Plant Physiol. (1982) 70, 573–578
0032-0889/82/70/0573/06/$00.50/0

Properties of a Mixed Function Oxygenase Catalyzing Ipomeamarone 15-Hydroxylation in Microsomes from Cut-Injured and *Ceratocystis fimbriata*-Infected Sweet Potato Root Tissues

Received for publication March 3, 1982 and in revised form April 28, 1982

MASAYUKI FUJITA, KAZUKO ÔBA, AND IKUZO URITANI
Laboratory of Biochemistry, Faculty of Agriculture, Nagoya University, Chikusa, Nagoya 464, Japan

ABSTRACT

Ipomeamarone 15-hydroxylase activity was found in a microsomal fraction from cut-injured and *Ceratocystis fimbriata*-infected sweet potato (*Ipomoea batatas* Lam. cv. Norin No. 1) root tissues and its optimum pH was 8.0. The enzyme reaction required O_2 and NADPH. The K_m values calculated for ipomeamarone and NADH were approximately 60 and 2 micromolar, respectively. NADPH alone had little effect on enzyme activity but activated the reaction in the presence of low concentrations of NADPH. Ipomeamarone 15-hydroxylase activity was strongly inhibited by *p*-chloromercuribenzoic acid and markedly suppressed by cytochrome *c* and *p*-benzoquinone. KCN was an activator rather than an inhibitor for the reaction. CO inhibited the activity strongly and its inhibition was partially reversed by light. CO difference spectra of the reduced microsomal fraction showed two absorption maxima at 423 and 453 nm; the latter maximum may be due to a cytochrome P-450. These results suggest that ipomeamarone 15-hydroxylase is a cytochrome P-450-dependent, mixed-function oxygenase.

Ipomeamarone 15-hydroxylase activity was not found in fresh tissue of sweet potato roots. However, the activity appeared and increased markedly in response to cut-injury or infection by *Ceratocystis fimbriata*, and reached a maximum after 24 to 36 hours of incubation. The increase in activity in the latter case was 3- to 5-fold higher than in the former. The time course patterns of development and successive decline in ipomeamarone hydroxylase activities were similar to those for cinnamic acid 4-hydroxylase activity, which had been described as a cytochrome P-450-dependent, mixed-function oxygenase. However, little substrate competition was found between ipomeamarone 15-hydroxylase and cinnamic acid 4-hydroxylase in our preparations.

When sweet potato root tissue is infected by *Ceratocystis fimbriata* E11. and Halst., various antifungal furano-sesquiterpenes belonging to the phytoalexins accumulate in the infected region (30). The main component is ipomeamarone (5). Ipomeamarone is hydroxylated to yield ipomeamaronol (10) by ipomeamarone 15-hydroxylase, (Fig. 1). (3). *In vivo* experiments indicated that ipomeamaronol was further converted to unidentified components which were somewhat hydrophilic (21). Accordingly, we suggested that 15-hydroxylation was the first step in ipomeamarone degradation.

In recent years there has been experimental evidence that Cyt P-450-dependent, mixed-function oxygenases participate in secondary metabolism in higher plants. These enzymes involve oxygenations of ent-kaur-16-ene as well as some of its oxidized derivatives in immature *Marach macrocarpus* seeds (19), cinnamic acid 4-hydroxylation in pea seedlings (28), sorghum seedlings (26), Jerusalem artichoke tubers (1), and cut-injured sweet potato roots (29), and 10-hydroxylation of geraniol and nerol in *Vinca rosea* seedlings (16, 18). The above reports have indicated that mixed-function oxygenation activities existed in microsomal fractions, required NADPH and O_2, were strongly inhibited by CO, and the CO inhibition was photoreversible.

This paper reports that ipomeamarone 15-hydroxylase also has general characteristics of a Cyt P-450-dependent, mixed-function oxygenase. Further, it deals with the relationship of ipomeamarone 15-hydroxylase with cinnamic acid 4-hydroxylase and the other microsomal enzymes, and discusses the physiological significance in sweet potato root tissue in response to cut-injury and infection by *C. fimbriata*.

MATERIALS AND METHODS

Plant Materials. Roots of sweet potato (*Ipomoea batatas* Lam. cv. Norin No. 1) were harvested at the Ito Farm, Shizuoka Prefecture, in the autumn and stored at 12 to 14°C. until use.

Reagents. Sources of chemicals and supplies were as follows: [1-^{14}C]sodium acetate (1 mCi, 54.2 Ci/mol) and [3-^{14}C]cinnamic acid (50 μCi, 55 Ci/mol), The Radiochemical Centre, Amersham; NADPH, NADH, $NADP^+$, and NAD^+, Sigma Chemical Company; glucose 6-P, glucose 6-P dehydrogenase, and Cyt *c*, Boehringer Mannheim GmbH; silica gel for TLC, Wakogel B-5F, (Wakojun-yaku Company); and ITLC (ITLC-SAF) paper, Gelman Instrument Company.

