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Characterization of Metabolic Pathway of Linoleic Acid 9-Hydroperoxide in Cytosolic Fraction of Potato Tubers and Identification of Reaction Products

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Abstract

Potato tubers are shown to contain a unique lipoxygenase pathway to form 9-hydroperoxy-10,12-octadecadienoic acid (9-HPODE) from linoleic acid. Here, we report the metabolic pathway of 9-HPODE in the cytosolic fraction and the characterization of enzymes involved in the conversion of metabolites. The analysis of enzymatic reaction products at pH 5.5 revealed the formation of 9-keto-10,12-octadecadienoic acid, 9-hydroxy-10,12-octadecadienoic acid, 9,10-epoxy-11-hydroxy-12-octadecenoic acid, 9,10,13-trihydroxy-11-octadecenoic acid, and 9,12,13-trihydroxy-10-octadecenoic acid. The cytosolic enzymes were separated by anion-exchange chromatography into two fractions E1 and E2, having molecular masses of 66 and 54 kDa, respectively. The enzyme fraction E1 only produced 9-keto-10,12-octadecadienoic acid, whereas E2 formed other products. The enzyme E1 showed higher reactivity with 13- and 9-hydroperoxide of α-linolenic acid than 9-HPODE, but no reaction with hydroxy fatty acids. In contrast, the enzyme E2 showed the highest reactivity with 9-HPODE, followed by hydroperoxides of α-linolenic acid and arachidonic acid. We also evaluated the antibacterial activity of hydroxy fatty acids against *Erwinia carotovora* T-29, a bacterium infecting potato tubers. Growth of the bacteria was suppressed more potently with 9- or 13-hydroxy fatty acids than dihydroxy or trihydroxy fatty acids, suggesting a role for the metabolites in the resistance of bacterial infection.

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Index Entries: Potato tuber; linoleic acid 9-hydroperoxide; hydroxy fatty acids; *Erwinia carotovora* T-29; bacterial infection.

Introduction

Lipoxygenases (LOXs) are a family of dioxygenases that catalyze the stereospecific insertion of molecular oxygen into unsaturated fatty acids containing at least one 1,4-*cis*, *cis*-pentadiene unit to form fatty acid hydroperoxides. Animal LOXs are involved in the production of eicosanoids, bioactive lipid mediators, through the arachidonate cascade by the action of distinct types of LOXs on arachidonic acid, an essential fatty acid in animal nutrition. In analogy with animals, higher plants are known to have a number of unique LOX isoforms that are different in terms of localization, reaction properties, and gene expression. Acting on C18-fatty acids such as linoleic acid and α-linolenic acid through the octadecanoid pathway, plant LOX pathways provide a variety of bioactive compounds called oxylipins serving as regulators of physiologic and defense-related processes *(1)*. Of these, α-linolenic acid 13-hydroperoxide, a LOX product in leaves, is known to be a precursor for the plant growth regulators including traumatin and jasmonic acid *(2)*.

On the other hand, the role of other LOXs is poorly characterized. Potato tubers have been shown to contain a unique LOX pathway to produce 9-hydroperoxides from linoleic acid and α-linolenic acid *(3,4)*. In potato tubers, the most abundant substrate for the LOX is linoleic acid, in contrast with α-linolenic acid in leaves. Even though the tissues of potato tubers have no arachidonic acid in the cell membranes, the potato enzymes show a 5-LOX activity for arachidonic acid to form 5-hydroperoxy-eicosatetraenoic acid (5-HPETE) as well as the unstable leukotriene A_4, an intermediate with epoxide *(4,5)*. The 5-LOX activity of potato tubers has been implicated to act on arachidonic acid released from the cell membranes of invading fungi, which would trigger a hypersensitive reaction against them *(6)*. Moreover, potato tubers have been reported to contain a novel enzymatic pathway by which fatty acid hydroperoxides from linoleic acid and α-linolenic acid are converted into novel unsaturated fatty acid with a butadienyl vinyl ether structure, giving colneleic acid *(7)* and colnelenic acid *(8)*, respectively.

In this article, we report on the characterization of the enzymes involved in the metabolism of linoleic acid 9-hydroperoxide, and on the identification of the reaction products formed enzymatically by the cytosolic fraction from the homogenates of potato tubers. In addition, we evaluated the antibacterial activity of hydroxy fatty acids against the bacterium infecting potato tubers.

Materials and Methods

Chemicals, Equipment, and Tubers

Linoleic acid, α-linolenic acid, arachidonic acid, and soybean LOX-1 isozyme from soybean seeds were obtained from Sigma (St. Louis, MO).

DEAE-Toyopearl 650M and TSKgel G3000SW columns for high-performance liquid chromatography (HPLC) were supplied by Tosoh (Tokyo, Japan). Silica gel 60 thin-layer chromatography (TLC) plates and a LiChrosorb C18 column for HPLC were purchased from Merck (Darmstadt, Germany). Silica gel (Wakogel C-200) for column chromatography was obtained from Wako (Osaka, Japan), silicone OV-1 for GC from GL Science (Tokyo, Japan), and 1% trimethyl chlorosilane in *N*,*O*-bis(trimethylsilyl)-trifluoroacetoamide for GC analysis from Pierce (Rockford, IL). All other chemicals were of reagent grade.

Potato tubers (*Solanum tuberosum*, var. Danshaku) were of commercial origin. *Erwinia carotovora* subspecies carotovora T-29, a bacterium with an ability to infect potato tubers, was kindly provided by Dr. S. Arase of the Laboratory of Plant Pathology at Shimane University.

Enzyme Assays

The enzyme activity for the metabolism of fatty acid hydroperoxide was determined for 3 min at 25°C by monitoring the decrease in the absorbance at 235 nm of the consuming 40 μ*M* 9-HPODE as a substrate in 1 mL of 0.1*M* potassium phosphate buffer (pH 5.5). The decreased amount was estimated by using the value of 23,600 as a molar coefficient *(9)*. The substrate for this assay, 9-HPODE, was prepared by incubating the enzyme preparation, the precipitated fraction from 30–50% ammonium sulfate saturation of potato tuber homogenates, at 4°C with 1 m*M* linoleic acid as substrate.

For the enzyme assay of potato tuber LOX, the enzyme preparation was incubated at 25°C for 2 min with 200 μ*M* linoleic acid as a substrate in 1 mL of 0.1*M* potassium phosphate buffer (pH 6.5) containing 0.2% Tween-20. The amount of the product was determined by monitoring the increase in the absorbance at 234 nm with the value of 25,000 as a molar coefficient *(10)*.

