Plant Physiol. (1995) 108: 1099–1107

Tomato Exo-(1→4)-β-D-Galactanase[1]

Isolation, Changes during Ripening in Normal and Mutant Tomato Fruit, and Characterization of a Related cDNA Clone

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An exo-(1→4)-β-D-galactanase was isolated from ripe tomato fruit (*Lycopersicon esculentum* Mill. cv Ailsa Craig and cv Better Boy) using anion-exchange, gel filtration, and cation-exchange chromatography. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the most active fraction revealed a predominant protein band at 75 kD and several minor bands. A 30-amino acid N-terminal sequence from this 75-kD protein showed a high degree of homology with other recently identified β-galactosidase/galactanase proteins from persimmon and apple fruits (I.-K. Kang, S.-G. Suh, K.C. Gross, J.-K. Byun [1994] Plant Physiol 105: 975–979; G.S. Ross, T. Wegrzyn, E.A. MacRae, R.J. Redgwell [1994] Plant Physiol 106: 521–528) and with the predicted polypeptide sequence encoded by the ethylene-regulated SR12 gene in carnation (K.G. Raghothama, K.A. Lawton, P.B. Goldsbrough, W.R. Woodson [1991] Plant Mol Biol 17: 61–71). The enzyme focused to a single band of β-galactosidase activity on an isoelectrofocusing gel at pH 9.8. The enzyme was specific for (1→4)-β-D-galactan substrates with a pH optimum of 4.5. The only reaction product detected was monomeric galactose, indicating that the enzyme was an exo-(1→4)-β-D-galactanase. β-Galactanase activity increased at the onset of ripening in normal fruit, but no similar increase was detected in the nonripening mutants *nor* and *rin*. A tomato homolog (pTomβgal 1) was isolated using the SR12 cDNA clone from carnation as a probe. This clone showed 73% identity at the amino acid level with β-galactosidase-related sequences from apple and asparagus and 66% identity with SR12. pTomβgal 1 is a member of a gene family. Northern analysis demonstrated that pTomβgal 1 expression was ripening related in normal fruits, with lower levels apparent in the nonsoftening mutants.

Tomato (*Lycopersicon esculentum* Mill.) ripening involves marked changes in fruit texture, and these events are thought to be intimately associated with cell-wall degradation. At the biochemical level the major polysaccharide fractions of the wall undergo significant modifications in structure. Some of the most apparent and well known of these occur in the pectic fraction, including polyuronide solubilization and depolymerization and a loss of wall arabinosyl and galactosyl residues (Gross and Wallner, 1979; Gross, 1984; Seymour et al., 1987). Several pectin-degrading enzymes have been isolated from tomato, the most predominant of which, in terms of levels of wall protein and activity, are PG and PE. The latter is responsible for the removal of methyl groups from galacturonic acid residues, which make up the backbone of pectins, and PG cleaves this backbone in an endo fashion. However, recent studies have shown that softening occurs even in tomato fruit in which PG (Smith et al., 1988, 1990) or PE (Tieman et al., 1992; Hall et al., 1993) is down-regulated using antisense RNA technology. The action of PG and PE in the wall appears to be tightly controlled, and perhaps their main role is during the later stages of ripening, when rapid liquefaction of the tissue occurs (Seymour et al., 1987; Huber and O'Donoghue, 1993).

Another important group of pectolytic enzymes are those that degrade the neutral sugar component, in which galactosyl and arabinosyl residues occur as side chains attached to a rhamnosyl unit in the main galacturonan backbone. Specific information concerning the structure of these side chains is sparse because of their complexity, but in many plant tissues, including those from tomato fruit (Seymour et al., 1990), chains of (1→4)-β-D-galactan and (1→5)-linked arabinofuranosyl units are common (Carpita and Gibeaut, 1993). Fleshy fruits, including tomato, show a loss of wall galactosyl residues during ripening (Bartley, 1976; Gross, 1984; Gross and Sams, 1984; Redgwell et al., 1992; Cutillas-Iturralde et al., 1993), and in tomato and kiwifruit, these changes are accompanied by an accumulation of free Gal (Gross, 1983; Ogawa et al., 1990). The loss of cell wall Gal has also been reported in nonfruit tissues such as senescing carnation petals (De Vetten and Huber, 1990). Evidence indicates that these events are likely to

[1] This work was funded jointly by Zeneca Plant Science and the Biotechnology and Biological Sciences Research Council.

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Abbreviations: CM-cellulose, carboxymethyl-cellulose; DAA, days after anthesis; PE, pectinesterase; PG, polygalacturonase.

result from the action of β-galactosidases/galactanases, which act on galactan-rich pectins.

In tomato, three different β-galactosidase activities have been detected and partially purified, but only one of the isoforms could degrade a (1→4)-β-D-galactan isolated from tomato cell walls (Pressey, 1983; Pressey and Himmelsbach, 1984). This enzyme was identified as an exo-(1→4)-β-D-galactanase. Detailed studies of the composition and structure of tomato cell wall polysaccharides by Seymour et al. (1990) demonstrated that galactans with this linkage predominate in tomato and undergo degradation during ripening. The free Gal measured in ripening tomato fruit is likely to result from this hydrolytic activity rather than altered metabolism (Kim et al., 1991). The role of β-galactanase in tomato fruit ripening remains unknown, but it has been speculated that it plays a key part in fruit softening. In the current paper we report the isolation of an exo-(1→4)-β-D-galactanase from tomato fruit, its properties, and its developmental regulation in normal and mutant fruit during ripening. We present amino acid sequence information concerning the β-galactanase protein and report the isolation and characterization of a β-galactanase-related cDNA clone from tomato fruit.

MATERIALS AND METHODS

Plant Material

Tomato plants (*Lycopersicon esculentum* Mill.) were grown in a heated glasshouse using standard culture practices. The fruits were harvested at different stages of maturity, as determined by time from anthesis, and/or at various stages during ripening as described in "Results." All fruit were used immediately after harvest.

Enzyme Extraction

Cell-wall-bound proteins were extracted from tomato pericarp with 1.0 M NaCl and precipitated with ammonium sulfate as described by Pressey (1983). Crude extracts were used for the estimation of β-galactosidase and β-galactanase activity during fruit development and ripening. In these experiments, flowers of cv Better Boy and cv Ailsa Craig were tagged at anthesis and fruit harvested at 20, 35, 38 to 40, 41 to 43, and 45 to 48 DAA, corresponding to immature green, mature green, breaker, orange, and red ripe stages of maturity, respectively. Fruits of the nonripening mutants *rin* and *nor* (in Ailsa Craig background) were harvested at 20, 45, 55, and 65 DAA. Developmental classification of the mutant cultivars was based on the appearance of locular tissue and seed development as described by Brecht (1987) and Su et al. (1984). Both mutants showed some color development in the pericarp 55 DAA and full color 65 DAA, orange for *nor* and yellow for *rin*. Three separate 100-g extractions were prepared from the pericarp of fruits at each stage of development.

