

Ceratocystis fimbriata 感染サトイモにおける過酸化脂質生成とその生成に関与する酸素に関する研究

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Studies on Lipid Peroxides and the Enzymes Which Are Involved in Their Production in Taro Tubers Infected by *Ceratocystis fimbriata*

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Abstract

Two enzymes, Lipoyxygenase (LOX) and lipid hydroperoxide converting enzyme (LHCE), which are responsible for the production of antifungal lipid peroxides, were detected in taro tubers infected by *Ceratocystis fimbriata*. The infected taro tubers contained two LOX isozymes (LOX-1, LOX-2). The main isozyme of LOX was LOX-2 that showed optimal activity at pH 5.5. The enzyme (LOX-2) showed similar magnitude of activity toward linoleic acid (100%) and linolenic acid (77%), but did not show any activity toward methyl linoleate (0%). It changed linoleic acid into 9- (9-LOOH) and 13-linoleate hydroperoxides (13-LOOH) in the ratio of 47:53. The infected taro tubers contained two isozymes of LHCE (LHCE-1, LHCE-2) with similar substrate specificity. When 9- or 13-LOOH of linoleic acid was used as the substrate of LHCE, main product was 9- or 13-hydroxy-octadecadienoic acid (LOH), respectively. 9,12,13-trihydroxyoctadecenoic acid (9,12,13-LOH) was produced as a minor product from 9-LOOH by LHCE. All of 9- and 13-LOOH, 9- and 13-LOH, and 9,12,13-LOH showed the similar toxicity toward both sweet potato and taro strains of *C. fimbriata*. On the other hand, the crude enzyme preparation (containing both LOX and LHCE) from the infected taro tubers converted linolenic acid into the peroxides compound(s), which inhibited the growth of sweet potato strain more severely than that of taro strain.

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Key words: lipoyxygenase, lipid hydroperoxide converting enzyme, taro, *Ceratocystis fimbriata*, antifungal compound.

INTRODUCTION

Various strains of *Ceratocystis fimbriata* have been isolated from black rot lesions on various plants such as taro and sweet potato. These strains show strict host specificity. Taro strain is pathogenic to taro but not to sweet potato, while sweet potato strain is pathogenic to sweet potato, but not to taro.

It has been reported with some plant host-parasite interactions that lipid peroxidation takes place in the early stage of infection and that the lipid peroxides produced in plants play the important roles in inhibiting the growth of invading parasite^{1-4,6,9,13-15,17}. We previously isolated 9,12,13-trihydroxy-(E)-10-octadecenoic acid (9,12,13-LOH) as an antifungal compound from taro tubers inoculated by *C. fimbriata*⁷. The structure of 9,12,13-LOH suggests that it is synthesized *in vivo* by following mechanisms; linoleic or linolenic acid was first peroxidized into hydroperoxide derivatives by lipoyxygenase (LOX). Subsequently, the hydroperoxide derivatives are changed into hydroxy fatty acid by lipid hydroperoxide converting enzyme (LHCE). LOX is a ubiquitous enzyme in higher plants¹⁶. Recently LHCE was detected in rice leaves infected by blast fungus and characterized by Ohta *et al.*^{10,11}

In this paper, we report detection and properties of LOX and LHCE in the taro tubers infected by

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C. fimbriata. In addition, we report the antifungal activity of lipid peroxides produced by the enzymes toward *C. fimbriata*.

MATERIALS AND METHODS

Chemicals. Lipoxygenase (from soybean, type I) was purchased from Sigma Chemical Co. DEAE-Toyopearl M-650 and Sephadex G-25 were from Toyo Soda Co. (Tokyo) and Pharmacia Fine Chemicals (Uppsala, Sweden), respectively. Trimethylsilylation reagent, Tri-sil, was obtained from PIERCE (Rockford, USA). All other chemicals were of reagent grade.

