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# A Pumpkin Cultivar Selected for Early Harvest Exhibits Decreased Pectin Hydrolase Activity

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## ABSTRACT

Ripening-associated changes in primary cell wall have been attributed to the activities of pectin hydrolases in several fruits, including tomato, melon, kiwifruit, starfruit, and raspberry. Using a sensitive method to quantify reducing sugars, we have examined the changes in polysaccharide-degrading enzyme activities and reducing sugar content of two pumpkin (*Cucurbita moschata*) cultivars, designated cultivar A and cultivar B, harvested at different times in the growing season. Pectin hydrolase activity in the cultivar B pumpkins harvested in October (1075.4 U/ g pumpkin) was elevated at least five-fold relative to activity in cultivar A pumpkins harvested in late August through September (89.1-359.4 U/ g pumpkin). The elevated pectin hydrolase activity did not correlate with the changes in levels of reducing sugars over the same harvest season. A decrease in the reducing sugar levels in raw pumpkin was observed throughout the harvest season with an approximately 50 % decrease between each harvest period. This change in the levels of reducing sugars was paralleled by the expression of α-amylase activity in raw pumpkin. This starch-hydrolyzing enzyme decreased from 705.9 U/ g pumpkin in cultivar A fruit harvested between late August and early September to 39.8 U/ g pumpkin in cultivar B fruit harvested in October. These results suggest that an increase in pectin hydrolase activity may not be a primary determinant in the ripening of pumpkin, as ripe pumpkins from two different cultivars have markedly different levels of pectinase activity at harvest.

## INTRODUCTION

The textural changes occurring during fruit ripening are attributed to the disassembly of the primary cell wall (Brady, 1987). Modifications of both covalent and noncovalent interactions between polysaccharides may play a role in the softening process (Fischer and Bennett, 1991). The ripening of many fruits, including starfruit (Chin et al., 1999), tomato (Crookes and Grierson, 1983), melon (Hadfield et al., 1998), raspberry (Iannetta

et al., 1999), and kiwifruit (Redgewell et al., 1992) has been correlated with the activities of endogenous cell wall-modifying enzymes. Among the enzymes implicated in ripening-associated process are the pectin-degrading enzymes that catalyze the hydrolytic depolymerization of polygalacturonide or pectin, a major component of the primary cell wall. Polygalacturonase (E.C. 3.2.1.15), pectin methylesterase (E.C. 3.1.1.11), and pectin lyase (E.C. 4.2.2.10) are pectinase isoforms which may independently or collectively contribute to the degradation of cell wall pectins.

Numerous studies have ascribed a role for polygalacturonase in early ripening events in tomato (Crookes and Grierson, 1983;Tigchelaar et al., 1978), melon (Hadfield et al., 1998), and raspberry (Iannetta et al., 1999), however genetic studies have demonstrated the expression of polygalacturonase activity to be insufficient in triggering the physical and chemical changes associated with fruit-ripening in tomato (Giovannoni et al., 1989; Smith et al., 1988; Smith et al., 1990). Furthermore, no correlation between polygalacturonase activity and decreased fruit firmness was observed during the maturation of banana fruit (Agravante et al., 1991). Other studies strongly intimate that enzyme-mediated pectin degradation is important in the later stages of ripening when deterioration of the fruit occurs (Chin et al., 1999; Kramer et al., 1992).

In contrast to tomato and kiwi fruit, pumpkin does not undergo extensive softening until the fruit is overripe. This study investigates the level of pectin hydrolase activity in mature pumpkin (*Cucurbita moschata*) to determine whether it is a primary determinant in the ripening of pumpkin. Two cultivars of *C. moschata*, one of which has been selected for its early maturing fruit which allows harvest before frost affects the crop, were analyzed. For each harvest time, raw fruit and canned pumpkin puree were analyzed for enzyme activities and concentration of reducing sugars.

