Partial Purification and Characterization of β-D-Galactosidase from Sweet Cherry, a Nonclimacteric Fruit†

Preston K. Andrews*

Department of Horticulture and Landscape Architecture, Washington State University, Pullman, Washington 99164-6414

Shulin Li

Department of Neuro-Oncology, Box 316, M. D. Anderson Cancer Center, University of Texas, 1515 Holcombe Boulevard, Houston, Texas 77030

β-D-Galactosidase (β-Gal, EC 3.2.1.23) was partially purified from sweet cherry (*Prunus avium* L. cv. Bing) fruit in four liquid chromatography steps, DEAE-Sephadex A-50, Sephadex G-75, and two Sephacryl S-200 columns. Partially purified β-Gal produced two protein bands with p*I* values of 4.2 and 4.5, based on native IEF electrophoresis. Both proteins showed high enzyme activity. β-Galase activity was severely inhibited by 1 mM Cu^{2+} in vitro but was stimulated by 1 mM GA_3, IAA, and Mg^{2+}. The K_m and V_{max} of β-Gal with *p*-nitrophenyl β-D-galactopyranoside as substrate were 1.25 mM and 5 $\mu mol \cdot mg^{-1} \cdot min^{-1}$, respectively. Maximum in situ β-Gal activity expressed on a specific protein basis occurred about 2 weeks prior to fruit maturity but at maturity when expressed on a fresh weight basis. Polyclonal antibodies made against the 57 kDa β-Gal reacted with four proteins from mature fruit. The estimated molecular masses of these proteins were 28, 43, 63, and 92 kDa. These results suggest that β-Gal may contribute to cell wall hydrolysis during sweet cherry fruit softening.

Keywords: *Antibody; cell wall; enzyme; Prunus avium, ripening*

INTRODUCTION

Fruit ripening is a coordinated series of biochemical and physical processes that result in synthesis and degradation of pigments, conversion of starch into sugars, changes in firmness, production of volatiles, and increased respiration in climacteric fruits (Speirs and Brady, 1991). Fruit firmness and texture are important attributes that define fruit quality and determine the fragility, storability, and shelf life of perishable commodities. While the mechanisms that regulate changes in firmness during ripening are not fully understood, the softening of fruit during ripening has been associated with alterations in the cell wall (Gross and Wallner, 1979; Mitcham et al., 1989; Greve and Labavitch, 1991) and middle lamella structure (Crookes and Grierson, 1983).

The hydrolysis of the pectin-rich middle lamella is catalyzed by polygalacturonase (PG, EC 3.2.1.15) and pectin methylesterase (PME, EC 3.1.1.11) (Huber, 1983; Fischer and Bennett, 1991). The loss of galactose from the hemicellulose of fruit cell walls may be catalyzed by galactanase (Pressey, 1983), or β-D-galactosidase (β-Gal, EC 3.2.1.23) (Bartley, 1974; Ranwala et al., 1992) but apparently not by PG (Wallner and Bloom, 1977).

β-Gal is a cell wall hydrolytic enzyme that has been reported in several developing fruits, including apple (Bartley, 1974; Wallner, 1978; Dick et al., 1990), tomato (Pressey, 1983), kiwi fruit (Wegrzyn and MacRae, 1992; Ross et al., 1993), muskmelon (Ranwala et al., 1992), apricot, plum, peach (Bouranis and Niavis, 1992), and avocado (De Veau et al., 1993). Its activity increased during the ripening and storage of several of these fruits (Wallner, 1978; Bouranis and Niavis, 1992; Ranwala et al., 1992). Multiple isoenzymes of β-Gal have been reported in tomato, muskmelon, avocado, and kiwi fruit (Pressey, 1983; Ranwala et al., 1992; De Veau et al., 1993; Ross et al., 1993).

All fruits in which β-Gal has been studied exhibit climacteric ripening behavior (Kays, 1991). The apparent absence of cell wall hydrolase activity in nonclimacteric fruits has led to the hypothesis that these fruits soften nonenzymatically (Huber, 1983). Purification of β-Gal has not been attempted from nonclimacteric fruits. This paper reports on the purification and characterization of β-Gal from sweet cherry (*Prunus avium* L.) fruit, a nonclimacteric fruit (Biale, 1960; Blanpied, 1972; Li et al., 1994).

MATERIALS AND METHODS

Plant Material. Sweet cherry (cv. Bing) fruits were collected from the R. B. Tukey Orchard at Washington State University in Pullman. Commercially mature, mahogany-colored, fruits were harvested in 1992. Fruits were immediately frozen and stored at −70 °C until extraction.

