Conversion of Oleic Acid to 10-Ketostearic Acid by a *Staphylococcus* Species[1]

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In the course of using microorganisms to produce value-added products from soybean oil or its fatty acids, a culture contaminant was isolated and tentatively identified as a *Staphylococcus* species. This microorganism converts oleic acid (*cis*-9-octadecenoic acid) to 10-ketostearic acid (10-keto-octadecanoic acid) in growing cultures. Bioconversion was studied in two different media at temperatures from 30 to 41°C, with shaking at 150 rpm. Cells were grown in 50 mL of media for 24 h and then incubated with 0.25 g oleic acid for 24 h. Optimum conditions have allowed better than 90% conversion with 85% recovery. Unreacted oleic acid and 10-hydroxystearic acid (10-hydroxy-octadecanoic acid) are the only other compounds present in the ethyl ether extract of the fermentation broth.

KEY WORDS: Bioconversion, oleic acid, 10-ketostearic acid, *Staphylococcus*.

Imported 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, therefore, command a premium price compared to major vegetable oils grown in the United States, *e.g.*, soybean oil. These major domestic oils have properties that cause them to be either too reactive toward atmospheric oxygen or not reactive enough for direct use in some industrial processes.

Molecular modifications, accomplished either chemically or enzymatically, may be useful in enhancing the reactivity of the vegetable oil by addition of functional groups into the fatty acid molecules. Chemical reactions often 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.

During experiments in which strains of *Bacillus pumilus* were being examined for their ability to hydroxylate oleic acid (*cis*-9-octadecenoic acid) (1), a culture contaminant was found which converted oleic acid to a different oxo-product. This microorganism was isolated on tryptone/glucose/yeast extract (TGY) agar plates and tentatively identified as a *Staphylococcus* species. This paper describes bioconversion of oleic acid to a more polar oxo-acid in growing cultures with yields greater than 90% and identification of that product as 10-ketostearic acid (10-keto-octadecanoic acid).

MATERIALS AND METHODS

Microorganism and media. The microorganism used in these experiments was a contaminant in a process using *B. pumilus* to hydroxylate oleic acid. The contaminated culture was streaked onto TGY (2) agar plates and incubated at 29°C for 24–48 h to isolate the organism. Isolated colonies were used to inoculate fresh TGY broth and were incubated at 29°C for 24–48 h. To verify purity, the broth culture was streaked onto TGY agar plates. Uniformity of colonial morphology of the streaked culture confirmed the purity of the isolate. An isolated colony was transferred to TGY agar slants, incubated at 29°C for 24–48 h and stored at 4°C. Microscopy showed the isolate to be a coccus. Therefore, for its identification, the isolate was characterized by the method of Kloos *et al.* (3).

Bioconversion experiments were run in TGY media and in the media used in the *B. pumilus* experiments (1), which consisted of 0.75% Difco proteose peptone No. 3 (Difco Labs, Detroit, MI), 0.75% tryptone, 0.2% KH_2PO_4, 0.2% K_2HPO_4, 0.5% NaCl, 0.01% $MgSO_4 \cdot 7H_2O$, 0.01% yeast extract, 0.01% beef extract and 0.1% dextrose at pH 7.2 (medium "B").

Growth curves for this microorganism were measured in medium "B". One-mL aliquots were aseptically removed at hourly intervals, diluted to 10 mL with fresh media, and optical densities at 600 nm were measured with a Beckman DU-70 Spectrophotometer (Beckman Instruments, Inc., Fullerton, CA).

Cultures were grown aerobically in 50 mL of media in 125-mL Erlenmeyer flasks shaken at 150 rpm at temperatures between 30–41°C. Oleic acid (0.28 mL, 0.5%; Nu-Chek-Prep, Inc., Elysian, MN; 99+% purity) was added to 24-h-old cultures and incubated for an additional 24 h. At the end of incubation, the broth was acidified to pH 2 with 9 N 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) as described previously (1). Hexane/ethyl ether/acetic acid (50:50:1, vol/vol/vol) was the TLC solvent system.

