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SOLUBLE LIPOXYGENASE ISOFORMS FROM TOMATO FRUIT

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Key Word Index—*Lycopersicon esculentum*; Solanaceae; tomato; fruit; lipoxygenase isoforms; isoelectric focusing; purification.

Abstract—Extraction of maximum lipoxygenase activity from tomato fruits was shown to require the presence of 0.1% Triton X-100. The detergent appeared to have a dual role improving recovery and preventing enzyme inactivation, especially during mechanical homogenization. Ultracentrifugation of total lipoxygenase activity revealed that the majority (96%) was of a soluble form, with very little associated with the membrane fraction. By ammonium sulphate fractionation and anion-exchange chromatography, soluble lipoxygenase was purified 46-fold. Separation of lipoxygenase activity by isoelectric focusing and detection by in-gel activity staining resulted in the identification of two predominant isoforms with pI values of 4.8 and 5.0, one of which appears to be ripening-specific. On examination of reaction products by HPLC, both isoforms appeared to exclusively produce 9-hydroperoxides.

INTRODUCTION

Lipoxygenase (LOX; EC1.13.11.12) is a dioxygenase which catalyses the incorporation of molecular oxygen into unsaturated fatty acids containing a *cis,cis*-1,4-pentadiene moiety. The enzyme is involved in the production of jasmonic acid and in the wound response. However, one other process with which it has been implicated is the production of flavour volatiles during fruit ripening [1]. In fresh tomatoes, the principal volatiles which contribute to flavour are hexenal and hexanal [2]. Both are thought to be synthesized from LOX-derived hydroperoxides (HPOs), *via* a pathway which involves HPO-lyase as outlined previously [3]. In tomato, linoleic and linolenic acids are the principal LOX substrates and the hydroperoxides formed will be 9- or 13-isomers depending on the position of oxygen insertion and hydrogen removal [4]. Indications are that the vast majority of HPOs formed by tomato LOX are the 9-isomers [5–7]. This situation creates an intriguing paradox owing to the regiospecificity of HPO-lyase, which only metabolises 13-HPOs [6]. Thus, characterization of the isoforms of LOX is essential to establish which ones are capable of catalysing the production of the 13-HPOs necessary for volatile production.

Tomato LOX was traditionally believed to be a soluble enzyme and it was partially purified and characterized as such [8–10]. Just recently, attention has focused on the membrane-associated form, which reportedly accounts for between 20 and 40% of total LOX activity, the remainder being cytosolic [11–13]. However, although two locations are now suggested as sites for tomato LOX, there is, as yet, no evidence for distinct soluble and membranous LOX proteins [14].

Here, we examine further the occurrence of soluble and membrane-associated tomato LOX forms and suggest that the actual proportion of membrane-associated LOX is much lower than previously reported. Furthermore, using isoelectric focusing (IEF) and in-gel activity staining, we show for the first time that soluble tomato LOX exists as multiple isoforms.

RESULTS AND DISCUSSION

Extraction and localisation of LOX

In order to optimize the recovery of total LOX from tomato fruit, by ensuring that both membrane-associated and soluble LOX forms were extracted, the effect of the non-ionic detergent, Triton X-100, in the extraction was examined (Fig. 1). Inclusion of the detergent increased the recovery of total LOX activity by *ca* eight-fold and this increased yield could be achieved by the inclusion of relatively low amounts (0.1%) of the detergent. Triton X-100 has previously been used in the extraction of LOX from a number of plant sources, including eggplant [15], kiwifruit [16], rice [17] and soya bean [18]. In most of these cases, the detergent was used at much higher concentrations (1–1.5%), presumably to solubilize membrane bound

