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A Lipoxygenase from Leaves of Tomato (*Lycopersicon esculentum* Mill.) Is Induced in Response to Plant Pathogenic Pseudomonads[1]

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ABSTRACT

Lipoxygenase (LOX) mRNA, enzyme protein, and enzyme activity were found to be induced in leaves of tomato (*Lycopersicon esculentum* Mill. cv Moneymaker) on inoculation with plant pathogenic bacteria. The rate of enzyme activity with linoleic or linolenic acid as substrate was approximately 10 times greater than that with arachidonic acid. Optimum activity was at pH 7.0. In the incompatible interaction, which was associated with a hypersensitive reaction (HR), a single band with relative molecular weight approximately 100,000 was revealed by probing western blots of enzyme extracts with antiserum raised against a pea lipoxygenase. Changes in the intensity of this band reflected the changes observed in LOX enzyme activity after bacterial inoculations. In the hypersensitive reaction, *i.e.* after inoculation with *Pseudomonas syringae* pv *syringae*, LOX mRNA was induced by 3 hours and enzyme activity began to increase between 6 and 12 hours and had reached maximum levels by 24 to 48 hours. In tomato leaves inoculated with *P. syringae* pv *tomato* (compatible interaction), LOX mRNA was induced later and enzyme activity changed only marginally in the first 24 hours, then increased steadily up to 72 hours, reaching the levels seen in the HR.

LOX[2] (linoleate:oxygen oxidoreductase, EC 1.13.11.12) is a dioxygenase catalyzing the hydroperoxidation of fatty acids containing a *cis,cis*-1,4-pentadiene structure, *e.g.* linoleic, linolenic, and arachidonic acids (see ref. 9 for a recent review). The enzyme is apparently ubiquitous in eucaryotes. In animals, the hydroperoxides produced from arachidonic acid by different LOXs form precursors of important classes of chemical messengers such as leukotrienes and lipoxins (1). In plants, no clearly defined physiological role has been demonstrated for LOX, but the hydroperoxides formed from linoleic and linolenic acids are further metabolized and produce several substances with pronounced physiological activity. Roles for LOX in plant growth and development, senescence, wound responses, and resistance against pathogens and pests have all been proposed (9). Arachidonic and eicosopentaenoic acids, which are present in *Phytophthora infestans* and are substrates for potato LOX, were shown to be apparent elicitors of the HR and phytoalexin accumulation in potato (4); in addition, there have been several reports of the earlier and greater induction of LOX enzyme activity in resistant rather than susceptible reactions in several pathosystems (see ref. 28 for review). We suggested (5, 27) that LOX activity might contribute directly to the membrane damage observed during HR. However, none of these reports contained information on the levels of LOX protein or transcripts and consequently very little is known about how changes in LOX activity might be regulated *in vivo*. Nevertheless, the picture that is emerging is that LOX may play an important, if still undefined, part in plant defense.

Plant defense responses occur not only in cells directly stimulated by pathogen contact but also in surrounding cells. Thus, biochemical studies involving biosynthetic enzyme capacity and labeled precursor feeding experiments (16, 20, 31) showed that, although phytoalexins accumulated in necrotic lesions at the site of pathogen invasion, their biosynthesis occurred, at least in part, in the surrounding healthy cells (3), with the synthesis in healthy cells perhaps becoming increasingly more important as the dying cells become metabolically incompetent. Recently, elegant experiments with defense gene-promoter/reporter gene-fusions showed that activation of defense genes such as chalcone synthase (25, 29) and chitinase (23) occurred in a zone of host cells outside the site directly treated with pathogen or elicitor preparations. These results raise the question of the nature of the intercellular signal, which is responsible for coordinating the defense responses in these adjacent host cells. Substances on the linoleic and linolenic acid cascades are possible candidates for such signaling molecules (9, 27, 32). Thus, some oxidized fatty acids are calcium ionophores (26); methyl jasmonate, a metabolite of the 13-hydroperoxide of linolenic acid, has been shown to affect gene expression in barley (15) and serves as a volatile signal for the induction of proteinase inhibitors (8); 12-oxo-phytodienoic acid, a precursor of jasmonate, has a structure similar to that of prostaglandin A_1; and another product of the LOX pathway is 12-oxo-*trans*-10-dodecenoic acid, proposed to be the component of the wound hormone traumatin (34). Linoleic acid caused necrosis and elicited phytoalexins in bean leaves (13) and it was speculated that the effect might be mediated by autoxidation to give the linoleic acid hydroperoxide. Thus, there is the potential for LOX activity and metabolites produced therefrom to play an important role in host-pathogen interactions. For the above reasons, it was decided to investigate the changes in LOX