## MATERIALS AND METHODS

### Materials

2,2'-bicinchoninic acid (BCA) was purchased from Fluka Chemical Corporation (Milwaukee, WI). All other chemical and biochemical reagents were supplied by Sigma (St. Louis, MO). Raw and canned pumpkin samples were generously donated by Nestle Corporation (Morton, IL). Raw pumpkin was obtained from field-grown crops in Morton. Canned pumpkin puree was also obtained from pumpkins grown and processed in Morton. During the canning process, the pumpkin was chopped into 4 to 8 inch pieces and wilted at 200 ºF to soften the flesh. The skin and seeds were removed and the pulp was ground in a mill and canned. Cultivar A, selected for its early maturing fruit, was harvested early in the season, between late August and early September (8/20-9/1), and during mid-season harvest, between middle to late September (9/12-9/20). Cultivar B was harvested between early to mid-October (10/15-10/20). In each harvest, the raw fruit and canned product were analyzed for carbohydrate content, and amylase and pectin hydrolase activities. The samples were pulverized with a hand held food processor for 5 min, resuspended in 0.4 M sodium carbonate buffer, pH 9.8, at 0.5 g fresh weight/ mL and diluted as indicated for analysis. Samples were kept on ice during experiments and stored at –20 ºC.

**Reducing sugar determination**

The relative concentrations of reducing and total carbohydrates were determined by the quantitative reduction of copper II (Waffenschmidt and Jaenicke, 1987). Pumpkin samples (0.25 mg fresh weight), in a final volume of 0.20 mL, were incubated in the presence of 1.38 mM copper (II) sulfate, 1.15 mM BCA, 3.0 mM L-serine, and 0.4 M sodium carbonate buffer, pH 9.8. Incubations were carried out at 100 ºC for 15 min on Costar 96 flat-bottom well plates (Fisher Scientific, Pittsburgh, PA) sealed with adhesive film and jacketed with a copper microplate heating aid. Samples were allowed to cool for 20 min prior to reading the optical density at 570 nm on a BioTek EL311 microplate autoreader (BioTek Instruments, Winooski, VT). The concentration of reducing sugar in each sample was determined by interpolation of a standard glucose (0 to 20 nmol) curve.

**Enzyme assays**

Pectinase and α-amylase activities were measured by spectrophotometric determination of the concentration of reducing sugars released when the appropriate polysaccharide substrate, amylose or pectin, was incubated with each sample (Mateos et al., 1992). Briefly, reaction mixtures (50 $\mu$L) containing 0.02 to 1.67 $\mu$g fresh weight pumpkin sample and 62.5 $\mu$g polysaccharide substrate were incubated for 30 min under the appropriate conditions for each enzyme activity. α-amylase activity was assayed in 0.1 M HEPES buffer, pH 7, at 27 ºC . Pectin hydrolase activity was assayed in 0.1 M sodium acetate buffer, pH 5 at 37 ºC . Activity units (U) are defined as $\mu$mol of glucose equivalents released per min.

**Statistical Analysis**

Data were subjected to analysis of variance (ANOVA) followed by Fisher's Least Significant Difference (LSD) post-test to determine the significance of differences between harvest means. Data are reported as the mean ± standard deviation (SD) of triplicate determinations for the ten samples collected in each harvest. The data were considered to be significantly different at $p < 0.05$.

## RESULTS

The pumpkin cultivar selected for early maturation of fruit and harvested between late August and late September (early and mid-season) exhibited relatively lower levels of pectin hydrolase activity than the pumpkin cultivar producing mature fruit in October (Figure 1). Pectin hydrolase activity in the late harvest pumpkins was 1075 U/g pumpkin and 717.1 U/ g pumpkin in raw and canned samples, respectively. Pumpkins harvested in early and mid-season showed no significant difference in pectin hydrolase activity per gram pumpkin. The temperature and pressure changes associated with the canning process had little to no effect on the relative pectin hydrolase activity in early and mid harvest (cultivar A) pumpkin, but resulted in a thirty percent decrease in the activity in the late harvest pumpkin (cultivar B) (Figure 1).