Preparation of inoculum. Taro and sweet potato strains of *C. fimbriata* were used. Inoculum (7×10^6 spores/ml) was prepared by the method of Kojima *et al.*⁹⁾

Preparation of taro tuber disks and inoculation. Taro tuber disks (1.2×0.2 cm) were prepared and were rinsed in distilled water to remove the latex from the surface. The rinsed disks were blotted with filter paper and incubated at 25°C for 25 hr in a moist chamber after inoculation with sweet potato or taro strain.

Preparation of peroxides of linolenic acid. The crude enzyme including both LOX and LHCE was prepared from the taro tubers incubated for 25 hr after inoculation by the method described below. The enzyme solution (10 ml, 2,000 units of LOX) was added to 20 ml of the solution containing 39 mg of linolenic acid in 0.1 M citrate- Na_2HPO_4 buffer, pH 5.5 and shaken at 25°C. After 12 hr shaking, 100 μl of conc. HCl was added to the reaction mixture and extracted with diethyl ether. The ether fraction was dried up under N_2 . The remained solid material was used as peroxides of linolenic acid for assay of antifungal activity.

Preparation of 9- and 13-hydroperoxy linolenic acids. 9-LOOH was prepared using potato lipoxygenase by following method. Crude potato lipoxygenase was prepared by the method of Sekiya *et al.*¹²⁾ Potato lipoxygenase (6,300 units) was added to 450 ml of 5 mM linoleic acid in 0.1 M acetate buffer, pH 5.5 containing 0.07% Tween 20 and incubated for 1 hr at 25°C by stirring and bubbling O_2 . Then the reaction mixture was adjusted at pH 4.0 with conc. HCl and extracted with diethyl ether. The extract was condensed and subjected to TLC of silica using a solvent (*n*-hexane : diethyl ether : acetic acid = 10 : 5 : 0.1). 9-LOOH was visualized under UV light and were scrapped off the silica gel plates and extracted with chloroform : methanol = 2 : 1. The extract was passed through a Ekicrodisc filter (Gelman Sciences Japan, Ltd.) to remove the silica gel powder and concentrated under N_2 .

13-LOOH was prepared using commercial soybean lipoxygenase as follows. Linoleic acid (1 g) was suspended in 30 ml of 0.1% Tween 20 and 1 N NaOH was added until the suspension became a clear solution. Then 400 ml of 0.1 M borate buffer (pH 9.0) and soybean lipoxygenase (10 mg) were added to the linoleic acid solution and incubated by stirring and bubbling O_2 at 0°C for 1 hr. After incubation, 13-LOOH was isolated from the reaction mixture by the same method as that used for 9-LOOH.

Both of 9- and 13-LOOH prepared as described above were purified further using a HPLC (LiChrospher Si 60 (4 mm i.d. $\times$ 250 mm, Cica-MERCK), solvent system; *n*-hexane containing 2.5% (v/v) *iso*-propyl alcohol and 0.2% (v/v) acetic acid). The purified 9- and 13-LOOH were stored as ethanol solution at -20°C and used within a month.

9- or 13-hydroxy of linolenic acid was prepared from 9- or 13-LOOH, respectively, by reducing with NaBH_4 . 9,12,13-Octadecenoic acid was purified as described in our preceding paper⁷⁾.

Extraction and assay of LOX and LHCE. Five disks (1.2×0.2 cm) were ground in 5 ml of 0.1 M Tris-HCl buffer (pH 7.5) containing 0.1% Tween 20 and sea sand using mortar and pestle and the homogenate was stood at 4°C for 1 hr. Subsequently, the homogenate was centrifuged at $43,700 \times g$ for 15 min at 4°C. The resultant supernatant was applied to a column (4.6 $\times$ 16 cm) of Sephadex G-25. The void volume fraction was used for assay of both LOX and LHCE.

The activity of LOX was assayed with spectrophotometrical procedure in our preceding paper⁸⁾ and one unit of LOX was defined as the amount of the enzyme that produce one μmol of lipid hydroperoxide per min under the assay conditions.

