Recovery of Hydroxy Fatty Acids from Lesquerella Oil with Lipases

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Two hydroxy acids, lesquerolic (53 wt%) and auricolic (4%), are present at significant quantities in *Lesquerella fendleri* seed oil. Results reported here indicate the selective release of hydroxy fatty acids during hydrolysis of this oil catalyzed by *Rhizopus arrhizus* lipase. For example, hydroxy acids composed 85–90 wt% of the free fatty acids released during lipolysis, as compared to 54% present overall in the oil. In addition, over 80% of the lesquerolic acid is released from the triglycerides. The reason for this lipase's success was determined to be its 1,3-positional specificity. The vast majority of lesquerella oil's hydroxy acids is at the 1- and 3-positions of its triglycerides, as confirmed by the compositional analysis of partial glycerides formed during lipolysis.

KEY WORDS: Hydrolysis, hydroxy acids, *Lesquerella fendleri*, lesquerolic acid, lipases, reverse micelles.

The U.S. Department of Agriculture has intensified its investigation of lesquerella as a potential new crop by cultivating *Lesquerella fendleri* in cooperation with private-sector partners in Arizona. *L. fendleri* oil contains about 54 wt% lesquerolic acid (14-hydroxy-11-eicosenoic acid) and 3% auricolic acid (14-hydroxy-11,17-eicosadienoic acid) (1), substances with possible industrial applications similar to those of ricinoleic acid (12-hydroxy 9-octadecaenoic acid) from castor oil (2–5). Prospects for developing *L. fendleri* as a new crop by the USDA have been reviewed recently (6,7).

Increasing the hydroxy acid content of lesquerella oil through breeding and obtaining highly enriched hydroxy acid fractions from the oil are essential goals of the lesquerella program. Carlson *et al.* (8) have recently concentrated lesquerolic acid by preparative chromatographic techniques. Here we report the employment of lipases (EC 3.1.1.3) to achieve a similar goal. By employing lipase types that differ in positional specificity for hydrolysis, more information on the location of fatty acid groups within lesquerella triglycerides was obtained. Others have investigated lipolysis of castor oil for recovery of ricinoleic acid, citing as advantages the elimination of high energy inputs and formation of estolides associated with traditional steam splitting processes (9–11). The same advantages apply to lipolysis as a step in the industrial processing of lesquerella oil.

MATERIALS AND METHODS

Refined *L. fendleri* oil was available from previous work (12). Its water content was reduced by treatment with 3 Å molecular sieves. The surfactant Aerosol-OT or AOT [sodium *bis*(2-ethylhexyl) sulfosuccinate] and lipases from *Candida cylindracea* and *Rhizopus arrhizus* were purchased from Sigma Chemical Co. (St. Louis, MO) and used without purification. All other materials were of high purity and used without further purification. Deionized water was used throughout.

Reactions were performed in reverse micellar medium. The medium was formed by adding an aqueous enzyme solution to a solution containing 10 volume percent *L. fendleri* oil and 100 mM AOT in isooctane. The water-surfactant molar ratio, or "w_o" value, of the medium initially was 8.0 and overall concentrations of lipase from *Candida cylindracea* and *Rhizopus arrhizus* were 7.81 and 1.5 units/mL, respectively, where one unit of activity equals the formation of 1 mM ester per hour from 100 mM linoleic acid, and 1-butanol in a reverse micellar medium containing 100 mM AOT at a w_o value of 9.23. All reactions were run at ambient temperature (22.5 ± 1°C). Aliquots were removed periodically and mixed with internal standard solution (50 mM lauric acid) and *bis*(trimethylsilyl) trifluoroacetamide (BSTFA)/trimethylchlorosilane (99:1, vol/vol) at 80°C for at least 10 min. This treatment produced trimethylsilyl (TMS) esters and ethers of free fatty acids and mono- and diglycerides, respectively, which were analyzed by gas chromatography (GC). Alternately, aliquots were treated with ethereal diazomethane to produce fatty acid methyl esters (FAME). The precision of the concentrations and weight percentages indicated in the figures is within ± 5%, with the exception of the diglyceride concentrations, which are precise to within ± 10%.

