STUDIES ON HOST-PARASITE INTERACTIONS

IN TARO TUBERS

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CONTENTS

ABBREVIATIONS

C6-NBD-PC	:	1-acy-2-[6-[(7-nitro-2,1,3-benzoxadiazol-4-yl)animo]-caproyl] phosphatidylcholine
^1H-^1H COSY	:	proton-proton correlation spectroscopy
Cyt c	:	cytochrome c
EIMS	:	electron ionization mass spectrometer
FABMS	:	fast atom bombardment mass spectrometer
GC-MS	:	gas chromatography mass spectrometer
HPLC	:	high performance liquid chromatography
IR	:	infrared spectrum
LHCE	:	lipid hydroperoxide converting enzyme
9- or 13-LOH	:	9- or 13- hydroxy linoleic acid
9,12,13-LOH	:	9,12,13- trihydroxy-10-octadecenoic acid
9- or 13-LOOH	:	9- or 13- hydroperoxy linoleic acid
LOX	:	lipoxygenase
MES	:	2-(N-monrpholino)-ethanesulforic acid
Mr	:	molecular weight
MS	:	mass spectrometer
NMR	:	nuclear magnetic resonance
TBA	:	thiobarubituric acid
Tris	:	tris(hydroxymethyl)aminomethane

GENERAL INTRODUCTION

All plants on the earth are continuously exposed to attacks by various kinds of pathogens such as virus, bacteria and fungi. When plants are infected by these pathogens, they defend themselves against the invaders using various strategies either singly or in combination. These defenses of host plants range from pre-formed antifungal compounds or physical barriers to defensive system induced in response to the attacks by pathogens. The induced defense reactions include hypersensitive necrosis[11-17], the production of phytoalexins (antifungal compounds)[18-20] and the induction of hydrolytic enzymes such as chitinase among others. Conversely, the pathogens counteract the host reactions by employing various strategies, again often in combination, such as secretion of toxins and of hydrolytic enzymes[1-10].

Ceratocystis fimbriata which belongs to Ascomycetes, is distributed throughout the tropical and temperate regions and causes black rot disease on various plants such as sweet potato, coffee, prune, cacao, oak, taro and almond[21,22]. In Japan, the species have been isolated from the lesions on sweet potato and taro. The strains of C. fimbriata isolated from different host plants show different host specificity. Such a specificity in the interactions between plants and parasites is expressed as the

results of mutual recognition between host and parasite. It is reasonably assumed that the similar mechanisms to that of the recognitions in other higher level of physiological phenomena such as immune response, development and pollination are operating in the interaction between host plant and parasite.

The host-parasite interactions between C. fimbriata and sweet potato have been studies extensively by Uritani and co-workers[21,22]. They elucidated many aspects of the complex interaction. They indicated that sweet potato roots infected by C. fimbriata accumulated furanoterpenoids as phytoalexins[22]. Furthermore, they demonstrated several other factors which are involved in determination of host-parasite specificity [21]. Contrary to the interaction between sweet potato and C. fimbriata, the one between taro and the fungus has scarcely studied. Therefore, the author studied the interaction between taro tubers and C. fimbriata in reference to production of antifungal compounds.

This thesis consists of three chapters. Chapter 1 deals with isolation and determination of antifungal compound produced in infected taro tubers. Chapter 2 is concerned with lipid peroxidation and its role in infected taro tubers. In chapter 3, the lipid peroxides and the enzymes which are involved in their production are described.

Chapter 1.

Isolation and identification of an antifungal compound in taro tubers infected with Ceratocystis fimbriata

INTRODUCTION

Various strains of C. fimbriata were isolated from black rot lesions on various plants such as taro and sweet potato. Host plants differ in their response to different strains.[21,22] When taro tubers(Colocasia antiquorum) are inoculated with endoconidial spore suspension of the taro strain of C. fimbriata, the spores germinate and invade the inner tissue, resulting in the appearance of symptoms of black rot. In contrast, when taro tubers are inoculated with the sweet potato strain of C. fimbriata, the spores germinate but hyphal extension is inhibited. No black rot symptoms develop but the inoculated surface turns a reddish color within 24 hr. These observations suggest that taro tubers show a hypersensitive reaction and may produce some antifungal compound(s) in response to inoculation with the sweet potato strain. The isolation of antifungal compounds from inoculated taro tubers had not been reported.

In this chapter, the author tried to isolate and identify an antifungal compound from taro tubers inoculated with the sweet potato strain of C. fimbriata.

MATERIALS AND METHODS

Preparation of inoculum.

The sweet potato strain of Ceratocystis fimbriata, incompatible with taro, was used in all experiments in this chapter. Inoculum (7×10^6 spores/ml) was prepared as described in [21].

Preparation of taro tuber disks and inoculation.

Disks(18 X 2 mm) of taro tubers(Colocasia antiquorum) were prepared and were rinsed in distilled water to remove the latex on the surfaces. The rinsed disks were blotted with filter papers and divided into three groups consisting of 5 disks. One group of disks was frozen immediately and stored at -30 °C for 24 hr (fresh disks), the second group of disks was dipped in the distilled water and incubated at 25 °C for 24 hr in moist chamber (wounded disks) and the third group of disks was inoculated by dipping in spore suspension and incubated at 25 °C for 24 hr in a moist chamber(inoculated disks). Each group of disks was extracted by shaking in 20 ml of acetone at 4 °C for 17 hr. The extract were applied to HPLC after being condensed.

Bioassay of antifungal activity.

Sample in solution were condensed in a test tube (12 X 120 mm) using a vacuum evaporator and finally lyophilized to remove the organic solvent completely. The dried sample was dissolved in 80 µl of 20 mM of 12-(N-morpholino)ethanesulfonic acid(MES)-NaOH buffer, pH 6.5, including 10 µl of water extract from sweet potato root (1 ml water per 1 g fresh weight root)as a nutrient. Then 10 µl of spore suspension was added to the solution and incubated at 25 °C on a shaker. After incubation for 4.5 hr, the growth of the spores was examined under a microscope and denoted by positive figures on an arbitrary scale. Zero denotes no germination.

Isolation of an antifungal compound from inoculated taro tuber slices.

The taro tubers were cut into 2 mm thick slices, which were rinsed in the distilled water to remove the latex on the surface and blotted with filter papers. The slices were kept in a moist chamber at 25 °C for 1 day and then inoculated with spore suspension. After being incubated at 25 °C for 2 days, the slices were boiled in ethanol for 10 min to inactivate the enzymes and then extracted with ethanol. The extract was evaporated to leave a water solution which was extracted twice with ethyl acetate. Ethyl acetate extract was condensed to dryness. The dried substance was dissolved in a small volume of 65% methanol containing 10 mM acetic acid and centrifuged to remove insoluble material. The resultant supernatant was subjected to HPLC on a reversed-phase column(Develosil ODS-5, 0.4 X 25 cm) with 65% methanol containing 10 mM acetic acid; flow 0.5ml/min and UV detector set

at 270 nm. The peak eluting at 15.5 ml was collected. Three mg of the purified compound was isolated from 3kg of the inoculated slices.

Spectral analysis.