Purification of Nonlabeled Ipomeamarone. Ipomeamarone was prepared from *C. fimbriata*-infected sweet potato root tissue by the method previously reported (22) with some modifications. The parenchymatous tissue of the roots was cut in blocks (1–1.5 cm thick). Tissue blocks were inoculated with *C. fimbriata* by procedures previously described (23) and incubated at 29°C for 3 d. After the incubation, 50 g of the infected regions (1 mm thick) were excised from the blocks, and homogenized in 125 ml of chloroform-methanol (1:1, v/v). Then the homogenate was passed through a glass filter, and the tissue residue was washed twice with 50 ml of the extracting solvent. The filtrate was shaken with 0.4 part of deionized H_2O, and the suspension separated into two layers by centrifugation. The chloroform layer was evaporated *in vacuo* and the resulting concentrate (crude terpene fraction) was applied to a silica gel TLC plate, which was developed with *n*-hexane-ethyl acetate (8:2, v/v). After the development, the silica gel-bearing ipomeamarone was scraped off with a spatula. Then the ipomeamarone fraction was extracted from the gel with ethyl acetate and the resulting extract was concentrated *in vacuo*. The concentrate was rechromatographed under the same conditions and the final ipomeamarone sample was purified by double development on the TLC plate with *n*-hexane-ethyl acetate (9:1, v/v).

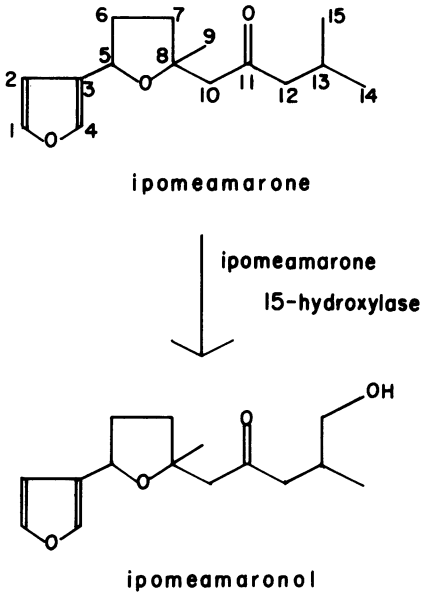

FIG. 1. Enzymic conversion from ipomeamarone to ipomeamaronol catalyzed by ipomeamarone 15-hydroxylase from cut-injured and *Ceratocytis fimbriata*-infected sweet potato root tissues.

Preparation of [^{14}C]Ipomeamarone as a Substrate for Ipomeamarone 15-Hydroxylase. The parenchymatous tissue of sweet potato roots was cut into blocks (1–1.5 cm thick). The pieces were inoculated with *C. fimbriata* and incubated at 29°C for 30 h. The 20 discs (20 mm diameter, 2 mm thick, including the infected region) were prepared from the tissue piece, and placed inoculated surface down on a stainless steel screen in a Petri dish. A [1-^{14}C]acetate solution (1 mCi, 18 μmol in a total volume of 1 ml) was evenly applied to the upper cut surfaces of the 20 discs, which were then incubated at 29°C for 6 h. [^{14}C]Ipomeamarone thus produced (8) was purified by the methods described for the isolation of nonlabeled ipomeamarone.

Preparation of Enzyme Solution. Enzyme solutions were prepared by the following method except for time course experiments of various enzyme activities. Roots were washed and sterilized with 0.05% NaOCl solution followed by a thorough washing with tap water. The parenchymatous tissue of the roots was sliced in 3-mm thick pieces with a meat slicer, and the slices (40 g) were incubated for 24 h at 29°C. The tissue was suspended in 100 ml of 50 mM Tris-HCl buffer (pH 8.5) containing 0.5 M sucrose, 1 mM EDTA, 1% (w/v) sodium isoascorbate, Polyclar AT (0.2 × fresh weight of tissue), and 0.5% (w/v) BSA and homogenized in a blender at maximum speed, six times each for 15 s. The homogenate was squeezed through two layers of nylon gauze and the filtrate was centrifuged at 10,000*g* for 15 min. The supernatant solution was applied to a Sephadex G-25 column (500 ml bed volume) previously equilibrated with 50 mM Tris-HCl buffer (pH 8.5) containing 0.5 M sucrose, 1 mM EDTA, and 1 mM DTT and eluted with the same buffer. The effluent solution was centrifuged at 100,000*g* for 2 h. The precipitate was suspended in 5 ml of 50 mM Tris-HCl buffer (pH 8.5) containing 0.5 M sucrose and 0.3 mM DTT and the suspension used for the enzyme solution. In time course experiments of various enzyme activities, the parenchymatous tissue of the roots was cut into blocks 1.0 to 1.5 cm thick. Then, cut-injured tissue samples were prepared from cut-injured surface region (1.0–1.5 mm thick) of the tissue blocks which had been incubated for various periods at 29°C. Diseased tissue samples were prepared from noninfected region (1.0–1.5 mm thick) adjacent to the infected region of the tissue blocks which were inoculated by *C. fimbriata* and incubated for various periods at 29°C. The enzyme solution was prepared by the method previously described except that the first centrifugation was performed at 300*g* for 10 min, instead of 10,000*g* for 15 min.

