Food Chemistry 128 (2011) 1072–1079

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Cell wall-modifying enzymes and firmness loss in ripening 'Golden Reinders' apples: A comparison between calcium dips and ULO storage

Abel Ortiz [a], Jordi Graell [b], Isabel Lara [a,*]

[a] Departament de Química, Unitat de Postcollita-XaRTA, Universitat de Lleida, Rovira Roure 191, 25198 Lleida, Spain
[b] Departament de Tecnologia d'Aliments, Unitat de Postcollita-XaRTA, Universitat de Lleida, Rovira Roure 191, 25198 Lleida, Spain

ARTICLE INFO

Article history:
Received 3 December 2010
Received in revised form 18 February 2011
Accepted 6 April 2011
Available online 12 April 2011

Keywords:
Apple
α-L-Arabinofuranosidase
β-Galactosidase
Calcium dips
Cell wall
Firmness
Pectate lyase
ULO storage

ABSTRACT

Calcium treatment and storage under ultra-low oxygen (ULO) conditions are common post-harvest practices aimed at delaying ripening-related softening of apple (*Malus* × *domestica* Borkh.) fruit, but the biochemical mechanisms underlying these effects have not been determined conclusively to date. In this study, commercially mature 'Golden Reinders' apples were dipped in 2% calcium chloride prior to storage at 1 °C and 92% RH under either regular air or ultra-low oxygen (ULO; 1kPa O_2:2kPa CO_2) for 19 or 31 weeks, and kept thereafter at 20 °C for 0, 7 or 14 days in order to simulate the usual marketing time. Cell wall composition and cell wall-modifying enzyme activities were determined in relation to fruit firmness. ULO-storage and calcium dips were effective for firmness preservation, seemingly due to decreased pectin solubilisation. β-Galactosidase, α-L-arabinofuranosidase and pectate lyase activities were correlated positively with firmness loss of 'Golden Reinders' fruit after storage.

1. Introduction

Standard quality specifications for commercialisation of apple (*Malus* × *domestica* Borkh.) fruit rely essentially on visual parameters such as size and surface colour. Yet these parameters are not sufficient to fit consumer's expectations, as apple preference is driven mainly by texture and flavour (Harker, Kupferman, Marin, Gunson, & Triggs, 2008; Jaeger, Andani, Wakeling, & MacFie, 1998). Since firmness is associated to juicy and crispy texture, firmer apples are generally more appreciated. Contrarily, soft apples can develop mealiness, a texture attribute causing a starch-like sensation in the mouth. However, while a substantial increase in apple acceptability was reported, as firmness rose up to 62 N (Harker et al., 2008), only small improvements in consumer acceptance resulted from further increases in firmness, suggesting that the enhancement of consumer acceptance in apples that are firm may rely on high levels of other attributes such as SSC and/or TA.

In addition to its role on sensory quality, firmness is also important for storage potential. Firmer fruit are more resistant to physical damage and infections during handling and storage, which is an economically relevant issue. Consequently, most post-harvest strategies have focused on delaying extensive fruit softening, and controlled atmosphere (CA) storage, mainly under ultra-low oxygen (ULO) concentrations, has been widely adopted as the technology of choice for apple storage. Unfortunately, CA storage often leads to partial suppression of flavour-contributing volatile compounds (Lara, Echeverría, Graell, & López, 2007; Ortiz, Echeverría, Graell, & Lara, 2010), which is a major drawback of CA technology often causing detrimental effects on the eating quality of produce.

Ripening-related softening of fruit is generally associated to the disassembly of middle lamella and primary cell walls (Brummell & Harpster, 2001; Goulao & Oliveira, 2008), which are composed of rigid cellulose microfibrils held in concert by networks of matrix glycans (hemicelluloses) and pectins, with varying levels of structural proteins and phenolics (Caffall & Mohnen, 2009). During ripening, these polysaccharides are extensively modified, mostly by the action of a large number of cell wall-localised proteins, resulting in solubilisation, depolymerisation and rearrangements of their associations, which eventually affect cell wall strength and lead to fruit softening (Brummell et al., 2001; Goulao, Cosgrove, & Oliveira, 2008). The real contribution of these modifying proteins to the softening process remains unclear despite numerous experiments on genetic modification of individual cell wall-related enzymes (Vicente, Saladié, Rose, & Labavitch, 2007). The degree of methylation of pectin is also a major factor determining pectin properties and textural attributes of fruit (Fraeye et al., 2009). During fruit development and ripening, pectinmethylesterase (PME, EC 3.1.1.11)-catalysed pectin demethylation may delay firmness loss,

* Corresponding author. Tel.: +34 973 702526; fax: +34 973 702924.
E-mail address: lara@quimica.udl.cat (I. Lara).

