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Degradation and solubilization of pectin by β-galactosidases purified from avocado mesocarp

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Three β-galactosidase (EC 3.2.1.23) isozymes were purified from the mesocarp of ripe fruit of *Persea americana* Mill. cv. Lula, and their effects on pectin derived from mature-green tomato fruit were investigated. The β-galactosidases had pI values of 5.0, 5.1 and 5.2, and molecular weights of 41, 49 and 54 kDa, respectively. There was a partial degradation of pectin resulting in the release of monomeric galactose upon treatment with avocado β-galactosidase. This degradation resulted in increased pectin solubility and decreased apparent average molecular size as determined by microfiltration and gel permeation by high-performance liquid chromatography. The increase in solubility was due, in part, to an apparent decrease in the ability of pectin molecules to aggregate together.

Key words – Avocado, β-galactosidase, fruit softening, galactose, pectin, *Persea americana*.

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Introduction

Fruit softening during ripening is a complex process that presumably involves structural changes in the walls of fruit cells. It is thought that these changes are brought about through the action of cell wall hydrolases degrading various wall polymers (Fischer and Bennett 1991), although other mechanisms may be involved (Gross 1990, Mitcham et al. 1989). One of the most studied hydrolases is polygalacturonase (PG) [endo (1→4)galacturonase (EC 3.2.1.15)]. PG cleaves α(1→4)-galacturonosyl linkages in pectin and until recently was generally considered to be the primary enzyme involved in the softening process. However, there is considerable evidence suggesting that PG is not exclusively responsible for the cell wall structural changes that occur during ripening (Huber 1984, Seymour et al. 1987, Smith et al. 1988, Tucker and Grierson 1982).

The failure of PG to play a significant role in early softening or to have the sole function in pectin degradation indicates that other cell wall hydrolases may be important. One group of cell wall hydrolases that may play a role in cell wall degradation are the glycosidases. For example, β-galactosidase is abundant during early softening in a variety of fruits (Fischer and Bennett 1991). It was suggested that β-galactosidase is involved in the degradation of tomato cell wall galactans (Pressey 1983). It is known that net cell wall galactosyl content decreases with ripening of many fruits (Gross and Sams 1984, Gross and Wallner 1979). Also, the amount of free galactose in tomatoes increases during ripening (Gross 1983).

Galactans, primarily in β(1→4)-linkages, are also constituents of side chains on the rhamnogalacturonan backbone (O'Neill et al. 1990). The galactosyl content of pectin has been shown to decrease during ripening (Gross 1984). It is possible that β-galactosidase is responsible for the degradation of these side chains (Pressey 1983, Redgwell et al. 1992, Seymour et al. 1990) and perhaps their removal brings about significant changes

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in how individual pectin molecules interact together and with other wall polymers, leading to decreased wall integrity. It has been shown that the decline in wall galactans precedes or accompanies increases in soluble polyuronide (Gross and Wallner 1979, Kim et al. 1991).

The present study was undertaken to characterize β-galactosidases in ripe avocado fruit, to study their ability to cleave galactosyl residues from pectin, and to determine the effect of galactosyl removal on pectin solubility.

Abbreviations – CDTA, *trans*-1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid; CSP, chelator soluble pectin; GC/EIMS-SIM, capillary gas chromatography/electron impact mass spectrometry-selective ion monitoring; IEF, isoelectric focusing; PG, polygalacturonase; PGA, polygalacturonic acid.

Materials and methods

Enzyme extracts were prepared from mesocarp of ripe fruit of *Persea americana* Mill. cv. Lula, obtained from Dr Robert Knight of the USDA/ARS, Miami, FL. Protein content was determined using the Bio-Rad Protein Assay Reagent (Bio-Rad Technical Manual No. 82-0275) with bovine serum albumin as the standard. The substrate was chelator soluble pectin (CSP), isolated from mature-green fruit of *Lycopersicon esculentum* Mill. (Gross 1984).