Next, we examined whether the increase in pectin hydrolase activity was accompanied by an increase in the reducing sugar content in pumpkins. The relative concentrations of reducing sugars in raw pumpkin as detected by the quantitative reduction of copper II decreased throughout the harvest season (Figure 2). An almost two-fold reduction in the concentration of reducing sugars in raw pumpkin is observed between each successive

harvest period, irrespective of cultivar differences. The canning process decreased the relative concentrations of reducing sugars in the early and mid-season harvest by approximately two and three-fold, respectively. There was no significant difference in the level of reducing sugars between raw and canned pumpkin harvested in October (late harvest). The relative concentrations of reducing sugars in canned pumpkin also decreased approximately two to three fold between early and mid-season harvest (Figure 2).

This decrease in the level of reducing sugars was inconsistent with the observed increase in pectinolytic activity and reports that pectinic degradation occurs at late growth stages in melon, raspberry, and tomato (Rose et al., 1998; Iannetta et al., 1999; Redgewell et al., 1992). In an effort to explicate the changes in the relative levels of reducing sugars in pumpkins harvested during different periods, the samples were analyzed for other polysaccharide hydrolase activities, including α-amylase (Figure 3). Raw pumpkin harvested early contained the highest levels of α-amylase activity (705.9 ± 117.1 U/g pumpkin) detected in this study. In raw pumpkin, α- amylase activity (Figure 3) decreased throughout the harvest season. Canned pumpkin showed variability in α-amylase activity throughout the harvest season. In mid-season and late harvest, α-amylase activities in raw and canned pumpkin were not significantly different. Activity in pumpkins of the early harvest decreased five-fold during the canning process to 141.2 U/g canned pumpkin.

## DISCUSSION

Mature fruit of the pumpkin cultivar selected for early ripening shows an approximately ten-fold lower activity of pectin hydrolase, an enzyme which is thought to play a critical role in cell wall disassembly during the ripening process, than pumpkin with the traditional-length growing season. The cultivar-associated increase in pectin hydrolase activity was not accompanied by a corresponding increase in reducing sugar content. Interestingly, a decrease in reducing sugar levels in raw pumpkin was observed throughout the harvest season with an approximately 50 % decrease between each harvest period. This change in the levels of reducing sugars was paralleled by the expression of α-amylase activity in raw pumpkin. This starch-hydrolyzing enzyme decreased from 705.9 U/ g pumpkin in fruit harvested between late August and early September to 39.8 U/ g pumpkin in fruit harvested in October. Cultivar-associated differences in the sensitivity of the polysaccharide hydrolyzing enzyme activities monitored in this study to the conditions of the canning process were observed. The conditions employed in the canning decreased the pectin hydrolase activity in late harvest pumpkins (cultivar B) by thirty percent relative to raw pumpkins from the same harvest and also decreased the α–amylase activity in the early harvest pumpkins (cultivar A) by eighty percent relative to raw pumpkins from the same harvest. This decrease in enzymatic activity in response to treatment at high temperatures is not surprising as most enzymes exhibit temperature-sensitivity due to the disruption of noncovalent interactions important for maintaining an active protein conformation.

The results of this study suggest that an increase in pectin hydrolase activity may not be a primary determinant in the ripening of pumpkin, as ripe pumpkins from two different cultivars demonstrate markedly different levels of pectinase activity at harvest. It has been demonstrated that the timing of expression of the relevant gene or gene families

may vary with species or cultivars (Crookes and Grierson, 1983; Hadfield et al., 1998; Iannetta et al., 1999). We show that the expression of pectin hydrolase activity is higher in mature pumpkins of cultivar B relative to mature pumpkins of cultivar A. In addition, pectin hydrolase activity shows little to no change between the early-season and mid-season harvested pumpkins of the early-ripening cultivar (cultivar A).

Several models have been proposed for the initiation of fruit ripening in which pectin hydrolase activity is not required. Redgewell et al. (1992) have demonstrated that ethylene produced in the initial stages of kiwifruit ripening is shortly followed by an increased swelling of cell wall materials without changing the primary structure of the polysaccharides. It is proposed that swelling increases the solubility of cell wall polysaccharides by allowing more flexibility in non-covalent associations between polymers and solvent than provided in the compact cell wall structure of unripe kiwifruit. In this regard, ripening-specific expansins, proteins thought to interrupt non-covalent interactions between cell wall and matrix polysaccharides, have been identified in tomato, strawberry, and melon (Rose et al., 1997) and putatively play a role in disrupting a critical structural component of the cell wall at the onset of fruit softening. Additionally, swelling may facilitate access of cell wall-degrading enzymes to their substrates (Redgewell and Fry, 1993). Nonetheless, these results suggest that increased pectin hydrolase activity is a consequence, not a cause, of cell wall disassembly.

