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Activity of β-Galactosidase and Polygalacturonase in Zucchini Squash (*Cucurbita pepo* L.) Stored at Low Temperatures

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Summary

When fruits are exposed to chilling temperatures, enzymatic systems are affected and normal cell metabolism is altered; cell wall enzymes are the least studied in this respect. Our objective is to determine the effect of storage temperature and/or kind of tissue on the activity of the enzymes polygalacturonase (PG) and β-galactosidase (β-gal) in zucchini squash (*Cucurbita pepo* L.) subjected to 2.5 and/or 12 °C for 16 days. Exocarp and endocarp samples were analyzed every 4 days for PG and β-gal activities. The highest β-gal activity was found in the exocarp at 12 °C; in both tissues β-gal decreased with time at 2.5 °C, but increased at 12 °C. PG activity was higher in the endocarp (highest activity at 2.5 °C) than in the exocarp (highest activity at 12 °C). It was concluded that PG activity in the exocarp constitutes an adequate system for studying the relationship between chilling injury and cell wall biological activity.

Key words: chilling injury, cold signaling, oligosaccharins, cell wall

Introduction

When fruits susceptible to chilling injury (CI) are stored at a critical temperature, a series of metabolic changes occur, some of which are protective to the cell, while others result in the visible symptoms of CI. There are some reviews dealing with the hypothetical basis of CI (*1–3*), but the biophysical mechanism is far from being elucidated yet. Development of CI involves either a stimulated or inhibited enzyme activity as has been found by several authors (*4–6*). Changes in cell wall metabolism have been found in response to cold storage of fruits. In cucumbers and peaches an increase in hot water-insoluble pectin and a decrease in hot water-soluble pectin have been found, which suggests a deesterification and copolymerization of pectin during the chilling process (*7,8*). Arpaia *et al*. (*9*) reported a higher content of uronic acids, galactose, arabinose, and rhamnose in kiwi fruits after storage at 1 °C. Gross and Wang (*10*) reported compositional changes in cell wall polysaccharides in chilled cucumber, and Brummell *et al*. (*11*) reported that cold storage of peaches affected the metabolism of cell wall polysaccharides by altering the activities of cell wall-related enzymes.

Among the major cell wall enzymes, PGs have been reported as having an enhanced activity in response to chilling treatments. Martínez-Téllez *et al*. (*12*) reported a gradual increase in PG activity in zucchini fruits as a function of the storage time at 2 °C; they found a maximum PG activity by day nine. Probably the high activity

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of PG was responsible for the fruit softening found at this combination of temperature and time, as there is evidence that suggests a main role of PG in the softening of fruits during the ripening process (*13*). Nevertheless, PG in such a system could have a more specialized function, as it has been demonstrated in the study of plant-pathogen interactions. Pathogen fungi produce pectinases that attack the homogalacturonan in the plant cell wall; PG is among the early secreted enzymes which have a central role in pathogenesis (*14*). Fungal PG exhibit both exo and endo activities, and they are coded by multigenetic families whose size seems to vary with the specificity of the interaction (*15*).

Endo-PG cleaves β-1,4-galacturonic acid residues in the homogalacturonan backbone and releases oligosaccharides of different molecular size. Some of these oligosaccharides, which are known as oligosaccharins, possess biological activity; even at very low concentrations they have the ability to induce the plant defense system. Thus, the plant cell wall possesses its own signals for defense, or endogenous elicitors (*15*). The signals are produced whether a pathogen attacks the plant or not, an example being the cell death phenomena that precede either wounding or low-temperature shocks (*16*). With such background in mind we propose a role of PG in the primary stage of the development of chilling injury in zucchini squash.

β-gal is an ubiquitous multigenetic enzyme with the ability to hydrolyze β-D-galactosyl, a non-reducing end residue from β-D-galactoside compounds. β-gal have been purified either from plants or fungi and their kinetic data have been determined in order to find a technological application for them in the hydrolysis of milk lactose (*17*,*18*).

It is known that xyloglucan is the natural substrate for β-gal in some dicotyledonous plants, but their role *in vivo* is not yet clear; presumably, they are involved in the changes the cell wall undergoes during the development and ripening of plants and fruits, respectively (*19–24*). It has been suggested that the galactan degradation carried out by these enzymes could determine structural changes and affect cell wall porosity, and that the increase in pore size would allow the access of other cell wall degrading enzymes to their substrate (*25*).