Purification. β-Gal was extracted by homogenizing 200 g of mature mesocarp tissue, previously thawed at 2 °C for 1 h, in cold water that included 1% (v/v) 2-mercaptoethanol and 5% (w/v) polyvinylpolypyrrolidone (PVPP) at a ratio of tissue to solution of 1:1 (w/v). The homogenate was centrifuged at 10000*g* for 20 min at 4 °C (Sorval Model RC-5 superspeed refrigerated centrifuge, GSA rotor, DuPont Instruments, Newtown, CT). The pellet was redissolved in 1 M NaCl that included 5 mM phenylmethanesulfonyl fluoride (PMSF). The pH was immediately adjusted to 6 with 1 N NaOH. After extraction for 16 h, the extraction solution was centrifuged at 20000*g* for 30 min at 4 °C (SS-34 rotor, Sorvall). The supernatant was collected and brought to 75% $(NH_4)_2SO_4$ saturation by the addition of solid $(NH_4)_2SO_4$ while stirring. After 20 h, the solution was centrifuged at 20000*g* for 20 min at 4 °C (SS-34 rotor). The pellet was dissolved in 20 mL of 40 mM Tris buffer (pH 8) and dialyzed against the same buffer

* Author to whom correspondence should be addressed [telephone (509) 335-3603; fax (509) 335-8690].

† H/LA Paper 93-11. Project 0937, College of Agriculture and Home Economics Research Center, Washington State University, Pullman.

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for 16 h in a dialysis bag with a MW cutoff of 1200−1400. The dialyzed enzyme extraction was centrifuged at 20000*g* to remove the precipitate.

Nineteen milliliters of centrifuged enzyme solution was applied to a 25 mm × 350 mm DEAE Sephadex A-50 (Pharmacia, Piscataway, NJ) packed column, which was preequilibrated with 40 mM Tris buffer (pH 8) that included 100 mM NaCl. The column was eluted with the Tris buffer (pH 8) that included 400 mM NaCl and 0.5% (v/v) 2-mercaptoethanol. The eluant was collected by an automatic fraction collector in 5 mL fractions (fraction collector 203, Pharmacia). β-Gal activity and protein purity of alternate fractions were determined spectrophotometrically at 410 nm (Hitachi Model 100−80 spectrophotometer, Mountain View, CA) and by SDS−PAGE, respectively, as described later. Six fractions with the highest activity were pooled and concentrated to 15 mL by vacuum freeze-drying (VirTis Co., Gardiner, NY). The concentrated solution was applied to a 25 mm × 300 mm Sephadex G-75 (Pharmacia) gel filtration column that was preequilibrated with 40 mM Tris buffer (pH 8) and eluted with 100 mM NaCl in the same Tris buffer. Forty 5 mL fractions were collected as previously described. Enzyme activity assays and verification of protein purity were performed.

Two additional 10 mm × 300 mm Sephacryl S-200 (Pharmacia) gel filtration column separations were performed following the same procedures as described for the G-75 gel filtration separation. A 2 mL fraction with peak activity was collected from the second Sephacryl S-200 elution and stored at −20 °C. All of the purification procedures were performed at 2 °C, and the flow rate for all elutions was 0.5 mL·min^{-1}.

Enzyme Activity Assay. β-Gal activity was assayed by detecting the product *p*-nitrophenol following hydrolysis of the substrate, *p*-nitrophenyl β-D-galactopyranoside. The reaction mixture consisted of 500 μL of 5 mM substrate in 40 mM sodium acetate buffer (pH 5.0) and 20−50 μL of enzyme solution. The reaction was allowed to proceed for 5−30 min at 37 °C and was terminated by adding 1.5 mL of 10 mM $Na_2B_4O_7$ (pH 9.2). The differences in enzyme solution volume and reaction time were due to differences in the activity of each sample. The concentration of liberated *p*-nitrophenol was determined by measuring the absorbance at 410 nm. One unit of enzyme activity was defined as that amount of enzyme catalyzing the liberation of 1 mol of *p*-nitrophenol·h^{-1} at 37 °C (Ranwala et al., 1992).

To examine developmental changes in β-Gal activity, fruits were sampled also at several fruit phenology stages, beginning with green fruit prior to lignification of the endocarp ("pit hardening") and ending when the fruit exocarp was dark red at normal commercial harvest. Samples also were collected when fruit color was straw, pink, and red.