Products were identified through GC-mass spectrometry (GC-MS), nuclear magnetic resonance (NMR) spectroscopy, Fourier transform infrared (FTIR) and melting point. GC-MS was performed under the following conditions: Hewlett Packard (HP) model 5890 GC (Hewlett Packard, Palo Alto, CA) equipped with a 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 MS was an HP 5970 with a mass selective detector.

High-performance liquid chromatography (HPLC) was performed with a Spectra-Physics SP8800 solvent delivery system (Spectra-Physics, San Jose, CA) to isolate pure material for further identification. A Dynamax 60-A silica column (250 mm × 21.4 mm i.d.) (Rainin Instrument Co., Inc., Woburn, MA) was used with the following timed solvent elution program: hexane/acetone (97:3, vol/vol) for 8

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min to 50:50 hexane/acetone at 20 min to 10:90 hexane/acetone at 30 min. Column flow was 6 mL/min with a 15% split to a Varex evaporative light scattering detector II (Varex Corporation, Rockville, MD) with a heater set at 105°C and 60 cc/min nitrogen flow through the nebulizer.

FTIR analysis of the methyl ester was run on a Perkin Elmer infrared Fourier transform Model 1750 spectrometer (Perkin Elmer, Inc., Oak Brook, IL) to confirm the presence of a ketone. Proton and ^{13}C NMR spectra were determined on the fatty acid dissolved in deuterated chloroform with a Bruker WM-300 spectrometer (Rheims, Tepten, Germany) operating at 300 MHz and 75.5 MHz, respectively.

RESULTS AND DISCUSSION

Anaerobic growth, inhibition of growth by furazolidone and lyphostatin, and sensitivity to novobiocin suggest strongly that the isolate is a species of *Staphylococcus* (4). Other biochemical and physiological characteristics suggest that the isolate belongs in the *Staphylococcus epidermidis* or in the *S. simulans* species groups (4). Further studies will be necessary to make an exact identification.

Growth curves for this microorganism at 31–32°C and 150 rpm run in three flasks indicated that maximum growth (beginning of the stationary phase) was reached at about 24 h (Fig. 1), yielding approximately 60 mg dry weight of cells from 50 mL media. Oleic acid was added to the fermentation broth at this steady-state. Bioconversion of oleic acid to a more polar product was accomplished after an additional 24 h, and continued incubation did not substantially increase yield. A white solid was obtained after extraction and solvent removal.

Structure determination. The electron impact spectrum of the methyl ester prepared with diazomethane gave a molecular ion of *m/z* 312 and an M − 31 ion at *m/z* 281 corresponding to loss of the methoxy group. Ions formed from α- or β-cleavage with respect to the oxo-group give characteristic fragmentation patterns that provide sufficient information to determine the position of the oxo-group (5). Large fragments corresponding to α-cleavage with ions *m/z* 199, 171 and 141, and to β-cleavage with ions *m/z* 214 (213 + H) and 156 (155 + H) place the keto group at the C-10 position.

Partial purification of the bioconversion product was accomplished during storage of an ethyl ether solution at refrigerator temperature. The cold liquid was poured off the white crystals that had precipitated. Final HPLC purification was performed to yield pure product according to GC analysis. Melting point of the white solid acid was 81–82.2°C, which is slightly less than the melting point for 10-ketostearic acid reported in the literature (82.5–83.4°C) (6).

FTIR of methyl ester solutions run in a 0.2-mm NaCl liquid cell showed absorption for the ester carbonyl at 1741 cm^{-1} in CS_2 and at 1742 cm^{-1} in CCl_4 and for the ketone carbonyl at 1716 cm^{-1} (CS_2) and 1718 cm^{-1} (CCl_4) (7). Proton and ^{13}C NMR further confirmed the identity of the bioconversion product. Resonance signals (ppm) and corresponding molecular assignments are given in Table 1.

Takatori *et al.* (8) investigated several authentic microorganisms, including *Micrococcus luteus, S. aureus* and *S. saprophyticus,* for their ability to convert oleic acid as a substrate to hydroxy and/or oxo fatty acids. Medium (thioglycollate), substrate concentration, incubation temperature (25–28°C) and time (7 d) were all different from those reported here. *M. luteus* was the only microorganism tested that could effect the conversion to an oxo-product. Under their conditions, the relative conversion of oleic acid to products was 84.5%, but 61.3% was 10-hydroxystearic acid and 23.2% was 10-oxo-stearic acid. In a second study, with brain-heart infusion-broth medium (9), 73.1% conversion was obtained, of which only 16.8% was 10-oxo fatty acid.