In light of the structural requirements of some oligosaccharins from xyloglucan (*14*), it is possible that during the exposure of fruits to chilling β-gal modifies the structure of such oligosaccharins, thus giving them their active form; however, this hypothesis lacks experimental support as there are not enough reports which relate CI to β-gal activity.

In this work PG and β-gal activities were monitored in the exocarp and endocarp from zucchini fruits stored at 2.5 and/or 12 °C through a 16-day period, in order to better understand their role in the chilling response of both tissues.

Materials and Methods

Fruit material

Field-grown Raven zucchini (*Cucurbita pepo* L.) were harvested and selected by size (17–20 cm) and quality attributes such as turgor, dark-green color, free of wounds, mechanical injuries, and insect infestation.

Storage conditions and sampling

After harvesting and sorting, zucchini fruits were divided into two groups of 50 fruits each. One was stored at 12 °C and the other at 2.5 °C, both at 85 % relative humidity (RH), for 16 days. Separately, two more groups, consisting of 15 fruits each, were stored at 12 °C and 2.5 °C, also at 85 % RH, for 16 days in order to evaluate the CI index.

In the course of a 16-day period, triplicate random samples were taken every 4 days from batches stored at both temperatures. Exocarp (3 mm thickness) was removed from fruits and sliced into pieces of 1 cm^2. The endocarp was cut into 1 cm^3 pieces. Samples thus obtained were placed in plastic bags and immediately stored at –70 °C until enzymatic analysis was made. Also, the CI index was determined every 4 days in the groups of fruits stored for that purpose.

Chilling injury index

CI index was evaluated according to Martínez-Téllez and Lafuente (*5*) by means of a subjective scale of visual symptoms based on both necrotic surface and pitting intensity, according to the following scale: 0, no pitting; 1, slight (10 % or less of the evaluated fruit); 2, medium (10–20 % of the evaluated fruit); and 3, severe pitting (>20 % of the evaluated fruit). CI symptoms were assessed 24 h after transferring fruits from storage chambers to room temperature (20 °C).

β-galactosidase activity in the exocarp and endocarp

β-gal determination was made according to Distler and Jourdian (*26*). Briefly, 20 g of frozen sample were placed in a beaker, then 2 g of polyvinyl polypyrrolidone (PVP) and 32 mL of 0.2 M phosphate buffer, pH=7.2, (unless otherwise specified all of the reactants used were from Sigma Chemical Co., USA) were added and the mix homogenated in an Ultra Turrax T25 homogenizer (Janke and Kunkel, IKA-Labortechnik, Germany). The homogenate was filtered through a miracloth, then the filtrate volume was measured and centrifuged at 19 500 rpm in JA-20 rotor at 4 °C for 30 min. The supernatant was filtered through Whatman paper no. 1. The filtrate constituted the crude extract in which β-gal activity was determined.

β-gal activity in crude extracts was determined in triplicate by incubating 20 μL of the crude extract with 25 μL of *p*-nitrophenyl-β-D-galactopyranoside as substrate, 10 μL of 1 % bovine serum albumine (BSA), and 45 mL of citrate-phosphate buffer, pH=4.3, at 37 °C for 1 h and then stopping the reaction with 1 mL of 0.25 M glycine buffer, pH=10, and finally measuring the absorbance at 400 nm in a Shimadzu spectrophotometer (Shimadzu Corp., Japan). A standard curve was constructed with *p*-nitrophenyl-β-D-galactopyranoside in order to quantify in each aliquot the *p*-nitrophenyl released by β-gal. Protein content in each extract was determined by Biuret. A unit of β-gal was defined as the quantity of en-

zyme that releases 1 μmol of *p*-nitrophenyl/(mg protein/mL) for 1 h, under the assay conditions.

PG activity

For PG activity determinations, the method described by Gross (*27*) was used. A mass of 10 g of frozen tissue was homogenated with 30 mL of cold 1 % sodium bisulphite for 30 s. The homogenate was filtered through miracloth and the filtrate was removed. The solid precipitate was resuspended in 30 mL of cold 1 % sodium bisulphite. This step was repeated twice. The residue was suspended in 30 mL of 1 M NaCl, pH adjusted to 6.0, and then held for 2 h at 2.5 °C with constant stirring. The suspension was centrifuged at 10 000 rpm, rotor JA-20, for 10 min at 4 °C. The supernatant was filtered through Whatman paper no. 1. From filtrates, aliquots of 5 mL were loaded onto Sephadex G-15 columns (Sigma Chemical Co., USA) which had previously been equilibrated with 50 mM sodium acetate, pH=4.4. Columns were eluted with 3.5 mL of 50 mM sodium acetate. The elutants were collected in glass vials and represented the crude extracts in which PG activity was determined.

