Biosynthetic Pathway of Diepoxy Bicyclic FA from Linoleic Acid by *Clavibacter* sp. ALA2

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ABSTRACT: The biosynthetic pathway of two bicyclic FA, 12:17,13:17-diepoxy-9(*Z*)-octadecenoic acid (DEOA) and 7-hydroxy-12:17,13:17-diepoxy-9(*Z*)-octadecenoic acid (hDEOA), by *Clavibacter* sp. ALA2 was investigated. When cultivated with linoleic acid as a substrate, the strain produced 12,13,17-trihydroxy-9(*Z*)-octadecenoic acid (THOA), DEOA, and hDEOA as well as other FA. To clarify the synthetic route to these bicyclic FA, the strain was cultivated with purified THOA as a starting substrate. THOA was consumed almost completely by the strain with sequential generation of DEOA and hDEOA. Moreover, the strain produced hDEOA when cultivated with purified DEOA. Therefore, it was confirmed that THOA was a precursor of these bicyclic FA and that hDEOA was generated from DEOA. Based on our previously reported result that linoleic acid is first converted to 12,13-dihydroxy-9(*Z*)-octadecenoic acid (DHOA) and the present results, the overall biosynthetic pathway for the diepoxy bicyclic FA from linoleic acid was postulated as: linoleic acid → DHOA → THOA → DEOA → hDEOA.

Paper no. J10149 in *JAOCS 79*, 369–372 (April 2002).

KEY WORDS: Bioconversion, biosynthetic pathway, *Clavibacter* sp. ALA2, diepoxy bicyclic fatty acid, linoleic acid, trihydroxy octadecenoic acid.

Hydroxy unsaturated FA are important industrial materials because the hydroxy groups give a FA special characteristics such as high viscosity and chemical reactivity. Owing to these special physical and chemical properties, hydroxy FA are used in a variety of products including resins, waxes, nylons, plastics, corrosion inhibitors, cosmetics, and coatings (1). Besides the special physicochemical properties, several hydroxy FA are known to have physiological properties such as antifungal activity (2–5). Currently, the only industrial source of hydroxy FA is castor oil and its derivatives.

Microbial production of hydroxy FA has received much recent attention, and there are now many reports dealing with production of hydroxy FA by microbial systems (6–14). Previously, Hou *et al.* (15,16) reported the production of a trihydroxy FA, 12,13,17-trihydroxy-9(*Z*)-octadecenoic acid (THOA) from linoleic acid by a new bacterial isolate, *Clavibacter* sp. ALA2. THOA also showed growth-inhibitory activity against plant pathogenic fungi (5). In addition to THOA, strain ALA2 also produces a variety of unique FA including tetrahydrofuranyl cyclic and diepoxy bicyclic FA (17,18).

These unusual FA are of interest because they have unique chemical structures and because they are expected to have some biological functions. To clarify the biological functions and to develop an industrial production process for these FA, it is important to understand their biosynthetic pathways. In this paper, the biosynthetic routes to the diepoxy bicyclic FA 12:17,13:17-diepoxy-9(*Z*)-octadecenoic acid (DEOA) and 7-hydroxy-12:17,13:17-diepoxy-9(*Z*)-octadecenoic acid (hDEOA) are examined. Here we report that THOA is a precursor for DEOA and hDEOA. A possible biosynthetic pathway for the diepoxy bicyclic FA from linoleic acid is discussed.

MATERIALS AND METHODS

Bacterial strain and chemicals. Clavibacter sp. ALA2 was used throughout the study (15,16). The strain was cultivated aerobically in a medium containing 0.5% dextrose, 1% tryptone, 1.5% yeast extract, 0.5% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.001% $FeSO_4$, 0.0014% $ZnSO_4 \cdot 7H_2O$, 0.0008% $MnSO_4$, and 0.001% nicotinic acid (pH 6.8) at 30°C. Linoleic acid and palmitic acid were purchased from Sigma (St. Louis, MO). Diazomethane was prepared by degradation of *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide (Sigma) with potassium hydroxide. All other chemicals were of highest purity unless otherwise stated.