Enzyme Assays. The basis of the assay for ipomeamarone 15-hydroxylase is the conversion of [^{14}C]ipomeamarone to ipomeamaronol, subsequent isolation of the product by chromatography, and assay for radioactive ipomeamaronol. Identification of the reaction product was previously reported by Fujita *et al.* (3). The standard reaction mixture contained 62 μM ipomeamarone, 50 mM Tris-HCl buffer (pH 8.0), 0.87 mM $NADP^+$, 1.5 mM glucose 6-P, 0.3 unit glucose 6-P dehydrogenase, 0.5 M sucrose, and microsomal fraction (1–4 mg of protein) in a final volume of 1.5 ml. The reaction was initiated by the addition of the microsomal fraction and incubated for 1.0 h at 30°C. Enzyme activity was linear for 1.5 h and proportional to enzyme concentrations up to 4 mg of protein. The reaction was terminated by the addition of 3.5 ml of chloroform-methanol (1:1, v/v), and then a small amount (approximately 1 μl) of crude terpene fraction was added for chromatographic markers. After shaking the suspension, the chloroform layer was recovered. After a second extraction with chloroform, the extracted solutions were combined and evaporated to dryness. The residue was dissolved in a small volume of chloroform-methanol (1:1, v/v) and the sample applied to ITLC paper. This was first developed to a height of 6 cm with *n*-hexane-ethyl acetate (6:4, v/v) and then to 15 cm with *n*-hexane-ethyl acetate (9:1, v/v). Ipomeamaronol was located on the chromatogram by comparison with marker terpenes. The chromatogram was either cut into 1-cm sections, or the portions of the chromatogram bearing the ipomeamaronol fraction were scraped from the paper. The pieces of the chromatograms and the ipomeamaronol fractions were placed in 5 ml of scintillator solution (0.08 g PPO and 4 mg POPOP per 5 ml of toluene), and the samples counted in a liquid scintillation spectrometer (Packard Tri-Carb model 3320).

Cinnamic acid 4-hydroxylase activity was assayed by method I of Tanaka *et al.* (29). NADPH:Cyt *c* oxidoreductase (NADPH-Cyt *c* reductase, EC 1.6.2.3) activity was determined by the method of Masters *et al.* (17). Antimycin A insensitive NADH: Cyt *c* oxidoreductase (NADH-Cyt *c* reductase, EC 1.6.2.1 [antimycin A insensitive]) activity was determined by the method of Mackler (15).

Inhibition of Ipomeamarone 15-Hydroxylase Activity by CO. A reaction mixture without substrate ([^{14}C]Ipomeamarone) was placed in a spitz glass, which was covered by aluminum foil; the container was sealed with a silicone stopper and filled with N_2 after evacuation. The gas exchange was repeated three times and calculated amounts of CO and O_2 were injected. The reaction was started by the addition of substrate. When the effect of light on CO inhibition was examined, the aluminum foil was removed and

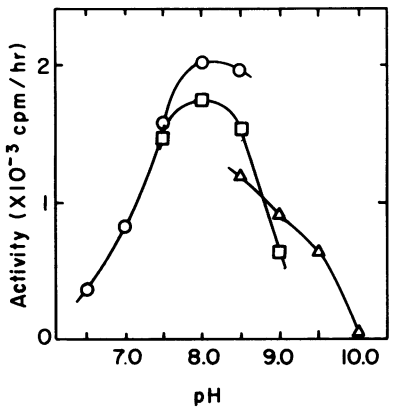

FIG. 2. Effect of pH of reaction mixture on ipomeamarone 15-hydroxylase activity. Enzyme activity was assayed as described in "Materials and Methods" with following buffer solutions: (○), 50 mM K-phosphate; (□), 50 mM Tris-HCl; and (△), 50 mM glycine-KOH.

Table I. *Effects of Pyridine Nucleotides on Ipomeamarone 15-Hydroxylase Activity*

Deletion	Addition	Relative Activity[b]
		%
None (complete)	None	100
NADPH rgs[a]	NADPH (1 mM)	61
NADPH rgs	NADH (1 mM)	3
NADPH rgs	NADPH (1 mM) + NADH (1 mM)	87
NADPH rgs	$NADP^+$ (1 mM)	3
NADPH rgs	NADPH (0.5 mM)	43
NADPH rgs	NADH (0.5 mM)	3
NADPH rgs	NADPH (0.5 mM) + NADH (0.5 mM)	85
NADPH rgs	None	4

[a] NADPH rgs, NADPH regenerating system (1 mM $NADP^+$, 2 mM glucose 6-P, and 0.3 unit glucose 6-P dehydrogenase).

[b] Relative activity was expressed as percentage of the activity of the complete system, which is described under "Materials and Methods."