LHCE activity was assayed by the method of Ohta *et al.*¹¹⁾ One unit of the activity was expressed

as the amount of the enzyme that causes disappearance of one μmol of hydroperoxy octadecadienoic acid per min at 25°C under the assay conditions.

DEAE-Toyopearl column chromatography. The inoculated taro tuber silices after incubated for 25 hr (80 g) was ground in 80 ml of TTG buffer (0.1 M Tris-HCl buffer (pH 7.5) with 0.1% Tween 20 and 10% (v/v) glycerol), including *iso*-ascorbic acid (0.8 g) and sea sand in a mortar. The homogenate was stood at 4°C for 1 hr, squeezed through 4 layers of gauze and centrifuged at $43,700 \times g$ for 15 min. The supernatant was passed through a Sephadex G-25 column and the effluent fraction was collected. The fraction was condensed by filtration through Amicon YM-10 filter and then applied to a column (2.3×10 cm) of DEAE-Toyopearl pre-equilibrated with TTG buffer. The column was washed with TTG buffer, and then eluted with 200 ml of a linear gradient of 0 to 0.3 M NaCl in TTG buffer. Every 3.8 ml was collected and assayed for LOX and LHCE activities and protein content.

Preparation and identification of LOX reaction products. LOX-2 after DEAE-Toyopearl column chromatography (30 units) was added to 10 ml of 2 mM linoleic acid in 0.1 M acetate buffer, pH 5.5, containing 0.1% Tween 20 and incubated at 25°C for 20 min. The reaction products were extracted with diethyl ether and condensed under N_2 . The remained substances were dissolved in ethanol and applied to HPLC. The peak of each product was collected and methylated with CH_3I and subsequently trimethylsilylated with Tri-Sil. The samples thus prepared were analyzed by Shimadzu GCMS-QP11000EX system with 2% OV-17 glass column; (tem.operation $160\text{--}240^\circ\text{C}$, $3^\circ\text{C}/\text{min}$. ion source 250°C , in let 20 eV .)

Preparation and identification of LHCE reaction products. 9- or 13-LOOH (5 mg) in 0.1 M phosphate buffer, pH 5.5 was incubated with 5 units of LHCE after DEAE-Toyopearl column chromatography at 25°C for 20 min. The reaction products were extracted with chloroform:methanol (2:1). Subsequently, products were purified by HPLC and analyzed by the same procedures as that for the products of LOX described above.

Analytical data ; Reaction products of 9-LOOH ; (1) methyl and trimethylsilylated product of 9-LOH, m/z 382[M](16.8) 225[M-157](71.2) 130[M-252](38) 73[M-309](100) (2) methyl and trimethylsilylated product of 9,12,13-LOH, m/z 461(14) 387[M-173](16) 259(56) 173[M-387](100).

Reaction products of 13-LOOH ; (1) methyl and trimethylsilylated product of 13-LOH, m/z 382[M] (18) 311[M-71](52) 130[M-252](51.2) 73[M-309](100).

Bioassay of antifungal activity. Antifungal activity of lipid peroxide were assayed as described in our preceding paper⁷⁾.

RESULTS

LOX in the infected taro tubers

LOX was extracted from taro tuber slices which were incubated for 25 hr after inoculation with

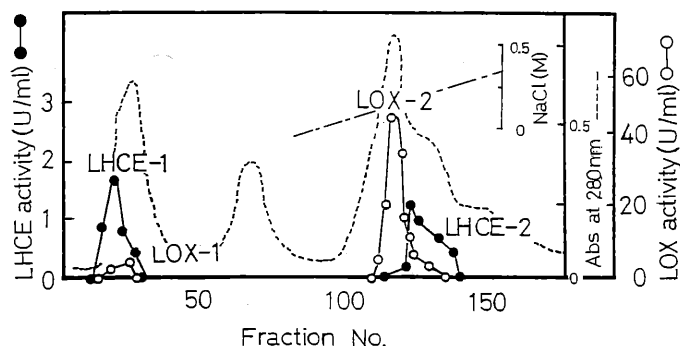


Fig. 1. Elution profiles of LOX and LHCE from taro strain-infected taro tubers on DEAE-Toyopearl column chromatography. Experimental conditions are described in the texts. ○: LOX activity, ●: LHCE activity, ... Abs. 280.