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[2] Abbreviations: LOX, lipoxygenase; HR, hypersensitive reaction; SE, specific elicitor; NSE, nonspecific elicitor; NSAID, nonsteroidal antiinflammatory drug.

activity in susceptible and resistant reactions of tomato to plant pathogenic bacteria. Tomato was chosen because it has several attributes that make it suitable for classic and molecular genetic studies: it is an important crop, has a small genome that has been extensively mapped, and is transformable by *Agrobacterium tumefaciens* (21). This is appealing when a long-term goal is to investigate control of gene expression.

The characteristics of a membrane-associated LOX from mature, green tomato fruit were reported recently (30), and the induction of LOX in leaves by elicitors from the interaction between tomato and the pathogenic fungus *Cladosporium fulvum* has been described (19). In this study, we report the differential induction of LOX activity in leaves of the tomato cv Moneymaker in response to inoculations with the nonhost pathogen *Pseudomonas syringae* pv *syringae* (which induces a hypersensitive resistance response), and *P. syringae* pv *tomato*, a pathogen of tomato causing the disease known as "bacterial speck."

MATERIALS AND METHODS

Plants, Bacteria, and Cultivation Conditions

Seeds of tomato (*Lycopersicon esculentum* Mill. cv Moneymaker) were purchased from Geissler Samen (Zürich, Switzerland). Seeds were germinated and transplanted to individual pots in a cool glasshouse (maximum temperature approximately 18°C) and transferred to a growth chamber with a 16-h photoperiod and day and night temperatures of 25 and 22°C, respectively. Seedlings were allowed 1 week after transfer to the growth chamber to acclimatize and then were inoculated at the three-leaf stage by infiltrating bacterial suspensions into leaves under reduced pressure as described (28). After inoculation, plants were returned to the growth cabinet and kept under constant illumination.

Pseudomonas syringae pv *syringae* isolate 35 and *P.s.* pv *tomato* isolate 293 were obtained from R. Grimm (Eidgenössische Forschungsanstalt für Obst-, Wein- und Gartenbau, CH-8820 Wädenswil). Bacteria were grown overnight in nutrient broth (Oxoid) supplemented with 1% (v/v) glycerol in an orbital shaker (25°C, 250 cycles min^{-1}). Bacteria were harvested by centrifugation (3000*g* for 10 min) and resuspended to $A_{660} = 0.06$ (approximately 1×10^8 colony-forming units mL^{-1}) in 1.4 mM KH_2PO_4, 2.5 mM Na_2HPO_4, pH 7.0 for inoculation into leaves.

Enzyme Extraction and Assays

Leaf material was harvested from whole plants, frozen in liquid nitrogen, and stored at −80°C until required. Leaf material (approximately 1 g) was ground with a mortar and pestle with 0.25 g of sand and 2 mL ice-cold extraction buffer (50 mM phosphate buffer, pH 7.0, 1% [w/v] PVP, 0.1% [v/v] Triton X-100 and 0.04% [w/v] sodium metabisulphite). Homogenates were centrifuged at 16,000*g* in Eppendorf tubes and the supernatant transferred to clean tubes and brought to 2.5 mL with 50 mM phosphate buffer, pH 7.0. The 2.5-mL aliquots were then loaded onto preprepared PD20 columns (Pharmacia) and eluted with 3.5 mL phosphate buffer. LOX assays were carried out immediately without freezing the samples.