Five-hundred grams of avocado mesocarp tissue were homogenized in one l of 100 m*M* Na-acetate, pH 5.0, containing 1.8 *M* NaCl and 1.5% (w/v) PVP using a Waring blender. The homogenate was stirred at 4°C for 1 h and then centrifuged at 12000 *g* for 30 min. The supernatant was filtered through Miracloth and solid $(NH_4)_2SO_4$ was added to the filtrate to 20% saturation. The suspension was then centrifuged at 12000 *g* for 30 min and the pellet discarded. Additional $(NH_4)_2SO_4$ was added to 50% saturation, and the precipitate was recovered by centrifugation (12000 *g* for 30 min). The supernatant from this fraction was dialyzed against 50 m*M* Na-acetate, pH 4.0, containing 50 m*M* NaCl for 14 to 16 h. PG activity was determined using 2-cyanoacetamide (Gross 1982) with polygalacturonic acid (Sigma Chem. Co.; 98% purity; PGA) as substrate. The pellet recovered from the 50% $(NH_4)_2SO_4$ fractionation was redissolved in 50 m*M* Na-acetate, pH 4.0, containing 50 m*M* NaCl. Undissolved material was removed by centrifugation (12000 *g* for 30 min). Pectolytic activity in this fraction was determined using 2-cyanoacetamide and also as described below. The concentration of NaCl used for resuspension (50 m*M*) was necessary because it was found that the pectolytic enzymes were not soluble in solutions of lower ionic strength (data not shown).

The protein recovered in the 50% $(NH_4)_2SO_4$ pellet was partially purified using a FPLC affinity column (2 × 10 cm) prepared from alginate and cross-linked with epichlorohydrin according to the procedure of Schols et al. (1990). The column was equilibrated in 50 m*M* Na-acetate (pH 4.0) containing 50 m*M* NaCl. The protein fraction was loaded on the column and unbound protein eluted using equilibration buffer. Bound proteins were then eluted from the column with 0.2 *M* Na-acetate (pH 8.0). All fractions were tested for pectolytic activity according to the procedure given below.

Active fractions from the alginate column were dialyzed against 1 m*M* Na-phosphate (pH 6.8) containing 100 m*M* NaCl for 14 to 16 h. The protein fraction was concentrated by ultrafiltration using a PM-10 membrane (Amicon, W. R. Grace & Co., Danvers, MA). This fraction was then loaded onto a FPLC hydroxyapatite column (1 × 10 cm). The column was equilibrated in 1 m*M* Na-phosphate (pH 6.8) containing 100 m*M* NaCl. Proteins were eluted from the column using a Na-phosphate gradient from 1 to 400 m*M*. Eluted fractions were collected and tested for their ability to degrade pectin either by performing native PAGE or isoelectric focusing (IEF) on aliquots from the enzyme extracts followed by electro-blotting the proteins into a native polyacrylamide gel containing 0.3% (w/v) tomato CSP. Alternatively, 0.3% CSP was replaced with 0.3% PGA. Native PAGE was performed using the procedure of Cruickshank (1987). IEF was performed using the mini-gel procedure of Robertson et al. (1987). IEF was initially performed in the pH range of 4.0 to 6.3, since it was determined that all activity was localized within that pH range (data not shown). The monomeric concentration of both the CSP-gel and PGA-gel was 10% (w/v); the ratio of bis-acrylamide to acrylamide was 3% (w/v). The electro-blotting buffer was 288 m*M* glycine and 37.4 m*M* Tris-HCl (pH 8.6). The voltage was 10 V with a transfer time of 2 h at 4°C. The CSP-gel was then placed in a solution containing 50 m*M* Na-acetate (pH 5.0) containing 50 m*M* NaCl and 0.02% NaN_3. The gel was incubated for 14 to 16 h, washed in 50% (v/v) methanol, and stained with 0.05% (w/v) aqueous toluidine blue 20 min. The gel was exhaustively washed with distilled water to visualize areas where pectin or polygalacturonic acid was degraded and had therefore diffused. These areas appeared as clear bands in a dark blue background.

