Ultrastructural and Changes in Pectin Composition of Sweet Cherry from the Application of Prefreezing Treatments

JESÚS ALONSO, MARÍA E. TORTOSA, WENCESLAO CANET, AND MARÍA T. RODRÍGUEZ

ABSTRACT: Thermal and calcium pretreatments applied to preserve the sweet cherry texture by the freezing/thawing process produced biochemical changes in the pectic substances and ultrastructural alterations to the cells and tissues, which were visible under scanning electron microscopy. Partial dehydration of the epidermic tissue caused by calcium (100 mM CaCl₂) and thermal (50 °C/10 min) pretreatment attenuated the surface damage produced by freezing. However, pretreatment at 70 °C/2 min caused partial destruction of the epidermic tissue and plasmolysis of the parenchymatic cells. After freezing, the cell walls in the parenchymatic tissue of the fruits pretreated with 100 mM CaCl₂ exhibited swelling as a result of gelling of the cell-wall pectic material. Thermal pretreatments increased the ethylenediaminetetraacetic acid (EDTA)-soluble pectin fraction and reduced the degree of pectin esterification. Thermal treatments at 70 °C, without immersion in calcium, reduced the water- and pectinase-soluble pectin fractions, whereas immersion in calcium prevented depolymerization of these fractions. Immersion in 100 mM CaCl₂ increased the water-soluble pectin fraction.

Keywords: Prunus avium L., pectin, freezing, thermal, and calcium treatments

Introduction

The seasonal nature of sweet cherry production and their short shelf life prompt the use of technological procedures (freezing, canning, dehydration) to make them available for the processing industry all year round. The exposure of vegetable tissues to low temperatures by the technological process of freezing causes molecular, cellular, and structural alterations in tissues, including disruption of the metabolic and enzymatic systems, protein denaturation, alteration of cell components, and loss of cell turgidity (Reid 1993; Somer and others 1998; Roy and others 2001).

During the freezing of fruits, ice begins to form in the extracellular medium and propagates via the cytoplasm when the cell membrane loses permeability. The decompartmentalization caused by ice crystals prevents the water from returning to the intracellular medium during thawing, causing loss of turgidity and hence of fruit texture (Reid 1993). To attenuate the damage from ice-crystal formation, it is recommended that the latent heat plateau during freezing be reduced and the product thawed slowly so that water can return to the semipermeable compartments left undamaged by the process (Grout and others 1989). The freezing process produces alterations in the product color and flavor and sensorially detectable changes in texture (Alonso and others 1995; De Ancos and others 2000; Lisiweska and Kniecik 2000).

The processing of fruits and vegetables has been related with the modification of cell wall physical state, macrostructure and microstructure, composition, and functionality (Kunzek and others 1999). The texture of sweet cherry depends on cell turgidity and the mechanical properties of the parenchymatic cell wall (Alonso and others 1994). The textural properties of these fruits have been related to the concentration of alcohol insoluble residues (Choi and others 2002) and the degree of polymerization of the pectin side chains and the number of bonds between the polymers (Batisse and others 1996). However, the softening of cherries during ripening does not seem to depend exclusively on pectin depolymerization (Batisse and others 1994; Kondo and Danjo 2001). In canned and frozen cherries, the texture changes have been associated with the composition of the pectin fractions and not with the quantity of pectin in the fruit (Alonso and others 1997; Carle and others 2001).

To enhance fruit and vegetable firmness and attenuate the loss of texture caused by freezing, thermal, and calcium pretreatments have been proposed (Alonso and others 1993, 1995; Paoletti and Menezatti 1993; Fuchigami and others 1995; Lamikanra and Watson 2004). According to Bartolome and Hoff’s theory (1972), thermal treatments at “low temperature” cause loss of membrane selective permeability, giving rise to a diffusion of cations to the cell wall. The increased presence of cations in the cell wall would activate the pectinesterase (PE) enzyme, increasing de-esterification of the pectins and facilitating the formation of divalent bridges between residues of galacturonic acid in the adjacent pectic chains. The divalent ion-pectin complex thus formed could act as an intercellular cement to give firmness to the tissues.

In fresh and frozen cherries, the application of calcium brining improved the firmness of the fruit caused partly by calcium absorption into the fruit and the possible implication of pectin methyl-esterase (Carle and others 2001). In previous works, our group observed that thermal and calcium pretreatments were effective for preventing the loss in firmness of frozen sweet cherries. These treatments increased pectinesterase activity and altered the cell wall pectin material. A decrease in the degree of esterification of pectins and an increase in the ethylenediaminetetraacetic acid (EDTA)-soluble pectin fraction and the Ca²⁺ cation content in the cell wall were observed (Alonso and others 1995, 1997). However,
the microstructural changes and damage produced by pretreatments and freezing have not been described to date.