^{1}H (500M Hz) and ^{13}C (125M Hz) NMR using GX500(Jeol); MS (DX303, Jeol):EIMS, GC inlet, 70eV and FABMS using a glycerol matrix. ^{1}H NMR data are in ppm/TMS and EIMS in m/z values.

9,12,13-trihydroxy-(E)-10-octadecenoic acid(compound-1).

ν_{max}^{KBr} cm^{-1}: 3300(br, OH), 2930, 2850(CH$_2$), 1710, 1410(COOH).
^{1}H NMR(CD$_3$OD): δ 0.91 (3H, t, J=6.5Hz, 18-CH$_3$), 1.1-1.7(20H, m, -CH$_2$-), 2.25(2H, br t, J=6.5Hz, H-2), 3.14(1H, ddd, J=1.5, 6.5 and 9.0Hz, H-13), 3.90(1H,t, J=6.5, H-12),4.05(1H, q, J=6.5Hz, H-9), 5.67(1H, dd, J=6.5 and 15Hz, H-11), 5.71(1H, dd, J=6.5 and 15Hz, H-10). ^{13}C NMR (CD$_3$OD): δ 15.2(C-8), 24.5, 27.0, 27.2, 27.2, 27.4, 31.1, 31.2, 31.3, 34.4, 36.3, 39.2, 11C, -CH$_2$-) 73.8, 76.7, 77.3(CH-OH), 131.9, 137.4(-CH=CH-), 159.8 (-COOH).

Preparation of compound-2.

To a solution of compound-1 (2 mg) in methanol (2 ml) was added excess CH$_2$N$_2$ in diethyl acetate. After 20 min, the solu-tion was dried up in vacuo to give compound-2 in quantitative yield.

Methyl ester of compound-1 (compound-2)

^{1}H NMR (CDCl$_3$): δ 0.86(3H, t, J=6.5Hz, 18-CH$_3$), 1.2 1.7 (20H, m, -CH$_2$-), 2.27(2H, t, J=6.5Hz, H-2), 3.41 (1H, m, H-13), 3.64(3H, s, COOCH$_3$), 3.89 (1H, t, J=6.5Hz, H-12), 4.08 (1H, br q, J=6.5Hz, H-9), 5.66 (1H, ddd, J=1.5, 6.5 and 15Hz, H-11), 5.76(1H, ddd, J=1.5, 6.5 and 15Hz, H-10).

Silylation of compound-2 for GC-MS.

Compound-2 was treated with Tri-Sil(1.5 M solution of trime-thylsilylimidazole in pyridine, PIERCE), and then the solution was directly loaded in MS: m/z 545[M-15](17.5), 460(100), 439(22), 387[M-173], 298(40), 259(95), 173[M-387](100).

Conversion of unsaturated fatty acid by lipoxygenase to anti-fungal compounds.

Spores(5 X 10^6) were incubated in 1.0 ml of 20 mM MES-NaOH buffer, pH6.5, containing 18 nmol of various fatty acids, 2 X 10^4 units of lipoxygenase(from soybean, Biozyme Laboratories) and 50 μl of sweet potato root water extract as a nutrient at 25 °C. In controls, boiled lipoxygenase was included instead of active lipoxygenase. After incubation for 17 hr, spores were examined for germination under microscope.

RESULTS AND DISCUSSION

The aceton extract from freshly prepared, wounded (incubated without inoculation) and inoculated taro tuber disks were applied to HPLC(ODS) and the resultant fractions were assayed for antifungal activity (Fig.1-1).
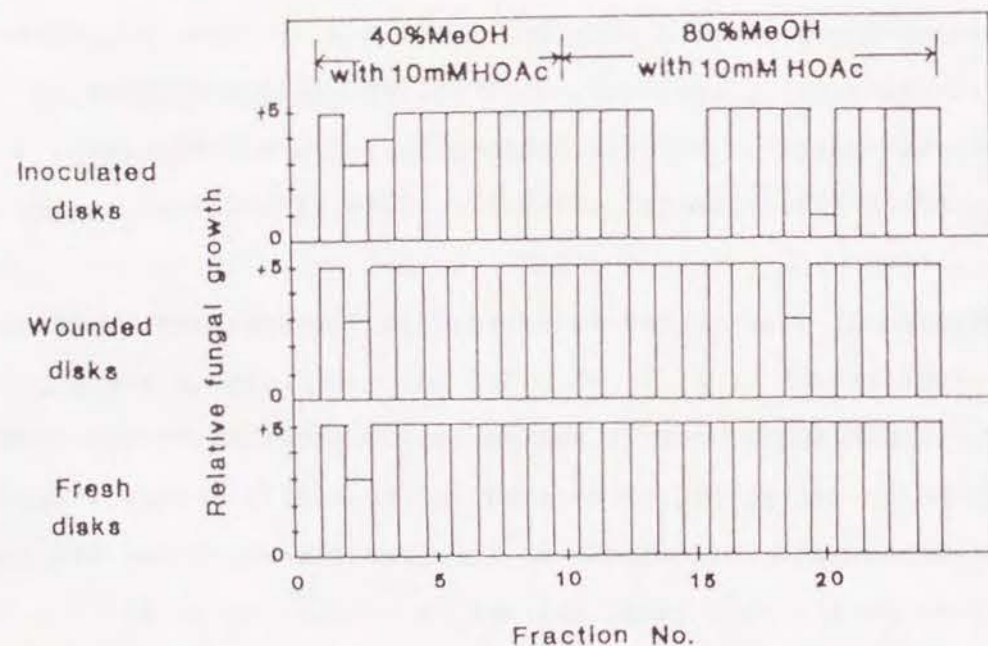

Figure 1-1. Demonstration by HPLC of production of antifungal compounds in the inoculated taro tubers. The acetone extracts from disks of fresh, wounded and inoculated taro tubers were subjected to HPLC and every 2 ml of eluate was fractionated and assayed for antifungal activity.

Fractions with antifungal activity were detected from all extracts. However, high antifungal activity fractions 14-15 was detected only in extracts from inoculated disks and showed the highest activity. No antifungal activity was detected in extracts from the fungus grown on potato-dextrose agar medium, suggesting that the antifungal compound(s) in the extract from inoculated disks were produced by taro tuber disks. Thus, it was demonstrated that taro tuber produced antifungal compound(s) in response to inoculation with the sweet potato strain of C. fimbriata.

Rechromatography of the above antifungal fraction from inoculated disks indicated that the fraction contained several antifungal compounds. An antifungal compound in the fraction was isolated. The structure of the compound(1) was established through IR, NMR and mass spectral analysis. The positive and negative ion FABMS gave (M+Na) and (M-H) peaks at m/z 353 and 329, respectively, indicating that the Mr of the compound is 330. IR demonstrated the presence of OH (3300 cm^{-1}), CH_2 (2930 and 2850) and COOH (1710).

