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Characteristics of a Membrane-Associated Lipoxygenase in Tomato Fruit[1]

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ABSTRACT

Microsomal membranes isolated from the pericarp of mature-green tomato (*Lycopersicon esculentum*) fruit rapidly metabolize exogenous radiolabeled linoleic acid into fatty acid oxidation products at 22°C. The reaction is strongly inhibited by *n*-propyl gallate, an inhibitor of lipoxygenase. The membranes also rapidly metabolize 16:0/18:2* phosphatidylcholine into radiolabeled oxidation products that comigrate on TLC plates with those formed from free linoleic acid. At 30°C, the formation of fatty acid oxidation products from 16:0/18:2* phosphatidylcholine is slower, and there is an initial accumulation of radiolabeled linoleic acid that is not evident at 22°C, which can be attributed to the action of lipolytic acyl hydrolase. Radiolabeled phosphatidic acid and diacylglycerol are also formed during metabolism of 16:0/18:2* phosphatidylcholine by the microsomal membranes, and there is no breakdown of either linoleic acid or phosphatidylcholine by heat-denatured membranes. When Triton X-100 treated membranes were used, the same patterns of metabolite formation from radiolabeled linoleic acid and 16:0/18:2* phosphatidylcholine were observed. Thus, the enzymes mediating the breakdown of these radiolabeled compounds appear to be tightly associated with the membranes. Collectively, the data indicate that there is a lipoxygenase associated with microsomal membranes from tomato fruit that utilizes free fatty acid substrate released from phospholipids. The microsomal lipoxygenase is strongly active over a pH range of 4.5 to 8.0, comprises approximately 38% of the total (microsomal plus soluble) lipoxygenase activity in the tissue, has an apparent K_m of 0.52 millimolar and an apparent V_{max} of 0.186 millimoles per minute per milligram of protein. The membranous enzyme also cross-reacts with polyclonal antibodies raised against soybean lipoxygenase-1 and has an apparent molecular mass of 100 kilodaltons.

Lipoxygenase (EC 1.13.11.12) is a dioxygenase that catalyzes the peroxidation of fatty acids containing a *cis,cis*-1,4-pentadiene configuration. Distinguishable isozymes of lipoxygenase have been described for a number of tissues in different plant species (8) and the best characterized of these are the three isozymes of soybean known as lipoxygenase-1, -2, and -3 (18). The various isozymes of lipoxygenase have different pH optima and are distinguishable on the basis of their isoelectric points or substrate positional specificity. In some cases as well, the isozymes are immunologically distinguishable. For example, soybean lipoxygenase-1 and -3 are immunologically distinct, whereas polyclonal antibodies to lipoxygenase-2 of soybean cross-react with lipoxygenase-1 and -3 (24). There is also evidence that the three lipoxygenase isozymes of soybean are encoded by different genes and that they have regions of homology with a lipoxygenase gene cloned from human leukocytes (10).

Most lipoxygenases clearly prefer free fatty acid substrates and show little reactivity toward esterified fatty acids. One notable exception is reticulocyte lipoxygenase, which readily attacks phospholipid fatty acids containing a *cis,cis*-1,4-pentadiene configuration (27). In plant tissues, the fatty acid hydroperoxides formed by the lipoxygenase reaction are further metabolized through one of two major pathways involving hydroperoxide lyase or hydroperoxide dehydrase (11). The hydroperoxide lyase pathway can lead to the formation of hexanal or *cis*-hexenal as well as traumatin (wound hormone) (11). The hydroperoxide dehydrase pathway results in the formation of allene oxide, which can be hydrolyzed to form ketols or can undergo rearrangement and cyclization leading ultimately to jasmonic acid, a compound known to facilitate senescence (32).

A precise physiological function for lipoxygenase has not been identified, although the enzyme has been implicated in growth and development, senescence, wounding, and pest resistance (11). Fractionation studies designed to establish the subcellular localization(s) of lipoxygenase have proven problematic in part because soluble lipoxygenase tends to adhere nonspecifically to membranes (29). *In situ* immunogold labeling has shown that lipoxygenase is randomly distributed throughout the cytoplasm in storage parenchyma cells of germinating soybean (31). As well, biochemical and immunological measurements have indicated that the activity of lipoxygenase is highest during the early stages of soybean germination suggesting that the enzyme may play a role in reserve mobilization (25). Fatty acid hydroperoxides, the initial product of the lipoxygenase reaction, have deleterious effects on membranes and proteins (15, 20), and their generally toxic nature has prompted the proposal that lipoxygenase may provide chemical defence against insect pests (28). The lipoxygenase reaction also results in the formation of activated oxygen (13, 17).

Most fractionation studies have indicated that lipoxygenase is in the cytoplasm and thus predominantly soluble, although there are some reports of particulate lipoxygenase activity. For example, lipoxygenase has been detected in both stroma and thylakoids of chloroplasts (4), in mitochondria (9), and in vacuoles (33). A membrane lipoxygenase has also been purified from tulip bulbs that is distinguishable by SDS-PAGE

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from lipoxygenase-1 of soybean (26). In the present study, we describe a lipoxygenase activity associated with microsomal membranes from tomato fruit that comprises 38% of the total (microsomal plus soluble) lipoxygenase activity in the tissue, appears to utilize free fatty acid released from membrane phospholipids as substrate and is immunologically related to its soluble counterpart and to soybean lipoxygenase-1.

