Pectin modifications and the role of pectin-degrading enzymes during postharvest softening of Jonagold apples

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Abstract

This study aimed at understanding softening in Jonagold apple (Malus x domestica Borkh.) fruits, by investigating pectin modifications and the evolution of pectin-modifying enzymes during postharvest storage and ripening. Jonagold apples were harvested at commercial maturity and stored at different temperatures and controlled atmosphere conditions for six months, followed by exposure to ambient shelf life conditions (20°C under air) for 2 weeks. The composition of the pectic material was analysed. Furthermore, the firmness and the ethylene production of the apples were assessed. Generally, the main changes in pectin composition associated with the loss of firmness during ripening in Jonagold apples were a loss of side chains neutral sugars, increased water solubility and decreased molar mass. Also, the activities of four important enzymes possibly involved in apple softening, β-galactosidase, α-
arabinofuranosidase, polygalacturonase and pectin methylesterase, were measured. Pectin-related enzyme activities highly correlated with ethylene production, but not always with pectin modifications.

**Keywords:** *Malus × domestica*, firmness, pectin, cell wall-modifying enzymes, controlled atmosphere

1. **Introduction**

Softening is one of the major changes that occur during ripening in climacteric fruits. Soft fruits are more susceptible to physical damage and fungal infections during handling. Consequently, to extend the storage life of fruits, considerable effort is made to maintain fruit firmness. This is often achieved by storing fruits at low temperatures, to reduce their metabolic rates. Additionally, apples are typically stored under controlled atmosphere (CA), usually with low O₂ levels and elevated CO₂ levels, to limit various oxidative processes that are implicated in the normal ripening process. Other technologies such as calcium dips (Ortiz, Graell, & Lara, 2011) and the use of ethylene inhibitors (Watkins, 2006) are also being employed to maintain fruit firmness during storage. Although efforts have been made to understand how low temperature and CA storage affect postharvest softening in apples (Johnston, Hewett, Hertog, & Roger Harker, 2001; Gwanpua et al., 2012), it is not clear how these postharvest treatments influence cell wall modifications during storage.

The cell wall of apple tissues, as for other fruits, is a Type I primary cell wall, which is made up of networks of xyloglucan-cellulose microfibrils, embedded in a matrix of pectin polysaccharides and structural proteins (Carpita & Gibeaut, 1993). The pectin matrix is responsible for maintaining cell wall integrity, by acting as a cross-link between the xyloglucan-cellulose microfibril frameworks. During ripening, various cell wall modifying enzymes may cause pectin polymers to undergo extensive structural modifications (Brummell & Harpster, 2001; Goulao & Oliveira, 2008), thereby loosening the cohesion between the cells. As a result of these changes in the pectin polymers, cell wall polymers are less bound together and become highly hydrated. The loss in cohesion of the pectin network is principally responsible for softening during ripening in most fruits (Fischer & Bennett, 1991).
Many enzymes are thought to be implicated in cell wall modifications during ripening in apples. Polygalacturonase (exo-PG EC 3.2.1.67 and endo-PG; EC 3.2.1.15), β-galactosidase (β-Gal; EC 3.2.1.23), α-arabinofuranosidase (α-AF; EC 3.2.1.55), pectin methylesterase (PME; EC 3.1.1.11), pectate lyase (PL; EC 4.2.2.2), endo-β-1,4-glucanases (EGase; EC 3.2.1.4) and β-xilosidase (β-Xyl; EC 3.2.1.37) activities have all been reported in apples (Goulao, Santos, de Sousa, & Oliveira, 2007; Wei, Ma, Shi, Qi, Zhu & Yuan, 2010). However, in most of these studies, only changes in enzyme activity were studied, whereas the structural modifications occurring at the (pectin) polymer level (as a consequence of enzyme activities) were not investigated. Therefore, the exact role played by the different enzymes during softening is not clearly understood.

The objective of this study is to understand the molecular basis of apple fruit softening during ripening. Several studies have revealed that very little non-pectolytic cell wall changes occur during ripening in apples (Fischer & Bennett, 1991). Furthermore, Goulao et al. (2007) show that in apples, the non-pectolytic enzymes (EGase, expansin and β-Xyl) were only important during fruit set and development. Consequently, non-pectolytic enzymes were not considered in the current study. We aimed at investigating modifications of the pectin polysaccharides that occur during postharvest ripening of Jonagold apples, and to elucidate the role of different pectin degrading enzymes (PG, PL, PME, β-Gal and α-AF) in catalysing these changes. Furthermore, the effect of storage conditions (temperature and gas composition of the atmosphere) on these changes was studied.

