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Cell Wall Degrading Enzyme Activity in Ripening Red Raspberry Fruit

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ABSTRACT

Activities of the cell wall degrading enzymes cellulase, polygalacturonase, and ß-galactosidase were determined on green, mottled, and red ripe 'Red Wing' raspberry (*Rubus idaeus*) fruit during the months of July, August, and September 1993. The enzyme activity, measured as µmoles of released product·g^{-1} of fruit·h^{-1} indicated the presence of polygalacturonase, cellulase, and ß-galactosidase in raspberry fruit. Enzymatic activities were highest in August and September for the three enzymes. In August maximum polygalacturonase activity was at the turning (mottled) stage of ripening (27 µmoles galacturonic acid·g^{-1}·h^{-1}), and maximum ß-galactosidase activity was measured in green fruit (6 µmoles of p-nitrophenol·g^{-1}·h^{-1}). Cellulase also showed the highest activity during the mottled stage in August and September (3 µmoles of glucose·g^{-1}·h^{-1}). Cellulase showed a pattern of increasing activity through time. In red raspberry cellulase, polygalacturonase, and ß-galactosidase appear to be involved in fruit softening during green to the mottled stages, but activity decreased in red ripe fruit.

RESUMEN

Se determinó la actividad de las enzimas degradadoras de la pared celular, celulasa, poligalacturonasa, y ß-galactosidasa, en frutos en estadío verde, moteado y rojo maduro de frambuesa (*Rubus idaeus*) cultivar 'Red Wing' durante los meses de julio, agosto y septiembre en 1993. La actividad enzimática, medida como µmoles del producto liberado·g^{-1} de fruto·h^{-1} indicaron la presencia de poligalacturonasa, celulasa, y ß-galactosidasa en los frutos de frambuesa. La actividad de las tres enzimas fue mayor en agosto y septiembre. En agosto, la máxima actividad de la poligalacturonasa fue durante el estadío cambiante (moteado) de maduración (27 µmoles de ácido galacturónico·g^{-1}·h^{-1}), y la máxima actividad de la ß-galactosidasa se midió en el fruto verde (6 µmoles de p-nitrofenol·g^{-1}·h^{-1}). La celulasa también mostró la mayor actividad durante el estadío moteado en agosto y en septiembre (3 µmoles de glucosa·g^{-1}·h^{-1}). La celulasa mostró un patrón de actividad creciente a través del tiempo. En la frambuesa en estadío rojo, la celulasa, la poligalacturonasa y la ß-galactosidasa parecieron estar involucradas en el ablandamiento del fruto durante los estadíos verde a moteado, pero la actividad disminuyó en el fruto rojo maduro.

Raspberries are a specialty crop with highest value as a fresh fruit for local and regional markets. Successful marketing involves postharvest practices to maintain freshness for as long as possible. Raspberries are highly perishable (3 day shelflife at 0 C), due to their high respiration rate (Kader, 1992) and fragile fruit structure (Jennings, 1988). These characteristics contribute to fruit softening and decay during storage, reducing product quality and marketability.

Polygalacturonase (PG), cellulase, ß-galactosidase, and pectin methyl esterase (PME) hydrolyze cell walls (Fischer and Bennett, 1991). These cell wall softening enzymes degrade the pectin fraction in cell walls, through intermediary steps, to glucose and galactose (Fischer and Bennett, 1991). Cell wall softening enzymes differ among fruit. PG, cellulase, and ß-galactosidase are found in tomatoes, apples, and avocadoes (Fischer and Bennett, 1991). PME has been found in strawberries (Barnes and Patchett, 1976) and cellulase in blackberries. In blackberry (Rubus spp), cellulase was reported to be the primary cause of fruit softening during ripening (Abeles and Takeda, 1989). Unlike other fruit, xylose is released as raspberry fruit softens (Gross and Sams, 1984).

Our objective in this study was to quantify cell wall degrading enzyme activity in ripening red raspberry fruit and to define their presence and possible involvement in fruit softening during ripening.

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MATERIALS AND METHODS

Fresh red raspberry fruit of the cultivar 'Red Wing' were harvested at sunrise from a commercial field in the Mesilla Valley in southern New Mexico. Fruit representing the maturity stages of green, mottled, and red ripe were harvested on 19 July, 6 August, and 3 September 1993. Green fruit were of small size, whitish-green color, with no signs of pink color. Mottled fruit had attained almost maximum size, and had a mixture of white, pink and red colors. Red ripe (commercially ripe) fruit were firm, fully red, and easily detached from the receptacle. The receptacle was not retained. Fruit were frozen at -20 °C within 2 h of harvest.

