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# Hydrolytic Activity and Ultrastructural Changes in Fruit Skins from Two Prickly Pear (*Opuntia* sp.) Varieties during Storage

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The activity of four cell wall hydrolases, pectinmethylesterase (PME), polygalacturonase (PG), cellulase, and $\beta$-galactosidase ($\beta$-Gal), was measured in fruit skins of two prickly pear varieties, Naranjona and Charola, during storage at 18 °C and 85−95% relative humidity (RH). In Naranjona (*Opuntia ficus indica*), of short postharvest life (ca. 2 weeks), PG, cellulase, and $\beta$-Gal increased their activity more than twice, whereas PME activity tended to increase only slightly during storage. In Charola (*Opuntia* sp.), of long postharvest life (ca. 2 months), only $\beta$-Gal increased its activity (77%), showing a high PG activity from the beginning of storage. Transmission electron microscopy observations showed middle lamella dissolution at the end of storage for both varieties. Naranjona showed a higher cell wall enzymatic activity than Charola, in agreement with their storability differences. Our results suggest that PG and cellulase in Naranjona and PG and $\beta$-Gal in Charola are the main enzymes responsible for cell wall hydrolytic and ultrastructural changes in skins of stored prickly pears.

**KEYWORDS:** ***Opuntia*** **sp.; cell wall; polygalacturonase; pectinmethylesterase; cellulase; $\beta$-galactosidase; nonclimacteric**

## INTRODUCTION

The semiarid regions of the central part of Mexico host the greatest genetic diversity of prickly pear in the world (*1*). The fruit is a many-seeded berry with a thick fruit wall (skin) enclosing a delicately flavored flesh with many seeds. The fruits are of different colors: green, yellow, orange, and red (*2*). On the basis of seasonal availability and developmental duration, there are prickly pears of early, intermediate, and late maturation (*1*). Also, there is variability in regard to the fruit storage life after harvest. The Naranjona variety can be stored for 2 weeks whereas Charola lasts 2 months at 18 °C.

At the beginning of prickly pear development, the receptacular tissue (skin) predominates over the locular one. During development, the pulp content increases and the skin proportion decreases (*3*), but at fruit maturity, the skin still comprises an important percentage of the whole fruit; a proportion of 48% was reported by Sawaya et al. (4). The skin gives resistance to the whole fruit during postharvest handling. So depending on the biochemical and physical characteristics, the prickly pear skin could account for the variation in storage life of fruits from different varieties. Prickly pear is a nonclimacteric fruit (*2*) in which relatively little work has been done to characterize cell wall postharvest changes. What happens in the cell wall and middle lamella from skin cells could be informative about the storage potential of the fruits. Structural changes which occur in the middle lamella and primary cell wall during ripening lead to cell separation and softening of tissue (*5*). Biochemical studies of cell wall changes during fruit ripening indicate that structural changes in pectin, hemicellulose, and cellulose are responsible for the alteration of the cell wall structure (*6*, *7*). Enzymes that influence the structure of these cell wall components may have important effects on the cohesiveness among cells during ripening. It is now generally accepted that textural changes during ripening are a composite effect of the action of cell wall hydrolases such as pectinmethylesterase (PME), polygalacturonase (PG), cellulase, and $\beta$-galactosidase ($\beta$-Gal) acting in concert (*8*). Changes in the activity of cell wall hydrolytic enzymes from prickly pear skin might give us explanatory information about which is (are) the enzyme(s) responsible for

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cell wall structural changes during storage, and about the great difference in the time of storability between Naranjona and Charola varieties studied in this work. To our knowledge, no reports on cell wall enzyme activity during storage of prickly pears have been published. Only the PME activity in pulp juice measured by Gurrieri et al. (*9*) and the PME extractability with NaCl solutions from prickly pear by Contreras-Esquivel et al. (*10*) have been reported. Thus, hereby we report changes in the enzymatic activity of several cell wall hydrolases and ultrastructural cell wall changes from prickly pear skins during fruit storage.