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doi:10.1016/j.foodchem.2011.04.016

as demethylated carboxyl groups can cross-link with divalent cations such as calcium, thus reinforcing the cell wall network and reducing its porosity (Brummell et al., 2001; Voragen, Coenen, Verhoef, & Schols, 2009).

Calcium applications thus have the potential to delay softening and to extend shelf life of apples, and therefore have an influence on texture, the other major attribute determining consumer acceptance of apples. They are also more economical and simple than CA technology, and have been shown recently to have beneficial effects on aroma biosynthesis by 'Golden Reinders' apples (Ortiz et al., 2010). Although these are interesting findings from a technological perspective, the optimisation of post-harvest handling requires a broader overview of changes induced by the procedures applied in each case. While calcium applications and CA storage have been shown to maintain apple firmness (reviewed in Johnston, Hewett, & Hertog, 2002), treatment effects on cell wall composition and related enzyme activities have received much less attention, and no conclusive results have been reported to date. The impact of post-harvest calcium dips on cell wall metabolism of air- and CA-stored 'Golden Reinders' apples was assessed herein. A two-week post-storage period at 20 °C was chosen to simulate the usual marketing time for these fruit.

2. Materials and methods

2.1. Plant material, calcium treatment and storage conditions

Apple (*Malus* × *domestica* Borkh., cv. Golden Reinders) fruit were harvested in 2007 at commercial maturity (139 days after full bloom), from 7 year-old trees grafted on M-9 EMLA rootstocks at the IRTA-Experimental Station in Mollerussa, in the area of Lleida (NE Spain). Ethylene production at harvest was 1.4 μl kg^{-1} h^{-1}; firmness and starch index averaged 72.3 N and 5.3, respectively. Immediately after harvest, fruit were randomly divided into four lots, two of which were dipped in a $CaCl_2$ solution (2%, w/v, in deionised water) at ambient temperature for 5 min. Subsequently, $CaCl_2$-treated and untreated apples were stored at 1 °C and 92% RH under either air or ultra-low oxygen (ULO) atmosphere (1kPa O_2:2kPa CO_2). O_2 and CO_2 concentrations were monitored continuously, and corrected automatically using N_2 from a tank and by scrubbing off excess CO_2 with a charcoal system. A humidifier was used to maintain RH to constant levels. Samples were taken after 19 or 31 weeks of storage, and placed at 20 °C for 0, 7 or 14 days in order to simulate commercial shelf life and final firmness of fruit reaching potential consumers.

2.2. Determination of calcium content

Seven days after removal from cold storage, samples of flesh tissue were taken (3 replicates × 2 apples/replicate), frozen in liquid nitrogen, freeze-dried, powdered, and kept at −80 °C until processing. One gramme of lyophilised powdered tissue was ashed in a muffle furnace at 500 °C for 2 h. Ashes were digested thereafter with 4 ml HCl:water (1:1, v/v) and heated at 70 °C until complete sample dehydration. Dried material was then resuspended in 2 ml HCl:water (1:1, v/v) for 15 min, filtered through 'Whatman 40 Ashless' paper filters, and the filtrate diluted to 50 ml in distilled water. Samples were then analysed by inductively coupled plasma emission spectroscopy (ICP-OES) in a 'Horiba Jobin Yvon ACTIVA' spectrometer, and results expressed as mg 100 g FW^{-1}.

2.3. Determination of flesh firmness

Skin tissue from two opposite sides was removed, and flesh firmness measurements were carried out individually on 15 fruit per treatment using a hand-held Effegi penetrometer equipped with an 11.1 mm-diameter probe with a convex tip. Results are given as N.