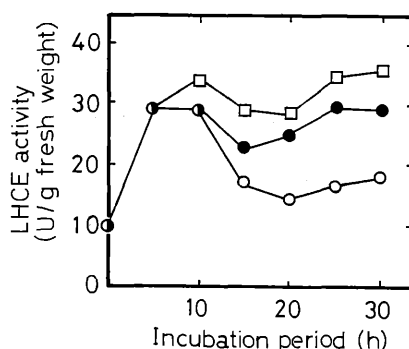


Fig. 2. Change in LHCE activity after inoculation. Experimental conditions are described in the texts. □: inoculated with taro strain, ●: inoculated with sweet potato strain, ○: wounded (no inoculation).

Table 1. Antifungal activity of 9-hydroperoxy (9-LOOH) and 13-hydroperoxy (13-LOOH) of linoleic acids against taro and sweet potato strains of *C. fimbriata*

Concentration of LOOH (mM)	Relative germ tube growth ^{a)}			
	Taro strain		Sweet potato strain	
	9-LOOH	13-LOOH	9-LOOH	13-LOOH
21.1	0	0	0	0
10.6	0	0	0	0
5.3	+1	+1	+1	+1
2.6	+1	+1	+1	+1
1.0	+3	+3	+3	+3
0	+5	+5	+5	+5

a) Relative germ tube growth was denoted by arbitrary scale. Zero indicated no germination.

taro strain of *C. fimbriata*, and analyzed by DEAE-Toyopearl column chromatography (Fig. 1). To peaks of LOX, (LOX-1 and LOX-2) were detected. The main component of LOX in infected taro tubers was LOX-2. LOX-2 showed optimal activity at pH 5.5. It showed the similar magnitude of activity toward linoleic acid (100%) and linolenic acid (77%) but not any activity toward methyl linoleate (0%). It converted linoleic acid into 9-hydroperoxy linoleic acid (9-LOOH) and 13-LOOH in the ratio of 47:53.

Induction of LHCE in inoculated taro tubers

LHCE activity was detected in fresh taro tubers and increased during the initial 10 hr, then decreased for the next 10 hr and finally increased again in non-inoculated (wounded) taro tubers during incubation (Fig. 2). LHCE activity was also changed in the tubers inoculated by taro or sweet potato strains in similar pattern to that in wounded tubers. However the magnitude of the changes in taro strain inoculated tubers was larger comparing with in sweet potato strain inoculated tubers and in wounded tubers.

Identification of reaction products of LHCE

The reaction products of LHCE with 9-LOOH and 13-LOOH as substrate were identified by using HPLC and GC-MS as described in MATERIAL and METHODS. The products showed the same Mass spectra as those of products prepared by Ohta *et al.*¹¹⁾ The products with 9-LOOH were 9-hydroxy linoleic acid (9-LOH) as a main product and 9,12,13-trihydroxy linoleic acid (9,12,13-LOH) as a minor product. A main product with 13-LOOH was 13-LOH.

Antifungal activities of hydroperoxy and hydroxy linoleic acids

No difference was observed in antifungal activity between 9-LOOH and 13-LOOH (Table 1). In addition, both LOOHs did not show differential toxicity toward sweet potato and taro strains (Table 1). There was also no difference in antifungal activity between 9-LOH and 13-LOH (Tables 1 and 2). 9-LOH and 13-LOH showed the similar magnitude of antifungal activity toward both strains (Table 2). 9,12,13-LOH also showed a similar magnitude of antifungal activity against both strains (Table 3).

