Production of 15-, 16- and 17-Hydroxy-9-Octadecenoic Acids by Bioconversion of Oleic Acid with *Bacillus pumilus*

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Several *Bacillus* strains were tested for their ability to hydroxylate oleic acid. Two strains—BD-174 and BD-226—converted oleic acid into a trio of hydroxy-octadecenoic acids. Bioconversion in 72–120 hr produced 5–11% of hydroxy acids relative to oleic acid as measured by gas chromatography. These acids were identified as the 15-, 16- and 17-hydroxy-9-octadecenoic acids by gas chromatography-mass spectrometry of trimethyl silyl derivatives of the product acids and their hydrogenated counterparts.

KEY WORDS: *Bacillus megaterium, Bacillus pumilus*, bioconversion, hydroxylation, oleic acid.

Certain vegetable oils, *e.g.*, castor and tung oils, have wide usage in industrial products because they contain fatty acids with unique chemical and physical properties. These oils command a premium price of two to five times that of soybean oil, a surplus agricultural commodity in the United States with an annual carry-over of 1.2 billion pounds. Soybean and other vegetable oils are either too viscous or too reactive toward atmospheric oxygen for applications in cosmetics, lubricants and chemical additives. For other uses, including coatings, detergents, polymers and certain additives and lubricants, reactivity of vegetable oils needs to be enhanced by introducing additional functionalities into the fatty acid molecules.

Molecular modifications may be accomplished either chemically or enzymatically. Chemical reactions are often run under severe conditions and produce random or mixed products. Reactions catalyzed by enzymes frequently can be carried out with better selectivity and under more moderate conditions. Enzymatic modification of surplus soybean or other vegetable oils or their fatty acids may provide products with potential industrial applications.

Soda (1) reported that a strain of soil bacterium converted oleic acid (*cis*-9-octadecenoic acid) to ricinoleic acid (12-hydroxy-*cis*-9-octadecenoic acid). Soda and Kido (2) have recently received a patent on this process. Various strains of *Bacillus pumilus* and *Bacillus megaterium* from the Agricultural Research Service (ARS) Culture Collection were evaluated for similar bioconversion activity. Two *B. pumilus* strains (BD-174 and BD-226) converted oleic acid to monoenoic hydroxy acids in more than trace amounts. These products were not the expected ricinoleic acid, but three isomeric hydroxy acids with the hydroxy group closer to the terminal end of the fatty acid.

MATERIALS AND METHODS

Microorganisms. Forty-seven *Bacillus* strains (26 *B. pumilus* and 21 *B. megaterium*) from the ARS Culture Collection were screened for their ability to hydroxylate oleic acid. Cultures were grown aerobically at 32°C in 50 mL of media in 125-mL Erlenmeyer flasks shaken at 150 rpm. Medium composition was (per liter): proteose peptone No. 3 (Difco Laboratories, Detroit, MI), 7.5 g; tryptone, 7.5 g; KH_2PO_4, 2 g; K_2HPO_4, 2 g; NaCl, 5 g; $MgSO_4{\cdot}7H_2O$, 0.1 g; dextrose, 5 g; yeast extract, 1 g; and beef extract, 0.1 g. The medium pH was adjusted to 7.2 with NaOH. Cell growth was monitored by optical density (OD) measurements taken at hourly intervals for 9 hr with a Beckman DU-70 Spectrophotometer (Beckman Instruments, Inc., Fullerton, CA). One-milliliter aliquots were aseptically withdrawn from the growing cultures at the designated times and diluted to 10 mL with fresh growth medium prior to OD measurement at 600 nm.

Bioconversion reaction. Oleic acid (0.28 mL, 0.5%) (Nu Chek Prep, Inc., Elysian, MN; 99+% purity) was added to 16- or 40-hour-old cultures and incubated at 32°C and 150 rpm. Aliquots were taken in preliminary experiments at 48, 72 and 96 hr after oleic acid addition to assess the extent of conversion. At the end of incubation, cultures were acidified to pH 2 with 9N sulfuric acid and extracted twice with equal volumes of diethyl ether. The combined ether fractions were washed with water and dried over anhydrous sodium sulfate. After filtering to remove the sodium sulfate, the solvent was removed with a rotary evaporator.

