Enzymatic Synthesis of Trierucin from High-Erucic Acid Rapeseed Oil[1]

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The lipase from *Candida rugosa* has been shown to discriminate against erucic acid. Advantage of this property has been taken to produce trierucin from high-erucic acid rapeseed (HEAR) oil. A method has been developed for extracting erucic acid from the oil as dierucin and subsequently enzymatically converting it to trierucin. Unrefined HEAR oil was hydrolyzed with lipase from *C. rugosa* to produce a mixture of free fatty acids and dierucin. Precipitation and filtration from cold ethanol gave 73% pure dierucin, free of fatty acids. This dierucin was treated in two ways to produce trierucin. First, in the presence of an immobilized lipase and a known amount of water, some trierucin is produced by interesterification. Second, a more efficient route to trierucin utilized *Rhizopus arrhizus* lipase to completely hydrolyze dierucin to erucic acid, which was then combined with an appropriate amount of dierucin in the presence of an immobilized lipase to produce trierucin in a quantitative yield.

KEY WORDS: *Candida rugosa*, dierucin, erucic acid, HEAR oil, lipase, Lipozyme, rapeseed, *Rhizopus arrhizus*, trierucin.

Erucic (*cis*-13-docosenoic) acid has many potential or well-established uses, either in the form of glycerides or as free fatty acid (1,2). At present, erucic acid-rich oils have been employed as lubricants. They may also be used in the continuous casting of steel and in transmission oil fluids (1), or in the manufacture of cosmetic products through the synthesis of waxes that could be used as a jojoba oil substitute (3). Erucic acid is a precursor for the synthesis of Nylon 1313 (4). A medicinal application has also been found for pure erucic acid—when administered in therapeutic doses, it can be used to treat the symptoms of the genetic disorder known as adrenoleukodystrophy (ADL) (5). The production of pure erucic acid in an efficient and economically viable manner is an area of research that has raised much interest lately(6).

The major erucic acid producing plants are rapeseed (*Brassica napus*), crambe (*Crambe abyssinica*), white mustard (*Sinapis alba*) and nasturtium (*Tropaeolum*) (7,8). This latter species is the only one to contain trierucin (8).

In this paper, as a logical step for future applications of erucic acid-rich oils, we have chosen to investigate the synthesis of trierucin. Rather than manipulating a plant's genetic makeup to force increased synthesis of erucic acid, we have instead opted to enzymatically produce trierucin from high-erucic acid rapeseed (HEAR) oil. To accomplish this goal, it was essential to first devise a process that would do two things—separate the erucic acid from other fatty acids, and then convert the erucic acid to trierucin. The first objective was investigated by using *Candida rugosa* lipase's discrimination against erucic acid (9), and for the second we used the expertise we gained with the synthesis of triolein (10). As part of this research work, an indirect approach to producing pure erucic acid from unrefined HEAR oil was investigated as well.

MATERIALS AND METHODS

HEAR oil. Unrefined HEAR oil was supplied by CanAmera Foods (Saskatoon, Canada). The fatty acid composition of HEAR oil was determined by a complete hydrolysis of the oil with a combination of the two lipases from *C. rugosa* and *Rhizopus arrhizus*. The resulting free fatty acids were identified by gas chromatography/mass spectrometry (GC/MS) of their methyl esters. The Instant Methanolic HCl kit from Alltech-Applied Science (Deerfield, IL) was used to synthesize the methyl esters. The methyl esters were identified by comparison with GC/MS [Hewlett-Packard (Palo Alto, CA) 5890 Gas Chromatograph and 5970 Mass Spectrometer with J&W Scientific (Folsom, CA) 30-m DB5 column] of standards. All fatty acids present, except behenic and palmitic, were quantitated by a previously published high-performance liquid chromatography (HPLC) method (11). Because behenic and palmitic acids do not absorb at 205 nm and are not concentrated enough for refractive index detection, they were instead quantitated from the ratio of their methyl esters to those of oleic and 11-eicosenoic acids. The oil was found to be composed of linolenic (13.1%), linoleic (12.8%), oleic (21.9%), 11-eicosenoic (9.8%), erucic (36.8%), behenic (4.6%) and palmitic (1.0%) acids.

Chemicals. All fatty materials for HPLC and GC/MS standards were purchased from Sigma Chemical Co. (St. Louis, MO). Chloroform was purchased from Anachemia (Montreal, Quebec, Canada). Ethanol, acetone and acetonitrile (HPLC grade) were obtained from Fisher Scientific (Montreal, Quebec, Canada).