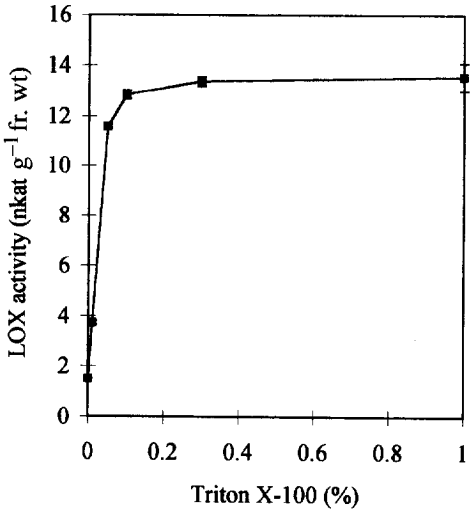

Fig. 1. Effect of Triton X-100 on LOX recovery.

activity; it is possible that Triton X-100 was having a similar effect here. However, previous reports have shown that the membrane-bound LOX in tomato fruit accounts for, at the most, 20–40% of total LOX activity [11–13], not the 90% as suggested by Fig. 1. This discrepancy could be accounted for by varietal differences in tomatoes employed in the various studies or possibly by the fact that the presence of Triton X-100 has effects additional to the simple solubilization of membrane-bound LOX. The presence of Triton X-100 may have prevented the association of LOX activity with the initial 10 000 *g* pellet fraction, thus increasing apparent yield. This is possible since it is known that LOX will stick non-specifically to membrane fractions [19]. An alternative explanation would be that Triton X-100 somehow stabilizes the LOX activity during the extraction process.

Triton X-100 could have been acting as a surfactant to reduce frothing and, hence, denaturation of the LOX enzyme during extraction. In order to examine this possibility, two extraction techniques, a Waring blender, in which frothing was likely to be maximal, and a pestle and mortar, in which frothing was likely to be minimal, were compared (Table 1). In the presence of Triton X-100, there was little apparent difference in yields of LOX activity using these two extraction techniques. However, in the absence of Triton X-100, yields were reduced by 48% using the pestle and mortar and by 91% using the blender. The protective action of Triton X-100 was further demonstrated by extracting LOX using a pestle and mortar in the absence of the detergent and, subsequently, subjecting this soluble LOX to homogenization with a Polytron in the presence or absence of Triton X-100 (Table 2). In the absence of detergent, LOX activity declined to 23% of its initial value following homogenisation. In comparison, in the presence of the detergent, the activity actually appeared to increase slightly. These results show that the inclusion of Triton X-100 in the extraction medium protected the LOX activity from denaturation during mechanical homogenisation. This may have been *via* a non-specific effect on frothing but the inclusion of Antifoam A, in place of Triton X-100, failed to protect the activity. The possibility thus exists that Triton X-100 has a more specific stabilizing effect on LOX activity but the nature of this, if it occurs, is unknown.

Table 2. Protection of LOX activity during Polytroning by Triton X-100. LOX was extracted from ripe tomato fruits using a pestle and mortar in the presence of 0.1 M Pi buffer pH 6.5 containing 1 mM EDTA. After centrifugation at 10 000 *g*, the supernatant was treated as shown. LOX activity (nka^{-1} fr. wt), values in parentheses are per cent control

Treatment	No addition	0.1% Triton X-100
No Polytron	4.33 (100)	4.33 (100)
Polytron (5 sec)	1.00 (23)	4.67 (108)

Since mechanical homogenisation dramatically reduces the recovery of tomato LOX activity this may have influenced previous reports on the relative quantities of soluble and membrane-bound enzyme in the fruit. This possibility was examined by comparing soluble and membrane-associated concentrations of the enzyme in fractions prepared from mechanically-homogenized or hand-homogenized samples in the presence or absence of Triton X-100 (Table 1). In

Table 1. Influence of homogenization method and detergent on recovery of LOX from tomato: (a) effects on total LOX, values in parentheses are per cent maximum recovered activity; (b) fractionation of total LOX by ultracentrifugation (180 000 *g*) where 180S = supernatant and 180 P = pellet, values in parentheses are per cent total LOX for each extract; (c) following washing in extraction buffer 180P from (b) was re-centrifuged providing supernatant (180S) and pellet (180P, resuspended in 1 ml 0.1 M phosphate pH 6.5, 2% Triton X-100)