A 10-mM stock solution of sodium linoleate substrate was prepared by adding 70 mg Tween-20, 70 mg linoleic acid (Fluka), and 4 mL oxygen-free water and homogenizing by drawing back and forth with a Pasteur pipette. This produced a milky emulsion that cleared on addition of approximately 0.55 mL 0.5 M NaOH. The volume was made up to 25 mL with oxygen-free water and dispensed into 2-mL screw-cap vials, which were flushed with argon and stored at −20°C. The standard assay was carried out in a total of 1 mL phosphate buffer, pH 7.0, containing 8 μL of substrate stock.

Two sets of inoculations with both *P. syringae* pv *syringae* and *P. syringae* pv *tomato* were carried out. The data presented are from one inoculation with each pathogen and are representative. Each time point is made up of the collective data for four replicates of three different enzyme concentrations giving ΔA_{234} in the linear range (*i.e.* 12 data values all measured at substrate excess), measured over a period of 2.5 to 4 min after an initial incubation of 1 min to allow the reaction rate to stabilize. Activity is reported as nkat mg^{-1} protein at 25°C, assuming the molar extinction coefficient for the conjugated diene product = 2.5×10^4 M^{-1} cm^{-1} (2). Error bars show the SD.

For pH profile determinations, sodium acetate buffer (pH 5.2 and 5.6), sodium phosphate buffer (pH 6.0–8.0), and borate/HCl (pH 8.4–9.2) were used with an enzyme extract from the incompatible combination (48 h after inoculation).

Substrate preference was determined under conditions of substrate limitation by measuring the rate of change of A_{234} with increasing amounts of linoleate, linolenate, or arachidonate. Linolenate and arachidonate substrates were prepared as 10-mM stocks as for linoleate.

Protein was determined using the Pierce bicinchroninic acid protein assay reagent (Pierce Europe b.v., Oud-Beijerland, Holland).

Electrophoresis and Western Blotting

Electrophoresis of enzyme extracts was carried out under denaturing conditions (12) using the mini-protean apparatus (Bio-Rad). Twenty micrograms of total protein was loaded per track. Proteins were blotted onto nitrocellulose membranes (Amersham) using an LKB Novablot electrophoretic transfer kit according to the manufacturer's instructions. Filters were blocked by washing for 3 × 15 min at room temperature in PBS (0.15 M NaCl in 0.01 M sodium phosphate buffer, pH 7.2) containing 0.3% (v/v) Tween 20 and 3% (w/v) BSA. The filter was incubated in antiserum (a kind gift of Dr. C. Domoney, John Innes Institute, Norwich, UK) containing antibodies to a LOX-B type isoform from *Pisum sativum* (6) in PBS with gentle shaking at 4°C overnight. The filter was washed for 3 × 15 min in PBS containing 0.3% (v/v) Tween 20 and incubated with goat anti-rabbit alkaline phosphatase conjugate (Fluka) and visualized with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

Mol wt standards were purchased from Sigma (MW-SDS-200 kit).

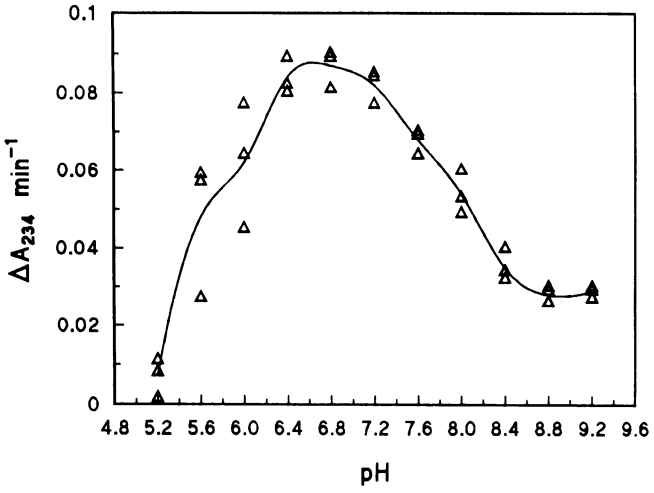

Figure 1. pH profile for activity of tomato leaf LOX. Activity is expressed as change in A_{234} min^{-1}. Three measurements were made at each pH value and the line is a smoothed curve through the means. Individual values are, however, shown in the figure to indicate the range.