Enzymes. The *C. rugosa* (L-1754) and *R. arrhizus* (L-4384) lipases were purchased from Sigma Chemical Co. Lipozyme™ IM-20, an immobilized 1,3-specific *Mucor miehei* lipase, was purchased from Novo (Bagsvaard, Denmark), and lipase SP 382, an immobilized nonspecific *Candida antartica* lipase, was a gift from Novo.

Analyses. The HPLC method used for monitoring all reactions has been published previously (11). The column used was a CSC-Spherisorb-ODS2, 5 μm, 25 × 0.46 cm from Chromatography Sciences Company (Montreal, Quebec, Canada). All chromatograms were monitored at 205 nm. The linear flow gradient of the mobile phase [acetone/acetonitrile (1:1)] was as follows (flow rate in mL/min): 0.0 (0.80), 6.0 (0.8), 10.0 (4.0), 55.0 (4.0), 59.0 (0.8) and 60.0 (0.8) min. The HPLC column was calibrated with the following standards: monoerucin, erucic acid, dierucin and trierucin, which had retention times of 5.6, 6.6, 10.8 and 49.0 min, respectively. Separation of 1,2-dierucin (14.55 min) from 1,3-dierucin (13.27 min) required a mobile phase of acetone/acetonitrile (2:3) at 3.6 mL/min.

Procedure for producing dierucin from HEAR oil (Scheme 1). To 40 g of HEAR oil (containing 15.8 g erucic acid) was added 16 mL water and 6.4 mL of a *C. rugosa* solution (0.01 g/mL). The oil was hydrolyzed under constant stirring (≈200 rpm) for 18 h at room temperature. This step resulted in the production of 10.6 g (from HPLC

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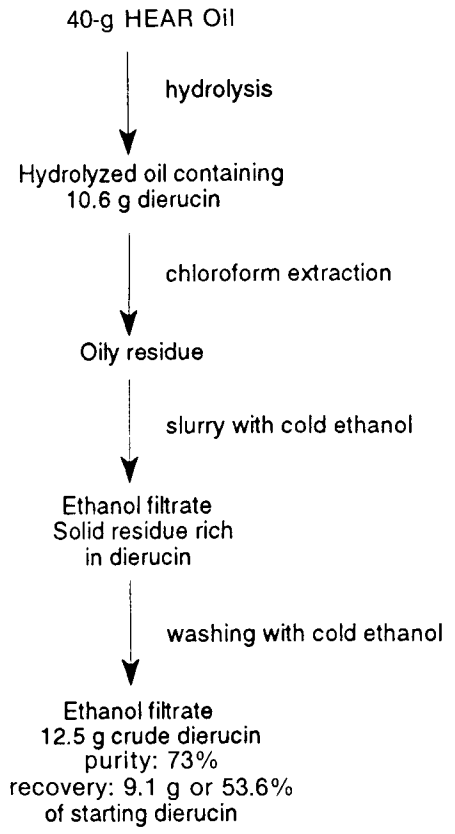

SCHEME 1

measurements) of dierucin contaminated with free fatty acids (linolenic, linoleic, oleic, 11-eicosenoic, palmitic, behenic and erucic acid). The hydrolyzed oil was extracted with 400 mL of chloroform/water (1:1). The chloroform layer was recovered, dried over magnesium sulfate, filtered and evaporated under vacuum. The oily residue was washed with 300 mL ice-cold ethanol yielding a solid, which was filtered to remove free fatty acids. The resulting solid was washed with another 300 mL cold ethanol to yield 12.5 g dierucin (purity 73%) equivalent to a total dierucin recovery of 9.1 g or 86% for this last step and 53.6% from the starting erucic acid present in the oil.

Procedure for producing trierucin from dierucin. Method 1: We added 0, 16, 24, 32, 48 and 64 μL of water to six Eppendorf vials, each containing 0.65 g of 60.5% pure dierucin and 0.05 g Lipozyme. The vials were closed, mixed at 60°C and the trierucin production was monitored by HPLC during 500 h.

Method 2: First, 4.164 g of 73% pure dierucin were hydrolyzed with the 1,3-specific lipase from *R. arrhizus* (in 13 mL water and 100 μL enzyme 0.01 g/mL) to produce free erucic acid, which was treated and worked up in similar fashion as was the dierucin produced from HEAR oil. Then an equimolar ratio of erucic acid and dierucin (73% pure) was reacted in the presence of one of the immobilized lipases at 60°C in a jacketed beaker under vacuum. When the lipase used was SP 382, 10 mmoles each (from HPLC analysis) of erucic acid and of dierucin were used, along with 0.5 g of SP 382. When the lipase was Lipozyme, 5.5 mmoles each of erucic acid and of dierucin were reacted in the presence of 0.5 g Lipozyme.

