Microbiological Transformations of Lipids: Acyl-Specific Hydrolysis of Lard by Yeasts

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The fatty acid and positional specificities of Saccharomyces cerevisiae (UI-SACCH) and Schizosaccharomyces octosporus (NRRL Y-854) in the hydrolysis of lard were studied by using gas-liquid chromatography. Synthetic triglycerides were used to determine the positional specificities of the lipases of both organisms. Palmitic acid is specifically cleaved from all three triglyceride ester positions by S. cerevisiae, while S. octosporus was able to cleave stearic acid at either position 1 or position 3 of the glycerol moiety. Preparative scale fermentation with 200 g of lard per liter yielded 48.4 g of palmitic acid per liter with S. cerevisiae and 42 g of stearic acid per liter with S. octosporus. The free fatty acids produced by microbial transformation of lard were characterized spectrally (H and C nuclear magnetic resonance and mass spectrometry) and chromatographically (thin-layer and gas chromatographies).

Lipids, in the forms of vegetable oils and animal fats, are abundantly available and renewable resources for microbial and enzymatic conversions to useful products. We are exploring the use of microbial transformations of such lipids as a means of adding value to chemicals (15). Lipids and lipid products have wide application in food and chemical industries. They are used as cooking oils, in margarines, and in ice cream (45) and as important oleochemicals such as lubricants (45), detergents (16), paints, inks (44), and plasticizers (33). Lard is an abundant animal fat of low economic value. Chemically, it consists of a mixture of triglyceride esters of palmitic, stearic, oleic, and smaller amounts of linoleic acids (14). Distributions of saturated and unsaturated fatty acids in the triglyceride structures found in lard are not random (14). Position 2 of glycerol is mainly found esterified with palmitate, while positions 1 and 3 are usually esterified with stearate and unsaturated fatty acids.

Microbial transformations have been exploited with many types of natural and synthetic compounds (8, 11, 17, 20, 23, 24, 34–40, 43, 46, 48, 52). With lard as a substrate, expected biotransformation reactions would include fatty acid hydrolyses catalyzed by lipases. Lipases are a ubiquitous group of enzymes produced by bacteria, yeasts, molds, and a few protozoa (32). This class of enzyme is being increasingly exploited for the hydrolysis, acylation, and interesterification of lipids and other esters. The lipolytic activities of lipases against long-chain triglycerides have been classified into three main groups (4). The first group displays 1,3-esterase specificity. A second group hydrolyzes all positions of the triglyceride esters equally well, and the third group of lipases displays high specificity for the hydrolysis of unsaturated fatty acid esters regardless of their position. Examples of these hydrolytic and fatty acid specificities with triglycerides are numerous.

This report describes the products obtained when lard undergoes hydrolysis by cultures of Saccharomyces cerevisiae (UI-SACCH) and Schizosaccharomyces octosporus (NRRL Y-854) and defines the specificities of enzymes within these microorganisms in the hydrolysis of triglycerides.

MATERIALS AND METHODS

General procedures. Melting points were recorded on a Thomas Hoover Unimelt capillary melting point apparatus and are uncorrected. Low-resolution mass spectra were obtained on a Trio-1 mass spectrometer (MS) linked with a 5890A Hewlett-Packard gas chromatograph (GC) equipped with a quadram (IBM 286-compatible) computer at an ionization voltage 70 ev. GC-MS (electron impact mode) was achieved on an OV-1 3% phenyldimethylsilicon column (6 m by 0.25 mm [outside diameter]; 80 to 100 mesh) with a helium carrier gas flow of 20 ml/min, and temperatures for the injector, column, and detector were held at 220, 180, and 300°C, respectively. Chemical ionization analysis was obtained with a Nermag R 10-10c spectrometer attached to a Girdel series 32 GC, with helium as the carrier gas and a 5% phenyldimethylsilicon column (25 m by 0.2 mm [inside diameter]) with 0.33-mm film). High-resolution mass spectra were obtained with a VG-ZAB-HF spectrometer. Nuclear magnetic resonance (NMR) spectral data were obtained with a Bruker NM 360-MHz high-field NMR spectrometer linked with an IBM Aspect-2000 processor, with 6.26 kHz as an internal deuterium lock and an 8.429-T cryomagnet. 1H and 13C NMR spectra were recorded at 360.134 and 90.556 MHz, respectively. Chemical shift values are reported in parts per million, and coupling constants are reported in hertz. Abbreviations for NMR signals are as follows: s, singlet; d, doublet; dd, double doublet; m, multiplet.