The bioconversion reported here is efficient in medium "B" and has produced 10-ketostearic acid in relative yields of better than 90% with 85% recovery over a range of temperatures from 30 to 38°C (Fig. 2). The bioconversion of oleic acid to 10-ketostearic acid was usually below 50% in TGY media over this range of temperatures, which is similar to the results reported by Takatori *et al.* (8,9). In

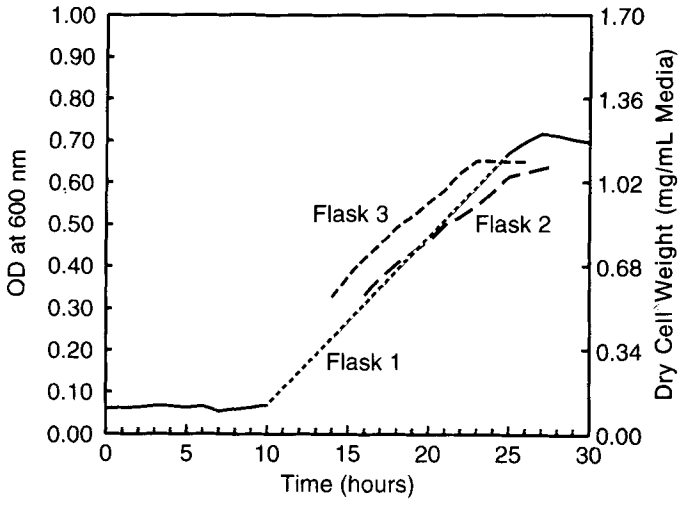

FIG. 1. Microorganism growth at 31–32°C and 150 rpm in medium "B". OD, optical densities.

TABLE 1

Proton and ^{13}C Nuclear Magnetic Resonance (NMR) Signals and Molecular Assignments for Bioconversion Product

NMR type	Resonance signal (ppm)	Hydrogens	Assignment
Proton	2.29–2.38	6	CH_2, α to carbonyls
	1.50–1.62	6	CH_2, β to carbonyls
	1.23–1.26	18	CH_2, C4-7, C13-17
	0.83–0.86	3	CH_3, terminal
^{13}C	14.06		C18
	22.61		C17
	23.78–23.87		C8, 12
	24.61		C3
	28.95–29.34		C4-7, C13-15
	31.79		C16
	34.03		C2
	42.72–42.80		C9, 11
	179.90		C1
	211.79		C10

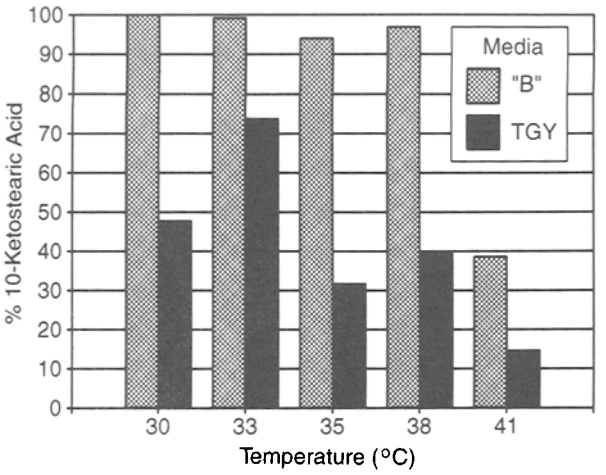

FIG. 2. Bioconversion of oleic acid to 10-ketostearic acid at various temperatures in "B" and tryptone/glucose/yeast extract (TGY) media.

both media, 10-hydroxystearic acid was less than 5% of the conversion products.

Addition of reactive functionalities to vegetable oil fatty acids may create new uses for a surplus U.S. agricultural commodity in nonfood industrial applications. Plasticizer, lubricant or detergent industries may find use for this 10-ketostearic acid as a precursor. Patents (10,11) have been issued describing use of keto-acid derivatives as grease thickeners.

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