Preparation and Separation of Enzymes

Peeled potato tubers (200 g) were homogenized in 120 mL of 0.1*M* sodium acetate buffer (pH 5.5) containing 2 m*M* sodium bisulfite and 2 m*M* ascorbic acid with a Waring blender at 4°C, filtered through gauze, and centrifuged at 15,000*g* for 20 min. Following operations were done at 4°C. After additional centrifugation at 100,000*g* for 1 h, the supernatant was dialyzed against 50 m*M* potassium phosphate buffer (pH 7.5). The resulting dialysate was used as the crude enzyme source for the enzymes involved in the metabolism of fatty acid hydroperoxides.

Further separation of the enzymes reacting with 9-HPODE was done by HPLC in a Shimadzu HPLC apparatus, model LC-4A. The clear supernatant was applied to a DEAE-Toyopearl column (8×250 mm). The column was washed with 2 bed vol of 10 m*M* Tris-HCl buffer (pH 8.0) containing 1 m*M* EDTA and 1 m*M* 2-mercaptoethanol. The enzyme-active fractions were eluted with a linear gradient of NaCl from 0 to 0.2*M* in the preceding mobile phase. The flow rate was set at 2 mL/mL, and the resulting effluent was collected every 4 min.

For separation of the enzymes by gel filtration by HPLC on a TSKgel G3000SW (8 × 600 mm), the column was eluted at a flow rate of 1 mL/min, and the effluent was collected every 0.5 mL as a fraction. The molecular weight of the enzyme was estimated by using standard proteins of ferritin (450 kDa), catalase (240 kDa), bovine serum albumin (BSA) (68 kDa), ovalbumin (45 kDa), chymotrypsin (25 kDa), and cytochrome *c* (12.5 kDa). The collected fraction was analyzed to determine the enzyme activity of LOX and the metabolism of fatty acid hydroperoxides.

For preparation of potato tuber LOX, peeled potato tubers (500 g) were homogenized as already described. After the homogenates were centrifuged at 15,000*g* for 20 min, the resulting supernatant was brought to 30–50% saturation with ammonium sulfate. The precipitated proteins were dialyzed against 50 m*M* potassium phosphate buffer (pH 7.5) at 4°C and used as the enzyme source of potato tuber LOXs.

Analysis of Reaction Products

The metabolites of fatty acid hydroperoxides were extracted with ethyl acetate after acidification with 0.2*N* HCl. The extract was evaporated to dryness, and the dried materials were dissolved in benzene. The reaction products were separated by TLC on silica gel 60 plates. The plates were developed with a mixture of hexane/benzene/chloroform/methanol/acetic acid (50:25:70:15:1 [v/v]). Reaction products were detected by spraying a solution of 10% cupric sulfate and 8% phosphoric acid followed by heating at 150°C for 20 min. For separation of the reaction products, reverse-phase HPLC (RP-HPLC) was run at a flow rate of 1 mL/min on a LiChrosorb C18 column (4 × 250 mm) using tetrahydrofuran/acetonitrile/water/acetic acid (200:360:440:0.5 [v/v]) as a mobile phase. The elution of fatty acid hydroperoxides was monitored at 232 nm for conjugated dienes or at 280 nm for conjugated trienes.

Each separated product was identified by gas chromatography-mass spectrometry (GC-MS) using a Hitachi M-80B apparatus equipped with a glass column (3 mm × 2 m) packed with Silicone OV-1. The column temperature was increased from 150 to 220°C at a rate of 10°C/min. For analysis by GC-MS, derivatives were identified using electron-impact (EI) mode and chemical ionization (CI) mode with gases of ammonia and isobutane.

Before injection into the column, the reaction products were reduced with sodium borohydride and methylated by treatment with etheral diazomethane. Then, their trimethylsilyl (TMS) ether derivatives were prepared by reaction with an equal volume of 1% trimethyl chlorosilane in *N*,*O*-bis(trimethylsilyl)-trifluoroacetoamide and dry pyridine.

Assay of Antibacterial Activity of Hydroxy Fatty Acids

The bacterium infecting potato tubers used here is the subspecies carotovora T-29 of *E. carotovora*. The bacteria were grown by shaking reciprocally in the trypticase soy broth soybean-casein digest medium (BBL Microbiology Systems). Growth was monitored by the increase in the absorbance

at 550 nm after shaking for 24 h at 30°C. Compounds to be tested were added to the bacterial culture by dissolving in ethanol.

Other Methods

The amount of proteins was determined essentially according to the methods of Lowry et al. *(11)* and its modification *(12)* using fatty acid–free BSA as a standard.

Preparation of hydroperoxy and hydroxy fatty acids was done as follows. Potato tuber LOXs were allowed to react with linoleic acid and α-linolenic acid to form the corresponding 9-hydroperoxides, 9-HPODE and 9-hydroperoxy-octadecatrienoic acid (9-HPOTE), respectively. These products were reduced by treating with sodium borohydride to give 9-hydroxides of the fatty acids, 9-HODE, and 9-hydroxy-octadecatrienoic acid, respectively. Alternatively, 13-hydroperoxides of linoleic acid and α-linolenic acid, 13-HPODE and 13-HPOTE, were produced by incubating with soybean LOX-1 at 4°C for 1 h in 0.1*M* Tris-HCl buffer (pH 8.5) containing 0.2% Tween-20. Moreover, the reduction of these compounds with sodium borohydride gave the corresponding 13-hydroxides, 13-HODE and 13-HOTE. For the preparation of 5-HPETE and 15-HPETE, potato tuber LOXs and soybean LOX-1 were allowed to react with arachidonic acid, respectively.

To determine antibacterial activity of hydroxy fatty acids, several types of hydroxy fatty acids with C18 were prepared and purified by column chromatography on Wakogel C-200. The purity of each compound was confirmed by TLC on silica gel 60 plates. To obtain ricinoleic acid, 12-hydroxy-9-octadecenoic acid, castor bean oil was hydrolyzed at 50°C for 30 min by 6% KOH in ethanol to give free form. After acidification with 2*N* HCl, the resulting monohydroxy fatty acid was extracted with a mixture of chloroform/methanol (2:1 [v/v]) and purified by silica gel column chromatography as just described. 9,10-Dihydroxy-octadecanoic acid and 9,10,12-trihydroxy-octadecanoic acid were prepared according to the method of Kenneth et al. *(13)*.

Results

Identification of Metabolites Formed From Linoleic Acid 9-Hydroperoxide

The soluble enzyme preparation from the centrifugation of potato tuber homogenates at 100,000*g* was allowed to react with 9-HPODE under the acidic conditions of pH 5.5. Analysis of the reaction products by TLC gave four major spots on the silica 60 plates (Fig. 1). By contrast, no reaction products were detected at an alkaline pH of 9.0 or with boiled enzyme preparations. The reaction products at pH 5.5 were separated by silica gel column chromatography and TLC. The purified reaction product was identified by combined analyses using ultraviolet (UV) spectroscopy, HPLC, and GC-MS.