In the ^{13}C NMR spectrum, one double bound (131.9 ppm and 137.4), three -CH(OH)-(73.8, 76.7 and 77.3) and twelve aliphatic carbons were identified. The data of $^{1}H-^{1}H$ COSY and the spin decoupling experiments showed the presence of a double bound in E-configuration ($J_{10,11}$=15 Hz) and the spin system (-CH_2-CH(OH)-CH=CH-CH(OH)-CH(OH)-CH_2-).

esterified with CH_2N_2 to afford the methyl ester (compound-2), followed by treatment with trimethylsilylimidazole to give the tris-TMSi derivative of 2. EIMS gave a parent peak at m/z 460 corresponding to M-100, which was possibly caused by McLafferty-type rearrangement (Fig. 1-2).
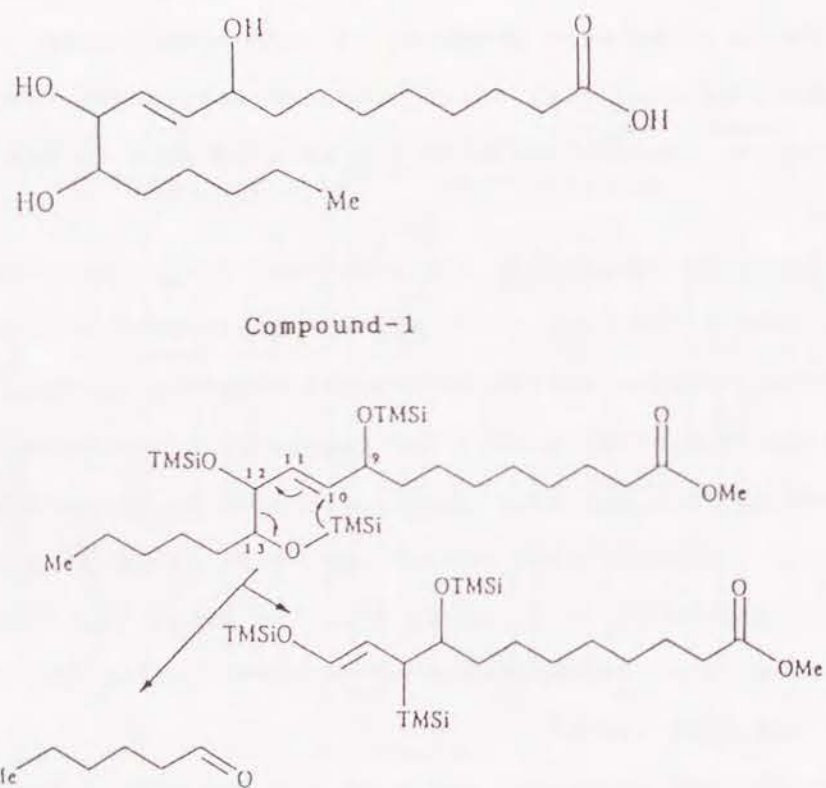

Compound-1

Figure 1-2. Mass-fragmentation of tris-TMSi of compound-2
at 70 eV.

The result strongly suggested that the double bound was at C-10. Thus, the structure of compound-1 was determined to be 9,12,13-trihydroxy-(E)-10-octadecenoic acid. This compound has

already been isolated from other plants; Oryza sativa inoculated with Pyricularia oryzae [23], Allium cepa [24] and Rudbeckia fulgida[25].

Fifty ppm of 9,12,13-trihydroxy-(E)-10-octadecenoic acid isolated from inoculated taro tubers inhibited completely the germination of spores of C. fimbriata. It was assumed from its structure that 9,12,13-trihydroxy-(E)-10-octadecenoic acid was produced from linolenic acid and/or linoleic acid by peroxidative reaction in vivo. Therefore, it was examined whether or not fatty acids such as linolenic acid changed to antifungal compounds by the action of lipoxygenase in vitro (Table 1-1).

Table 1-1 Conversion by lipoxygenase of unsaturated fatty acids
         to antifungal compounds

Additions of mixture		
Fatty acid	Lipoxygenase	Relative fungal growth
None	active	+ 9
None	boiled	+ 9
Linolenic acid (18:3)	active	0
Linolenic acid	boiled	+ 9
Linoleic acid (18:2)	active	0
Linoleic acid	boiled	+ 8
Oleic acid (18:1)	active	+ 8
Oleic acid	boiled	+ 7
Palmitic acid (16:0)	active	+ 9
Palmitic acid	boiled	+ 8

Spores were incubated in the medium containing various fatty acid and lipoxygenase or boiled lipoxygenase at 25 °C. After incubation for 17 hr, the growth of spores was examined under microscope.

Antifungal activity was detected in the assay mixtures
containing lipoxygenase and linolenic acid with three double
bonds or linoleic acid with two double bonds, while antifungal
activity was not detected in the assay mixture containing the
enzyme and oleic acid with one double bond or palmitic acid, a
saturated fatty acid. HPLC analysis showed that lipoxygenase
produced not a single, but many antifungal peroxidized products
from linolenic acid and linoleic acid. Furthermore, the increase
of lipoxygenase activity and accumulation of lipid peroxides take
place in taro tubers in response to inoculation (in Chapter 2).
These results pointed out the important roles of peroxidation of
lipids in the defense reaction of taro tubers inoculated with C.
fimbriata as in other host-parasite interactions[23,26,27].

Involvement of peroxidation of lipids in early host defense
reaction seems to be reasonable and general in the incompatible
host-parasite interactions from the following consideration. In
most of incompatible host-parasite interactions, hypersensitive
necrosis of host tissues occurs in the early stage of infection
[11,28]. In those tissues, the cellular membranes must be disor-
ganized and the lipids in such membranes become susceptible to
peroxidation [29,30]. The lipid peroxides thus formed show anti-
fungal activity against invading parasite.

SUMMARY

An antifungal compound has been isolated from tubers of
taro (Colocasia antiquorum) inoculated with black rot fungus
(Ceratocystis fimbriata) and identified as 9,12,13-trihydroxy
-(E)-10-octadecenoic acid.

Chapter 2.

Lipid peroxidation and its role in taro tubers
infected by Ceratocystis fimbriata

INTRODUCTION

In chapter 1, it was demonstrated that 9,12,13-trihydroxy-(E)-10-octadecenoic acid was produced as an antifungal compound in infected taro tubers. It is reasonable to assume from the structure of 9,12,13-trihydroxy-(E)-10-octadecenoic acid that it is produced from linolenic acid and/or linoleic acid by a peroxidative reaction in vivo.

Lipid peroxidation has been reported with some plant host-parasite interactions [23,26,29-33]. In some interactions [31,34], lipid peroxidation appears to be initiated by the production of active oxygen species by plant cells in response to the incompatible pathogen. Among the active oxygen species, the superoxide anion(O_2^-) was suggested to serve as a trigger for the formation of more reactive oxygen species such as the hydroxyl radical($OH \cdot$) and singlet oxygen($^1O_2$) [34]. Each of these species can react with polyunsaturated fatty acids to form these peroxides. Alternatively, the involvement of lipoxygenase in lipid peroxidation has been suggested in some host-parasite interactions [30,35].

In this chapter, lipid peroxidation and O_2^- generation were examined with taro tubers inoculated by taro strain (compatible or pathogenic) or sweet potato strain (incompatible or non-pathogenic) of C. fimbriata. The changes in the activities of phospholipase A_2 and lipoxygenase in inoculated taro were also studied. Phospholipase A_2 was included since it was assumed to be involved in the release of unsaturated fatty acids such as linolenic acid and linoleic acid from C-2 position of glycerophospolipid [36]. In addition, the effect of lipid peroxides toward growth of the two strains was examined in vitro in the relation to host specificity of C. fimbriata.