Thin-layer chromatography (TLC) was performed on 0.25-mm-thick layers of silica gel GF254 (Merck) prepared on glass plates (5 by 20 cm and 20 by 20 cm) with a Quikfit Industries (London, United Kingdom) spreader. Plates were air dried and activated at 120°C for 1 h prior to use. Developed chromatograms were visualized under UV by fluorescence after spraying with 0.1% (wt/vol) 2',7'-dichlorofluorescein (Aldrich) in methanol and then spraying with para-anisaldehyde (4-methoxybenzaldehyde; Aldrich) visualizing reagent (60 ml of CH3OH, 0.5 ml of H2SO4, and 0.5
ml of para-anisaldehyde (7), heated at 105°C to develop a brown color. GC was performed with a Hewlett-Packard 5890A GC linked to a Hewlett-Packard 3390A integrator. Nitrogen was used as both the carrier and makeup gas, and hydrogen and oxygen (air) were used as a source of energy for the flame ionization detector. Nitrogen, air, hydrogen, and makeup gas flows were 30, 260, 20, and 10 ml/min. A Supelcowax column (30 m by 0.25 mm [inside diameter] with 0.20-mm film) (Supelco, Inc., Bellefonte, Pa.) was used, and column, injector, and detector temperatures were maintained at 180 to 210 (5°C min), 220, and 300°C, respectively. Column head carrier gas and hydrogen pressures were kept at 35 lb/in². Retention times (Rf) for palmitic, stearic, oleic, and linoleic acid methyl esters were 2.89, 4.63, 4.93, and 5.61 min, respectively.

**Microorganisms.** All microorganisms are maintained in the culture collection at the College of Pharmacy, University of Iowa, and were originally isolated in our laboratories, obtained from Northern Regional Research Laboratories, Peoria, Ill., or purchased from the American Type Culture Collection. Cultures were maintained on Sabouraud maltose agar slants or no. 5 agar medium (3 slants consisting of 0.1% [wt/vol] yeast extract, 0.1% [wt/vol] beef extract, 0.2% [wt/vol] tryptone, 1 mg of FeSO₄·7H₂O per liter, 1% glucose, and 2% agar. The medium was adjusted to pH 7.2 before it was autoclaved, and all cultures were stored at 4°C.

**Chemicals.** Synthetic triglycerides were obtained from Sigma (St. Louis, Mo.), including 1,3-dipalmitoyl-2-oleoyl-glycerol (POP), 1,2-dipalmitoyl-3-oleoyl-glycerol (PPO), 1,3-dioleoyl-2-palmityl-glycerol (POG), 1,2-dioleoyl-3-palmitoyl-glycerol (OOG), tripalmitin (PPP), 1,2-distearoyl-3-oleoyl-glycerol (SSO), 1,3-dioleoyl-2-stearoyl-glycerol (OSO), 1,2-dioleoyl-3-stearoyl-glycerol (OOS), tristearin (SSS), and 1,2-distearoyl-3-palmitoyl-glycerol (SSP). Commercially available refined lard was purchased from Gay’s Locker and Meat Market (Iowa City, Iowa). About 50 g of lard was purified from free fatty acids and mono- and diglycerides on a silica gel (J. T. Baker, Phillipsburg, N.J.) column (10 by 45 cm) which was eluted with 5% acetone in petroleum ether. Fractions containing only triglycerides were collected, dried over anhydrous Na₂SO₄, and evaporated to dryness under vacuum to yield 41.5 g of a purified lard preparation.