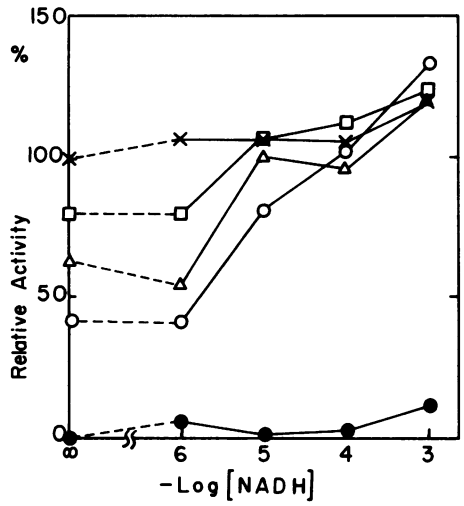

FIG. 3. Effect of combinations of NADPH with NADH on ipomeamarone 15-hydroxylase. Reactions were carried out as described in "Materials and Methods" in mixtures containing both NADPH and NADH which were in various concentrations as follows: (●), 0 M NADPH; (○), 1 μM NADPH; (△), 5 μM NADPH; (□), 10 μM NADPH; and (×), 1 mM NADPH. Concentration of NADPH was expressed, assuming that $NADP^+$ added was immediately reduced to NADPH by glucose 6-P dehydrogenase. Relative activity was expressed as percentage of activity with 1 mM NADPH and no NADH.

the spitz glass illuminated with an incandescent lamp (500 w) at a distance of approximately 30 cm.

Measurement of Cyt P-450. The concentration of Cyt P-450 was determined from the CO difference spectrum of a sample reduced by the addition of either sodium dithionite or NADPH, using 91 mM^{-1} cm^{-1} of the extinction coefficient, based on the method of Omura and Sato (24).

Determination of Protein. To remove BSA from the enzyme solution completely, it was diluted with 20 volumes of suspension buffer, and the suspension centrifuged at 100,000*g* for 2 h. The resulting pellet was suspended in distilled H_2O and the suspension used for protein determination. Protein was determined by the method of Lowry *et al.* (14) with BSA as a standard.

RESULTS

Properties of Enzyme. Ipomeamarone 15-hydroxylase activity was localized in the microsomal fraction. The effect of pH on the enzyme activity was examined over a range of pH 6.5 to 10.0. The optimum pH of the reaction was 8.0, as shown in Figure 2.

Table II. *Effects of Reagents on Ipomeamarone 15-Hydroxylase Activity*
DTT and EDTA were removed from reaction mixtures

Addition	Final Concentration	Relative Activity[a]
	mM	%
None (complete)		100
KCN	1	148
α,α′-Dipyridyl	0.1	108
α,α′-Dipyridyl	1	106
p-Chloromercuribenzoic acid	0.19	14
$HgCl_2$	1	19
$CuCl_2$	1	12
$MgCl_2$	1	94
Cyt *c*	1	11
p-Benzoquinone	0.25	25
p-Benzoquinone	0.5	8

[a] Relative activity was expressed as percentage of the activity of the complete system.

Table III. *Inhibition of Ipomeamarone 15-Hydroxylase Activity by Carbon Monoxide and Its Reversal by Light*

Gas Mixture	Relative Activity[a]
	%
Air	100
N_2 (100%)	25
O_2 (15%) + N_2 (85%)	90
CO (15%) + O_2 (15%) + N_2 (70%)	36
CO (15%) + O_2 (15%) + N_2 (70%) + Light	70

[a] Relative activity was expressed as percentage of the activity of air control.

The enzyme requires NADPH for the activity; $NADP^+$ was ineffective (Table I). NADH alone was not effective, but the addition of NADH together with subsaturating levels of NADPH stimulated the enzyme activity over the levels observed with NADPH alone (Fig. 3). In the absence of NADPH, the activity was negligible in spite of the presence of NADH. But the presence of 1 μM NADPH, simultaneous addition of NADH stimulated enzyme activity markedly. As the concentration of NADPH was increased, however, the stimulation by NADH decreased, and in the presence of 1 mM NADPH, the stimulation by NADH was not detected. These results seem to reflect the synergistic interaction between components of the NADPH-dependent electron transport system and those of the NADH-dependent electron transport system in microsomes. In higher plants, similar results have been reported by Hasson and West (4).

Substrate saturation curves for ipomeamarone and NADPH followed Michaelis-Menten kinetics. The calculated K_m values were as follows: ipomeamarone, approximately 60 mM; NADPH, approximately 2 μM, which was determined by additions of appropriate concentrations of $NADP^+$ in the NADPH-generating system.

Effects of various reagents on the enzyme activity are shown in Table II. KCN, which is a typical inhibitor for heme enzymes except for Cyt P-450, stimulated the activity of the hydroxylase; however, α,α′-dipyridyl (1 mM), which inhibited cinnamic acid 4-hydroxylase activity from pea seedlings by 27% (28), did not inhibit ipomeamarone 15-hydroxylase. *p*-Chloromercuribenzoic acid, $HgCl_2$, and $CuCl_2$, inhibitors of sulfhydryl enzymes, markedly inhibited enzyme activity. Addition of Cyt *c* or *p*-benzoquinone, which accept electrons from the microsomal electron transport system, caused marked decreases in enzyme activity. Potts *et*

