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Production and identification of a novel compound, 7,10-dihydroxy-8(*E*)-hexadecenoic acid from palmitoleic acid by *Pseudomonas aeruginosa* PR3

Jae-Han Bae · Deuk-Soo Kim · Min-Jung Suh · Sei-Ryang Oh · In-Jung Lee · Sun-Chul Kang · Ching T. Hou · Hak-Ryul Kim

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Abstract Hydroxy fatty acids are considered as important value-added product for industrial application because of their special properties such as higher viscosity and reactivity. Microbial production of the hydroxy fatty acids from various fatty acid substrates have been actively studied using several microorganisms. The new bacterial isolate *Pseudomonas aeruginosa* (PR3) had been reported to produce mono-, di-, and tri-hydroxy fatty acids from different unsaturated fatty acids. Of those, 7,10-dihydroxy-8(*E*)-octadecenoic acid (DOD) and 7,10,12-trihydroxy-8(*E*)-octadecenoic acid (TOD) were produced from oleic acid and ricinoleic acid, respectively. Based on the postulated common metabolic pathway involved in DOD and TOD formation by PR3, it was assumed that palmitoleic acid containing a singular 9-*cis* double bond, common structural property sharing with oleic acid and ricinoleic acid, could be utilized by PR3 to produce hydroxy fatty acid. In this study, we tried to use palmitoleic acid as substrate for production of hydroxy fatty acid by PR3 and firstly confirmed that PR3 could produce 7,10-dihydroxy-8(*E*)-hexadecenoic acid (DHD) with 23% yield from palmitoleic acid. DHD production was peaked at 72 h after the substrate was added to the 24-h-culture.

Keywords *Pseudomonas aeruginosa* · Palmitoleic acid · Dihydroxy fatty acid · Bioconversion

J.-H. Bae · D.-S. Kim · M.-J. Suh · H.-R. Kim (✉)
Department of Animal Science and Biotechnology,
Kyungpook National University,
Daegu 702-701, South Korea
e-mail: hakrkim@knu.ac.kr

S.-R. Oh
Natural Medicine Research Center, KRIBB,
Daejeon, South Korea

I.-J. Lee
Division of Plant Biosciences, Kyungpook National University,
Daegu, South Korea

S.-C. Kang
Department of Biotechnology, College of Engineering,
Daegu University,
Gyungsan City, South Korea

C. T. Hou
Microbial Genomic and Bioprocessing Research Unit, National Center for Agricultural Utilization Research, ARS, USDA,
Peoria, IL 61604, USA

Introduction

Hydroxy fatty acids have gained industrial interests because the hydroxyl group gives fatty acid special properties such as higher viscosity and reactivity compared with other normal fatty acids (Bagby and Calson 1989). These special chemical properties enable hydroxyl fatty acid (HFA) to have a wide range of industrial applications including resins, waxes, nylons, plastics, lubricants, cosmetics, and additives in coatings and paintings. In addition, HFAs are reported to contain antimicrobial activities against plant pathogenic fungi and some bacteria (Bajpai et al. 2004; Hou and Forman 2000; Kato et al. 1984; Shin et al. 2004).

Recently, much effort has been focused on the microbial production of the hydroxy fatty acids from various fatty acid substrates. These hydroxy fatty acids produced in microbial systems are classified into three types: monohydroxy (Hou 1994; Hou and Bagby 1992; Kim et al. 2000b), dihydroxy (Hou and Bagby 1991; Chang et al. 2006; Kim et al. 2000c), and trihydroxy fatty acids (Hou 1996; Kim et al. 2000a; Kuo et al. 2001). Among the microbial strains used for HFA

production, *Pseudomonas aeruginosa* PR3 converted linoleic acid to the equimolar mixture of 9,10,13-trihydroxy-11(*E*)-octadecenoic acid (9,10,13-THOD) and 9,12,13-trihydroxy-10(*E*)-octadecenoic acid (9,12,13-THOD) (Kim et al. 2000a). PR3 also produced 7,10-dihydroxy-8(*E*)-octadecenoic acid (DOD) from oleic acid (Hou et al. 1991) and triolein (Chang et al. 2006). Its production was improved to more than 80% yield through modifying culture conditions (Kuo et al. 2001). 10-Hydroxy-8(*E*)-octadecenoic acid was identified as an intermediate leading to formation of DOD in this bioconversion (Hou and Bagby 1992; Kim et al. 2000b). Ricinoleic acid was successfully used as a substrate to produce 7,10,12-trihydroxy-8(*E*)-octadecenoic acid (TOD) with a yield of 35% by PR3 (Kuo et al. 1998).