SDS−PAGE. Enzyme purity was examined at each purification step by SDS−PAGE (Mini-PROTEAN II Cell, Bio-Rad, Richmond, CA) with 12% resolving and 4.5% stacking gel or with a precast 4−15% gradient denatured gel (Mini-PROTEAN II Ready Gel, Bio-Rad). The Laemmli (1970) buffer system was used. Following electrophoresis, the gel was fixed in 7% (v/v) acetic acid and 40% (v/v) methanol for 1 h, then stained with brilliant blue G-colloidal (Sigma Chemical Co., St. Louis, MO) for 1 h, and destained with 25% (v/v) methanol for 48 h. Protein standards (Bio-Rad) were included in each SDS−PAGE.

Isoelectric Focusing (IEF) of Native β-Gal. Vertical mini-slab (Mini-PROTEAN II Cell, Bio-Rad) gel IEF was conducted by loading 1−2 μg of β-Gal purified on the Sephacryl S-200 column in each lane, using procedures described by Robertson et al. (1987) with the following modifications to the running conditions to obtain a narrow band: 200 V for 2 h, followed by 400 V for 5 h. The system was kept on ice during the run. After the run, the entire gel was soaked in 1% (v/v) trichloroacetic acid for 2 h. Two duplicate lanes of the gel were stained by the same protocol as for SDS−PAGE, previously described. The other two identical lanes of the gel were separated. Each lane, about 2 cm wide, was divided into nine pieces along the pH gradient for either pH determination or β-Gal activity assay. Gel pieces were incubated in individual test tubes with 500 μL of 10 mM enzyme substrate, *p*-nitrophenyl β-D-galactopyranoside in 40 mM sodium acetate buffer (pH 5) for 30 min at 37 °C and assayed for enzyme activity as previously described.

Protein Determination. The total protein content at each step was determined according to Bradford's (1976) method using Bio-Rad reagents.

β-Gal Characterization. Buffers of pH 3−8 were prepared by adjusting mixtures of acetate, phosphate, and Tris buffers (20 mM) with 0.1 N HCl or 0.1 N NaOH. The substrate for β-Gal, *p*-nitrophenyl β-D-galactopyranoside, was added to each buffer solution to a final concentration of 5 mM. To each 500 μL of pH-adjusted substrate was added 20 μL of β-Gal, purified from the second Sephacryl S-200 column. β-Gal activity was measured as previously described.

A series of 2-fold substrate solution dilutions beginning at 10 mM were prepared for determining the kinetics of β-Gal. Twenty microliters of β-Gal, purified from the second Sephacryl S-200 column, was added to 500 μL of each substrate dilution at pH 5 and 37 °C. The K_m and V_{max} were determined using the Michaelis−Menten equation.

Inhibitors/Cofactors. Plant growth regulators, metallic cations, and the β-D-glucosidase substrate were examined to determine their effects on β-Gal activity. Gibberellic acid (GA_3), indole-3-acetic acid (IAA), Cu^{2+}, and Mg^{2+} were separately dissolved in 500 μL of 5 mM *p*-nitrophenyl β-D-galactopyranoside in 40 mM sodium acetate buffer (pH 5.0) to a final concentration of 1 mM. The β-D-glucosidase substrate, *p*-nitrophenyl β-D-glucopyranoside, was dissolved in 500 μL of 40 mM sodium acetate buffer (pH 5.0), without *p*-nitrophenyl β-D-galactopyranoside, to a final concentration of 1 mM. Twenty microliters of β-Gal, purified from the second Sephacryl S-200 column, was added to each reaction vessel. The reactions were allowed to proceed for 8 min at 37 °C and then were terminated, and β-Gal activity was measured, as previously described for the enzyme activity assay.

GA_3 (Pro-Gibb, Abbott Laboratories, North Chicago, IL), which is applied to sweet cherry orchards in the Pacific Northwest of North America to delay fruit maturity and inhibit softening (Beers et al., 1993), was applied at 20 mg·L^{-1} to fruits in situ both at pit hardening and at the straw-color stages, 48 and 58 days after anthesis, respectively. Water was used as the control. The spray was applied until runoff. Fruit samples were collected 4, 8, and 16 days after the final treatment. Mesocarp tissue was assayed for β-Gal specific activities at each sampling according to the methods previously described.

Antibody Production. Polyclonal antibodies were produced against the purified 57 kDa β-Gal antigen by subcutaneous injections of two rabbits. The initial booster included a mixture of 100 μL of fraction 10 from the Sephadex G-75 column plus 300 μL of fraction 5 from the second elution of the Sephacryl S-200 column and 400 μL of Freund's complete adjuvant. The second booster was injected 15 days later. It was produced by subjecting the G-75 and S-200 gel filtration fractions to SDS−PAGE, as previously described, followed by staining with brilliant blue G-colloidal for 1 h. The β-Gal bands were sliced from the gel, vacuum freeze-dried, ground to a fine powder, dissolved in 400 μL of 40 mM sodium acetate buffer (pH 5), and mixed with 600 μL of Freund's incomplete adjuvant. The third booster was identical to the second and was injected 10 days later. Each booster contained about 70 μg of protein. Four days after the third booster, each rabbit received an intermuscular injection of 1 mL of xylene, and the blood was collected from the heart. The antiserum was isolated by centrifuging at 900*g*.

