Endo- β -mannanase Activity in Tomato and Other Ripening Fruits

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Abstract. High amounts of endo- β -mannanase (EC 3.2.1.78) activity were extracted from tomato (*Lycopersicon esculentum* Mill.) fruits when a high-salt-containing buffer was used. Two pI forms of the fruit enzyme were identified, one being much more basic than the many seed isoforms. The number of isoforms increased if a protease inhibitor was not used during extraction. The enzyme was found in the ripe fruits of many other species, and was particularly active in those of muskmelon (*Cucumis melo* L. Cantalupensis Group) and watermelon [*Citrullus lanatus* (Thunb.) Matsum. and Nak.]. In most fruits, enzyme activity was localized in the skin and the epidermal and subepidermal regions. The enzyme in several fruits had a molecular weight of $\approx 40,000$ and reacted immunologically with the tomato seed endo- β -mannanase antibody.

Endo- β -mannanase (EC 3.2.1.78) is present in the seeds of monocots, dicots, and gymnosperms, often in numerous isoforms (Dirk et al., 1995). It is an *endo* enzyme that hydrolyzes mannans, galactomannans and glucomannans, and may be involved in the germination of some seeds (Bewley, 1997a, b). A major role is in the post-germinative mobilization of hemicellulosic cell-wall reserves in seeds of endospermic legumes (Reid et al., 1977) and other species (Bewley and Reid, 1985). Cell-wall mannan polymers also are present in tuber (Franz, 1979; Sugiyama et al., 1973), bulb (Matsuo and Mizuno, 1974), and fruit tissues (Lecas and Brillouet, 1994; Redgwell et al., 1997; Rose et al., 1998; Tong and Gross, 1988).

The activity of endo- β -mannanase has been studied extensively in germinating and germinated tomato seeds (Groot et al., 1988; Nonogaki et al., 1992; Toorop et al., 1996; Voigt and Bewley, 1996). The enzyme has been purified (Nonogaki et al., 1995) and a cDNA clone obtained (Bewley et al., 1997). It is not present in developing tomato seeds (unpublished) but is in the surrounding fruit tissue during ripening (Bewley et al., 2000; Pressey, 1989).

Using both improved assay (Downie et al., 1994; Toorop et al., 1996) and extraction tech-

niques, we have confirmed in this paper that the enzyme is present in tomato fruit, but, unlike that in seeds, requires a high salt buffer for its extraction. Endo- β -mannanase has not been reported to be present in the fruits of other

species, even though its mannan substrate is present in their cell walls. Using our improved high-salt extraction technique, we set out to determine how widespread is the presence of this enzyme in fruits.

Materials and Methods

Fruits of tomato were obtained from a local supplier (market-ready cv. Trust) or grown under ambient daylight in a greenhouse at the university (as was the processing cv. Heinz 902). One other unknown cultivar of a field-grown market-ready 'Beef' tomato fruit was obtained from a local supermarket, as were several fruit of other species (Table 1), for which the cultivars are also unknown.

Protein/enzyme extraction. The combined skin, inner and outer pericarp regions of a tomato fruit (1–1.5 g) were removed and frozen in liquid nitrogen. These were ground to a powder in an ice-cold mortar and pestle, with washed sea sand, and 1.5–2 mL of one of the following buffers was added: acidified water pH <2 (Pressey, 1989); 0.1 M Hepes-NaOH pH 8 (low salt) or McIlvaine pH 5 (achieved using 0.2 M Na₂HPO₄ and 0.1 M citric acid) (McIlvaine, 1921) plus 0.5 M NaCl (high salt). After a clearing spin at full speed in an Eppendorf microfuge at 4 °C, the supernatant was adjusted to a known volume (usually 2 mL) and aliquots were used for assaying endo- β -mannanase activity.

Table 1. Endo- β -mannanase activities in different regions of ripened fruits of several species. Some enzyme extracts were also run on IEF gels and the activities of the isoforms determined on an overlay gel containing substrate. All fruits were mature unless otherwise noted.