Purification of THOA and DEOA. Strain ALA2 was aerobically cultivated in 50 mL of the medium described above for 1 d at 30°C, and then 125 μL (100 mg) of linoleic acid was added to the culture. The culture was incubated for an additional 3 d. After incubation, 2 mL of 6 N HCl was added to the culture, and the lipids were extracted with 50 mL of ethylacetate, and then with 50 mL of diethyl ether by using a separation funnel. The lipid extracts were combined, and the solvent was evaporated to obtain a crude lipid extract (typically 0.2–0.3 g from 50-mL culture).

The crude lipid extract (approximately 2 g) was suspended in 30 mL of *n*-hexane and applied on a column (2.5 cm i.d. ×

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35 cm height) packed with silica gel 60 (70–230 mesh; EM Science, Gibbstown, NJ). After washing the column with 800 mL of *n*-hexane/ethyl acetate (9:1 vol/vol), DEOA was eluted with 400 mL *n*-hexane/ethyl acetate (8:2 vol/vol). The column was further washed with 800 mL *n*-hexane/ethyl acetate (2:8 vol/vol) and then eluted with 400 mL ethyl acetate to recover THOA (approximately 75% purity by GC).

When necessary (e.g., to obtain a highly pure lipid sample for establishing standard lines for quantification by GC), the DEOA and THOA fractions were further purified by Silica Gel 60 F_{254} preparative TLC (EM Science) using toluene/1,4-dioxane (95:5 vol/vol) and toluene/1,4-dioxane (7:3 vol/vol), respectively.

Microbial conversion of FA. Strain ALA2 was cultivated in 50 mL of the medium at 30°C for 2 d. A 125-µL (100 mg) aliquot of linoleic acid, purified THOA (approximately 75% purity) or purified DEOA (approximately 90%) was added to the culture, and incubation continued. An extended cultivation time of 2 d and the use of only a limited amount of substrate were designed to produce better biotransformation. Intermittently, a portion (0.5 mL) of the mixture was withdrawn into a microcentrifuge tube. A 20-µL volume of 6 N HCl and 0.1 mL of 10 mg/mL palmitic acid in ethyl acetate (internal standard) were added. The lipids were extracted twice with 0.5 mL of ethyl acetate, dried under nitrogen gas, and converted into methyl esters using diazomethane.

The FAME were analyzed with a gas chromatograph (Model 5890; Hewlett-Packard, Palo Alto, CA) equipped with a capillary column (SPB-1, 15-m × 0.32 mm, 0.25 µm film thickness; Supelco, Bellefonte, PA). Helium was used as carrier gas at 1 mL/min. The initial temperature of the column was 160°C, which was heated at a rate of 5°C/min to 210°C, and then kept at 210°C for 30 min. Detection was done by an FID with hydrogen, helium (make-up gas), and air at 27, 29 and 445 mL/min, respectively.

The amounts of linoleic acid, THOA, and DEOA were calculated from the peak area relative to that of palmitic acid (internal standard) using standard lines drawn using known amounts of the authentic FA. Linear relationships were found between peak area ratio and weight area ratio for these three FA. We were not able to make a standard line for hDEOA, however, owing to the difficulty in obtaining a pure authentic sample (the amount of hDEOA in the crude lipid extract was very small). As a compromise, the quantification of hDEOA was done using the standard line for DEOA, although it may differ from the true amount.

RESULTS AND DISCUSSION

Figure 1 shows typical gas chromatograms of FA composition during the conversion reaction of linoleic acid by strain ALA2. At 48 h, linoleic acid was completely consumed, and several new peaks of THOA, DEOA, and hDEOA appeared. Further incubation (at 96 h) resulted in consumption of THOA, decrease of DEOA, and increase of hDEOA. A time course of the changes in the amount of the FA is shown in Figure 2A. From 2 mg/mL of linoleic acid, 1.15 mg/mL of THOA and 0.17 mg/mL of DEOA were formed at 48 h; thereafter, THOA and DEOA gradually decreased to 0.1 and 0.06 mg/mL, respectively, generating hDEOA to 0.23 mg/mL at 96 h.

