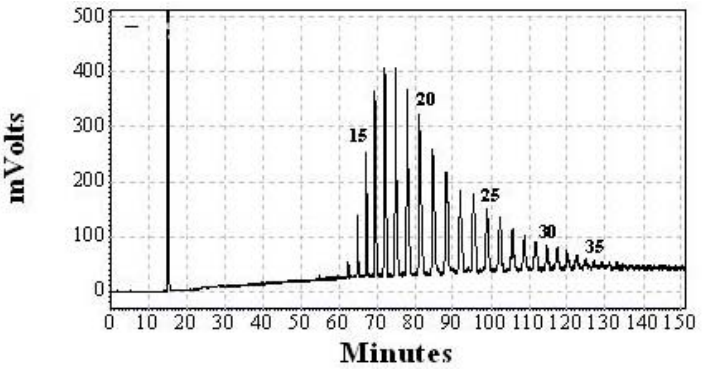

Fig. 3. HPAEC-ELSD chromatogram of precipitate (High-DP, DP 13-42) from pH 2 precipitation of 4.5 h digest of 2% LiPGA. Numbers indicate the DP of designated peaks.

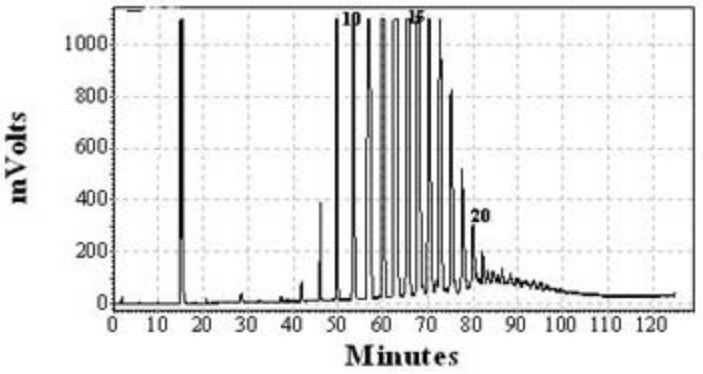

Fig. 4. HPAEC-ELSD chromatogram of precipitant (Medium-DP, DP 7-21) from 50 mM NaOAc and 22.5% EtOH precipitation of pH 2 supernatant. Numbers indicate the DP of designated peaks.

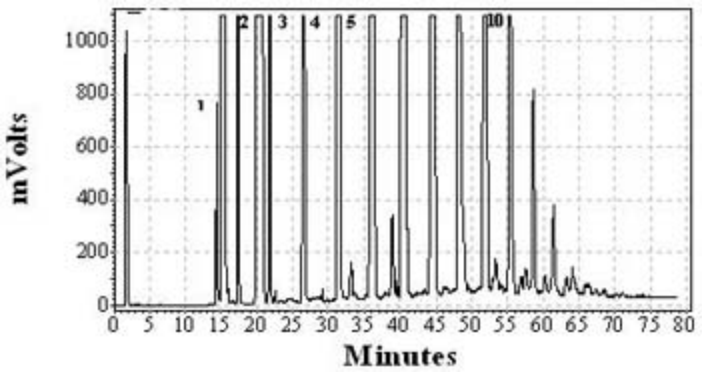

Fig. 5. HPAEC-ELSD chromatogram of supernatant (Low-DP, DP 1-13) from 50 mM NaOAc and 22.5% EtOH precipitation of pH 2 supernatant. Numbers indicate the DP of designated peaks.

Comparison of the GA oligomers present in each of the different size-classes indicates they could be useful for testing assorted HG functional properties and in characterizing the mode of action of different isoforms of EPG present in commercial pectolytic enzyme mixtures. The Medium-DP size-class could be especially helpful since digestion of this material will demonstrate the initial fragment size produced by EPG action on larger oligomers and the utility of using EPG to estimate demethylated block size in pectin treated with the different PMEs present in citrus fruit. Table 1 demonstrates the power of the HPLC separation and ELSD detection method employed since the mass and molar amount of individual oligomers can now be estimated.