Extract	(a) Total LOX (nkat^{-1} g fr. wt)	(b) 180S (nkat^{-1} g fr. wt)	180P	(c) 180S (nkat^{-1} g fr. wt)	180P
WB	1.77 (9)	0.82 (46)	0.95 (54)	0.06	0.50
WB + Triton	19.22 (100)	18.50 (96)	0.72 (4)	0.42	0.05
P & M	10.07 (52)	9.18 (91)	0.89 (9)	0.29	0.44
P & M + Triton	17.53 (91)	16.83 (96)	0.70 (4)	0.50	0.03

the absence of detergent, extraction using the Waring blender gave apparent ratios of soluble to membrane-bound LOX activity of *ca* 1:1. When the pestle and mortar was used in the absence of Triton X-100, the absolute amount of membrane-bound LOX activity was the same as that found in the sample extracted using the Waring blender. However, the absolute activity in the soluble fraction was more than one order of magnitude greater. The net result of this difference was that in this case the ratio of soluble to membrane-bound activity now appears to be 10:1. This result suggests that mechanical homogenization results in the inactivation of the soluble LOX activity, but has little or no effect on the membrane-associated fraction. In the presence of Triton X-100, both extraction techniques gave equivalent absolute amounts of soluble and membrane-bound LOX activity and, in this case, the ratio of the two was *ca* 24:1. The inclusion of other 'protective' agents, EGTA and 7% sucrose, did not affect these values. Washing the 180 000 *g* 'microsomal pellet' with 0.1% Triton X-100 resulted in the 'solubilization' of most of this membrane fraction.

Taken together, these findings suggest that homogenization and extraction conditions could combine to misconstrue the apparent proportion of soluble to membrane-bound LOX activities. More realistic values can be obtained if extractions are made in the presence of low concentrations of Triton X-100 or if homogenization is carried out using a pestle and mortar. In this instance, soluble LOX appears to account for up to 96% of the total activity. The remaining 4% of LOX activity was pelletable at 180 000 *g* but was easily solubilized by 0.1% Triton X-100, indicating that its association with the membrane fraction was not particularly strong. It remains to be seen whether or not this represents a true membrane-bound isoform or simply a fraction of the soluble LOX that becomes associated with the membrane during extraction. However, it is apparent that the bulk of tomato LOX activity can be solubilized using a Waring blender in the presence of Triton X-100. The nature of this soluble LOX activity was further investigated.

Partial purification of soluble LOX

Having found that quantitatively the most important LOX was the soluble form, it was decided to characterize this further. As a first step, a simple purification protocol utilizing ammonium sulphate precipitation and anion-exchange chromatography (Fig. 2) was carried out. Effective chromatography required the presence of 0.1% Triton X-100, which appeared to prevent LOX from binding non-specifically, to other proteins and eluting with them as multiple peaks. Together, these steps removed 99% of contaminating proteins and resulted in a 46-fold purification (Table 3).

