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β-Galactosidases in Ripening Tomatoes[1]

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ABSTRACT

Tomatoes (*Lycopersicon esculentum* L.) contained a high level of β-galactosidase activity which was due to three forms of the enzyme. During tomato ripening, the sum of their activities remained relatively constant, but the levels of the individual forms of β-galactosidase changed markedly. The three enzymes were separated by a combination of chromatography of DEAE-Sephadex A-50 and Sephadex G-100. During ripening of tomatoes, β-galactosidases I and III levels decreased but the β-galactosidase II level increased more than 3-fold. The three enzymes were optimally active near pH 4, and all were inhibited by galactose and galactonolactone. However, the enzymes differed in molecular weight, K_m value with p-nitrophenyl-β-galactoside, and stability with respect to pH and temperature. β-Galactosidase II was the only enzyme capable of hydrolyzing a polysaccharide that was isolated from tomatoes and that consisted primarily of β-1, 4-linked galactose. The ability of β-galactosidase II to degrade the galactan and the increase in its activity during tomato ripening suggest a possible role for this enzyme in tomato softening.

During fruit ripening, the most apparent cell-wall change is an increase in water-soluble polyuronide (6–8, 15). The solubilization of polyuronide is generally attributed to the action of polygalacturonase, which appears in many fruits near the onset of ripening (6, 15, 19). Another change that occurs, at least in apples, strawberries, and tomatoes, is a pronounced loss of galactose from the cell walls. This process does not appear to be related to cell-wall degradation by polygalacturonase (9, 17), but possibly to a galactanase. Bartley (2) found a β-galactosidase in apples that degraded galactan and increased in activity during apple ripening. Tomatoes also contain β-galactosidase, but it has been concluded that this enzyme is not responsible for the hydrolysis of cell wall galactans (5). Furthermore, Gross and Wallner (5) were unable to detect galactanase activity in tomatoes at any stage of ripeness. The present paper shows that the β-galactosidase activity in tomatoes is due to three enzymes and that one of the β-galactosidases hydrolyzes tomato galactan.

MATERIALS AND METHODS

Plant Material. Tomatoes (*Lycopersicon esculentum* Mill.) were grown both in the greenhouse (cv Tropic) and in the field (cv Better Boy). The fruit were harvested at mature green and ripe stages and extracted immediately after harvest.

Enzyme Assays. β-Galactosidase was assayed by measuring the rate at which it hydrolyzes *p*-nitrophenyl-β-galactoside (Sigma). The reaction mixture consisted of 0.5 ml of 0.1 M citrate (pH 4.0), 0.4 ml of 0.1% BSA, 0.1 ml of diluted enzyme, and 0.4 ml of 13 mM substrate. After 15 min at 37°C, the reactions were terminated by the addition of 2 ml of 0.2 M sodium carbonate, and the liberated *p*-nitrophenol was measured at 415 nm. One unit of β-galactosidase was defined as the amount that hydrolyzed 1 μmol of *p*-nitrophenyl-β-galactoside/15 min.

Galactanase was assayed by measuring the release of reducing sugars from a galactose-rich polysaccharide isolated from tomatoes (see below). The reaction mixture consisted of 0.4 ml of 0.1 M sodium acetate (pH 4.0), 0.4 ml of enzyme solution diluted with 0.2% BSA, and 0.2 ml of 1% polysaccharide. After 1 h at 37°C, the reaction was terminated by heating in boiling water, and the solution was analyzed for reducing groups by the arsenomolybdate method (13). The reaction rates in the β-galactosidase and galactanase assays were linear with respect to both enzyme concentration and incubation time.

The protein content of chromatographic fractions was measured by absorbance at 280 nm, whereas that of concentrated crude and purified enzyme solutions was measured by the Bradford method (4).