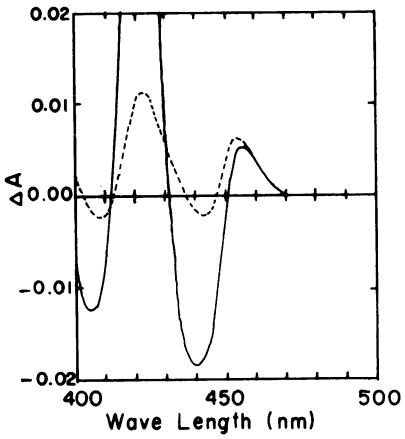

FIG. 4. CO difference spectra of a reduced microsomal fraction from diseased sweet potato root tissue. Microsomal fraction (10,000*g* for 15 min; 100,000*g* for 2 h) was prepared from diseased tissue taken from noninfected regions (1.0–1.5 mm thick) adjacent to infected regions of tissue blocks (1.0–1.5 cm thick) which had been inoculated with *C. fimbriata* and incubated for 24 h at 29°C. Microsomal fraction (3.6 g tissue/ml, 2.4 mg microsomal protein/ml) containing NADPH-regenerating system (1 mM $NADP^+$) was divided equally into two cells. Base line of equal light absorbance was first plotted, then CO was bubbled into sample cell for 2 min and difference spectrum (- - -) was recorded. After recording difference spectrum, a small amount of solid dithionite was added to both cells and difference spectrum (—) was again recorded.

al. (26) previously reported that *p*-benzoquinone was an effective inhibitor of cinnamic acid 4-hydroxylase from sorghum seedlings. Thus, the above results strongly suggest that ipomeamarone 15-hydroxylase activity has components of the microsomal electron transport system.

Effects of composition of gas phase on this enzyme activity are shown in Table III. The O_2/N_2 value was adjusted to 0.18 and 0, and relative activities, which are expressed as percentage of the activity at 0.25 of O_2/N_2 value (air), decreased 10% and 75%, respectively. The decrease indicates an O_2 requirement for ipomeamarone 15-hydroxylase activity. Since activity was detected at 0% O_2/N_2 it is suspected that removal of O_2 from the reaction system was not complete. The presence of 15% CO caused 60% inhibition and the inhibition was relieved 62% by light. The above results strongly indicate that ipomeamarone 15-hydroxylase is a Cyt P-450-associated, mixed-function oxygenase.

CO Difference Spectra of Microsomal Fractions from Sweet Potato Root Tissue. The presence of CO-binding pigment in the microsomal fraction from sweet potato root tissue was confirmed by the difference spectrum of the microsomal fraction from diseased tissue (Fig. 4). One absorbance peak was seen at 453 nm when NADPH was used as the reducing agent and the spectrum measured immediately. However, within a few minutes another absorbance peak at 423 nm gradually increased to approximately 1.5-fold of the peak at 453 nm. When sodium dithionite was added as a reducing agent, the peak at 423 nm appeared faster and became more prominent; two additional peaks at 540 and 573 nm also were detected. The peaks at 423, 540, and 573 nm corresponded closely to those in the CO difference spectra of peroxidase from other sources (6, 13). The presence of peroxidase in sweet potato root tissue was previously reported by Kawashima and Uritani (11, 12). The peak at 453 nm in the CO difference spectrum provides direct evidence for a Cyt P-450 in this system. The Cyt P-450 concentrations calculated from the peaks at 453 nm in fresh tissue, cut-injured tissue, and diseased tissue were 1.2, 7.8, and 16 pmol/g of tissue weight, respectively. The intensities of the *A* at 453 nm seem to be associated with activities of ipomeamarone 15-hydroxylase and cinnamic acid 4-hydroxylase (Fig. 5, A and B).

Effects of Cinnamic Acid on Ipomeamarone 15-Hydroxylase Activity and of Ipomeamarone on Cinnamic Acid 4-Hydroxylase Activity. Effects of cinnamic acid on ipomeamarone 15-hydroxylase activity and those of ipomeamarone on cinnamic acid 4-hydroxylase activity are shown in Tables IV and V, respectively. When the reaction mixture contained 36 μM [^{14}C]ipomeamarone as the substrate for ipomeamarone 15-hydroxylase, the simultaneous addition of 0- to 28-fold concentration of nonlabeled cinnamic acid hardly influenced the formation of ipomeamaronol. Conversely, when the reaction mixture contained 3.6 μM [^{14}C]-cinnamic acid as the substrate for cinnamic acid 4-hydroxylase, the simultaneous addition of 0- to 278-fold concentrations of nonlabeled ipomeamarone decreased in a little measure the formation of 4-hydroxycinnamate; in the case of 1 mM of final concentration the activity was 70% of the control. The above two experiments were performed under the same conditions except for the concentrations of ipomeamarone, cinnamic acid, and enzyme solution. The above results suggest that these two hydroxylase activities depend on separate enzymes and not on a single hydroxylase.