2. Materials and methods

2.1. Fruits

Jonagold apple (*Malus x domestica* Borkh.) fruit were harvested in 2012 from Van der Velpen Orchard, located at Opvelpsestraat, Bierbeek, Belgium. The fruit were harvested at commercial maturity on 22nd September 2012, and transported immediately to the Flanders Centre of Postharvest Technology (VCBT), Belgium, where the storage experiments were done. The optimal picking date was determined by the
VCBT, using a combination of firmness, starch index, soluble solids content, skin colour and respiration rates (Streif, 1996).

2.2. Storage experiments

The fruit were randomly divided into three batches, and stored under the following different conditions for six months: 1 °C in CA (1 kPa O₂ and 3 kPa CO₂), which is the recommended optimal storage condition for long term storage of Jonagold apples; 1 °C in normal air (20.8 kPa O₂ and 0.03 kPa CO₂), to study the effect of CA, and 4 °C in CA, to study the effect of temperature. At the end of storage, the apples were placed under ambient shelf life conditions (20.8 kPa O₂, 0.03 kPa CO₂ and 20 °C) for two weeks. All measurements were done immediately after harvest, every two months during storage (for all conditions), and every seven days when the fruit were placed under ambient shelf life conditions. For each sampling time, 10 fruit were used in each determination of firmness and ethylene production. For enzyme activity assays and pectin characterization, five and four fruit were used, respectively.

2.3. Measurement of firmness

Flesh firmness was measured according to ASAE Standards (2001), using an LRX Universal Testing Machine (Lloyd Instruments, UK), equipped with a load cell of 500 N. A self-cutting cylindrical plunger with a surface of 1 cm², with a diameter of 11.3 mm, was attached to the load cell and allowed to move at a constant speed of 8 mm s⁻¹ towards the fruit. The firmness was taken as the maximum force (N) needed for the plunger to penetrate the fruit to a depth of 8 mm. Two measurements were taken on the equator, one at the blush side and one at the green side, and the average was taken as the firmness value.

2.4. Measurement of ethylene production

Ethylene production was measured according to Bulens, Van de Poel, Hertog, de Proft, Geeraerd, and Nicolai (2011). An apple was initially enclosed in a jar of 1.7 L and flushed for 3 hours with humidified gas with the same composition as the atmosphere and temperature under which the apple was stored. The
inlet and outlet of the jars were then closed and 3 mL gas samples were withdrawn from the jars and
analysed by injecting into a CompactGC (Interscience, Louvain-la-Neuve, Belgium) gas chromatograph.
Calibration was done by ethylene standards ranging from 50 μg L$^{-1}$ to 50 mg L$^{-1}$. The rate of ethylene
production was obtained by doing a second measurement after 18 h for measurements done at 1 °C in CA,
or 3 h for measurements done under ambient shelf life conditions.

2.5. Pectin characterization

Characterization of the pectin from each of the four fruits was done by carrying out the following steps.

2.5.1. Extraction of alcohol insoluble residue (AIR)

The cell wall material was isolated from the apple tissue as alcohol insoluble residue (AIR) using the
method described by McFeeters and Armstrong (1984). About 50 g of crushed apple was homogenised in
320 mL 95 % (v/v) ethanol using a mixer (Buchi mixer B-400, Flawil, Switzerland). The suspension was
filtered (Machery-Nagel MN 615 Ø 90 mm) and the residue was again homogenised in 160 mL 95 %
(v/v) ethanol. After another filtration step, the residue was homogenised in 160 mL acetone. The AIR was
obtained by drying the final residue overnight at 40 °C.