RNA Isolation and Northern Blot Analysis

RNA was extracted from frozen, stored samples by the hot-phenol method. Tissue ground in liquid nitrogen was extracted (10 mL to 1 g) with a 1:1 mixture of water saturated phenol:2 × NETS (200 mM NaCl, 2 mM Na_2EDTA, 20 mM Tris-HCl, pH 7.5, 1% [w/v] SDS) that had been preheated to 80°C. The slurry was shaken for 30 to 60 s then placed on ice for 5 to 10 min and the phases separated by centrifugation. The aqueous phase was further extracted with water-saturated phenol and, after separating by centrifugation, made 0.3 M with sodium acetate (pH 5.2) and precipitated with 2 volumes of ethanol. The pellet was collected by centrifugation and redissolved in 6 mL of water in a Corex tube. An equal volume of 5 M LiCl was added and the tube incubated on ice for 4 h. The RNA pellet was collected by centrifugation and redissolved in 500 μL water, precipitated again with ethanol, and the pellet redissolved in a small volume of water. Samples (15 μg/track) were separated in a 1% agarose formaldehyde denaturing gel and capilliary blotted (14) onto Hybond-N membranes (Amersham). Before blotting, the gel was stained with ethidium bromide and viewed under UV light to confirm that similar quantities of ribosomal RNAs were present in each track. A random-primed labeling kit (Boehringer) was used with a 2.2-kilobase bean (*Phaseolus vulgaris*) LOX cDNA clone (EMBL accession No. X63521, 76% identity at the nucleotide level to soybean LOX-3 sequences; B.M. Meier, unpublished results) to prepare a ^{32}P-labeled probe. Hybridization was carried out overnight at 65°C in 1 M NaCl containing 10% (w/v) dextran sulphate, 0.8% (w/v) SDS, and 150 μg mL^{-1} denatured salmon sperm DNA. The most stringent wash was 30 min at 50°C in 2 × SSC (1 × SSC = 0.15 M NaCl, 15 mM Na_3citrate, pH 7.0). Exposure was overnight at −80°C against Fuji RX x-ray film. The autoradiograms were scanned with a Beckman model CDS 200 scanning densitometer and the peak areas integrated electronically.

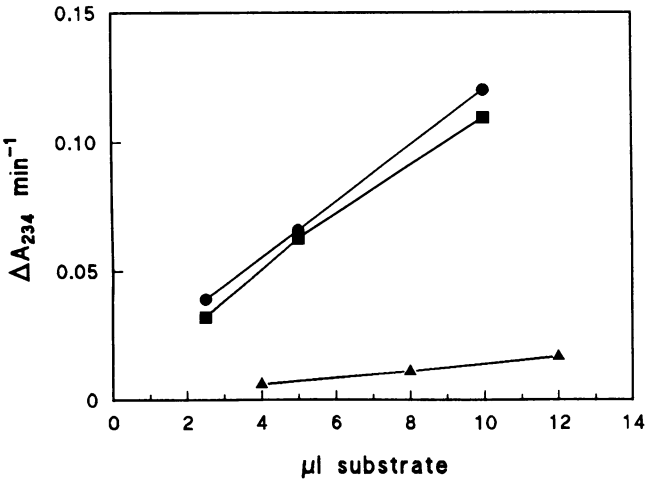

Figure 2. Substrate preference of tomato leaf LOX. ●, Linoleic acid; ■, linolenic acid; ▲, arachidonic acid. Activity is expressed as rate of change in A_{234} min^{-1}. Each point is the mean of three values.

