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Cell wall disassembly during papaya softening: Role of ethylene in changes in composition, pectin-derived oligomers (PDOs) production and wall hydrolases

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ABSTRACT

Cell wall disassembly in ripening climacteric fruit is a highly complex process where ethylene plays a crucial role. Ethylene inhibitors can be used to explore the changes in the cell wall matrix and cross-linked polysaccharides in ethylene-regulated processes. The results of applying the ethylene receptor blocking inhibitor 1-methylcyclopropene (1-MCP) and the ethylene-releasing compound ethephon (2-chloroethylphosphonic acid) indicate that softening of 'Maradol' papaya fruit is dependent on ethylene. When fruit were induced to ripen extensively by exposure to a high dose of ethephon, 1-MCP inhibited the subsequent softening dramatically, but when inhibition of the ethylene response was caused by application of 1-MCP, subsequent fruit treatment with ethephon promoted extensive loss of galactose from the water-soluble polysaccharides, but this was not accompanied by fruit softening. The cell wall changes accompanying normal fruit softening were pectin solubilization and polyuronide depolymerization and these processes occurred simultaneously. Polygalacturonase likely is responsible for the ripening-associated changes in 'Maradol' papaya fruit texture and pectin polymer integrity. An increase in extractable fruit polygalacturonase follows the increased presence of pectin-derived oligosaccharides.
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1. Introduction

Papaya fruit, as with most climacteric fruits, have a short shelf life. One of papaya's postharvest limitations is its high sensitivity to mechanical damage and diseases, which negatively impacts yield and quality during handling, storage, transportation, marketing and, hence, consumer satisfaction. No estimates of wholesale and retail losses of 'Maradol' papaya are reported in Mexico, however estimation of postharvest losses in Hawaiian papaya fruit due to parasitic diseases, physiological disorders, mechanical damage and over-ripening, are as high as 75% (Paull et al., 1997). In each of these situations, both ethylene and alterations in the integrity of cell walls are involved in one way or another.

Ethylene is the key regulator of ripening-related changes in papayas and its synthesis can be stimulated by external biotic or abiotic factors and fruit developmental events. In either case, ethylene activates a cascade of signals that ultimately act at the level of gene expression to influence pathogen defense (Klee, 2002) and fruit ripening (Giovannoni, 2001). Examination of ethylene-regulated cell wall degradation has important implications for the control of fruit softening. Therefore, ethylene inhibitors (Blankenship and Dole, 2003) can be used to study many aspects of metabolism, including changes in cell walls and texture, in harvested papaya fruit.

'Maradol' papaya fruit is an economically important tropical fruit in Mexico. Changes in cell wall enzymes have been described in other cultivars of papaya. These enzymes included polygalacturonase (PG), pectin methylesterase (PME), β-galactosidase (β-gal), xylanase and cellulase (Paull and Chen, 1983; Lazan et al., 1995; Manenoi and Paull, 2007). Lazan et al. (1995) reported an increase in pectin solubility and depolymerization during ripening. These are two independent but related events where PG plays an important role. Zhao et al. (1996) described an important correlation between changes in PG and xylanase activity, papaya fruit softening, and a decrease in the apparent average molecular mass of pectin.

The aim of this research was to define cell wall metabolism and enzymes involved in the process of postharvest softening of 'Maradol' papaya. We used ethylene and 1-MCP in individual or sequential applications, to determine whether the main changes in matrix and cross-linked polysaccharides are dependent on ethylene production and responses. In addition, an analysis of fruit

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content of several cell wall-degrading enzymes (CWDEs) was carried out and parallels between changes in these enzymes, ethylene concentration and responses, and fruit firmness were identified. In addition, the identification and preliminary analysis of pectin-derived oligosaccharides (PDOs) in ripening 'Maradol' papayas provided additional data supporting the conclusion that ethylene responses, pectin metabolism, and fruit softening are developmentally related in this fruit.

2. Materials and methods

2.1. Promotion and inhibition of fruit ripening

'Maradol' papayas (*Carica papaya* L) at the 1/4 ripening stage (20–25% of skin yellowing) were harvested in June 2006 from a commercial orchard located in Tepic, Nayarit, Mexico. Hermaphrodite fruit free of mechanical injury were selected, washed and disinfected by immersion in 0.25% Captan for 3 min in order to decrease fungal disease incidence during storage. The fruit were dried at room temperature and divided into sub-samples for treatments. For fruit ripening inhibition (treatment with 1-MCP), SmartFresh™ (Agrofresh Rohm & Haas Inc.) was used. Fifty fruit were placed into a stainless steel, hermetically sealed container (0.238 m^3) and treated with the amount of the Smartfresh compound calculated to produce a 300 nL L^{-1} concentration of 1-MCP gas (concentration not verified by GC) allowing fruit exposure for 12 h at 20 °C. For ripening induction (treatment with ethephon), a solution equivalent to 2.5 g L^{-1} of 2-chloroethyl phosphonic acid (ethephon) was used; fifty fruit were dipped for 3 min at 20 °C. Then, 12 h after 1-MCP or 24 h after ethephon application, 25 fruit from each treatment were treated with the respective ripening antagonist; i.e., 1-MCP-treated fruit were treated with ethephon and ethephon-treated fruit were treated with 1-MCP, as described. Fruit were then stored to simulate marketing conditions (20 ± 2 °C, 85% RH) and allowed to continue postharvest development. Fifty untreated fruit served as a control.