In this study, we wanted to obtain information on the microstructure alterations in different fruit tissues produced by freezing/thawing and by the thermal and calcium prefreezing treatments and to relate them to the changes that take place in the pectin composition. The aim was to have effective pretreatments to preserve the loss of texture produced during freezing and attenuate the structural damage in the tissues.

Materials and Methods

Raw material and processing

The sweet cherry (Prunus avium L.) cv Pico Colorado is included in the Picota group. This group embraces 4 traditional late varieties of sweet cherries from the Jerte valley (Spain) harvested without a stem. The fruits were harvested at an optimum stage of ripeness and selected as commercial grade I. To survey the homogeneity of the population used, only the fruits with a diameter of 22 to 25 mm and homogeneous color were used. The average weight of the fruits was 6.65 ± 0.61 g; the soluble solid content was 21.46% ± 1.71%; and the color parameters were L = 25.83 ± 1.58, a = 19.76 ± 2.24, and b = 7.47 ± 2.03. After selection, the samples were divided into 3 groups: 2 for thermal treatment at 50 °C/10 min and 70 °C/2 min, and 1 as a control. After preheating, each group was further divided into another 2 groups, and the fruits were immersed for 10 min in cool solutions at 0 °C (1:10w/v) containing 0.5% citric acid, 0.03% ascorbic acid, pH 3 with or without 100 mM CaCl₂. The samples were air dried, and freezing was by forced convection of liquid nitrogen vapor. The initial sample temperature was 3.1 °C, and a temperature of −70 °C was maintained in the medium until the thermal center of the fruits reached −24 °C. After the cherries had been frozen, they were packed in stratified polyethylene bags (800 g/bag) and sealed with a low vacuum of 50 Kpa to prevent oxidation and damage from surface dehydration. Samples of the cherry fruits were taken before freezing and after 1 mo of storage at −24 °C. The fruits were slowly thawed in the sealed bags at 5 °C for 14 h, then tempered to 22 °C.

Scanning electron microscopy

Cylindrical pieces of 4-mm dia were cut from the equatorial zone of the fruits before and after the freezing-thawing process. The specimens were 1st fixed for 2 h in formaldehyde-acetic acid (FAA) and then dehydrated through a graded ethanol series between 70% and 100% with changes every 15 min, and dried using the critical point method. After the specimens had been mounted, they were metallized with a 400Å-thick platinum sheet in a P-S1 diode sputtering system. Scanning electron microscopy (SEM) inspection and observation of the samples were carried out with a Hitachi (Ontario, Canada) S-2500 microscope. Microphotographs were taken with a Mamiya camera (Barcelona, Spain) fitted to the SEM, using an Ilford 6 × 9 cm FP-4 film that was processed as normal. Samples were analyzed between 36 and 2100 magnifications.

Extraction of alcohol insoluble solids (AIS) and fractionation and determination of the degree of esterification of the pectic material

The method described by Facteau (1982) was used for this. Eighty grams of stoned fruits were homogenized in an Omnimixer (Sorvall®, Madrid, Spain) with 95% ethanol to obtain a final concentration of 70%. The residue was filtered on Whatman paper (nr 1), washed with 70% ethanol, and then resuspended and incubated for 1 h at 70 °C in 100 mL of 70% ethanol. The residue was washed with 300 mL of 70% ethanol and then with 100 mL of acetone. The filtrate was stove-dried at 65 °C, pulverized by a mortar, and stored until used.

To obtain the different pectin fractions, 100 mg of AIS was used. Samples were shaken in 100 mL of distilled water for 12 h and centrifuged at 3500 × g for 5 min. Water-soluble pectins were contained in the supernatant and the precipitated material was resuspended in 100 mL of EDTA 50 mM and shaken for 12 h. After another centrifugation, the supernatant containing EDTA-soluble pectins was separated, and the precipitated materials were resuspended in 100 mL of fungal pectinase (Fluka) 0.05% at pH 5 and shaken for 1 h at room temperature. The supernatant resulting from centrifugation of the digest contained pectins solubilized by pectinase. The process was carried out in duplicate and each fraction was assayed 3 times for its uronic acid contents (Scott 1979). The whole process was carried out at 0 °C to avoid carbonization of the samples.