The disassembly of pectin is thought to initially involve the loss of covalently-associated galactose residues (Rose et al., 1998, Chin et al., 1999). Galactose is among the neutral sugars in the side chains linking the polymer to the polysaccharide matrix in plant cell walls. The loss of neutral sugar residues results in the increased solubility of the polysaccharide (Rose et al., 1998; Chin et al., 1999) which is believed to facilitate the pectinase-mediated deglycosylation and depolymerization in late ripening stages (Rose et al., 1998; Hadfield et al., 1998). In this regard, Ptitchkina et al. (1994) have estimated that pumpkin pectin contains only 50 % galacturonate, suggesting an unusually high concentration of neutral sugar residues relative to citrus pectins which contain approximately 80 % galacturonate.

We did not detect a concomitant increase in free galactose by monitoring the relative concentration of reducing sugars in the two pumpkin cultivars. In fact, a decrease in the relative reducing sugar concentration is observed with each successive harvest period. However, the method employed is not specific for galactose and would be influenced by the release of any sugar capable of undergoing oxidation. Interestingly, we detected a decrease in $\alpha$-amylase activity that paralleled the changes in relative reducing sugar concentration and may provide a plausible explanation for the higher levels of reducing sugar observed in the pumpkins of the earlier harvests. Therefore, we cannot conclusively eliminate the possibility that galactose release occurred over the harvest season but was obscured by $\alpha$-amylase-released glucose from starch.

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Figure 1. Pectin hydrolase activity in raw and canned pumpkin as a function of harvest. Data are the mean ± S.D. for 10 samples measured in triplicate. Bars labeled with a single asterisk(*) were not significantly different from each other ($p > 0.05$).