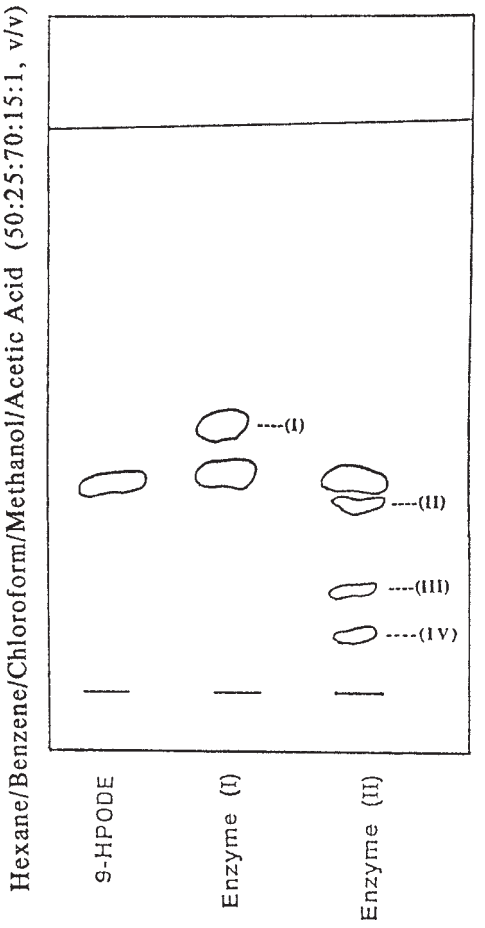

Fig. 1. TLC analysis of reaction products from 9-HPODE by soluble fraction of potato tubers. The soluble fraction after centrifuging at 100,000*g* for 1 h at 4°C was allowed to react at 25°C for 15 or 30 min with 40 μ*M* 9-HPODE in 0.1*M* potassium phosphate buffer (pH 5.5) or 0.1*M* glycine-NaOH buffer (pH 9.0). The resulting products were extracted with ethyl acetate and dissolved in benzene. Portions were subjected to analysis by TLC as described in Materials and Methods.

The absorption spectrum of compound I showed a maximum absorption at 277 nm, which is characteristic of conjugated trienes. When reduced with sodium borohydride, this product showed a shift of the absorption maximum to 232 nm, indicating the presence of conjugated dienes in the reduced compound I. The reduced product was found to coelute with 9-HODE by RP-HPLC. GC-MS analysis in the EI mode of compound I as a derivative of methyl ester gave a molecular ion of 308 *m/z* and characteristic fragment ions of 123 *m/z*, 151 *m/z*, and 185 *m/z* (Fig. 2). Moreover, the mass spectrum of the derivative of TMS ether and methyl ester of compound I after reduction with sodium borohydride was found to be the same as for compound II, indicating the formation of 9-HODE from compound I by the

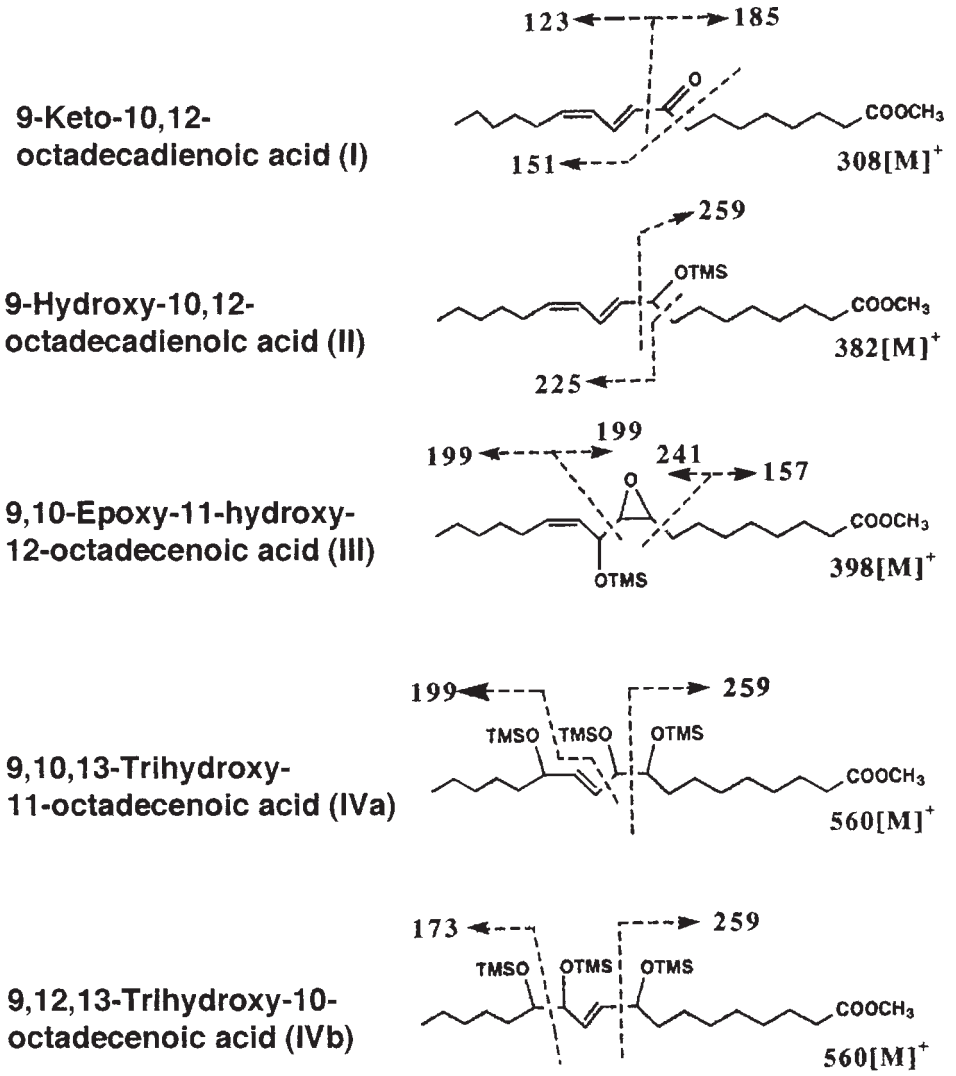

Fig. 2. Mass spectra of methyl ester–TMS ether derivatives in EI mode. Sites for the cleavage of compounds to generate mass spectral fragment ions are shown for the derivatized metabolites formed from 9-HPODE by the soluble enzyme preparation of potato tubers. Derivatization of metabolites and analysis by GC-MS were done as described in Materials and Methods.

reduction. Thus, compound I was identified as 9-keto-10,12-octadecadienoic acid. Compound II had an absorption maximum at 232 nm, reflecting the existence of conjugated dienes. Compound II was eluted at the same retention time as 9-HODE. GC-MS analysis of methyl ester and TMS ether derivative of this compound in the EI mode confirmed the presence of 382 *m/z* as a molecular ion and characteristic fragment ions of 225 *m/z* and 259 *m/z*. Analysis in the CI mode with ammonia gas gave prominent ions

of 293 *m/z* ($M+NH_4$–90) and 293 *m/z* (M+1-90). Hence, compound II was identified as 9-HODE.