## MATERIALS AND METHODS

**Materials**. Naranjona prickly pear (*Opuntia ficus indica*) and Charola (*Opuntia* sp.) were harvested at their mature-green (MG) stage of ripening in San Luis de la Paz, Guanajuato State, and Agua Gorda, Zacatecas State, México, respectively. Undamaged fruits were selected on the basis of size uniformity. They were washed with running water and stored in a bioclimatic chamber (model Acros, Apodaca, Nuevo León) at 18 °C (± 1 °C) where the RH ranged from 85% to 95%. Preliminary trials on prickly pears, harvested at the MG stage and stored under these conditions, showed postharvest lives of about 2 weeks and about 2 months for Naranjona and Charola varieties, respectively. For enzymatic analysis, Naranjona fruits were sampled every 3 days during a total period of 15 days, whereas Charola fruits were sampled every 12 days for a 60 day period of storage. Seven replications (i.e., fruits analyzed) were done every sampling day. Skin samples were frozen in liquid nitrogen and stored at −70 °C until evaluation. Transmission electron microscopy observations were done at the beginning (MG stage) and at the end of storage for both varieties.

**Enzyme Extraction.** The method described by Barrett and Gonzalez (11), with modifications in the salt concentration and centrifugation speed, was used to extract all enzymes. Cell wall enzymes were extracted by homogenizing prickly pear skin in 1 M NaCl at 4 °C for 1.5 min in a blender (model LO-4, Sunbeam Mexicana, Tlalnepantla, México State). The homogenate was centrifuged at 20000*g* for 30 min at 4 °C and filtered through Whatman no. 4 paper. The supernatant protein was precipitated with ammonium sulfate at 85% saturation, stirred for 1 h, and centrifuged at 20000*g* for 10 min. The protein pellet was resuspended in 30 mL of deionized water and used for enzyme assays.

**Enzyme Assays.** PME activity was determined according to Hagerman and Austin (*12*), on the basis of the color change of a pH indicator during the PME-catalyzed reaction. As the ester bonds are hydrolyzed, acid groups are produced and the pH is lowered, causing the indicator dye to change color. This change is continuously monitored spectrophotometrically. In a cuvette, 1 mL of 0.5% citrus pectin (87.6% galacturonic acid. 9.8% methoxy groups, CAS No. 9000-69-5, Sigma Chemical Co., St. Louis, MO) was mixed with 0.15 mL of bromothymol blue solution and 0.25 mL of deionized water. The reaction was started by adding 0.1 mL of enzymatic extract. The starting pH of the assay was 7.5. The reaction was carried out at 30 °C and monitored at 620 nm. One unit (U) of PME activity is defined as the amount of enzyme required to release 1 $\mu$mol of $H^+$/h at 30 °C.

PG activity was assayed spectrophotometrically according to Gross (*13*) on the basis of the hydrolytic release of reducing groups from polygalacturonic acid. Enzyme extract (0.08 mL) and 0.08 mL of 0.5% polygalacturonic acid in sodium acetate buffer, pH 5.0, were mixed and incubated at 37 °C for 2 h. A 1 mL sample of cold 100 mM borate buffer (pH 9) was used to terminate the reaction, and then 0.2 mL of 1% 2-cyanoacetamide was added. Samples were mixed and immersed in a boiling water bath for 10 min. After equilibration to 25 °C, the absorbance at 276 nm was measured to quantify released reducing groups using a Beckman model DU 640 spectrophotometer. Blanks were prepared by heating the enzymatic extract for 10 min in a boiling water bath before its addition to the reaction mixture. A unit of PG activity is defined as the amount of enzyme that released 1 $\mu$mol of reducing groups/h at pH 5.0 at 37 °C.

Cellulase activity was evaluated in a reaction mixture containing 0.12 mL of a 0.5% carboxymethylcellulose solution in 50 mM sodium acetate, pH 5.0, and 0.08 mL of enzymatic extract. After incubation at 37 °C for 2 h, the reaction was stopped, and reducing sugars were measured by the 2-cyanoacetamide technique as reported by Gross (*13*). One unit of cellulase activity is defined as the amount of enzyme that released 1 $\mu$mol of reducing groups/h at pH 5.0 at 37 °C.