Enzyme Extraction. Pericarp tissue (500 g) from fresh tomatoes was homogenized with 700 ml of cold water. All of the subsequent steps were conducted at about 3°C. The suspension was stirred for 30 min, and solid NaCl was added to a final concentration of 1.0 M. It was adjusted to pH 6 with 0.5 N NaOH and stirred for an additional 1 h. The suspension was then centrifuged at 8000*g* for 20 min, and ammonium sulfate was added to the supernatant solution to 80% of saturation. The precipitated proteins were collected by centrifugation, dissolved in 30 ml of water, and dialyzed against 0.15 M NaCl overnight. This solution was then centrifuged to remove a small amount of insoluble material, and the supernatant represented the crude extract.

Column Chromatography of the Enzymes. The following columns were used for separating and purifying the galactosidases: a 5 × 70 cm column of DEAE-Sephadex A-50 equilibrated with 0.05 M sodium acetate (pH 6.0) containing 0.10 M NaCl; a 2.5 × 90 cm column of Sephadex G-100 equilibrated with 0.15 M NaCl; and a 2.5 × 40 cm column of CM-Sephadex C-50 equilibrated with 0.05 M sodium acetate (pH 5.2) containing 0.25 M NaCl. Each column was eluted with the equilibration solution. The enzymes in the fractions were concentrated with either an Amicon Corp. model 52 or 202 unit and PM-10 membranes.

Isoelectric Focusing. Isoelectric focusing was performed with a Desaga/Brinkmann TLE double chamber according to the manufacturer's instructions. A glass plate (20 × 20 cm) was coated with a suspension of 7 g Sephadex G-75 superfine in 100 ml of 2% pH 2 to 10 isolytes (Brinkmann Instruments). The layer was dried at room temperature until fine cracks appeared along the edges. The enzyme solutions were concentrated to about 3 ml by ultrafiltration with an Amicon model 52 cell and a PM-10 membrane, and were dialyzed against 2% glycine (pH 6.5). Sephadex G-75 was added to each solution to produce a fairly liquid suspension.

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A trough was cut out of the gel layer in the middle of the plate and filled with the enzyme suspension. Focusing was conducted at 4°C at 200 v for 12 h and then at 800 v for 2 h. Strips (1 cm) of the gel were cut parallel to the electrodes, suspended in a small volume of 0.15 M NaCl, and assayed for β-galactosidase.

Carbohydrate Analysis. Neutral sugars and uronic acid were measured by the anthrone (16) and *m*-hydroxydiphenyl (3) methods, respectively. Neutral sugar compositions were determined by GC according to Albersheim *et al.* (1). After hydrolysis of the polysaccharides with 2 N trifluroacetic acid at 121°C for 1 h, the sugars were converted to alditol acetates and separated on a column of GP 3% SP-2340 on 100/200 Supelcoport (Supelco, Inc.) at 225°C.

Isolation of Galatose-Rich Polysaccharide. Cell walls were isolated from green tomatoes as described previously (14). The washed cell walls (1 g) were suspended in 200 ml of 0.1 M glycine (pH 3.5) containing 0.1 M NaCl. Polygalacturonase II (86 units) and pectinesterase II (210 units) were added, and the suspension was incubated at 37°C with stirring for 1 h (see Pressey and Avants [14] for the details concerning the isolation of the pectic enzymes). The suspension was filtered and the filtrate was boiled 5 min to inactivate the enzymes. The solution was clarified by centrifugation and concentrated to 10 ml by ultrafiltration with an Amicon PM-10 membrane. The concentrated solution was then chromatographed on a 2.5 × 40 cm column of Bio-Gel A-15 m (200–400 mesh) equilibrated with 0.15 M NaCl. The fractions were analyzed for carbohydrates by the anthrone (16) and the *m*-hydroxydiphenyl (3) methods. Most of the anthrone-positive material was eluted near the exclusion limit of the gel, whereas most of the uronic acid was eluted near the inclusion limit of the gel. The fractions corresponding to the anthrone-positive peak were pooled and concentrated to 10 ml by ultrafiltration. The polysaccharide was precipitated by adding 2 volumes of ethanol to the solution, collected by centrifugation, and dried. The yield was 48 mg.