Analysis of products. Bioconversion was monitored by gas chromatography (GC) and thin-layer chromatography (TLC). Extracted lipids were esterified with diazomethane and then injected into a Hewlett Packard model 5890 gas chromatograph (Hewlett Packard, Palo Alto, CA) equipped with a flame ionization detector, a Supelco SPB-1 capillary column (15 m × 0.25 mm id; 1 μm thickness; 1 mL/min helium flow; Supelco, Inc., Bellefonte, PA), and a Hewlett Packard 3396A integrator. GC runs were isothermal at 230°C. Yields were calculated from GC percentages relative to palmitic acid, which was added to the broth as an internal standard before extraction.

TLC analyses were performed on 5 cm × 20 cm Silica Gel 60 (0.25 mm thickness) plates (EM Science, Cherry Hill, NJ) developed in toluene/dioxane/acetic acid (79:14:7, v/v/v). The chromatograms were visualized with sulfuric acid spray and charring by means of a heat gun.

Product identification. Products were identified by GC-mass spectrometry (GC-MS), Fourier transform infrared (FTIR) and ozonolysis. GC-MS was performed under the following conditions: HP 5890 GC, 15 m × 0.25 mm DB-1 capillary column; splitless injection at 120°C with a 2 min hold; temperature programmed at 20°C/min to 200°C and then 10°C/min to 270°C. The mass spectrometer was an HP 5970 with a mass-selective detector. Trimethylsilyl (TMS) derivatives were prepared by standard techniques with Trisil-TBT reagent (Pierce Chemical Co., Rockford, IL) (3).

After conversion to methyl esters with 5% HCl-MeOH, individual hydroxy acids were isolated by silica high-performance liquid chromatography (HPLC) with 20% ethyl ether in hexane as effluent. The HPLC system consisted of a Spectra Physics (San Jose, CA) 8800 solvent

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delivery system, an Econosphere 250 mm × 4.6 mm id silica column (5 μ), and a Kratos Spectroflow 757 UV detector (Kratos, Ramsey, NJ). Solvent flow was 1 mL/min, and UV detection was at 215 nm.

Double bond position was determined by reductive ozonolysis (4) and GC-MS of the products. FTIR spectra of the hydroxy methyl esters in CS_2 were obtained with a Perkin-Elmer Infrared Fourier Transform Model 1750 Spectrometer (Perkin-Elmer, Inc., Oak Brook, IL) and were used to assess the configuration of the double bond and to further confirm the presence of hydroxyl groups.

RESULTS AND DISCUSSION

During the screening of *Bacillus* for hydroxylating capability, only two strains of the 47 cultured, BD-174 and BD-226, converted oleic acid to hydroxy acids in more than trace amounts (> 1%). In initial experiments, substrate was added at 40 hr after inoculation. Subsequent growth curves for the two *B. pumilus* strains having hydroxylating potential indicated that maximum cell density was reached between 9 and 10 hr for BD-174 (OD, 0.37) and between 8 and 9 hr for BD-226 (OD, 0.32). Oleic acid, then, was added to the culture medium after overnight growth (16 hr) in subsequent experiments. Under conditions of this study, *B. pumilus* substituted hydroxyl groups at the first, second or third carbon atoms away from the terminal methyl group of oleic acid to produce 15-, 16- and 17-hydroxy-9-octadecenoic acids. Hydroxylation did not occur by hydrolysis at the double bond of oleic acid, at the terminal position, or at the C12 position to produce ricinoleic acid.