Time Courses of the Development of the Activities of Ipomeamarone 15-Hydroxylase and Some Other Microsomal Enzymes. Time courses of development of the activities of ipomeamarone 15-hydroxylase and several other microsomal enzymes together with protein content in response to cut-injury and infection by *C. fimbriata* are shown in Figure 5, A and B, respectively. Both ipomeamarone 15-hydroxylase and cinnamic acid 4-hydroxylase were undetectable in fresh tissue, but their activities appeared and increased dramatically in response to cut-injury or infection; they reached a maxima after 1.0 to 1.5 d, and then declined. Interestingly, maximal activity of ipomeamarone 15-hydroxylase was approximately 5-fold higher in response to infection than in response to cut-injury (Fig. 5), but the maximal activity of cinnamic acid 4-hydroxylase in the former case was similar to the latter case (Fig. 5.), and was sometimes up to 2-fold higher. Levels of NADPH-Cyt *c* reductase and NADH-Cyt *c* reductase, the marker enzymes of mammalian microsomes, increased and reached maxima in 1.0 to 1.5 d after incubation in response to cut-injury or infection, then they gradually declined. Protein content was higher in infected tissues.

DISCUSSION

The enzyme, ipomeamarone 15-hydroxylase, which specifically required NADPH as its cofactor and molecular oxygen as a substrate for the hydroxylation of ipomeamarone, has the properties expected of a mixed function oxygenase. The activity was found in the microsomal fraction and was inhibited by *p*-chloromercuribenzoic acid, $HgCl_2$, and $CuCl_2$, inhibitors of sulfhydryl enzymes, and was strongly reduced in the presence of Cyt *c* and *p*-benzoquinone, both nonphysiological electron acceptors of microsomal electron transport systems. KCN did not inhibit, but instead activated this hydroxylase activity. A stimulation by cyanide has been reported in aniline *p*-hydroxylation from rat liver microsomes (9). This enzyme activity was inhibited by CO and the inhibition was partially reversed by light. The above results indicate that a microsomal electron transport system containing Cyt P-450 is a component of ipomeamarone 15-hydroxylase. Although the participation by Cyt P-450-dependent, mixed-function oxygenases has been reported in some secondary metabolism systems in higher plants, their involvement in phytoalexin metabolism has not been previously reported.

CO difference spectra of reduced microsomal fractions from cut-injured or diseased tissue gave an absorbance maximum around 453 nm but such spectra were scarcely detectable in fresh tissue preparations. This absorbance maximum was thought to be associated with a pigment such as Cyt P-450 which exists in

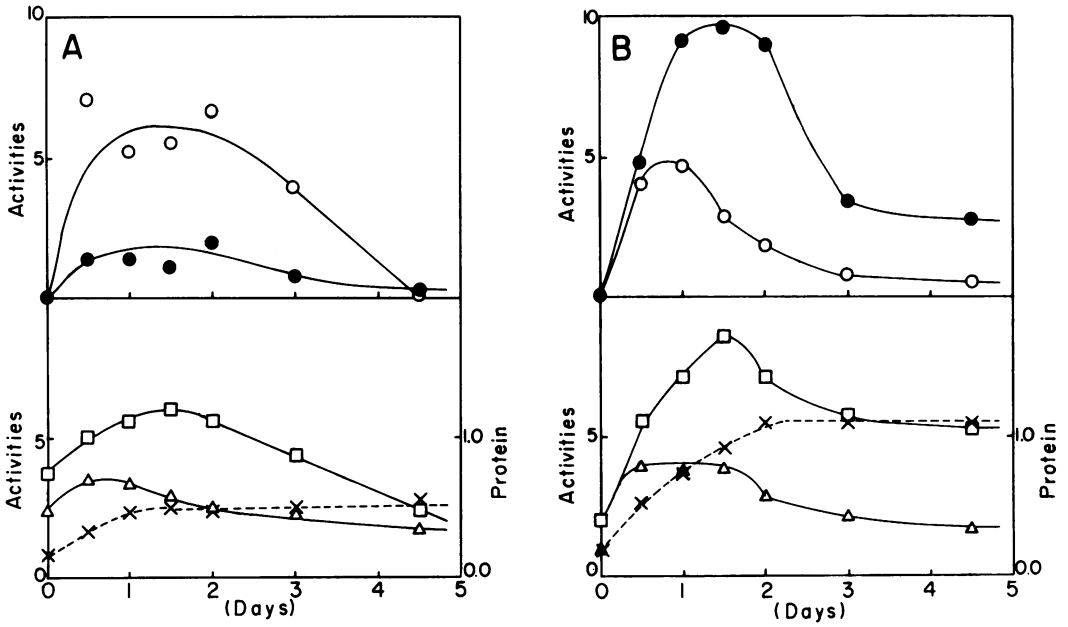

FIG. 5. Time courses of development of activities of ipomeamarone 15-hydroxylase and certain mirosomal enzymes and protein content in tissues in response to cut-injury (A) and *C. fimbriata* infection (B). Enzyme solution was prepared and activities were assayed as described in "Materials and Methods." (●), ipomeamarone 15-hydroxylase activity (nmol/g tissue weight·h); (○), cinnamic acid 4-hydroxylase activity (nmol/g tissue weight·h); (△), NADPH-Cyt *c* reductase activity (Δ A_{550}/g tissue weight·min); (□), NADH-Cyt *c* reductase activity (△)A_{550}/g tissue weight·min); (×), protein content (mg/g tissue weight).