Western Blot Analysis. The total protein of mature fruit was isolated following procedures of Schuster and Davies (1983). Three grams of mesocarp tissue was used in the isolation. The protein content was determined according to Bradford's (1976) method using Bio-Rad reagents.

A precast 4−15% gradient denatured gel (Mini-PROTEAN II Ready Gel, Bio-Rad) was employed for protein separation. Six micrograms protein was loaded in each lane. A vertical mini-slab system (Mini-PROTEAN Cell II, Bio-Rad) was used to separate the proteins at a constant 200 V for about 1 h. The separated proteins were transferred from the gel to poly(vinylidene difluoride) (PVDF) membrane using the Trans-Blot cell (Bio-Rad) with wire electrodes at a constant 30 V in 25 mM Tris buffer (pH 8.3) for 16 h as described by Towbin et al.

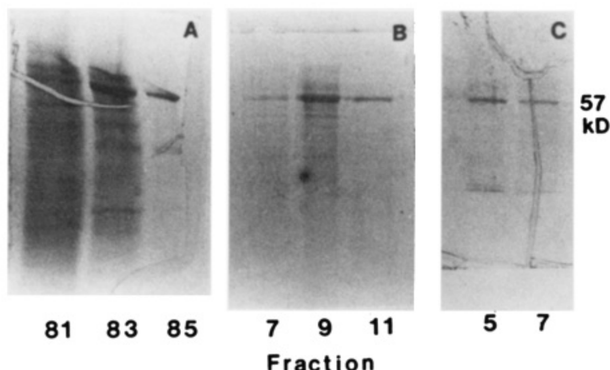

Figure 1. SDS–PAGE analysis of β-Gal peak activity fractions from DEAE-Sephadex A-50 (A), Sephadex G-75 (B), and first Sephacryl S-200 (C) columns. All gels were stained with brilliant blue G-colloidal.

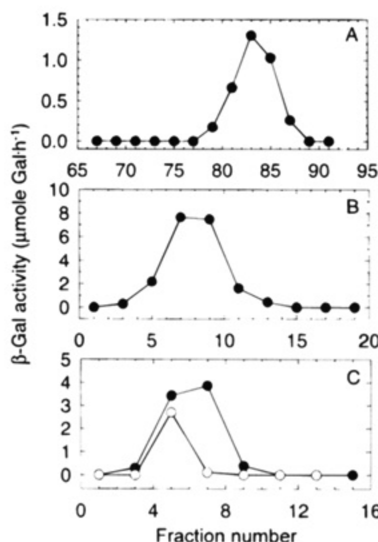

Figure 2. Elution profiles of β-Gal from DEAE-Sephadex A-50 (A), Sephadex G-75 (B), and first (●) and second (○) elutions from Sephacryl S-200 (C) columns. (Gal = galactose.)

(1979). The PVDF membrane was immunoblotted with the β-Gal antibody followed by incubation with goat anti-rabbit IgG conjugated with horseradish peroxidase (Sigma Chemical Co., St. Louis, MO). The color of the target enzymes appeared after incubation with the substrate, 4-chloro-1-naphthol, at 21 °C for 10–15 min. Prestained SDS–PAGE standards (Bio-Rad) were used to estimate molecular weight. The bands were photographed after the membrane was dried.

RESULTS AND DISCUSSION

The purification of β-Gal was completed in the following five steps: $(NH_4)_2SO_4$ fractionation, DEAE–Sephadex A-50 anion exchange chromatography, and three gel filtration chromatographic steps. β-Gal specific activity was increased only 22-fold from that of the crude extract. This was low in comparison with the findings of Ranwala et al. (1992), mainly due to the very high activity of our crude enzyme extract. Other explanations for the apparent low degree of purification are that β-Gal might have required a cofactor for high activity, which was satisfied in the crude extraction but not after purification, or the multiple liquid chromatographic steps might have resulted in loss of enzyme activity.

The dominant band on SDS–PAGE from the peak β-Gal activity fractions from the DEAE-Sephadex anion exchange column was a 57 kDa polypeptide, with fraction 85 being nearly pure (Figures 1A and 2A). This fraction showed very high β-Gal activity (Figure 2A).