Yields were determined both by total recovery weights and by GC percentages, with palmitic acid as an internal standard (Table 1). Average recovery of ethyl ether-soluble material from the culture broths incubated from 0 to 5 days after addition of oleic acid was greater than 85% (186–245 mg) of the substrate weight added. Lowest recovery was 74% in the five-day sample, possibly due to oxidation and/or catabolism of the substrate. Recoveries calculated from the palmitic acid internal standard were lower than the weighed values. Differences range from +1 to −14%, with an average difference of −6%. These differences may be due to ether-soluble material that does not elute from the GC column even at elevated temperatures. Peaks other than oleic and these hydroxy acids were less than 3% of the total GC area. The relative percent conversion in individual cultures of oleic acid to the three hydroxy acids determined by GC reached between 5 and 11% from 72 to 120 hr after substrate was added. The reason for the smaller percent conversion by BD-226 at 120 hr is not known. Aliquots taken during extended incubations (up to 12 days) contained hydroxy acids in relative yields greater than 20% in several experiments, but with subsequent transfers of the cultures, this activity has not been sustained.

The three underivatized hydroxy methyl esters were not completely resolved by the GC conditions used. TMS derivatives, however, separated into three peaks and gave relative amounts of 1.5:1:5.1 for the 15-, 16- and 17-hydroxyoctadecenoic acids, respectively.

The position of the hydroxyl groups was determined by GC-MS of the TMS derivatives of the products and of the catalytically reduced saturated hydroxy acids. Mass spectra show prominent ions from α-cleavage of the molecular backbone on both sides of the TMS group and a rearrangement ion (5). For the 15-isomer, these fragments were *m/z* 341, 312 and 145; for the 16-isomer, *m/z* 355, 326 and 131; and for the 17-isomer, *m/z* 369, 340 and 117 (Fig. 1). GC-MS of the ozonization fragments identified methyl azelaldehydate, thus confirming a Δ9 unsaturation. Fragmentation patterns consistent with the expected molecular structure of the corresponding hydroxy-aldehyde products also were observed. The absence of a band at 10.36 μ in the FTIR spectrum of the methyl ester of the hydroxy acid indicates that the double bond retains the *cis* configuration during hydroxylation. The broad band near 3 μ confirmed the presence of the hydroxyl moiety.

As far as we can determine, this is the only hydroxylation at these positions reported in the literature for this species of *Bacillus*. Hydroxylation of fatty acids at ω2, ω3 and ω4 was described for a cell-free enzyme system isolated from *B. megaterium* (6,7). This system hydroxylated both n-saturated and monounsaturated fatty acids at the three carbon atoms adjacent to the terminal methyl group. No hydroxylation of the terminal carbon was reported, and the most abundantly hydroxylated position was that of two carbons removed from the methyl terminus. Similarly, we found no 18-hydroxy-octadecenoic acid. However, the most abundant hydroxylation product from incubation of these two *B. pumilus* strains with oleic acid was 17-hydroxyoctadecenoic acid.

TABLE 1

Lipid Recovery and Relative Percent Conversion of Oleic Acid to Hydroxy Acids by BD-174 and BD-226[a]

Hours after substrate added	Weight (mg)				Percent hydroxy acids	
	By balance		By internal standard			
	BD-174	BD-226	BD-174	BD-226	BD-174	BD-226
0	209	215	212	218	—	—
24	222	241	222	219	2.6	0
48	245	232	213	224	8.4	3.0
96	221	212	200	194	9.4	6.5
120	186	205	168	175	9.0	3.9

[a]Average of duplicate runs.