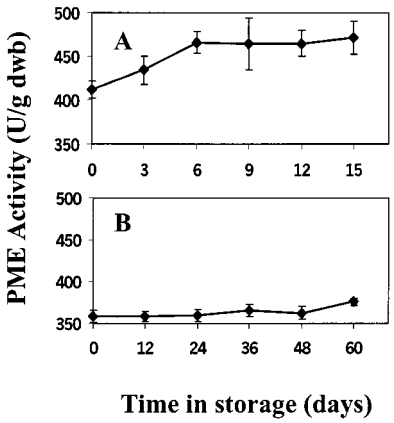


**Figure 1.** PME activity from the skin of prickly pear harvested at the mature-green stage and stored at 18 °C and 85–95% RH: (A) Naranjona variety (*O. ficus indica*), (B) Charola variety (*Opuntia* sp.). Each value is the average of seven replications. Vertical bars are the standard error of the mean.

$\beta$-Gal activity was assayed using the method described by Pressey (*14*). The reaction mixture consisted of 0.5 mL of 50 mM sodium acetate buffer, pH 5.0, containing 0.05% (*p*-nitrophenyl)-$\beta$-D-galactopyranoside substrate and 0.02 mL of enzyme extract in 0.23 mL of deionized water. After 15 min of incubation at 37 °C, the reaction was stopped by adding 1 mL of 1 N ammonium hydroxide containing 2 mM EDTA. The amount of liberated *p*-nitrophenol was determined by measuring the optical density at 400 nm. One unit of $\beta$-Gal activity is defined as the amount of enzyme that hydrolyzes 1 $\mu$mol of (*p*-nitrophenyl)-$\beta$-D-galactopyranoside to *p*-nitrophenol and D-galactose/h at pH 5.0 at 37 °C.

To estimate enzymatic activity changes during storage, regression analysis was done using the S-Plus 2000 software (Math Soft, Inc.).

**Transmission Electron Microscopy (TEM)**. TEM was done according to Ben-Arie et al. (*15*) with modification (4% paraformaldehyde was included in the fixation solution). Small pieces of prickly pear skin were cut and fixed in 2% glutaraldehyde + 4% paraformaldehyde in 0.1 M cacodylate buffer for 5 h at 4 °C. Then they were rinsed with cacodylate buffer and postfixed in 1% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.5) for 2 h at room temperature. The tissues were then dehydrated through graded alcohol series (10−100%). Samples were placed in propylene oxide, embedded in epoxic resin containing propylene oxide (1:1) for 1 day, and finally placed in resin, which polymerized in an oven at 60 °C for 36 h. Sections were cut using an ultramicrotome at 60−90 nm thickness, contrasted with uranyl acetate and lead citrate for observation with a JEOL-1010 transmission electron microscope operating at 80 kV.

## RESULTS AND DISCUSSION

PME, PG, cellulase, and $\beta$-Gal have been the most studied hydrolytic enzymes related to softening and cell wall structural changes during fruit ripening (*11*, *16*, *17*). Our results show that their changes during storage were quite different between varieties. **Figure 1** shows that the PME activity from Naranjona was 412 U/g dry weight basis (dwb) at the MG stage of ripening, increased significantly during the first days in storage at a rate of 9 U/day ($P < 0.001$), and then remained constant. In Charola, the PME activity remained constant at about 360 U/g dwb, during the entire storage period (**Figure 1B**). In **Figure 2A**, it can be seen that the PG activity from Naranjona was 1.2 U/g dwb at the MG stage and increased significantly during storage

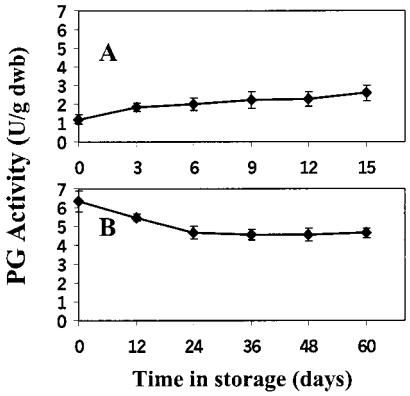


**Figure 2.** PG activity from the skin of prickly pear harvested at the mature-green stage and stored at 18 °C and 85–95% RH: (A) Naranjona variety (*O. ficus indica*), (B) Charola variety (*Opuntia* sp.). Each value is the average of seven replications. Vertical bars are the standard error of the mean.