A standard curve was constructed with D-galacturonic acid. For the PG assay, 200 μL of crude extract and 200 μL of 0.025 % polygalacturonic acid were added to the test tubes. In a control tube, 200 μL of 50 mM sodium acetate were added instead of the extract. Tubes were vortexed and held at 30 °C for 1 h. Reaction was stopped by adding 1 mL of cold borate buffer, pH=10; then 200 μL of 1 % cyanoacetamide were added. Tubes were placed in a boiling water bath for 10 min, cooled to room temperature and the absorbance was measured at 276 nm in a Shimadzu spectrophotometer (Shimadzu Corp., Japan). From the standard curve the reducing sugars content was calculated and expressed as μmol of D-galacturonic acid. The value obtained in the control tube was subtracted from each assay tube. Protein content was determined by the Bradford method. A unit of PG was defined as the quantity of enzyme that releases 1 μmol of D-galacturonic acid/(mg protein/mL), in 1 h, under the assay conditions.

Experimental design and statistical analysis

The experimental design was completely randomized. Frozen samples were randomized and PG and β-gal residual activities were determined. Data were analyzed by a factorial arrangement in NCSS software (*28*) at $p<0.05$. An ANOVA test was made in order to determine the effect of storage temperature and time, as well as tissue type, on PG and β-galactosidase activities.

Results and Discussion

β-galactosidase activity

The ANOVA test showed that both temperature and kind of tissue had a significant effect on β-gal activity ($p<0.05$) in zucchini fruits. The temperature-tissue interaction was not significant. Fig. 1 shows that β-gal activity was approximately 30 % higher in the exocarp than in the endocarp. Because of the lack of reports on β-gal activity in zucchini fruits, it was impossible to make comparisons. The role of β-gal in plant tissue is not yet clear; in plants they show different patterns of activity in each stage of development, so probably their role *in vivo* is aimed at cell differentiation. In fruits, they could be involved in ripening mechanisms, but there is not enough evidence to prove it.

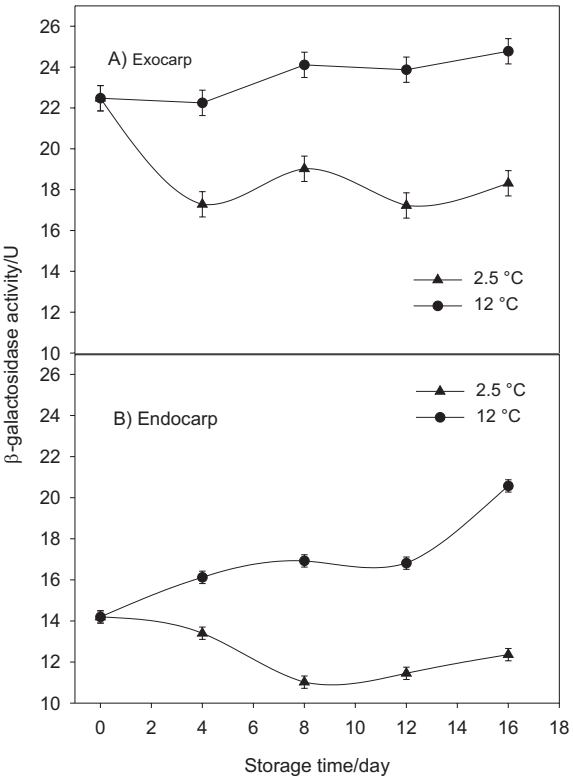

Fig. 1. Effect of storage temperature and time on β-galactosidase activity in exocarp and endocarp of zucchini squash stored at 2.5 or 12 °C for 16 days ($p<0.05$)

In this work, if β-gal is involved in the normal softening mechanisms of the tissue as a result of senescence, a higher activity in the exocarp than in the endocarp would be reasonable, as it is in the exocarp where the softening is more apparent. The lack of data about differences in chemical composition between cell walls from both zucchini exocarp and endocarp tissue makes it difficult to correlate this enzymatic activity with changes in cell wall composition.

Fig. 1 also shows the effect of storage temperature on β-gal activity in zucchini fruit. In general, there was a higher activity in fruits stored at 12 °C than in those at 2.5 °C. Such behavior is considered normal and attributed to a reduced speed in metabolism due to the lower temperature.