Three GC procedures were used in the analysis of aliquots. In the first, a capillary column (25 m length; 0.25 mm i.d.) was used with a stationary phase of neutral polarity (methyl 65% phenyl silicone, 0.1 mm film thickness) from Quadrex (New Haven, CT) and an oven temperature program of 100 to 365°C at 10°C/min, followed by a 5-min hold at 365°C. The inlet and detector (flame ionization detector, FID) temperatures were set at 380°C. Helium carrier gas flow was split at a ratio of 55:1. Most sample components, including partial glyceride-TMS ethers (but not triglycerides) were resolved and eluted by this system.

A second GC method was employed for more exact determination of free fatty acid from diazomethane-treated aliquots. A 30 m × 0.32 mm i.d. polar capillary column of SP-2340 stationary phase (0.20 mm thickness; Supelco, Bellefonte, PA) was utilized. The oven temperature program consisted of a 1-min hold at 180°C, followed first by a 3°C/min ramp to 210°C, then a 15°C/min ramp to 250°C, and a 7-min hold at 250°C. Injector and FID temperatures were both at 250°C. The flow of helium carrier gas was split at a ratio of 28:1.

The third GC method, for diazomethane-treated samples, was used to determine the relative amounts of fatty acid, partial glyceride and triglyceride. The 3.41 m × 0.32 mm i.d. capillary column was of GB-1 nonpolar stationary phase. The oven temperature program consisted of a 1-min hold at 100°C, a 15°C/min ramp to 365°C and a 4-min hold at the final temperature. The injector temperature and FID were held at 325 and 350°C, respectively. The flow of helium carrier gas was split at a ratio of 5.33:1.

In the hydrolytic reaction with *C. cylindracea* lipase, a precipitate formed at longer reaction times. To determine the components present in the precipitate, it was solubilized in acetone/chloroform (1:1, vol/vol) with the aid of sonication. The solution was then analyzed by Super-

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critical Fluid Chromatography (SFC) in a model 600 chromatograph with a 10 m × 50 μm column of nonpolar SB-Methyl-100 stationary phase of 0.25 μm thickness from Dionex (Salt Lake City, UT). The carrier fluid was high-purity carbon dioxide from Air Products (Tamaqua, PA). The oven temperature was held at 100°C, and the carrier fluid pressure was held at 125 atm, then ramped at 5 atm/min to 325 atm. Detection was achieved with an FID held at 325°C, carrier flow was split *via* timed split injection.

RESULTS AND DISCUSSION

Lesquerella fendleri lipolysis was carried out by a 1,3-positional specific lipase (from *Rhizopus arrhizus*) and a random lipase (from *Candida cylindracea*) for isolation of the oil's hydroxy fatty acids. The reactions were conducted in reverse micelles, which are nanometer-sized colloidal dispersions of aqueous/polar material in lipophilic organic solvent formed by the action of surfactant molecules. The properties of reverse micelles and the advantages of employing them for enzymology have been thoroughly reviewed (13). Reverse micelles have been utilized for a variety of lipase-catalyzed reactions, such as hydrolysis (14), interesterification (15) and esterification (16,17). Our reaction medium was phosphate buffer (pH 6.9) dispered in lesquerella oil/isooctane solution (10:90, vol/vol) by the surfactant (AOT). The initial water-surfactant (molar) ratio, or "w_o" value, was 8.0. This ratio is important because it controls the size of dispersions (13). Other reaction conditions were noted above. No effort was made here to optimize the composition of the medium. Lipases from *C. cylindracea* and *R. arrhizus* were present at overall concentrations of 7.81 and 1.54 U/mL, respectively (U defined in the Materials and Methods section).

Time course of reaction. Figure 1 depicts changes in concentration with time of free fatty acid (FFA), monoglyceride (MG) and diglyceride (DG). The figure demonstrates that for the conditions employed *C. cylindracea* and *R. arrhizus* lipases hydrolyze lesquerella oil at similar rates. However, when the enzyme concentrations noted above are considered, it is apparent that the latter lipase type hydrolyzes lesquerella oil more rapidly. This is due to the stronger discrimination of *C. cylindracea* lipase against hydroxy acids.