Enzyme Purification

After tissue was extracted with 1.0 M NaCl and proteins were precipitated with ammonium sulfate, tomato β-galactosidase isoforms were further purified by separation on columns of DEAE-Sephadex A-50 (4.4 × 90 cm) and Sephadex G-100 (2.1 × 85 cm) as described by Pressey (1983). The active fractions were pooled, dialyzed against 50 mM sodium acetate/acetic acid buffer, pH 5.2, and applied directly onto a fast protein liquid chromatography column of CM-cellulose (2.6 × 18 cm) (CM-52; Whatman) that had been equilibrated with the same buffer. The column was washed with 100 mL of the equilibration buffer at 1 mL min^{-1}. Proteins were eluted from the column by a linear 0 to 0.5 M NaCl gradient in 50 mM sodium acetate/acetic acid, pH 5.2 (550 mL). In all cases active fractions from the various columns were concentrated by ultrafiltration on PM10 membranes (Amicon, Beverly, MA).

Enzyme Assays

β-Galactosidase activity was assayed by measuring the rate at which it hydrolyzed *p*-nitrophenyl-β-D-galactopyranoside in accordance with procedure of Pressey (1983). Other glycosidase activities were assayed using the appropriate *p*-nitrophenyl glycoside substrates. One unit of activity was defined as the amount that released 1 μmol *p*-nitrophenol min^{-1} at 37°C. For β-galactanase activity, the reaction mixture consisted of 0.4 mL of 0.1 M sodium acetate/acetic acid buffer, pH 4.0, 0.4 mL of enzyme solution diluted with 0.2% BSA, and 0.2 mL of 1% polysaccharide substrate (2 mg/assay). Samples were incubated at 37°C for up to 4 h, and the Gal released was estimated using the Gal-dehydrogenase procedure (Kurz and Wallenfels, 1974) and verified by GC. The monosaccharides were converted to their alditol acetates and separated by GC on an SP-2330 (Supelco UK, Poole, Dorset, UK) wide-bore capillary column (30 m × 0.75 mm i.d.) at 200 to 240°C at 4°C min^{-1}.

Protein Determination

Protein concentrations (0–20 μg) were measured by the dye-binding method of Bradford (1976). BSA was used as a standard.

SDS-PAGE

SDS-PAGE was performed as described by Laemmli (1970) using a resolving gel of 10% acrylamide and a 4% stacking gel. Staining was with Coomassie blue.

IEF

Analytical IEF was performed on a Pharmacia flat-bed apparatus FBE-3000 using precast Pharmacia CleanGel IEF. The gel was rehydrated according to the manufacturer's instructions and contained 10% (w/v) sorbitol, 10% (v/v) carrier ampholytes, pH 3 to 10, and 6.25% (v/v) Pharmalyte pH 8 to 10.5. Electrode strips were soaked in 0.25 M Hepes for the anode and 1.0 M NaOH for the cathode. The gel was run according to the manufacturer's instructions at 10°C, and the pH gradient was determined by Pharmacia pI markers (pH 5.2–10.25). The focused gel was divided

such that one section was stained for protein by Coomassie blue R-250 and the other was stained for β-galactosidase activity with 4-methylumbelliferyl-β-D-galactopyranoside (0.3 mM) in 0.2 M sodium citrate/citric acid buffer, pH 4.0. The buffered substrate was applied in an overlay of filter paper on the surface of the gel, and activity was visualized under UV light after 15 min of incubation.

Protein Sequencing

Twenty micrograms of partially purified β-galactosidase II was run on SDS-PAGE and the resolved proteins were blotted from the gel onto a polyvinylidene difluoride membrane (ProBlott, Applied Biosystems) in 10 mM 3-(cyclohexylamino)-1-propane-sulfonic acid, 10% (v/v) methanol, pH 11. The blot was stained for 1 min in 0.1% Coomassie blue R-250 and destained in 50% (v/v) methanol. Protein bands were cut out and sequenced from the N terminus using an Applied Biosystems 470A gas-phase protein sequencer equipped with a 120-A on-line phenylthiohydantoin analyzer.

Polysaccharide Substrates

Spruce galactan (60% Gal, 30% Man, and 10% Glc) was obtained from Dextra Laboratories (Reading, UK). Other substrates were obtained from Sigma, and the tomato galactan was isolated as follows. Cell walls were prepared from unripe tomato fruits (Seymour et al., 1987) and a galactan-rich polysaccharide was isolated from this material using purified tomato PG and PE as described by Pressey and Avants (1973) and Pressey and Himmelsbach (1984). The galactan was purified by chromatography on a column of Bio-Gel A-15m (2.6 × 67 cm) eluted with 0.15 M NaCl at a flow rate of 0.5 mL min^{-1}. This yielded two major peaks of neutral sugar (peaks 1 and 2). Fractions from each peak were pooled, concentrated by ultrafiltration, and dialyzed against 50 mM sodium phosphate buffer, pH 6.5, prior to loading onto a column of DEAE-Sephadex A-50 (2.6 × 20 cm), which had been equilibrated with the same buffer. The sample was washed with 200 mL of 50 mM sodium phosphate buffer, pH 6.5, and sugars were eluted with a step gradient of 0.125, 0.250, and 0.50 M NaCl (200 mL of each NaCl concentration) in 50 mM sodium phosphate buffer, pH 6.5, at a flow rate of 2 mL min^{-1}. This step resolved peak 2 into two neutral sugar fractions (A and B), but no further separation of peak 1 occurred. Both the spruce galactan and the tomato galactan fractions were characterized by ^{13}C-NMR and showed predominantly (1→4)-β-linkages between galactosyl residues. Neutral sugars and uronic acids in the column fractions were detected by the phenol:sulfuric acid (Dubois et al., 1956) and *m*-hydroxydiphenyl (Blumenkrantz and Asboe-Hansen, 1973) methods, respectively. Sugar composition of the polysaccharides was determined after hydrolysis in H_2SO_4 (Selvendran et al., 1979), and monosaccharides were separated by GC as described above. TLC of saccharide mixtures was performed according to the method of Buckeridge and Reid (1994). Prior to TLC, samples were passed through columns of Dowex 50W-X8 (H^+ form), freeze dried, and dissolved in 10% (v/v) isopropanol.

cDNA Isolation and Characterization

A cDNA library from ripening tomato fruit (cv Ailsa Craig) constructed in λgt11 was purchased from a commercial source (Clontech, Palo Alto, CA). To screen the library, duplicate Hybond N^+ (Amersham) filter lifts from 15 plates, each containing approximately 24,000 plaques, were probed with ^{32}P-labeled pSR12, a clone isolated from senescent carnation petals (Raghothama et al., 1991). The filters were hybridized in Blotto (0.25% [w/v] skim milk powder, 5× SSPE, and 0.01% [w/v] SDS) at 55°C overnight and washed in 6× SSC, 0.1% (w/v) SDS at the hybridization temperature. Several positive plaques were isolated. After preparation of λ DNA and subcloning into pUC19 (Sambrook et al., 1989), one of these clones, with an insert of about 3 kb (pTomβgal 1), was fully sequenced from both directions by the dideoxynucleotide method.