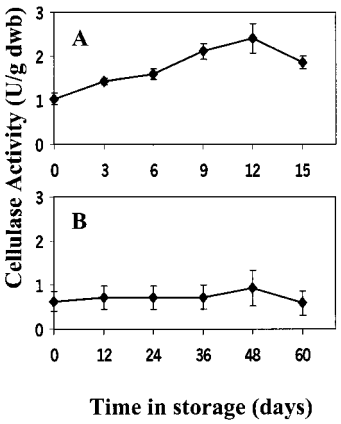


**Figure 3.** Cellulase activity from the skin of prickly pear harvested at the mature-green stage and stored at 18 °C and 85–95% RH: (A) Naranjona variety (*O. ficus indica*), (B) Charola variety (*Opuntia* sp.). Each value is the average of seven replications.Vertical bars are the standard error of the mean.

at a rate of 0.08 U/day ($P < 0.001$). In Charola (**Figure 2B**), the PG activity was 6.3 U/g dwb at the MG stage, decreased significantly at a rate of 0.05 U/day ($P < 0.001$) during the first 24 days in storage, and remained unchanged thereafter. In **Figure 3A**, it is shown that the cellulase activity from Naranjona was 1.0 U/g dwb at the MG stage and increased significantly at a rate of 0.11 U/day ($P < 0.001$) up to the 12th day in storage; thereafter the activity decreased. In Charola, the cellulase activity (**Figure 3B**) was 0.6 U/g dwb at the MG stage and remained low and constant during storage. **Figure 4A** shows that the $\beta$-Gal activity from Naranjona was 45 U/g dwb at the MG stage and tended to increase slightly during the first 9 days in storage but increased significantly at the end of storage ($P < 0.05$), attaining a 2.5-fold increase. In Charola, the $\beta$-Gal activity at the MG stage (**Figure 4B**) was 140 U/g dwb and increased significantly in storage at a rate of 1.6 U/day ($P < 0.001$).

As the storage time progressed, Naranjona prickly pear skin showed an increased activity in PG, cellulase, and $\beta$-Gal by a factor higher than 2, whereas PME tended to increase only slightly. In the Charola variety, $\beta$-Gal showed an increment in activity of 77% during storage, whereas the PG activity was maximum at the beginning of storage, declining by 36% during the first 24 days, and staying constant thereafter.

**Figure 5** shows cell wall ultrastructural changes during storage of both prickly pear varieties. Naranjona and Charola

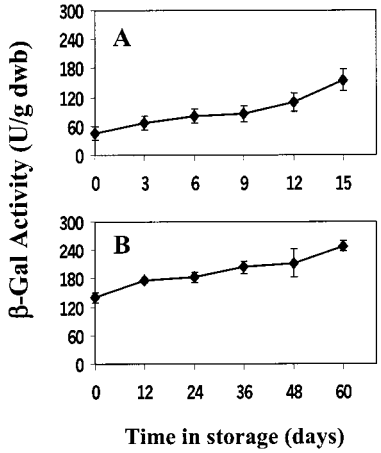


**Figure 4.** $\beta$-Gal activity from the skin of prickly pear harvested at the mature-green stage and stored at 18 °C and 85–95% RH: (A) Naranjona variety (*O. ficus indica*), (B) Charola variety (*Opuntia* sp.). Each value is the average of seven replications. Vertical bars are the standard error of the mean.

fruits both at the MG stage exhibited an intact middle lamella as can be seen in the electron-dense region between primary cell walls from adjacent cells (**Figure 5A1,B1**). However, the middle lamella showed dissolution by the end of the storage period for both varieties, at day 12 for Naranjona and at day 60 for Charola (**Figure 5A2,B2**). An appearance of cell separation and intercellular spaces is evident at the end of storage. This suggests the action of pectinases on the middle lamella during prickly pear storage, in agreement with enzyme activity measurements (**Figures 1 and 2**). TEM observations also showed degradation in the primary cell wall in 12 day stored Naranjona (**Figure 5A2**) but not in the primary cell wall from 60 day stored Charola (**Figure 5B2**). This is in agreement with results for cellulase activity, which increased in Naranjona but remained low and constant in Charola. Crookes and Grierson (*18*) reported a postharvest dissolution of the middle lamella and a disruption of the primary cell wall during ripening of tomato, similar to those observed in Naranjona during storage.

