Available online at www.sciencedirect.com
SCIENCE DIRECT
Phytochemistry 67 (2006) 13–18
PHYTOCHEMISTRY
www.elsevier.com/locate/phytochem

Detection of uronic oxidase activity in ripening peaches

Dario Cantu [a], L. Carl Greve [b], Susan Lurie [c,*], John M. Labavitch [b]

[a] Department of Agricultural Sciences and Technologies, University of Milan, Milan, Italy
[b] Department of Plant Sciences, University of California, Davis, CA 95616, USA
[c] Department of Postharvest Science, Volcani Center, ARO, Bet Dagan 50250, Israel

Received 2 August 2005; received in revised form 2 October 2005
Available online 23 November 2005

Abstract

Uronic acid oxidase activity was found in an extract from harvested peaches that was incubated with citrus pectin at pH 8.5. The product of this reaction was identified by GC–MS analysis to be galactaric acid. The reaction was linear at 37 °C for up to 20 h, and the pH optimum was 8.5. The activity found in firm peaches one day after harvest did not change as the peaches softened over 5 days to eating softness. The incubation conditions were those suitable for monitoring the activity of pectate lyase, but instead of finding an increase in galacturonosyl residue reducing groups due to generation of pectin-derived oligosaccharides, uronic acid oxidase catalyzed the oxidation of the aldehyde reducing functions to carboxyl groups.

Keywords: *Prunus persica*; Peach; Cell wall enzyme; Ripening; Softening; Uronic acid oxidase

1. Introduction

Plants contain a variety of enzymes that break down the substantial array of pectic polysaccharides found in their cell walls. *Endo*-polygalacturonase (PG) has been found in many fruits in which ripening is accompanied by solubilization and digestion of pectin (Pressey and Avants, 1973). When pure PGs are incubated with polygalacturonic acid, an unmethylated homogalacturonan, the enzyme acts at random along the polymer backbone and the digestion products are almost exclusively short oligomers containing 2–5 GalA residues. However, pectin catabolism in ripening fruits generally results in a heterogeneous mixture of shorter and longer oligomers as well as soluble, polymeric molecules. This is undoubtedly due to the structural complexity of the cell wall's pectin polymer complement and the inability of pectolytic enzymes to reach all potential sites of action. The pectin breakdown-generated oligomers may serve as signals that influence the expression of fruit genes locally (Lurie, unpublished; Melotto et al., 1994; Ridley et al., 2001). *Exo*-PGs have also been reported in extracts of fruits (e.g., Pressey and Avants, 1973; Downs et al., 1992). *Exo*-PG generally catalyzes the hydrolysis of single GalA residues away from a pectic polymer or oligomer, acting at the non-reducing ends of the substrates. Both *exo*- and *endo*-PGs act only on de-esterified sites in a pectin substrate, thus pectin methyl esterase (PME) is often an important contributor to overall pectin degradation (Brummell and Harpster, 2001). A third pectin polymer-degrading enzyme, pectate lyase (PL), was once thought to be present mainly in microorganisms, but has been reported to be in several fruits in the past several years (Martin-Rodriguez et al., 2002), including peaches (Trainotti et al., 2003).

In studying the role of PG in abscission of citrus leaf explants Riov (1974) found an enzyme activity that oxidized the GalA residues released from polymeric substrate by the PG, as well as the residual polygalacturonic acid (PGA) substrate's reducing ends. This enzyme was named uronic acid oxidase (UAO; Riov, 1974). It has been identified in many plant tissues, with the highest activity reported in citrus peel (Pressey, 1993). In citrus fruit and in citrus

* Corresponding author. Tel.: +97239683606; fax: +97239683622.
E-mail address: slurie43@agri.gov.il (S. Lurie).

0031-9422/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.phytochem.2005.10.003

leaf abscission layers the activity increased with ethylene treatment (Huberman and Goren, 1982). Citrus is a non-climacteric fruit that does not soften greatly during ripening. However, if the activity in peach, a climacteric fruit, were enhanced by ethylene and UAO were present in ripening fruits, its action could have potentially interesting physiological implications.