RNA Extraction and Northern Blotting

Total RNA was extracted from tomato leaves, roots, and fruit pericarp as described by Smith et al. (1986) except that contaminating carbohydrate and phenolic compounds were removed by differential cetyltrimethylammonium bromide precipitation (Murray and Thompson, 1980). The RNA samples (15 μg) were separated on formaldehyde-agarose (1.5%) gels and transferred to Hybond N^+ membrane. Prehybridization and hybridization steps were conducted in 5× Denhardt's solution, 5× SSC at 65°C, and the membrane was probed with radiolabeled pTomβgal 1. Following hybridization, blots were washed twice in 3× SSC, 0.1% (w/v) SDS at 65°C for 20 min and once in 0.3× SSC, 0.1% (w/v) SDS at 65°C for 10 min.

RESULTS

Changes in β-Galactanase Activity during Development and Ripening

β-Galactanase activity showed a significant ripening-related response in normal tomatoes, with a 4-fold increase in activity between mature green and ripe fruit (Fig. 1A). This increase was observed at the breaker stage of development in Better Boy but at the orange ripe stage for Ailsa Craig. Enzyme activity in the mutants *nor* and *rin* remained at the base level of normal green fruits and did not alter significantly throughout development. In contrast to these findings, total β-galactosidase activity (Fig. 1B) showed no marked ripening-related changes and levels were similar in both normal and mutant fruits.

β-Galactosidase Isoforms and β-Galactanase Purification

Chromatography of a crude extract from ripe Ailsa Craig tomatoes on DEAE-Sephadex A-50 yielded two peaks of β-galactosidase activity, A and B (Fig. 2A). Peak A was resolved into two peaks of activity by gel permeation on Sephadex G-100 (Fig. 2B). Calibration of Sephadex G-100 was conducted with β-galactosidase (*Escherichia coli*, 116 kD), BSA (66 kD), ovalbumin (45 kD), carbonic anhydrase (29 kD), and Cyt *c* (12.4 kD). The earlier eluted peak (β-

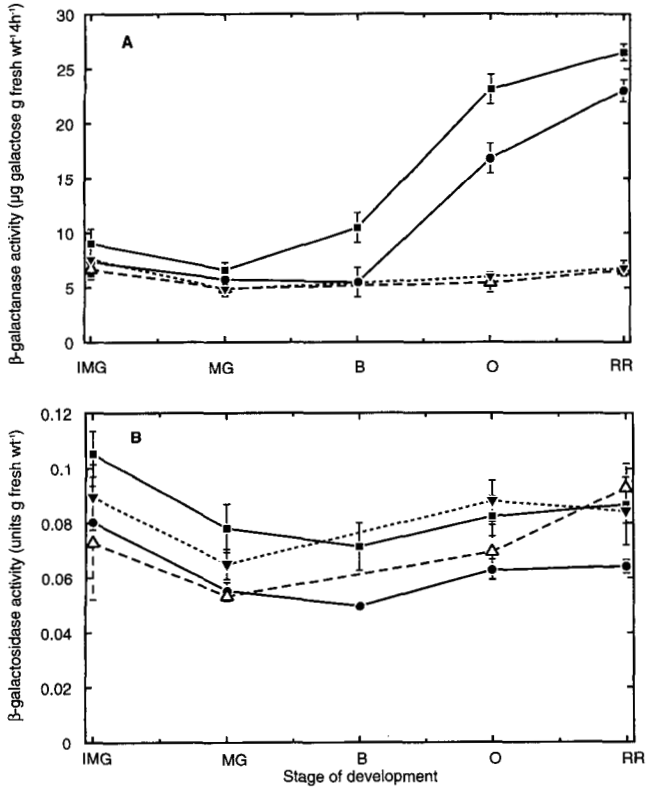

Figure 1. Changes in β-galactanase (A) and β-galactosidase (B) activities during fruit development and ripening. β-Galactanase activity was measured during a 4-h period using (1→4)-β-D-galactan-rich polysaccharide from spruce as the substrate. Normal fruit cv Better Boy (■) and cv Ailsa Craig (●) were harvested at immature green (IMG), mature green (MG), breaker (B), orange (O), and red ripe (RR) stages of development. The mutants *nor* (▼) and *rin* (△) were harvested at physiologically equivalent stages of maturity as judged by the appearance of locular tissue and seed development (see "Materials and Methods").

galactosidase I, fractions 20–23) had an apparent molecular weight of 125,000 and the second peak (β-galactosidase II, fractions 25–31) had an apparent molecular weight of 61,000. Peak B from DEAE-Sephadex (β-galactosidase III) eluted at a position on Sephadex G-100 similar to that of β-galactosidase II, with an estimated molecular weight of 72,000. Unlike Ailsa Craig, ripe Better Boy tomato fruit showed equal proportions of β-galactosidase activity in peaks A and B, on DEAE-Sephadex A-50, and fractionation of peak A on Sephadex G-100 also resulted in equivalent levels of β-galactosidase I and II, corresponding to the findings of Pressey (1983). Further purification of β-galactosidase II was achieved on CM-cellulose on which activity was eluted at 0.18 M NaCl (Fig. 2C). Only β-galactosidase II showed activity against the tomato or spruce (1→4)-β-D-galactan substrates (Table I). In Better Boy fruit, β-galactosidase II activity against *p*-nitrophenyl-β-D-galactopyranoside increased approximately 4-fold during ripening from 1.71 to 5.93 units kg^{-1} pericarp, whereas levels of β-galactosidase I and III decreased (data not shown).