The observed enhancement in the activity of the cell-wall-degrading enzymes in Naranjona suggests that none of the hydrolytic enzymes acts alone to bring about cell wall structural changes, as reported by Barka et al. (*16*) for tomato softening. In Charola, we observed a predominance of PG and $\beta$-Gal. Our results show that different varieties from the same type of fruit show differences in the activity behavior of the cell-wall-degrading enzymes. Huber (*6*) and Seymour and Gross (*7*) reported that different fruits may have different enzymes responsible for their softening. We also found differences in activity for the same type of enzyme between varieties during storage. The Naranjona variety showed activities of cellulase and PME higher than those of Charola (about 2−3- and 0.15−0.25-fold, respectively), whereas Charola showed activities of PG and $\beta$-Gal higher than those of Naranjona (about 2−5- and 1.5−2.5-fold, respectively).

In Naranjona, it seems that cell wall structural changes might be due to a differential contribution and concerted action of PG, cellulase, PME, and $\beta$-Gal. Barrett and Gonzalez (*11*), working with the nonclimacteric cherry fruit, concluded that the integrated action of PG, PME, $\beta$-Gal, and possibly other enzymes appears to be required to break down the closely packed structure of the fruit cell wall, although they did not detect cellulase activity at any time during maturation and storage.

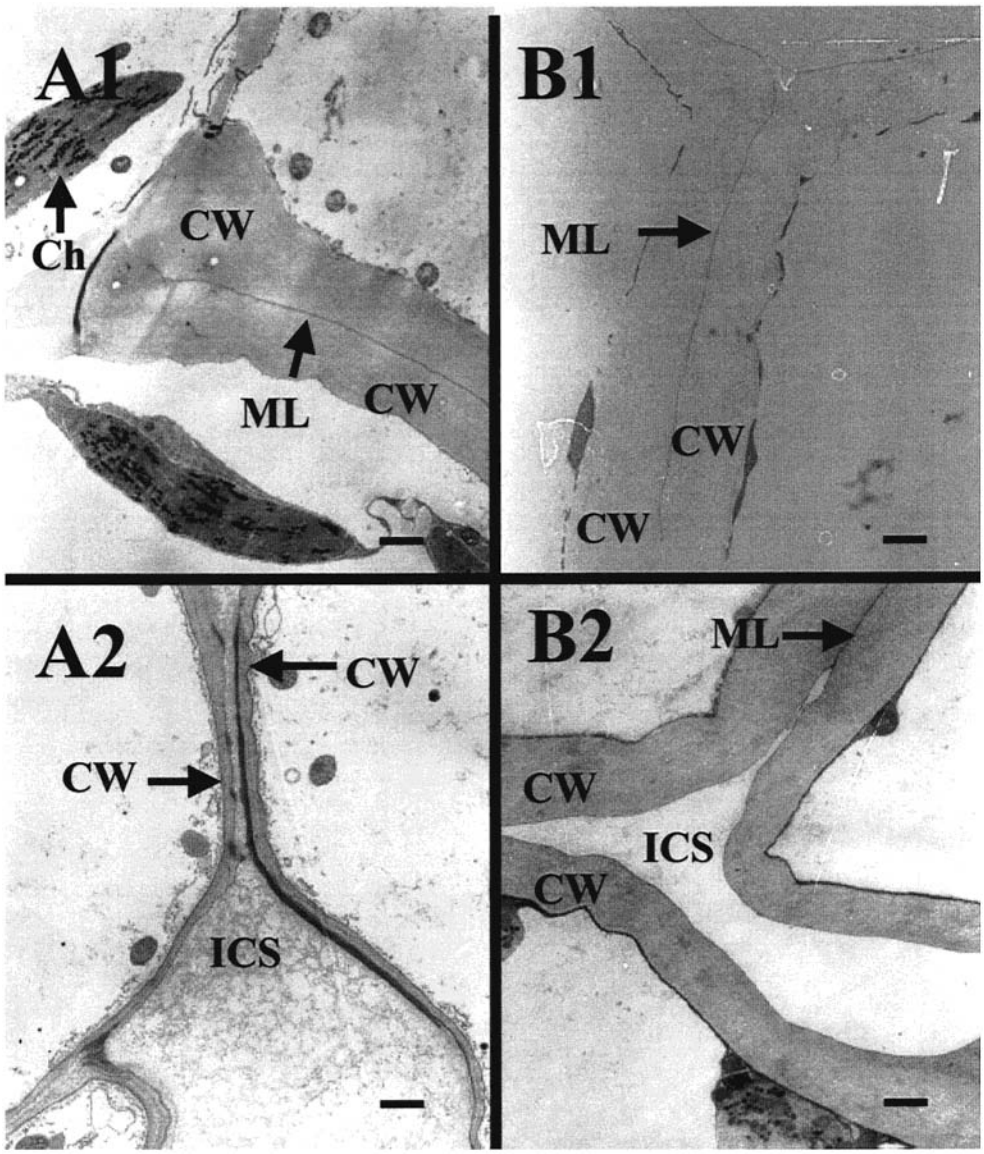


**Figure 5.** Changes in cell wall ultrastructure during storage of two varieties of prickly pear at 18 °C: (A1) mature-green Naranjona variety (0 days in storage), (A2) 12 day stored Naranjona variety, (B1) mature-green Charola variety (0 days in storage), (B2) 60 day stored Charola variety. CW = cell wall, ML = middle lamella, ICS = intercellular space, and Ch = chromoplast. The magnification of all micrographs is 6000× (reproduced at 90% of the original size). Bar = 1 $\mu$m.

In Charola, PG and $\beta$-Gal activities were predominant, whereas cellulase activity was relatively low. PG and $\beta$-Gal have shown increments in activity during ripening of nonclimacteric fruits. The PG activity increased in peppers (*19*), while $\beta$-Gal activity enhancements have been observed in cherries (*11*). Also, a predominant PG and $\beta$-Gal activity was observed in mango by Ketsa et al. (*20*), which suggested that these are important enzymes in the softening of ripening mango. Furthermore, in papaya and mango (two climacteric fruits), $\beta$-Gal appeared to be more closely related to softening than PG and PME (*21*, *22*).