Each experimental unit consisted of 40 to 50 berries. For analysis, fruit were thawed at room temperature and homogenized with di water. Amount of water used was based on fruit consistency. July harvested, green and mottled fruit were diluted 1:3 (wt:vol), and mature red fruit were diluted 1:2. August- and September-harvested green and mottled fruit were diluted 1:4, and mature red fruit was diluted 1:3. After homogenization, the mixture was centrifuged at 900 x g for ten minutes.

The cell wall degrading enzymes, cellulase, polygalacturonase, and ß-galactosidase, were assayed for each sample. For cellulase, samples consisted of 100 µL of supernatant plus 900 µL of substrate (2% low viscosity carboxymethyl-cellulose (CMC) sodium salt in 0.05 M acetate buffer, pH 4.5). A 100 µL (5.1 units) 1% cellulase solution (Sigma, Saint Louis, MO. Cellulase [EC 3.2.1.4 from *Aspergillus niger*]) plus 900 µl of 2% CMC substrate was used as a standard. The enzyme-substrate treatments were incubated for 24 h at 37 °C. Cellulase activity was not detectable when enzyme-substrate solution was measured at an earlier time. The reaction was stopped with 1 mL dinitrosalicylic acid (DNS) reagent (2.5 g DNS, and 15 g Na-K tartrate in 2 N NaOH) (Miller, 1959). The mixtures were then heated for 5 min at 100°C. Deionized water (4 mL) was added to the reaction tubes before measuring the absorbance of the samples with a spectrophotometer (Sequoia-Turner, Model 690, Chicago, Ill) set at 490 nm.

For polygalacturonase, 100 µL of supernatant was added to 900 µL of substrate (1% polygalacturonic acid in 0.05 M acetate buffer, pH 4.5). A standard solution with 25 µL (3.0 units) of pectinase (Sigma,{EC 3.2.1.15., from *Aspergillus niger*}), plus 75 µL H_2O and 900 µL of 1% polygalacturonic acid substrate was utilized to compare units of activity. Reducing sugars were measured as previously described.

ß-galactosidase activity in fruit homogenate samples was measured by mixing 100 µl of supernatant with 900 µl of substrate (0.05 M acetate buffer, pH 4.5, 0.015 M NaCl, 0.06% bovine serum albumin (BSA), and 1% p-nitrophenyl-ß-D-galactopyranoside). The controls were 100 mL water plus 900 mL of substrate, and 25 mL (3.0 units) of pectinase, combined with 75 µL H_2O and 900 mL of substrate. These treatments were incubated for 1 h at 37 °C, and the reaction was stopped with 1 mL of 0.2 M Na_2CO_3. Four mL of di water were added before measuring absorbance at 400 nm to determine the p-nitrophenol groups released and activity expressed as µmol·g fresh weight^{-1}·h^{-1}.

Each treatment was replicated three times per experiment and three experiments were conducted for each enzyme tested. A split plot design was used with sampling date (month) as the main plot, and maturity stages (green, mottled, and mature red fruit) as the subplots. The experiment was analyzed using analysis of variance (ANOVA) for each enzyme. Mean separations were determined using Fishers $LSD_{.05}$.

RESULTS AND DISCUSSION

Cellulase activity depended on sampling date and ripeness stage (Fig. 1). Mottled fruit had more cellulase activity than green or red fruit. In all but September, red fruit had more cellulase activity than green fruit. Fruit harvested in July had less enzyme activity than those harvested in August or September. Ethylene increases in red raspberry (Burdon and Sexton, 1990), paralleling cellulase activity. Ethylene may trigger *de novo* synthesis of raspberry cellulase activity during ripening as has been shown for avocado (Burdon and Sexton, 1990).

Differences in cellulase activity among sampling dates may be due to temperature or amounts of available water. Dopico et al. (1993) found that avocadoes stored at 7 °C had delayed softening and mRNAs for cellulase and PG were inhibited. In our study, mean temperatures in the harvest location were 35.5, 34.1, and 31.5 °C in July, August and September, respectively. Temperatures for maximal cellulase activity may have been exceeded in July, resulting in reduced activity. Additionally, the 0.5 cm of rainfall (91% of rainfall between July and September) may have diluted cellulase concentrations in July-harvested raspberries. This effect may have been greater in riper fruit, resulting in red fruit harvested in July having less cellulase activity than red fruit from later harvests.

Increased cellulase activity in mottled fruit relative to green or red fruit may be due to changes associated with cessation of growth and the onset of maturation. Different cellulase isozyme forms may be appearing with ripening raspberries (Abeles and Biles, 1991; Fischer and Bennett, 1991). The type of isozyme present at the time of assay influences cellulase activity (Pressey, 1977).