The CM-cellulose fractions containing the most β-galactanase activity were analyzed by SDS-PAGE (Fig. 3A). A major band at 75 kD was always the predominant protein in fractions containing high levels of activity (Fig. 3B), although several minor bands at 66, 41, 30.5, and 29 kD were also present. Attempts were made to purify the enzyme further by affinity chromatography on lactose-agarose (Pierce) and by IEF. No further purification was ob-

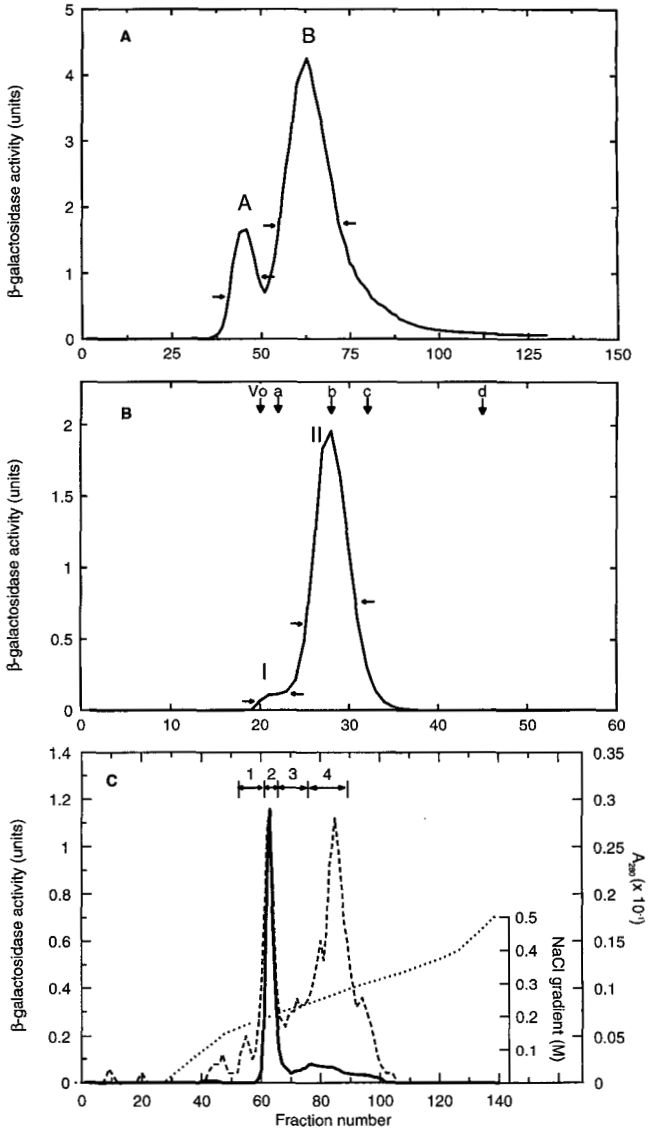

Figure 2. Purification of a β-galactosidase/β-galactanase from ripe Ailsa Craig tomatoes. A, DEAE-Sephadex A-50. The applied sample was a crude extract eluted with 50 mM sodium acetate buffer, pH 6, containing 0.1 M NaCl. B, Sephadex G-100. Resolution of peak A (DEAE-Sephadex) into two peaks of β-galactosidase activity. Column was equilibrated and run with 0.15 M NaCl at a flow rate of 0.75 mL/min. Vo, Void volume; other elution standards include: a, *E. coli* β-galactosidase (116kD); b, BSA (66 kD); c, ovalbumin (45kD); and d, Cyt *c* (12.4 kD). C, CM-cellulose. Peak II from Sephadex G-100 was applied to the column in 50 mM sodium acetate, pH 5.2, and eluted with a linear NaCl gradient of 0 to 0.5 M NaCl (. . . .). ——, β-Galactosidase activity; – – – –, A_{280}. Arrows indicate pooled fractions.

Table I. *Release of galactose by tomato fruit β-galactosidase II acting on a range of plant oligosaccharides and polysaccharides*

Substrate was incubated at 37°C for 1 h with 0.05 unit of β-galactosidase activity.

Compound	Linkage Position of Terminal β-galactosyl Residue on Aglycone	Activity[a]
		μg Gal released h^{-1}
β-Lactose	4	4.5
Galactobiose[b]		
Gal-(1→6)-β-Gal	1,6	0
Gal-(1→3)-α-Gal[b]	1,3	0
Gal-(1→3)-α-Gal-(1→4)-β-Gal[b]	1,3	0
Gal-(1→3)-α-Gal-(1→4)-β-Gal-(1→3)-α-Gal[b]	1,3	0
Gum guar	(1→6)-α-D-Gal; (1→4)-β-D-Man	0
Gum arabic	1,3; 1,6	2.3
Locust bean gum	(1→6)-α-D-Gal; (1→4)-β-D-Man	2.3
Xylan	—	0
Arabinogalactan	1,3; 1,6	0
Spruce galactan	1,4	60.0
Tomato cell-wall material[c]	1,4	28.0
Tomato galactan[d]		
Peak 1 (1:6.2)	1,4	48.7
Peak 2A (1:3.9)	1,4	42.0
Peak 2B (1:2.9)	1,4	28.0

[a] Substrate concentration = 0.2% unless otherwise stated. [b] Substrate concentration is 10 mM. [c] Gal represents about 15% of the cell-wall material (Seymour et al., 1990). [d] Ara:Gal ratios are shown in parentheses.

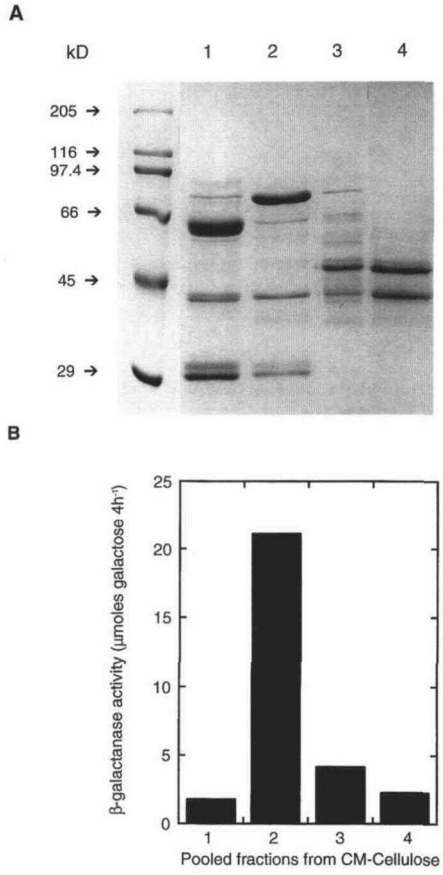

Figure 3. A, SDS-PAGE of pooled column fractions from ion-exchange chromatography on CM-cellulose (see Fig. 2C for identification of samples). Pool 1, Fractions 53 to 61; pool 2, fractions 62 to 65; pool 3, fractions 66 to 76; pool 4, fractions 77 to 89. B, β-Galactanase activity in pooled fractions from the CM-cellulose column. β-Galactanase activity was measured during a 4-h period using (1→4)-β-D-galactan-rich polysaccharide from spruce as a substrate.

tained by the affinity step (data not shown). A single band of β-galactosidase activity was obtained on the IEF gel with an apparent pI of 9.8. This active band was cut out of the IEF gel and run on SDS-PAGE, giving a predominant protein band at 75 kD and two faint bands at 29 and 30.5 kD. The 41-kD protein band appeared to be absent (Fig. 4).

