Characterization and Activity Change of Farnesol Dehydrogenase in Black Rot Fungus-infected Sweet Potato

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NADP-dependent farnesol dehydrogenase was isolated from black rot fungus-infected sweet potato root tissue and purified almost to homogeneity with several purification steps including Toyopearl HW-60 (S) chromatography. The enzyme was estimated to have a molecular weight of 90,000 and consists of two identical subunits. *trans,trans*-Farnesol was oxidized to *trans,trans*-farnesal at the highest level among various kinds of alcohol in the presence of NADP by the enzyme, but the enzyme showed broad substrate specificity. The SH-group in the enzyme participated in its activity. The enzyme was present in small amounts in fresh tissue, but in response to the infection the activity was increased, accompanied with the accumulation of furano-sesquiterpenoids. The activity was increased also in response to cut injury, although in a lesser amount.

When infected by the black rot fungus, *Ceratocystis fimbriata* and some other pathogens, various kinds of sesquiterpenoids such as ipomeamarone^{1,2)} are accumulated in the infected region of sweet potato root tissue,^{3,4)} and play a role in protecting the tissue from microbial penetration as phytoalexins.^{5,6)} The *in vivo* and *in vitro* experimental results demonstrated that the mevalonate pathway, the route from acetyl CoA to farnesol was involved in the biosynthetic pathway of sesquiterpenoids as the first half step, and the enzymes in this step were induced in response to the infection, prior to sesquiterpenoid accumulation.^{7,8)}

A few experimental observations^{9~12)} have been obtained to elucidate the second half step of the pathway, the route from *trans, trans*-(*t,t*)-farnesol to furano-sesquiterpenoids such as dehydroipomeamarone,¹³⁾ ipomeamarone^{1,2)} and ipomeamaranol.^{14,15)} Since Oguni

and Uritani⁹⁾ indicated that ¹⁴C-farnesol was efficiently converted to ¹⁴C-ipomeamarone in black rot fungus-infected tissue, we assumed that the initial step of farnesol metabolism might be the dehydration of farnesol to farnesal, and recognized the presence of NADP-dependent farnesol dehydrogenase in diseased tissue.

This paper deals with the purification and characterization of the above farnesol dehydrogenase in diseased tissue and the change of the activity in sweet potato root tissue in response to black rot fungus infection and cut injury.

MATERIALS AND METHODS

Materials. Roots of sweet potato (*Ipomoea batatas* Lam., cv. Norin No. 1) were harvested at the Kariya Farm, Aichi Prefecture in the autumn and stored at 13 ± 1°C until used.

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Abbreviations: farnesol or *t,t*-farnesol, *trans,trans*-farnesol; *c,t*-farnesol, *cis,trans*-farnesol; DTT dithiothreitol.

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The farnesol purchased (Fluka AG·Buchs SG, Switzerland) was composed of the *trans*, *trans*-form and the *cis*, *trans*-form, which were separated by TLC using *n*-hexane–EtOAc (7:3, v/v) as the developing solvent.

t,t-Farnesol was synthesized with the oxidation of *t,t*-farnesol by activated MnO₂.¹⁶⁾

Preparation of crude enzyme solutions from diseased tissue for purification and for analysis of changes with time. Sweet potato roots were perpendicularly cut into slices (2 cm thick), which were inoculated on the cut surfaces with a spore suspension of *Ceratocystis fimbriata* Ell. et Halst., and incubated at 29°C under high humidity. After 48 hr of incubation, non-infected tissue (1 to 1.5 mm thick) adjacent to the infected region was collected and cut finely. The tissue pieces (80 g) were homogenized at about 2°C in 160 ml of 100 mM potassium phosphate buffer (pH 7.5) containing 0.5 M sucrose, 10 mM mercaptoethanol, 10 mM EDTA and 10% (16 g) Polyclar AT with a blender three times each for 15 sec. The homogenate was squeezed through two layers of nylon gauze, the filtrate centrifuged at 300 × *g* for 10 min. The supernatant solution was passed through a Sephadex G-25 column preequilibrated with 20 mM potassium phosphate buffer (pH 8.0) containing 0.5 M sucrose and 1 mM dithiothreitol (DTT) (called standard buffer), and eluted with standard buffer yielding the protein fraction, which was centrifuged at 10,000 × *g* for 20 min, and the supernatant solution was used as the crude enzyme solution.

For the analysis of the course of infection or injury, the tissue slices with or without inoculation were incubated, then 10 g of adjacent non-infected tissue pieces were used to prepare the crude enzyme solution, in the same way as shown above.