Species		Enzyme activity		Isoforms
Common name	Scientific name	Tissue	(pkat/gfw)	(pI)
Tomato	<i>Lycopersicon esculentum</i> Mill.	Skin	2845	5.5, 9.1
		Outer pericarp	1592	---
		Inner pericarp	699	---
Muskmelon	<i>Cucumis melo</i> L. Cantalupensis Group	Skin	1056	5 (strong) 7.0–7.5, 5 (weak) 5.5–6.0
		Flesh	86.4	---
Watermelon	<i>Citrullus lanatus</i> (Thunb.) Matsum. and Nak.	Skin	507.5	7.0
		Flesh	85.8	---
Nectarine	<i>Prunus persica</i> (L.) Batsch	Skin	93.5	5.8, 6.3
		Flesh	93.9	---
Peach	<i>Prunus persica</i> (L.) Batsch.	Skin	32.5	0 ^z 4.9
		Flesh	24.9	19.93 ^z ---
Apricot	<i>Prunus armeniaca</i> L.	Skin	0	20.58 ^z 3.9
		Flesh	0	8.60 ^z ---
Orange	<i>Citrus sinensis</i> (L.) Osbeck	Skin + flesh	67.5	5.2, 5.5
Apple	<i>Malus $\times$domestica</i> Borkh.	Skin	1.8	6.0
		Flesh	12.8 ^y	16.22 ^x ---
Cherry	<i>Prunus avium</i> L.	Skin	6.0	0 ^z ---
		Flesh	36.8	0 ^z 5.2, 5.5
Kiwifruit	<i>Actinidia deliciosa</i> C.S Liang & A.R. Ferguson	Skin + flesh	4.6	---
Cucumber	<i>Cucumis sativus</i> L.	Skin	0.7 ^y	5.0
		Flesh	0 ^y	---
Grape	<i>Vitis vinifera</i> L.	Skin + flesh	0	---
Plum	<i>Prunus domestica</i> L.	Skin + flesh	0	---
Strawberry	<i>Fragaria $\times$ananassa</i> Duchesne	Skin + flesh	0	---
Pear	<i>Pyrus communis</i> L.	Skin + flesh	0	---
Pepper (red, green and yellow)	<i>Capsicum frutescens</i> L.	Skin + flesh	0	---
Banana	<i>Musa $\times$paradisiaca</i> L.	Skin + flesh	0	---
Mango	<i>Mangifera indica</i> L.	Skin + flesh	0	---

^zOverripe.

^yGreen.

^xRed.

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Endo- β -mannanase activity assay and isoform determination. A variation of the gel-diffusion assay of Downie et al. (1994) and Toorop et al. (1996) was used. A 0.5-mm-thick gel of 0.1% (w/v) locust bean gum (Sigma Chemical Co., St. Louis) in McIlvaine buffer (pH 5) and 1.2% agarose (Sangon, Scarborough, Ont.) was poured on Gel-Bond (Amersham Pharmacia Biotech, Baie d'Urfé, Que.) and 2-mm-diameter wells punched out 2 cm apart. Into the wells, 2 μ L of sample for assay were pipetted, or serial dilutions of *Aspergillus niger* endo- β -mannanase standard (Megazyme, Bray, Ireland). The gel was incubated at 25 °C overnight in a humid atmosphere before washing, with shaking, at $\approx$ 30 rpm, in McIlvaine buffer (pH 7) for 30 min, 0.5% w/v Congo red (Sigma) in water for 30 min, water for 2 min, 80% (v/v) ethanol for 10 min, McIlvaine buffer (pH 7) for 1–2 h, and finally in 1 M NaCl until color development was complete. The gel was scanned into a Bitmap format and printed out before the diameters of the clearing zones were measured and activity expressed in relation to the *A. niger* standards.

Isoelectric focusing and identification of the isoforms was achieved using the method of Toorop et al. (1996). Combined skin and outer pericarp tissue of cv. Trust tomato fruit (0.7 g) was extracted in 2 mL McIlvaine buffer (pH 5) plus 0.5 M NaCl, in the presence or absence of 1% plant protease inhibitor (Sigma). The extract was desalted using a Microcon-10 (10 kDa cut-off) concentrator (Amicon, Beverly, Mass.) and $\approx$ 15 μ g loaded onto the IEF gel. An extract from 66-h germinated tomato seeds (Toorop et al., 1996) was used for comparison. A wide range (Fig. 2) pI gel was used to identify all isoforms and the pI of the basic

isoform confirmed using a narrow-range (pI 8–10) gel (not shown).