Table 2. Antifungal activity of 9-hydroxy (9-LOH) and 13-hydroxy (13-LOH) of linoleic acids against taro and sweet potato strains of *C. fimbriata*

Concentration of LOOH (mM)	Relative germ tube growth ^{a)}			
	Taro strain		Sweet potato strain	
	9-LOH	13-LOH	9-LOH	13-LOH
21.1	0	0	0	0
10.6	0	0	0	0
5.3	+1	+1	+2	+1
2.6	+3	+3	+3	+3
1.0	+5	+5	+5	+5
0	+5	+5	+5	+5

a) Relative germ tube growth was denoted by arbitrary scale. Zero indicated no germination.

Table 3. Antifungal activity of 9,12,13-octadecenoic acid (9,12,13-LOH) against taro and sweet potato strains of *C. fimbriata*

Concentration of 9,12,13-LOH (mM)	Relative germ tube growth ^{a)}	
	Taro strain	
	Sweet potato strain	
9.1	0	0
7.6	+2	+2
6.1	+2	+2
3.0	+8	+8
1.5	+8	+8
0	+8	+8

a) Relative germ tube growth was denoted by arbitrary scale. Zero indicated no germination.

Table 4. Antifungal activity of the mixture of peroxides of linolenic acid produced by crude enzymes (includes LOX and LHCE) from taro strain-inoculated taro tubers against taro and sweet potato strains of *C. fimbriata*

Concentration of linolenic acid ^{b)} (mM)	Relative germ tube growth ^{a)}	
	Taro strain	
	Sweet potato strain	
56	+0.5	0
42	+3	0
28	+4	+1
14	+5	+1
Controls ^{c)}		
active enzyme only	+10	+10
linolenic acid only	+10	+10
boiled enzyme only	+10	+10
boiled enzyme and linolenic acid	+8	+8

a) Relative germ tube growth was denoted by arbitrary scale. Zero indicated no germination.

b) The germ tube growth was examined in the assay mixture containing the linolenic acid at the concentrations indicated and enzyme preparation.

c) In controls, assays were carried out in the mixtures containing active enzyme only, linolenic acid (56 mM) only, boiled enzyme only or boiled enzyme and linolenic acid (56 mM).

Differential antifungal activity of mixtures of linolenic acid peroxides toward two strains

Linolenic acid was peroxidized by the crude enzyme from infected taro tubers which contained both LOX and LHCE. HPLC analysis indicated that the reaction product contained several unknown products in addition to 9-LOOH, 13-LOOH, 9-LOH, 13-LOH and 9,12,13-LOH (data not shown). The peroxidized linolenic acid thus prepared showed differential toxicity toward taro and sweet potato strains; 42 mM of the mixture inhibited completely germ tube growth of sweet potato strain, while it inhibited moderately that of taro strain (Table 4).

DISCUSSION

The present study demonstrated that taro tubers infected by *C. fimbriata* contained LOX and LHCE. The two enzymes, LOX and LHCE, converted, *in vitro*, linolenic acid into 9, 12,13-trihydroxyoctadecenoic acid, which was isolated as one of antifungal compounds from the infected taro tubers in our preceding paper⁷⁾. LHCE in the infected taro tubers was very similar to the enzymes in rice leaves reported by Ohta *et al.*¹¹⁾ Both taro tubers and rice leaves contained two isozymes. Both enzymes from two plants converted linolenic acid into the same peroxides.

LHCE activity changed in the similar pattern in three taro tuber samples; wounded, sweet potato strain-infected and taro strain-infected taro tuber disks (Fig. 2). Activity increased during the initial 10 hr, and decreased during the next 10 hr. Finally the activity increased again. Only slight difference was observed in the activity of the first peak at 10 hr after inoculation among the three taro tuber samples. Subsequently, the difference in the activity in three samples became largers; the activity in taro strain infected tubers was the highest and the activity in wounded tubers was the lowest. Such induction pattern of LHCE suggested that the increased activity during the initial 10 hr was due to wounding and the subsequent change was due to infection.