2.5.2. Determination of galacturonic acid (GalA) content and degree of methoxylation (DM)

To measure the galacturonic acid (GalA) content, pectin was first completely hydrolysed using
concentrated sulphuric acid, as described by Ahmed and Labavitch (1977), and the concentration of GalA
was then measured spectrophotometrically. The degree of methoxylation (DM) was determined as the
ratio of the molar amount of methyl-esters to the molar amount of GalA residues. The amount of methyl-
esters was determined by saponification of the ester bonds with NaOH as described by Ng and Waldron,
(1997), and spectrophotometrically measuring the amount of methanol released using the method by
Klavons and Bennett (1986).
The cell wall material was fractionated into water soluble pectin (WSP), chelator soluble pectin (CSP) and sodium carbonate soluble pectin (NSP) by sequentially extracting the AIR with water, a chelating agent and an alkaline solution, respectively. The WSP fraction was obtained using the method described by Sila, Smout, Elliot, Van Loey, and Hendrickx (2006). 45 mL of boiling distilled water was added to 0.25 g of AIR on a heated stirring plate. The mixture was allowed to boil for 5 min, then cooled and filtered (Machery-Nagel MN 615 Ø 90 mm). The filtrate was adjusted to 50 mL using distilled water, and the residue was used for further fractionation. CSP was extracted by adding 45 mL 0.05 M cyclohexane-trans-1,2-diamine tetra-acetic acid (CDTA) in 0.1 M potassium acetate pH 6.5 (Chin, Ali, & Lazan, 1999) to the residue, and was left for 6 h at 28 °C, while shaking. The mixture was filtered (Machery-Nagel MN 615 Ø 90 mm) and the filtrate was adjusted to 50 mL using the 0.05 M CDTA solution. NSP was extracted from the resulting residue by stirring in 45 mL 0.05 M Na₂CO₃ solution, containing 0.02 M NaBH₄ for 16 h at 4 °C. The mixture was re-incubated at 28 °C for 6 h (Chin et al., 1999) and filtered. The filtrate was adjusted to 50 mL using the 0.05 M Na₂CO₃ solution, while the residue was discarded. The GalA content of all three fractions were measured as described earlier.

2.5.4. Neutral sugar analysis

The neutral sugar content of the WSP fractions was analysed as described in Houben, Jolie, Fraeye, Van Loey, and Hendrickx (2011). Briefly, this was done by first completely hydrolysing lyophilized WSP, in 4 M trifluoroacetic acid (TFA) at 110 °C for 1.5 h. After cooling in an ice bath, TFA was removed from the digested sample by evaporating under nitrogen at 45 °C. The samples were then diluted to 0.1 % w/v, and the different neutral sugars were quantified by high-performance anion exchange chromatography (HPAEC) using a Dionex system (DX600), equipped with a GS50 gradient pump, a CarboPac™ PA20 column, a CarboPac™ PA20 guard column, and an ED50 electrochemical detector (Dionex, Sunnyvale, USA). Prior to sample injection the system was equilibrated for 5 min using 100 mM NaOH and for
additional 5 min using 4 mM NaOH. Samples (10 μL) were injected and eluted for 20 min at a flow rate of 0.5 mL/min with 4 mM NaOH at 30 °C, followed by column regeneration (for 10 min) using 500 mM NaOH. Commercial neutral sugar standards at varying concentrations (1-10 mg L⁻¹) were used as external standards for identification and quantification. To correct for degradation of the monosaccharides during the acid hydrolysis step, mixtures of the sugar standards were subjected to the aforementioned hydrolysis conditions, and the peak areas were compared to those of untreated standard mixtures (Houben et al., 2011).

2.5.5. Analysis of molecular mass distribution

Changes in the molecular mass (MM) distribution of the WSP fractions were studied using size exclusion chromatography (SEC). The separation was performed on series of three identical columns of TSK gel (GMPWXL, 7.8 mm × 30 cm, Tosoh Bioscience) with a TSK guard column (PWXL, 40 × 6 mm) and the eluent was monitored using a reflective index (RI) detector (Shodex RI-101, Showa Denko K.K., Kawazaki). A 100 μL of 0.25 % WSP fraction solubilized in the eluent overnight and filtered through 0.45 μm filter before being injected by an autosampler (G1329A, Agilent technologies, Diegem, Belgium). The eluent (0.1M Acetic acid buffer, pH 4.4 with 0.1 M NaCl to assure a stable pH and minimize ionic interactions) was prepared using demineralized water (organic free, 18 MΩ cm resistance), filtered (0.1 μm) and degased by an on-line degasser of the HPLC system (Agilent technologies 1200 Series, Diegem, Belgium). The flow rate was 0.5 mL min⁻¹ and the columns were kept at 35 °C. All samples were allowed to stir overnight before usage and pullulan standards with MM ranging from 180 to 788,000 Da (Varian Inc, Palo Alto, California) were eluted to estimate the MM of the pectin fractions. It should be kept in mind that due to the fact that the separation on a SEC column is based the hydrodynamic volumes, pullulan and the WSP polymer molecules having a similar hydrodynamic volume might differ in their MM. Hence, the standard curve only allows rough estimations of MM of pectins (Fraeye et al., 2009).
2.6. Analysis of cell wall enzyme activity

PG, PL, PME, β-Gal and α-AF enzymes were extracted and assayed, at harvest, after every two months during storage, and after every seven days during ambient shelf life exposure. Five biological replicates were taken for each time point, in which the enzymes were extracted and assayed separately for each fruit.