The bioconversion pathway involved in DOD production by PR3 from oleic acid (C18:1, 9-*cis*) containing a *cis*-double bond at carbon 9 was postulated as that oleic acid was first converted to 10(*S*)-hydroxy-8(*E*)-octadecenoic acid (HOD) during which one hydroxyl group was introduced at C10, and a double bond was shifted from C9 *cis* to C8 *trans*. The resulting plausible intermediate HOD was then subject to another hydroxylation at C7 resulting into complete formation of DOD (Kim et al. 2000b). The reaction mechanism involved in TOD production from ricinoleic acid [12-hydroxy-9(*Z*)-octadecenoic acid] was similar to that of DOD production from oleic acid except that the intermediate and final products were 10,12-dihydroxy-8(*E*)-octadecenoic acid (DHOD) and TOD, respectively, rather than HOD and DOD. This difference was originated from that ricinoleic acid contained a hydroxyl group pre-introduced at C12, and this hydroxyl group was retained during the reaction (Kim et al. 2000c). These results suggested that the enzyme system commonly involved in the production of DOD and TOD from their corresponding substrates contained substrate specificity for fatty acid carrying a singular *cis*-double bond at carbon 9. Considering the structural properties, it is possible to imagine that other fatty acids carrying a singular *cis*-double bond at carbon 9 can be utilized as a substrate for HFA production by PR3.

These augmented us to address that palmitoleic acid, fatty acid of 16 carbons carrying a *cis*-double bond at carbon 9, could be utilized as a substrate for the production of hydroxyl fatty acid by PR3. In this paper, we first report that a novel dihydroxy fatty acid was produced from palmitoleic acid by PR3.

Materials and methods

Chemicals

Palmitoleic acid (C16:1,9-*cis*), with more than 99% purity by GC, and elaidic acid (C17:0) were purchased from Nu-Chek Prep (Elysian, MN, USA). A mixture of trimethylsilylimidazole (TMSI) and pyridine was purchased from Supelco (Bellefonte, PA, USA). All other chemicals were reagent grade and were used without further purification. Thin-layer precoated Kieselgel $60F_{254}$ plates were obtained from EM Science (Cherry Hill, NJ, USA). Other chemicals were purchased from Sigma Chemical (St Louis, MO, USA), unless mentioned otherwise.

Microorganism and bioconversion

P. aeruginosa NRRL strain B-18,602 (PR3) was kindly provided by Dr. Hou of United States Department of Agriculture/National Center for Agricultural Utilization Research (USDA/NCAUR). The strain was aerobically grown at 28°C with shaking at 200 rpm in a 125-ml Erlenmeyer flask containing 50 ml of standard medium. The standard medium used hereafter contained (per liter) 4 g dextrose, 2 g K_2HPO_4, 2 g $(NH_4)_2HPO_4$, 1 g NH_4NO_3, 0.5 g yeast extract, 0.014 g $ZnSO_4$, 0.01 g $FeSO_4{\cdot}7H_2O$, and 0.01 g $MnSO_4{\cdot}7H_2O$. The medium was adjusted to pH 7.0 with diluted phosphoric acid. For the production of hydroxy fatty acids, palmitoleic acid substrate (0.3 g) was added to the 24-h-old culture, followed by additional incubation for 72 h. At the end of cultivation, the culture broth was acidified to pH 2 with 6 N HCl, followed by immediate extraction twice with an equal volume of ethyl acetate and diethyl ether. The solvent was evaporated from the combined extracts with a rotary evaporator. The values presented in time-coursed production of target compound were the average of duplicate. The error range was within 10% of average value.