Products III and IV have no characteristic UV absorption spectrum reflecting conjugated diene or triene compounds. The mass spectrum of the methyl ester and TMS ether derivative of compound III gave a molecular ion of 398 *m/z* and fragment ions of 157 *m/z*, 199 *m/z*, and 241 *m/z*. Moreover, the mass spectrum of the same derivative in the CI mode showed prominent ions at 259 *m/z* (241+NH_4) and 416 *m/z* ($M+NH_4$). Hence, compound III was proved to be 9,10-epoxy-11-hydroxy-12-octadecenoic acid. Compound IV in TLC plates was converted into the derivative of methyl ester and TMS ether. The derivative showed two peaks of IVa and IVb by separation by GC-MS. The mass spectrum of the derivative of the earlier peak IVa in the EI mode gave a molecular ion of 560 *m/z* and fragment ions of 199 *m/z* and 259 *m/z*. Mass analysis with the CI mode using isobutane provided characteristic ions of 259 *m/z* and 545 *m/z* ($M–CH_3$). Thus, compound IVa was identified as 9,10,13-trihydroxy-11-octadecenoic acid. In addition, the derivative of compound IVb gave a molecular ion of 560 *m/z* as well as fragment ions of 173 *m/z* and 259 *m/z*. The characteristic ions of 545 *m/z* ($M–CH_3$) and 259 *m/z* were detected by mass analysis with the CI mode. Compound IVb was determined as 9,12,13-trihydroxy-10-octadecenoic acid.

Properties of Separated Enzyme Preparations Involved in Metabolism of Linoleic Acid 9-Hydroperoxide

When the subcellular localization of the enzyme activity in the metabolism of linoleic acid 9-hydroperoxide was determined in the homogenate of potato tubers, it was found that almost 80% of the total enzyme activity was recovered in the cytosolic fraction. This enzyme fraction was separated into two enzyme-active fractions by HPLC on a DEAE-Toyopearl column by eluting with a linear gradient of NaCl from 0 to 0.2*M* (Fig. 3). Fraction I was eluted without absorption to the anion-exchange column, whereas fraction II was eluted with about 0.1*M* NaCl after absorption to the column. The resulting separated enzyme fraction was applied to the gel filtration column by HPLC using TSKgel G3000SW (Fig. 4). Based on the elution time of the enzyme activity, the molecular weights are estimated as 66 and 54 kDa for enzymes E1 and E2, respectively.

To determine the reaction products by the separated enzyme preparations, each enzyme fraction after gel filtration was separately incubated with 9-HPODE as a substrate (Fig. 5). The reaction products were extracted and analyzed by TLC. Enzyme E1 obtained after gel filtration only produced 9-keto-10,12-octadecadienoic acid as compound I, whereas enzyme E2 gave other reaction products corresponding to 9-hydroxy-10,12-octadecadienoic acid (II), 9,10-epoxy-11-hydroxy-12-octadecenoic acid (III), and trihydroxy fatty acids (IV).

The optimal pH of the separated enzyme preparations was determined as shown in Fig. 6. Enzyme E1 showed the highest enzyme activity at pH 5.0 when the increase in the absorbance at 280 nm of forming conju-

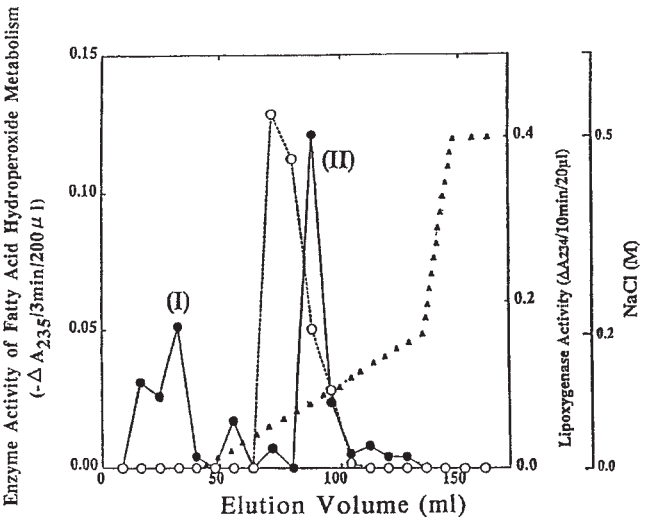

Fig. 3. Separation of soluble enzyme fraction by HPLC on DEAE-Toyopearl 650M column. The soluble fraction after centrifuging at 100,000*g* was loaded onto a DEAE-Toyopearl column. After washing with 2 bed vol of 10 m*M* Tris-HCl buffer (pH 8.0), the enzymes were eluted at a flow rate of 2 mL/min with a linear gradient of NaCl concentration from 0 to 0.2*M*. The effluent was collected in 4-mL fractions. The portions were analyzed for the assays of LOX activity (○) with linoleic acid as a substrate and the enzyme activity in the metabolism of 9-HPODE (●). The gradient of NaCl concentration is also shown (▲). Other details are described in Materials and Methods.