Preparative IEF was performed to further purify the enzymes using a pH range of 4 to 6.3, using the same procedure as described for analytical IEF. Protein bands, having the same pI values as previously observed bands having pectolytic activities, were cut from the gel and electro-eluted (Andrews 1985) using 288 m*M* glycine, 37.4 m*M* Tris-HCl (pH 8.6) for 24 h at 4°C. Bands were tested individually for glycosidase activity using the following *p*-nitrophenyl-glycoside derivatives (Pharr et al. 1976): α-D-galactosidase, β-D-galactosidase, α-L-arabinosidase, α-D-mannosidase, α-D-glucosidase, β-D-glucosidase, β-D-xylosidase and α-L-rhamnosidase.

The pectolytic extract was also subjected to two-dimensional electrophoresis to determine purity and molecular weight. IEF, in the pH range of 4 to 6.3, was performed as described before, followed by SDS-PAGE

(Schagger and von Jagow 1987). Run time for SDS-PAGE was about 1.5 h at 75 V. Protein bands were visualized by silver staining (Wray et al. 1981).

The ability of the isolated avocado β-galactosidases to degrade pectin was determined by examining changes in molecular size of CSP after β-galactosidase treatment. Pectin degradation was initiated by the addition of 0.05 units of avocado β-galactosidase to solutions containing 30 mg CSP in 30 ml of 50 m*M* Na-acetate (pH 5.0) containing 50 m*M* NaCl and 0.02% NaN_3. Since a significant proportion of CSP may have been CDTA (Mort et al. 1991), the amount of CSP (30 mg) was determined colorimetrically by the phenol-sulfuric acid assay of Dubois et al. (1956). After a 24 h incubation at 37°C, the solution was heated at 60°C, rather than 100°C, for 15 min to stop the reaction, but minimize potential pectin alterations caused by boiling. A control sample was run identically except with heat denatured avocado β-galactosidase. Samples were passed through a series of 4 filters having an increasingly smaller pore size (1.2, 1.0, 0.45, and 0.22 µm). The diameter of the filters was 2.4 cm. Filters were washed with 10 ml of 50 m*M* Na-acetate (pH 5.0) containing 50 m*M* NaCl. The amount of carbohydrate in the filter wash and in the filtrates was determined colorimetrically (Dubois et al. 1956). The filtrates were further analyzed by gel permeation HPLC using Synchropak GPC 4000, 1000, and 100 columns run in series with a refractive index detector (Fishman et al. 1989). The eluant was 50 m*M* NaCl. The amount of carbohydrate injected into the HPLC was 3 mg. Fractions from HPLC were collected and total carbohydrate content determined colorimetrically (Dubois et al. 1956). Total column volume was determined using a monosaccharide, and the CSP fractions eluting at that retention time were prepared for GC/EIMS-SIM analysis to determine monomeric product(s) of β-galactosidase treatment. Alditol acetate derivatives were made according to the procedure of Blakeney et al. (1983) and analyzed by GC/EIMS-SIM (Gross 1984).

Results

Five hundred grams of ripe avocado mesocarp yielded about 630 mg of soluble protein. Approximately 110 mg of this protein was precipitated by increasing the $(NH_4)_2SO_4$ concentration from 20% to 50% saturation. This latter fraction had no detectable PG activity as determined by the 2-cyanoacetamide assay (data not shown). However, it did have pectolytic activity as determined by native PAGE followed by electro-blotting into a CSP-gel (data not shown). PG activity was found in the supernatant after the 50% $(NH_4)_2SO_4$ fractionation using the 2-cyanoacetamide assay (data not shown).

This avocado preparation was further purified using an alginate affinity column. Two protein fractions were eluted from the column (Fig. 1). The first fraction was devoid of pectolytic activity. However, the second fraction was active against pectin but not polygalacturonic acid as indicated by the results obtained from native PAGE followed by electro-blotting into a CSP-gel and into a PGA-gel (data not shown). The second peak also tested negative for PG activity as determined by the 2-cyanoacetamide assay using PGA as substrate. Approximately 13 mg of protein were recovered in the second fraction.