**Screening and fermentation procedure.** Biotransformation experiments were performed by the two-stage shaken culture (6) procedure and culture medium described elsewhere (27). The medium was adjusted to pH 6.8 before the addition of purified lard (5 g) and sterilization by autoclaving for 15 min. Each 25 ml of medium contained in steel-caps, 125-ml DeLong culture flasks was inoculated with a microorganism, incubated at 28°C, and shaken at 250 rpm on New Brunswick Scientific G-25 gyratory shakers. Controls consisted of sterile media containing the same amount of substrate and incubated under the same conditions as well as fermentations in which the organism was incubated under identical conditions without a substrate.

**Analytical methods.** Samples (3 ml) were taken from fermentations after 3, 7, 10, and 15 days; acidified to pH 2 (6 N HCl); and extracted with equal volumes of ethyl acetate-methanol (9:1 [vol/vol]). After evaporation of the organic solvent, the residue was redissolved in ethyl acetate and spotted on 0.25-mm silica gel TLC plates that were developed in petroleum ether-diethyl ether-formic acid (9:1:0.1 [vol/vol]). Spots were visualized under 366 nm of UV light by spraying with 0.1% [wt/vol] 2’,7’-dichlorofluorescein in methanol and/or by spraying developed plates with 0.5% para-anisaldehyde reagent (7) and heating for 5 min at 105°C. Portions of extracts were dissolved in methanol and methylated by the addition of 100 μl of BF₃·CH₃OH complex (25). The methylated products were subjected to GC analyses.

**RESULTS**

Specific hydrolysis of palmitic acid from lard by *S. cerevisiae* (UI-SACCH). Five grams of lard was added to 25 ml of LD medium (27) contained in 125-ml DeLong flasks. The sterilized flasks were inoculated with *S. cerevisiae* and incubated for 11 days. As the fermentations progressed, a white, waxy, semicrystalline pellet was gradually formed and was clearly visible in the fermentation flasks. When the fermentation was harvested, the fermentation beer was decanted from the waxy pellet. The pellet was washed with acetone and dissolved in petroleum ether. The solids were removed from the fermentation beer by centrifugation at 5,000 × g, and the supernatant was extracted three times with 200 ml of petroleum ether. The combined extract was washed with an equal volume of distilled water, dried over anhydrous Na₂SO₄, and concentrated to a viscous solution (10 ml). This solution was then added dropwise to 100 ml of acetone to obtain 1.21 g of metabolite 1 as a white amorphous powder.

Metabolite 1 was identified as palmitic acid as follows: melting point, 59 to 61°C; TLC *Rf*, 0.63; GC *Rf* of methyl ester, 2.89 min; chemical ionization/CH₄ mass spectrum, *m/z* 257 (M⁺ + 1); ¹H NMR (CDCl₃) δ 0.89 (3H, t, CH₃), 1.27 (2H, m, H₆), 1.60 (2H, m, H₄), 2.29 [20-H, m (CH₂)₃], 3.41 (2H, m, H₃), and 4.25 (2H, t, H₂). ¹³C NMR (CDCl₃) δC 13.94(C₆), 22.63(C₄), 31.90(C₃), 29.19 to 29.66(C₃ to C₁₈), 25.01(C₂), 34.34(C₁), 49.95(C₁), and 174.10(C₂).

Specific hydrolysis of stearic acid from lard by *S. octosporus* (NRRL Y-854). The same fermentation protocol was followed as described above for *S. cerevisiae*, and during the course of the fermentation, a white, waxy, semicrystalline pellet formed in the culture flask. The reaction was terminated after 11 days and worked up as described above for the isolation of metabolite 1. Following extraction and crystallization from acetone, 1.05 g of pure metabolite 2 was obtained as a white amorphous powder.

Metabolite 2 was identified as stearic acid as follows: melting point, 69 to 70°C; TLC *Rf*, 0.61; GC methyl ester *Rf*, 4.63 min; EI (DIP) mass spectrum, *m/z* 284 (M⁺); ¹H NMR (CDCl₃) δ 0.89 (3H, t, CH₃), 1.27 (2H, m, H₆), 1.64 (2H, m, H₄), 2.04 to 2.36 (22H, m, H₃ to H₅), 2.03 (2H, m, H₂), 3.36 (2H, m, H₂), and 5.03 (2H, t, H₂). ¹³C NMR (CDCl₃) δC 14.26(C₆), 23.29(C₄), 25.66(C₃), 29.89 to 30.34(C₃ to C₁₈), 32.61(C₂), 34.79(C₅), 46.99(C₃), and 174.10(C₂).