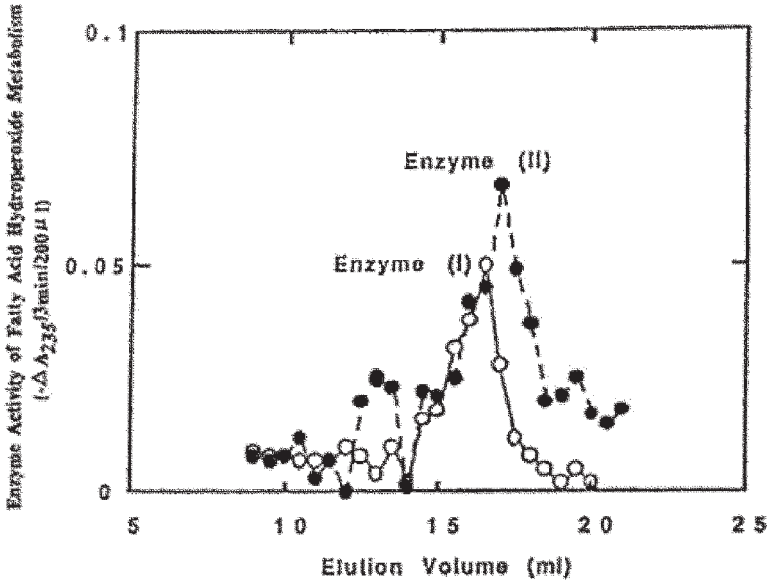

Fig. 4. Gel filtration of enzymes in metabolism of 9-HPODE by HPLC on TSKgel G3000SW column. Each enzyme fraction (1 mg of protein) after separation by anion-exchange chromatography as described in Fig. 3 was separated by gel filtration chromatography on a TSKgel G3000SW column by elution at a flow rate of 1 mL/mL with 10 m*M* Tris-HCl buffer (pH 7.5) containing 0.1*M* sodium sulfate. The effluents were collected every 0.5 mL and subjected to determination of enzyme activity for the metabolism of 9-HPODE as described in Materials and Methods. (○) Enzyme E1; (●) enzyme E2.

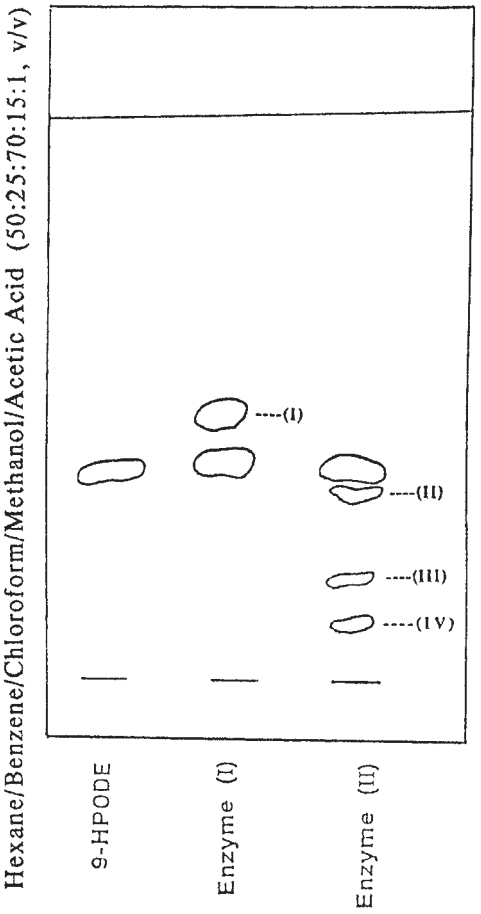

Fig. 5. TLC analysis of reaction products from 9-HPODE by enzyme preparations after gel filtration by HPLC. Enzyme E1 or E2 obtained after elution on a TSKgel G3000SW column was incubated at 25°C for 30 min with 40 μ*M* 9-HPODE. For assays of enzymes E1 and E2, the enzyme reactions were done using 0.1*M* potassium phosphate buffer (pH 6.0) and 0.1*M* sodium acetate (pH 5.0), respectively. The resulting products were extracted with ethyl acetate and analyzed by TLC as described in Materials and Methods.

gated trienes reflecting the product with ketodienes was monitored with 9-HPODE as a substrate. Alternatively, enzyme E2 also had an optimal pH of 5.0 for assay of the decrease in the absorbance at 235 nm, whereas there was no enzyme activity when following the increase in the absorbance at 280 nm (Fig. 6).

Moreover, we determined the substrate specificity of the enzyme preparations, E1 and E2, for a variety of fatty acid hydroperoxides (Fig. 7). Enzyme E1 catalyzed the formation of the compounds with ketodienes more preferentially with 13-HPOTE and 9-HPOTE than 9-HPODE. However, enzyme E1 had no reactivity with hydroxy fatty acids including 9-HODE and 13-HODE, indicating no dehydrogenase activity. In sharp