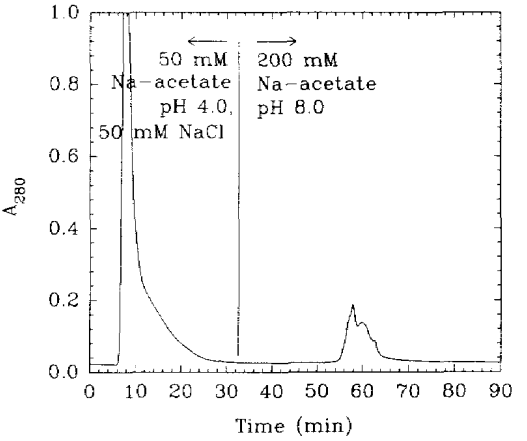

Fig. 1. Alginate-FPLC profile of crude avocado preparation. The first and second fractions were eluted with 50 m*M* Na-acetate (pH 4) containing 50 m*M* NaCl and 0.2 *M* Na-acetate (pH 8.0), respectively.

The second fraction from alginate chromatography was further purified using hydroxyapatite. Two peaks eluted from this column, one during loading and washing and a second during the Na-phosphate gradient (Fig. 2). Both peaks were collected and tested for pectolytic activities using native PAGE followed by electro-blotting onto a CSP-gel. Pectin degradative and solubilization activity was associated with peak 2 but not

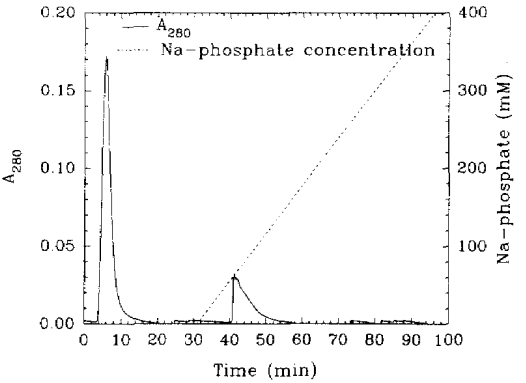

Fig. 2. Hydroxyapatite-FPLC profile of the second fraction from alginate-FPLC. Peak 1 eluted in 1 m*M* Na-phosphate (pH 6.8) containing 100 m*M* NaCl and peak 2 during the Na-phosphate gradient.

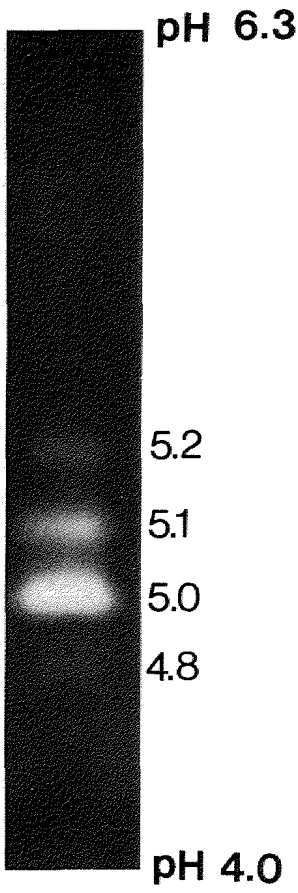

Fig. 3. Photograph of CSP-gel showing 4 pectolytic enzymes found in peak 2 from hydroxyapatite FPLC. Peak 2 was first subjected to IEF in the pH range 4 to 6.3 and then electro-blotted on CSP-gel. The gel was stained with toluidine blue after an overnight incubation with 50 m*M* Na-acetate (pH 5.0) containing 50 m*M* NaCl, 0.02% NaN_3 and 5% tomato CSP. Clear areas indicate areas of pectin degradation. Numbers on right side of photograph refer to the pH values of the corresponding IEF gel.

with peak 1 (data not shown). Approximately 4 mg of protein eluted in the second peak, from the 13 mg loaded on the column. Peak 2 proteins were subjected to IEF and then electro-blotted into a CSP-gel. The results are illustrated in Fig. 3. There were four areas where pectin became more soluble due to enzymatic activity. The pIs of the 4 pectolytic enzymes were approximately 5.2, 5.1, 5.0, and 4.8.

Preparative IEF was performed on peak 2 from the hydroxyapatite column. Four protein bands having the same pIs as above were cut from the gel, electroeluted, and tested for various glycosidase activities. The 3 most basic bands tested positive for β-galactosidase activity using *p*-nitrophenyl-β-D-galactoside as substrate; no other glycosidase activities were detected. Only α-galactosidase activity (EC 3.2.1.22) was detected in the most acidic band.