RESULTS

Separation and Purification of Tomato β-Galactosidases. Chromatography of the crude extract of ripe Tropic tomatoes on DEAE-Sephadex A-50 yielded two peaks of β-galactosidase activity (Fig. 1). Fractions 15 through 21 (peak A) were pooled, as were fractions 23 through 30 (peak B), and each pooled mixture was concentrated to 10 ml by ultrafiltration. The concentrated enzyme solutions were then chromatographed on the Sephadex G-100 column. Peak A was resolved into two peaks of β-galactosidase activity (Fig. 2). The earlier eluted enzyme was designated β-galactosidase I (fractions 12 through 14) and the later eluted enzyme, β-galactosidase II (fractions 16 through 21). Peak B could not be further resolved with Sephadex G-100, and the enzyme associated with this peak was designed β-galactosidase III. The three enzymes were further purified by chromatography on CM-Sephadex C-50. The elution volumes on this column were 34, 145, and 52 ml for β-galactosidase I, II, and III, respectively. A summary of the overall purification procedure is given in Table I.

β-Galactosidases in Green and Ripe Tomatoes. The procedure for extracting and separating the three β-galactosidases was applied to measuring the individual enzymes in the green and ripe fruit of the two cultivars of tomatoes (Table II). The total activity was somewhat higher in Better Boy than in Tropic fruit. β-Galactosidase III was the major form of the enzyme in both cultivars and its activity decreased during ripening. In contrast, β-galactosidase II, whose activity was lowest in green fruit, increased in activity by more than 3-fold during ripening.

Extractabilities of the β-Galactosidases. Possible association of the β-galactosidases with the cell walls was determined from the solubilities of the enzymes. Ripe tomatoes were extracted sequentially with water at pH 4.0 and 1.0 M NaCl at pH 6.0. The proteins in the two extracts were precipitated with ammonium sulfate, dissolved, dialyzed, and analyzed for the three β-galactosidases. β-Galactosidase I was completely extracted with water. Most of β-galactosidase III (65%) was extracted with water, but only 36% of β-galactosidase II was soluble in water.

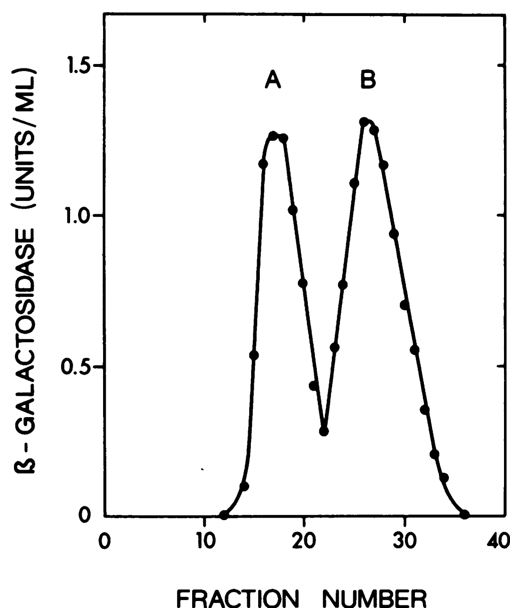

FIG. 1. Elution profile of β-galactosidase activity fractionated from a crude extract of ripe Tropic tomatoes by use of a DEAE-Sephadex A-50 column. The flow rate was 50 ml/h and the fraction volume was 20 ml.

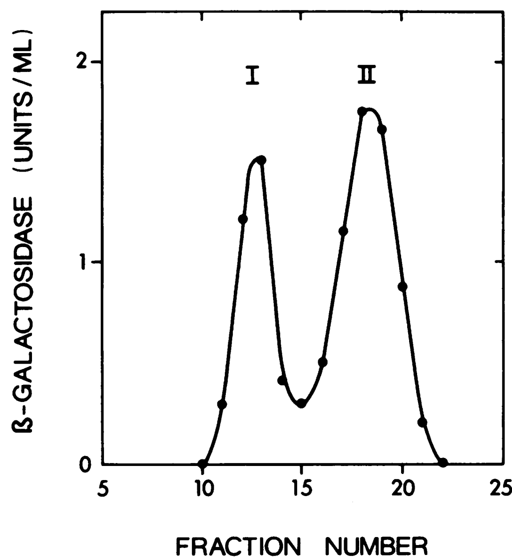

FIG. 2. Resolution of peak A (Fig. 1) into two peaks of β-galactosidase activity by a Sephadex G-100 column. The flow rate was 22 ml/h and the fraction volume was 13 ml.