2.6.1. Enzyme extraction

PG was extracted using the method first used by Pressey (1986). Crushed apple samples were first freed from reducing groups. This was done by washing with cold distilled water (1:1.5 [w/v]), at a pH of 3.0 (adjusted using 0.1 M HCl), and stirring mixture for 15 min at 4 °C. The homogenate was centrifuged at 14,600 x g for 30 min, and the resulting pellet was again washed with cold distilled water. PG was extracted by dissolving the washed pellet in a solution of 1.2 M NaCl (1:1 [w/v]), containing 1 % (v/v) Triton X-100 and 1 % (w/v) Polyvinylpolypyrrolidone (PVPP), and stirring the homogenate for 3 h at 4 °C, while ensuring the pH was kept at 6 throughout extraction.

PL was extracted using a slight modification of the protocol used by Payasi and Sanwal (2003). 5 g of crushed apple sample was mixed with 10 mL of 0.05 M sodium phosphate buffer (pH 7.0), containing 0.02 M freshly prepared cysteine-HCl, 1 % (w/v) PVPP and 1 % (v/v) Triton X-100. After centrifugation at 20,000 x g for 30 min, 90 % ammonium sulphate precipitation of the supernatant was carried out and the resulting solution was stirred for 2 h at 4 °C.

PME was extracted using a modification of the method used by Ly-Nguyen, Van Loey, Fachin, Verlent, & Hendrickx, (2002). This was done by adding a 0.2 M Tris buffer, containing 1.0 M NaCl (pH 7.5) to crushed apple sample (1:1 [w/v]), and stirring the mixture for 2 h at 4 °C.
To extract β-Gal, the method used by Tateishi, Inoue, and Yamaki (2001) was used, with slight modifications. 0.5 g of crushed apple sample was mixed with 1 mL of 10 mM borate buffer pH 9.0, containing 1.0 M NaCl and 0.1 % (v/v) Triton X-100 (1:1 [w/v]), and stirring the mixture for 2 h at 4 °C.

α-AF was extracted using the method used by Tateishi and Kanayama (1996), with slight modifications. A 20 mM borate buffer, containing 2.0 M NaCl, 2 % (v/v) Triton-100, 3 mM ZnCl₂ and 2 % (w/v) sucrose was used. 1 mL of the buffer was added to 0.5 g of crushed apple sample, and the mixture was stirred for 2 h at 4 °C. In all cases, after the extraction, the homogenate was centrifuged at 20,000 x g for 30 min and the supernatant was taken as the crude extract.

2.6.2. Enzyme assays

PG activity was determined by measuring the amount of reducing groups released from the hydrolysis of polygalacturonic acid, using 2-cyanoacetamide (Gross, 1982). 0.2 mL of the enzyme extract was mixed with 0.8 mL of 0.5 % polygalacturonic solution in 0.05 M sodium acetate buffer, pH 5.2. The mixture was incubated at 37 °C for 1 h, and 2 mL of 0.1 M borate buffer (pH 9.0) was added to stop the reaction. To measure the PG activity, 400 mL of 1 % 2-cyanoacetamide was added to the reaction mixture and boiled for 10 min, cooled, and the absorbance read at 276 nm and 22 °C. One unit of PG activity was defined as the amount of enzyme producing 1 μmol of reducing groups per minute under the assay conditions.

PL activity was assayed following the protocol of Payasi, and Sanwal (2003), which is based on the thiobarbituric acid (TBA) procedure of Waravdekar and Saslaw (1959). 1.5 mL of the enzyme extract was incubated in a medium comprising of 2.5 mL 1 % (w/v) polygalacturonic acid in 0.05 M Tris-HCl buffer (pH 8.5) and 1.0 mL of 0.01 M CaCl₂, for 3 h at 37 °C. 0.3 mL of ZnSO₄·7H₂O was added to stop the reaction, followed by 0.3 mL of 0.05 M NaOH. The resulting precipitate was removed by centrifuging at 1,500 x g for 10 min. 1.5 mL TBA was added to 2.5 mL of the supernatant, followed by 0.75 mL 0.1 M HCl and 0.25 mL water. The mixture was heated in an oil bath at 100 °C for 30 min, cooled and the
absorbance was read at 550 nm. One unit of PL activity was defined as the amount of enzyme causing a change in absorbance of 0.01 per min under the assay conditions.