Figure 4 is a photograph of a 2-D electrophoresis gel of the preparation containing the 4 active pectolytic enzymes. IEF, in the range of 4 to 6.3, is the first direction, SDS-PAGE is the second. The 4 enzymes appear as single polypeptides. The 3 β-galactosidases, pIs 5.2, 5.1 and 5.0, had molecular weights of 49, 55, and 41 kDa, respectively. The purported α-galactosidase, pI 4.8, had a molecular weight of 25 kDa.

The purified β-galactosidase isozyme with a pI value of 5.0 was tested for its ability to degrade CSP. This isozyme was chosen since it had the highest recoverable activity in the preparation as determined by the *p*-nitrophenyl-β-D-galactosidase assay. After a 24 h incubation, the sample was passed through the series of filters. The results are illustrated in Fig. 5. When CSP was treated with heat-denatured β-galactosidase most of the pectin was retained on one of the 4 filters. Only about a third was of a small enough molecular size to pass through all filters. When CSP was treated with active β-galactosidase, most of it was degraded to a form which allowed it to pass through all filters.

The two resulting filtrates were further analyzed using gel permeation HPLC to detect any additional variations in average molecular size (Fig. 6). The product which passed through the filters had a distinctly lower average molecular size after treatment with active β-galactosidase compared to CSP treated with heat-denatured β-galactosidase. Monosaccharide standards eluted from the HPLC columns at a retention time of 49 to 51 min (data not shown). Alditol acetate derivatives of monosaccharides in the CSP fractions eluting at that retention time were analyzed by GC/EIMS-SIM. Results revealed that treatment of CSP with β-galactosidase resulted in the release of galactose. After a 24 h incubation with β-galactosidase, approximately 50 μg of monomeric galactose was recovered in the filtrate from a starting material of 30 mg CSP. Galactose was the only monosaccharide released from CSP after β-galactosidase treatment. No galactose was recovered from the CSP fraction treated with heat-denatured β-galactosidase.

Discussion

The role of β-galactosidase in cell wall degradation during softening has been uncertain. The results of Gross and Wallner (1979) indicated that β-galactosidase was not responsible for the decline in the galactose content of tomato fruit cell wall during ripening since a tomato β-galactosidase did not degrade a tomato galactan. It was suggested that the loss of cell wall galactan was due to turnover and insertion of galactan-poor polymers in the cell wall during ripening (Knee 1978, Knee et al. 1977, Lackey et al. 1980).

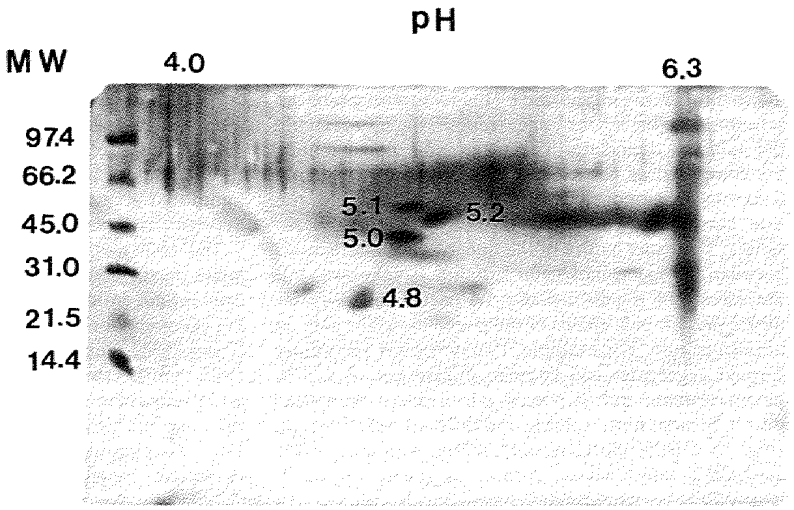

Fig. 4. Photograph of silver-stained two dimensional gel of peak 2 proteins from hydroxyapatite FPLC. IEF, in the pH range 4 to 6.3, was the first direction and SDS-PAGE the second. Molecular weight markers are on the left. Those enzymes which had pectolytic activity are identified by their pI values.