The degree of esterification was measured by assaying for methanol released from the different fractions by basic de-esterification. Methanol was assayed by gas chromatography (McFeeters and Armstrong 1984) with use of a stainless-steel Carbowax (15%) 1500 (4 m × 1/8 in) column over a Chrom. W, 80/100 mesh, and n-propanol as an internal standard. The degree of esterification of the pectic material was expressed as (moles of methanol/moles of galacturonic acid) × 100%.

Statistical analysis

Statistical processing of the results was by 1-way variance analysis, using the least significant difference (LSD) test with a 95% confidence interval for comparing the means.

Results and Discussion

Effect of pretreatments and freezing on the microstructure

The epidermic tissue of the cherry is made up of small polygonal cells (40- to 50-μm) covered by a thin hydrophobic cuticle approximately 1 μm thick. The surface of the fruit was dotted with small drops of epicuticle waxes (Figure 1c). The stomatal density of sweet cherry fruit ranged from 100 to 200 stomata/cm. In the raw sweet cherries, the fixed stomata were in a semi-closed ostiole position (Figure 1b) and consisted of 2 guard cells approximately 25 to 30 μm in length surrounded by a pair of subsidiary cells about 40 μm in length. The subepidermic tissue consisted of 2 to 3 layers of small cells with wide cell walls and without intercellular spaces (Figure 1a). Loose parenchymatic tissue was found inside the fruit consisting of large globular cells (100- to 300-μm) with triangular intercellular space morphology (Figure 1a).

Extraction of alcohol insoluble solids (AIS) and fractionation and determination of the degree of esterification of the pectic material

The method described by Facteau (1982) was used for this. Eighty grams of stoned fruits were homogenized in an Omnimixer (Sorvall®, Madrid, Spain) with 95% ethanol to obtain a final concentration of 70%. The residue was filtered on Whatman paper (nr 1), washed with 70% ethanol, and then resuspended and incubated for 1 h at 70 °C in 100 mL of 70% ethanol. The residue was washed with 300 mL of 70% ethanol and then with 100 mL of acetone. The filtrate was stove-dried at 65 °C, pulverized by a mortar, and stored until used.
The calcium and thermal pretreatments caused changes and damage to the cells and tissues. The pretreatment with 100 mM CaCl₂ partially dehydrated the epidermic tissue of the fruit. This was reflected in dimpling of the cuticle and the appearance of small cracks in criss-cross patterns (Figure 2a). After this pretreatment, no alterations were detected in the subepidermic and parenchymatic tissues, whose appearance was similar to that of the fresh controls.

Dehydration was also detected, yet more pronounced, in the epidermic tissue of fruit treated at 50 °C/10 min with (Figure 2b) or without calcium (Figure 2c). The epidermal cell and stomata dehydration caused by both treatments could be compared with the turgidity of epidermal cells in the fresh control (Figure 1c). However, the parenchymatic tissue of fruit treated at 50 °C/10 min consisted of turgid cells with triangular intercellular spaces similar to those found in the fresh control (Figure 2d), and the cells did not exhibit any differences regarding the presence/absence of 100 mM CaCl₂ in the cool solutions.

The greatest change was in the fruit treated at 70 °C/2 min (Figure 3). Partial destruction of the epidermic tissue caused the formation of craters on the surface of the fruit (Figure 3a). The epicuticle waxes had a lumpy appearance and the cuticle exhibited cracks and scaling. The craters observed on the surface of the fruit could be the result of destruction of the stomatal cells (guard and subsidiary) and consequent exposure of the substomal chambers. This postulation is based on the small number of stomata found in the fruit pretreated at 70 °C/2 min and the high degree of dehydration of the stomatal cells (Figure 3d). There was formation of intercellular spaces between the subepidermal cells and plasmolysis of parenchymatic cells, with large and irregular intercellular spaces (Figure 3b and 3c).

SEM revealed freezing-induced damage to all tissues. In the frozen controls, there was dehydration and plasmolysis of the epidermal and subepidermal cells, whereas the cuticle exhibited scaling and areas of separation of the epidermic tissue (Figure 4a). Intracellular spaces were formed in the subepidermic cells, with areas of separations from the parenchymatic tissue (Figure 4a). There was also plasmolysis of the parenchymatic tissue cells, with enlargement of the intercellular spaces (Figure 4b).