Figure 1 demonstrates that with *C. cylindracea* lipase the concentration of MG and DG approach zero. The same holds true for triglycerides (TG) (data not shown). But their loss after *ca.* 25 h is not accompanied by a corresponding gain in FFA. Mass balances account for >95% of the fatty acid groups only up to 20 h. The disappearance of FF groups is accounted for by the formation of a viscous precipitate starting at about 20 h. The precipitate was collected and solubilized in a chloroform/acetone mixture after several minutes of sonication. Analysis of this mixture by GC and SFC showed significant amounts of glycerol, surfactant (AOT), fatty acid (especially lesquerolic acid), MG, DG and TG. However, no precipitation occurred during hydrolysis catalyzed by *R. arrhizus* lipase. The cause of the precipitation is related to the larger amounts of glycerol formed during hydrolysis by *C. cylindracea* lipase. Previously, it was shown that glycerol (16) or ethylene glycol (17) can be cosolubilized with straight-chain FFA in reverse micelles without precipitation or phase separation for a variety of conditions. When the same experiments were repeated with hydroxy acids, we did not encounter cosolubilization, instead, a precipitate formed. In contrast, hydroxy acids and straight-chained alkyl alcohols or benzyl alcohol were cosolubilized in reverse micelles without precipitation. The exact mechanism causing the precipitation is unknown.

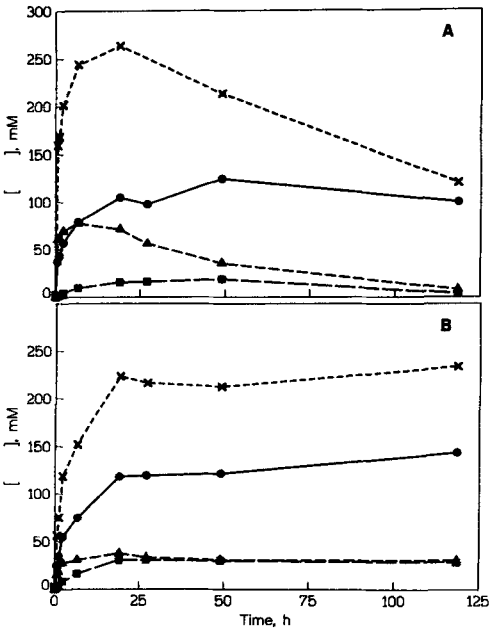

FIG. 1. Change in concentration of free fatty acids and partial glycerides during the course of hydrolysis directed by lipase from A, *C. cylindracea*; and B, *R. arrhizus*. The concentrations of ●, free fatty acids; ■, monoglycerides; and ▲, diglycerides are plotted. x Molarity of fatty acid groups among the free acids and partial glycerides.

Figure 1B depicts concentration changes for FFA, MG and DG formed during hydrolysis catalyzed by the position-specific lipase from *R. arrhizus*. In contrast to the results obtained with random lipase (Fig. 1A), MG and DG concentrations remained constant at 30 mM after one day. In addition, the FFA concentration slowly increased in a linear fashion after reaching a plateau at *ca.* 125 mM. This apparently is caused by acyl migration (see below). Thus, barring acyl migration, thermodynamic equilibrium appears to be reached for this reaction after about one day.

Production of free hydroxy fatty acids. Figure 2 illustrates changes in lesquerolic acid concentration with time for each reaction. Hydrolysis by *R. arrhizus* lipase produced 120 mM lesquerolic acid, which is about 80% of the lesquerolic acid content in the whole oil (heavy horizontal line at *ca.* 136 mM). This result suggests that lesquerolic acid occurs predominately at the 1- and 3-positions of *L. fendleri* triglycerides because *R. arrhizus* lipase is 1,3-positional specific. Analysis of MG and DG formed during the reactions confirms this result. Of the partial glycerides, *C. cylindracea* lipase-catalyzed hydrolysis produced only significant amounts of 1-monolesquerolin and 1,3-dilesquerolin. [The presence of these MG and DG species and the slow production of free lesquerolic and auricolic acids (see below) demonstrate *C. cylindracea* lipase's discrimination against hydroxy acids.] Additional proof was provided by *R. arrhizus* lipolysis, which produced no 2-monolesquerolin but did yield a DG species

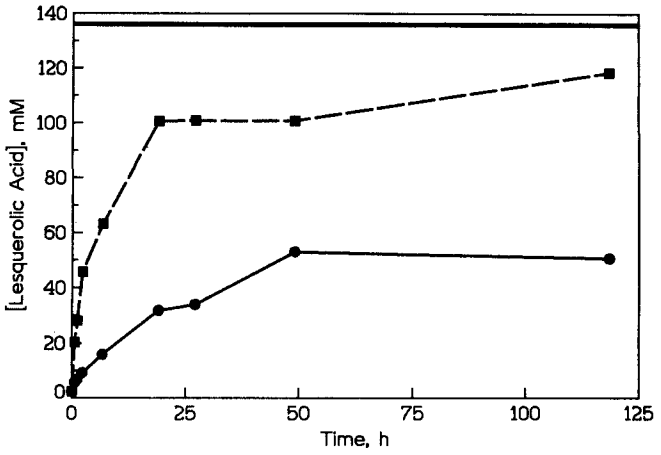

FIG. 2. Change in concentration of lesquerolic acid during course of hydrolysis. The maximal amount of lesquerolic that can be liberated is 136 mM, which is noted by the thick horizontal line. Lipase from ●, *C. cylindracea*; and ■, *R. arrhizus*.