Dierucin purification. The HPLC semi-preparative column used for the purification of ethanol-washed dierucin was CSC-Spherisorb-ODS2 (5 μm, 25 × 1.0 cm) purchased from Chromatography Sciences Company. The mobile phase consisted of acetone/acetonitrile (1:1) at a flow rate of 4.0 mL/min for 25 min and a column temperature of 35°C. All runs were monitored at 205 nm. Ethanol-washed dierucin (1.8 g) was dissolved in 10 mL acetone, injected on the column in 200-μL aliquots, and the dierucin peak was collected between 18.5 and 21.5 min. All dierucin eluates were combined, and most of the solvent was removed under reduced pressure. The resulting saturated dierucin solution was cooled at −20°C, whereupon a solid precipitated, which was removed by filtration to yield 0.51 g of purified dierucin.

A mass spectrum was obtained by chemical ionization with methane. An *m/z* 732 peak with 3% relative intensity was observed for the dierucin molecular ion.

As 1,2-dierucin is not available commercially, it was synthesized *in situ* by heating 1,3-dierucin at 70°C for 48 h. A new peak appeared in its HPLC chromatogram, 1 min after the 1,3-dierucin peak. Considering the fact that 1,2-diolein has a retention time slightly longer than that of 1,3-diolein (12), it is presumed that the new peak is 1,2-dierucin and that this compound is not present in the 1,3-dierucin product.

RESULTS AND DISCUSSION

To produce trierucin from HEAR oil, it was evident that a means of concentrating the erucic acid in the glycerides of the oil was of paramount importance. Since our previous work (9) clearly demonstrated that the lipase from *C. rugosa* was discriminating against erucic acid, it was decided to use this selectivity to elaborate an enzymatic approach to synthesizing trierucin.

When HEAR oil (Fig. 1A) is hydrolyzed by the lipase from *C. rugosa*, one obtains a product (Fig. 1B) that contains free fatty acids (linolenic, linoleic, oleic, 11-eicosenoic, palmitic, behenic and erucic) and 1,3-dierucin. Approximately 15% of the erucic acid in the original oil is in the free form, and 85% is found in the form of 1,3-dierucin. The high area of the free fatty acids' peak comes from the lower flow rate during this part of the separation.

As shown in Figure 2, the production of dierucin from HEAR oil increases for the first 18 h and then levels off at a concentration corresponding to 85% of the erucic acid present in the starting oil. A plausible explanation for the discrimination of this lipase against erucic acid can be proposed. In HEAR oil, erucic acid is found almost exclusively on the primary hydroxyls of glycerol (7,8,13). When erucic acid is attached to both positions 1 and 3 on the same glyceride and another fatty acid is present at position 2, hydrolysis may only occur at position 2. On the other hand, when erucic acid is found only at position 1 or 3 on a glyceride molecule, then complete hydrolysis may occur. If this hypothesis is proven valid, this would indicate that in HEAR oil, 85% of the erucic acid is found at both positions 1 and 3 on the same glyceride, 15% being found only at position 1 or 3 on a glyceride molecule. It is also possible that more erucic acid is present on both primary hydroxyls, but acyl migration (14) produces 1,2-dierucin that can be hydrolyzed.