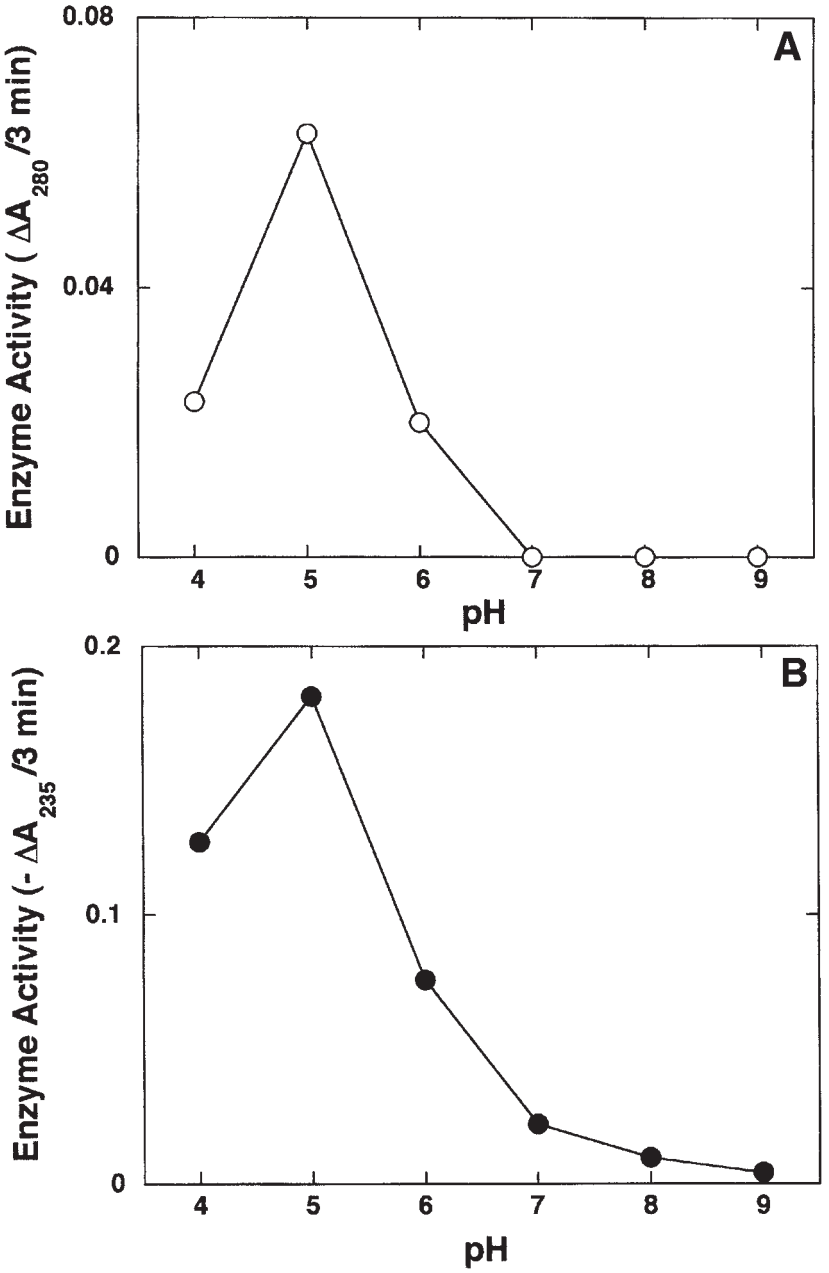

Fig. 6. Optimal pH of enzyme preparations in metabolism of 9-HPODE. The enzyme preparations, E1 and E2, after separation of the soluble fraction by anion-exchange chromatography were used for determination of the enzyme activity in the metabolism of 9-HPODE at different pH values. The enzyme reaction was done in either 0.1*M* sodium acetate buffer (pH 4.0–5.0), 0.1*M* potassium phosphate buffer (pH 6.0–7.0), or 0.1*M* glycine-NaOH buffer (pH 8.0–9.0). Other details are described in Materials and Methods. **(A)** The enzyme activity of E1 was determined by monitoring the increase in the absorbance at 280 nm. **(B)** The enzyme activity of E2 was determined as the decrease in the absorbance at 235 nm.

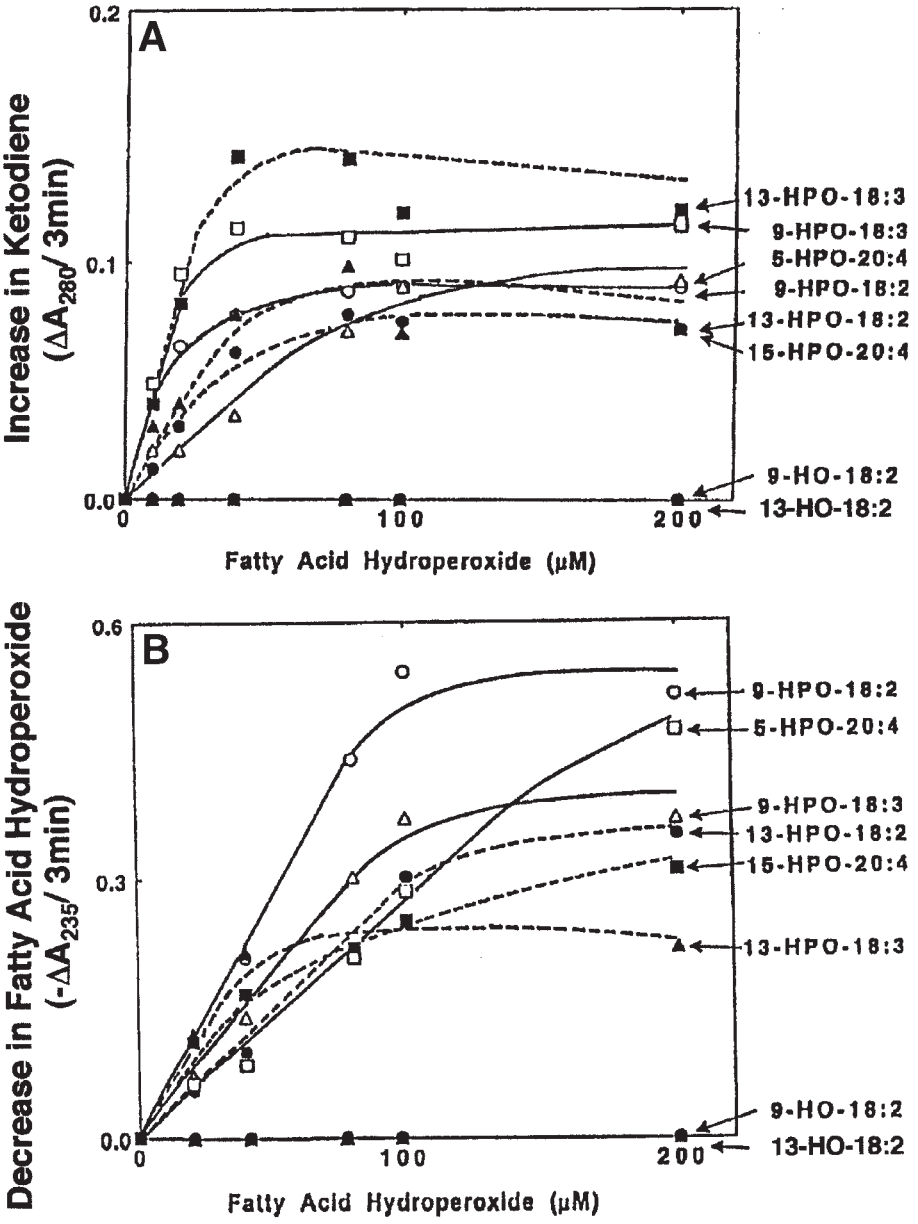

Fig. 7. Substrate specificity of separated enzyme preparations for reaction with variety of fatty acid monohydroperoxides or monohydroxides. **(A)** Enzyme E1 was allowed to react with increasing concentrations of fatty acid monohydroperoxides or monohydroxides in 0.1*M* sodium acetate buffer (pH 5.0). The enzyme activity of E1 was assayed by the increase in the absorbance at 280 nm of forming ketodienes. **(B)** Enzyme E2 was incubated with increasing concentrations of the same substrates in 0.1*M* sodium acetate buffer (pH 5.0). The enzyme activity was determined by following the decrease in the absorbance at 235 nm of conjugated dienes. Other details are described in Materials and Methods. 9-HPO-18:2, 9-HPODE; 13-HPO-18:2, 13-HPODE; 9-HPO-18:3, 9-HPOTE; 13-HPO-18:3, 13-HPOTE; 5-HPO-20:4, 5-HPETE; 15-HPO-20:4, 15-HPETE; 9-HO-18:2, 9-HODE; 13-HO-18:2, 13-HODE.