After the proteins were transferred from SDS-PAGE to polyvinylidene difluoride membrane, a 30-amino acid N-terminal sequence was obtained from the 75-kD protein and shorter sequences (15 amino acid residues or less) were obtained from the N terminus of the 41- and 29-kD proteins. The 30.5-kD protein was N-terminally blocked. The N-terminal sequence of the 75-kD protein showed strong homology with other recently identified β-galactosidase/β-galactanase enzymes from persimmon and apple (Kang et al., 1994; Ross et al., 1994) and a senescence-related protein encoded by the SR12 gene from carnation (Raghothama et al., 1991) (Fig. 5). No sequence homology could be detected between the 41- or 29-kD proteins and known β-galactosidases/β-galactanases.

Catalytic Properties of the Enzyme

The pH optimum and K_m for β-galactosidase II activity against *p*-nitrophenyl-β-D-galactopyranoside were deter-

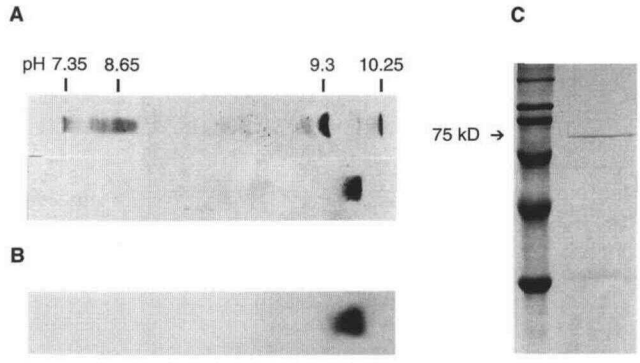

Figure 4. IEF (A) and detection (B) of β-galactosidase activity. C, Analysis by SDS-PAGE of the active gel band shown in B.

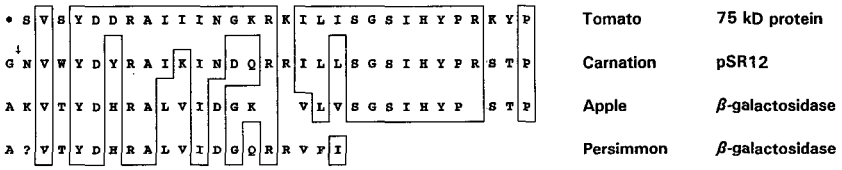

Figure 5. Comparison of the N-terminal amino acid sequence of the 75-kD protein from tomato with the translated sequence of a senescence-related cDNA from carnation petals (Raghothama et al., 1991) and the N-terminal amino acid sequences of β-galactosidases/galactanases from apple (Ross et al., 1994) and persimmon (Kang et al., 1994). The arrow (↓) indicates the predicted signal sequence cleavage site for pSR12 as determined using the program SigSequence (Rockefeller University) based on the method of Von Heijne (1986).

mined in reaction mixtures containing 0.007 units of enzyme activity. The enzyme was active in McIlvaine's buffer between pH 2.6 and 6.0 but optimally at pH 3.5. The pH optimum for the hydrolysis of tomato galactan polysaccharide (peak 1, Table I) by β-galactosidase II was 4.5. The enzyme exhibited Michaelis-Menten kinetics with a K_m value of 1.77 mM and V_{max} of 29.2 units mg^{-1} protein.

Substrate Specificity

The substrate specificity of β-galactosidase II was tested using a range of disaccharides, trisaccharides, *p*-nitrophenyl-glycosides, and polysaccharides (Table I). The enzyme was active against *p*-nitrophenyl-β-D-galactopyranoside but showed no activity against other *p*-nitrophenyl substrates, including α-D-galactopyranoside, α- and β-D-glucopyranoside, α- and β-D-mannopyranoside, α-L-arabinofuranoside, β-D-xylopyranoside, and α-L-rhamnopyranoside. Activity was recorded against 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside and 4-methylumbelliferyl-β-D-galactopyranoside. With respect to the polysaccharide substrates, the enzyme was most active against the tomato and spruce (1→4)-β-D-galactans, although the extent of Gal release was affected by galactan composition in the case of tomato. No activity was recorded against substrates with terminal nonreducing (1→3)- and (1→6)-linked Gal residues, e.g. larchwood arabinogalactan. The enzyme also showed no detectable ability to hydrolyze the disaccharide Gal-(1→6)-β-Gal and hydrolyzed lactose only slowly. Evidence that the enzyme is exo acting came from TLC of the reaction products in which only monomeric Gal was released from the galactan substrates (data not shown) and product release was linear with time up to 1 h for tomato galactan (Table II).

Table II. *Effect of incubation time on β-galactanase activity*

Two milligrams of substrate were incubated at 37°C with purified enzyme containing 0.05 unit of β-galactosidase (37°C) activity.

Substrate	Incubation Time	Gal Released
	h	*μg/2 mg substrate*
Tomato galactan (peak 2A; see Table I)	0	0
	0.25	11.3
	0.5	19.6
	1	36.3
	4	71.5
Spruce galactan	0	0
	1	53.2
	4	95.1

Isolation of a β-Galactanase-Related Clone from Tomato Fruit

The SR12 cDNA clone from carnation was used as a probe to screen a cDNA library from ripening tomato fruit. A tomato homolog was isolated and named pTomβgal 1. This clone had an insert size of 2945 bp with an open reading frame from nucleotides 75 to 2579. The deduced amino acid sequence of pTomβgal 1 cDNA shared 73% identity with β-galactosidase clones from apple and asparagus (EMBL accession No. S41889) and 66% identity with the SR12 (Fig. 6). If translation of the pTomβgal 1 sequence is initiated at the first in-frame Met, this would yield a 93.3 kD polypeptide of 835 amino acids. This polypeptide contains a hydrophobic leader sequence that is likely to be cleaved at or close to the Ser residue, position 23. This is in agreement with the N-terminal amino acid sequence data from our β-galactanase protein. After this signal peptide is removed, a predicted 90.8 kD polypeptide will remain with a pI of 6.6. The amino acid sequence of the tomato β-galactanase protein and this cDNA clone differ in 6 amino acids over a total of 30 N-terminal residues.

pTomβgal 1 Expression in Normal and Mutant Tomato Fruit

There was no detectable pTomβgal 1 gene expression in unripe Ailsa Craig fruit, but as the fruit ripened pTomβgal 1 message accumulated (Fig. 7). The transcript size was estimated at 2.9 kb. pTomβgal 1 message was not detected in *rin* 45 DAA, but the transcript was apparent in fruit harvested after a further 20 d. In contrast, *nor* fruit showed higher levels of message 45 than 65 DAA. The pTomβgal 1 message was not detectable in tomato leaves but was apparent in root tissues (Fig. 7).