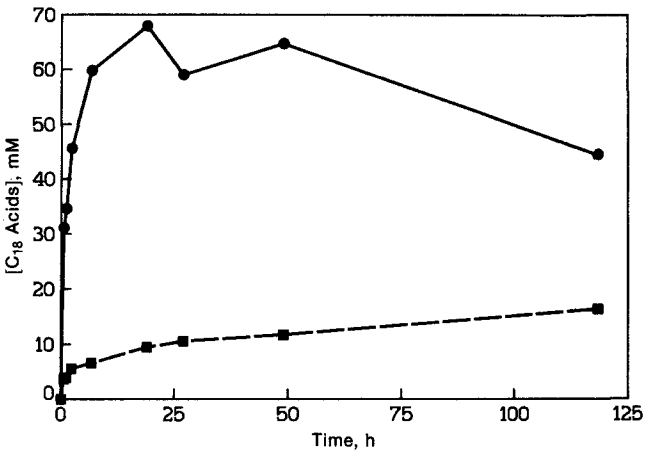

FIG. 4. Change in concentration of the liberated fatty acids eighteen-carbon units in length (C_{18}'s) during the course of hydrolysis. The maximum amount of C_{18}'s that can be released is 131 mM. Lipase from ●, *C. cylindracea*; and ■, *R. arrhizus*.

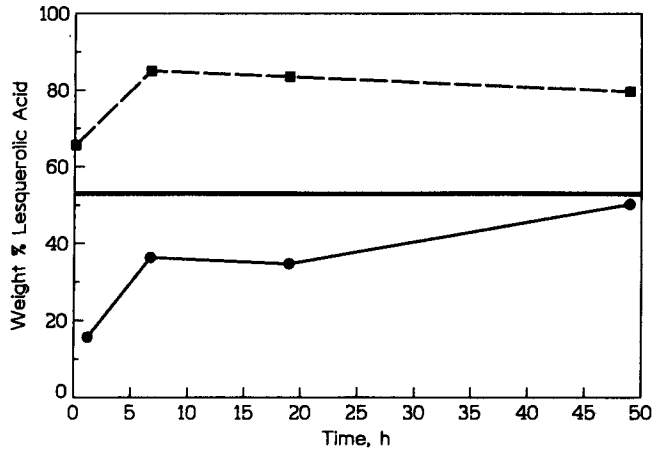

FIG. 3. Change in wt% lesquerolic acid among the free fatty acids during the course of hydrolysis. Thick horizontal line represents concentration of lesquerolic acid among lesquerella oil's fatty acid groups. Lipase from ●, *C. cylindracea*; and ■, *R. arrhizus*.

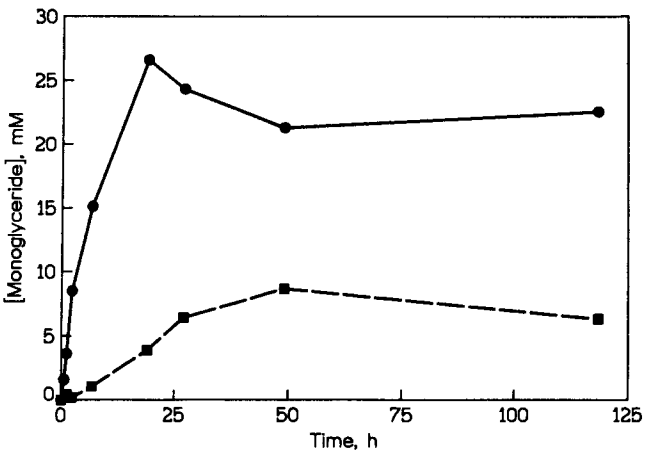

FIG. 5. Change in concentration of monoglyceride species during hydrolysis catalyzed by *R. arrhizus* lipase. ■, C_{18} 1-monoglycerides; and ●, C_{18} 2-monoglycerides. No other monoglyceride species were present at appreciable quantities.

with a hydroxy acid at the 1-position and a C_{18} unsaturated acid at the 2-position. The lower concentration of free lesquerolic acid for hydrolysis by *C. cylindracea* lipase (Fig. 2) is due partly to the formation of the precipitate (discussed above).