Pressey (1983) identified 3 β-galactosidases in tomato fruit. Two of the 3 enzymes were unable to hydrolyze cell wall galactans in vitro and their activities declined during ripening. However, the third β-galactosidase did degrade cell wall galactan and its activity increased during ripening. Furthermore, Seymour et al. (1990) have attributed the loss of tomato wall galactosyl residues during ripening to the cleavage of $\beta(1\rightarrow4)$-linkages. This is consistent with a β-galactosidase being responsible for the net loss of cell wall galactosyl residues.

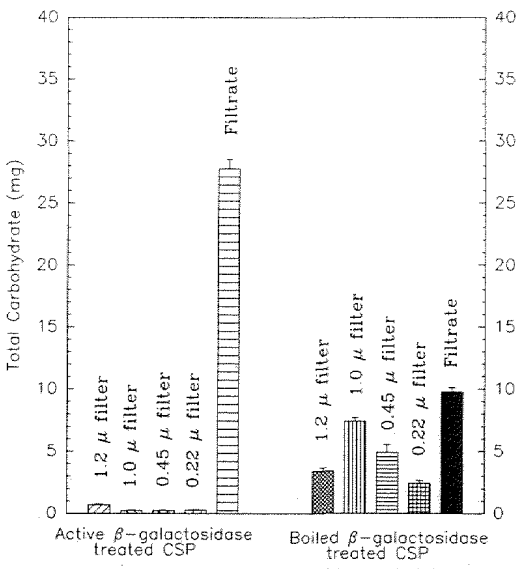

Fig. 5. Effect of avocado β-galactosidase, pI 5.0, on the amount of pectin retained on a series of filters having decreasing pore size, i.e., 1.2, 1.0, 0.45, and 0.22 μm.

We have isolated 3 β-galactosidases from ripe avocado fruit. All three degrade and solubilize pectin, unlike the 3 β-galactosidases isolated from tomato fruit. The avocado β-galactosidases have similar pIs, ranging from 5.0 to 5.2. These values are lower than those reported for tomato β-galactosidases, which ranged from 6.7 to 7.8 (Pressey 1983). The avocado β-galactosidases also have lower molecular weights than those of tomato. The avocado isozymes molecular weights ranged from 41 to 54 kDa whereas the tomato fruit β-galactosidases ranged from 62 to 144 kDa (Pressey 1983). The strong affinity of the 3 avocado β-galactosidases for alginate and their insolubility at low ionic strength (Pressey 1983) suggests that they are wall-associated enzymes.

One of the current theories on the structure of pectin

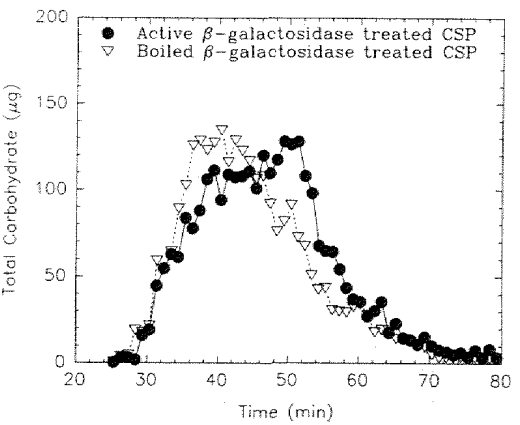

Fig. 6. Gel permeation HPLC profile of the pectin product after treatment with active and boiled avocado β-galactosidase, pI 5.0.