This study describes our preliminary examination of the UAO in a melting flesh peach. A key feature of the report is a GC–MS-based analysis that confirms the oxidation of C-1 aldehyde functions of GalA residues at the reducing termini of a pectin substrate. This conversion of GalA residues to galactaric residues is the action ascribed to UAO in earlier reports. Our unfractionated fruit protein extract used for UAO activity measurement undoubtedly contains pectolytic enzymes. Thus, whether this peach UAO interacts with polymeric or oligomeric substrates in planta is not yet clear.

2. Results and discussion

UAO activity in the enzyme extract from peach fruit was demonstrated by identifying galactaric acid residues by GC–MS analysis of the complete reaction mixture. UAO acts at the C-1 aldehyde function that is exposed when the GalA residue at a reducing end is in the open ring configuration, with the aldehyde being oxidized to the corresponding galactaric acid (Fig. 1, step 1). The methanolysis reaction utilized here methylates the C-6 carboxyl functions of the reducing-end GalA residue and all other GalA residues in the substrate and products as well as the new C-1 carboxyl function generated by UAO action at the (former) reducing-end residue (Fig. 1, step 2). The methanolysis reaction also cleaves almost all of the glycosidic linkages in the starting pectin substrate and any oligomers present, thus generating methyl glycosides. The only exception is that UAO-generated galactaric acid residues are converted to C-1, C-6 dimethyl esters, the only non-glycoside monomers present in the mixture of products. Subsequent reduction with NaBorodeuteride reduces all carboxyl methyl esters to hydroxyl functions, adding 2 deuterium atoms to each alcohol function generated (Fig. 1, step 3). These are subsequently acetylated to generate *per*-acetylated derivatives of galactitol and methyl-galactosides (Fig. 1, step 4). The resulting galactitol residues in the derivatized preparation will carry four extra mass units, as compared to a galactitol that had originated from a galactosyl residue in the reaction product mixture, and two more mass units than any galictitol residues produced by reduction of the GalA carboxyl residues. The additional 2 mass units result because galactaric acid residues initially have two carboxyl functions, at C-1 and C-6, and both are reduced with deuterium. The reduction would leave the methyl glycosides of the GalA residues that had been reduced to Gal intact, the final product being methyl glycosides of 2, 3, 4, 6-tetra-acetyl galactose. The galactitol hexa-acetate residues derived from galactaric acid can generate a diagnostic 291 AMU fragment from either carbons 1–4 or 3–6, because of the symmetrical addition of deuterium (Fig. 2) and the fact that fragmentation following electron impact is equally likely between carbons 2 and 3 or 3 and 4. Additionally, the GLC retention time of the acetylated galactaric acid derivative is that of reduced and fully acetylated Gal residue, whereas the tetra-acetyl methyl galactosides elute much earlier in the GLC program. Thus, the formation of galactaric acid residues, hence evidence of UAO action, can be inferred from the substantial enrichment of the 291 fragment and its coincidence with the GC retention time for galactitol hexa-acetate. This analytical protocol supports an unambiguous conclusion that the product of the reaction is a galactaric acid residue, a conclusion that can only be inferred from the measured decrease in reducing ends as the reaction proceeds. There could be consequences for cell wall structural relationships or ion-binding capacity if insoluble pectins were UAO substrates and, thus, bear galactaric residues at their (former) reducing ends. However, speculation on this point would be groundless without additional studies.

UAO activity in the enzyme extract from peach fruit was linear in the reaction mixture for at least 18 h (Fig. 3). At the end of that period either the substrate had been exhausted or the rate of reducing end generation due to (hypothetical) pectolytic activity was balanced by reducing end oxidation catalyzed by UAO. Previous studies of UAO have measured activity at 30 or 37 °C for periods only up to 1 h (Pressey, 1993; Riov, 1975). The stability of a partially purified enzyme was found to be quite high, not losing activity during storage at −10 °C for months (Pressey, 1993).