ANOVA test for β-gal, both in the exocarp and endocarp, showed that the temperature-storage time interaction was significant ($p<0.05$). Fig. 1A shows that β-gal activity in the exocarp of fruits stored at 12 °C increased gradually as a function of time and was higher than that found in fruits stored at 2.5 °C in the course of 16 days. This result is consistent with the idea that a lower temperature either delays or inhibits enzymatic reactions (*29*).

In the endocarp (Fig. 1B), β-gal activity was, in general, higher at 12 °C than at 2.5 °C. At 12 °C there was a gradual increase, reaching on day 8 a value which stayed constant until day 12; after that there was an abrupt increase. In the endocarp of fruits at 2.5 °C β-gal activity decreased for the first 8 days, then increased gradually to a value near that of day 4.

The activity pattern of β-gal as a function of both time and storage temperature was similar in both kinds of tissues, and it seems to be due to a temperature-inhibition mechanism. Results suggest that β-gal does not have a specialized function in the CI mechanism in zucchini. However, further work is required in this respect.

Polygalacturonase activity

The temperature-tissue interaction had a significant effect on PG activity ($p<0.05$). The highest PG activity was found in the endocarp of fruits stored at 12 °C (Fig. 2). PG is the most active enzyme in fruit ripening (*13*). Zucchini is a non-climacteric fruit so it might not be reasonable to relate PG activity with ripening of harvested fruits; instead, the term senescence could be more adequate. A higher activity in the endocarp can be due to the possibility that the senescence pattern is from the core to the exocarp. In tomato, during the postharvest ripening process, it has been demonstrated that endo--PG activity follows a circular pattern and it appears in the exocarp before it appears in the endocarp, which seems to be the result of differences in ethylene sensitivity in different tissues (*30*).

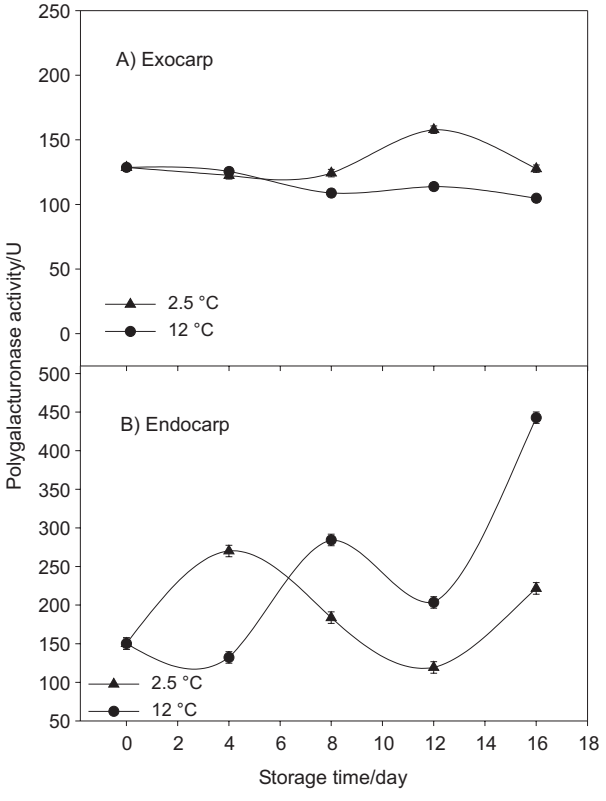

Fig. 2. Effect of storage temperature and time on polygalacturonase activity in exocarp and endocarp of zucchini squash stored at 2.5 or 12 °C for 16 days ($p<0.05$)

The minor PG activity in the endocarp at 2.5 °C (Fig. 2) can be explained in terms of the temperature dependence of the enzymatic reactions (*29*).

Fig. 2 also shows that PG activity was higher in the exocarp in zucchini fruits stored at 2.5 °C than in those at 12 °C. Such a result confirms what was reported by Martínez-Téllez *et al*. (*12*) and shows that the pattern of PG activity in relation to temperature is not as expected, which is discussed below.

The ANOVA test showed a significant interaction between temperature and storage time for PG activity in the exocarp from zucchini fruits ($p<0.05$). Fig. 2A shows a decreasing tendency of PG activity with storage time at 12 °C, whereas at 2.5 °C a transient increase occurred with a maximum at 12 days. A temperature of 12 °C does not cause chilling injury in zucchini, as was reported by Balandrán-Quintana *et al*. (*31*) and Soto-Cerón (*32*), and confirmed here (Fig. 3); however, a delay in the senescence processes is conceivable at such temperature.