Enzyme purification. The crude enzyme solution was precipitated by 40 to 64% saturation of ammonium sulfate and the precipitate was collected by centrifugation and suspended in 5 ml of standard buffer. The suspension was passed through Sephadex G-25 column previously equilibrated with the above buffer. The effluent was applied to a DE-52 column (3.0 × 6.0 cm) that had been equilibrated with standard buffer. After passing 20 ml of the buffer, the farnesol dehydrogenase-containing fraction was eluted with a linear gradient of 150 ml each of standard buffer and the above buffer containing in addition 0.15 M KCl.

This fraction was concentrated by ultrafiltration using an Amicon PM30 membrane up to 5 ml, changing the buffer to 20 mM potassium phosphate buffer (pH 8.0) containing 0.2 M KCl and 1 mM DTT. The concentrate was applied to a column (2.6 × 38.0 cm) of Toyopearl HW-60 (S) (Toyo Soda Co.) preequilibrated with the same buffer and eluted with the above buffer by an HPLC pump (Hitachi 635) at the rate of 1 ml per min. The fraction containing the enzyme was concentrated by ultrafiltration as above. During concentration the buffer was changed to the buffer without 0.2 M KCl. The concentrate was applied

to a 2',5'-ADP-Sepharose 4B column (1.0 × 3.6 cm) previously equilibrated with 20 mM phosphate buffer containing 1 mM DTT and eluted with a linear gradient of 10 ml each of the above buffer and the same buffer containing in addition 3 M KCl. The enzyme solution thus obtained was concentrated by ultrafiltration up to 1 ml, changing the buffer to 20 mM phosphate buffer (pH 8.0) containing 1 mM DTT. Finally, the enzyme-containing concentrate was stored at –80°C until used, and diluted by the above buffer for the enzymatic assay.

Enzyme assay. The reaction medium was composed of 20 mM potassium phosphate buffer (pH 8.0) with or without 0.5 M sucrose, 0.3 mM *t,t*-farnesol in 5 μl acetone, an aliquot of the enzyme solution, and 0.5 mM NADP in 0.3 ml of final volume. Prior to the enzymatic reaction, the medium without NADP was incubated at 25°C for about 5 min until a constant value for the absorbance at 340 nm was obtained. Then the reaction was started by adding the NADP solution. The initial increase in the absorbance was monitored by a spectrophotometer, and 1 unit of the enzyme activity was designated as the enzyme amount that reduced 1 nmol of NADP per min.

Polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis was carried out, and farnesol dehydrogenase activity was located in the gel by staining in the dark at 25°C in 5 ml of 20 mM potassium phosphate buffer (pH 8.0) containing 2 mM *t,t*-farnesol, 0.25 mM NADP, 0.01% of phenazine methosulfate, and 0.01% of *p*-nitroblue tetrazolium. Protein staining was done by Coomassie brilliant blue R.

Determination of protein content. Protein content was determined by the method of Lowry *et al.*¹⁷⁾

Determination of furano-sesquiterpenoid content. Furanos sesquiterpenoid content in the infected region was determined by the method of Hyodo *et al.*¹⁸⁾

RESULTS

Occurrence of farnesol dehydrogenase in diseased tissue

The crude enzyme solution from diseased tissue was incubated at 30°C with shaking for 60 min with 2.5 mM *t,t*-farnesol in 0.6 ml acetone, 1 mM NADP, and 10 mM NaF in 15 ml of total volume. The reaction was stopped by adding 15 ml acetone, and *n*-hexane (15 ml) was shaken with the mixture. The upper layer was evaporated *in vacuo* yielding an oily substance, which was applied to TLC plates and separately developed by *n*-hexane–EtOAc

(9:1) and CHCl_3 -ethyl ether (19:1). In each case, one spot was detected on the TLC plate by UV-absorption under a UV-lamp, and by a positive reaction with 2,4-dinitrophenylhydrazine. Furthermore, the R_f values (the former, 0.96; the latter, 0.87) coincided with those of the authentic *t,t*-farnesal. When *c,t*-farnesol was used as the substrate, *c,t*-farnesal was proven to be the reaction product by the same procedures. Thus, it was demonstrated that farnesol dehydrogenase occurred in diseased tissue.

Purification of farnesol dehydrogenase

DE-52 column chromatography easily separated NADP-dependent farnesol dehydrogenase from NAD-dependent alcohol dehydrogenase,

which was eluted first by passing the buffer alone as the initial step (see MATERIALS AND METHODS). As shown in Table I, NADP-dependent farnesol dehydrogenase was purified about 1000 fold by ammonium sulfate salting-out, DE-52 chromatography, Toyopearl permeation chromatography, and 2',5'-ADP Sepharose 4B affinity chromatography, and the yield was about 15%. According to the polyacrylamide zymograms of the final sample, one sharp peak of enzyme staining appeared and accorded with the main stained protein band, which was accompanied by two faint bands (see Fig. 1). Thus, this sample was regarded as the almost purified enzyme and used for the characterization of the enzyme.