RESULTS

Optimum pH Determination

Determination of the pH profile of enzyme activity from the hypersensitive reaction with linoleic acid as substrate showed a fairly well-defined hyperbola with a suggested optimum between pH 6.4 and 7.2 (Fig. 1). Below about pH 6.4, it is difficult to keep the substrate in solution, which makes assays at low pH values difficult to perform. This is reflected in the range of individual measurements compared with the range at pH 6.4 and above (Fig. 1). Based on this activity profile, LOX activity was measured routinely at pH 7.0.

Substrate Preference

The rate of substrate conversion was approximately nine times greater with linoleic and linolenic acids than it was for arachidonic acid (Fig. 2).

Induction of LOX in Response to Bacteria

Endogenous LOX activity in healthy uninoculated leaves was low, but was found to increase after inoculation with the tomato pathogen *P. syringae* pv *tomato* (susceptible or compatible interaction), or *P. syringae* pv *syringae* (nonhost pathogen that induces an HR in an incompatible interaction). In both cases, an increase in activity was seen by 6 h after inoculation, but in the incompatible interaction the rate of increase in activity was much greater over the first 24 h after inoculation than in the compatible interaction (Fig. 3, a and b). Maximum levels were reached in the incompatible interaction by 48 h. In the susceptible reaction, LOX activity began to increase rapidly only after 24 h and reached a maximum by 72 h (Fig. 3b). Hypersensitive cell collapse occurred from approximately 12 h after inoculation with *P. syringae* pv *syringae* and necrosis in the susceptible reaction could be observed approximately 48 h after inoculation with *P. syringae* pv *tomato*.

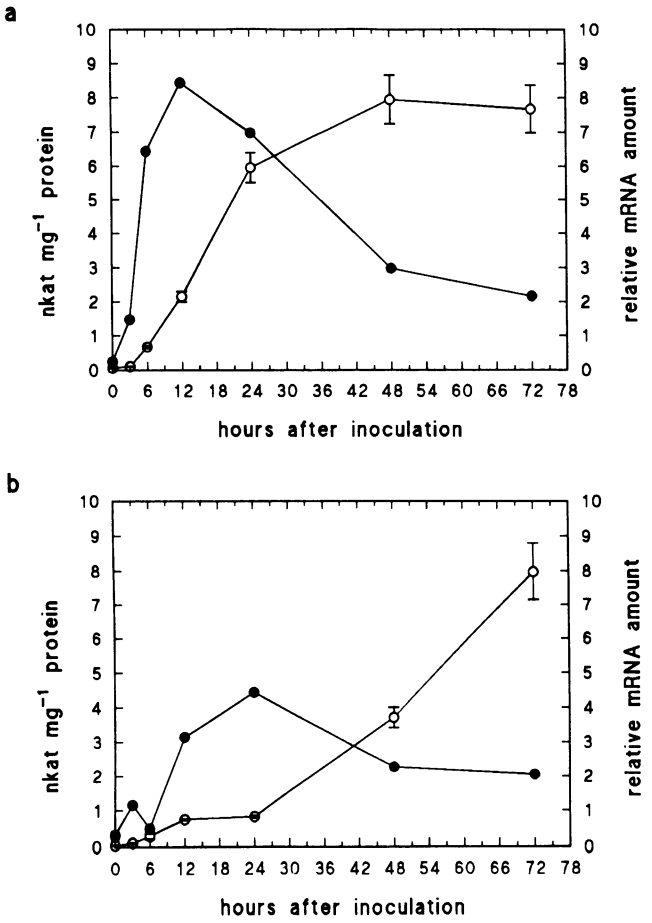

Figure 3. Time course of changes in LOX enzyme activity (○) and relative amount of LOX mRNA (●) in leaves inoculated with (a) *P. syringae* pv *syringae* (HR), and (b) *P. syringae* pv *tomato* (susceptible reaction). Enzyme activity is expressed as nkat mg^{-1} protein. Error bars show the SD.