To further purify β-Gal, fractions 81–86 were pooled (Figure 2A), concentrated by vacuum freeze-drying, and separated on a Sephadex G-75 gel filtration column eluted with 100 mM NaCl in Tris buffer (pH 8.0). Peak β-Gal activity was again detected (Figure 2B). Associated with peak activity was a more purified β-Gal with a 57 kDa molecular mass (Figure 1B).

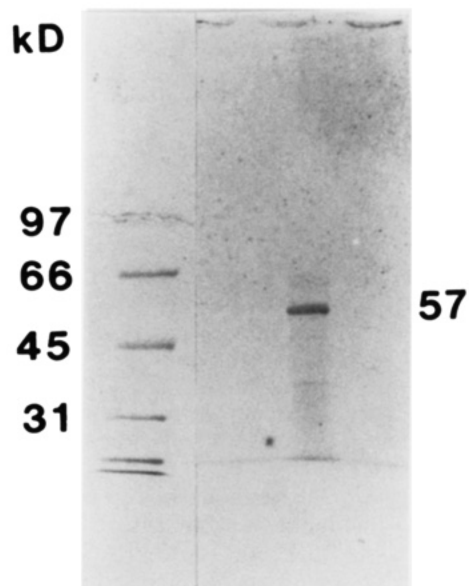

Figure 3. SDS–PAGE analysis of the β-Gal peak activity fraction 5 from the second elution on the Sephacryl S-200 column. Molecular mass was estimated with 18.5–97 kDa SDS–PAGE standards (left lane). The gel was stained with brilliant blue G-colloidal.

Because of the presence of minor bands shown on the SDS–PAGE after the first two steps (Figure 1B), another purification step was performed. A larger molecular mass range (5–250 kDa) gel filtration solid phase (Sephacryl S-200) was used for this separation. Fractions 7–11 with peak β-Gal activity from the G-75 column (Figure 2B) were combined, loaded on the S-200 column, and eluted with the same buffer used to elute the G-75 column. The elution profile is shown in Figure 2C. The first S-200 column separation resulted in a nearly pure enzyme (Figure 1C).

To remove the remaining ambiguous bands, a second S-200 gel filtration was performed, but only a slight improvement in enzyme purity was achieved (Figure 3). A sharp peak of activity was obtained in this elution profile (Figure 2C). Although five purification steps were performed, the enzyme was only about 90% purified, with a few faint bands remaining (Figure 3).

Although it has been suggested that β-Gal is an important fruit softening enzyme (Pressey, 1983), its purification and characterization from fruit has been rare compared with other cell wall hydrolases, e.g., PG, PME, and carboxymethyl cellulase (C_x-cellulase) [see Fischer and Bennett (1991)]. The purification of β-Gal, however, has been reported from radish seeds (Sekimata et al., 1989), mung beans (Kundu et al., 1990), and carrot cell suspension cultures (Konno et al., 1986, 1988; Konno and Katoh, 1992). While partial purification of β-Gal from tomato (Pressey, 1983) and muskmelon fruit (Ranwala et al., 1992) has been achieved, purification to near homogeneity has been achieved only recently in apple (Dick et al., 1990), kiwi fruit (Ross et al., 1993), and avocado (De Veau et al., 1993). These fruits are all climacteric (Kays, 1991).

As with other enzymes, varied molecular masses have been reported for β-Gal purified from different plant species. Mung beans contained four β-Gals, referred to as Gal I, Gal II, Gal III, and Gal IV, with molecular

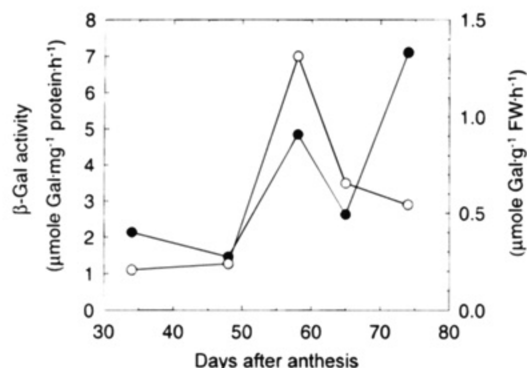

Figure 4. Enzyme activity of β-Gal on total specific protein (○) and tissue fresh weight (FW) (●) bases during sweet cherry fruit development in 1992. (Gal = galactose.)