After freezing, the surface of the fruit pretreated with 100 mM CaCl₂ was smoother and less scaly than that of the frozen control (Figure 5a). Possibly dehydration of the epidermic tissue caused by treatment with 100 mM CaCl₂ prevented the ice crystals from becoming large enough to overcome the cell flexibility. The ostioles of the stomata exhibited an opening (Figure 5c) with a pronounced dehydration of the guard and subsidiary cells. The cell walls surrounding the parenchymatic cells appeared to have become thicker and there were fewer intercellular spaces (Figure 5b).

After freezing, the epidermic tissue was more intact in cherries pretreated at 50 °C/10 min than in the frozen controls (Figure 6). Epidermal dehydration caused by the pretreatment, 100 mM CaCl₂, must have provided some protection from damage by the ice crystals. The stomas of these fruits were dehydrated but not damaged by the application of the thermal treatment (Figure 6c). Nonetheless, scaling, small surface cracks, and lumpy epicuticle waxes were observed in the cuticle (Figure 6b). The parenchymatic cells showed evidence of plasmolysis and partial gelling of the cell walls in fruit treated with 100 mM CaCl₂ following thermal treatment (Figure 6a).

After freezing, the surface of the cherries pretreated at 70 °C/2 min exhibited dehydration and serious damage, with cracking of the cuticle and lumpy formation of the epicuticle waxes (Figure 7c). There was plasmolysis of the subepidermal cells and almost complete separation
of the epicarp from the mesocarp (Figure 7a). The parenchymatic tissue cells showed evidence of plasmolysis and an increase in the size and number of intercellular spaces (Figure 7b). The fruits pretreated at 70 °C/2 min with the presence of 100 mM CaCl₂ in the cool solutions exhibited a swelling of the cell wall (Figure 7d).

Changes in pectin composition

Pectic material was analyzed after freezing, and the results therefore reflect the overall changes brought about by pretreatment and freezing. Total uronic acid levels obtained ranged from 300 to 450 μg/mg of AIS (Table 1), which is similar to the values reported in other analyses of pectic material in cherries (Alonso and others 1995). The addition of calcium to the thermal pretreatments at 70 °C/2 min increased significantly (P = 0.05) the total concentration of uronic acid levels in AIS. A partial depolymerization was observed in the fruits treated at 70 °C/2 min without any calcium application. Both thermal and calcium pretreatment reduced the degree of esterification of the total pectic material. De-esterification of pectic material increased when the treatment temperature was increased from 50 °C to 70 °C, and a synergic effect was observed when the 2 treatments (thermal and calcium) were applied consecutively, obtaining maximum and significant de-esterification (P = 0.05) in the fruits treated at 70 °C/2 min with immersion in 100 mM CaCl₂ (Table 1).

The water-soluble pectin fraction accounted for 35% of total uronic acids, which is within the range of 5% to 35% found for cherries during ripening (Fils-Lycaon and Buret 1990). Cherries heated at 70 °C/2 min not immersed in 100 mM CaCl₂ exhibited a sharp and significant (P = 0.05) decrease in the water-soluble pectin fraction. However, a significant (P = 0.05) increase in the water-soluble pectin fraction and a significant (P = 0.05) decrease in esterification were observed in the same cherries after immersion in CaCl₂ (Table 1).

The principal changes in pectic material took place in the EDTA-soluble fraction. At the higher treatment temperature (70 °C), the uronic acids in the EDTA-soluble fraction increased significantly (P = 0.05) at 256% compared with the control concentration, and there was a 30% to 45% decrease in esterification (Table 1).

Levels of pectinase-soluble uronic acids decreased significantly (P = 0.05) with the higher treatment temperature, although this decrease was attenuated by subsequent immersion in CaCl₂. Pretreatments caused no significant (P = 0.05) change in the degree of esterification of the pectinase-soluble pectin fraction.

Discussion

SEM enabled us to examine, over a wide range of magnifications, the physical damage to the ultrastructure of cherries by ice crystalization and recrystallization during freezing and frozen storage, and also to view certain kinds of damage and physical alteration to the cell wall from thermal and calcium pretreatments.

The preservation of the texture of frozen vegetables from the application of thermal treatments at low temperature has frequently been studied. The application of thermal pretreatments with or without calcium aims to preserve part of the loss in texture produced by freezing and de-freezing processes. The effectiveness of these treatments has been related to alterations in the pectic material. However, changes produced in the microstructure from the application of these treatments are not frequently studied. Thus, treatments at temperatures of 70 °C, which are effective in preserving the texture of numerous frozen vegetable products (references), caused considerable damage in the internal and external structure (Figure 3) of delicate fruits like the cherry. On the other hand, thermal treatments at 50 °C, although they caused a pronounced dehydration of the epidermic tissue, did not appear to alter the internal structure of the parenchymatic tissue (Figure 2). We should ask whether these treatments at low temperatures, with or without a subsequent application of calcium, will be effective in altering the composition of the pectic material to preserve the texture of the fruits in the freezing process.