MATERIALS AND METHODS

Reagents.

1-acy-2-[6-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]-caproyl] phosphatidylcholine (C_6-NBD-PC) was purchased from AVANTI POLAR-LIPIDS, INC. Lipoxygenase (from soybean) and superoxide dismutase (from bovine erythrocyte) (SOD) were purchased from Biozyme Laboratories, Ltd. and Wako Pure Chemical Industries, Ltd., respectively.

Preparation of inoculum.

Taro and sweet potato strains of C. fimbriata were used. The taro strain is pathogenic to and compatible with taro, while the sweet potato strain is non-pathogenic to and incompatible

with taro. Inoculum (7 X 10^6 spores/ml) was prepared as described in Chapter 1.

Preparation of taro tuber disks and inoculation.

Disks (2 X 0.2 cm) of taro tubers (Colocasia antiquorum Schott) were prepared and were rinsed in distilled water to remove the latex from the surface. The rinsed disks were blotted with filter paper and divided into 3 groups, each consisting of 5 disks. One group of disks was dipped in distilled water and incubated at 25 °C in a moist chamber (wounded disks). The other two groups were inoculated by dipping in a spore suspension of either the taro or sweet potato strain and incubated at 25 °C in a moist chamber. To monitor the contribution of the fungus to the values determined in the following sections, the fungi were grown on filter paper disks containing water extract from taro tubers as nutrient and used as samples. The taro tuber extract was prepared by homogenizing 100 g of taro tubers in 100 ml of water and used after diluting 5-times by water.

Determination of lipid peroxides.

Five disks (2 X 0.2 cm) were homogenized in 15 ml of methanol with sea sand in a mortar. Then, 15 ml of chloroform was added to the homogenate and mixed well. The homogenate was centrifuged at 1,160 X g for 10 min. The supernatant was evaporated to dryness and resuspended in 2 ml of water using a sonicator. The resulting suspension was extracted with 5 ml and then 2

ml of ethyl acetate. The combined ethyl acetate extracts were evaporated to dryness using a rotatory evaporator. The residue was dissolved in 0.5 ml of methanol. The amount of lipid peroxides in the methanol solution was determined using the thiobarubituric acid (TBA) reaction of Yagi et al. [37] with the some modifications. The sample (0.5 ml) was mixed with 0.5 ml of 0.67% TBA and 1.5 ml of 0.05 N HCl and subsequently boiled in a water bath for 30 min. The mixture was cooled in tap water, then 2 ml of n-buthanol were added and the solution was mixed vigorously. The mixture was centrifuged at 1,670 X g for 10 min. The fluorescence of the supernatant was measured at 515 and 553 nm, excitation and emission wavelengths, respectively. The amount of peroxides was calculated using 1,1,3,3-tetraethoxypropane as a standard and denoted as amount of malondialdehyde.

In all experiments in this chapter where lipid samples were evaporated using evaporator, N_2 gas was introduced into vessel to return to atmospheric pressure after evaporation to prevent oxidation of samples.

Assay of superoxide anion (O_2^-) generation.

Extracelluar O_2^- was assayed based on its ability to reduce cytochrome c (Cyt c) or nitroblue tetrazolium (NBT) using the method of Doke with some modifications [38,39]. In the assay using Cyt c, 5 disks (1.2 X 0.2 cm) were incubated in 3 ml of 10 mM 2-(N-morpholino) ethanesulfonic acid (Mes)-NaOH buffer (pH 6.5) including 20 μM Cyt c (oxidized form) and 10 mM NaN_3 at 25 °C

on a shaker. Reduction of Cyt c was monitored by the absorbance

at 550 nm, 5, 10 and 20 min after initiation of incubation. In

order to verify Cyt c reduction by O_2^-, superoxide dismutase

(SOD) (350 units/ml of assay mixture) was added to the reaction

solution from which NaN_3 was omitted. In the assay using NBT, 5

disks were incubated in 3 ml of 10 mM Mes-NaOH buffer (pH 6.5)

containing 0.05% NBT at 25 °C for 20 min on a shaker. Subsequent-

ly, an aliquot (1.5 ml) of the solution was withdrawn and centri-

fuged. The resultant supernatant was heated at 85 °C for 15 min

and then cooled to room temperature. Its absorbance at 580 nm

was recorded to measure the reduced formazan. In the control

assay, SOD (350 units/ml) was included in the reaction mixture.

Extraction and assay of phospholipase A_2.

Five disks (1.2 X 0.2 cm) were homogenized in 10 ml of 0.1 M

tris-HCl buffer (pH 7.5) containing 0.3 M sucrose in a mortar

with a pestle. The homogenate was centrifuged at 95,000 X g for

70 min. All the enzyme activity was recovered in the precipitate

fraction. The precipitate was resuspended in 5 ml of the homoge-

nizing medium described above using a teflon homogenizer and used

as enzyme preparation. Phospholipase A_2 was assayed fluorimetri-

cally according to a reported method [40] with some modifica-

tions. The reaction mixture contained the fluorescent phospho-

lipid (C_6-NBD-PC, 6.3 µM), KCl(100 µM), $CaCl_2$ (2 mM), Mes-NaOH

buffer (80 mM, pH 6.0) and enzyme in 1.0 ml of final volume. The

reaction was carried out at 25 °C and the fluorescence increase

was monitored at 470 nm (excitation) and 540 nm (emission) con-

tinuously as a function of time. The calibration curve was made

as follows. Varying amounts of C_6-NBD-PC were hydrolyzed in 1.5 N

methanolic KOH solution by heating at 70 °C for 30 min. The

hydrolysate was neutralized with HCl and then its fluorescence

was measured with the same settings as the samples. The enzyme

activity was expressed as nkat per g fresh weight. Since sub-

strate specificity of the enzyme was not examined, the enzyme

activity measured by this method might be that of other lipase

showing phospholipase A_2 activity.

Extraction and assay of lipoxygenase.

Lipoxygenase was extracted and assayed by the method of

Ocampo et al. with some modifications [35]. Five disks (1.2 X

0.2 cm) were ground in 7 ml of 0.1 M phosphate buffer (pH 7.5)

containing EDTA (1 mM), polyclar AT (0.3 g), and sea sand with

mortar and pestle and centrifuged at 43,700 X g for 10 min. The

resultant supernatant was used as enzyme preparation. The reac-

tion mixture contained 700 ul of 0.1 M citrate-Na_2HPO_4 buffer (pH

5.5) and 100 ul of 2.5 mM linoleic acid in 50 mM phosphate buffer

(pH 9.0) containing 0.25% Tween 20. The reaction was started by

the addition of enzyme and the change in absorbance at 234 nm was

followed. The amount of peroxides produced was calculated by

using an absorbance coefficient of 25,000 $M^{-1}cm^{-1}$.

Effect of peroxides of linolenic acid produced by lipoxygenase on growth of spores.