From Figures 1 and 2A, one can speculate that DEOA was derived from THOA *via* biocatalytic conversion. However, because of the many small peaks of other FA, the results were not conclusive to confirm this speculation. To make sure of

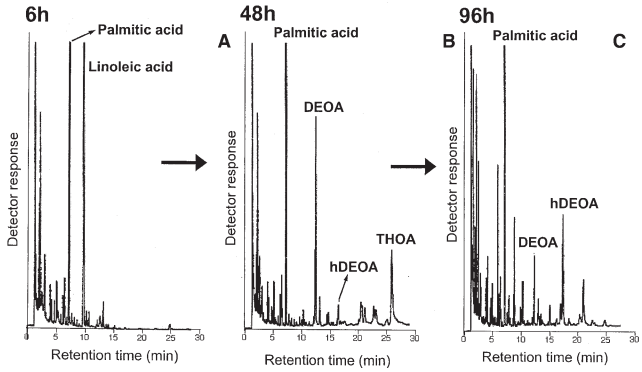


FIG. 1. Gas chromatographic analyses of FA composition during the conversion of linoleic acid. The chromatograms of the lipid extracts at 6, 48, and 96 h after the addition of linoleic acid are shown. DEOA, 12:17,13:17-diepoxy-9(*Z*)-octadecenoic acid; hDEOA, 7-hydroxy-12:17,13:17-diepoxy-9(*Z*)-octadecenoic acid; THOA, 12,13,17-trihydroxy-9(*Z*)-octadecenoic acid.

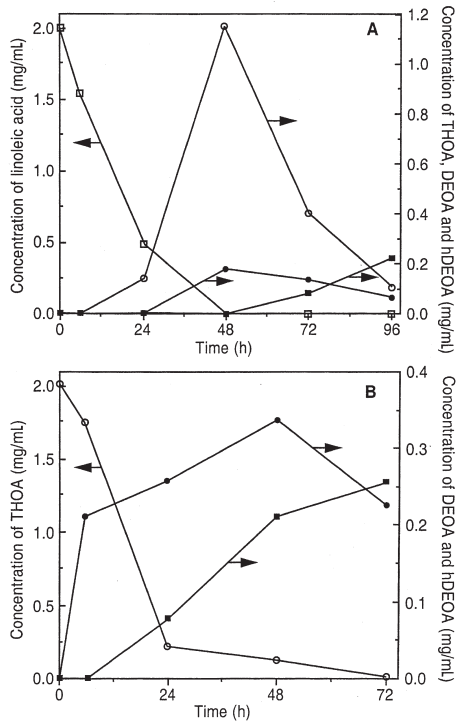


FIG. 2. Time-course change in FA during the bioconversion of linoleic acid (A) and THOA (B) as substrates. Symbols: (○), THOA; (●), DEOA; (□), linoleic acid, (■), hDEOA. For abbreviations see Figure 1.

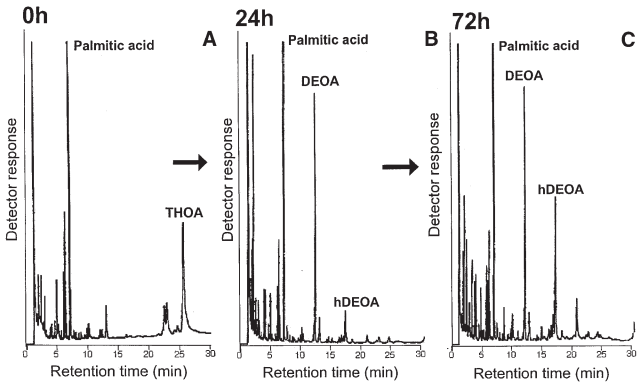


FIG. 3. GC analyses of FA composition during the conversion of THOA. Chromatograms of the lipid extracts at 0, 24, and 72 h after the addition of THOA are shown. For abbreviations see Figure 1.

the formation pathway of diepoxy bicyclic FA, we performed the conversion reaction using purified THOA as the starting substrate. Figure 3 presents gas chromatograms recorded during the reaction with THOA as the substrate. As expected, THOA was consumed completely in 24 h, and DEOA was generated as a major product. Afterward, the peak of DEOA decreased, and the peak of hDEOA appeared. The quantitative analyses of the FA during the reaction (Fig. 2B) also illustrate the rapid consumption of THOA with formation of DEOA in 6–24 h, and the subsequent generation of hDEOA. By starting from 2 mg/mL of THOA, 0.23 mg/mL of DEOA and 0.26 mg/mL of hDEOA were formed in 72 h. The yields of DEOA and hDEOA from THOA were approximately 12 and 13%, respectively. These yield values are reasonable considering the yields of DEOA and hDEOA from THOA in Figure 2A (from 48 to 96 h). Furthermore, when cultivated with purified DEOA, the strain produced hDEOA (Fig. 4). These results, strongly suggested that THOA was a precursor of DEOA and that hDEOA was formed from DEOA by hydroxylation at C7.