Analysis of products

The extracted reaction products were analyzed by thin-layer chromatography (TLC) and gas chromatography (GC). The TLC was developed with a solvent system consisting of toluene is to dioxane is to acetic acid (79:14:7 v/v/v). Spots were visualized first by iodine vapor and then by spraying the plate with 50% sulfuric acid and heating in a 100°C oven for 10 min. For GC analysis, the samples were first methylated with diazomethane for 5 min at room temperature, followed by derivatization with a mixture of TMSI and pyridine (1:4 v/v) for at least 20 min at room temperature. The TMS-derivatized sample was analyzed with Shimadzu GC-17A (Shimadzu Seisakusho, Kyoto, Japan) equipped with a flame-ionization detector and a capillary column (SPB-1™, 15 m×0.32 mm i.d., 0.25 μm thickness from Supelco). GC was run with a temperature gradient of 20°C/min from 70 to 200°C, holding for 1 min at 200°C, and then 0.7°C/min to 240°C, followed by holding for 15 min at 240°C (nitrogen flow rate = 0.67 ml/min).

Injector and detector temperatures were held at 250 and 270°C, respectively. Elaidic acid (C17:0) was used as an internal standard for quantification. For purification of a single product from crude extract, the extract was fractionated by column chromatography (1.2 cm i.d. ×25 cm length) packed with silica gel 60. Elutions were carried out sequentially using 50 ml of hexane, 50 ml hexane/ethylacetate (80:20 v/v), 50 ml hexane/ethylacetate (50:50 v/v), and 100 ml chloroform/methanol (50:50 v/v). Most of the target product (>95%) were eluted from the chloroform/methanol (50:50 v/v) fraction.

Chemical structure of the purified target product was determined through GC/mass spectrometry (GC/MS), nuclear magnetic resonance (NMR), and Fourier transform infrared (FTIR) spectroscopy. Electron-impact (EI) mass spectra were obtained with a Hewlett Packard (Avondale, PA, USA) 5890 GC coupled to a Hewlett Packard 5972 Series Mass Selective Detector. The column outlet was connected directly to the ion source. Separation was carried out in a methylsilicone column (30 m×0.25 mm i.d., 0.25 μm film thickness) with a temperature gradient of 20°C/min from 70 to 170°C, holding for 1 min at 170°C and 5°C/min up to 250°C, followed by holding for 15 min (helium flow rate = 0.67 ml/min). Trimethylsilyl (TMS) derivatives for GC/MS analysis of the purified compound pre-methylated with diazomethane were prepared as mentioned above. Proton and ^{13}C NMR spectra were determined in deuterated chloroform with a Varian-500 spectrometer (Billerica, MA, USA), operated at a frequency of 400 and 100 MHz, respectively. FTIR analysis of the free acid product was run as films on KBr on a Perkin Elmer Infrared Fourier Transform Model 1750 spectrometer (Perkin Elmer, Oakbrook, IL, USA).

Results

Production and isolation of products

P. aeruginosa PR3 converted palmitoleic acid to a mixture of several products including one major peak with GC retention time mostly being within 10 min (Fig. 1). This major product was isolated from the chloroform/methanol (50:50 v/v) fraction of the column chromatography. The purified product was identified as a single major peak (>95%) by GC and revealed as one major spot on TLC analysis (Fig. 2).