Spores (5 X 10^6) of taro or sweet potato strain were incubated at 25 °C for 1 hr in 950 μl of 0.1 M Mes-NaOH buffer, (pH 6.5) containing different amounts of linolenic acid and 100 μl of taro tuber water extracts (1 g fresh weight per 1 ml of water) on a shaker. During this incubation time, no germ tubes appeared. Subsequently, 2 X 10^4 units of lipoxygenase (from soybean, Biozyme Laboratories) in 50 μl was added to the spore suspension and incubated for additional 3.5 hr. Considering the labile nature of peroxides, lipoxygenase was added to the assay mixtures after pre-incubation. In control 1, linolenic acid was omitted, while lipoxygenase was omitted in control 2. After incubation, spores were examined for germination and germ tube growth under a microscope. Germ tube growth was quantified on an arbitrary scale.

Effect of lipid peroxides-containing fractions prepared from taro tubers inoculated by either taro or sweet potato strain on growth of spores.

Taro tuber slices (0.2 cm thick) were incubated at 25 °C for 36 hr after inoculation with spores of taro or sweet potato strain. The incubated slices (10 g) were extracted twice by shaking in 20 ml ethyl acetate at 4 °C, the first time for 3 hr and the second time for 1 hr. The ethyl acetate extracts were combined, evaporated to dryness and redissolved in 2 ml of ethyl acetate. The ethyl acetate solution was washed twice with 2 ml

of water. Then the ethyl acetate solution was evaporated to dryness and redissolved in ethanol. Peroxide contents of the ethanol solution was determined by the TBA reaction described above. Various volumes of the ethanol solution were evaporated to dryness in test tubes and redissolved in 900 μl of 0.1 M Mes-NaOH buffer (pH 6.5) containing 100 ul of taro tuber water extract. To the solutions, spores (7 X 10^6) of taro or sweet potato strain, which had been incubated at 25 °C for 1 hr and washed with water, were added and incubated at 25 °C for 5 hr. Thereafter, the growth of the spores was examined under a microscope and quantified by relative values as above.

RESULTS

Lipid peroxidation in inoculated taro tubers.

Lipid peroxidation measured by TBA reaction was compared among wounded taro tuber disks and inoculated disks by either taro or sweet potato strain of C. fimbriata (Fig. 2-1). Under the present conditions, the spores of both strains started to germinate 3 hr after inoculation and the surfaces of the taro tuber inoculated by either strains turned reddish in color after 10 hr. Lipid peroxidation began to increase about 10 hr after inoculation coinciding with the development of a reddish color and increased similarly in both inoculated treatments, although

the level of peroxides was slightly higher in the taro strain-inoculated disks than in the sweet potato strain-inoculated disks throughout the incubation period. In wounded disks, the surface did not turn reddish color throughout the incubation period, and only a small transient peak of lipid peroxidation was observed at about 7 hr and lipid peroxidation thereafter remained at a lower and relatively constant level. Lipid peroxidation was negligible in both filter paper disks inoculated by taro or sweet potato strain.
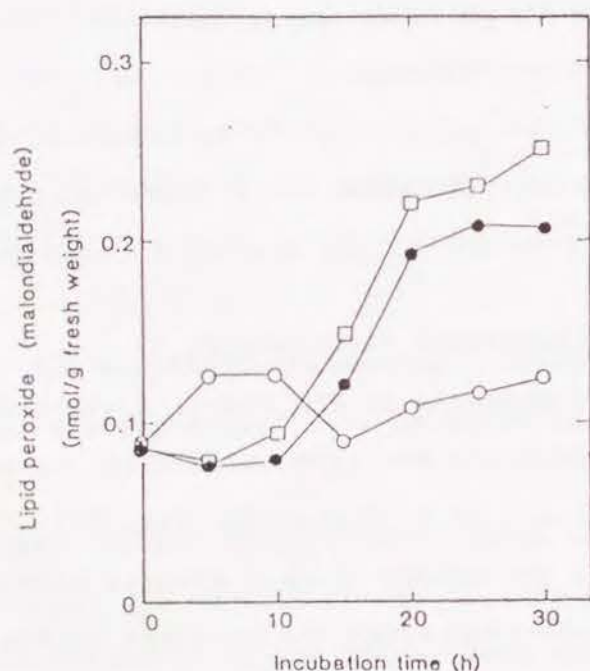

Fig.2-1 Lipid peroxidation in taro tuber disks wounded (incubated without inoculation, —O—), inoculated by taro (—□—) and sweet potato strain (—●—) of C. fimbriata.

Examination of generation of O_2^- in inoculated taro tubers.

Generation of O_2^- in inoculated disks was examined. Since the biomembrane showed little permeability to O_2^- [41], the method measured only O_2^- generated outside the cells. O_2^- generation was assayed with disks inoculated by either taro or sweet potato stain at 5 hr intervals until 30 hr after inoculation. Some reduction of Cyt c or NBT was detected with all disks tested. However, the reduction was not inhibited at all by SOD added to the reaction mixture. Therefore, it was concluded that the reduction was not caused by O_2^-. In the assay using NBT, the disks in the assay mixture were stained blue within 30 min of incubation. However, wounded disks (control) were stained rather stronger than disks inoculated by taro or sweet potato strain. Moreover, the intensity of the staining did not change during incubation. These results suggest that even if reduction of NBT in the cells on the surface of the disks was actually due to O_2^-, the O_2^- generation in these disks was not due to inoculation but just to wounding.

Phospholipase A_2 activity in inoculated taro tubers.

In disks inoculated by taro strain, phospholipase A_2 activity increased, without a lag phase, to reach a plateau within 10 hr after inoculation and, subsequently, began to decrease after 20 hr to initial level (Fig. 2-2). In the sweet potato strains-inoculated disks, phospholipase A_2 activity increased more slowly, plateauing at about 20 hr but reached a lower level than

in the taro strain-inoculated disks. In wounded disks, phospho-
lipase A_2 did not change significantly throughout the incubation
period. Only negligible activity of phospholipase A_2 was detect-
ed with filter paper disks inoculated by taro or sweet potato
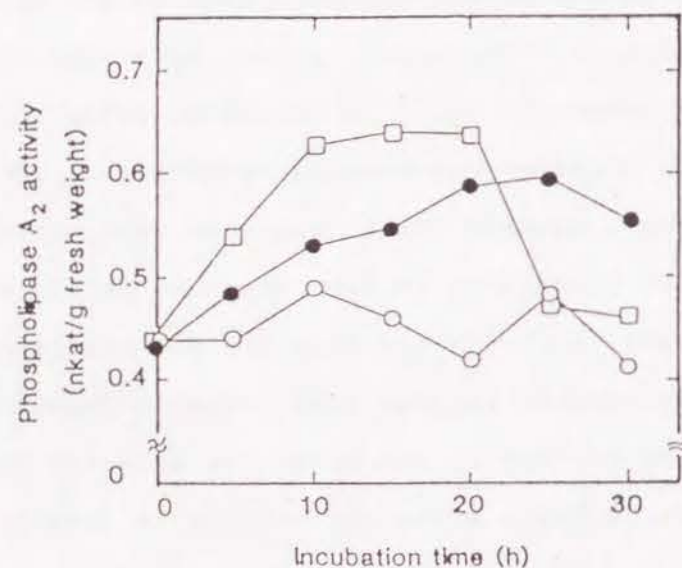strain.

Figure 2-2. Phospholipase A_2 activity in taro tuber disks
wounded (—O—), inoculated by taro (—□—) and sweet potato
strain (—●—) of C. fimbriata.

Lipoxygenase activity in inoculated taro tubers.