One test of functionality of these narrow-range size-classes is the biological activity demonstrated by the ability of GA oligomers to elicit plant defense responses (Simpson et al., 1998). Results from treating fresh strawberry fruit with the different GA oligomer size-classes showed that strawberries, cv. Festival, exhibited reduced decay when treated with the medium DP oligomers (30% fruit with lesions, Fig. 6A) compared to low DP oligomers (93% fruit with lesions), high DP oligomers (86% fruit with lesions), or the two buffers (100% and 77% fruit with lesions for the buffer with and without ethanol, respectively) after 6 d in storage at 15°C.

Table 1. Concentration of individual oligomers present in the Medium-DP size-class.

Peak DP	Area %	µMol mL^{-1}
7	0.12	2.44
8	0.70	14.00
9	3.10	61.96
10	7.56	151.26
11	14.07	281.40
12	17.73	354.56
13	17.01	340.24
14	14.71	294.14
15	10.07	201.34
16	6.46	129.20
17	4.05	80.92
18	2.24	44.82
19	1.28	25.54
20	0.70	14.04
21	0.21	4.16

Strawberries, cv. Camino Real, from a second harvest had similar results (Fig. 6B), with fruit treated with medium DP oligomers having the least decay (43%) compared to low (70%) or high DP oligomers (73%), and the two buffers (63

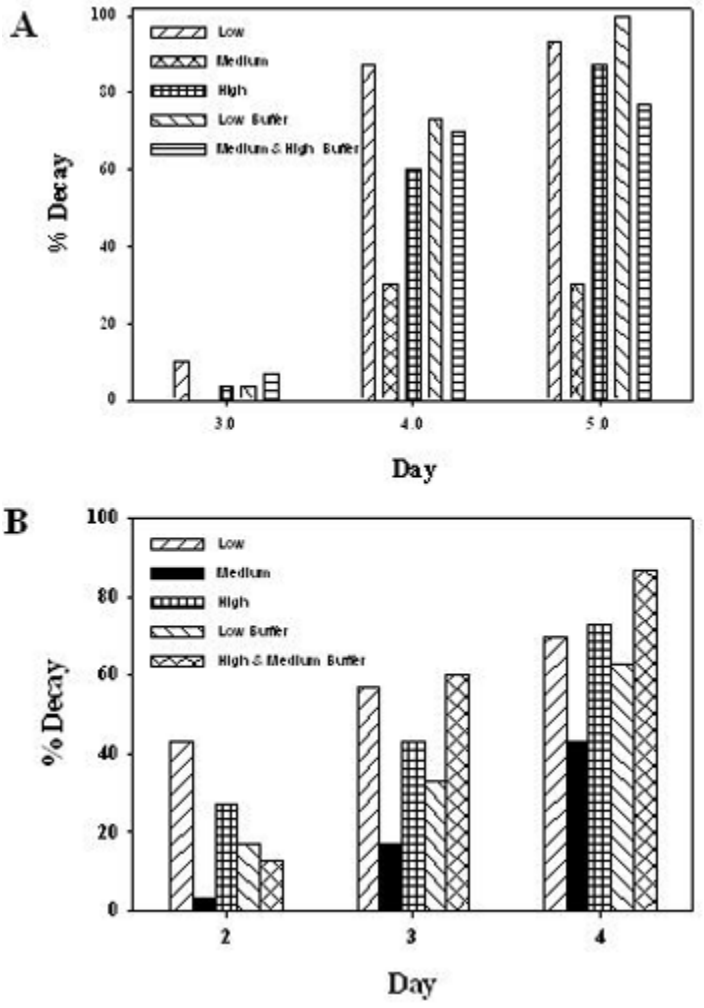

Fig. 6. Percent decay on whole strawberry fruit after dipping into low-, medium- or high-DP GA size classes and their corresponding buffer controls (low-DP buffer = 50 mM LiOAc, 50 mM NaOAc and 22.5% EtOH; medium- and high-DP buffer = 50 mM LiOAc). A. cv. Festival stored at 15°C. B. cv. Camino Real stored at 19°C.

and 87% with and without ethanol, respectively) after 4 d storage at 19°C.

Conclusions

Differential precipitation following enzymatic digestion of LiPGA with EPG resulted in three different populations of GA oligomers. Some overlap of oligomers present in the size-classes did occur. The ability of the HPAEC-ELSD methods to separate, detect and quantify the individual oligomers present in each size class was demonstrated. This will aid in characterizing EPG activity and pectin fine structure. Treating freshly harvested strawberry fruit with the medium-DP size-class illustrated biological activity and a potential application in slowing decay and extending shelf life of an extremely perishable commodity.