The pH optimum of the enzyme activity was found to be pH 8.5, but activity was measured over a broad pH range. Activity at pH 6.5 was only 11% of the maximum activity, but at pH 7.5 it was 52% and at pH 10 it was 72% of the activity at pH 8.5 (Fig. 4). Riov (1975) found maximal activity in enzyme isolated from citrus leaf abscission zones to be at pH 8 and Pressey (1993) found the activity in citrus peel to be highest at pH 8.5.

According to Riov (1975), in addition to GalA, the enzyme described could oxidize glucuronic acid, as well as the reducing terminal GalA residues of PGA. Several neutral sugars, including galactose, glucose, mannose, and xylose were not oxidized by the citrus peel enzyme (Pressey, 1993). Riov suggested that UAO's preferred substrate was GalA produced as part of catabolism of pectin polymers, a role similar to that proposed for microbial UAO (Swoboda and Massey, 1966), particularly the bacterial enzyme (Chang and Feingold, 1970). However, Pressey (1993) found greater activity as the degree of polymerization of the substrate increased, with the greatest activity measured using PGA. Our analysis also suggests that PGA is a good substrate for the enzyme from peaches (data not shown). However, because it would not be surprising if the UAO-containing enzyme extract used also contained *endo*- and *exo*-PG (e.g., Downs et al., 1992; Pressey and