PME was assayed using the method described by Ly-Nguyen et al. (2002). 0.25 mL of enzyme extract was added to a solution of 0.35 % apple pectin, containing 0.117 M NaCl. The reaction mixture was incubated at 30 °C, while titrating with 0.01 M NaOH to maintain the pH at 7.5 using an automatic pH-stat (718 STAT titrino, U Metrohm). One unit of PME activity was defined as the amount of enzyme required to release 1 μmol of carboxyl group per min, under the assay conditions.

β-Gal was assayed following the protocol used by Tateishi et al. (2001). The reaction mixture, which constituted 0.4 mL of the enzyme extract, 0.5 mL of 0.1 M citrate buffer (pH 4.0), 0.1 mL BSA solution and 0.4 mL of the 13 mM p-nitro phenyl-β-D-galactopyranoside, was incubated for 15 min at 37 °C. The reaction was stopped by adding 2 mL of 0.2 M Na₂CO₃ solution and the absorbance was measured at 400 nm. One unit of enzyme activity was defined as the amount of enzyme that hydrolyses the release of 1 μmol of p–nitrophenol from the substrate per min, under the assay conditions.

α-AF were assayed using the method used by Tateishi and Kanayama (1996). 0.4 mL of the enzyme extract was incubated in a reaction mixture consisting of 0.5 mL of 0.1 M citrate buffer (pH 4.0), 0.1 mL BSA solution and 0.4 mL of the 3.6 mM p-nitrophenyl-α-L-arabinofuranoside, at 37 °C. The reaction was stopped after incubation for 15 min by adding 2 mL of 0.2 M Na₂CO₃ solution, and the absorbance was measured at 400 nm. One unit of enzyme activity was defined as the amount of enzyme that hydrolyses the release of 1μmol of p–nitrophenol from the substrate per min, under the assay conditions.

2.7. Statistical analysis

Significant differences \((P < 0.05)\) between means were investigated using one-way ANOVA, followed by ranking using the Tukey's honestly significant difference criterion (The Mathworks Inc., Natick, USA).

3. Results and discussion
3.1. Effect of storage temperature and controlled atmosphere on the evolution of firmness and ethylene production in Jonagold apples

Firmness of the fruit decreased with time during storage, with fruit that were stored at 1 °C in normal air undergoing the fastest breakdown in firmness (Fig. 1). Apple softening is usually sigmoid, in which there is initially a slow softening phase, followed by a rapid softening phase and, finally, a slow softening phase (Johnston et al., 2001). Fruit stored at 1 °C under CA conditions were able to maintain their firmness throughout the 6 month storage period, and did not enter into the rapid softening phase. This is because during CA storage, there is inhibition of various oxidative reactions (such as respiration and ethylene synthesis) that are very important in ripening. The firmness of fruit stored at 4 °C in CA conditions did not change considerably during the first four months of storage, but thereafter there was a rapid breakdown in firmness as the fruit enter into the rapid softening phase. The fruit stored at 1 °C in normal air had a rapid breakdown in firmness, such that the initial slow softening phase was not evident. This is because the relatively high oxygen levels in normal atmosphere help to accelerate ripening by increasing the rate of oxidative breakdown reactions within the fruit. When the fruit were transferred to ambient shelf life conditions, there was a rapid increase in the rate of softening, except for the fruit that were stored at 1 °C in normal air. At the end of storage, fruit stored at 1 °C in normal air had already entered the last softening phase, as the fruit approach final firmness values, after which no significant further breakdown in firmness can occur (Johnston et al., 2001).

The rate of ethylene production in the fruit was generally very low during CA storage (Fig. 1), because low O₂ level inhibits the final reaction in the ethylene synthesis pathway (oxidation of 1-aminocyclopropane-1-carboxylic acid to ethylene). The fruit stored at 1 °C in normal air showed a relatively higher rate of ethylene production, due to the relatively high O₂ level present in normal air. The high rates of ethylene production during storage in normal air, and also during ambient shelf life, coincided with rapid softening. This is similar to observations made in other studies (Johnston et al., 2001; Gwanpua, Verlinden, Hertog, Van Impe, Nicolai, & Geeraerd, 2013) where strong and negative correlations were established between rates of softening and ethylene production. Many other studies
have revealed that ethylene may have an important role in initiating and promoting apple softening (for example, Watkins, Nock, & Whitaker, 2000). During late shelf life, ethylene production reached peak values, and started decreasing since ethylene production is shut down as fruits enter senescence. This decline was most evident for the fruit stored under normal atmosphere, as they already became overripe by the time they were placed under ambient shelf life conditions.