References

Baldwin, E. A., R. H. Briggs. 1988. Cell-wall lysing enzymes and products of cell-wall digestion elicit ethylene in citrus. Physiol. Plant. 73:58-64.

Benen, J. A. E., H. C. M. Kester, and J. Visser. 1999. Kinetic characterization of *Aspergillus niger* N400 endopolygalacturonases I, II and C. Eur. J. Biochem. 259:577-585.

Cameron, R. G. and K. Grohmann. 2005. Separation, detection and quantification of glacturonic acid oligomers with a degree of polymerization greater than 50. J. Liq. Chromatogr. Relat. Technol. 28:559-570.

Cameron, R. G., G. Luzio, S. W. Kauffman, and K. Grohmann. 2004. Characterization of esterified blocks in pectin homogalacturonan regions after de-esterification with the thermally tolerant pectin methylesterase from citrus fruit. Proc. Fla. State Hort. Soc. 117:410-415.

Catoire, L., M. Pierron, C. Morvan, C. Herve du Penhoat, and R. Goldberg. 1998. Investigation of the action patterns of pectinmethylesterase isoforms through kinetic analyses and NMR spectroscopy. J. Biol. Chem. 273:33150-33156.

Denes, J.-M., A. Baron, C. M. G. C. Renard, C. Pean, and J.-F. Drilleau. 2000. Different action patterns for apple pectin methylesterase at pH 7.0 and 4.5. Carbohydr. Res. 327:385-393.

Grasdalen, H., A. K. Andersen, and B. Larsen. 1996. NMR spectroscopy of the action pattern of tomato pectinesterase: generation of block structure in pectin by a multiple-attack mechanism. Carbohydr. Res. 289:105-114.

Grohmann, K., R. G. Cameron, and B. S. Buslig. 1995. Fractionation and pretreatment of orange peel by dilute acid hydrolysis. Bioresour. Technol. 54:129-141.

Hotchkiss, A. T. and K. B. Hicks. 1990. Analysis of oligogalacturonic acids with 50 or fewer residues by high-performance anion-exchange chromatography and pulsed amperometric detection. Anal. Biochem. 184:200-206.

Limberg, G., R. Korner, H. C. Bucholt, T. M. I. E. Christensen, P. Roepstorff, J. D. Mikkelsen. 2000a. Analysis of different de-esterification mechanisms for pectin by enzymatic fingerprinting using endopectin lyase and endopolygalacturonase II from *A. niger*. Carbohydr. Res. 327:293-307.

Limberg, G., R. Korner, H. C. Bucholt, T. M. I. E. Christensen, P. Roepstorff, and J. D. Mikkelsen. 2000b. Quantification of the amount of galacturonic acid residues in block sequences in pectin homogalacturonan by enzymatic fingerprinting with exo- and endo-polygalacturonase II from *Aspergillus niger*. Carbohydr. Res. 327:321-332.

Luzio, G. 2004. Determination of galacturonic acid content of pectin using a microtiter plate assay. Proc. Fla. State Hort. Soc. 117:416-421.

Parenicová, ˇL., J. A. E. Benen, H. C. M. Kester, and J. Visser. 1998. *pgaE* encodes a fourth member of the endopolygalacturonase gene family from *Aspergillus niger*. Eur. J. Biochem. 251:72-80.

Simpson, S. D., D. A. Ashford, D. J. Harvey, and D. J. Bowles. 1998. Short chain oligogalacturonides induce ethylene production and expression of the gene encoding aminocyclopropane 1-carboxylic acid oxidase in tomato plants. Glycobiology 8:579-583.

Taylor, A. J. 1982. Intramolecular distribution of carboxyl groups in low methoxyl pectins—A review. Carbohydr. Polym. 2:9-17.

Willats, W. G. T., C. Orfila, G. Limberg, H. C. Buchholt, G.-J. W. M. Van Alebeek, A. G. J. Voragen, S. E. Marcus, T. M. I. E. Christensen, J. D. Mikkelsen, B. S. Murray, and J. P. Knox. 2001. Modulation of the degree and pattern of methyl-esterification of pectic homogalacturonan in plant cell walls. J. Biol. Chem. 276:19404-19413.