Properties of the β-Galactosidases. Various properties of the β-galactosidases were determined in reaction mixtures containing 0.1 unit of each enzyme. β-Galactosidases I and III hydrolyzed *p*-nitrophenyl-β-galactoside at pH between 2 and 5.5, but optimally at about pH 3.8. β-Galactosidase II was active between pH 3 and 6.5 but was optimally active at pH 4.2. The activities were independent of the buffer, being identical for Mes-acetate, glycine-KCl, and citrate buffers. Similarly, the activities were not affected by 10 mM (final concentrations) EDTA, 1 mM Ca^{2+}, Mg^{2+}, Mn^{2+}, and Zn^{2+}. All three enzymes were completely inhibited by 0.1 μM Hg^{2+}.

Table I. *Summary of the Purification of Tomato β-Galactosidases from Ripe Tropic Fruit*

Step	β-Galactosidase I			β-Galactosidase II			β-Galactosidase III		
	Protein	Activity	Specific activity	Protein	Activity	Specific activity	Protein	Activity	Specific activity
	mg	*units*	*units/mg*	*mg*	*units*	*units/mg*	*mg*	*units*	*units/mg*
$(NH_4)_2SO_4$ pellet	96.4	372[a]		96.4	372[a]		96.4	372[a]	
DEAE-Sephadex A-50	14.4	157[b]		14.4	157[b]		19.8	184	9
Sephadex G-100	3.6	50	14	1.7	85	50	3.7	152	41
CM-Sephadex C-50	0.8	34	43	0.4	62	155	1.0	123	123

[a] Total β-galactosidase activity in the ammonium sulfate pellet.
[b] Total β-galactosidase activity in peak A.

Table II. *Levels of the Three β-Galactosidases in Green and Ripe Fruit of Two Cultivars of Tomatoes*

The details of the analyses are described in the text. The results represent the means of three experiments ± SE.

Tomatoes	β-Galactosidase I	β-Galactosidase II	β-Galactosidase III
		units/500 g tomatoes	
Tropic			
Green	61 ± 5	26 ± 7	234 ± 18
Ripe	48 ± 4	87 ± 16	196 ± 16
Better Boy			
Green	66 ± 12	28 ± 4	264 ± 10
Ripe	47 ± 10	105 ± 11	220 ± 26

The β-galactosidases were inhibited by both galactose and D-galactonalactone. The latter reagent was converted to its most inhibitory form according to Levvy and Conchie (12). The concentrations of D-galactonolactone for 50% inhibition were 210, 70, and 140 μM for β-galactosidase I, II, and III, respectively. L-Galactonolactone was not an effective inhibitor at concentrations as high as 5 mM. The concentrations of galactose for 50% inhibition were 11, 2, and 15 mM for β-galactosidases I, II, and III, respectively. Glucose, mannose, and arabinose at 100 mM concentrations did not inhibit the enzymes.

The thermal stabilities of the enzymes were determined by heating the reaction mixtures for 5 min at various temperatures prior to addition of the substrate. The temperatures for 50% inactivation were 52, 48, and 52°C, for β-galactosidases I, II, and III, respectively. The effect of pH on stability was also determined. A mixture of 0.1 ml enzyme solution and 0.1 ml of 0.1 M citrate (pH 3–6.5) was heated for 5 min at 50°C, mixed with 0.5 ml of 0.1 M citrate (pH 4) and substrate, and assayed for residual activity. Stability was maximum at pH 5.7, 5.0, and 5.4 for β-galactosidases I, II, and III, respectively.

The mol wt of the enzymes were estimated by gel filtration on Sephadex G-100. The standard proteins and their elution volumes on this column were as follows: Cyt *c*, 362 ml; α-chymotrypsinogen, 324 ml; ovalbumin, 263 ml; BSA, 226 ml; and BSA dimer, 171 ml. The elution volumes and calculated mol wt for the enzymes were 157 ml and 144,000, 232 ml and 62,000, and 220 ml and 71,000 for β-galactosidases I, II, and III, respectively.