PG was affected by ripeness stage and harvest date (Fig. 2). The highest PG activity was observed in the mottled stage during the months of August and September. In all months but July, PG activity was higher in red than in green fruit. In July, PG activity in green fruit was similar to mottled and higher than red fruit. This differs from what has been observed in other fruits (tomatoes and apples) where PG activity increased with ripening (Wallner and Walker, 1975; Fischer and Bennett, 1991). High temperature has been shown to suppress PG mRNAs in tomato (Picton and Grierson, 1988). The high temperature in July as recorded in this study, may have suppressed PG mRNA in raspberry.

ß-galactosidase (BG) activity differed among harvest dates and ripeness stages (Fig. 1C). For all harvest dates, BG activity decreased as raspberries matured from the green to the mottled stage. In July, BG activity was higher at the red than mottled stage, but not different at these stages in August and

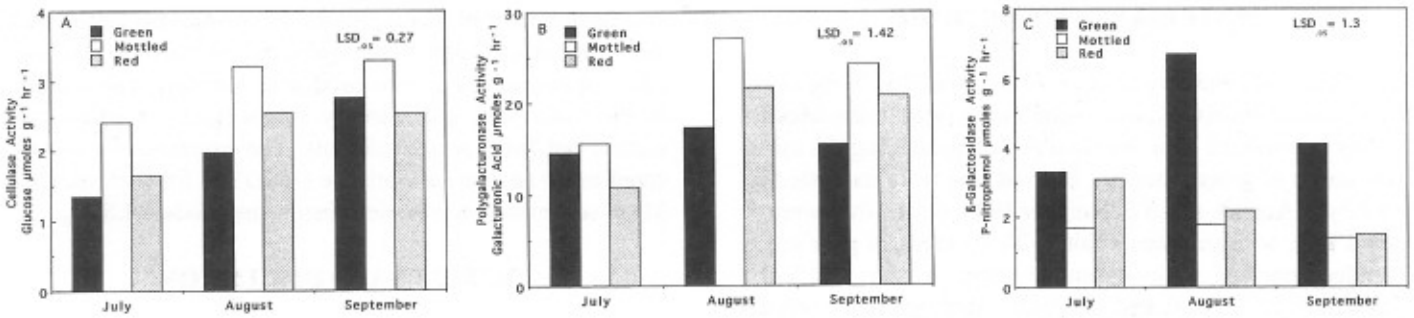

Fig. 1. Cell wall degrading enzyme activity of ripening raspberry. A) Cellulase activity in ripening raspberry fruit. B) Polygalacturonase (PG) activity in ripening red raspberry fruit. C) ß-galactosidase (BG) activity in ripening red raspberry fruit. Values represent three replicates, each consisting of an extract made from 40-50 berries. Mean separations were determined using Fishers $LSD_{0.05}$.

September. These results are similar to reports where higher BG activity was observed in green tomato fruit (Wallner and Walker, 1975). Other reports, however, indicate more activity during the mottled and mature red stages in other fruits. For instance, BG activity increases with maturity in apples (Bartley, 1974) and in peppers (Biles et al., 1993). Pressey (1983) found total BG activity was high in tomatoes while only one BG isozyme increased during ripening.

In raspberries, the most common sugar released during cell wall breakdown was xylose (Gross and Sams, 1984). Wallner and Walker (1975) hypothesized that BG released side-chain neutral sugar residues like galactose, that initiated color change in tomato. Later, Gross (1985) and Kim et al. (1987) found that galactose can promote ethylene formation in tomatoes. Unlike galactose, xylose did not promote ethylene formation (Gross, 1985; Kim et al., 1987). However, some galactose residues may be produced by BG in raspberry that stimulate some other ripening mechanisms.

Activity of cell wall degrading enzymes in maturing raspberry fruit differed from that of ripening tomatoes (Wallner and Walker, 1975), apples (Fisher and Bennett, 1991), and peppers (Biles et al., 1993). The enzyme activity patterns found in raspberry fruit suggest that BG starts cell wall changes at the green stage by releasing galactose side-chain residues. After these changes, cellulase and PG became more active in the mottled stage, and declined in the red fruit.

In other fruits, such as apple and tomato, PG activity is thought to influence fruit softening more than cellulase (Gross, 1990; Fisher and Bennett, 1991). Interpretation of our data indicates that PG and cellulase have similar activity levels in red raspberries. Both enzymes appear important in raspberry fruit softening, with activity peaking in mottled fruit. In mottled raspberries, PG and cellulase may loosen cell walls for growth and cell expansion (Labavitch, 1975). Softening of red raspberries may be continued because of increased cell membrane permeability (Gross, 1990) and/or ethylene (Guerrero-Prieto, 1994). These changes would lead to accelerated loss of membrane integrity, stimulated cell wall enzyme activity, and ultimately to very soft fruit. The increased PG and cellulase activities in raspberry fruit coincide with color change, indicating a possible role for these enzymes in raspberry softening.

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