FIG. 1. Polyacrylamide Gel Zymograms of Farnesol Dehydrogenase Preparation from Diseased Sweet Potato.

A and B: The zymograms shown by a densitometer on activity staining and protein staining, respectively. The methods are described in MATERIALS AND METHODS. The peaks at R_m 0.65 in A and 0.61 in B indicate farnesol dehydrogenase. The peak at R_m 0.82 in A shows DTT, which was stained non-enzymatically.

Molecular weight and subunits

The molecular weight of the enzyme was calculated from the straight line obtained by several marker proteins using Toyopearl HW-60 (S) permeation chromatography (see Fig. 2), and estimated to be 90,000. According to the SDS-polyacrylamide gel electrophoresis, one strong band appeared accompanied by two faint bands, and the former was regarded as the subunit of the enzyme, and the molecular weight was estimated to be 47,000, using six marker proteins. Thus, the enzyme was concluded to consist of two identical subunits.

The effect of pH on the activity

The enzyme activity toward the NADPH-generating reaction inclined to be high on the alkaline side, and the optimum pH was above pH 9.5 (Fig. 3). The enzyme activity in the

TABLE I. PURIFICATION OF FARNESOL DEHYDROGENASE FROM DISEASED TISSUE

Step	Protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)	Fold
Sephadex G-25 chromatography and centrifugation	412.80	7.430	0.018	100.0	1.0
$(\text{NH}_4)_2\text{SO}_4$ 40~65% saturation	111.00	4.773	0.043	62.5	2.4
DE-52 chromatography	7.43	2.080	0.280	27.1	15.6
Toyopearl permeation chromatography	1.52	1.687	1.110	17.3	61.7
2',5'-ADP Sepharose 4B affinity chromatography	0.06	1.101	18.342	14.5	1019.0

FIG. 2. Determination of the Molecular Weight of Farnesol Dehydrogenase by Toyopearl Permeation Chromatography.

Marker Proteins: cytochrome *c* from horse heart (Cyt *c*, 11,700), myoglobin from equine skeletal muscle (17,200), ovalbumin from hen's egg (43,000), malate dehydrogenase from pig heart (MDH, 70,000), lactate dehydrogenase from beef muscle (LDH, 140,000), alcohol dehydrogenase from yeast (ADH, 140,000), catalase from beef liver (240,000), and apoferritin from horse spleen (460,000). Elution solvent: 20 mM potassium phosphate buffer (pH 8.0) containing 0.2 M KCl and 1 mM DTT. Elution was performed at 4°C and at the rate of 1 ml per min, and the detection was carried out by the absorbance at 280 nm.

FIG. 3. Effect of pH on the Activity of Farnesol Dehydrogenase.

Right and left sides: NADPH generating reaction and the reverse reaction, respectively.

○---○, ●—● and □---□: potassium phosphate buffer (pH 6.0~8.0), Tris-HCl buffer (pH 7.5~9.0) and NH₄OH-HCl buffer (pH 8.5~10.5), respectively.

FOH and FHO: farnesol and farnesal, respectively.

reverse reaction, however, appeared high in the neutral range, and the optimum pH was 7.0 to 7.5.

Substrate specificity

NADP was absolutely required for the activity, and NAD could not be substituted for NADP. Various kinds of alcohol were tested for the activity. The maximum activity was given when *t,t*-farnesol was used and the activity was decreased in the following order (Table II): *c,t*-farnesol (C₁₅) > geraniol (C₁₀) > citronerol (C₁₀) > nerol (C₁₀) > decanol (C₁₀). Benzylalcohol, glycerol and ethanol showed no activity. This enzyme was capable of oxidizing C₁₅- to C₁₀-isoprenoid and aliphatic components. The *K_m* value for *t,t*-farnesol was 15.6 μM, but that for geraniol was 729.0 μM. Thus, this enzyme is reasonably cal-

TABLE II. SUBSTRATE SPECIFICITY OF FARNESOL DEHYDROGENASE FROM DISEASED TISSUE

Substrate (0.3 mM)	Activity (units)	Relative activity (%)	<i>K_m</i> (μM)
<i>trans,trans</i> -Farnesol	1.25	100.0	15.6
<i>cis,trans</i> -Farnesol	1.04	83.2	—
Geraniol	0.72	57.6	729.0
Citronerol	0.47	37.6	—
Nerol	0.45	36.0	—
Decanol	0.12	9.6	—
Benzylalcohol	0.00	0.0	—
Glycerol	0.00	0.0	—
Ethanol	0.00	0.0	—

TABLE III. EFFECT OF INHIBITORS ON FARNESOL DEHYDROGENASE ACTIVITY

DTT was contained in the enzyme sample, but the concentration ($3.3 \sim 6.6 \times 10^{-6}$ mM) was negligible compared to those of the above reagents.