3.2. Changes in pectic polymers during softening of Jonagold apples stored under different conditions

3.2.1. The evolution of the degree of methoxylation (DM)

The DM of the AIR had a mean value of about 85 % before storage, but did not change significantly with time during storage and subsequent exposure to ambient shelf life conditions. This is in agreement with other studies on apples in which the DM of the AIR remained unchanged during ripening (Massiot, Baron, & Drilleau, 1996; Billy et al., 2008). In this study, the DM of the AIR for fruit stored under normal air were not significantly different from those stored under CA conditions, although the fruit stored under normal air were much softer at the end of 6 months storage. However, the DM of the WSP fraction was about 65 %, on average, much lower than those of the AIR. This suggest some demethoxylation may occur in solubilised pectin.

3.2.2. Pectin solubilisation

The evolution of the WSP, CSP and NSP fractions during storage and subsequent exposure to shelf life conditions is shown in Fig. 2. The relative portions of the WSP fraction increased with time during CA storage, and the fruit stored under normal air had higher proportion of the WSP than those stored under CA. Similar observations were made by other authors (Yoshioka, Aoba, & Kashimura, 1992; Billy et al., 2008). The WSP fractions of the fruit stored under normal air did not undergo any increase after 4 months storage, and even when the fruit were transferred to ambient conditions, because by the fourth month of storage the fruit had soften to the final firmness value (Fig. 1). Both the CSP and NSP fractions decreased
during ripening, most likely because they are inter-converted to WSP. Decrease in CSP fractions during postharvest ripening in apples was also observed by Yoshioka et al. (1992).

3.2.3. Neutral sugar profile evolution

The neutral sugar composition (fucose (Fuc), rhamnose (Rha), arabinose (Ara), galactose (Gal), glucose (Glc), xylose (Xyl), mannose (Man) and galacturonic acid GalA) of the WSP fraction during storage and shelf life exposure for the different treatments is shown in Fig. 3. It was observed that the amount of Gal and Ara Rhamnogalacturonan I (RG-I) pectin side chain sugars decreased during ripening, an observation in agreement with other studies (Gross & Sams 1984; Massiot et al., 1996; Peña & Carpita, 2004). After 4 months of storage, the Gal and Ara content of the WSP extracted from the fruit stored under normal air were lower than those from fruit stored under CA. However, when the fruit were transferred to ambient shelf life conditions, the Gal and Ara of the CA stored fruit rapidly went down to values similar to those measured from fruit stored under normal air. This trend was also reflected in the firmness changes shown in Fig. 1. The composition of the other neutral sugars did not change significantly during ripening, except for Xyl and Man that also decreased during ripening. Furthermore, the total neutral sugar content in the WSP decreased during storage and when the fruit where placed under ambient shelf life conditions, with pectin from apples stored under normal air having the lowest amount of total sugars after 6 months storage. Decrease in total sugar content during ripening in apples and other climacteric fruits was also reported by Gross and Sams (1984), and Peña and Carpita (2004). Since the neutral sugars of the side chains of RG-I within the WSP fractions were lost during ripening, the ratio of the GalA to neutral sugars, which is a measure of the pectin linearity, was higher for fruit stored under normal air than those stored under CA. This was also reflected in a decrease in the degree of branching of RG-I, DBr, defined as (Ara+Gal)/Rha, during ripening. Furthermore, the increase in the WSP fractions during ripening was a result of pectin solubilisation, possibly due to the loss of neutral sugars in the side chain of RG-I. Neutral sugar side chains act as points of attachment between the pectin polymer and the xyloglucan-cellulose
microfibril framework (Brummell & Harpster, 2001), and therefore as pectin loses its neutral sugars, it becomes less bound to the cell wall. Lo Scalzo, Forni, Lupi, Giudetti, and Testoni (2005) reported that polyuronides poor in neutral sugars are preferentially released during ripening.

3.2.4. Molar mass changes

As the size exclusion column separates the polysaccharides based on their hydrodynamic volume, shifts in the location of the peak are representative of changes in the hydrodynamic volume of the polymer. A shift in the peak location of the WSP fraction to the right suggests depolymerization during ripening (Fig. 4). However, significant changes were observed only in fruit that were stored under normal air, which had undergone substantial softening (Fig. 1). The relative decrease in molar mass could also be a consequence of the loss of side chains neutral sugars, and not necessarily a result of depolymerisation of the main pectin backbone because of the extremely high pectin degree of methoxylation. Other studies have shown that apple fruits do not undergo substantial depolymerisation during ripening (Yoshioka et al., 1992; Fischer, Arrigoni, & Amadò, 1994).