masses of 67, 83, 96, and 44 kDa, respectively, as determined by nondenaturing gel filtration chromatography (Kundu et al., 1990). SDS–PAGE analysis of these mung bean β-Gal isoenzymes indicated that the proteins were about half the molecular masses of the gel filtration chromatography method. Therefore, it was suggested that these isoenzymes were dimers (Kundu et al., 1990). A β-Gal purified from apple fruit had estimated molecular masses of 161 and 146 kDa by gel permeation and SDS–PAGE, respectively (Dicks et al., 1990). A 104 kDa β-Gal I isoenzyme, estimated by gel filtration from carrot cell suspension cultures, was 50 kDa by SDS–PAGE (Konno et al., 1988), again suggesting that this β-Gal isoenzyme is a dimer. A β-Gal isolated from lily pollen had a very high molecular mass of 450 kDa (Singh and Knox, 1985). A β-Gal II isoenzyme isolated from carrot cultures was 65 and 60 kDa by gel filtration chromatography and SDS–PAGE, respectively (Konno and Katoh, 1992), suggesting a monomeric protein. In tomato fruit, three β-Gals (Gal I, Gal II, and Gal III) have been estimated to be 144, 62, and 71 kDa according to the enzyme activity peaks from gel filtration chromatography (Pressey, 1983). De Veau et al. (1993) isolated three proteins with β-Gal activity with estimated molecular masses of 41, 49, and 54 kDa by SDS–PAGE. A β-Gal isolated from kiwi fruit had a molecular mass of 59 kDa by both gel permeation chromatography and SDS–PAGE (Ross et al., 1993). A previously purified β-Gal from kiwi fruit had a molecular mass of 145 kDa (Ogawa et al., 1990), which may correspond to the β-Gal I (Gal I) from tomato fruit (Pressey, 1983) and the 146 kDa β-Gal from apple fruit (Dick et al., 1990). The β-Gal isolated from sweet cherry fruit had an estimated molecular mass of 57 kDa by SDS–PAGE (Figure 3), similar in molecular mass to the β-Gal II (Gal II) of tomato fruit (Pressey, 1983), the β-Gal II secreted from carrot cell suspension cultures (Konno and Katoh, 1992), and the 59 and 54 kDa isoenzymes from kiwi fruit (Ross et al., 1993) and avocado (De Veau et al., 1993), respectively. Therefore, the sweet cherry β-Gal may be a β-Gal II isoenzyme, since estimations of molecular mass can vary up to ± 5 kDa as a result of SDS–PAGE conditions, and even more when compared with estimates by gel filtration chromatography.

The specific activity of β-Gal on a protein basis began increasing between 48 and 58 days after anthesis (Figure 4). Maximum activity occurred at 58 days after anthesis, 10 days earlier than maximum activities of PG, PME, and C_x-cellulase (Andrews and Li, 1994). The declining β-Gal activity on a fresh weight basis compared to protein basis at 74 days was due to a substantial increase in protein content between 65 and 74 days from 0.141 to 0.459 mg·g^{-1} fresh weight, respectively. β-Gal was more closely related to the initial decrease in fruit firmness than were the activities of PG, PME, and C_x-cellulase (Andrews and Li, 1994).

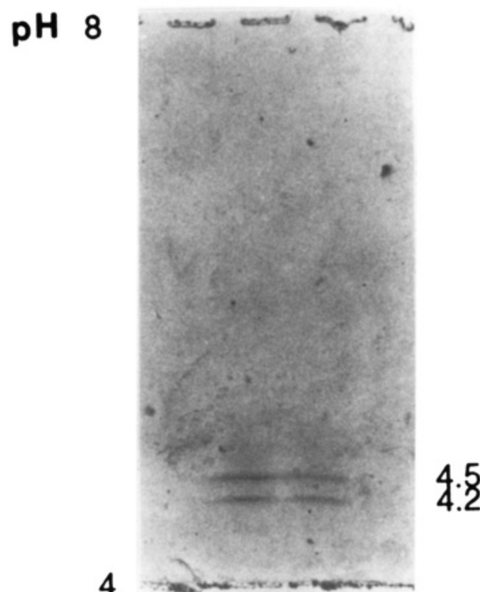

Figure 5. Duplicate lanes of native IEF electrophoresis of purified β-Gal protein. The gel was stained with brilliant blue G-colloidal.

The role of glycosidases in fruit ripening has received minor attention, despite detection of glycosidase activity in climacteric apple, tomato, apricot, peach, muskmelon, and kiwi fruit (Bartley, 1974; Pressey, 1983; Bouranis and Niavis, 1992; Ranwala et al., 1992; Wegrzyn and MacRae, 1992) and nonclimacteric pepper and orange fruits (Gross et al., 1986; Burns, 1990). It has been proposed that the glycosidase, β-Gal, may be responsible for the in vivo solubilization of galactose that occurs during fruit ripening (Fischer and Bennett, 1991). Partially purified isoenzymes of β-Gal from *Cucumis pepo* L. fruit degraded components of the fruit cell walls (Ranwala et al., 1992). Similar degradation of native cell wall polysaccharides was reported for a putative 60 kDa β-Gal purified from kiwi fruit (Ross et al., 1993). The 2-fold increase in β-Gal activity during ripening of kiwi fruit, however, did not account for the total loss in cell wall galactose (Wegrzyn and MacRae, 1992; Ross et al., 1993). A 5-fold increase in β-Gal activity was reported for apple fruit (Ogawa et al., 1990), while a 6-fold increase in specific activity was measured in sweet cherry fruit (Figure 4).