Western blots and tissue prints. A polyclonal rabbit (*Oryctolagus cuniculus* L.) antibody to a purified tomato seed endo- β -mannanase (Nonogaki et al., 1995) was generously provided by Dr. Yukio Morohashi, Saitama Univ., Japan. Equal amounts of fruit protein (usually 25 μ g) were loaded and separated on SDS-PAGE gels (Laemmli, 1970) and transferred to nitrocellulose membranes by western blotting (Towbin et al., 1979) before being challenged with a 10^{-4} dilution of the primary antibody. Detection of binding of the antibody to endo- β -mannanase was achieved using a donkey (*Equus asinus* L.)-anti-rabbit HRP-labeled secondary antibody, and detection was by the enhanced chemiluminescence (ECL) method (Amersham Pharmacia Biotech, Baie d'Urfé, Que.).

Localization of endo- β -mannanase within fruits was determined by placing freshly-cut surfaces of the fruit on a 0.5-mm-thick polyacrylamide (10% T, 0.9% C) activity gel containing 0.1% (w/v) locust bean (*Robinia pseudoacacia* L.) gum in McIlvaine buffer (pH 5) and 0.5 M NaCl. After incubation at 37 °C in a humid atmosphere for $\approx$ 1 h, the gels were stained with Congo red and developed as above.

Results and Discussion

Extraction of endo- β -mannanase from tomato fruits. In the only report previous to our studies on endo- β -mannanase activity in tomato fruits, Pressey (1989) extracted 1 kg of pericarp tissue with acidified water and obtained very low amounts of enzyme activity. Using this method we were unable to

detect any activity in gram quantities of red-ripe tissue (Fig. 1). Some activity was detected in the skin and pericarp of one processing and two market-ready cultivars of tomato using 0.1 M Hepes-NaOH buffer pH 8, but most activity was obtained using McIlvaine buffer pH 5 with high salt added (0.5 M NaCl) as extraction buffer (Fig. 1). The Hepes buffer with salt added also extracted large amounts of activity, and McIlvaine buffer alone extracted $\approx$ 50% of the activity obtained in the presence of salt (not shown). The requirement for high salt in the extraction medium suggests that the enzyme is insoluble in situ, probably because it is associated with the pectin components of cell walls. The pH of extraction was unimportant because Hepes-NaOH buffer (pH 8) plus salt was as effective as the McIlvaine buffer (pH 5) plus salt. Nor did the pH of the extraction buffer affect the activity of the enzyme in the gel assay; adjusting the pH of the high-salt extracts from 5 to 8, or vice-versa, following grinding and centrifugation did not affect enzyme activity (not shown). The insoluble nature of endo- β -mannanase in the tomato fruit contrasts with the situation in the seed (Toorop et al., 1996; Voigt and Bewley, 1996) and seeds of other species, e.g. *Datura ferox* L. (Sanchez and de Miguel, 1997) and lettuce (*Lactuca sativa* L.) (Halmer and Bewley,

Fig. 1. Activity of endo- β -mannanase in extracts from red-ripe fruit tissues of tomato (combined skin and outer and inner pericarp) using two market-ready cultivars (cv. Trust, greenhouse grown, and an unknown field-grown cultivar) and one processing cultivar (Heinz 902). Extraction was in acidified water, low-salt Hepes-NaOH buffer (pH 8) or McIlvaine buffer (pH 5) plus high-salt (0.5 M NaCl). Error bars: $\pm$ variability of duplicate samples.

Fig. 2. Isoelectric focussing (IEF) gel separation of the isoforms of high-salt tomato fruit endo- β -mannanase extracts. Extraction was conducted in the presence (+) or absence (–) of protease inhibitors. The isoforms extracted from intact germinated (66 h) tomato seeds are shown for comparison.

1979), where it is highly soluble in water or low-salt buffers.

Isoforms of the tomato fruit endo- β -mannanase. The tomato fruit endo- β -mannanase obtained by Pressey (1989) reportedly had a single pI of 9.3 by isoelectric focusing. Our results confirm the basic nature of this isoform (our estimate is pI 9.1), but we also detected a more acidic isoform (Fig. 2) in the region to which all of the major isoforms of the seed migrate (Fig. 2; pI <6). During extraction of the fruit enzyme, the presence of a protease inhibitor results in only the additional pI 5.5 isoform, whereas, in its absence, there was a third isoform of $\approx$ pI 4.7. We presume that this additional isoform was the result of limited proteolysis during extraction, and was not present within the fruit.