Reaction rates for the enzymes were measured at *p*-nitrophenyl-β-galactoside concentrations of 0.19 to 3.8 mM. The double reciprocal plots were linear for all three enzymes at these substrate concentrations. The K_m values were calculated to be 0.32, 0.77, and 0.43 mM for β-galactosidases I, II, and III, respectively.

Isoelectric focusing of the purified β-galactosidases yielded single bands of activity for each enzyme. The isoelectric points were 6.7, 7.8, and 6.8 for β-galactosidases I, II, and III, respectively.

Properties of the Galactose-Rich Polysaccharide. The polysaccharide isolated from tomato cell walls was eluted near the exclusion limits of Bio-Gel-A-15 m (Fig. 3); hence, its mol wt was estimated as about 5,000,000. It contained 22% uronic acid, 3.5% rhamnose, 0.8%, xylose, 1.2% glucose, 14.5% arabinose, and 58% galactose. The ^{13}C NMR spectrum of the polysaccharide indicated that the galactose residues were β-1,4-linked in chains of degree of polymerization greater than six. (The structural studies on this polysaccharide will be presented in a separate publication.)

Hydrolysis of the Polysaccharide by the β-Galactosidases. The DEAE-Sephadex A-50 fractions of an extract from ripe tomato were analyzed for hydrolytic activity with the tomato polysaccharide as the substrate by measuring the release of reducing groups (13). The only activity detected was associated with fractions corresponding to peak A (Fig. 1). When the fractions corresponding to peak A were combined and chromatographed on Sephadex G-100 (Fig. 2), the polysaccharase activity was found to be associated with β-galactosidase II. The pH optimum for the hydrolysis of the polysaccharide by β-galactosidase II was identical to that for the substrate *p*-nitrophenyl-β-galactoside (pH 4.2). However, the polysaccharase activity was inhibited by citrate and ethylene glycol bis(β-aminoethylether)-*N,N,N′,N′*-tetraacetic acid, whereas *p*-nitrophenyl-β-galactosidase activity was not affected by these chelating agents. This finding suggests that a divalent cation is required for β-galactosidase II to hydrolyze the polysaccharide; however, Ca^{2+}, Mg^{2+}, Mn^{2+}, and Zn^{2+} added to the reaction mixture did not increase the activity. The endogenous level of the cofactor must have been adequate for maximal activity.

The reaction mixture was increased to 5 ml to provide sufficient sample for the identification of the reducing carbohydrates released by β-galactosidase II from the polyaccharide. After 4 h at 37°C, the solution was heated, centrifuged, and applied to a 2.5 × 40 cm column of Bio-Gel A-15 m in water. About one-fourth of the anthrone-positive material remained unchanged in molecular size, but the remainder was eluted near the inclusion limit of the gel (Fig. 3). The fractions for each peak were pooled, and the pooled mixtures were evaporated to 4 ml and analyzed both for uronic acid by the hydroxydiphenyl method (3) and for neutral sugars by the GC method (1). The first peak consisted of 41% uronic acid, 6.4% rhamnose, 25% arabinose, 1.4% xylose, 2.2% glucose, and 24% galactose. The second peak consisted of 4% arabinose and 96% galactose. The galactose in this peak was present as the free sugar because identical values for galactose were obtained for TFA-hydrolyzed and unhydrolyzed aliquots. Thus, fragments released from the polysaccharide by β-galactosidase II were either completely hydrolyzed during the reaction or β-galactosidase II is an exoenzyme. It is not clear if the arabinose released is related to β-galactosidase action or not.