Two bands were observed on the IEF native enzyme gel, with isoelectric points (p*I*) of 4.2 and 4.5 (Figure 5). β-Gal from other species was reported to have neutral to slightly basic p*I* values (Pressey, 1983; Sekimata et al., 1989; Konno and Katoh, 1992; Ross et al., 1992); however, the β-Gal isoenzymes recently purified from avocado fruit had p*I* values of 5.0–5.2 (De Veau et al., 1993). The presence of two bands might indicate that the native enzyme was composed of a dimer with each subunit of similar molecular mass and having different p*I* values or of two distinct isoenzymes. Charge differences resulting from different amino acid compositions or levels of phosphorylation may account for these differences. On the basis of the position of the bands on the IEF gel (Figure 5) and the β-Gal activity of each piece (Figure 6A), it was concluded that one or both of these bands were β-Gal. If β-Gal is a dimer, one or both of the subunits exhibited a catalytic response. It is also possible that a functionally unrelated protein

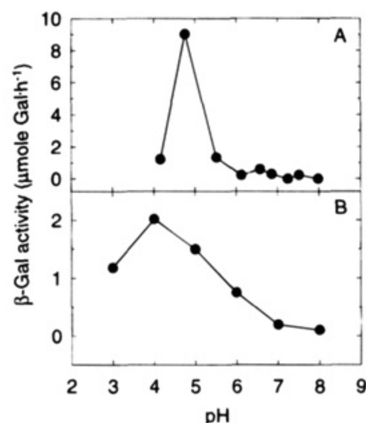

Figure 6. β-Gal activity of nine pieces cut along the pH gradient of IEF gel (A) (see Figure 5) and of purified β-Gal at different pH (B).

Table 1. Effect of Metals, Plant Growth Regulators, and p-Nitrophenyl β-D-Glucopyranoside on β-Gal Activity

compound	enzyme activity (mol of p-nitrophenol·h^{-1})
control[a]	1.31
Cu^{2+}	0.19
Mg^{2+}	3.30
GA_3	4.52
IAA	2.82
p-nitrophenyl β-D-glucopyranoside[b]	0.0

[a] Contains β-Gal substrate, p-nitrophenyl β-D-galactopyranoside, only. [b] Contains β-D-glucosidase substrate only.

of identical molecular mass coeluted with the functional monomeric β-Gal. The β-Gal from sweet cherry fruit was of similar molecular mass to other monomeric β-Gal II isoenzymes (Pressey, 1983; Konno and Katoh, 1992). Additionally, β-Gal II (Gal II) was the only isoenzyme from tomato fruit capable of degrading cell wall components (Pressey, 1983).

The assay for pH optimum indicated that maximum β-Gal activity occurred at pH 4.0 (Figure 6A). The reported pH optima of 3.2–4.4 for β-Gal activity (Pressey, 1983; Sekimata et al., 1989; Dick et al., 1990; Kundu et al., 1990; Konno and Katoh, 1992; Ranwala et al., 1992; Ross et al., 1993) corresponded to the pH 4 optimum for sweet cherry fruit β-Gal.

The K_m and V_{max} determined from the Michaelis–Menten equation were 1.25 mM and 5.0 μmol·mg^{-1}·min^{-1}, respectively. This K_m of β-Gal was higher than the 0.17, 0.46, and 0.77 mM K_m values of β-Gals from carrot cell suspension cultures (Konno and Katoh, 1992), radish seeds (Sekimata et al., 1989), and tomato fruit (Pressey, 1983), respectively. It was lower, however, than the 1.66 mM K_m of soluble and NaCl-released β-Gal partially purified from muskmelon fruit (Ranwala et al., 1992) and the 2.63 mM K_m of mung bean seed β-Gal II (Kundo et al., 1990). The 5.36 μmol·mg^{-1}·min^{-1} V_{max} of radish seed β-Gal (Sekimata et al., 1989) was similar to the 5.0 μmol·mg^{-1}·min^{-1} V_{max} for sweet cherry fruit β-Gal. The substrate used in all of these kinetic studies was p-nitrophenyl β-D-galactopyranoside. Some differences in K_m and V_{max}, however, can be partially accounted for by slight differences in reaction conditions.