**Determination of the specificity of the lipases in *S. cerevisiae* (UI-SACCH) and *S. octosporus* (NRRL Y-854).** The specificities of *S. cerevisiae* and *S. octosporus* lipases were determined by employing the two-stage fermentation protocol and using different synthetic triglycerides as substrates. Stage I was started by incubating each organism separately in a medium consisting of (each in weight per volume) 2% glucose, 0.5% yeast extract, 0.5% peptone, 0.5% NaCl, and 0.5% K₂HPO₄, adjusted to pH 6.8 before autoclaving for 15 min. Each culture was centrifuged after 48 h, and the cells were resuspended in 10 ml of LD medium (27) containing 0.05% (wt/vol) yeast extract and incubated again for 12 h before the addition of 50 mg of one of the mentioned triglycerides contained in 0.1 ml of N,N-dimethylformamide. Samples (1 ml) were taken from the incubation mixtures every 1, 2, and 3 h, acidified with 6 N HCl, and extracted with 2 ml of ethyl acetate-methanol (9:1 [vol/vol]); the organic solvent was evaporated to dryness, spotted on TLC.
plates, methylated (10, 25), and subjected to GC analyses. The triglycerides POP, PPO, OPO, OOP, PPP, and SSP were used with *S. cerevisiae* (UI-SACCH), while SSO, OSO, OOS, SSS, and SSP were used with *S. octosporus* (NRRL Y-854).

**Quantitative determination of fatty acids in lard.** Fifty milligrams of lard was hydrolyzed by refluxing for 2 h with 10 ml of a 10% (wt/vol) solution of aqueous NaOH. The reaction mixture was acidified to pH 4 with 2 N HCl, extracted twice with 3 ml of ethyl acetate, dried over anhydrous Na$_2$SO$_4$, and evaporated to dryness under vacuum to yield 43 mg of a mixture of free fatty acids. A 10-mg sample of the isolated fatty acid mixture was methylated with boron trifluoride-methanol as described previously (10, 25) and analyzed by GC. Standard curves for palmitic, stearic, oleic, and linoleic acid methyl esters were established over a range of 0.2 to 2.0 mg, and peak areas were used for the quantitative determination of fatty acids.

**DISCUSSION**

Lard is a complex mixture of various glycerol triesters. Little work has appeared in the literature concerning microbiological transformation of lard as an abundant and potentially useful renewable resource. In this work, screening experiments included 11 microbial genera representing 25 species which were examined for their abilities to convert lard to useful products. These included (number screened) Bacillus (2), Candida (11), Cylindrocarpon (1), Hansenula (1), Mycobacterium (1), Nocardia (1), Pseudomonas (2), &tomycyces (2), Schizosaccharomyces (1), Sarcina (1), and Streptomyces (2) species. Of these, the best apparent transformations (TLC) were achieved with two yeasts, *S. cerevisiae* (UI-Sacch) and *S. octosporus* (NRRL Y-854). Each of these microorganisms was then utilized for preparative scale incubations to provide sufficient amounts of metabolites for structural identification.

A total of 1.21 g (24.2% yield) of pure palmitic acid (metabolite 1) was obtained from 5 g of purified lard with *S. cerevisiae* by a simple extraction and precipitation technique. This highly efficient reaction affords 48.4 g of a pure fatty acid product per liter. On the basis of the total amount of palmitic acid present in the sample of lard hydrolyzed by *S. cerevisiae*, the maximum theoretical yield of palmitic acid would be 50 g/liter. The fatty acid product was identified as palmitic acid by comparison of spectral (mass spectrum and $^1$H and $^{13}$C NMR) and physical properties of the isolated compound with authentic palmitic acid. The metabolite methyl ester was also identical to authentic methyl palmitate by GC analysis ($R_t$, 2.89 min).