DISCUSSION

A loss of cell wall galactosyl residues is a characteristic of ripening in many types of fruit, including apple, melon, avocado, kiwifruit, and tomato (Bartley, 1976; Gross, 1984; Gross and Sams, 1984; De Veau et al., 1993; Ross et al., 1993), and has also been recorded in the senescent petals of carnation (De Vetten and Huber, 1990). To date the role of

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TOMAA                                S VSYDDRAIII NGKRKILISG SIHYPRKYP                  30
 TOM   ......MGFW MAMLLMLLLC LWVSCGI.AS VSYDHKAIIV NGQRKILISG SIHYPRSTPE MWPDLIQKAK   63
APPLE  ....MGVGIQ TMWSILLLFS CIFSAAS.AS VSYDHKAIII NGQKRILISG SIHYPRSTPE MWPDLIQKAK   65
ASPARA ....MALKLV LMLMVALLAA VWSPPAVTAS VTYDHKSVII NGQRRILISG SIHYPRSTPE MWPDLIQKAK   66
 SR12  MLCGKENNVM KMMLVYVFVL ITLISCVYGN VWYDYRAIKI NDQRRILLSG SIHYPRSTPE MWPDIIEKAK   70
                     ..          .  * **   ... *....**.** ********** ****.*.***

 TOM   EGGVDVIQTY VFWNGHEPEE GKYYFEERYD LVKFIKVVQE AGLYVHLRIG PYACAEWNFG GFPVWLKYVP   133
APPLE  DGGLDVIQTY VFWNGHEPSP GNYYFEERYD LVKFIKLVQQ EGLFVNLRIG PYVCAEWNFG GFPVWLKYVP   135
ASPARA DGGLDVIQTY VFWNGHEPSP GQYYFGGRYD LVRFLKLVKQ AGLYAHLRIG PYVCAEWNFG GFPVWLKYVP   136
 SR12  DSQLDVIQTY VFWNGHEPSE GKYYFEGRYD LVKFIKLIHQ AGLFVHLRIG PFACAEWNFG GFPVWLKYVP   140
       .. .****** ********   * ***. *** **.*.*.. .  **  .**** *. ******* **********

 TOM   GISFRTNNEP FKAAMQKFTT KIVDMMKAEK LYETQGGPII LSQIENEYGP MEWELGEPGK VYSEWAAKMA   203
APPLE  GIAFRTDNEP FKAAMQKFTE KIVSMMKAEK LFQTQGGPII LSQIENEFGP VEWEIGAPGK AYTKWAAQMA   205
ASPARA GIHFRTDNGP FKAAMGKFTE KIVSMMKAEG LYETQGGPII LSQIENEYGP VEYYDGAAGK SYTNWAAKMA   206
 SR12  GIEFRTDNGP FKEKMQVFTT KIVDMMKAEK LFHWQGGPII LNQIENEYGP VEWEIGAPGK AYTHWAAQMA   210
       ** ***.* * **  *  **  *** *****  *   ****** * *****.** .*.  * .**  *  *** **

 TOM   VDLGTGVPWI MCKQD.DVPD PIINTCNGFY CDYFTPNKAN KPKMWTEAWT AWFTEFGGPV PYRPAEDMAF   272
APPLE  VGLDTGVPWI MCKQE.DAPD PVIDTCNGFY CENFKPNKDY KPKMWTEVWT GWYTEFGGAV PTRPAEDVAF   274
ASPARA VGLNTGVPWV MCKQD.DAPD PVINTCNGFY CDYFSPNKDN KPKMWTEAWT GWFTGFGGAV PQRPAEDMAF   275
 SR12  QSLNAGVPWI MCKQDSDVPD NVIDTCNGFY CEGFVPKDKS KPKMWTENWT GWYTEYGKPV PYRPAEDVAF   280
         *  ****. ****. * **  .* ****** *  * *     ******* ** .* *..*  * * ***** **

 TOM   AVARFIQTGG SFINYYMYHG GTNFGRTSGG PFIATSYDYD APLDEFGSLR QPKWGHLKDL HRAIKLCEPA   342
APPLE  SVARFIQSGG SFLNYYMYHG GTNFGRTAGG PFMATSYDYD APLDEYGLPR EPKWGHLRDL HKAIKSCESA   344
ASPARA AVARFIQKGG SFINYYMYHG GTNFGRTAGG PFISTSYDYD APIDEYGLLR QPKWGHLRDL HKAIKLCEPA   345
 SR12  SVARFIQNGG SFMNYYMFHG GTNFE.TTAG RFVSTSYDYD APLDEYGLPR EPKYTHLKNL HKAIKMCEPA   349
        ****** ** ** ****.** ****. * .*  *  ****** **.**.*  *  **. ** .* *.*** ** *

 TOM   LVSVDPTVTS LGNYQEARVF KSESGACAAF LANYNQHSFA KVAFGNMHYN LPPWSISILP DCKNTVYNTA   412
APPLE  LVSVDPSVTK LGSNQEAHVF KSESD.CAAF LANYDAKYSV KVSFGGGQYD LPPWSISILP DCKTEVYNTA   413
ASPARA LVSGEPTITS LGQNQESYVY RSKSS.CAAF LANFNSRYYA TVTFNGMHYN LPPWSVSILP DCKTTVFNTA   414
 SR12  LVSSDAKVTN LGSNQEAHVY SSNSGSCAAF LANYDPKWSV KVTFSGMEFE LPAWSISILP DCKKEVYNTA   419
       *** .. .*  **  **  *   * *  **** ***.       * *    .  **.**.**** ***  *.***

 TOM   RVGAQSAQM. .KMTPVSRGF SWESP.NEDA ASHEDDTFTV VGLLEQINIT RDVSDYLWYM TDIEIDPTEG   479
APPLE  KVGSQSSQV. .QMTPVHSGF PWQSFIEETT SSDETDTTTL DGLYEQINIT RDTTDYLWYM TDITIGSDEA   481
ASPARA RVGAQTTTM. .KMQYLG.GF SWKAYTEDTD ALND.NTFTK DGLVEQLSTT WDRSDYLWYT TYVDIAKNEE   480
 SR12  RVNEPSPKLH SKMTPVISNL NWQSYSDEVP TADSPGTFRE KKLYEQINMT WDKSDYLWYM TDVVLDGNEG   489
       .*          *  .  .  *     .          *      * **.  *  *  *****  *       *