Even though Cu^{2+} and Mg^{2+} both are divalent cations, Cu^{2+} nearly eliminated the activity of β-Gal, while Mg^{2+} doubled its activity relative to the control (Table 1). In vitro inhibition of β-Gal has been widely studied. Silver was found to completely inhibit β-Gal activity in muskmelon fruit (Ranwala et al., 1992) and nearly so in mung

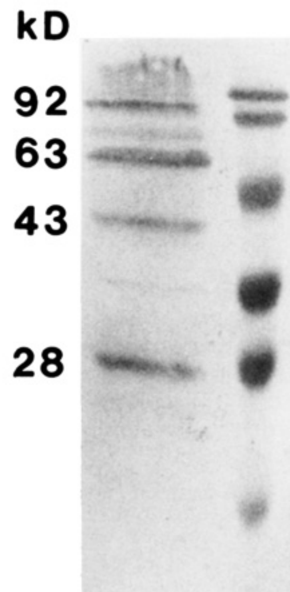

Figure 7. Western blot analysis of total protein isolated from mature sweet cherry fruit with polyclonal antibodies produced against the purified 57 kDa β-Gal. Molecular masses were estimated with prestained 18.5–106 kDa SDS–PAGE standards (right lane).

bean (Kundo et al., 1990) and radish (Sekimata et al., 1989) seeds. Mercury completely inhibited activity in tomato (Pressey, 1983) and muskmelon (Ranwala et al., 1992) fruit and carrot cell suspension cultures (Konno and Katoh, 1992), with nearly complete inhibition in mung bean (Kundo et al., 1990) and radish (Sekimata et al., 1989) seeds. Up to 50% inhibition of activity by Cu^{2+} was found in carrot cell cultures (Konno and Katoh, 1992) and mung bean seeds (Kundo et al., 1990) and nearly complete inhibition in radish seeds (Sekimata et al., 1989). In sweet cherry fruit, the inhibition by Cu^{2+} was 85% (Table 1). While the mechanism of inhibition by Cu^{2+} is unknown, the oxidation of sulfhydryl groups is one possibility. Colbalt and Fe^{3+} eliminated β-Gal activity in muskmelon fruit (Ranwala et al., 1992). Neither stimulatory nor inhibitory effects on β-Gal activity were found for Ca^{2+}, Mg^{2+}, Mn^{2+}, or Zn^{2+} in most species (Pressey, 1983; Kundo et al., 1990; Konno and Katoh, 1992; Ranwala et al., 1992); however, in radish seeds Mn^{2+} almost completely inhibited β-Gal activity (Sekimata et al., 1989). In sweet cherry fruit Mg^{2+} strongly promoted β-Gal activity (Table 1). This divalent cation may be a cofactor for β-Gal in sweet cherry fruit. Since certain chelating agents inhibited polysaccharase activity from hydrolyzing a galactose-rich polysaccharide isolated from tomato cell walls, Pressey (1983) suggested that a divalent cation may be required for β-Gal II (Gal II) activity.

Both GA_3 and IAA increased β-Gal activity 2-fold relative to the control (Table 1). In situ exogenous applications of 20 μg·mL^{-1} GA_3 applied to straw-colored fruit resulted in a significant reduction of β-Gal activity of mature fruit 16 days later (data not shown). Other researchers have not examined the effects of plant growth regulators on β-Gal activity. The in vivo product of β-Gal, galactose, strongly inhibited β-Gal activity of tomato fruit (Pressey, 1983) and mung bean seeds (Kundo et al., 1990). β-Gal did not utilize p-nitrophenyl β-D-glucopyranoside as a substrate.

Polyclonal antibodies raised against the 57 kDa sweet cherry β-Gal reacted with four polypeptides in the total protein isolated from mature fruit (Figure 7). These proteins had molecular masses of 28, 43, 63, and 92 kDa

using prestained standards. Antibodies have not been reported to be raised against β-Gal purified from other species. The 63 kDa protein may correspond to the purified β-Gal estimated to be 57 kDa by SDS–PAGE (Figure 3). The other proteins may be isoenzymes of β-Gal or functionally unrelated proteins that share sufficient homology at the epitope to react with the antibody.

CONCLUSIONS

The evidence presented here suggests that β-Gal isolated from sweet cherry fruit is similar to the β-Gal II isoenzyme isolated from other species. While its in vivo substrate in sweet cherry fruit still must be identified, it may function in hemicellulose degradation, with greatest hydrolytic activity occurring in fruit about 2 weeks prior to maturity. This suggests that β-Gal may contribute to softening of nonclimacteric sweet cherry fruit.

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