MATERIALS AND METHODS

Plant Material and Fractionation

Tomato fruit (*Lycopersicon esculentum* L. cv Caruso) were grown under greenhouse conditions and harvested at the mature-green stage. Pericarp tissue was cut into small pieces (≈10 mm^3) and suspended (≈1 g/mL) in cold homogenizing buffer (100 mM Mops, 10 mM EGTA, and 7% sucrose at pH 7.6). The suspension was homogenized with a Polytron tissue homogenizer (Brinkmann Instruments) for four 20 s periods at a setting of 5 with 50 s intervening cooling periods. The resulting homogenate was filtered through four layers of cheesecloth, and the filtrate was centrifuged at 12,100*g* for 20 min. The supernatant was centrifuged at 180,000*g* for 60 min to yield a pellet of microsomal membranes and a soluble fraction. The microsomal pellet was resuspended in an equivalent volume of cold homogenizing buffer and recentrifuged at 180,000*g* for 60 min. The resulting pellet was resuspended in 4 mL of homogenizing buffer. The soluble fraction was passed through a 300,000 D cut-off filter (Omega cell 150 [60 mm diameter] Filtron Technology Corp.) using nitrogen gas for pressure to remove any residual membrane. Protein was measured by the method of Bradford (3) using bovine serum albumin as a standard. Density gradient fractionation of the microsomal membranes and measurements of marker enzymes were carried out as described previously (23).

Phospholipid Metabolism

Phospholipid metabolism was measured using radiolabeled 16:0/18:2* phosphatidylcholine [L-α-(1-palmitoyl, 2-linoleoyl-[1-^{14}C] phosphatidylcholine (2.06 GBq/mmol), New England Nuclear] as substrate. The basic assay mixture contained 50 mM Hepes (pH 7.0), 150 mM KCl, 0.2 mM EGTA, 1 mM $MgCl_2$, 45 to 100 μg of microsomal membrane protein, and 1.8 kBq of [1-^{14}C]-16:0/18:2* phosphatidylcholine in 0.1% Triton X-100 in a final volume of 0.5 mL. In some experiments, 250 μM $CaCl_2$ was added as well to give a free Ca^{2+} concentration of 40 μM. The assay mixture was incubated at 22°C (or 30°C) for varying periods up to 60 min, and the reaction was terminated by the addition of 0.1 mL of 4 N HCl. Lipids were extracted by the addition of 1.5 mL of 2:1 chloroform:methanol (v/v), and the lipid extract was analyzed for radiolabeled phosphatidic acid, diacylglycerol, free fatty acids, and remaining phosphatidylcholine by TLC essentially as described previously (2). An aliquot of the lipid extract (0.7 mL) was transferred to a test tube, dried under N_2, redissolved in 30 μL of 2:1 (v/v) chloroform:methanol, and spotted on Whatman LK5D plates. The plates were partially developed in chloroform:acetic acid:methanol:water (70:25:5:2 v/v) to a distance of 4 inches, completely dried under N_2, and then fully developed in petroleum ether:diethyl ether:formic acid (70:30:1 v/v). Regions of the plate corresponding to phosphatidic acid, diacylglycerols, free fatty acids, and phosphatidylcholine were scraped, mixed with 5 mL of scintillation fluid (Aquamix, ICN) and counted in a Beckman LS6800 Scintillation Counter. The separated lipids were identified using authentic standards.

The water-soluble radiolabeled metabolites formed from choline-labeled phosphatidylcholine by microsomal membranes were measured as described previously (21). The reaction mixture contained 4 kBq of L-dipalmitoyl[choline-methyl-^{3}H] phosphatidylcholine (2.1 TBq $mmol^{-1}$, New England Nuclear) and 600 μg of microsomal membrane protein in a final volume of 1 mL. The reaction was allowed to proceed for 60 min at 22°C. In addition, the prospect that phosphorylcholine could be converted to choline by a nonspecific phosphatase associated with the microsomal membranes was tested by adding 2 kBq of radiolabeled phosphorylcholine (methyl-^{14}C, 2.22 GBq $mmol^{-1}$, New England Nuclear) to the same reaction mixture and measuring the levels of radiolabeled choline and radiolabeled phosphorylcholine at the end of the reaction after separating them by TLC as described previously (19).

Lipoxygenase Assays

Lipoxygenase activity was measured spectrophotometrically or by monitoring the conversion of radiolabeled linoleic acid to oxidation products. For measurements of radiolabeled linoleic acid breakdown, the reaction mixture contained 50 mM Hepes (pH 7.0), 150 mM KCl, 0.2 mM EGTA, 1 mM $MgCl_2$, 45 to 100 μg of microsomal or soluble fraction protein and 1.8 kBq of [U-^{14}C]linoleic acid (35 GBq $mmol^{-1}$, New England Nuclear) in a final volume of 0.5 mL. The reaction mixture was incubated at 22°C for varying periods up to 60 min and then terminated by the addition of 0.1 mL of 4 N HCl. The radiolabeled reaction products were extracted, separated by TLC, and quantified by the same procedure that was used for measuring the products of 16:0/18:2* phosphatidylcholine metabolism. In some experiments, 100 units of soybean lipoxygenase-1 (Sigma) rather than microsomal membranes were used as a source of enzyme. For pH profile determinations, 50 mM Hepes was replaced by 130 mM phosphate, Tris, or Ches[2] adjusted to specific pH values.