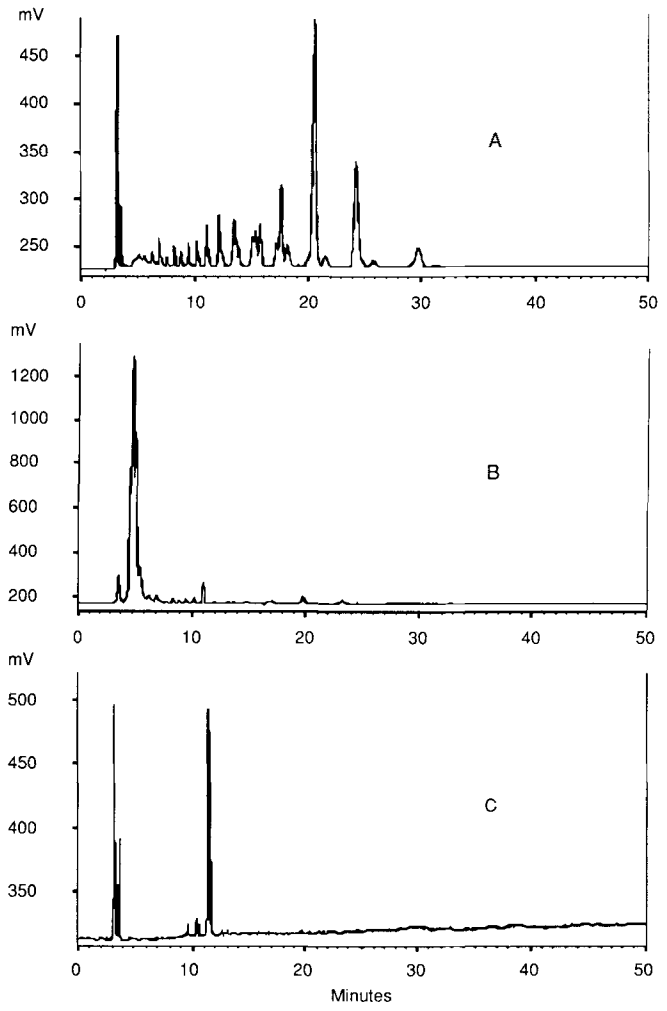

FIG. 1. High-performance liquid chromatography chromatograms for A, starting high-erucic acid rapeseed (HEAR) oil (peaks represent triglycerides of different molecular weight); B, hydrolyzed HEAR oil (peaks at 4–6 min are free fatty acids, peak at 11 min is 1,3-dierucin); C, ethanol-washed dierucin (peaks at 9.5–10.5 min are diglycerides, peak at 11 min is 1,3-dierucin).

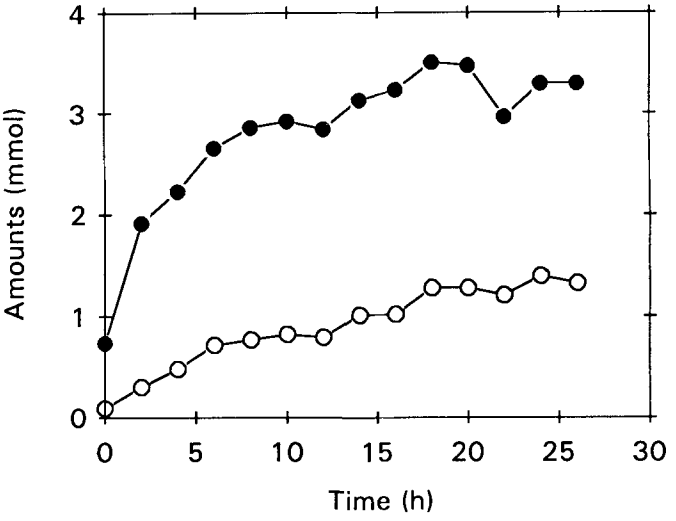

FIG. 2. Time course of the hydrolysis of 10 g of high-erucic acid rapeseed oil with 4.0 mL water and 1.6 mL of a 0.01 g/mL solution of *Candida rugosa* lipase. ○, Erucic acid, ●, dierucin.

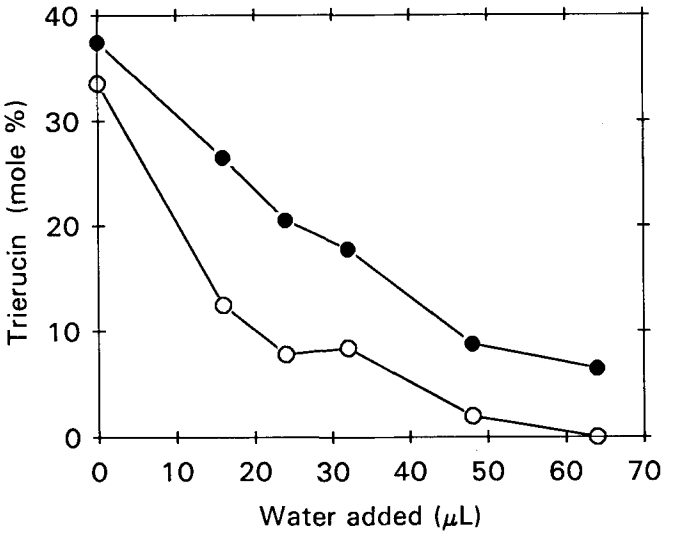

FIG. 3. Effect of the amount of water added on the interesterification of 1,3-dierucin. Trierucin concentration after 96 h (○) and 264 h (●).