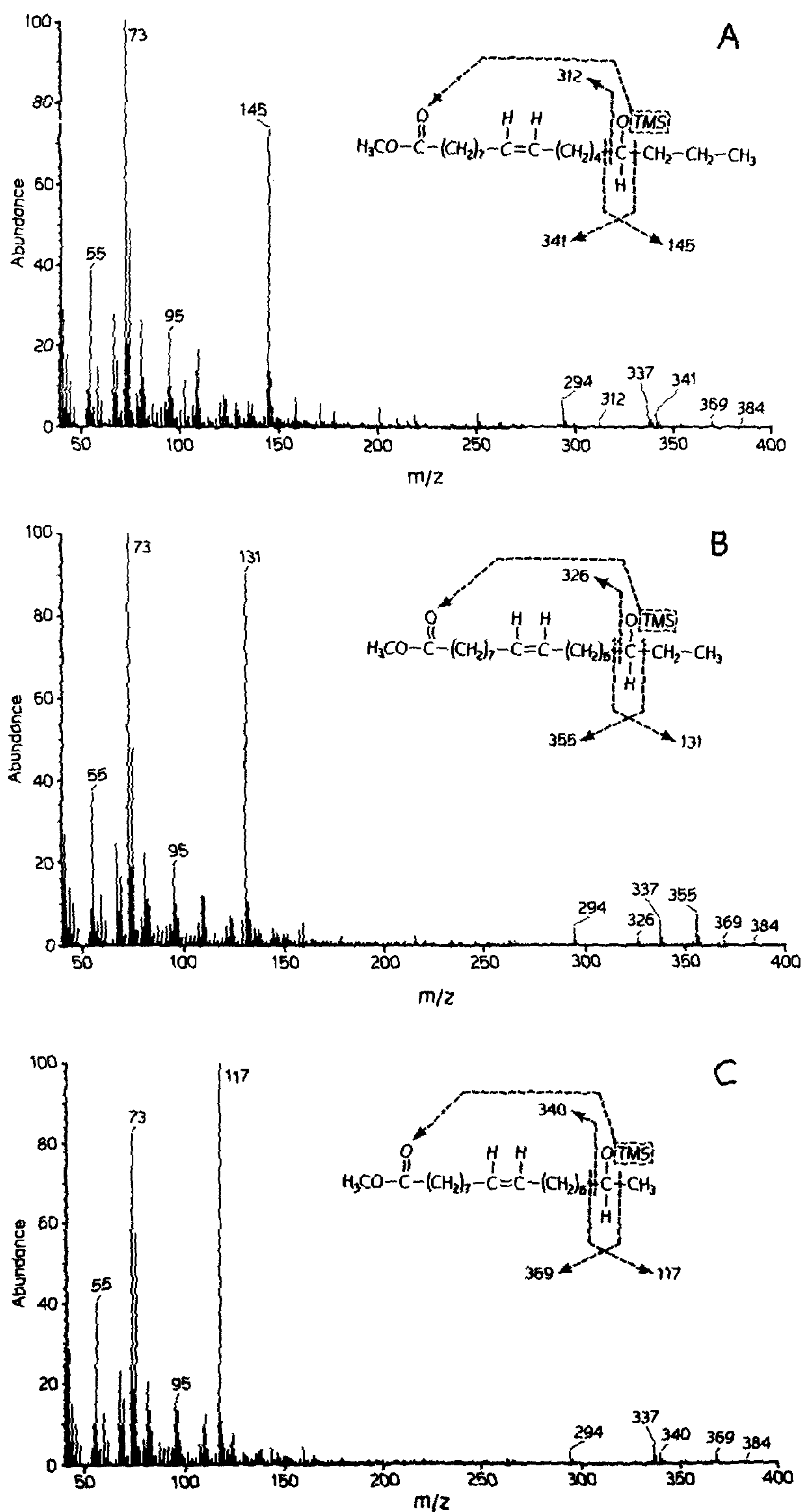

FIG. 1. Mass spectra of TMS derivatives of 15- (A), 16- (B) and 17- (C) hydroxyoctadecenoic acids with fragmentation patterns corresponding to identifying ions.

The mechanism proposed for the *B. megaterium* system, an enzyme-substrate complex with binding sites effectively rendering both end carbons unreactive and activating the 15-, 16- and 17-carbon sites (7), also would be compatible with the enzyme system studied here. The reason for the difference in enzyme specificity for producing more 16- or 17-hydroxy acid between the two *Bacillus* species is not known. Elucidation of the mechanism and isolation of the enzyme(s) responsible for these hydroxylations may provide the ability to do site-directed syntheses. Biological introduction of a hydroxyl group at the 15-, 16- or 17-position of oleic acid while maintaining the double bond moiety provides an additional reactive site, which may be exploited to yield specialty products, value-added products or chemical and enzymatic intermediates for further processing into industrially useful materials, *e.g.*, polyesters for coatings or macrolactones for odor compounds.

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