In both disks inoculated by taro strain and by sweet potato
strain, lipoxygenase activity began to increase about 10 hr after
inoculation (Fig. 2-3). This incubation time (10 hr) correlated
well with the time of development of a reddish color on the
inoculated surface and with time of increase of peroxidation in
those disks (Fig. 2-1). In the later incubation period, 25 to 30
hr, the lipoxygenase activity in the taro strain-inoculated disks
was almost twice as high as that in the sweet potato strain-
inoculated disks (Fig. 2-3). No significant change in lipoxyge-
nase activity was detected in wounded disks during incubation.
Filter paper disks inoculated by both strains showed only negli-
gible lipoxygenase activity.

Effect of peroxides of linolenic acid on growth of spores.

It was indicated in chapter 1 that unsaturated fatty acid
such as linolenic and linoleic acids were converted by the action
of lipoxygenase into various species of peroxides with antifungal
activity in vitro. In this chapter, therefore, it was examined
if the peroxides of linolenic acid produced by the action of
lipoxygenase exhibited differential inhibitory activity towards
the growth of taro and sweet potato strains of C. fimbriata
(Table 2-1). There was not clear difference in sensitivity to
lipid peroxides of linolenic acid between the two strains, al-
though the sweet potato strain seemed to be slightly more sensi-
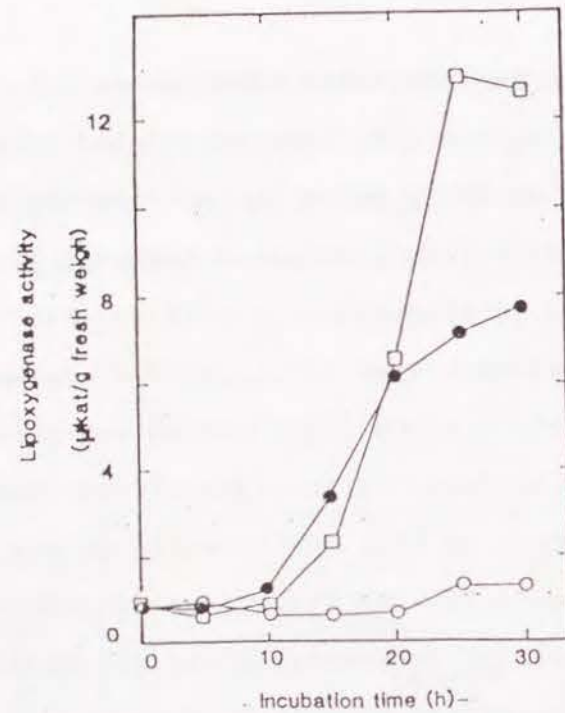tive than the taro strain.

Figure 2-3. Lipoxygenase activity in taro tuber disks wounded
(—O—), inoculated by taro (—□—) and sweet potato strain
(—●—) of C. fimbriata.

Table 2-1. Effect of peroxides of linolenic acid produced by
lipoxygenase on the growth of spores of taro and sweet potato
strains of C. fimbriata

Concentration of linolenic acid added (mM)	Relative germ tube growth	
	Taro strain	Sweet potato strain
7.1	_a)	-
6.4	-	-
3.2	-	-
2.8	+ 1	-
2.5	+ 1	-
2.1	+ 3	+ 1
1.8	+ 4	+10
0 (control 1)	+10	+ 8
7.1(control 2)	+ 8	

a) A minus sign(-) denotes no germination of spores.

Effect of lipid peroxides-containing fractions from inoculated
taro tubers on growth of spores.

Lipid peroxides-containing fractions prepared from taro
tubers inoculated by either taro or sweet potato strain were
assayed for inhibition of growth of spores of both strains (Table
2-2). The growth of both strains was inhibited in assay media
containing the low concentration of peroxides. However, signifi-
cant difference in the sensitivity to peroxides was not detected
between the two strains.

Table 2-2. Effect of lipid peroxides-containing fractions pre-
pared from taro tubers inoculated by taro or sweet potato strain
of C. fimbriata on the growth of spores of both strains.

Concentration of lipid peroxides (as concentration of malondialdehyde) (μM)	Relative germ tube growth			
	Fraction from taro strain inoculated tubers		Fraction from sweet potato strain inoculated tubers	
	Taro strain	Sweet potato strain	Taro strain	Sweet potato strain
0.72	_a)	-	-	-
0.65	-	-	-	-
0.59	-	-	-	-
0.52	-	-	-	-
0.46	+ 0.5	+ 0.5	+ 0.5	-
0.39	+ 1	+ 0.5	+ 1	+ 1
0.33	+ 2	+ 0.5	+ 3	+ 2
0.26	+ 4	+ 3	+ 4	+ 2
0 (control)	+10	+10	+10	+10

a) A minus sign (-) denotes no germination of spores.

DISCUSSION

It has been reported that enhanced lipid peroxidation occurs in incompatible host-parasite interactions undergoing hypersensitive necrosis but not in compatible interactions [30,35]. In contrast, lipid peroxidation took place in a similar magnitude and similar time-course pattern in both taro tubers inoculated by taro strain (compatible or pathogenic) and by sweet potato strain (incompatible or non-pathogenic) of $\underline{C}$. $\underline{fimbriata}$. Phospholipase A_2 and lipoxygenase were also induced similarly in both tubers inoculated by the two strains. This result also differed from those of other host-parasite interactions [30,35,42]; in these cases, lipoxygenase increased in incompatible interactions, while it did not increase in compatible interactions. One possible explanation for the differences mentioned above might be that no distinct necrosis occurs in taro tubers inoculated by either strain of $\underline{C}$. $\underline{fimbriata}$.

Two reaction pathways have been reported for the peroxidation of lipids [30,31,34,35]; one involves active oxygen species and the other is mediated by lipoxygenase. In the light of the results of this chapter, lipid peroxides seem to be produced through the latter pathway in inoculated taro tubers. In several repeated assays, inoculation-induced generation of O_2^- was not detected by either of two methods. But reasonable activities of phospholipase A_2 and lipoxygenase were detected and they changed in a manner accounting for the production of lipid peroxides in disks inoculated by both strains (Figs. 2-2 and 2-3). Filter paper disks inoculated by either strain gave negligible values of lipid peroxidation and activities of phospholipase A_2 and lipoxygenase, indicating that fungal contribution to the presented values can be neglected (Figs. 2-1,2-2,2-3).

The hypersensitive reaction is assumed to be an early reaction of host which results from its incompatible recognition and triggers the host resistance reactions [27,28,43]. During the early process of hypersensitive reaction, peroxidation of membrane lipid was found to occur [34]. Thus, it is inferred that lipids peroxidation is involved in host defense reaction through the induction of the hypersensitive reaction.

There is a clear difference between taro and sweet potato strains in pathogenicity to taro tubers, as mentioned in the introduction. Nevertheless, such a clear difference was observed neither in the magnitude and time course pattern of lipid peroxidation nor in induction of phospholipase A_2 and lipoxygenase between taro tuber disks inoculated by either of two strains. Consequently, it is unlikely that a different growth behavior (pathogenicity) of taro and sweet potato strains on taro tubers could be ascribed to the difference in the initiation time or magnitude of lipid peroxidation or hypersensitive reaction in the tissues. In addition, there was no clear difference in sensitivity to lipid peroxides between the two strains (Tables 2-1 and

2-2). Therefore, the different pathogenicity of the two strains is not attributable to the different sensitivity of lipid peroxides accumulated in the infected taro tubers.