A total of 1.05 g (21% yield) of pure stearic acid (metabolite 2) was obtained from 5 g of purified lard with *S. octosporus*. This highly efficient reaction affords 42 g of pure stearic acid per liter. On the basis of the total amount of stearic acid present in the sample of lard hydrolyzed by *S. octosporus*, the maximum theoretical yield of stearic acid would be 44 g/liter. The isolated fatty acid product was completely identified as stearic acid on the basis of chemical and spectral (mass spectrum and $^1$H and $^{13}$C NMR) comparisons with authentic stearic acid. The metabolite methyl ester was also identical to authentic methyl stearate by GC and cochromatography ($R_t$, 4.63 min).

Enzymatic hydrolyses of palmitic and stearic acids from lard by *S. cerevisiae* and *S. octosporus*, respectively, were obviously very highly fatty acid specific. The positional specificities of the lipases of the two microorganisms were examined by incubating each microorganism with a series of triglycerides of known composition. The triglyceride SSP was hydrolyzed by both microorganisms. *S. cerevisiae* catalyzed the cleavage of palmitic acid from POP, PPO, OPO, OOP, and SSP. Thus, with these substrates, palmitic acid is released from all three positions of the glycerol moiety. On the basis of the substrates examined, the enzymes of *S. cerevisiae* are positionally nonspecific but palmitic acid specific. *S. octosporus* catalyzed the hydrolysis of stearic acid from SSO, OOS, SSS, and SSP but not from OSO. This result suggests that the enzyme(s) of *S. octosporus* is positionally, 1- and 3-glyceride, and stearic acid specific.

The search for new and useful microbial lipases continues as new applications and needs appear. The positional and fatty acid specificities of microbial lipases are well known. *Penicillium roquefortii* and *Aspergillus niger* exhibit selective cleavage of butyric and caprylic acids from equimolar mixtures of tributylin and tricaprylin, respectively (42). *Candida lipolytica* lipase specifically hydrolyzes butyric acid from butterfat (51). The extracellular lipase of *Geotrichum candidum* releases mainly the unsaturated fatty acids from several natural fats (1, 22) and oleic acid from synthetic triolein (2). *G. candidum* lipase was reported to be cis-9- and cis,cis-9,12-fatty acid specific, and these acids were hydrolyzed regardless of their triacylglycerol positions (32). *Staphylococcus aureus* lipolytic activity plays an important role in pathogenesis resulting in significant increases of oleic acid in plasma (13, 50) and consequently causing an inhibition of protein-oxidative phosphorylation (9, 29). *S. aureus* lipase was nonspecific in hydrolyzing the acyl-glycerol bond regardless of either the position or fatty acid involved (47). The lipases of *G. candidum* (22), *Candida cylindraceae* (5), *Corynebacterium acnes* (19), *Penicillium cyclopium* (30), and *S. aureus* (47) nonspecifically cleave fatty acids from triglycerides. *A. niger* (30), *Rhizopus arrhizus* (41), *Mucor javanicus* (21), and *Rhizopus delemar* (30) specifically hydrolyze fatty acids located on positions 1 and 3 of triglycerides. The fatty acid specificities of lipases from *C. cylindraceae* (5), *Candida paralipolytica* (31), and *P. cyclopium* (30) exhibited differences in the rates of hydrolysis of various long-chain saturated and unsaturated fatty acid esters.

Lipases also catalyze interesterification reactions with triglycerides. Interesteerification is an important process that is used in the oil and fat industry to modify the composition and, therefore, the physical properties of triglyceride mixtures (16). Hydrolysis and resynthesis of glycerides are reversible processes that cause intra- and intermolecular acyl migration, yielding interesterified products (12, 28). Intramolecular acyl migrations to positions 1 and 3 are catalyzed by 1,3-glyceride-specific lipases with the production of triglycerides that are unobtainable by chemical interesterification.

Lard is more abundantly available as a renewable lipid feedstock because of changes in dietary habits in the United States. The high yielding nature and the high specificities of the hydrolytic enzymes of these yeasts render the biotransformation of lard into valuable and usable pure fatty acids practical. Efforts to isolate and characterize the lipases in these organisms are under way.

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REFERENCES