Structure determination

The EI GC/MS data of TMS derivative of the methylated product are given in Fig. 3. This was consistent with the TMS derivative of a methylated C16 dihydroxy monoenoic

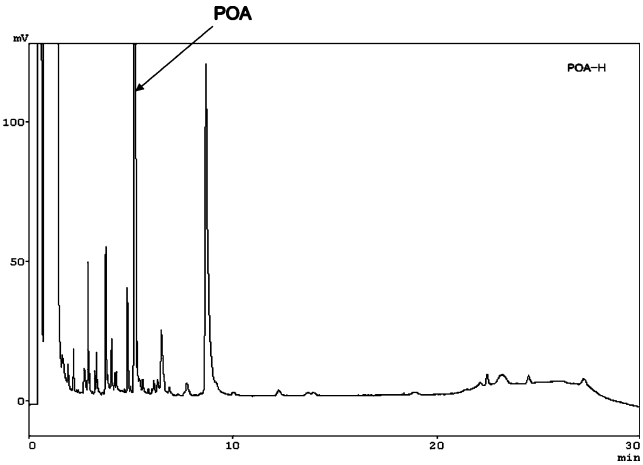

Fig. 1 Gas chromatogram of the crude extract produced from the bioconversion of palmitoleic acid by PR3. Reaction and GC running conditions are explained in the Materials and methods section

fatty acid with a molecular mass of 444. The locations of hydroxyl group were apparent from the fragments observed in the EI spectrum of the TMS derivative of the methylated fatty acid. The intense fragment arising from the alpha cleavage to the derivatized hydroxyl group toward the methyl end gave fragments containing TMS at m/z 187 and both two TMS and a double bond at 315 m/z. Other two intense fragments arising from alpha cleavage to the derivatized hydroxyl group toward the methylated carboxyl end were observed at 231 m/z containing TMS and at 359 m/z containing both two TMS and a double bond. These fragments allocated the hydroxyl groups at C7 and C10 and a double bond at C8–9.

Fig. 2 Thin-layer chromatography of the crude extract and purified product from the bioconversion of palmitoleic acid by PR3. *lane 1* palmitoleic acid, *lane 2* crude reaction products, *lane 3* purified product. Running and developing conditions are given in the Materials and methods section

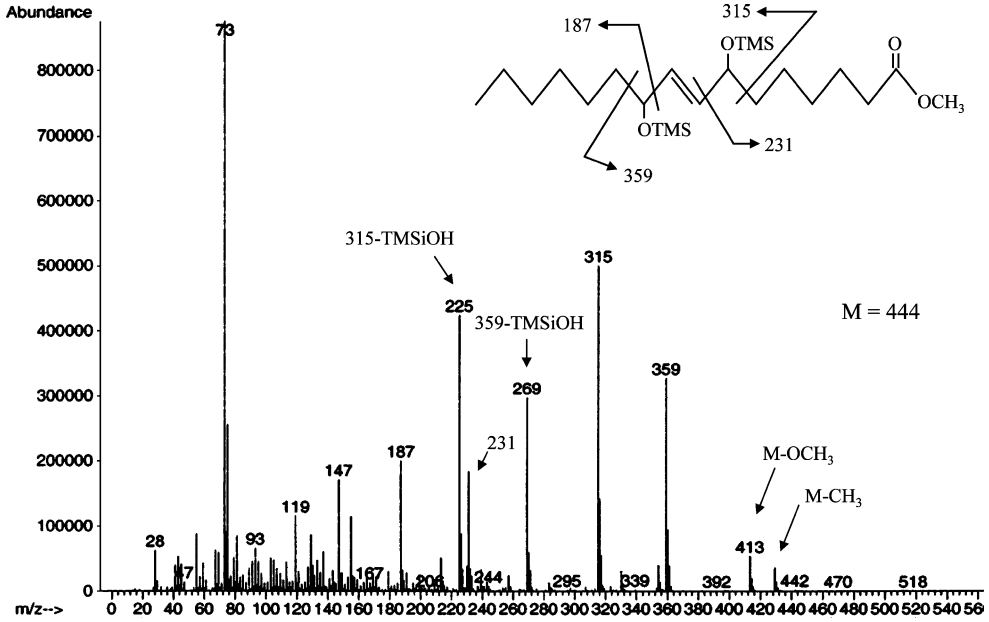

Fig. 3 Electron-impact mass spectrum of TMS derivative of the methylated purified product. Conditions for compound separation are explained in the Materials and methods section

FTIR analysis The purified product was subjected to infrared (IR) analysis (Fig. 4). The presence of hydroxyl group was indicated by the broad strong IR absorption at 3,400 cm^{-1} region. Absorption observed at 1,710 cm^{-1} represented carbonyl group and the absorption at 970 cm^{-1} indicated that the unsaturation was *trans* (Kim et al. 2000b).