Reagent (mM)	Activity (units)	Inhibition (%)
<i>N</i> -Ethylmaleimide (0.05)	0.00	100.0
ICH ₂ CONH ₂ (0.05)	0.74	28.8
CuCl ₂ (0.05)	0.00	100.0
ZnCl ₂ (0.05)	0.00	100.0
MgCl ₂ (1.00)	1.02	1.9
EDTA (3.33)	0.53	49.0
H ₂ O	1.04	0.0

led NADP-dependent farnesol dehydrogenase.

The effect of inhibitors on the activity

The activity was inhibited by some SH-group inhibiting reagents such as *N*-ethylmaleimide, monoiodoacetamide, CuCl_2 and ZnCl_2 at relatively low concentrations, indicating that the SH-group in farnesol dehydrogenase participated in its activity. EDTA also inhibited the enzyme activity to some extent, which suggests the participation of some metal ions in the activity (see Table III).

Changes in the activity of farnesol dehydrogenase in response to black rot fungus infection and cut injury

In response to black rot fungus infection and cut injury, the activity of farnesol dehydrogenase was increased, although a little activity was detected in fresh tissue without incubation. With infection, the activity increased with a lag of several hr and reached a plateau 2 days after the inoculation. With cut

injury, the activity was increased at a similar level until 1 day of incubation, thereafter reaching a plateau. Following the increase in the activity, furano-sesquiterpenoids were produced in diseased tissue. However, these were not detected at any time in cut-injured tissue (see Fig. 4).

DISCUSSION

There are many reports on terpene alcohol dehydrogenases in higher plants. Among them, an NADP-dependent farnesol dehydrogenase has been reported to be present in orange flavedo.¹⁹⁾ However, the report showed only the presence of this enzyme, but did not describe the purification and characteristics. On the other hand, NADP-dependent geraniol dehydrogenase was detected in orange juice vesicles,²⁰⁾ and actively oxidized some monoterpenic alcohols in the presence of NADP, and at a slower rate farnesol, too. The behavior of this enzyme with pH and inhibitors resembled those of the sweet potato enzyme. Furthermore, another report²¹⁾ described the dehydrogenation of borneol, a cyclic-monoterpene alcohol, by NADP-dependent dehydrogenase isolated from sage. However, this was different from the NADP-dependent monoterpene alcohol dehydrogenases in orange in several enzymatic features. NADP-dependent alcohol dehydrogenase samples were isolated from tea seeds²²⁾ and strawberry seeds,²³⁾ and enzymatically characterized. The natures of the latter were similar to those of the sweet potato enzyme, although the substrate specificity was not exactly the same, and the capability of oxidizing farnesol was not investigated in both seed enzymes.

It was demonstrated that furano-sesquiterpenoids such as ipomeamarone,^{1,2)} dehydroipomeamarone¹³⁾ and ipomeamarone^{14,15)} were converted from farnesol,^{9,12,24)} which were produced by the mevalonate pathway,^{7,8)} in diseased sweet potato root tissue. Thus, to elucidate the initial reaction of the conversion of farnesol to furano-sesquiterpenoids should be important. One possi-

FIG. 4. Changes in the Activity of Farnesol Dehydrogenase and the Content of Furano-sesquiterpenoids in Response to Black Rot Fungus Infection or Cut Injury.

●—● and ○—○: the activity of farnesol dehydrogenase in black rot fungus-infected and cut-injured tissues, respectively.

■—■ and □—□: the content of furano-sesquiterpenoids in black rot fungus-infected and cut-injured tissues.

bility may be the production of farnesal by NADP-dependent farnesol dehydrogenase. Recently, Burka *et al.*¹²⁾ found the occurrence of 9-hydroxyfarnesol in HgCl₂-treated sweet potato. Hence, the above enzyme may participate in the conversion of 9-hydroxyfarnesol to 9-hydroxyfarnesal, in the alternative way. Since the enzyme is formed especially in response to black rot fungus infection, it is very likely that the enzyme is involved in the formation of the furano-ring in furano-sesquiterpenoids as mentioned above.

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