Substrate for the lipoxygenase spectrophotometric assay was prepared by dissolving 100 μL of Tween 20 in 20 mL of 50 mM Mops (pH 7.0), adding 20 μL of linoleic acid, mixing thoroughly, and incubating the substrate mixture under N_2 for 10 min at 22°C. The reaction mixture contained 1 mL of substrate, 100 μg of microsomal membrane protein or 75 μg of soluble fraction protein, and 50 mM Mops (pH 7.0) in a final volume of 3.5 mL, and the change in absorbance at 234 nm was followed over a 3 min period.

In Vivo Radiolabeling of Phosphatidylcholine

Seeds (≈0.7 g) of *Phaseolus vulgaris* L. (cv Kinghorn) were imbibed overnight in 2.5 mL of distilled water containing 37 MBq of phosphorus-32 (10.6 TBq/mg P, ICN) in order to label endogenous phosphatidylcholine. The seeds were frozen

[2] Abbreviation: Ches, 2-[N-cyclohexylamino]-ethanesulfonic acid.

in liquid nitrogen and ground to a powder using a mortar and pestle. Lipids were exhaustively extracted from the homogenate using 2:1 (v/v) chloroform:methanol. The organic phase was dried down under N_2, and the lipids were taken up in 150 μL of 2:1 (v/v) chloroform:methanol. The lipids were separated by TLC using a chloroform:acetic acid:methanol:water (70:25:5:2 v/v) solvent system, and phosphatidylcholine was identified using pure phosphatidylcholine. The radiolabeled phosphatidylcholine was extracted from the silica using 2:1 (v/v) chloroform:methanol, dried down under N_2, and taken up in 1.2 mL of Mops homogenization buffer.

Antibody Preparation and Western Blot Analysis

Soybean lipoxygenase-1 (Sigma) was further purified on native 8% acrylamide gels. The major active band was identified by activity-staining (6) and electroeluted using a Bio-Rad model 422 Electro-Eluter. Polyclonal antibodies against the purified lipoxygenase protein were raised in chicken using the immunization and IgG isolation protocol described by Jensenius *et al.* (12). The antibody preparation was further purified by affinity chromatography. For this purpose, soybean lipoxygenase-1 (Sigma), purified on 8% SDS-PAGE gels, was electroeluted and coupled to sufficient CNBr-activated sepharose 4B to make a 2 mL column (Affinity Chromatography, Pharmacia Fine Chemicals, 1979). IgG (10 mg) was dissolved in 0.5 mL of coupling buffer (0.5 M NaCl, 0.1 M $NaHCO_3$ [pH 8.3]), and loaded onto the column. Purified antilipoxygenase IgG was eluted with NaI (2.5 M) in 0.5 M Tris (pH 7.5), and used as the source of primary antibody.

For Western blots, 50 μg of microsomal membrane protein or soluble fraction protein and 10 μg of soybean lipoxygenase-1 (Sigma) were separated on 8% SDS-PAGE gels, transferred to nitrocellulose paper using a transfer buffer consisting of 25 mM Tris (pH 8.2), 192 mM glycine, 20% methanol (v/v), and 0.2% SDS. The transfer was performed overnight in a Bio-Rad transblot apparatus at 14 V. The blots were removed and incubated in BSA buffer (25 mM Tris [pH 7.5], 140 mM NaCl, 0.5% BSA, 0.05% Tween 20) for 1 h on an orbital shaker. The blots were then treated for a further 1 h with primary antibody against lipoxygenase (1:670 in BSA buffer). Following four 10-min washes in BSA buffer, the blots were treated for 1 h with secondary antibody (rabbit antichicken IgG conjugated to alkaline phosphatase [ICN] diluted 1:5000 in BSA buffer) and subsequently washed once with BSA buffer, once with BSA buffer containing 1% Triton X-100, 5 mM EDTA, and twice more with BSA buffer. Bound antibody was visualized by incubating the blot in 20 mL of alkaline phosphatase buffer (100 mM Tris [pH 9.5], 100 mM NaCl, 5 mM $MgCl_2$) containing 132 μL of 3-bromo-4-chloro-3-indonyl-phosphate (50 mg mL^{-1}) and 66 μL of nitroblue tetrazolium (50 mg mL^{-1}). The color reaction was stopped by placing the blot in 20 mM Tris (pH 8.0), containing 5 mM EDTA.

RESULTS

Microsomal membranes isolated from the pericarp of mature-green tomato fruit proved capable of metabolizing radiolabeled linoleic acid, a substrate for lipoxygenase. Within 5 min at 22°C, levels of linoleic acid declined by ~90%, and the reaction was strongly inhibited by 2 mM *n*-propyl gallate (Fig. 1). When the membranes were heat-denatured, there was no detectable metabolism of linoleic acid, and this together with the fact that *n*-propyl gallate is a known inhibitor of soluble lipoxygenase (14) suggests that these membranes contain an active lipoxygenase-like activity. The products of linoleic acid metabolism (fatty acid oxidation products) migrated as a discrete band in the TLC separation protocol (Fig. 2) and were formed in a temporal pattern that reciprocated the breakdown of linoleic acid over time (Fig. 1). As well, the formation of these fatty acid oxidation products was strongly inhibited in the presence of 2 mM *n*-propyl gallate (Fig. 1). It is noteworthy, however, that the products of linoleic acid metabolism by soybean lipoxygenase-1 did not comigrate with the products of linoleic acid metabolism by tomato fruit microsomes (Fig. 2). This suggests that there is further metabolism of the initial product of the lipoxygenase reaction by the microsomal membranes. This contention is supported by the finding that when peroxidized linoleic acid formed by soybean lipoxygenase-1 is metabolized by tomato fruit microsomes, the product comigrates with the product of linoleic acid metabolism by the same membranes.