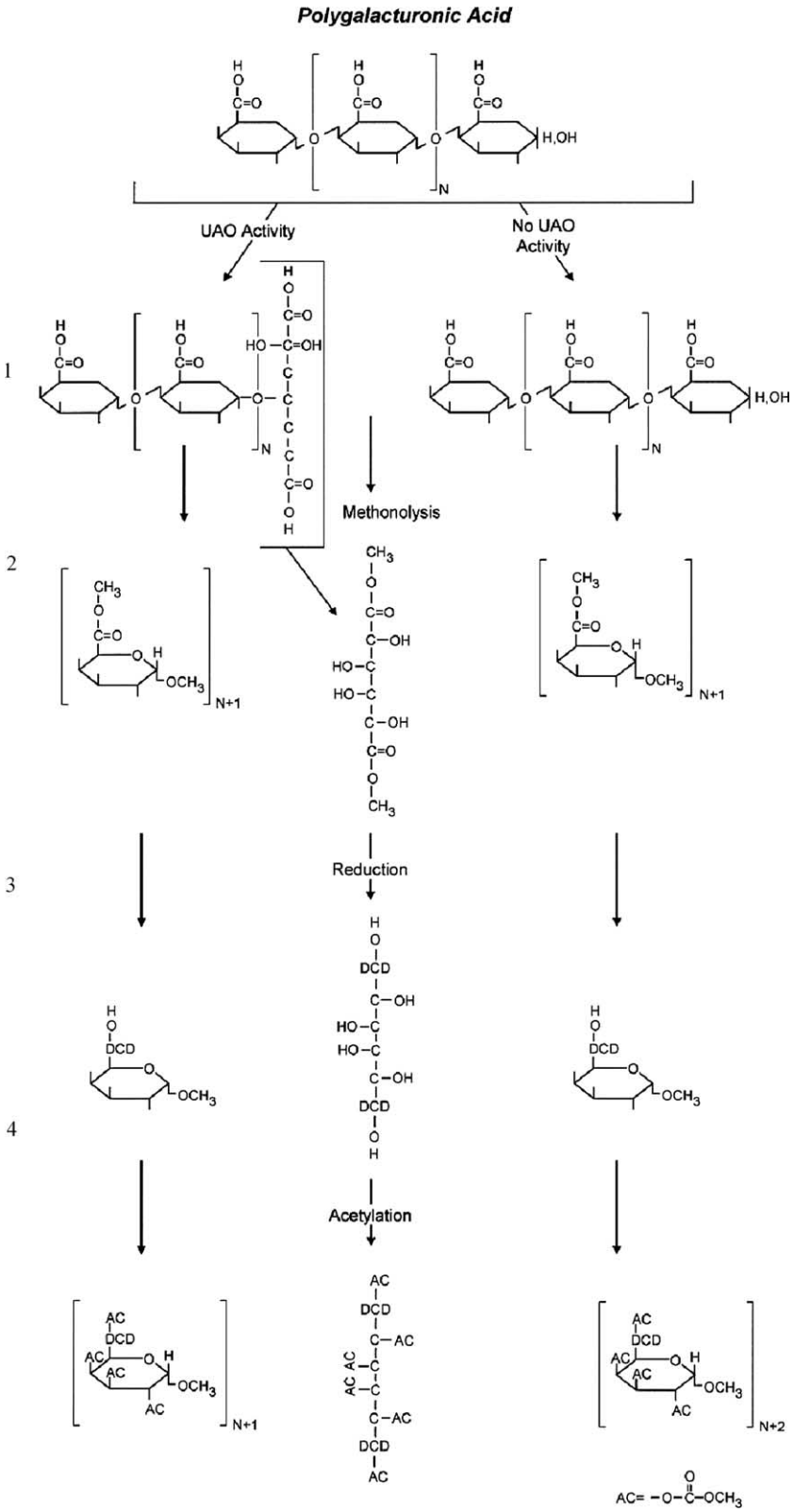

Fig. 1. Derivatization of GalA residues and the galactaric acid residue generated by peach UAO action on pectin substrate. Starting with the substrate (and, potentially, oligosaccharides produced by PG present in the peach fruit protein extract), UAO action (left side) converts reducing end GalA residues to galactaric acid residues (1). In the absence of UAO (right side) reducing end GalA residues are not altered. Methanolysis (2) generates methyl esters on the C-1 carboxyl of the galactaric acid residue and the C-6 carboxyls of the galactaric acid residue and the GalA residues in both the UAO products and un-oxidized poly- and oligomeric-GalAs. The treatment also methanolyses glycosidic linkages, generating methyl glycosides. Reduction with sodium borodeuteride (3) converts all carboxyl methyl esters to the corresponding alcohol functions and converts only the galactaric acid di-ester to galactitol. Acetylation (4) then replaces the protons on the six available hydroxyls on galactitol and the four available hydroxyls on the Gal-methyl glycosides with acetyl groups.