Freezing-induced plasmolysis of epidermal cells and damage to the cuticle were attenuated by calcium pretreatment and low-temperature thermal pretreatment (50 °C/10 min). Partial dehydration of the epidermic tissue caused by both kinds of treatment presumably reduces the size of the ice crystals that form during freezing, thus preventing them from overcoming the membrane and cell flexibility. The increased compartmentalization of the epidermic tissue cells after freezing presumably favors the preservation of turgidity and hence a texture more akin to that of the fresh product.

The thickening of cell walls observed in the fruits treated with calcium (Figure 5b) should be related to the gelling of cell wall pectic material. The reduced degree of esterification in the pectin and the increase of divalent cations in the cell wall of the fruit treated with CaCl₂ would favor the formation of divalent bridges between residues of galacturonic acid in the adjacent pectic chains, causing geling of part of the pectic material in the cell wall. However, the treatment with calcium, without the application of thermal treatments, did not produce any significant alterations (P = 0.50) in the composition of the cherry pectic material except for the uronic acid content in the water-soluble pectin fraction (Table 1). In eggplant, the decrease in pectin esterification by vacuum infusion of fungal PE and calcium has been related to cell integrity (Banjongconsin and others 2004). In canned cherries, Carle and others (2001) improved the firmness of the fruits by adding calcium. This suggests the implication of PE activity and changes in pectin composition.

On the other hand, the application of calcium together with the thermal treatments decreased the total esterification of the pectins and prevented their depolymerization (Table 1).
The application of thermal treatments at low temperature (50 °C to 70 °C) did not produce any changes in the composition of the cherry pectic material. However, pretreatments at 70 °C/2 min produced serious damage in the delicate tissues of these fruits and are therefore not recommended. The isolated use of calcium was not effective in significantly altering the composition of the pectic material. However, if it was used with thermal treatments at low temperature, it had a synergistic effect and decreased the degree of esterification of water-soluble and EDTA-soluble pectins, thereby favoring the formation of calcium bridges and preventing the depolymerization of pectins. Accordingly, the use of both treatments together can be recommended in the freezing process of cherries. Complementary studies must be done to adjust the calcium concentration and treatment time depending on the variety and calorific value of the fruits.

**Conclusions**

-probably, the alterations produced by the thermal treatment and the freezing facilitate the penetration and diffusion of calcium in the inner tissues. The significant decline (P = 0.05) in esterification of water-soluble and EDTA-soluble pectins in samples pretreated thermally and with calcium could be explained by the increase in PE activity that was observed when the temperature was increased from 50 °C to 70 °C (Alonso and others 1993) or when the calcium concentration of the pretreatment was increased (Alonso and others 1995). Membrane damage from freezing and the thermal treatments must facilitate the enzyme-substrate interaction. The reduced esterification of the pectic material favors the formation of calcium bridges between the residues of galacturonic acid in the adjacent pectic chains and increases the EDTA-soluble fraction. Four different PE isoforms were purified in sweet cherries (Alonso and others 1996). Two of them were more thermostable and retained 100% of their activity after heating for 1 min at 60 °C and 70 °C, respectively, and the 4 isoforms were activated by 0.5 to 1 mM CaCl₂ concentration (Alonso and others 1996).

Depolymerization of the pectic material in the samples treated at 70 °C/2 min (Table 1) may be of enzymatic origin caused by polygalacturonase (PG). The damage from the thermal treatments and the freezing could facilitate the action of the PG enzyme on the substrate, causing a reduction in water-soluble uronic acids in samples treated at 70 °C/2 min. A low degree of polymerization produces soft fruit and fewer interactions between polymers (Batisse and others 1996). It may be that the effect of immersion in 100 mM CaCl₂ prevented depolymerization and the loss of pectic material resulting from the thermal treatment through inhibition of PG activity by Ca²⁺. Then again, it is known that freezing/thawing inhibits PG activity in cherries (Alonso and others 1993). Thus, any depolymerization of cherry pectic material by PG must have taken place after thermal treatment and before freezing the fruits.

**References**