If PG is involved in the normal senescence process of the exocarp, it would at least have to show a more or less constant value of its activity in fruits at 12 °C; however, the opposite was true: a 20 % decrease took place over a 16-day period (Fig. 2A). Fruits stored at 12 °C did not show CI symptoms during the 16 days (Fig. 3) and there was no perceptible softening by sight and touch. Although the fruit texture was not measured in this study, in previous work we had never found significant differences in firmness in zucchini stored at 12 °C for 12 or more days, with an average firmness value of 5.8 kgf (*32*). We have also found in zucchini fruits stored at 2.5 or 12 °C that the cell wall's galacturonic acid content was almost twofold in fruits stored for 16 days with respect to that at day zero, irrespective of the temperature (*33*). So the results of this study suggest that PG in the exocarp is not involved in the senescence process in zucchini, at least during the stage in which the softening is not yet apparent. However, the decreasing pattern of PG at 12 °C suggests that during the early storage stages the enzyme had a different role, probably contributing to some acclimation mechanism.

Fruits stored at 2.5 °C showed evident CI symptoms after 8 days of storage (Fig. 3). At 12 days the softening was totally manifested and coincided with the maximum in PG activity (Fig. 2A). There is evidence which suggests that the PG-mediated pectin degradation does not have a significant contribution to fruit softening until the last ripening stages, when PG is the main factor responsible for pectin depolymerization (*13*). It is then possible that the maximum activity of PG at 12 days and 2.5 °C is the main cause of the softening found in the exocarp tissue. At 16 days of storage at 2.5 °C the tissue was totally damaged (Fig. 3) and PG activity decreased (Fig. 2A). Probably at that time there was an extensive degradation of pectin in the tissue so PG activity was no longer required.

In the endocarp there were significant differences in the time-temperature interaction ($p<0.05$). At 12 °C, the PG activity at 16 days was almost 3-fold that at 0 days (Fig. 2B). Such behavior is the opposite of that found for the exocarp at the same temperature (Fig. 2A) and suggests that at 12 °C the metabolic changes which took

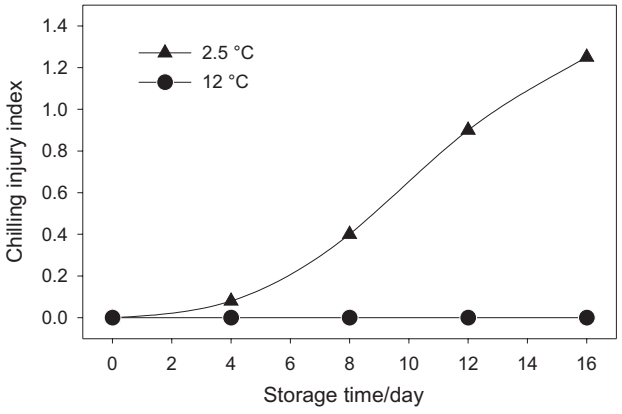

Fig. 3. Development of chilling injury in zucchini squash stored at 2.5 or 12 °C for 16 days

place in the endocarp require a higher PG activity than those which operate in the exocarp. Also supported is the idea that the senescence pattern in zucchini fruits, in terms of cell wall degradation, takes place from the core to the outer surface.

Fig. 2B shows a sinusoidal pattern of PG activity in the endocarp of zucchini at 2.5 °C, with a maximum and a minimum at 4 and 12 days, respectively, which means that over a 16-day period at 2.5 °C PG activity was in a range with an approximate mean of 189 U; however, the sinusoidal behavior suggests that during the first days of exposure to chilling, PG had another more specialized role than solely to break down pectin. It is possible that some pectic oligomers released at that stage acted as signaling molecules in the acclimation mechanisms. It is worth noting that 8 days before CI was not visibly present in fruits at 2.5 °C (Fig. 3), so the possibility of irreversibility was latent, and precisely at that time PG had a maximum activity.

The next burst in PG activity in the endocarp at 2.5 °C occurred after 12 days (Fig. 2B), but CI progressed to levels at which reversibility was impossible, probably because such a burst was due to extensive tissue degradation.

Conclusions

The data presented in this study have shown that kind of tissue, temperature and time of storage had a significant effect on PG and β-galactosidase activities in zucchini fruits. In both exocarp and endocarp tissue, changes of β-galactosidase activity as a function of time and temperature were attributed to both the normal senescence and temperature inhibition processes. The gradual increase in PG activity in the exocarp at 2.5 °C suggests that PG is activated in the early stages of exposure to chilling. The exocarp and PG could be an adequate system to study the relationship between CI and cell wall biological function.

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