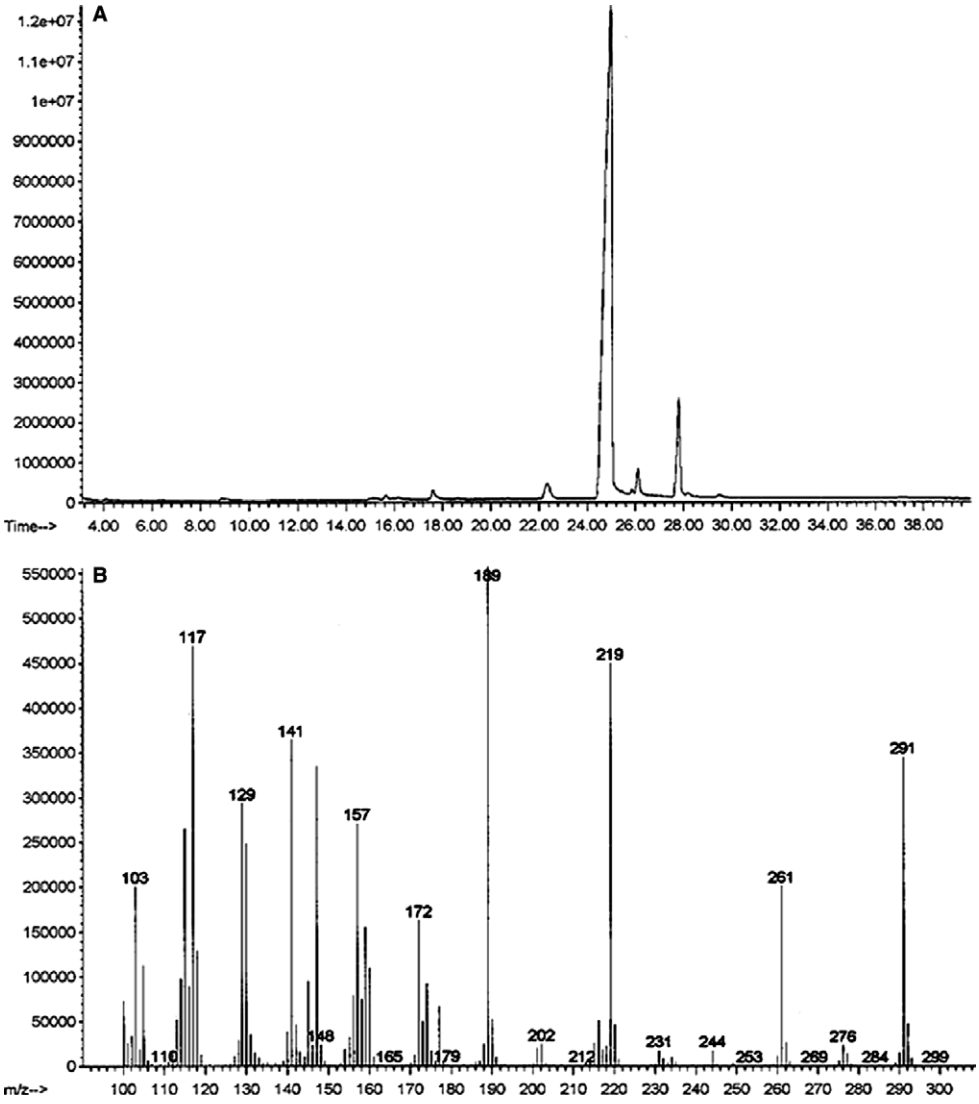

Fig. 2. Panel A: Total ion chromatogram of the GLC analysis of the galactitol hexa-acetate generated from galactaric acid derivatized as described in Fig. 1. The peak at 24.735 min is coincident with galactitol hexa-acetate generated from reagent Gal (not shown). Panel B: Mass spectrum of the peak at 24.735 in the GLC separation shown in panel A, showing the prominent peak at mass 291, indicative of a 4-carbon fragment from carbons 1–4 or 3–6 including a deuterium reduced carboxyl function.

Avants, 1973; Trainotti et al., 2003), it is possible that first pectin hydrolysis and then oxidation of PG-generated pectin oligomers and monosaccharide GalA were occurring in the reaction mixture.

The UAO activity that was extractable in buffer containing 1 M NaCl did not change appreciably as the peach fruit softened (Fig. 5). This is in contrast to *endo*- and *exo*-PG activities, which increased in activity as Hermoza, a melting flesh peach fruit, ripened and softened (Zhou et al., 2001). However, pectin methyl esterase activity changed little during ripening of the same peaches (Lurie et al., 2003; Zhou et al., 2001), although pectin methylation decreased as ripening proceeded. This is an indication that a steady state level of in vitro enzyme activity can correlate with decreasing substrate in vivo. Therefore, a role for UAO in the pectin modification accompanying peach fruit softening cannot be ruled out, even though its activity did not increase as softening proceeds.

Pectin oligomers are produced in plant tissue both during ripening and during pathogenesis by fungi and bacteria. There is considerable interest in these oligomers as possible signals, both for developmental processes and for regulation of pathogen defense reactions (Cote and Hahn, 1994). Tomato fruit pectin-derived oligomers have been found to enhance ripening in tomatoes (Melotto et al., 1994), and also to induce biosynthesis of defense molecules (Boudart et al., 1995). It has been thought that pectin oligomers were produced by the action of fruit and/or pathogen PGs. However, Spiro et al. (1993) identified bioactive oligomers containing between 10 and 15 GalA residues plus a galactaric acid residue at the reducing end following fungal *endo*-PG treatment of PGA. These oxidized,

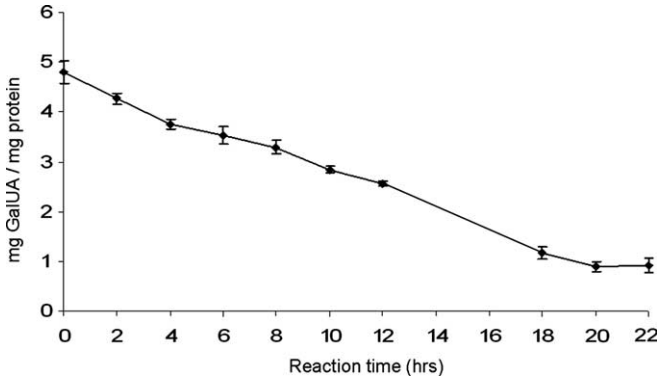

Fig. 3. Activity of uronic acid oxidase from peach fruit during incubation at pH 8.5 and 37 °C. The reaction was continued for up to 22 h and aliquots were taken at different times and assayed for reducing ends. The continuously decreasing reducing group concentration indicates the persistence of UAO action over the first 18 h of the incubation period.