Figure 3 plots the wt% of lesquerolic acid in the released acids with lipolysis time. The lesquerolic acid content of *L. fendleri* oil is shown by the heavy horizontal line. This content is nearly matched by the wt% obtained from hydrolysis by the random lipase at 50 h. But for hydrolysis by *R. arrhizus* lipase, Figure 3 shows that 85% of FFA is lesquerolic acid. Acyl migration probably accounts for the small linear decrease in lesquerolic acid wt% after 5 h (see below). The lower wt% lesquerolic acid early in both reactions is due to the preference of each enzyme for other fatty acids (most unsaturated octadecanoic acids) over lesquerolic acid. The results illustrated in Figures 2 and 3 show the advantages of employing positional specific lipases for recovery of lesquerolic acid.

Two other hydroxy fatty acids were found in lesquerella oil at significant quantities—auricolic acid at 2.6 wt% and ricinoleic acid at 0.4%. Release of these acids parallels that of lesquerolic acid and, likewise, they are generated at higher concentrations during hydrolysis by *R. arrhizus* lipase.

Nonhydroxy fatty acids. Most of the nonhydroxy fatty acids in *L. fendleri* (43 wt%) are C_{18} acids; stearic (18:0; 2%), oleic ($18:1^9$; 16%), vaccenic ($18:1^{11}$; 2%), linoleic (18:2; 8%) and linolenic (18:3; 15%); or C_{16} acids; palmitic (16:0; 1.3%) and palmitoleic (16:1; 0.8%). Trace amounts (<0.5%) of other fatty acids have also been detected in *L. fendleri* oil (18).

Figure 4 demonstrates the release of C_{18} with time. After 1 day, 70 mM of C_{18} (of a possible 130 mM) are released by *C. cylindracea* lipase. The decrease at later times is due to loss from solution *via* precipitation as discussed. Hydrolysis by *R. arrhizus* lipase releases only 10 mM C_{18} FFA in one day, indicating the prevalence of C_{18} for the 2-position of the triglyceride molecules. This also is reflected by the synthesis of large quantities of 2-monooctadecanoates, as shown in Figure 5, for hydrolysis by *R. arrhizus* lipase. The formation of C_{18} monoglycerides

as by-products, which are of commercial value as food and cosmetic emulsifier (19,20), further enhances the favorability of 1,3-specific lipolysis for treatment of lesquerella oil.

The slow linear increase of C_{18} FFA (Fig. 4) and the formation of C_{18} 1-monoglycerides (Fig. 5) after 20 h for *R. arrhizus* lipase-catalyzed hydrolysis is attributed to acyl migration. Other evidence supports this conclusion, including the slow linear rate of glycerol formation (data not shown). Similar trends have been observed in reverse micelles for lipolysis of rapeseed and meadowfoam oils (D. Hayes and R. Kleiman, unpublished data) and esterification of 2-monoglycerides (17). Acyl migration, believed to occur among DG and MG, can be promoted by a variety of sources, such as water (21,22) and surfactant concentration (17). We suggest removing or denaturing the enzyme at the onset of equilibrium to prevent release of the octadecenoic acids which migrate to the 1- and 3-positions.

During the beginning of the reaction, free linolenic and linoleic acids are present in large amounts relative to oleic and vaccenic acid. This is due to preferential catalysis by both lipase types toward more unsaturated acids. But after the initial period has been completed for both reactions, the ratio of oleic to linoleic to linolenic acid in the FFA, MG and DG is nearly identical to the same overall ratio in the oil. But results indicate that the proportion of stearic and vaccenic acids present at the outer triglyceride positions are greater than those of their Δ9 C_{18} counterparts. Hence, the positional composition of lesquerella oil acids mirrors that observed for many other oils; *i.e.*, saturated, longer-chain or unusual fatty acids (such as hydroxy acids and those with unusual double bond positions) are commonly encountered at the 1- and 3-positions, whereas common unsaturated fatty acids are found at the 2-position (23–25).

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