However, the results in this chapter demonstrated definitely that lipid peroxides with antifungal activity are produced in taro tubers in response to infection by C. fimbriata. The lipid peroxides thus formed must be involved in the host defense reaction, although some other factor(s) seems to contribute to host-parasite specificity between C. fimbriata and taro tubers.

SUMMARY

When taro tubers (Colocasia antiquorum Schott) were inoculated by taro strain (compatible or pathogenic) or sweet potato strain (incompatible or non-pathogenic) of C. fimbriata, lipid peroxidation, which was measured by the thiobarubituric acid reaction, took place to a similar magnitude and in similar time-course pattern in both tubers. Throughout the incubation period, no generation of superoxide anions (O_2^-) due to inoculation by either strain was detected in the tubers. The activities of phospholipase A_2 and lipoxygenase changed in a manner accounting for the production of lipid peroxides observed in taro tubers inoculated by both strains. The sweet potato and taro strains showed the similar sensitivity to the toxicity of peroxides of linolenic acid and lipid peroxides from inoculated taro tubers.

Chapter 3.

Lipid peroxides and the enzymes which are involved in their production in taro tubers infected by Ceratocystis fimbriata

INTRODUCTION

As mentioned in chapter 1, 9,12,13-trihydroxy-(E)-10-octadecenoic acid (9,12,13-LOH) is produced as an antifungal compound in infected taro tubers. This compound has been reported that anti-conidial germination compounds[48] and elicitors [44] in interactions between rice plant and Pyricularia oryzae. The structure of 9,12,13-LOH suggests that this compound is biosynthesized through the following pathway ; linoleic or linolenic acid is first peroxided into hydroperoxide derivative by lipoxygenase (LOX). Subsequently, the hydroperoxide derivative is changed into hydroxy fatty acid by lipid hydroperoxide converting enzyme (LHCE). LOX is a ubiquitous enzyme in higher plants [50]. LHCE was recently detected in rice leaves infected by blast fungus and characterized by Ohta et al. [46,47].

In this chapter, LOX and LHCE in the taro tubers infected by C. fimbriata were investigated in the terms of production of antifungal peroxides.

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. Trimethylsilyation 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 $\underline{C}$. $\underline{fimbriata}$ were used. Inoculum (7×10^6 spores/ml) was prepared as in Chapter 1.

Preparation of taro tuber disks and inoculation.

Taro tuber disks (1.2×0.2 cm) were prepared and inoculated as described in the preceding chapters.

Preparation of peroxides of linolenic acid

The enzyme solution 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 shaked 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 linoleic acids

9-LOOH was prepared using potato lipoxygenase by the following method. The crude potato lipoxygenase was prepared by the method of Sekiya $\underline{et}$ $\underline{al}$. [49]. Potato lipoxygenase(6,300 units) was added to 450 ml of 5 mM linoleic acid in 0.1M acetate buffer, pH 5.5 containing 0.07% Tween 20 and incubated for 1 hr at 25 °C with 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($\underline{n}$-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 1N NaOH was added until the suspension became a clear solution. Then 400 ml of 0.1M borate buffer (pH 9.0) and soybean lipoxygenase (10 mg) were added to the linoleic acid solution and incubated with 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. x 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 solutions at -20 °C and used within a month.

9- or 13-Hydroxy linoleic acid was prepared from 9- or 13-LOOH, respectively, by reducing with $NaBH_4$. 9,12,13-Octadecen-oic acid was purified as described in Chapter 1.

Extraction and assay of LOX and LHCE

Five disks (1.2x0.2cm) were ground in 5 ml of 0.1M 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 x g for 15 min at 4 °C. The resultant supernatant was applied to a column (4.6 X 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 Chapter 2 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.[47]. 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 taro tuber slices (80 g) after incubated for 25 hr after inoculation were 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 x 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 X 10cm) 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 (LOX in peak 2) after DEAE-Toyopearl column chromatography (30 units) was added to 10 ml of 2 mM linoleic acid in 0.1M 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_2N_2 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 160-240 °C ,3 °C /min .ion source 250 °C, in let 20eV.)

Preparation and identification of LHCE reaction products

9- or 13-LOOH (5mg) in 0.1 M phosphate buffer, pH 5.5 was incubated with 5 units of LHCE after DEAE-Toyoperl column chromatography at 25 °C for 20min. 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 trimethylsilized 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 was assayed as described in Chapter 1.

RESULTS

LOX in the infected taro tubers

LOX was extracted from taro tuber slices which were incubated for 25 hr after inoculation with taro strain of C.fimbriata, and analyzed by DEAE-Toyopearl column chromatography (Fig.3-1). Two 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.
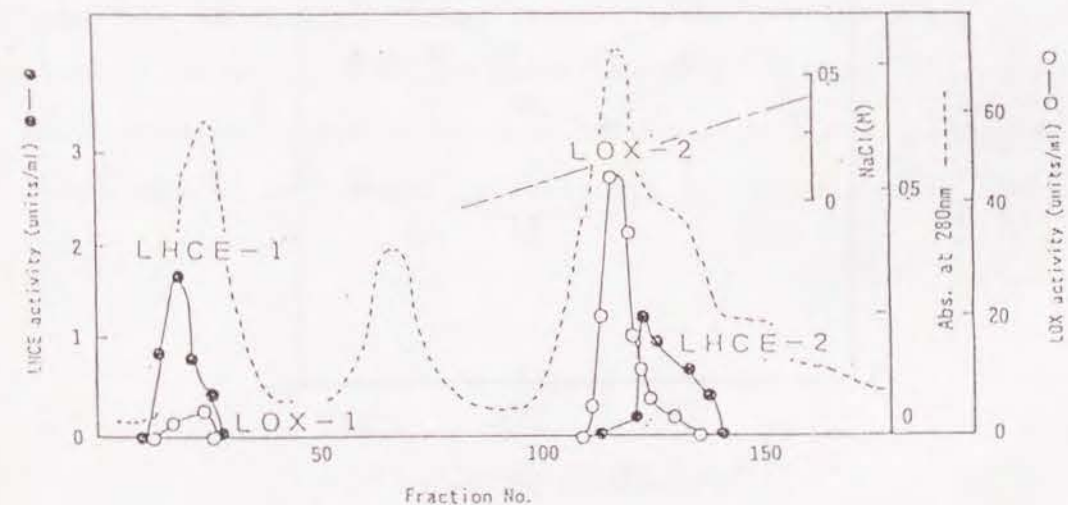

Figure 3-1. Elution profiles of LOX and LHCE from taro strain-infected taro tubers on DEAE-Toyoperl column chromatography.
—O— LOX activity, —●— LHCE activity, ——— OD_{280}