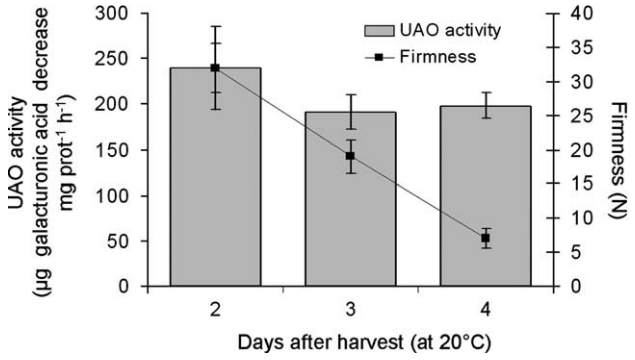

Fig. 5. The activity of uronic acid oxidase in peach fruit proteins extracted in 1 M NaCl-containing buffer and fruit firmness at different times after harvest. Fruits were held at 20 °C and assayed for firmness daily. Three fruits of a similar firmness were taken for enzyme activity analysis. Incubation time was 18 h at 37 °C.

potentially endogenous UAO-modified oligomers had activity in bioassays, although their activities were less than those of corresponding oligomers with a GalA residue at their reducing ends (Spiro et al., 1998). Therefore, peach UAO may be part of a complex program of cell wall pectin disassembly that has implications for fruit ripening-related texture change or/and changes in pectin-derived oligomer populations that influence the overall coordination of the ripening process.

3. Experimental

3.1. Plant material

Fruit (*Prunus persica* cv "breeding line 90, 1–4") were harvested from the experimental orchard at the University of California, Davis and held at 20 °C for ripening. Six fruit were chosen daily for testing of firmness on two peeled sides of the fruit using a firmness tester with an 11 mm probe (Guss Fruit Texture Analyzer, Strand, South Africa). The three fruits that were closest in firmness were peeled, diced and their tissue frozen at −20 °C for further analysis.

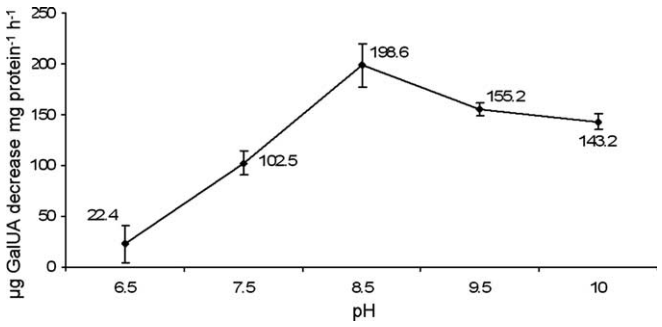

Fig. 4. The activity of uronic acid oxidase from peach fruit at different pH values, measured as the decrease in reducing end groups. Incubation time was 18 h at 37 °C. The numbers in the figure refer to enzyme activity at a particular pH.

3.2. Enzyme extraction and uronic acid oxidase activity determination

Extracts were prepared similarly to preparation for pectic lyase (Martin-Rodriguez et al., 2002). Frozen fruit flesh (20 g) was powdered in liquid nitrogen in a mortar and then 20 ml of cold 2% polyethyleneglycol and 0.2% sodium bisulfite was added. Once the slurry was melted, it was filtered through two layers of Miracloth©. The solid residue was washed twice with cold 0.2% sodium bisulfite, filtered and suspended in 16 ml cold 50 mM Na acetate buffer (pH 5.0) containing 0.5 M NaCl. After stirring 1 h at 4 °C the material was centrifuged at 16,000 rpm for 20 min at 4 °C. Following filtration as above, the supernatant was diluted 1:1 with cold 50 mM Na acetate buffer (pH 5.0) and used as crude enzyme extract.