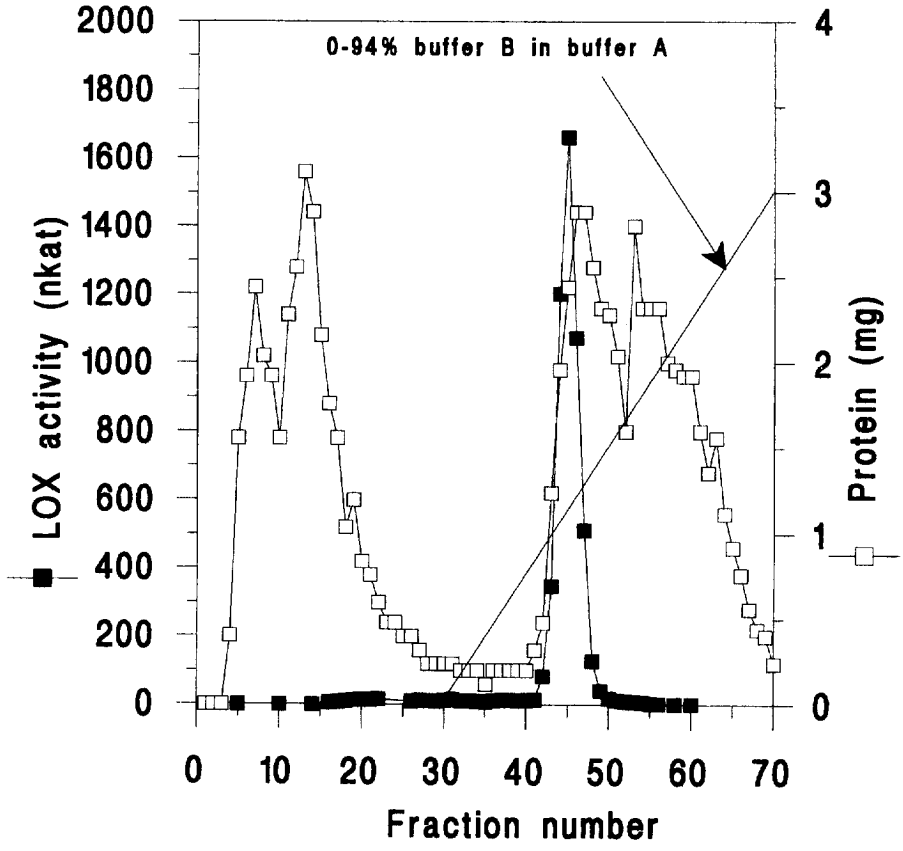

Fig. 2. Elution profile of LOX from anion-exchange column.

Table 3. Partial purification of soluble tomato LOX

Purification step	Total activity (nkat)	Total protein (mg)	Specific activity (nkat mg^{-1} protein)
Crude	8020	501	16.0
$(NH_4)_2SO_4$ ppt	5730	181	31.7
1st Econo Q	5250	13.5	388.9
2nd Econo Q	4200	5.71	735.6

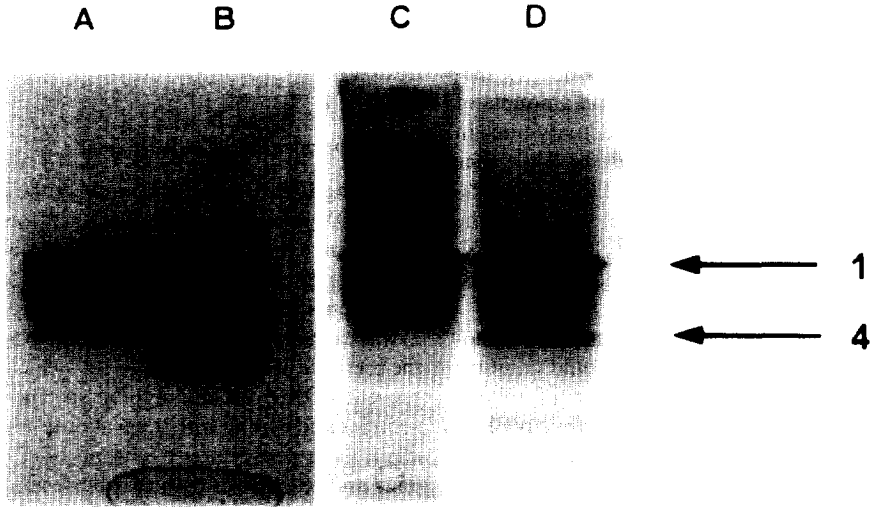

Fig. 3. Separation of LOX isoforms by vertical IEF. Gels were stained for LOX activity (lanes A and B) and then protein (C and D). Sample A and C was from green fruits, while B and D was from ripe fruits. 1 = isoform 1 (pI 5.0), 4 = isoform 4 (pI 4.8).