3.3. The role of pectin-modifying enzymes in softening of Jonagold apples

The evolution of the activity of the different pectin-modifying enzymes during storage at different conditions, and subsequent ambient shelf life exposure, is shown in Fig. 5. Using the extraction and activity measurement conditions, as mentioned in the materials and methods, no activity could be detected for PL, suggesting that this enzyme may not be involved in ripening of ‘Jonagold’ apples. Although it has been suggested that PL may play an important role in pectin depolymerisation during ripening of climacteric fruits, its accumulation during ripening has mainly been reported in banana (Payasi, & Sanwal, 2003). Activities of PG, PME, β-Gal and α-AF could all be measured and were always the highest for the fruit stored under normal air, followed by those stored at 4°C in CA. A correlation plot for the different parameters characterising the pectin polymer, the firmness, the ethylene production and the activities of all four enzymes is shown in Fig. 6. From this plot, there was a strong and negative correlation between the different enzymes activities and the firmness. Also, the activities of all four
enzymes were positively correlated to the rate of ethylene production. Further, the firmness was positively
correlated with the neutral sugar content of the WSP fraction, the degree of branching of the pectin chain,
while it was negatively correlated with the amount of the WSP fraction. Moreover, the WSP fraction was
negatively correlated with the neutral sugar content and the molar mass of the pectin. This implies
solubilisation is most likely a consequence of both loss of side chain neutral sugars of RG-I and pectin
depolymerisation.

The high enzyme activities in fruit stored under normal air correlated with very high rates of ethylene
production (Fig. 1). Ethylene has been shown to play a key role in controlling ripening, by acting as a
regulator for the synthesis of various enzymes implicated during ripening (Watkins et al., 2000; Wei et
al., 2010).

β-Gal activity increased throughout ripening, with fruit stored at 1°C under normal air showing the
highest activity, followed by those stored at 4°C in CA. This trend was clearly reflected in the loss of Gal
during storage at different conditions (Fig. 4). The loss in Gal was highest in fruit stored at 1°C under
normal air. It has been suggested that one of the first events that occur during softening in apples is the
loss of Gal from side chains of RG-I (Peña & Carpita, 2004), which could explain why β-Gal activity
increased during early ripening. α-AF activity did not change so much during storage, except for fruit
stored at 1°C under normal air. PME activity increased during ripening, but did not result to any
significant changes in the DM of the AIR during ripening (result not shown). However, the DM of the
WSP fraction was much lower than that of the AIR, suggesting PME may not be able to access pectin that
are tightly bound together, but can easily interact and act on solubilised pectin. Increased PME activities
during ripening were also reported by Wei et al. (2010) and Goulao et al. (2007) in apples. Although
pectin with lower DM is preferentially hydrolysed by PG (Tieman, Harriman, Ramamohan, & Handa,
1992), demethoxylation of pectin creates additionally negatively charged carboxyl groups that can form
ionic bonds with chelating agents (Van Buggenhout, Sila, Duvetter, Van Loey, & Hendrickx, 2009),
thereby increasing the firmness of the fruit. PG activity was initially very low during storage, and only
increased after about 4 months of storage. The remarkable differences between the estimated molar mass
of the WSP for fruit stored in normal air and those stored at 4°C in CA after 4 months storage (Fig. 5A) were not comparable to the differences between their PG activity. This raises a doubt as to whether the decrease in molar mass of WSP observed during ripening was a consequence of extensive loss of side chain neutral sugars, rather than a result of depolymerisation by PG activity.

The activities of β-Gal and PME reached a peak when the fruit were placed under ambient shelf conditions, and this correlated with ethylene production, which is likely because ethylene plays a key role in regulating the activities of these cell wall-modifying enzymes. The peak value of the enzyme activities appears to be related to the storage conditions, with apples that were stored under optimal conditions (1°C, under CA) attaining the lowest peak values, while those stored at 1°C under normal air reached the highest peak values. Furthermore, the decline in the activities of these enzymes during shelf life exposure suggests that the loss of Gal from the side chains of RG-I and pectin de-methoxylation are events mostly associated with early ripening. This was confirmed by the decline in the rate at which Gal was lost from the WSP fraction in shelf life (Fig. 3), and the fact that the DM of the WSP was lower than the DM of AIR, but did not undergo any significant change with time during storage and shelf life exposure. Contrarily, the activities of α-AF and PG (except for fruit that were stored at 1 °C under normal air) continued to increase even after 15 days under ambient shelf conditions. This suggests that the loss of Ara from the side chains of RG-I, and pectin depolymerisation could be events associated with late ripening. From Fig. 3, the Ara content of the WSP fraction continue to decrease during shelf life, except for apples that were stored at 1°C under normal air. Also, there was a shift in the peaks of the molar mass distribution towards the right, except for fruit that were stored at 1°C under normal air (which had already soften to their final firmness values), suggesting that depolymerisation may occur during shelf life.