NMR analysis The purified free acid was subjected to proton and ^{13}C NMR analysis to conform the elucidated structure of the purified sample. Resonance signals (ppm) and corresponding molecular assignments are given in Table 1. Resonance signal of olefinic protons (–CH=CH–) obtained from proton NMR was observed at 5.63 ppm with coupling constant of 15.4 Hz strongly indicating *trans*-configuration across the double bond. Two tertiary protons (–CH–O–) were observed at 4.08 ppm. Because of the downfield shift, compared to alcohols adjacent to saturated carbons and the doublet of triplet multiplicity, the hydroxyl-bearing carbons were suggested to be vicinal to the double bond. ^{13}C NMR confirmed the presence of the following carbons: carbonyl carbon at around 178.7 ppm (C1), a double bond between 133.67 (C8) and 133.95 (C9), two –CHOH– carbons at 72.35 ppm (C7) and at 72.54 (C10). Other protons and carbons were as shown in the table. The data obtained from GC/MS, FTIR, and proton and ^{13}C NMR confirmed that the purified compound was 7,10-dihydroxy-8 (*E*)-hexadecenoic acid (DHD).

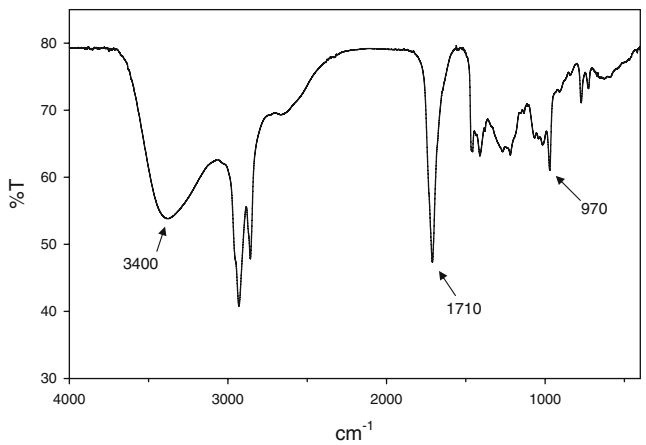

Fig. 4 FTIR analysis of the purified DHD. Analytical conditions are explained in the Materials and methods section

Table 1 Proton and ^{13}C nuclear magnetic resonance signals and molecular assignments for DHD[a]

Carbon number	Resonance signal (ppm)	
	^{1}H	^{13}C
1		178.70
2	2.32	33.89
3	1.62	25.37
4	1.42	29.16
5	1.42	24.54
6	1.70	36.67
7	4.08	72.35
8	5.63 ($J_{8,9}$ = 15.4 Hz)	133.67
9	5.45	133.95
10	4.08	72.54
11	1.70	37.01
12	1.26	24.85
13	1.26	28.79
14	1.26	31.79
15	1.26	22.57
16	0.87	14.05

[a] *DHD* 7,10-dihydroxy-8(*E*)-hexadecenoic acid

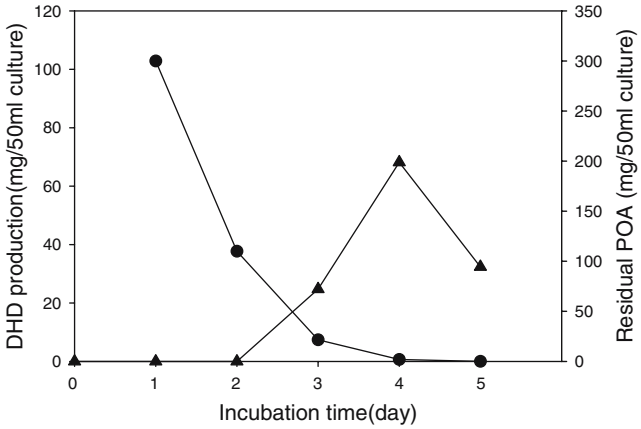

Fig. 5 Time-coursed production of DHD from palmitoleic acid by PR3. Palmitoleic acid (300 mg) was added to the culture at 24 h after incubation started. *Circles* and *triangles* represented the concentration of residual palmitoleic acid (POA) and DHD production, respectively. See the Materials and methods section for other reaction conditions