Extract (2 ml) was mixed with an equal volume of 0.36% citrus pectin (degree of esterification 60%, Sigma Chemical Co., product P-9135) in 50 mM Tris–HCl buffer (pH 8.5), and incubated at 37 °C with 0.2 ml toluene added to prevent bacterial development. Incubation was performed for 2, 6, 8, 10, 12, 18, 20, 22 h, with aliquots taken for reducing sugar determination at intervals. The reaction was found to be linear for up to 20 h. In further experiments, 18 h was the incubation time chosen. Controls of enzyme extract in absence of substrate and substrate in absence of enzyme were run in reaction buffer each time. Determination of reducing end concentration involved the 2-cyanoacetamide assay described by Gross (1982). GalA was used as a standard. Protein content in the enzyme extract was determined using Bio-Rad reagent (Hercules, CA). One UAO activity unit was defined to be a 1 µg decrease in GalA reducing equivalents per mg protein per h. To quantify the reducing end content at time 0, enzyme extract was boiled for

10 min and pectin substrate was added once the enzyme had cooled. For determination of pH optimum 100 mM Tris–Bis Propane buffers (pH 6.5, 7.5, 8.5 and 10) were used as reaction buffer. For each experiment, separate enzyme preparations were made from three different samples of frozen fruit. Each preparation and its controls were run in triplicate, results were averaged and standard deviations calculated.

3.3. GC–MS analysis of products

For GC–MS analysis, 1 ml aliquots of a reaction mixture were thoroughly dried in a stream of filtered air and drying was completed by several additions/evaporations of acetone. Anhydrous 2 N methanolic HCl (1 ml) was added and methanolysis was performed by placing the sealed tube containing the sample in a heating block (17 h at 85 °C; Bhat et al., 1991). After evaporations (as above) in methanol and acetone, samples were reduced by adding 1 ml ethanol:water (1:1) containing 10 mg/ml sodium borodeuteride and incubating overnight at room temperature in a sealed tube. Multiple evaporations with methanol:acetic acid (9:1) and methanol were used to remove borate and, thus, avoid the inhibition of acetylation that might be caused by borate blockage of hydroxyl groups. The samples were completely dried and acetylated in 0.02 ml 1-methyl imidazole (used as acetylation catalyst) and 0.2 ml acetic anhydride, incubated at room temperature for 10 min. (Blakeney et al., 1983). To separate the products to be subjected to MS analysis from by-products of the reactions performed, samples were diluted with 2 ml water and then 3 ml dichloromethane were added. The samples were then mixed and the upper, aqueous phase was collected and discarded. This partitioning step was repeated 3×, the washed dichloromethane layer was evaporated to dryness, dissolved in 0.1 ml of acetone, and subjected to GC–MS analysis. Samples of control reaction mixtures (boiled enzyme plus substrate, substrate alone) were also subjected to the derivatization and GC–MS analysis.

Samples were analyzed using a Hewlett-Packard 6890 gas chromatograph interfaced to a model 5973 mass-selective detector. The column used was a 30 m, 0.25 mm i.d., DB-23 capillary column with a 25 μm film (J&W Scientific). The oven was programmed from 160° to 210° at 2°/min, with a 10 min hold at 210°. He was used as carrier gas at a flow rate of 1 ml/min.

References

Bhat, U.R., Mayer, H., Yokuta, A., Hollingsworth, R.I., Carlson, R.W., 1991. Occurrence of lipid A variants with 27-hydroxyocatosanoic acid in lipopolysaccharides from members of the family Rhizobiaceae. J. Bacteriol. 173, 2155–2159.

Blakeney, A.B., Harria, P.J., Henry, R.J., Stone, B.A., 1983. A simple and rapid preparation of alditol acetates for monosaccharide analysis. Carbohydr. Res. 113, 291–299.