Induction of LHCE in inoculated taro tubers

LHCE activity was detected in fresh taro tubers and in-
creased 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.3-2). 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 magni-
tude of the changes in the taro strain inoculated-tubers was
larger compared with those in the sweet potato strain
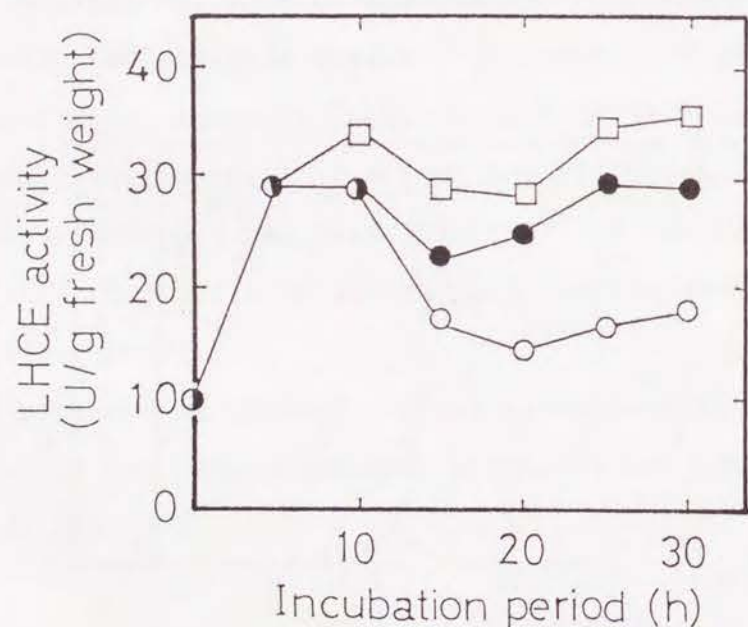inoculated-tubers and in the wounded tubers.

Figure 3-2. Change in LHCE activity after inoculation.
—□— inoculated with taro strain, —●— inoculated
with sweet potato strain, —○— wounded(no inoculation)

Identification of reaction products of LHCE

The reaction products of LHCE with 9-LOOH and 13-LOOH as
substrates were identified by using HPLC and GC-MS as described
in MATERIALS and METHODS. The products showed the same Mass spec-
tra as those of products prepared by Ohta et al.[47]. 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 have been observed in antifungal activity
between 9-LOOH and 13-LOOH (Table 3-1). In addition, both LOOHs
did not show differential toxicity toward sweet potato and taro
strains (Table 3-1). There was also no difference in antifungal
activity between 9-LOH and 13-LOH (Table 3-1 and 3-2). 9-LOH and
13-LOH showed the similar magnitude of antifungal activity toward
both strains (Table 3-2). 9,12,13-LOH also showed a similar
magnitude of antifungal activity against both strains (Table 3-
3).

Table 3-1.
    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.

Table 3-2.
    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 LOH (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 3-3.
    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.

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 several unknown products were produced besides 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 3-4).

Table 3-4.
    Antifungal activity of the mixture of peroxides of linolenic
acid produced by crude LOX 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).

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 anti-
fungal compounds from the infected taro tubers in Chapter 1. LHCE
in the infected taro tubers was very similar to the enzymes in
rice leaves reported by Ohta et al.[47]; 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. 3-2). 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 inocu-
lation among the three taro tuber samples. Subsequently, the
difference in the activity in three samples became lagers; the
activity in the taro strain infected-tubers was the highest and
the activity in the 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 the
taro strain infected-tuber disks was higher than that in the
sweet potato strain infected-tuber disks. The result is consist-
ent with the results of time course analysis of lipid peroxide
accumulation in the 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 strain (in Chapter 2). One possible explanation for the differences in LHCE activity and lipid peroxide accumulation in taro tuber disks infected by the two strains is that taro strain invade into more inner cells in the disks than sweet potato strain.

The enzyme preparation containing LOX and LHCE produced various peroxides from linolenic acid in vitro; 9-LOOH, 13-LOOH, 9-LOH, 13-LOH and 9,12,13-LOH . When antifungal activity of the above peroxides were examined separately, (Tables 3-1,3-2,3-3), 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 that 9-LOH was more toxic to Magnaporthe grisea than 9-LOOH.[47]

Differential toxicity was demonstrated with a mixture of linolenic acid peroxides prepared by using the crude enzyme from infected taro tubers which contained both LOX and LHCE (Table 3-4). Sweet potato strain was inhibited more severely than taro strain. This result is apparently contradictory to the results in Table 3-1, 3-2 and 3-3, 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. The unknown oxidized products included in the peroxidized linolenic acid frac-

tion might be responsible for differential toxicity. Alternatively, the differential toxicity might appear through the interactions among the mixed peroxides.

SUMMARY

Two enzymes, lipoxygenase (LOX) and lipid hydroperoxide converting enzyme (LHCE), which are responsible for the production of antifungal lipid peroxides, were detected in taro tubers infected by C. 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- and 13-LOOH of linoleic acid were used as the substrates of LHCE, main products were 9- and 13-hydroxyoctadecadienoic 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 lino-
lenic acid into the peroxides compound(s), which inhibited the
growth of sweet potato strain more severely tan that of taro
strain.

When taro tubers are inoculated by spores of the taro strain
of $\underline{C}$. $\underline{fimbriata}$, the spores germinate and invade the inner tis-
sue, resulting in the appearance of symptoms of black rot. In
contrast, when taro tubers are inoculated by spores of the sweet
potato strain, the spores germinate but hyphal extension is
inhibited, resulting in no development of symptoms. The main
objectives of this thesis were to elucidate how host plant (taro)
and parasite ($\underline{C}$. $\underline{fimbriata}$) recognize each other and how host
defends itself against invasion of parasite.

It was demonstrated that antifungal compounds were produced
in taro tubers infected by the sweet potato strain. One of
antifungal compounds was isolated and identified to be 9,12,13-
trihydroxy-($\underline{E}$)-10-octadecenoic acid, which is assumed to be
produced from linolenic acid or linoleic acid by the peroxidative
reaction. The subsequent analysis indicated that lipid peroxida-
tion took place and lipid peroxides were accumulated in taro
tubers infected by both strains to similar magnitude and in simi-
lar time-course pattern. Superoxide anions(O_2^-) seemed not to be
involved in the peroxidative reaction for antifungal compound
production. Phospholipase A_2, lipoxygenase(LOX) and lipid hy-
droperoxide converting enzyme(LHCE) were detected in infected
taro tubers and their activities were changed during incubation
in the manners accounting for production of lipid peroxides. No

significant difference, however, was not observed in induction
patterns of those enzymes between tubers infected by two strains.

But lipid peroxides isolated from infected taro tubers and
produced from linolenic acid and linoleic acid _in vitro_ using the
enzymes showed strong antifungal activity. When individual
peroxide was separately assayed, they inhibited similarly the
growth of two strains. However, peroxide mixture which were
produced from linolenic acid using crude enzyme preparation from
infected taro tubers inhibited the growth of sweet potato strain
more severely than that of taro strain.

The results presented in this thesis indicate that lipid
peroxidation occur in the early stage of infection in taro tubers
infected by _C. fimbriata_ and that the lipid peroxides produced by
peroxidative reaction are involved in defense reaction of host
plant. However, lipid peroxides seem not to play a direct role
in host-parasite specificity since the difference was not clear
in their toxicity to two strains and in the induction patterns of
the enzymes such as phospholipaseA_2, LOX and LHCE which are
responsible for their production, in taro tubers infected by two
strains.

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