is that it is composed of discrete macromolecular subunits (Fishman et al. 1989). These macromolecular subunits form large aggregates held together by noncovalent interactions with the strength of the noncovalent interactions being influenced by the ionic strength of the solution. The higher the ionic strength, the more readily the subunits dissociate. The macromolecular subunits are composed of polysaccharide side chains covalently linked to a rhamnogalacturonan backbone. Some of the side chains are composed of $\beta(1\rightarrow4)$-galactosyl residues. The exact nature of the noncovalent interactions between the macromolecular subunits is unknown, but the effects of β-galactosidase on pectin solubility indicate that the side chains of the pectin molecule play an important role. For example, tomato pectin apparently formed aggregates too large to diffuse out of a 10% polyacrylamide gel. Upon β-galactosidase treatment pectin became more soluble and diffused out of the gel (Fig. 3). Furthermore, pectin, treated with an inactive avocado β-galactosidase, formed aggregates in which nearly 70% were too large to pass through a filter with a pore size of 0.22 μm (Fig. 5). Upon a 24 h incubation with an active β-galactosidase, pectin molecules were no longer able to form these large aggregates; pectin molecules were sufficiently degraded to allow 97% to pass through a 0.22 μm filter. When 30 mg of pectin was treated with β-galactosidase for 24 h only 50 μg of released galactose was recovered, a loss from pectin of less than 0.2% of the total carbohydrate. This loss was apparently large enough to significantly disrupt the noncovalent forces holding pectin aggregates together in 50 m*M* NaCl.

It should be noted that the galactosyl content of pectin is greater than 10% of total pectin carbohydrate (Gross and Wallner 1979). There are several explanations for the failure of avocado β-galactosidase to remove more of the galactose in 24 h. It is possible that not all of the galactose was available as a substrate for β-galactosidase. There are several reasons for this to be the case. For example, the β-galactosidases isolated from avocado in our study were apparently specific for $\beta(1\rightarrow4)$-galactosyl linkages. These β-galactosidases would presumably remove terminal galactosyl residues sequentially from the side chains until confronted with a residue or linkage they could not cleave. Any $\beta(1\rightarrow4)$-linked galactosyl residue internal to this glycosyl residue would be unavailable to the enzyme. These glycosyl residues could potentially be galactose in $\beta(1\rightarrow6)$-linkages or arabinosyl residues (O'Neill et al. 1990). $\beta(1\rightarrow6)$-galactosyl residues are minor components of pectin (O'Neill et al. 1990). However, arabinosyl residues are present in significant amounts (O'Neill et al. 1990, Gross 1984). It should be noted that *p*-nitrophenyl-α-L-arabinosidase(s) was detected in our crude avocado enzyme preparation (unpublished data). We did not determine the role this enzyme may play in pectin degradation, however, it could assist β-galactosidase in removal of the side chains. It is also possible that the CDTA used to extract CSP from fruit may directly or indirectly inhibit or block β-galactosidase activity in certain regions of the pectin polymer. It is known that CDTA will bind to pectin in significant amounts. Mort et al. (1991) reported that as much as 50% of the dry weight of CSP was actually CDTA that was not removed during dialysis of CSP. We did not directly measure the CDTA content of our CSP preparation. However, we found that about 40% of its dry weight was not carbohydrate (data not shown).

The ability of β-galactosidase to degrade and solubilize pectin could partially explain how fleshy fruits may soften, at least to some extent, in the absence of PG (Giovannoni et al. 1989, Grierson and Tucker 1983, Gross 1984, Huber 1984, Knee 1978, Seymour et al. 1987, Smith et al. 1988, Tucker and Grierson 1982). β-Galactosidase is an enzyme that appears early in fruit ripening and prior to the appearance of PG (Pressey 1983, Watkins et al. 1988, Wegrzyn and MacRae 1992). A second galactose-containing polysaccharide, associated with the cellulosic fraction, also exhibits a decrease in galactosyl content during fruit ripening (Gross 1984). It is possible that one or more of the β-galactosidases active against pectin is also involved in the degradation of this galactose-containing polymer.

The involvement of the purported avocado α-galactosidase in pectin degradation and/or fruit softening is unclear. Galactosyl residues in $\alpha(1\rightarrow4)$-linkages are known to exist in rhamnogalacturonan II (O'Neill et al. 1990). However, this pectic polysaccaride is only a minor component of the cell wall and is thought not to play a structural role (O'Neill et al. 1990). The ability of the putative avocado α-galactosidase to degrade pectin in vitro suggests that either $\alpha(1\rightarrow4)$-galactosyl residues exist in other cell wall polysaccharides, or that this enzyme, which was assayed using the artificial substrate *p*-nitrophenyl-α-D-galactopyranoside, has a different activity in vivo.

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