 TOM   FLNSGNWPWL TVFSAGHALH VFVNGQLAGT VYGSLENPKL TFSNGINLRA GVNKISLLSI AVGLPNVGPH   549
APPLE  FLKNGKSPLL TIFSAGHALN VFINGQLSGT VYGSLENPKL SFSQNVNLRS GINKLALLSI SVGLPNVGTH   551
ASPARA FLKTGKYPYL TVMSAGHAVH VFINGQLSGT AYGSLDNPKL TYSGSAKLWA GSNKISILSV SVGLPNVGNH   550
 SR12  FLKKGDEPWL TVNSAGHVLH VFVNGQLQGH AYGSLAKPQL TFSQKVKMTA GVNRISLLSA VVGLANVGWH   559
       **  *  * * *. **** .. ** **** *   **** * *  .*    .  * *.. .**   ***.*** *

 TOM   FETWNAGVLG PVSLNGLNEG TRDLTWQKWF YKVGLKGEAL SLHSLSGSPS VEWVEGSLVA QKQPLSWYKT   619
APPLE  FETWNAGVLG PITLKGLNSG TWDMSGWKWT YKTGLKGEAL GLHTVTGSSS VEWVEGPSMA EKQPLTWYKA   621
ASPARA FETWNTGVLG PVTLTGLNEG KRDLSLQKWT YQIGLHGETL SLHSLTGSSN VEWGEAS... QKQPLTWYKT   617
 SR12  FERYNQGVLG PVTLSGLNEG TRDLTWQYWS YKIGTKGEEQ QVYNSGGSSH VQWGPPAW.. .KQPLVWYKT   626
       ** .* **** *. * *** *  .*.    *  *  *  **    .    **  *.*          **** ***

 TOM   TFNAPDGNEP LALDMNTMGK GQVWINGQSL GRHWPAYKSS GSCSV.CNYT GWFDEKKCLT NCGEGSQRWY   688
APPLE  TFNAPPGDAP LALDMGSMGK GQIWINGQSV GRHWPGYIAR GSCGD.CSYA GTYDDKKCRT HCGEPSQRWY   690
ASPARA FFNAPPGNEP LALDMNTMGK GQIWINGQSI GRYWPAYKAS GSCGS.CDYR GTYNEKKCLS NCGEASQRWY   686
 SR12  TFDAPGGNDP LALDLGSMGK GQAWINGQSI GRHWSNNIAK GSCNDNCNYA GTYTETKCLS DCGKSSQKWY   696
        *.** *. * ****.  *** ** ******  ** *       ***   * *  * . . **    **  **.**

 TOM   HVPRSWLYPT GNLLVVFEEW GGDPYGITLV KREIGSVCAD IYEWQPQLLN WQRLVSGKFD RPLRPKAHLK   758
APPLE  HIPRSWLTPT GNLLVVFEEW GGDPSRISLV ERGTALDAKK L*........ .......... .......... ...........   731
ASPARA HVPRSWLIPT GNFLVVLEEW GGDPTGISMV KRSVASVCAE VEELQPTMDN WRTKAYG... ...RPKVHLS   750
 SR12  HVPRSWLQPR GNLLVVFEEW GGDTKWVSLV KRTIA..... .......... .......... ...........   731
       *.***** *  **.***.*** ***   . .*  *  .

 TOM   CAPGQKISSI KFASFGTPEG VCGNFQQGSC HAPRSYDAFK .....KNCVG KESCSVQVTP ENFGGDPCRN   823
APPLE  .......... .......... .......... .......... .......... .......... ..........   731
ASPARA CDPGQKMSKI KFASFGTPQG TCGSFSEGSC HAHKSYDAFE QEGLMQNCVG QEFCSVNVAP EVFGGDPCPG   820
 SR12  .......... .......... .......... .......... .......... .......... ..........   731

 TOM   VLKKLSVEAI CS        835
APPLE  .......... ..        731
ASPARA TMKKLAVEAI CE        832
 SR12  .......... ..        731
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Figure 6. Alignment of pTomβgal 1 (TOM) deduced amino acid sequence with the 30 N-terminal amino acid residues from the tomato β-galactanase (TOMAA) and deduced amino-acid sequences of apple β-galactosidase (APPLE, Ross et al., 1994), asparagus β-galactosidase (ASPARA, EMBL accession No. S41889), and the carnation SR12 (Raghothama et al., 1991) sequences. Identical amino acids are represented by asterisks and conserved substitutions are represented by dots.

these changes in fruit ripening is unclear, but speculations focus on their involvement in cell wall loosening during extension growth (Valero and Labrador, 1993) and fruit softening (De Veau et al., 1993; Ross et al., 1993, 1994). To test these ideas it is important to identify and characterize the enzyme activities involved. Pressey (1983) partially purified three β-galactosidase isoforms from ripening tomato fruit and demonstrated that one of these enzymes was capable of degrading a (1→4)-β-D-galactan from tomato cell walls in an exo fashion. Since then, other workers have described the isolation of galactan-degrading enzymes from ripening avocado (De Veau et al., 1993) and kiwifruit (Ross et al., 1993) and very recently from persimmon (Kang et al., 1994) and apple (Ross et al., 1994). In addition, Buckeridge and Reid (1994) described the purification and properties of an exo-(1→4)-β-D-galactanase from the cotyledons of germinated lupin seeds. In this paper we report the characteristics and developmental regulation of an isoform of tomato β-galactosidase that degrades tomato cell wall (1→4)-β-D-galactans.