4. Conclusion

High β-Gal activity, concomitant with the loss of Gal from the side chain of RG-I, is a very important event during early ripening of Jonagold apples. PME appears not to play an important role in ripening of Jonagold apples, since the DM of pectin did not change throughout ripening, although in vitro measured
PME activity increased with ripening. PG activity only increased late in ripening, suggesting that this enzyme may be involved during late ripening in this apple cultivar. Some decrease in molar mass of water soluble pectin was observed during softening, which could either be a result of pectin depolymerisation, a consequence of the extensive loss of side chain neutral sugars, or both. Furthermore, the solubilisation of pectin seems to occur before decrease in molar mass of pectin. Loss of neutral sugars, an increase of pectin solubility and a decrease water soluble pectin molar masses are all changes associated with softening during ripening of Jonagold apples. The activities of the cell wall enzymes were strongly correlated with the rate of ethylene production, but not always with changes in pectin. In this study we observed mainly changes occurring in the WSP fraction. However, some very interesting modifications may occur in the other fractions, and future research could focus on investigating these changes. Furthermore, measuring in vitro enzyme activities does not necessarily give accurate information about changes in pectin/cell wall polysaccharides, and the texture changes. Therefore, research aimed at understanding the mechanism of softening should entail both studies on enzyme activities and the actual changes occurring in the pectin polymer.

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References


Figure captions

Fig. 1. Firmness and ethylene production of Jonagold apples during storage at different conditions, followed by ambient shelf life exposures (grey region). The points are the means of 10 measurements, with the error bars denoting the standard error. For each time point measurement, means with different letters are significantly different, while * connotes means that are different from initial value. CA and NA denote controlled atmosphere and normal air respectively.

Fig. 2. Changes in the water soluble pectin (WSP), chelator soluble pectin (CSP) and sodium carbonate soluble pectin (NSP) fractions of the alcohol soluble residues (AIR) Jonagold apples during storage at different conditions, followed by exposure to ambient shelf life conditions. The points are the means of four measurements, with the error bars denoting the standard error. For each time point measurement, means with different letters are significantly different, while * connotes means that are different from initial value. CA and NA denote controlled atmosphere and normal air respectively.

Fig. 3. Changes in the Rhamnogalacturonan I side chain neutral sugars content of the water soluble pectin of Jonagold apples, during storage at different conditions and subsequent exposure to ambient shelf life conditions (grey region). The points are the means of five measurements, with the error bars denoting the standard error. For each time point measurement, means with different letters are significantly different, while * connotes means that are different from initial value. CA denotes controlled atmosphere, NA denotes normal air.

Fig. 4. Changes in the molar mass distribution of the water soluble pectin of Jonagold apples after 4 months storage at different conditions (A), after 6 months storage at different conditions (B) and after 7 days subsequent exposure to ambient shelf life conditions (C). The lines are the mean signal of 4 biological replicates, CA denotes controlled atmosphere, and NA denotes normal air.
The evolution of β-galactosidase (β-Gal), α-arabinofuranosidase (α-AF), pectin methylesterase (PME), polygalacturonase (PG), activities in Jonagold apples during storage at different conditions, followed by ambient shelf life exposures (grey region). The points are the mean of five measurements, with the error bars denoting the standard error. For each time point measurement, means with different letters are significantly different, while * connotes means that are different from initial value. CA denotes controlled atmosphere, NA denotes normal air.

Fig. 6 Correlation plot between the firmness, ethylene production (Eth), β-galactosidase (β-Gal), α-arabinofuranosidase (α-AF), pectin methylesterase (PME), polygalacturonase (PG) activities, degree of demethoxylation of the alcohol insoluble pectin residues (DM AIR), the water soluble pectin (WSP), chelator soluble pectin (CSP), the sodium carbonate soluble pectin (NSP) fractions, degree of branching of the WSP fraction (DBr), molar mass of the WSP fraction (MM), the rhamnose (Rha), arabinose (Ara) and galactose (Gal) content of the side chains of the WSP fractions.
Figure 1
Figure 2

Figure 3
Figure 4
Figure 5
Figure 6