Western blots after SDS-PAGE of enzyme extracts showed that enzyme protein was induced in a similar manner to measurable LOX activity (Fig. 4). There is a clear signal (M_r approximately 100,000) in extracts from 24, 48, and 72 h after inoculation in the incompatible interaction, *i.e.* those time points at which LOX activity was high (Fig. 3a). In the compatible interaction at 72 h after inoculation, the antiserum highlighted two weak, sharp bands of similar size at M_r approximately 100,000 (data not shown). This might indicate that different LOX isoforms are induced in the hypersensitive and susceptible reactions. However, a number of other bands were also highlighted by the antiserum and it cannot be considered completely specific for LOX protein.

Probing of northern blots with a bean LOX cDNA clone showed induction of homologous transcripts between 3 and 6 h after inoculation in the HR, peaking at 12 h and subsequently declining (Fig. 5a). In the susceptible reaction, LOX transcripts were induced later, reaching a maximum around 24 h after inoculation of approximately half that in the incompatible interaction (Fig. 5b). Densitometer scans of the autoradiograms showed that in both the susceptible and resistant reactions, accumulation of LOX transcripts preceded the induction of enzyme activity (Fig. 3, a and b).

DISCUSSION

Our results show a clear differential induction of LOX activity in tomato leaves in the hypersensitive and susceptible reactions. This is in agreement with several other pathosystems in which an earlier induction of LOX enzyme activity in the resistance rather than in the susceptible response has been reported (5, 10, 17, 18, 33). In this report, we demonstrate the accumulation of LOX mRNA and enzyme protein in addition to showing increases in enzyme activity. In *P. vulgaris* inoculated with *P. syringae* pv *phaseolicola,* it was suggested that LOX activity might be involved in initiating membrane damage, leading to the hypersensitive necrosis of affected cells (5, 27). In this way, it resembles the role of LOX in destroying organelle membranes during reticolucyte maturation to erythrocytes (11). However, an alternative suggestion is that LOX is induced to remove free fatty acids released from membranes as a result of free radical-initiated membrane lipid peroxidation (10). A further potential role for LOX in host-pathogen interactions is the production of signal molecules (1).

Indications that LOX may be important in plant defense have also come from studies using elicitors. Thus, elicitor induction of LOX activity was shown in leaves of tomato (19) and in suspension-cultured cells of bean (24) and tobacco (22). In tomato, LOX activity and lipid peroxidation were associated unequivocally with the resistance response, because a SE from *Cladosporium* induced neither in a cultivar susceptible to the fungal race from which the elicitor was derived (19). However, necrosis in response to SE, but not NSE, from *Cladosporium fulvum* was light dependent, even though LOX activity and lipid peroxidation were also induced in the dark. The NSAID piroxicam, which inhibits LOX, reduced electrolyte leakage caused by NSE from *C. fulvum* but not by SEs. Taken together, these data suggest that the mechanisms of cell death induced by SEs and NSEs might be different and that at least a further factor, in addition to LOX and lipid peroxidation, was necessary in the case of SE. Ellis (7) found that piroxicam and other NSAIDs inhibited elicitor-induced

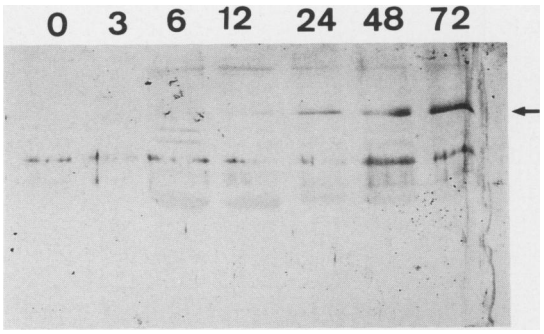

Figure 4. Western blot after SDS-PAGE of the enzyme extracts shown in Figure 3a for the HR, probed with LOX antiserum raised against a LOX-B type isoform from pea. The arrow indicates the position of the presumed LOX band at M_r approximately 100,000.