The tomato fruit microsomal membranes also proved capable of metabolizing exogenous 16:0/18:2* phosphatidylcholine at 22°C. The major radiolabeled metabolites were fatty acid oxidation products, which increased progressively with time over a 90 min reaction period (Fig. 3). These metabolites were identified as fatty acid oxidation products based on the finding that they comigrated in the TLC separation protocol with the products formed from radiolabeled linoleic acid by microsomal lipoxygenase (Fig. 2). Phosphatidic acid also comigrated with these fatty acid oxidation products. However, in

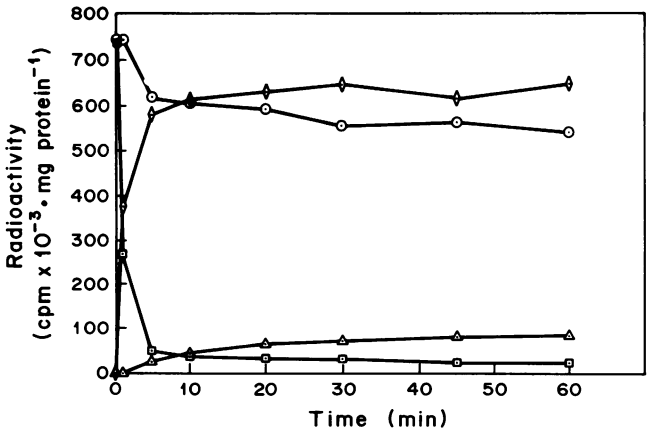

Figure 1. Effect of *n*-propyl gallate on the metabolism of [U-^{14}C] linoleic acid by microsomal membranes isolated from mature-green tomato fruit. The reaction was carried out at 22°C. (○), Changes in linoleic acid with time in the presence of 2 mM *n*-propyl gallate; (□), changes in linoleic acid with time in the absence of *n*-propyl gallate; (△), formation of fatty acid oxidation products over time in the presence of 2 mM *n*-propyl gallate; (◇), formation of fatty acid oxidation products over time in the absence of *n*-propyl gallate. Data are from one of three separate experiments all showing the same results.

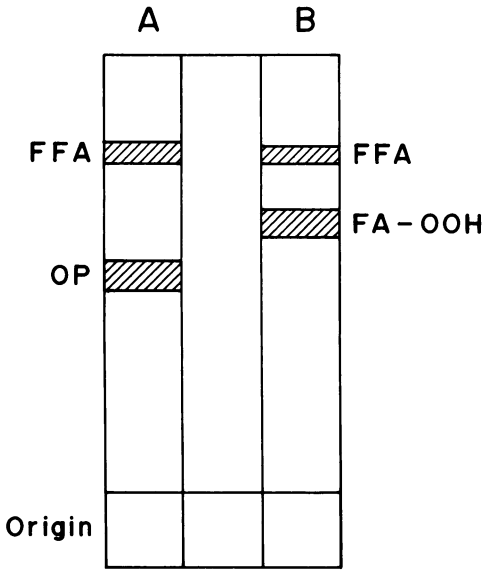

Figure 2. Diagrammatic illustration of the separation of products of [U-^{14}C]linoleic acid metabolism by microsomal membranes from mature-green tomato fruit (A) and by purified soluble soybean lipoxygenase (B). FFA, free fatty acids; OP, oxidation products; FA-OOH, peroxidized fatty acid.

parallel experiments in which ^{32}P-labeled phosphatidylcholine purified from bean cotyledon tissue was administered to the microsomal membranes from tomato fruit, phosphatidic acid comprised less than 1% of the radiolabeled metabolites formed over a 90 min reaction period at 22°C. The fatty acid oxidation products formed from ^{32}P-labeled phosphatidylcholine in these experiments were not radiolabeled and hence did not interfere with the detection of radiolabeled phosphatidic acid. Thus it can be concluded that phosphatidic acid comprises <1% of the radiolabeled metabolites of 16:0/18:2* phosphatidylcholine. The only other detectable radiolabeled metabolite of 16:0/18:2* phosphatidylcholine was diacylglycerol, which was formed at much lower levels than fatty acid oxidation products (Fig. 3). The same pattern of metabolite formation was also observed when 40 μM Ca^{2+}, which has been shown to stimulate membrane-associated phospholipase D and phosphatidic acid phosphatase (22), was included in the assay mixture, although the degree of phosphatidylcholine degradation was enhanced in the presence of Ca^{2+}. When the membranes were heat-denatured, there was no detectable metabolism of 16:0/18:2* phosphatidylcholine or of [U-^{14}C] linoleic acid.