2.4. Extraction, fractionation and uronic acid analysis of cell wall materials

Samples of flesh tissue were taken from six apples per treatment (2 fruit/replicate × 3 replicates), frozen in liquid nitrogen, freeze-dried, and powdered. Weight loss after lyophilisation was consistently around 82%. Cell wall materials (CWM) were extracted from lyophilised tissue (3 g) according to Redgwell, Melton, and Brasch (1992). Samples were homogenised in 20 ml phenol:acetic acid:water (2:1:1, w/v/v) (PAW) for 20 min. After centrifugation at 4000*g* and 4 °C for 45 min, the pellet was resuspended in 10 ml water and centrifuged again. The PAW and water wash supernatants were combined and intensively dialysed (mol wt. cut-off 7000) for 2 days against Milli-Q water at 4 °C. The dialysate was centrifuged at 4000*g* and 4 °C for 45 min to sediment out the precipitate formed during the dialysis. The supernatant (henceforth, PAW-soluble fraction; PAW_{sf}) was recovered, lyophilised and weighed. The pellet obtained after PAW extraction and water wash was subsequently washed twice in acetone, recovered by vacuum-filtration through Whatman grade 4 paper filters, lyophilised and weighed to determine yield of CWM, expressed as % (w/w) FW. For further fractionation, CWM (100 mg) from each replicate were extracted sequentially with water, 0.05 M cyclohexane-*trans*-1,2-diamine tetra-acetate (CDTA), 0.05 M Na_2CO_3, and 4 M KOH as described previously (Selvendran & O'Neill, 1987), in order to fractionate water-soluble pectin, loosely-bound pectin, covalently-bound pectin and matrix glycans (hemicelluloses), respectively. Each fraction was dialysed (mol wt. cut-off 7000) for 2 days against Milli-Q water at 4 °C, filtered through Miracloth, lyophilised and weighed. Yields are expressed as % (w/w) CWM. For uronic acid content determination, 30–35 mg of the CDTA- and Na_2CO_3-soluble fractions were pre-hydrolysed in 1 ml of 12 M H_2SO_4 for 1 h at 37 °C, prior to dilution in 11 ml distilled water and further hydrolysis at 100 °C for 2 h. Uronic acid content in the hydrolysate was measured by the *m*-hydroxydiphenyl method (Blumenkrantz & Asboe-Hansen, 1973), using galacturonic acid as a standard, and results were expressed as % (w/w).

2.5. Extraction and assay of cell wall-modifying enzyme activities

For the extraction of polygalacturonase (exo-PG; EC 3.2.1.67 and endo-PG; EC 3.2.1.15), pectinmethylesterase (PME; EC 3.1.1.11), pectate lyase (PL; EC 4.2.2.2) and endo-1,4-β-D-glucanase (EGase; EC 3.2.1.4), 100 mg of freeze-dried flesh tissue was homogenised (10%, w/v) in extraction buffer prepared according to Lohani, Trivedi, and Nath (2004). PG activity was determined on apple pectin (d.e. 70–75%) as described previously (Pathak & Sanwall, 1998), with galacturonic acid (GalUA) as a standard. One unit (U) of PG activity was defined as the liberation of 1 μmol of GalUA min^{-1}. PME activity was measured as described by Hagerman and Austin (1986), with the reaction mixture containing crude enzyme extract, pectin and bromothymol blue prepared as described by Alonso, Howell, and Canet (1997). One unit (U) of PME activity was defined as the decrease of one unit of A_{620} min^{-1}. PL activity was assayed according to Moran, Nasuno, and Starr (1968) as modified by Lohani et al. (2004). One unit (U) of PL activity was defined as the increase of one unit of A_{235} min^{-1}. For the assessment of EGase activity, the DNS method (Miller, 1959), with carboxymethylcellulose [1.3% (w/v) in 20 mM Tris–HCl, pH 7.0] as the assay substrate, was used to determine the amount of reducing sugars released, using glucose as a standard. One unit (U) of EGase activity was defined as the release of 1 μmol of glucose min^{-1}.