Table IV. *Effect of Cinnamic Acid on Ipomeamarone 15-Hydroxylase Activity*

Reaction mixture contained 36 μM [^{14}C]ipomeamarone as the substrate of ipomeamarone 15-hydroxylase. Reaction conditions are described under "Materials and Methods."

Addition	Final Concentration	Relative Activity[a]	
		Experiment 1	Experiment 2
	mM	%	
None (complete)		100	100
Cinnamic acid	0.1		97
Cinnamic acid	0.5	93	95
Cinnamic acid	1.0		94

[a] Relative activity was expressed as percentage of the activity of the complete system.

Table V. *Effect of Ipomeamarone on Cinnamic Acid 4-Hydroxylase Activity*

Reaction mixture contained 3.6 [^{14}C]cinnamic acid as substrate of cinnamic acid 4-hydroxylase. Otherwise, the incubation conditions were the same as those for the experiments described in Table IV.

Addition	Final Concentration	Relative Activity[a]	
		Experiment 1	Experiment 2
	mM	%	
None (complete)		100	100
Ipomeamarone	0.1	89	88
Ipomeamarone	0.5	76	88
Ipomeamarone	1.0	75	73

[a] Relative activity was expressed as percentage of the activity of the complete system.

mammalian liver microsomes and also appears to occur in plants (25). The content of Cyt P-450 calculated from the absorbance at 453 nm seemed to reflect the amount of ipomeamarone 15-hydroxylase and cinnamic acid 4-hydroxylase activities in fresh tissue, cut-injured tissue, and diseased tissue. However, the *A* at 453 nm may not only be due to Cyt P-450 associated with ipomeamarone 15-hydroxylase and cinnamic acid 4-hydroxylase but to other, as yet unknown systems as well; the existence of various kinds of Cyt P-450 has been reported in mammalian sources such as rabbit liver (7). In higher plants, as far as we know, data suggesting the existence of multiple Cyt P-450 forms have been reported previously only by Reichhart *et al.* (27) and Coolbaugh *et al.* (2). In this paper, substrate competition studies were carried out on ipomeamarone 15-hydroxylase and cinnamic acid 4-hydroxylase activities using nonlabeled ipomeamarone and cinnamic acid. These experiments showed almost no substrate competition among the two enzyme activities. This suggests a narrow specificity for substrates in Cyt P-450 in plants, as indicated by West (31). Further, ipomeamarone 15-hydroxylase activity in diseased tissue was 3- to 5-fold higher than that in cut-injured tissue, but cinnamic acid 4-hydroxylase activity in diseased tissue was approximately the same as, or a little larger than, that in cut-injured tissue. These results suggest that multiple Cyt P-450 forms are formed in both cut-injured and *C. fimbriata*-infected sweet potato root tissues.

In this laboratory the pathway for ipomeamarone synthesis has been studied in *C. fimbriata* infected sweet potato root tissue (30); but much remains to be known about the metabolism of the compound. When one thinks physiologically about responses of sweet potato root tissue to *C. fimbriata* infection, the fate of ipomeamarone is important because ipomeamarone is toxic not only to *C. fimbriata* but also to sweet potato root tissue itself. *In vivo* experiments have yielded evidence that ipomeamarone accumulates in the infected surface region (21). Evidence was also presented that the ipomeamarone was converted to unidentified water soluble small fragments (20). Assuming that these small fragments of ipomeamarone are nontoxic and are derived from ipomeamaronol, such results and those presented here lead us to suggest that ipomeamarone 15-hydroxylation may serve in a mechanism of detoxification of ipomeamarone in sweet potato root tissue.

Acknowledgments—The authors wish to thank Dr. T. Kosuge, Department of

Plant Pathology, University of California at Davis, for carefully reading the manuscript and Dr. Y. Tanaka, RI Center, Nagoya University, and Dr. H. Inoue, at this laboratory, for helpful advice and discussions.