The microsomal membranes were washed after isolation in order to remove cytosolic contaminants. The prospect that soluble enzymes nonspecifically adhering to the membranes might account for the metabolism of 16:0/18:2* phosphatidylcholine or [U-^{14}C]linoleic acid was examined further by treating the microsomes with 0.36% (W/V) Triton X-100 for 30 min at 4°C. When the partially solubilized membranes were centrifuged at 180,000*g* for 1 h after the Triton X-100 treatment, the resulting pellet, which contained ≈50% of the original protein, retained the capability to metabolize 16:0/18:2* phosphatidylcholine. The major metabolites were again fatty acid oxidation products, and there was also a progressive accumulation of diacylglycerol. The Triton X-100-treated membranes also retained the capability to rapidly metabolize [U-^{14}C]linoleic acid. Only about 17% of the initial linoleic acid-metabolizing activity was present in the solubilized fraction obtained from the Triton X-100 treatment.

In an effort to confirm that the formation of fatty acid oxidation products from 16:0/18:2* phosphatidylcholine was attributable to the action of microsomal lipoxygenase on radiolabeled linoleic acid released from the phospholipid substrate, the effects of *n*-propyl gallate on the metabolism of 16:0/18:2* phosphatidylcholine were examined. In three separate experiments, less than 1% of the exogenous 16:0/18:2* phosphatidylcholine was broken down over 90 min in the presence of 2 mM *n*-propyl gallate, and there was no significant accumulation of radiolabeled metabolites (data not shown). The basis for this effect is not clear, but it may reflect nonspecific inhibition by *n*-propyl gallate of one or more of the reactions liberating free linoleic acid from the phospholipid substrate. When the microsomal membranes metabolized 16:0/18:2* phosphatidylcholine at 30°C, there was an initial rapid accumulation of radiolabeled free linoleic acid with time that was not evident at 22°C (Figs. 3 and 4). Levels of free linoleic acid subsequently declined, and there was a corresponding increase in levels of fatty acid oxidation products (Fig. 4). These temporal relationships are consistent with the contention that microsomal lipoxygenase prefers free to esterified linoleic acid as substrate. The formation of fatty acid oxidation products is much lower at 30°C than at 22°C suggesting that lipoxygenase activity is considerably reduced at the higher temperature (Figs. 3 and 4), and it is presumably for this reason that there is an initial accumulation of free linoleic acid at 30°C. There was also a progressive accumulation of radiolabeled diacylglycerol over the 90 min reaction period at 30°C (Fig. 4).

Further evidence indicating that the microsomal lipoxygenase utilizes free rather than esterified linoleic acid as substrate was obtained from spectrophotometric measurements of ac-

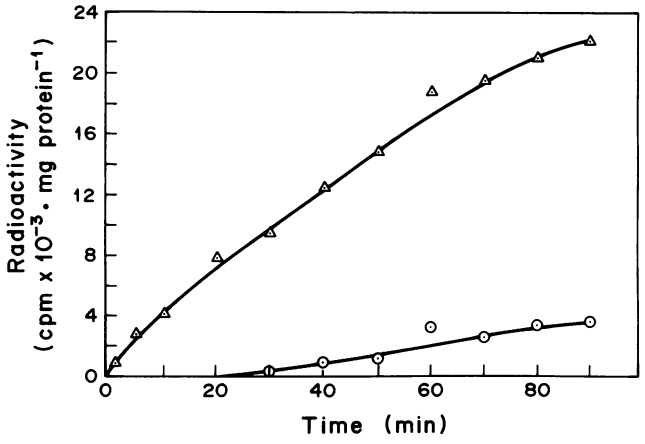

Figure 3. Time-dependent formation of radiolabeled metabolites from 16:0/18:2* phosphatidylcholine at 22°C by microsomal membranes isolated from mature-green tomato fruit. (△), Fatty acid oxidation products; (○), diacylglycerol. Data are from one of three separate experiments all showing the same results.

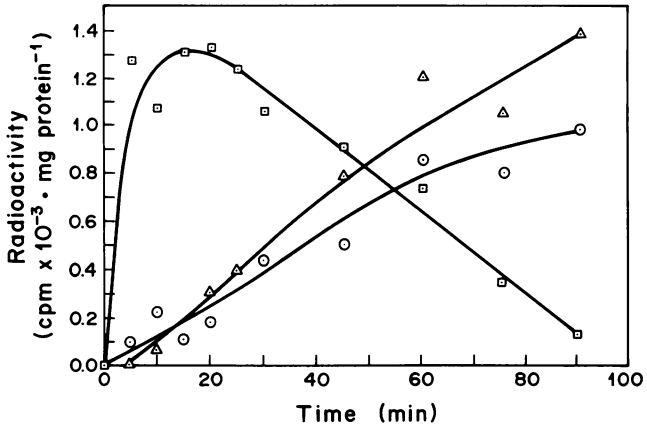

Figure 4. Time-dependent formation of radiolabeled metabolites from 16:0/18:2* phosphatidylcholine at 30°C by microsomal membranes isolated from mature-green tomato fruit. (△), Fatty acid oxidation products; (○), diacylglycerol; (□), free fatty acids. Data are from one of three separate experiments all showing the same results.