As mentioned above, LHCE activity at second peak in taro strain infected tuber disks was higher than that in sweet potato strain infected tuber disks. This result is consistent with the results of time course analysis of lipid peroxide accumulation in taro tubers infected by the two strains; more lipid peroxides were accumulated in the taro tuber disks infected by taro strain than in the taro tuber disks infected by sweet potato strains⁸⁾. One possible explanation for the differences in LHCE activity and lipid peroxide accumulation in taro tuber disks infected by the two strains might be that taro strain invade into more inner cells in the disks than sweet potato strain.

LOX and LHCE produced various peroxides from linolenic acid; 9-LOOH, 13-LOOH, 9-LOH, 13-LOH and 9,12,13-LOH. We examined antifungal activity of the above peroxides separately (Tables 1-3). All peroxides showed strong antifungal activity. However, there was no difference in toxicity among them. In addition, they inhibited similarly the growth of sweet potato and taro strains of *C. fimbriata*. The results were in contrast to the result by Ohta *et al.* that 9-LOH was more toxic to *Magnaporthe grisea* than 9-LOOH.

Differential toxicity was demonstrated with linolenic acid peroxidized by the crude enzymes from infected taro tubers which contained both LOX and LHCE (Table 4). Sweet potato strain was inhibited more severely than taro strain. This result apparently is contradiction to the results in Table 1, 2 and 3, which indicated that individual peroxide of linolenic acid such as 9-LOOH, 13-LOOH, 9-LOH, 13-LOH and 9,12,13-LOH did not show differential toxicity. There are some possible explanation for the contradiction. The unknown oxidized products included in the peroxidized linolenic acid fraction might be responsible for differential toxicity. Alternatively, the mixture of individual peroxide might show differential toxicity.

The present paper demonstrated definitely the occurrence of LOX and LHCE which are involved in production of lipid peroxides. The lipid peroxides produced by the enzymes had antifungal activity. The activity of LHCE increased in taro tubers in response to infection by *C. fimbriata*. These results suggest that these enzymes and their products, namely, lipid peroxides play role in the host defense reaction.

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和 文 摘 要

升井洋至・中山実生子・大鶴 勝: *Ceratocystis fimbriata* 感染サトイモにおける過酸化脂質生成とその生成に関与する酵素に関する研究

抗菌活性をもつ過酸化脂質の生成に関与する lipoyxygenase (LOX) と lipid hydroperoxide converting enzyme (LHCE) が *C. fimbriata* 感染サトイモ中に検出された。感染サトイモ中には 2 つの LOX (LOX-1, LOX-2) が含まれていた。LOX-2 が主要酵素であり、至適 pH は 5.5 で、linolenic acid と linoleic acid に対してほぼ同じ活性を示した。本酵素は linoleic acid をほぼ同量の 9-hydroperoxy linoleic acid (9-LOOH) と 13-hydroperoxy linoleic acid (13-LOOH) に変換した。感染サトイモ中には 2 つの LHCE (LHCE-1, LHCE-2) が含まれていた。2 つの LHCE の基質特異性は同じで、9-LOOH と 13-LOOH をそれぞれ 9-hydroxy octadecenoic acid (9-LOH) と 13-hydroxy octadecenoic acid (13-LOH) に変換した。9,12,13-trihydroxy octadecenoic acid (9,12,13-LOH) も 9-LOOH より LHCE により微量生成物としてつくられた。これらの過酸化脂質は同じ強さの抗菌活性を示し、*C. fimbriata* の 2 つの菌株の生育を同じ程度に阻害した。一方、感染サトイモより調製した粗酵素液 (LOX と LHCE を含む) を linolenic acid に作用させてできた過酸化脂質は *C. fimbriata* のサツマイモ菌株をサトイモ菌株よりも強く阻害した。