From these observations, a procedure for obtaining dierucin from HEAR oil was devised as specified under the Materials and Methods section. Thus, from 40 g of HEAR oil, 12.5 g of 73% pure dierucin was produced. Figure 1C shows that the other components present are mainly diglycerides (9.5–10.5 min).

Two alternative methods for producing trierucin were selected as outlined previously. With Method 1, dierucin in the presence of Lipozyme was treated with various amounts of water (Fig. 3), and the trierucin production was monitored at 96 and 264 h. All reactions had reached equilibrium at 264 h. Clearly, the best trierucin yield was obtained when no water was added [Lipozyme already contains 8–10% w/w water (15)]. Moreover, with no added water, approximately 50% of the erucic acid initially present as dierucin was converted to trierucin at equilibrium. As the initial water concentration was steadily increased, final trierucin concentration decreased in linear fashion (Fig. 3). This may represent hydrolysis or interesterification with other glycerides present. Furthermore, the rate of trierucin production also seems to be impaired by the additional water, as indicated by the low trierucin production at 96 h. When no excess water was added, approximately 46% of the available erucic acid equivalents had been incorporated into trierucin, whereas in the reaction with the largest amount of added water no measurable trierucin was produced in the same time frame, indicating a slow initiation of the reaction that can give 8% at 264 h.

While this simple procedure did lead to partial conversion of dierucin into trierucin, the relatively long reaction times and low product yields meant that a better and far more efficient method was required. We have previously shown that high triglyceride synthesis yields are obtained when stoichiometric amounts of substrates are used in the presence of an immobilized lipase without solvent at 60°C under reduced pressure (10). This exact same approach

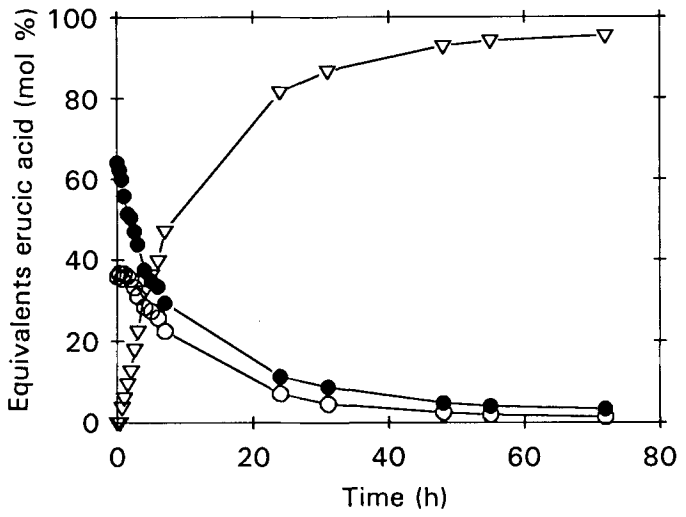

FIG. 4. Time course of trierucin synthesis from dierucin and erucic acid with SP 382. ▽, Trierucin; ●, Dierucin; ○, Erucic acid.

was utilized in formulating Method 2. Thus, stoichiometric amounts of free erucic acid (from hydrolysis of 73% pure dierucin with the 1,3-specific lipase from *R. arrhizus*) and dierucin were reacted in the presence of the nonspecific lipase SP 382. After 72 h, maximum trierucin production was attained with 95% of the erucic acid equivalents being incorporated into trierucin (Fig. 4). This procedure was repeated with Lipozyme. In this instance, a slightly lower yield of the desired triglyceride was obtained, 91.5% of all erucic acid equivalents being converted to trierucin in 96 h (data not shown). These results are consistent with our previous findings on triolein synthesis from diolein and oleic acid (12).

The final phase of this work involved improving the purity of the product dierucin, which could yield erucic acid of purity suitable for the treatment of ADL. Painuly and Grill (6) recently purified erucic acid by means of various HPLC and recrystallization procedures. The erucic acid used in their investigations was initially 90% pure and their product was 99% pure. Their yield ranged from 55 to 65%. A drawback of their procedure is that minimum purity of the starting erucic acid required is 90%.

Using only ethanol-purified dierucin derived from HEAR oil and a semi-preparative HPLC column, we purified dierucin to 99% with a 28% yield. The purity was assessed by HPLC and by MS. With HPLC, only one single, symmetrical peak was detected in the chromatogram. This peak had a retention time of 10.75 min, which coincides with the retention time of 1,3-dierucin, and the molecular weight of the constituent was assessed by MS. From this point on, it would be a simple matter to produce high-purity free erucic acid simply by hydrolyzing the dierucin with a 1,3-specific lipase, as reported here.

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