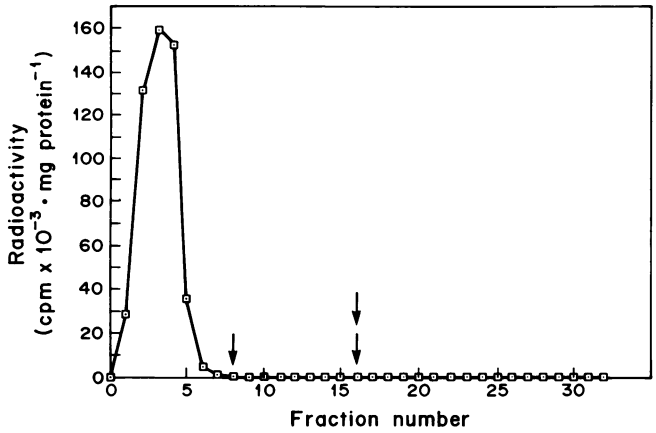

Figure 5. Analysis of radiolabeled water-soluble products of choline-labeled phosphatidylcholine metabolism by microsomal membranes isolated from mature-green tomato fruit. The reaction was allowed to proceed for 30 min. The water-soluble products were separated on a Dowex-1 × 8 ($HC0O^-$) anion exchange column. (□), Choline; single arrow, elution position for glycerophosphorylcholine; double arrow, elution position for phosphorylcholine. Data are from one of three separate experiments all showing the same results.

tivity. The microsomal enzyme showed no detectable activity over a 3-min reaction period when phosphatidylcholine (16:0/18:2; 18:2/18:2), phosphatidic acid (18:0/20:4), or diacylglycerol (18:2/18:2/; 18:3/18:3) were utilized as substrates, whereas in the presence of free linoleic acid (18:2) the specific activity was 226 μmol substrate oxidized min^{-1} mg $protein^{-1}$ (Table I). There was clear evidence for lipoxygenase-mediated metabolism of radiolabeled linoleic acid derived from 16:0/18:2* phosphatidylcholine (Figs. 3 and 4), and thus the fact that there was no detectable lipoxygenase activity with these sources of esterified linoleic acid over a 3-min reaction period in the spectrophotometric assay (Table I) presumably reflects the need to generate free linoleic acid before the enzyme becomes active. As well, there was no detectable activity in the presence of oleic acid, which is not a substrate for lipoxygenase (Table I). The data in Table I were obtained by measuring the change in absorbance at 234 nm over a 3 min period attributable to the formation of peroxidized fatty acid. The experiments with radiolabeled linoleic acid indicated that the microsomal membranes from tomato fruit are capable of further metabolizing peroxidized fatty acids (Fig. 2). The fact that an increase in absorbance at 234 nm can be measured during the initial stages of the reaction is consistent with the contention that the radiolabeled oxidative metabolites are derived from peroxidized linoleic acid.

Diacylglycerol can be formed from phosphatidylcholine either through the sequential actions of phospholipase D and phosphatidic acid phosphatase or directly by phospholipase C. These possibilities were evaluated by analyzing the water-soluble metabolites formed when [choline-methyl-^{3}H] phosphatidylcholine was metabolized by microsomal membranes from tomato fruit. Virtually all of the water-soluble radioactivity was associated with choline, the expected product of phospholipase D activity, whereas radiolabeled phosphorylcholine and glycerophosphorylcholine, which would be formed by the direct actions of phospholipase C and lipolytic acyl hydrolase, respectively, on choline-labeled phosphatidylcholine, were not detectable (Fig. 5). Nor was there any conversion of radiolabeled phosphorylcholine to choline by the microsomes indicating that the absence of radiolabeled phosphorylcholine is not attributable to its conversion to choline by a nonspecific phosphatase (data not shown). Indeed, it has been noted previously that phosphorylcholine in

Table I. *Substrate Preferences of Microsomal Lipoxygenase from Mature-Green Tomato Fruit*

Activity was measured spectrophotometrically at 22°C over a 3 min period. Means ± SE for $n = 3$ separate experiments are shown.

Substrate	Activity	
	μmol min^{-1} mg protein^{-1}	*μmol min^{-1} g fresh wt^{-1}*
Linoleic acid (18:2)	226 ± 63	34 ± 5.9
Phosphatidylcholine (16:0/18:2)	ND[a]	ND
Phosphatidic acid (18:0/20:4)	ND	ND
Diacylglycerol (18:2/18:2)	ND	ND
Diacylglycerol (18:3/18:3)	ND	ND
Oleic acid (18:0)	ND	ND

[a] Not detectable.

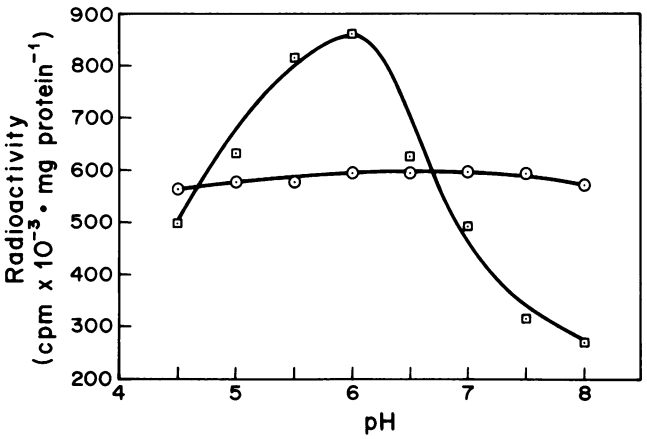

Figure 6. pH dependence of microsomal lipoxygenase (○) and soluble fraction lipoxygenase (□) from mature-green tomato fruit. Activity was assayed in phosphate buffer at 22°C using [U-^{14}C]linoleic acid and is expressed as the loss of radiolabeled substrate. Data are from one of two experiments showing the same results.

higher plant cells is metabolically inert unless it is used for synthesis of phosphatidylcholine and is resistant to hydrolysis by nonspecific phosphatases (1). These observations collectively indicate that the formation of diacylglycerol is attributable to the sequential actions of phospholipase D and phosphatidic acid phosphatase.