Gardner *et al.* (18) found 12,13-dihydroxy-9-octadecenoic acid (DHOA) in the crude lipid extract of strain ALA2 cultivated with linoleic acid. Therefore, it is likely that linoleic

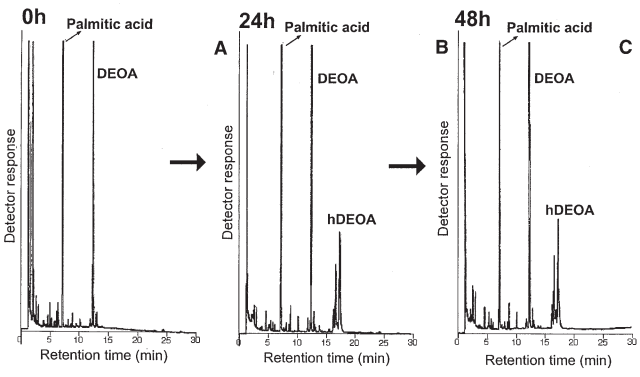
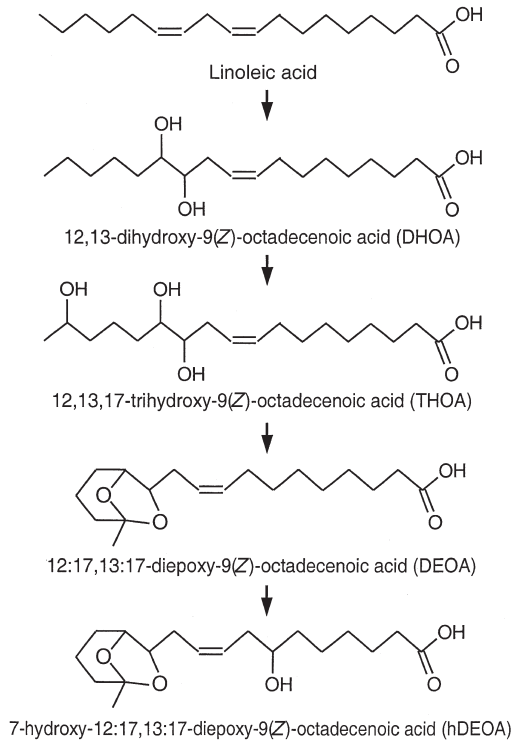


FIG. 4. GC analyses of FA composition during the conversion of DEOA. Chromatograms of the lipid extracts at 0, 24, and 48 h after the addition of DEOA are shown. For abbreviations see Figure 1.



SCHEME 1

acid is first converted to DHOA, and then into THOA by further hydroxylation at C17. Based on this consideration and the present results, the overall biosynthetic pathway of the diepoxy bicyclic FA from linoleic acid is postulated to be as shown in Scheme 1. First, linoleic acid is hydroxylated at C12 and C13 (either sequentially or simultaneously) to DHOA, which is further converted into THOA. THOA is then transformed to DEOA, then into hDEOA by another hydroxylation at C7.

A notable reaction step in the postulated pathway would be the formation of the bicyclic ring (i.e., formation of DEOA from THOA). Since the bicyclic moiety of DEOA is an intramolecular ketal structure, THOA itself is not likely the direct precursor for DEOA. In other words, THOA might be first converted into another intermediate, and then into DEOA. A reasonable candidate for such an intermediate for DEOA might be a ketone-diol, possibly 12,13-dihydroxy-17-keto-9-octadecenoic acid, of which the keto group at C17 reacts with the vicinal diol group at C12 and C13 to form the bicyclic ring. We tried to detect this ketone-diol, which we expected would elute faster than THOA on GC, if present. However, no such compound was detected during the conversion of THOA (Fig. 3), suggesting that the keto-diol might be converted into the ring too quickly to be accumulated as an intermediate.

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[Received November 6, 2001; accepted January 10, 2002]