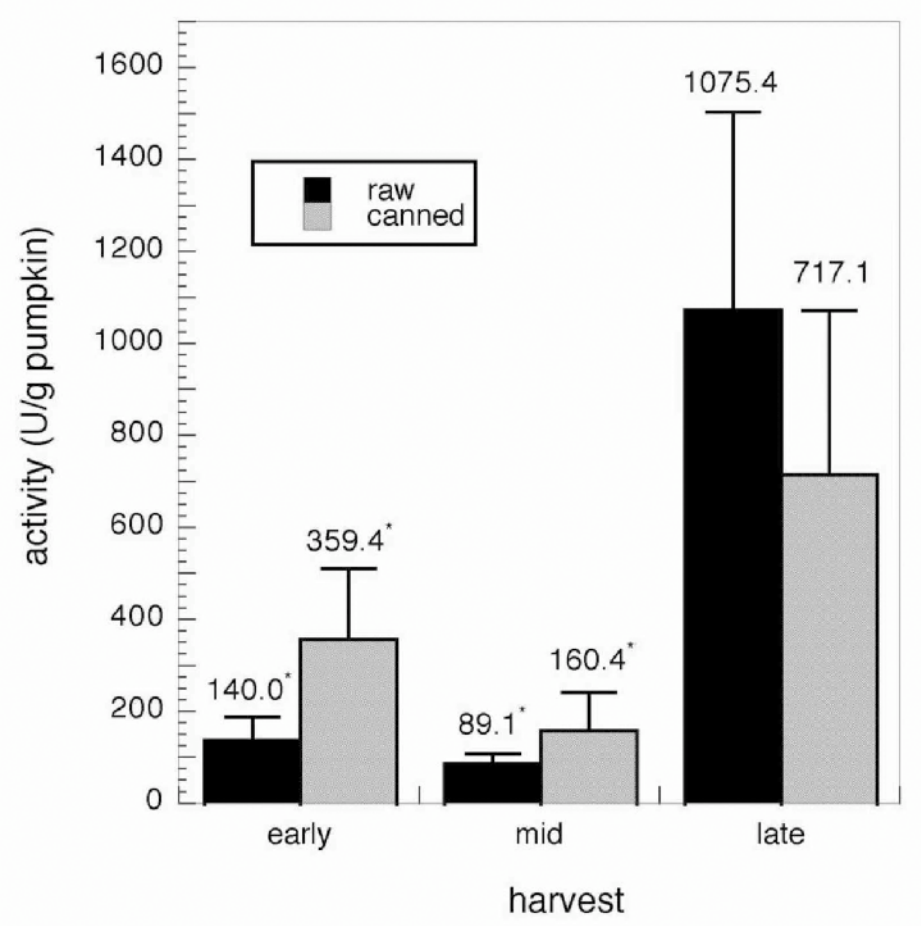

Figure 2. Reducing sugar content of raw and canned pumpkin as a function of harvest. Data are the mean ± S.D. for 10 samples measured in triplicate. Bars labeled with a single asterisk (*) were not significantly different (p = 0.322).

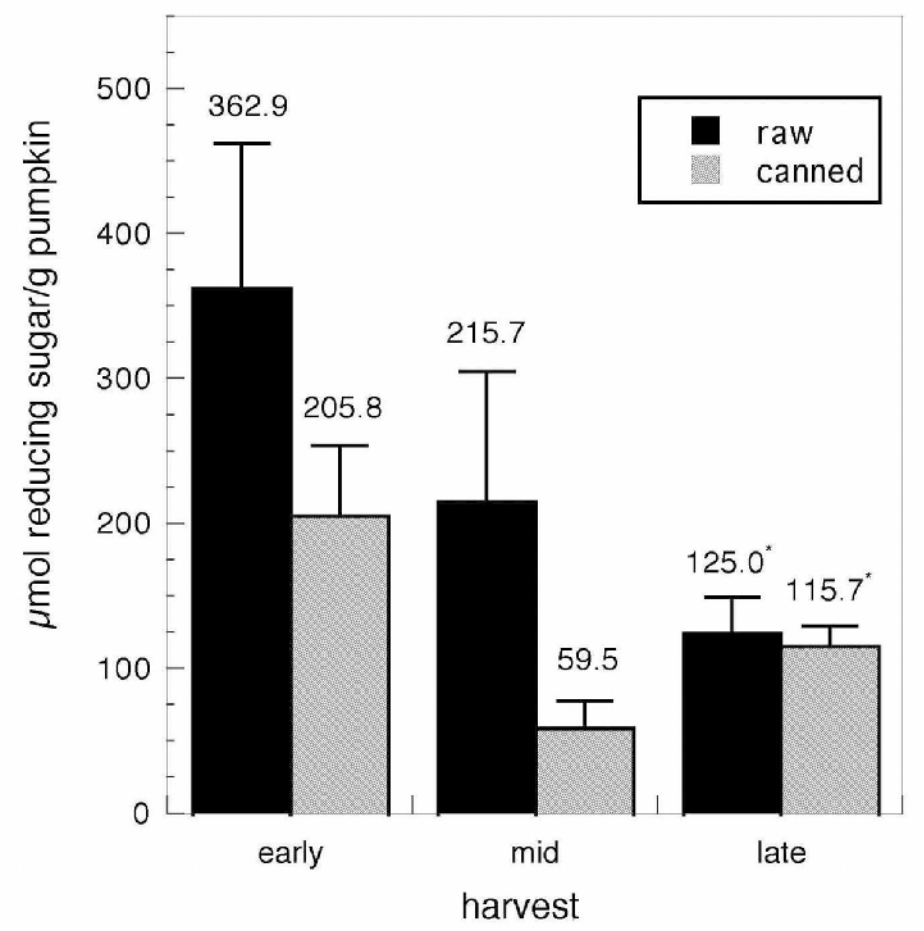

Figure 3. α-amylase activity in raw and canned pumpkin as a function of harvest. Data are the mean ± S.D. for 10 samples measured in triplicate. Bars labeled with a single asterisk (*) were not significantly different (p = 0.681). Bars labeled with a double asterisk (**) were not significantly different (p = 0.194).