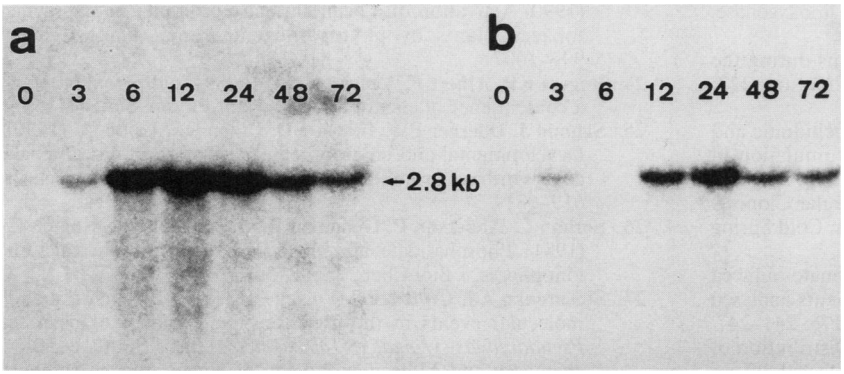

Figure 5. Northern blot of total RNA isolated from leaves after inoculation and probed with a bean lipoxygenase cDNA. The numbers indicate the time of sampling after inoculation in hours. a, With *P. syringae* pv *syringae* (incompatible interaction); b, with *P. syringae* pv *tomato* (compatible interaction).

browning and phytoalexin accumulation in potato tuber tissue, but they also showed that the NSAIDs were effective free radical scavengers as well as inhibitors of LOX activity. Whether piroxicam inhibits LOX activity in these systems *in vivo* or exerts its effect as a free radical scavenger has yet to be shown.

The LOX activity we found to be induced in tomato leaves by bacteria showed a similar pH profile to the soluble LOX described by Todd *et al.* (30) from tomato fruits, and it is possible that there is primarily an induction of this LOX isoform during the HR. The membrane-associated LOX reported by Todd *et al.* (30) had a broad pH optimum over the whole range they tested (pH 4.5–8.0).

The results of the western blots were not completely satisfying because the antiserum also reacted with some other proteins in the leaf extracts (Fig. 4); even though ponceau red staining revealed that the majority of proteins in the extracts did not cross-react with the antiserum (data not shown). Cross-reactivity notwithstanding, in the incompatible interaction a band that was recognized by the antiserum to pea LOX, and was at the correct M_r for LOX, increased in a manner similar to LOX enzyme activity. The induction was preceded by accumulation of LOX mRNA (Fig. 3, a and b, and Fig. 5). In the susceptible reaction, the presence on western blots of two sharp bands of similar size at M_r approximately 100,000 might suggest that different LOX isoforms are induced in the compatible compared with the incompatible interaction. However, in view of the cross-reactivity with other proteins in the extracts, in a pattern that does not fit with possible breakdown products, conclusions must be tentative.

The increased LOX activity in response to pathogen inoculation correlated with induction of a protein recognized by LOX antiserum and preceded by accumulation of LOX mRNA is compatible with the suggestion that the response is regulated, at least in part, at the levels of gene activation and *de novo* enzyme synthesis. LOX has not been reported from prokaryotes and, because bacterial mRNA is difficult to prepare in an undegraded form, it seems extremely improbable that either the activity measured or the 2.8-kilobase transcripts that accumulated originated from the bacteria.

Due to the nature of the inoculation method used, *i.e.* vacuum infiltration, which gives rise to a mosaic of hypersensitive flecks interspersed with healthy cells, the primary site of LOX induction is not known. It would be interesting to localize the response predominantly to either necrotic or healthy cells. However, because visible necrotic flecks occurred in the HR at approximately 12 h after inoculation but enzyme activity reached a maximum some hours later, it seems likely that some synthesis occurs in the healthy, as well as in the dying, cells. Localization of the response would help greatly in interpreting the role of LOX in host-pathogen interactions.

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