DISCUSSION

There is increasing evidence that fruit ripening is accompanied by a decrease in galactose content of cell walls and that this loss of galactose represents a separate process in the degradation of

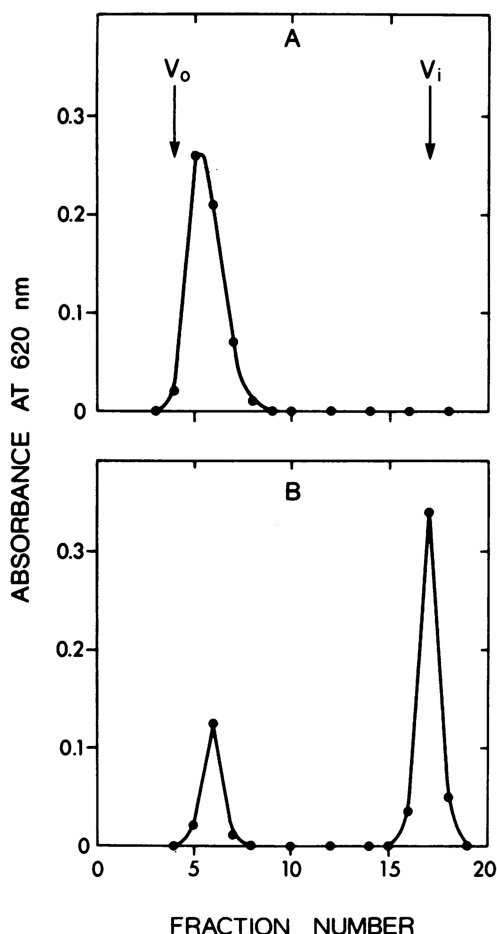

FIG. 3. Elution profile of the galactose-rich polysaccharide fractionated with a Bio-Gel A-15 m column. A, Sample of the original polysaccharide; B, sample of the polysaccharide after reaction with β-galactosidase II. The void volume (V_o) and included volume (V_i) were determined with blue dextran (Sigma) and sucrose, respectively. The flow rate was 24 ml/h and the fraction volume was 13 ml.

the cell walls responsible for fruit softening. Substantial declines in cell-wall galactose have been reported for ripening apples (8), strawberries (10), and tomatoes (17). Bartley (2) demonstrated that apples contain a β-galactosidase that increased in activity during fruit ripening. This enzyme could hydrolyze galactan and therefore may be involved in the loss of galactose from apples. However, the situation in tomatoes appeared to be more complicated. It was reported that tomatoes contain high β-galactosidase activity but that this enzyme does not release galactose from either isolated cell walls or a galactose-rich polysaccharide (11, 18).

The results of the present study confirm that tomatoes contain a high level of β-galactosidase activity. However, the results also indicate that this activity is not due to a single enzyme, as Gross and Wallner (5) assumed, but to three forms which can be separated by a combination of chromatography on DEAE-Sephadex A-50 and Sephadex G-100. Two of the enzymes (β-galactosidases I and III) have similar properties except for the 2-fold difference in mol wt, suggesting that β-galactosidase I is a dimer of β-galactosidase III. Only one of the forms, β-galactosidase II, could hydrolyze the galactose-rich polysaccharide isolated from tomatoes. Because the galactan in this polysaccharide was established to be β-1,4-linked, the enzyme was a β-1,4-galactosidase.

There may be several reasons why Gross and Walner (5) did not detect galactanase activity in their extracts of ripe tomatoes. First, they prepared extracts from frozen fruit. The present study (data not shown) indicated that such fruit yield lower amounts of β-galactosidase II than fresh tomatoes. Second, they extracted and assayed β-galactosidase activity in citrate buffers. The present study showed that citrate inhibited activity of the enzyme if the substrate was a galactan but not if it was *p*-nitrophenyl-β-galactoside.

Failing to detect galactanase in tomato extracts, Lackey *et al.* (11) proposed that the loss of galactose from the cell walls may be due to decreased synthesis of galactan. Knee *et al.* (10) had demonstrated the turnover of a galactose-containing cell wall fraction during strawberry fruit development. Also, Knee (9) suggested that changes in apple cell wall structure were due to enhanced turnover and formation of different polymers rather than to degradative reactions. But any loss of galactan from cell walls would require its hydrolysis to galactose, which may then be utilized in respiration or in th: formation of other polysaccharides. In the present study, β-galactosidase II was able to degrade galactan in tomato cell walls, and its activity did increase during fruit ripening. Considered together, therefore, these two findings can explain the loss of galactose from tomatoes during ripening.

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