Time-coursed production of DHD from palmitoleic acid by PR3

The time course study of DHD production was carried out for the time specified after palmitoleic acid was added to the 24-h-old culture. Production of DHD was time dependently increased and peaked at 72 h after the addition of palmitoleic acid as substrate, after which production was decreased (Fig. 5). Concentration of residual palmitoleic acid decreased immediately after addition of substrate.

Discussion

Structural analysis of the major product produced from palmitoleic acid by *P. aeruginosa* PR3 confirmed that strain PR3 could introduce two hydroxyl groups on carbons 7 and 9 with shifted migration of 9-*cis* double bond into 8-*trans* configuration resulting in the formation of DHD (Fig. 6).

There was very limited trial to use palmitoleic acid as substrate for microbial conversion. *Flavobacterium* sp. DS5 produced 10-keto and 10-hydroxy products from palmitoleic acid (Hou 1995) and a filamentous fungus *Trichomonas* sp AM076 was reported to convert palmitoleic acid to a small amount of 9,12-hexadecadienoic acid (Shirasata et al. 1998). However, there was no identified dihydroxy fatty acid product from those microorganisms.

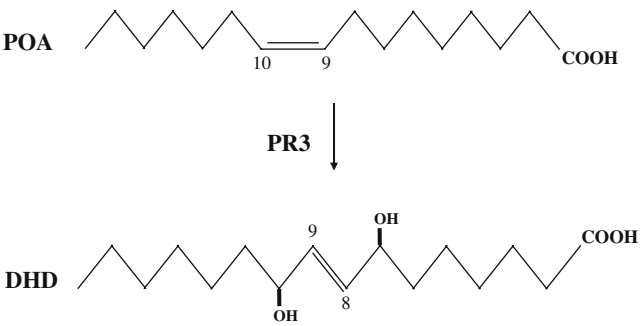

Fig. 6 Postulated bioconversion pathway of palmitoleic acid leading to DHD by strain PR3. POA and DHD represented palmitoleic acid and 7,10-dihydroxy-8(*E*)-hexadecenoic acid, respectively

Production yield (23%) of DHD from palmitoleic acid was relatively low compared to those of DOD (70%) from oleic acid (Chang et al. 2006) and TOD (45%) from ricinoleic acid (Kuo et al. 2001). This result strongly suggested that strain PR3 could further metabolize DHD as carbon and energy source because DHD production was relatively low and peaked at 72 h (Fig. 5) and concentration of residual palmitoleic acid decreased steeply within 24 h after it was added to the culture. This was also confirmed by the fact that microbial growth, supplemented with palmitoleic acid, continued further, but not with control (data not shown). However, it was not clear whether rapid consumption of palmitoleic acid was caused only through formation of DHD or not.

The postulated bioconversion pathway of palmitoleic acid leading to DHD was very similar to those of DOD production from oleic acid (Kim et al. 2000b) and TOD from ricinoleic acid (Kim et al. 2000c). All pathways included introduction of two hydroxyl groups next to 8-*trans* double bond migrated from 9-*cis* configuration. These results strongly suggested that enzyme system involved in DHD production from palmitoleic acid was the same as those for DOD and TOD production from their corresponding substrates. Based on these results, it was assumed that substrate specificity of this enzyme system could be attributed to the location of *cis* double bond in the fatty acid, although this fact should be verified from further detailed enzymatic study. In conclusion, *P. aeruginosa* PR3 was able to convert palmitoleic acid to 7,10-dihydroxy-8 (E)-hexadecenoic acid with 23% yield possibly using common enzyme system involved in DOD and TOD production. Optimization of environmental conditions for DHD production and industrial uses of DHD are currently under investigation.

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