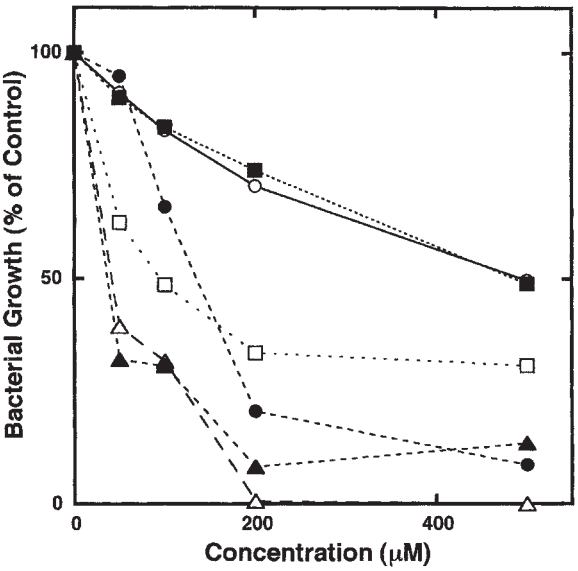

Fig. 8. Antibacterial activity of different types of hydroxy fatty acids against *E. carotovora* T-29. The bacterium was grown by shaking at 30°C for 24 h. After the addition of different types of hydroxy fatty acids to the bacterial cultures, antibacterial activity was determined by monitoring the absorbance at 550 nm as an index of bacterial growth. Other details are described in Materials and Methods. (○) Linoleic acid; (●) 9-HODE; (△) 13-HODE; (▲) 12-hydroxy-9-octadecenoic acid; (□) 9,10-dihydroxy-octadecanoic acid; (■) 9,10,12-trihydroxy-octadecanoic acid.

contrast, enzyme E2 showed higher reactivity with 9-HPODE and 9-HPOTE even though the enzyme can react with other 13-hydroperoxy isomers as well as 5-HPETE from arachidonic acid. This enzyme E2 exhibited no reaction with hydroxy isomers.

Antibacterial Activity of Hydroxy Fatty Acids

Using *E. carotovora* T-29, a bacterium species infecting potato tubers, we evaluated the antibacterial activity of a variety of hydroxy fatty acids with C18, as shown in Fig. 8. Bacterial growth was more potently inhibited by the addition of hydroxy fatty acids in the order of monohydroxy fatty acids, dihydroxy fatty acids, and trihydroxy fatty acids. The effect of linoleic acid without hydroxyl moiety is similar to that of trihydroxy fatty acid, which is much less potent than monohydroxy fatty acid. Acting on linoleic acid, potato tuber LOXs catalyze the formation of 9-HPODE. The reduced product 9-HODE was identified as a metabolite of our enzyme reaction mixture. The antibacterial activity of 9-HODE was much more potent among the compounds tested here. Similarly, a more potent effect was found with 13-HODE, suggesting no specific regiospecificity for the position of hydroxy group.

Discussion

Potato tuber LOXs have recently been cloned by different groups *(14,15)*. The enzymes are well known to react with linoleic acid and α-linolenic acid to produce 9-hydroperoxide of their polyunsaturated fatty acids *(3,4)*. However, the most abundant polyunsaturated fatty acid for the substrate of potato tuber LOXs is linoleic acid from membrane galactolipids and phospholipids. Therefore, the predominant product of potato tuber LOXs should be linoleic acid 9-hydroperoxide. The role of LOXs acting on the C-9 residue is poorly understood in contrast to LOXs in generating 13-hydroperoxide, which is present in plant leaves and soybean seeds *(2)*. As an initial aim, we tried to separate and characterize the metabolic enzymes of fatty acid hydroperoxides in potato tubers. The present study attempted to employ the enzyme preparations from the cytosolic fraction.

The crude enzymes from the supernatant after centrifuging at 100,000*g* from the homogenates of potato tubers gave four reaction products at an acidic pH of 5.5 on reacting with 9-HPODE. Analyses by absorption spectrum, HPLC, and GC-MS allowed identification of these enzymatic reaction products. The metabolites were identified as 9-keto-10,12-octadecadienoic acid, 9-HODE, 9,10-epoxy-11-hydroxy-12-octadecenoic acid, 9,10,13-trihydroxy-11-octadecenoic acid, and 9,12,13-trihydroxy-10-octadecenoic acid. The formation of these products is thought to be owing to the enzymatic reaction as shown in Fig. 1 because the boiled enzyme showed no reaction products from linoleic acid. Moreover, the nonenzymatic reduction of linoleic acid 9-hydroperoxide to the corresponding 9-hydroxide did not account for the production of monohydroxide, suggesting the very low level of the nonenzymatic reductants in the homogenates of potato tubers. Similarly, previous studies using crude enzymes from preparations from potato tubers reported the enzymatic reaction of converting linoleic acid hydroperoxides into some oxygenated fatty acid derivatives including epoxy and trihydroxy groups at pH 5.0–7.0 *(16)*.

To our knowledge, 9-keto-10,12-octadecadienoic acid has not been described in potato tubers before. This substance can be distinguished from colneleic acid on the basis of several lines of evidence. First, colneleic acid was reported to have an absorption maximum at 250 nm, whereas our compound I showed a maximum at 277 nm. Second, the product obtained after reduction with sodium borohydride coeluted with known 9-HODE by RP-HPLC. Third, mass spectral analysis of the derivative with TMS ether and methyl ester revealed that the reduced compound I with sodium borohydride was consistent with that of the corresponding 9-HODE derivative even though the mass spectrum of the methyl ester derivative of the parent compound I gave the same parent ion at 308 *m/z* as that of colneleic acid.

Thus, our crude enzyme preparations from potato tubers did not generate any reaction spots corresponding to divinyl ether derivatives on the TLC plates even when the enzymatic reaction was done at an alkaline pH

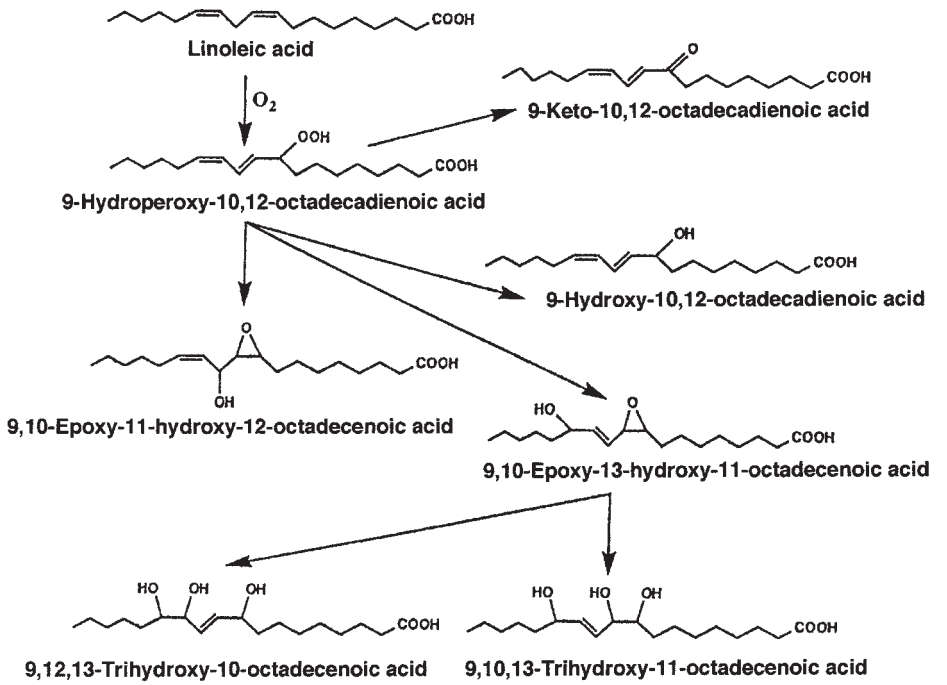

Fig. 9. Proposed metabolic pathways of linoleic acid 9-hydroperoxide in the cytosol fraction of potato tubers.