The microsomal lipoxygenase displays essentially unchanged activity over the pH range 4.5 to 8.0, whereas lipoxygenase in the soluble fraction has a pH optimum at ≈6.0 (Fig. 6). The data in Figure 6 were obtained with phosphate buffer, and when Ches or Tris were used to examine the pH range 7.5 to 9.0, no additional peaks in activity were observed for either enzyme. The microsomal enzyme comprises ≈38% of the total (microsomal plus soluble) lipoxygenase activity in the tissue and has a specific activity ≈2.5-fold higher than that for the soluble enzyme. Measured values (obtained using the spectrophotometric assay) for specific activity at pH 7.0 are 226 ± 63 and 89 ± 13 (SE for $n = 3$) μmol linoleic acid oxidized min^{-1} mg protein^{-1} for the microsomal and soluble enzymes, respectively, at 22°C. (This difference in specific activity is underestimated in Fig. 6 because of the presence of substantially more unlabeled endogenous linoleic acid in the membranes than in the soluble fraction). Values for the apparent K_m and V_{max} of the microsomal enzyme calculated from a Lineweaver-Burk plot with linoleic acid as substrate are 0.52 mM and 186.3 μmol min^{-1} mg protein^{-1}, respectively (Fig. 7). Gradient fractionation of the isolated microsomes and measurements of marker enzyme activity indicated that particulate lipoxygenase is associated predominantly with endoplasmic reticulum and chloroplast membranes (data not shown).

Microsomal and soluble lipoxygenases from the tomato fruit both cross-reacted with polyclonal antibodies raised against soybean lipoxygenase-1 (Fig. 8). Equal amounts of microsomal and soluble protein were loaded in Figure 8, and the lower intensity of the Western blot for the soluble enzyme is consistent with the ≈2.5-fold difference in specific activities of the two enzymes. The tomato microsomal and soluble lipoxygenases are of comparable molecular mass to soybean lipoxygenase-1 (Fig. 8) and, based on the mobilities of prestained protein standards (which give less accurate molecular mass determinations than unstained standards) in Western blots, have an apparent molecular mass of 100 kD. There are also two lipoxygenase degradation products evident in the Western blots, one for the membranes at 10.1 kD and one for the soluble fraction at 22 kD.

DISCUSSION

Lipoxygenase has traditionally been regarded as a soluble enzyme, of which there are a number of isozymes (7, 10, 11, 18, 32), and although there are several reports of membrane-associated lipoxygenase activity (5, 6, 16, 17, 26), the putative membranous form(s) of the enzyme is not well characterized. In the present study, we provide evidence for a microsomal lipoxygenase in mature-green tomato fruit that is immunologically related to its soluble counterpart and to soybean lipoxygenase-1. The microsomal enzyme utilizes linoleic acid, but not oleic acid, as a substrate and is inhibited by *n*-propyl gallate, a known inhibitor of soluble lipoxygenase (14). The enzyme also appears to be intimately associated with the microsomal membranes and not simply an adhering cytosolic contaminant inasmuch as it remains in the sedimentable material after partial solubilization of the membranes with Triton X-100. This procedure has been used previously to further purify isolated microsomal and plasma membranes resulting in an ≈2-fold enrichment in lipid-degrading enzymes associated with microsomes (22) and in the H$^+$-ATPase of plasmalemma (30). The distinction between the soluble and membrane-associated lipoxygenase activities is also evident from their respective response to pH. The membranous activity proved to be essentially insensitive to pH over the range 4.5 to 8.0, whereas the soluble enzyme displayed a clear peak in activity at pH ≈6.0. The broad pH optimum of the microsomal lipoxygenase activity may reflect the presence of more than one isozyme. For example, aggregate soluble lipoxygenase activity from rice embryos has an optimum pH range of

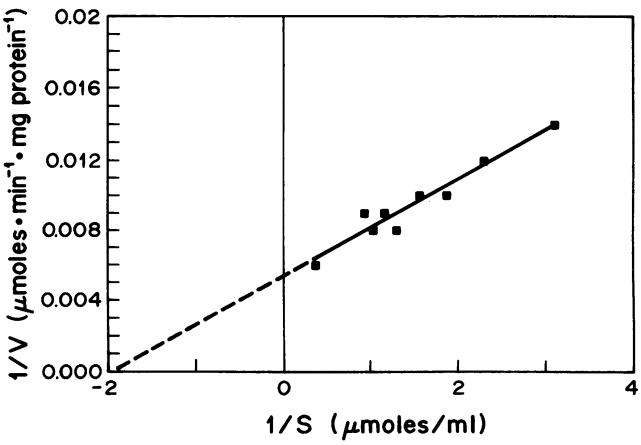

Figure 7. Lineweaver-Burk plot for microsomal lipoxygenase from mature-green tomato fruit. Activity was assayed spectrophotometrically at 22°C over a 3 min period using linoleic acid as substrate. The plot was fitted to the points by linear regression analysis. $R^2 = 0.93$.