Endo- β -mannanase in other fruits. We extended the study to determine if this enzyme is present in fruits of other species (Table 1). These were chosen from a local supermarket and, when possible, they were visually and tangibly ripe. For convenience, all extracts were made using high-salt McIlvaine buffer (pH 5), and segments of skin and inner fruit tissues were used for the assay. Of the fruits exhibiting enzyme activity, watermelon and orange yielded similar activities following high and low salt extractions; no fruit yielded lower activity when the high-salt buffer was used (not shown). The amount of activity differed greatly among species, with two cucurbit fruits, muskmelon and watermelon, clearly containing the most activity, although not as much as tomato, and largely within the skin and 2–3 mm subepidermal region (Table 1). Another member of the same family, the cucumber, exhibited little endo- β -mannanase activity, but only unripe green fruits were available. In many, but not all fruits, the greatest activity was in the outer regions, as in the tomato (Bewley et al., 2000). For some species that contained little or no activity in the ripe state, e.g., cherry, grape, plum, peach, apricot, and banana, overripe fruits were used. In apricot, there was a resultant increase in enzyme activity, but in all other fruits there was either a decline or no change. There was no enzyme activity in ripe or overripe red or green grape cultivars, and the activity in ripe red and green cultivars of apple was similar (Table 1). Some of the fruits that exhibited no endo- β -mannanase activity contain mannans in their cell walls, e.g., grape (Lecas and Brillouet, 1994), and strawberry (Redgwell et al., 1997); perhaps in these tissues a β -mannoside mannohydrolase (exo- β -mannanase) (McCleary, 1982), rather than an endo-mannanase, is involved in their degradation.

The antibody to tomato seed endo- β -mannanase was used in western blots against extracts from other fruits (Fig. 3). A number of high molecular weight bands cross-reacted in all fruit samples, but this also occurred using the pre-immune serum. Bands of molecular weight similar to that of the tomato seed enzyme were present in extracts from the outer regions of watermelon and muskmelon and from orange, and of slightly higher mol mass (43–45 kDa) in those of peach and

Fig. 3. Western blot of SDS-PAGE-separated high-salt buffer extracts from the outer regions of the fruit of various species, challenged with germinated tomato seed endo- β -mannanase antibody. Equal amounts of protein (25 μ g) were loaded in each lane.

Fig. 4. Tissue prints of segments of fruits of several species obtained by laying the tissue on gels containing galactomannan substrate and high-salt (0.5 M NaCl) for 1 h at 37 °C. Lighter areas indicate high enzyme activity. (a) Peach, (b) nectarine, (c) watermelon, (d) plum (no activity), (e) orange, and (f) muskmelon. Arrows indicate the skin of the fruit segments.

nectarine. In some lanes, more minor bands of lower molecular weight (20,000–30,000) were visible; the significance of these is unknown. Nevertheless, endo- β -mannanases of molecular weight similar to that in tomato are present in fruits of several other species, and are of sufficient homology to be recognized by the polyclonal antibody prepared from tomato seeds. No immunoreactive bands were detected in extracts of plum (Fig. 3), a fruit that exhibits no endo- β -mannanase activity.

Isoelectric focussing on a pH 4.5–8.5 IEF gel revealed one to several isoforms (Table 1). No other fruit contained a form having a basic pI as high as that in tomato.

The tissue print technique was used to confirm the location of the enzyme in several fruits; the lighter regions of the activity gel indicate enzyme activity (Fig. 4). The watermelon and muskmelon slices exhibited most activity, which was localized in the outer regions. The skin of the orange also contained considerable activity; activity was present more generally in the nectarine and peach. Consistent with the lack of activity in the enzyme assay, the plum fruit gave no response on the tissue print.

This work has shown the presence of endo- β -mannanase in the fruit of many species. It requires a high-salt buffer for extraction and it is almost exclusively located in the outermost

tissues, probably in association with cell walls. It is absent from the fruit of other species, however, even though they contain mannose polymers in their cell walls.

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