of 9.0. This result was in contrast with those of earlier studies describing the enzymatic production of colneleic acid, a divinyl ether derivative *(7,17)*. We have no definite explanation for the discrepancy among them. Possible explanations could be the difference of varieties of potato tubers, the enzyme stability during the handling of the enzymes, or the method of subcellular fractionation.

Based on our studies, we can propose the metabolic pathway in the cytosol fraction of 9-HPODE as an enzymatic reaction product of potato tuber LOXs as described in Fig. 9. When the enzyme preparation was incubated with 9-HODE, we failed to detect 9-keto-10,12-octadecadienoic acid. This finding suggests no conversion of 9-hydroxide into the corresponding 9-ketodiene. The current study confirmed the formation of 9,10-epoxy-11-hydroxy-12-octadecenoic acid, which cannot be converted into other compounds because the epoxide moiety in that compound is more stable. We detected two types of trihydroxy-octadecenoic acids that can be easily formed through an intermediate of 9,10-epoxy-13-hydroxy-11-octadecenoic acid. This intermediate is likely to be so unstable in the homogenate of potato tubers that it was not detected in the present homogenates.

The crude cytosolic enzyme preparations were separated into two fractions, enzymes E1 and E2, by anion-exchange column chromatography when the enzyme activity was monitored at pH 5.5. The enzyme E1 prepared after partial purification with gel filtration only produced 9-keto-

10,12-octadecadienoic acid. Previously, some LOXs were described as being able to catalyze the formation of keto-fatty acids by acting on the LOX products in soybeans *(10)*. However, potato tuber LOXs were completely separated from enzyme I. Hence, the involvement of LOXs in the further conversion of 9-HPODE into 9-keto-10,12-octadecadienoic acid can be excluded here. Enzyme E2 was found to be responsible for the generation of trihydroxy fatty acids. These compounds should be formed through the intermediate with epoxide moiety, which can be spontaneously hydrolyzed to two types of trihydroxy fatty acids. Further purification of these enzymes was not successful because the inactivation occurred easily even though the enzyme preparations were stored at pH 7.5 in the presence of 20% glycerol at –30°C. It would be important to find the optimal conditions under which the enzymes remain stable during purification procedures and storage.

We cannot exclude the occurrence of other enzymes in metabolizing the fatty acid 9-hydroperoxide in potato tubers because we only assayed at an acidic pH of 5.5. In addition, the enzyme assay at different pH values might detect other enzymes as well as with the use of other subcellular fractions such as particular fractions. More recently, molecular cloning of a divinyl ether synthase was done from tomato cDNA *(18)*. According to that study, this enzyme needs to be solubilized with Triton X-100R, indicating the preferential localization at the membrane fractions. By contrast, we used the cytosolic fraction after centrifuging at 100,000*g*. This might explain why we failed to detect the enzyme activity of colneleic acid.

A group of bioactive compounds that are formed in plants from the LOX pathways are referred to as oxylipins, including jasmonic acid, traumatin, and other related compounds *(19)*. In the LOX pathway leading to the formation of 13-hydroperoxide of linoleic acid or α-linolenic acid, some key enzymes are involved in the metabolism of these acids, such as allene oxide synthase to form the precursor of jasmonic acid and hydroperoxide lyase to cleave 13-HPODE to generate short chain aldehydes contributing to odors or aromas in leaves and fruits. Recent advances in molecular biologic approaches have revealed that these enzymes have been shown to be members of a family of cytochrome P-450 enzymes as well as divinyl ether synthase *(18,20)*. More recently, Hamberg *(21)* reported the biosynthesis of new cyclopentenone fatty acids through a new type of allene oxide from 9-HPODE in potato sprouts and roots as well as developing potato tubers. Our enzyme preparation of E2 appears to be different from allene oxide synthase because the chemical structure of the proposed intermediate with epoxide, as shown in Fig. 9, is not identical to the allene oxide of Hamberg's *(21)* report. The current study implies the presence of potentially novel pathways to metabolize 9-HPODE in different preparations depending on the tissues, stages of growth, or subcellular localization in potato.

The biologic role of the LOX pathway in forming 9-hydroperoxy fatty acids in potato tubers has been unclear in comparison with other LOX pathway providing 13-hydroperoxide, which is the precursor for several

growth regulators as well as compounds with antimicrobial activities. The present study evaluated the antibacterial activity of several hydroxy fatty acids against *E. carotovora* T-29, which can infect potato tubers (Fig. 8). One of the products of potato tuber homogenates, 9-HODE, exhibited more potent antibacterial activity than dihydroxy or trihydroxy fatty acids against the bacterium species. Moreover, monohydroxy fatty acids such as 13-HODE and 12-hydroxy-9-octadecenoic acid were also as potent as 9-HODE. These observations indicate the common features of monohydroxy fatty acids without selectivity for the position of hydroxy group. In agreement with our studies, 9-HODE was reported to be identical to the bioactive compound isolated from rice extracts by monitoring the antifungal activity against rice blast *(22)*. We did not test the other metabolites identified here because of the lower amounts in the extracts. Further studies need to be conducted.

Regarding the modes of action of hydroxy fatty acids, one possible action might be caused by the direct action of hydroxy fatty acids on the infecting bacterium. Alternatively, another presumed action might be the indirect function of these substances to serve as an elicitor for inducing the production of phytoalexins in plants. In the case of hydroperoxides of fatty acids, the enzymatic cleavage of these compounds by hydroperoxide lyase is implicated in allowing the formation of short chain alkenals and oxoacids *(23)*. Therefore, we also tested the antibacterial activity of some available aldehydes against *E. carotovora* T-29 and found a dose-dependent inhibition of bacterial growth by *n*-decanal, *n*-octanal, and *n*-hexanal (data not shown). Further studies in potato tubers will be necessary in order to identify the hydroperoxide lyase acting on 9-HPODE.

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