2.2. Ethylene concentration, respiration rate and firmness

Three fruit were weighed, individually placed into glass containers and flushed continuously with CO_2-free humidified air. The system was kept at a pressure of 101 kPa and temperature of 20 °C. One milliliter of headspace gas was injected into a gas chromatograph (Model 3300, Varian Inc.). Thermal conductivity detector for CO_2 and flame ionization detector for ethylene were used. Fruit firmness was determined at four points around the fruit equator (3 fruit per treatment), using a penetrometer (Chatillon DFGS-100, automatic base TCD-200, 8 mm diameter tip). Maximum force for tip insertion to a depth of 1.5 cm at speed of 5.2 mm s^{-1} was recorded.

2.3. Cell wall yield and isolation

Fruit used for firmness determinations also were used for wall preparation. A wedge-shaped, longitudinal slice was cut from each of the 3 fruit in a given treatment. Seeds and peel were removed from the tissue wedges and the tissue was sliced into cubes of approximately 3 mm on each side. A sample of 100 g of cubed flesh from the pooled tissue was homogenized in an Ultraturrax with 300 mL of 95% ethanol to dissolve low molecular weight sugars and organic acids and the slurry was boiled with continuous stirring for 45 min to ensure enzyme inactivation and prevention of autolytic breakdown of wall polymers when isolated cell wall material was extracted subsequently (Rose et al., 1998). The insoluble material was filtered through glass fiber filter, recovered and sequentially washed with 250 mL of ethanol, 250 mL of chloroform:methanol (1:1, v/v), and 250 mL of acetone. Insoluble, acetone-washed material was oven-dried at 37 °C, weighed and stored in a desiccator until use. This crude extract was named alcohol insoluble solids (AIS).

2.4. Cell wall composition analysis

Two mg of AIS was dissolved in H_2SO_4 and assayed for uronic acid (UA) content as described by Ahmed and Labavitch (1977). Spectrophotometric evaluation of UA concentrations was calibrated using a galacturonic acid (GalA) (Sigma) standard curve. For total sugars (TS), three mg of AIS were stirred with 3 mL of 67% H_2SO_4 for 4 h and aliquots were assayed by the anthrone reaction according to Yemm and Willis (1954). Spectrophotometric evaluation of TS concentrations was calibrated using a glucose (Sigma) standard curve. The non-cellulosic neutral sugar (NS) composition was obtained from two mg of AIS residue hydrolyzed with 2 mol L^{-1} trifluoroacetic acid at 121 °C for 1 h. After hydrolysis the samples were centrifuged (clinical centrifuge, ca, 2000 × g). The pellets were dissolved in 67% H_2SO_4 and assayed using the anthrone reagent to evaluate AIS cellulose content, with a 67% H_2SO_4-solution of cellulose powder used for a standard curve. The TFA-soluble supernatants were dried and converted to alditol acetates (Blakeney et al., 1983). For analysis, they were injected into a gas chromatograph (Model 3800, Varian Inc.) with a 30 m × 0.25 mm i.d. capillary column (model DB-23, J & W Scientific, Folsom, CA) as described in Carrington et al. (1993). Results were expressed as mol%, calculated using standards of rhamnose (Rha), fucose, arabinose (Ara), xylose (Xyl), mannose, galactose (Gal), glucose (Glc) and *myo*-inositol as internal standard (Sigma).

2.5. Cell wall fractionation and fraction composition analysis

The AIS was fractionated into pectin- and hemicellulose-enriched samples as follows. Duplicate samples of approximately 200 mg of AIS were weighed and each sample was sequentially extracted with (1) water, (2) EDTA in 0.05 mol L^{-1} sodium acetate pH 6.5, (3) 0.05 mol L^{-1} Na_2CO_3 containing 0.02 mol L^{-1} $NaBH_4$, (4) 4% KOH containing 0.1% $NaBH_4$ and (5) 24% KOH containing 0.1% $NaBH_4$. The fractions were obtained by suspending the AIS powder in 20 mL of the extraction solvent and stirring for 12 h at room temperature. Solubilized material was separated by centrifugation at 8500 × *g* until a compacted pellet was sedimented. The supernatant was transferred and the pellet was extracted again with 10 mL of the same solution, stirred for 4 h, and then centrifuged as described. The second supernatant was combined with the first for each extractant. The water-soluble fraction was designated WSF, the EDTA-soluble fraction as ESF, the Na_2CO_3-soluble fraction as SCSF, and the 4% KOH- and 24% KOH-soluble fractions as 4KSF and 24KSF, respectively. All extracted fractions were filtered through GF/A (Whatman glass fiber filter) to eliminate insoluble solids and the ESF, SCSF, 4KSF and 24KSF were dialyzed 4 times against distilled water with changes each 8 h. The 4KSF and 24KSF were neutralized with concentrated glacial acetic acid prior to the dialysis step. All fractions were assayed in triplicate for UA and TS or in duplicate for NS composition, as described above. After colorimetric and chromatographic assays, fractions were frozen, freeze-dried and stored at −20 °C until use.

2.6. Size exclusion chromatography (SEC)

Lyophilized samples were dissolved in the appropriate running buffer and fractionated on a size-exclusion column (1.5 cm × 80 cm)

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