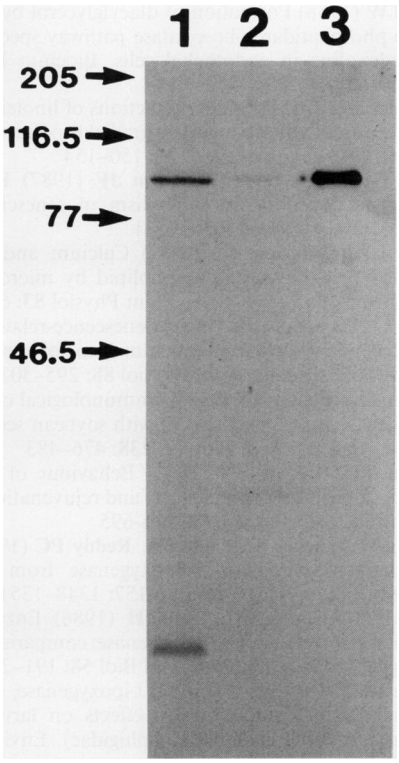

Figure 8. Immunological detection of lipoxygenase by Western blot analysis. Lane 1, 50 μg of microsomal membrane protein from mature-green tomato fruit; lane 2, 50 μg of soluble fraction protein from mature-green tomato fruit; lane 3, 10 μg of soybean lipoxygenase-1 (Sigma). Molecular mass markers are indicated in kD.

4 to 7 that is attributable to three isozymes with pH optima of 4.5, 5.5, and 7.0 (32).

Several lines of evidence indicate that the microsomal lipoxygenase prefers free rather than esterified fatty acids as substrate. First, the oxidation products from 16:0/18:2* phosphatidylcholine comigrate on TLC plates with those formed from radiolabeled linoleic acid by the microsomal membranes, which suggests that they are free fatty acid oxidation products rather than oxidation products of esterified linoleic acid. Second, when the microsomal membranes metabolize 16:0/18:2* phosphatidylcholine at 30°C rather than 22°C, there is an initial accumulation of radiolabeled free linoleic acid that subsequently declines coincident with a corresponding rise in fatty acid oxidation products. Finally, in spectrophotometric assays carried out for a short reaction period (3 min), there was no detectable activity when linoleic acid esterified to phosphatidylcholine, phosphatidic acid, or diacylglycerol was used as a substrate, whereas in the presence of free linoleic acid the specific activity was ≈226 μmol substrate oxidized min^{-1} mg $protein^{-1}$. Deesterification of peroxidized linoleic acid from 16:0/18:2* phosphatidylcholine is not precluded, but in view of the strong preference of the microsomal lipoxygenase for free fatty acid substrate, this contribution to the pool of fatty acid oxidation products formed from 16:0/18:2* phosphatidylcholine by the microsomal membranes is likely to be minimal.

The generation of free linoleic acid from 16:0/18:2* phosphatidylcholine by the microsomal membranes appears to involve the sequential formation of phosphatidic acid, diacylglycerol and, finally, free fatty acids. Moreover, the enzymes mediating the formation of these metabolites appear to be tightly associated with the membranes inasmuch as the pattern of radiolabeled metabolite formation by membranes partially solubilized with Triton X-100 is the same as that for native membranes. The progressive accumulation of radiolabeled diacylglycerol during the reaction can be attributed to the concerted actions of phospholipase D and phosphatidic acid phosphatase rather than phospholipase C. This is evident from the finding that radiolabeled choline, the expected water-soluble product of phospholipase D, was liberated when choline-labeled phosphatidylcholine was used as a substrate, whereas radiolabeled phosphorylcholine, the expected water-soluble product of phospholipase C, was not detectable. Lipolytic acyl hydrolase, the plant enzyme that deesterifies fatty acids from lipids, is known to be able to utilize phospholipids and diacylglycerol as substrate, although diacylglycerol appears to be the preferred substrate (7). For tomato microsomal membranes, however, radiolabeled glycerophosphorylcholine, the expected water soluble product resulting from the action of lipolytic acyl hydrolase on choline-labeled phosphatidylcholine, was not detectable. This indicates that the radiolabeled linoleic acid formed during the metabolism of 16:0/18:2* phosphatidylcholine is released either from phosphatidic acid or diacylglycerol.

Western blotting has indicated that the tomato microsomal and soluble lipoxygenase enzymes have the same molecular mass (≈100 kD) as soybean lipoxygenase-1. The various isozymes of soybean lipoxygenase are known to have similar molecular masses (10). This may also be true for putative isozymes of lipoxygenase in tomato fruit and could account for the fact that only a single major lipoxygenase band was evident in SDS-PAGE Western blots for microsomal membranes and soluble protein. Given the strong preference of the microsomal lipoxygenase for free rather than esterified fatty acid substrate, the enzyme presumably remains essentially inactive until there is deesterification of phospholipid fatty acids. The membranous lipoxygenase may contribute to the loss of membrane function accompanying tomato fruit ripening and senescence inasmuch as the peroxidized products of the lipoxygenase reaction are known to have deleterious effects on membranes and proteins (15, 20). As noted previously for lipoxygenases generally (11), the microsomal lipoxygenase may also play a role in the development of flavor compounds in the ripening tomato fruit. As ripening progresses and the membrane phospholipids are degraded, the resulting free fatty acids would presumably be more readily available to the membrane-associated enzyme than the soluble enzyme.

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