This compared favourably with other reports on tomato LOX purification. For soluble LOX, published degrees of purification include 26-fold [9], 27-fold [8], 35-fold [10] and 300-fold [7]. A membrane-associated LOX has been purified 49-fold [12].

Separation of soluble LOX isoforms by IEF

The partially purified tomato LOX was subjected to vertical IEF in conjunction with in-gel LOX activity staining to examine the isoform profile. Historically, IEF has proved to be an effective way of separating LOX activities into constituent isoforms, successes include pea [20], soya bean leaf [21] and cucumber [22]. Two major isoforms in tomato fruits could be identified, along with two further small bands (Fig. 3). All four isoforms had acidic pI values between 4.8 and 5.0 and M_rs, estimated by SDS-PAGE, of 97 000. These pI values are in close accordance with the value of 5.1 previously reported for a single soluble LOX band separated by IEF over a much broader ampholyte range [14]. The small differences in charge between the isoforms in Fig. 3 could explain why, on chromatofocusing [14], we could only resolve a single peak of LOX activity. Two isoforms of tomato LOX have previously been identified in the membrane fraction [17]; this is the first report of isoforms from the soluble phase.

The fact that all four isoforms were attributable to genuine LOX activity was confirmed by finding (a) that band formation required the presence of a suitable unsaturated LOX substrate and, that substituting oleic acid for linoleic acid, prevented any band formation; (b) that band formation occurred in the presence of cyanide, thus ruling out heme-catalysed oxidation [23]; (c) the bands were able to produce HPOs (see next section); (d) the bands cross-reacted with a LOX polyclonal antibody raised against soya LOX protein. This antibody successfully recognised isoforms 1–3 in their native states in IEF gels, but isoform 4 was not recognized unless it was subject to denaturing electrophoresis on SDS-PAGE gels.

Examination of the isoform profiles from green and ripe fruits revealed that the isoform profile changed during ripening (Fig. 3). Green fruits were dominated by isoform 1. Ripe fruits, in contrast, had lower amounts of isoform 1 and contained mostly isoform 4. Total LOX activity remained fairly constant from green-breaker fruits to red-ripe fruits and only seemed to fall significantly in over ripe fruits (data not shown).

By excising stained bands corresponding with isoforms 1 and 4, we obtained sufficient protein to

Table 4. *N*-terminal sequence homology between isoform 1 and *tomlox A* previously sequenced [24]

Position	*tomlox A*	Isoform 1
1	V	V
2	G	G/L
3	G	I
4	L	L
5	I	I
6	G	G
7	G	K
8	H	H
9	H	H
10	D	D

attempt *N*-terminal sequence analysis. Isoform 1 showed considerable sequence homology to a *tomlox A* previously sequenced [24] (Table 4). Unfortunately, sequence data could not be obtained for isoform 4 since this appeared to be *N*-terminally-blocked. This result, along with the differential responses of isoforms 1 and 4 to the LOX antibody would suggest that these represent different LOX proteins and, perhaps, that they are the products of separate genes.

Investigation of hydroperoxide products of LOX isoforms

Bands corresponding with isoforms 1 and 4 were separated by IEF, detected by activity staining, excised from the gel and then macerated. This mulch was then incubated with linolenic or linoleic acid and the HPOs produced were then separated by HPLC. Both isoforms appeared to produce 9-HPO exclusively, with no detectable 13-HPO present. This observation is consistent with the fact that crude tomato LOX has been found to produce predominantly 9-HPOs both in our studies and in those of several other groups [5–7]. However, it is possible that these isoforms are capable of 13-HPO production *in vivo*, as a number of factors *in vitro* have been shown to influence HPO isomer formation by other LOXs. These include pH [25], oxygen tension [9] and LOX-binding to membranes [26]. The possible role of these isoforms in tomato fruit flavour volatile formation thus remains intriguing.