LITERATURE CITED

1. BENVENISTE I, JP SALAUN, F DURST 1977 Wounding-induced cinnamic acid hydroxylase in Jerusalem artichoke tuber. Phytochemistry 16: 69–73
2. COOLBAUGH RC, SS HIRANO, CA WEST 1978 Studies on the specificity and site of action of α-cyclopropyl-α-[*p*-methoxyphenyl]-5-pyrimidine methyl alcohol (ancymidol), a plant growth regulator. Plant Physiol 62: 571–576
3. FUJITA M, K ÔBA, I URITANI 1981 Ipomeamarone 15-hydroxylase from cut-injured and *Ceratocystis fimbriata*-infected sweet potato root tissues. Agric Biol Chem 45: 1911–1913
4. HASSON EP, CA WEST 1976 Properties of system for the mixed function oxidation of kaurene and kaurene derivatives in microsomes of the immature seed of *Marah macrocarpus*. Plant Physiol 58: 473–478
5. HIURA M 1941 Studies on storage and rot of sweet potato (2). Rep Gifu Agric Coll 50: 1–5
6. HORIE S 1964 On the carbon monoide-shift of absorption spectrum of cytochrome a_3. J Biochem (Tokyo) 56: 113–112
7. IMAI Y, C HASHIMOTO-YUTSUDO, H SATAKE, A GIRARDIN, R SATO 1980 Multiple forms of cytochrome P-450 purified from liver microsomes of phenobarbital- and 3-methylcholanthrene-pretreated rabbits. J Biochem 88: 489–503
8. INOUE H, I URITANI 1979 Biosynthetic correlation of various phytoalexins in sweet potato root tissue infected by *Ceratocystis fimbriata*. Plant Cell Physiol 20: 1307–1314
9. KAMATAKI T, M KITADA, K CHIBA, H KITAGAWA, Y IMAI, R SATO 1980 Enhancement by cyanide of aniline p-hydroxylation activity in rat liver microsomes. Biochem Pharmacol 29: 1141–1147
10. KATO N, H IMASEKI, N NAKASHIMA, I URITANI 1971 Structure of a new sesquiterpenoid, ipomeamaronol, in diseased sweet potato root tissue. Tertrahedron Lett 13: 843–846
11. KAWASHIMA N, I URITANI 1963 Occurrence of peroxidases in sweet potato infected by the black rot fungus. Agric Biol Chem 27: 409–417
12. KAWASHIMA N, I URITANI 1965 Some properties of peroxidase produced in sweet potato infected by the black rot fungus. Plant Cell Physiol 6: 247–265
13. KEILIN D, EF HARTREE 1951 Purification of horseradish peroxidase and comparison of its properties with those of catalase and methaemoglobin. J Biochem 49: 88–104
14. LOWRY OH, NJ ROSEBROUGH, AL FARR, RJ RANDALL 1951 Protein measurement with Folin phenol reagent. J Biol Chem 193: 265–275
15. MACKLER B 1967 Microsomal DPNH-cytochrome *c* reductase. Methods Enzymol 10: 551–553
16. MADYASTHA KM, TD MEEHAN, CJ COSCIA 1976 Characterization of a cytochrome P-450 dependent monoterpene hydroxylase from higher plant *Vinca rosea*. Biochemistry 15: 1097–1102
17. MASTERS BSS, CH WILLIAMS JR, H KAMIN 1967 The preparation and properties of microsomal TPNH-cytochrome *c* reductase from pig liver. Methods Enzymol 10: 565–573
18. MEEHAN TD, CJ COSCIA 1973 Hydroxylation of geraniol and nerol by a monooxygenase from *Vinca rosea*. Biochem Biophys Res Commun 53: 1043–1048
19. MURPHY PJ, CA WEST 1969 The role of mixed function oxidases in kaurene metabolism in *Echinocystis macrocarpa* Greene endosperm. Arch Biochem Biophys 133: 395–407
20. ÔBA K, I URITANI 1981 Mechanism of furano-terpene production in sweet potato root tissue injured by chemical agents. Agric Biol Chem 45: 1635–1639
21. ÔBA K, K ÔGA, I URITANI 1982 Metabolism of ipomeamarone in sweet potato root slices after treatment with mercuric chloride or infection with *Ceratocystis fimbriata*. Phytochemistry In press
22. OGUNI I, I URITANI 1974 Dehydroipomeamarone as an intermediate in the biosynthesis of ipomeamarone, a phytoalexin from sweet potato root infected with *Ceratocystis fimbriata*. Plant Physiol 53: 649–652
23. OGUNI I, K OSHIMA, H IMASEKI, I URITANI 1969 Biochemical studies on the terpene metabolism in sweet potato root tissue with black rot. Agric Biol Chem 33: 50–62
24. OMURA T, R SATO 1967 Isolation of cytochrome P-450 and P-420. Methods Enzymol 10: 556–561
25. PETER RR, DS BENDALL 1975 Cytochrome components of plant microsomes. Eur J Biochem 55: 333–341
26. POTTS JM, R WEKLYCH, EC CONN 1974 The 4-hydroxylation of cinnamic acid by sorghum microsomes and the requirement for cytochrome P-450. J Biol Chem 249: 5019–5026
27. REICHHART D, JP SALAÜN, I BENVENISTE, F DURST 1979 Induction by manganase, ethanol phenobarbital, and herbicides of microsomal cytochrome P-450 in higher plant tissues. Arch Biochem Biophys 196: 301–303
28. RUSSELL DW 1971 The metabolism of aromatic compounds in higher plants. J Biol Chem 246: 3870–3878
29. TANAKA Y, M KOJIMA, I URITANI 1974 Properties, development and cellular localization of cinnamic acid 4-hydroxylase in cut-injured sweet potato. Plant Cell Physiol 15: 843–854
30. URITANI I, K ÔBA, A TAKEUCHI, K SATO, H INOUE, R ITO, I ITO 1981 *In* RL Ory, ed, Antinutrients and Natural Toxicants in Foods. Food and Nutrition Press Inc., Connecticut, pp 1–16
31. WEST CA 1980 Hydroxylases, monooxygenases, and Cyt P-450. Biochem Plants 2: 317–364