The contribution of the different cell-wall-degrading enzymes on the changes in cell wall structure must be differential and dependent on the type of reaction carried out by a determined enzyme. PME catalyzes the de-esterification of galactosyl uronate methyl esters of pectin to their free carboxyl groups, whereas endo-PG cleaves $\alpha$-(1,4)-galacturonosyl linkages in demethylated pectin. Demethylation by PME results in a greater number of carboxyl groups which may facilitate PG activity on pectin. Cellulase refers to enzymes able to hydrolyze the *endo*-$\beta$-1,4-glucan links which can be found in cellulose and hemicellulose (xyloglucans). But because plant cellulase has not been clearly shown to degrade crystalline cellulose, its name is recently also referred to as *endo*-$\beta$-1,4-glucanase (*23*). For the purpose of this paper, we decided to refer to this enzyme as cellulase. On the other hand, $\beta$-Gal is responsible for the degradation of terminal $\beta$-1,4-linkages from galactans.

Interestingly, the PG activity in Charola was maximum at the beginning of storage and decreased thereafter. However, this variety exhibits a long postharvest life (about 2 months). So, it is suggested that this enzyme might be regulated during storage. This regulatory mechanism might disappear with PG extraction as suggested for glycosylhydrolases (PG and $\beta$-Gal) by Gallego and Zarra (*24*). A high PG activity might make up for a relatively low PME and cellulase activity in Charola, since the degree of pectin demethylation is important for the action of PG as suggested by Koch and Nevins (*25*).

Cell-wall-degrading enzyme activities in nonclimacteric fruits have been poorly studied. From the few works reported, it can be seen that cellulase activity has not shown consistent results

in nonclimacteric fruits. While the activity of cellulase was not detected during cherry ripening (*11*), this enzyme exhibited an activity enhancement during strawberry and pepper fruit ripening (*26*−*28*). On the other hand, most of the works about cell-wall-degrading enzyme activity in nonclimacteric fruits indicate that the extent of the increase is lower than that observed in climacteric fruits. We observed increments in activity of 2−3-fold in prickly pear, whereas Barka et al. (*16*) and Ketsa et al. (*20*) reported increments of 5−6-fold in tomato and mango, respectively.

From the regression analysis, the rate of changes in activity during storage showed that Naranjona had a higher cell wall enzymatic activity than Charola, in agreement with their differences in storage life. In Charola, the limited $\beta$-Gal activity in concert with a regulated PG activity might contribute to the long storage term of this variety, whereas the increments in activity of the *endo*-acting enzymes (PG and cellulase) might explain the short storage term of Naranjona. So, from the present work, it is suggested that PG and cellulase in Naranjona and PG and $\beta$-Gal in Charola are the main responsible enzymes affecting cell wall ultrastructural changes in prickly pear.

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