EXPERIMENTAL

Plant material. Tomato (*Lycopersicon esculentum* Mill. cv. Ailsa Craig) plants were grown under glasshouse conditions and fruit were harvested at the ripening stage required. Locular tissue was removed and the pericarp was then used directly or frozen in liquid N_2 and stored at $-70°$.

LOX extraction. Diced pericarp was homogenized using either a Waring blender or a pestle and mortar in 0.1 M Pi buffer pH 6.5 and 1 mM EDTA (1 ml g^{-1} tissue) in the presence or absence of 0.1% (w/v) Triton X-100. The homogenate was centrifuged at 10 000 *g* for 20 min. Where indicated (Table 1), this crude extract was subjected to ultracentrifugation at 180 000 *g* for 1 hr and separated into soluble (supernatant) and microsome (pellet) frs.

LOX purification. Crude LOX was fractionated by the addition of $(NH_4)_2SO_4$. Enzyme activity was precipitated at 30–55% satn and the pellet collected by centrifugation at 10 000 *g* for 50 min. Protein was resuspended in 0.1 M Pi buffer pH 6.5 containing 30% (v/v) glycerol and frozen at $-20°$ until further use. The LOX pellet was then thawed and desalted by passage through a column of Sephadex G-25M (PD-10, Pharmacia) previously equilibrated with 20 mM Pi buffer pH 7.5 containing 0.1% (w/v) Triton X-100 (Buffer A). This sample was loaded onto two Econo Q (Bio-Rad) columns connected in series, which had been previously equilibrated with Buffer A. LOX was eluted by a linear gradient of Buffer A, containing 0.5 M NaCl (Buffer B), as indicated in Fig. 2. The active frs from two Econo Q column runs were then pooled and desalted as described above. Protein was then loaded onto a single Econo Q column. Active frs were then pooled and concd by ultrafiltration in an Amicon C-50 concentrator. Enzyme could then be stored in the presence of 30% (v/v) glycerol at $-20°$ until required.

Vertical IEF. Vertical IEF was performed using a Bio-Rad mini Protean gel system [27] but with the following modifications. Ampholytes used were in the range pH 4.5–5.4 (Pharmalyte, Pharmacia), 0.1% Triton X-100 was included in the gels and the gel temp. was maintained at *ca* 4° during the 5 hr run regime of ref. [21]. Following IEF, gels were stained for LOX activity by a method slightly modified from ref. [28]. Linoleic acid (100 μl) and *o*-dianisidine were mixed in 15 ml EtOH, before adding 85 ml 0.1 M Pi buffer 6.5. Gels were incubated for *ca* 30 min at room temp and then rinsed in dist. H_2O. Gels were then stained to detect proteins using Brilliant Blue G perchloric acid soln (Sigma).

LOX assay. LOX activities were determined at 25° using a Clark-type oxygen electrode. The reaction mixt. (3 ml) contained 0.1 M Pi buffer pH 6.5 (2.5–2.79 ml), 670 μM linoleic acid (200 μl) and LOX (10–300 μl). Linoleic acid substrate was prepd as described in ref. [29].

Hydroperoxide analysis. Following identification by in-gel activity staining, LOX isoforms were cut from the gel and macerated in 200 μl of 20 mM Pi buffer pH 7.5 containing 0.1% Triton X-100. The gel homogenate was then incubated for 20 min at room temp. in the presence of 0.1 M Pi buffer pH 6.5 and 1.3 mM linoleic acid, in a final vol. of 20 ml. O_2 was gently bubbled through the reaction mixt. throughout the incubation. HPOs were recovered from the reaction mixt. and sepd by HPLC using a method based on ref. [30] employing the same prepn and running buffers using a Texil 10 μm silica particle column. The identity of the HPOs was confirmed using standard 13-HPO

produced using soya bean LOX, [30] and 9-HPO produced by crude tomato LOX. In each case, confirmation of HPO identity was also carried out using the $NaBH_4$-induced shift in HPLC elution, as described previously [30].

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