Boudart, G., Dechamp-Guillaume, G., Lafitte, C., Ricart, G., Barthe, J.P., Mazau, D., Esquerre-Tugaye, M.T., 1995. Elicitors and suppressors of hydroxyproline-rich glycoprotein accumulation are solubilized from plant cell walls by endopolygalacturonase. Eur. J. Biochem. 232, 449–457.

Brummell, D.A., Harpster, M.H., 2001. Cell wall metabolism in fruit softening and quality and its manipulation in transgenic plants. Plant Mol. Biol. 47, 311–340.

Chang, Y., Feingold, D., 1970. D-glucaric acid and galactaric acid catabolism by *Agrobacterium tumefaciens*. J. Bacteriol. 102, 85–96.

Cote, F., Hahn, M.G., 1994. Oligosaccharins: structures and signal transduction. Plant Mol. Biol. 26, 1375–1411.

Downs, C.G., Brady, C.J., Gooley, A., 1992. Exopolygalacturonase protein accumulates late in peach ripening. Physiol. Plant. 85, 133–140.

Gross, K.C., 1982. A rapid and sensitive spectrophotometric method for assaying polygalacturonase using 2-cyanoacetamide. Hort. Sci. 17, 933–934.

Huberman, M., Goren, R., 1982. Is uronic acid oxidase involved in the hormonal regulation of abscission in explants of citrus leaves and fruits. Physiol. Plant. 56, 168–176.

Lurie, S., Zhou, H.W., Lers, A., Sonego, L., Alexandrov, S., Shomer, I., 2003. Study of pectin esterase and changes in pectin methylation during normal and abnormal peach ripening. Physiol. Plant. 119, 287–294.

Martin-Rodriguez, M.C., Orchard, J., Seymour, G.B., 2002. Pectate lyases, cell wall degradation and fruit softening. J. Exp. Bot. 53, 2115–2119.

Melotto, E., Greve, L.C., Labavitch, J.M., 1994. Cell wall metabolism in ripening fruit (VII. Biologically active pectin oligomers in ripening tomato (*Lycopersicon esculentum* Mill.) fruits). Plant Physiol. 106, 575–581.

Pressey, R., 1993. Uronic acid oxidase in orange fruit and other plant tissues. Phytochemistry 32, 1375–1379.

Pressey, R., Avants, J.K., 1973. *Endo*- and *exo*-polygalacturonase in peach fruit. Plant Physiol. 52, 252–259.

Ridley, B.L., O'Neill, M.A., Mohnen, D., 2001. Pectins: structure, biosynthesis, and oligalacturonide-related signaling. Phytochemistry 57, 929–967.

Riov, J., 1974. A plygalacturonase from citrus leaf explants: role in abscission. Plant Physiol. 53, 312–316.

Riov, J., 1975. Metabolism of uronic acids in plant tissues. Plant Physiol. 55, 602–606.

Spiro, D., Kates, K., Koller, A., O'Neill, M., Albersheim, P., Darvill, A., 1993. Purification and characterization of biologically active 1,4-linked α-D-oligogalacturonides after partial digestion of polygalacturonic acid with endopolygalacturonase. Carbohydr. Res. 247, 9–20.

Spiro, D., Ridley, B., Eberhard, S., Kates, K., Mathieu, Y., O'Neill, M., Mohnen, D., Guerun, J., Darvill, A., Albersheim, P., 1998. Biological activity of reducing-end-derivatized oligogalacturonides in tobacco tissue cultures. Plant Physiol. 116, 1289–1298.

Swoboda, B., Massey, V., 1966. On the reaction of the glucose oxidase from *Aspergillus niger* with bisulfite. J. Biol. Chem. 241, 3409–3416.

Trainotti, L., Zanin, D., Casadoro, G., 2003. A cell wall oriented genomic approach reveals a new and unexpected complexity of the softening in peaches. J. Exp. Bot. 54, 1821–1832.

Zhou, H.W., Lurie, S., Ben Arie, R., Dong, L., Weksler, A., Lers, A., 2001. Intermittent warming of peaches reduces chilling injury by enhancing ethylene production and enzymes mediated by ethylene. J. Hort. Sci. Biotechnol. 76, 620–628.