In the present study, three isoforms of β-galactosidase were isolated from red ripe fruit of cv Better Boy and cv Ailsa Craig, only one of which (β-galactosidase II) showed an ability to degrade (1→4)-β-D-galactan-rich polysaccharides. These findings are in close agreement with those of Pressey (1983). The activity of β-galactosidase II increased approximately 4-fold during ripening, whereas that of β-galactosidases I and III decreased, the former being virtually absent in ripe cv Ailsa Craig (Fig. 2B). In green fruit, β-galactosidase III was the most abundant isoform and the only one detectable in the yellow-ripe stage of the *rin* mutant (data not shown). However, in crude extracts of the latter, β-galactanase activity was measured (Fig. 1A), indicating that β-galactosidase II activity is present but probably masked by the abundance of isoform III. Since β-galactosidases I and III possess no obvious cell wall-

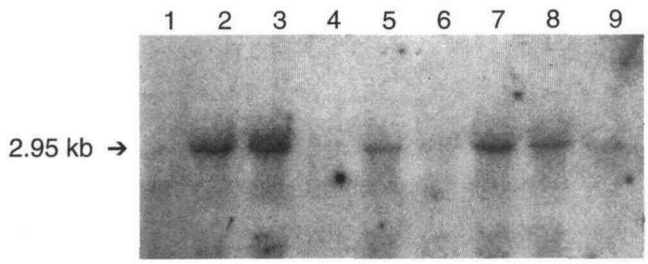

Figure 7. Northern analysis of pTomβgal 1 expression in unripe (1), breaker (2), and red ripe (3) fruit, leaves (4), and roots (5) of normal (Ailsa Craig) tomato plants. pTomβgal 1 message detected in *rin* 45 (6) or 65 (7) DAA and *nor* 45 (8) and 65 (9) DAA. Each lane contained 15 μg of total RNA extracted from tomato pericarp, leaf, and root tissues.

degrading activity, they presumably act on other substrates, perhaps being involved in the degradation of galactolipids or glycoproteins (Dey and Campillo, 1984).

After β-galactosidase II was purified by anion-exchange, gel-filtration, and cation-exchange chromatography, SDS-PAGE of the most active column fractions revealed several protein bands. There is strong evidence that the predominant band (M_r 75,000, Fig. 3A) is the β-galactosidase/β-galactanase: (a) The N-terminal amino acid sequence of the 75-kD protein shares a very high degree of homology with the N-terminal sequences from persimmon (Kang et al., 1994) and apple fruit (Ross et al., 1994) β-galactosidases/β-galactanases. Strong homology is also shown with the translated sequence of a carnation petal senescence-related cDNA clone, pSR12 (believed to encode a β-galactosidase; Woodson, 1994). (b) No other protein band was consistently associated with enzyme activity. (c) Gel-filtration chromatography indicates an enzyme with a molecular weight >60,000. (d) Putative β-galactosidase/β-galactanase cDNA clones isolated from apple (Ross et al., 1994), asparagus (EMBL accession No. S41889), and carnation (Raghothama et al., 1991) encode for mature polypeptides of 78.5, 90.6, and 82.8 kD, respectively. (e) Other proteins in the most active fractions (41 and 29 kD, Fig. 3A) show no N-terminal sequence homology to known β-galactosidases/β-galactanases. When β-galactosidases/β-galactanases have been purified from other sources, more than one protein band has appeared on SDS-PAGE. This is clearly apparent in the case of apple (77.5, 44 and 32 kD; Ross et al., 1994), persimmon (44 and 34 kD; Kang et al., 1994), and kiwifruit (67, 46, and 33 kD; Ross et al., 1993).

The affinity of this tomato β-galactosidase for substrates containing (1→4)-β-D-galactan and the presence of only monomeric reaction products indicate that this enzyme is an exo-(1→4)-β-D-galactanase in vivo. Evidence from kiwifruit (Ross et al., 1993), avocado (De Veau et al., 1993), and apple (Ross et al., 1994) indicates that other fruits possess similar hydrolytic enzymes, but their activities in vitro appear insufficient to account for the removal of wall Gal during fruit ripening. In contrast, tomato β-galactanase is highly active against its native substrates. During ripening, 1 g of tomato fruit pericarp tissue loses about 1.26 mg of wall Gal during 10 d (Seymour et al., 1990; Kim et al., 1991). Based on our measurements of β-galactanase activity against tomato cell wall material (Table I), 1 g of ripe pericarp has sufficient activity (assuming β-galactanase is one-third of total β-galactosidase activity [Fig. 2]) to release this amount of wall Gal within 5 d. These calculations indicate that this β-galactanase could be responsible for the removal of cell wall Gal during tomato fruit ripening. The comparative inability of the β-galactanase to degrade the purified tomato galactan substrates, in comparison with its activity against the cell wall material, is difficult to explain. However, we observed a much higher level of Gal release from a crude galactan preparation (242.4 μg of Gal released in 1 h from 2 mg of substrate by 0.05 unit of β-galactosidase activity) prior to purification by ion-exchange chromatography. The reason for these different effects is not clear.

The cDNA clone pSR12 from carnation (Raghothama et al., 1991), which showed close homology at the amino acid level with the tomato β-galactanase, was used successfully as a probe to isolate a tomato homolog. The clone isolated, pTomβgal 1, was closely related to recently published β-galactosidase cDNA sequences from apple (Ross et al., 1994) and asparagus and to SR12. The predicted N-terminal amino acid sequence of pTomβgal 1 is very similar, but not identical, to that of the 75-kD protein isolated from tomato fruit. Two further β-galactanase-related cDNA clones have been obtained from the tomato library using pTomβgal 1 as a probe. All of these clones are closely related but again show minor differences in nucleotide/amino acid sequence (data not shown). These findings indicate that pTomβgal 1 is a member of a gene family. A similar observation was recently reported for apple β-galactosidases.

The pTomβgal 1 clone encodes a predicted mature protein of approximately 90 kD. This is larger than the β-galactanase protein from tomato fruit, which has an apparent molecular mass on SDS-PAGE of 75 kD. However, there is evidence to indicate that β-galactosidase/β-galactanase proteins undergo some form of processing, either at the RNA level or during extraction: (a) In apple the size of β-galactosidase as predicted from the mature protein sequence was 78.5 kD, whereas an N-terminal amino acid sequence closely related to this clone was obtained from a 44-kD polypeptide. (b) The SR12 protein product had a mass of 70 kD on SDS-PAGE when purified from carnation petals (Jiang et al., 1994), but the SR12 transcript encodes a mature polypeptide of 79.6 kD. The northern analysis indicates that pTomβgal 1 is a ripening-related gene. However, the pattern of pTomβgal 1 expression is not fully consistent with measurements of β-galactanase activity, particularly in the mutants, and its relationship to β-galactanase activity requires further study.

We can conclude from the protein purification and cDNA library screening that pTomβgal 1 is a member of a gene family and that this family member shows ripening-related gene expression in normal fruit. The role of β-galactosidases/β-galactanases in fruit ripening is not known, and we are now investigating their role in tomato fruit ripening using genetically modified plants.

ACKNOWLEDGMENTS

We acknowledge Pat Barker (Babraham Research Station, Cambridge, UK) for the protein sequencing work and are grateful to Prof. M. Venis for his advice concerning the preparation of the

manuscript. We would also like to thank Dr. W.R. Woodson (Purdue University, West Lafayette, IN) for the generous gift of the carnation pSR12 clone to K.H.

Received December 15, 1994; accepted March 19, 1995.
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The EMBL/GenBank/DDBJ accession number for the sequence reported in this article is X83854.

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