Isolation of Erucic Acid from Rapeseed Oil by Lipase-Catalyzed Hydrolysis

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ABSTRACT: Three lipases were compared for their ability to hydrolyze high erucic acid rapeseed oil, with the objective of concentrating the erucic acid in a single glyceride fraction. Lipase from *Pseudomonas cepacia* released all fatty acids rapidly and did not result in selective distribution of erucic acid. *Geotrichum candidum* lipase released C20 and C22 fatty acids extremely slowly, resulting in their accumulation in the di- and triglyceride fractions. Less than 2% of the total erucic acid was found in the free fatty acid (FFA) fraction. Lipase from *Candida rugosa* released erucic acid more slowly than C20 and C18 fatty acids at 35°C but only resulted in a limited accumulation of the erucic acid in the di- and triglyceride fractions. However, when hydrolysis catalyzed by *C. rugosa* lipase was carried out below 20°C, the reaction mixture solidified and was composed solely of FFAs and diglycerides. The diglyceride fraction contained approximately 95% erucic acid while about 20% of the total erucic acid was found in the FFA fraction. It is concluded that hydrolysis at low temperature with *C. rugosa* lipase results in a higher purity of erucic acid in the glyceride fraction than can be obtained with *G. candidum* lipase, but with considerable loss of erucic acid to the FFA fraction.
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KEY WORDS: *Candida rugosa,* erucic acid, *Geotrichum candidum,* lipase, rapeseed oil.

Erucic acid (C22:1, δ13) is a natural fatty acid which occurs in the storage triglycerides (TG) of plants of the family Brassicaceae. Rapeseed is a member of this genus and is grown in several countries for its oilseed. Rapeseed oil, which contains a high content of erucic acid (more than 40%), is becoming important for industrial applications. Erucic acid and its derivatives are currently used as slip agents in plastics and as a component in biodegradable lubricating oils (1). The use of erucic acid is likely to increase because of international concerns over biodegradability and renewability of natural resources.

Erucic acid can be isolated from rapeseed oil fatty acids by fractional distillation or multiple solvent crystallization at low temperature (2). In the case of fractional distillation, temperatures of up to 255°C must be utilized which may result in undesirable by-product formation (3). The use of lipases with fatty acid selectivity could provide a milder and more convenient route to erucic acid purification. Lipase from *Geotrichum candidum* is well known for its preference for C18 fatty acids which contain a *cis* double bond in the δ9 position (4). It has recently been shown that this lipase only poorly utilizes fatty acids which are longer than 18 carbons (long-chain fatty acids) even if a *cis* double bond is located at the δ9 position in the fatty acid chain (5). This property was exploited to prepare material containing up to 85% erucic acid from high erucic acid rapeseed oil (HEAR oil) fatty acids by an esterification reaction (6). Lipase from *Candida rugosa* (formerly *C. cylindracea*) also exhibits some fatty acid selectivity and was previously shown to release long-chain fatty acids more slowly than C16 and C18 acids from fish oil TG (7,8). More recently, it was demonstrated that this enzyme hydrolyzed esters containing erucic acid more slowly than esters containing C16 or C18 fatty acids (5,9,10).

The object of the work described here is to compare and evaluate the usefulness of selected lipases for the preparation of erucic acid by hydrolysis of HEAR oil.

MATERIALS AND METHODS

Materials. Geotrichum candidum lipase was obtained from Biocatalysts Plc (Glamorgan, United Kingdom). *Pseudomonas cepacia* lipase was obtained from Amano Pharmaceutical (Troy, VA). Lipase from *C. rugosa* was a gift of Enzeco (New York, NY). HEAR rapeseed oil was a generous gift of Calgene Chemical (Skokie, IL). BSTFA [*bis*(trimethylsilyl) trifluoroacetamide] was purchased from Regis Chemical Co. (Morton Grove, IL).

Hydrolysis. The following conditions were used throughout, unless otherwise stated: HEAR oil (5 g) was stirred magnetically at 600 rpm with 3.5 mL of 50 mM phosphate buffer, pH 7, in which was dissolved 100 mg of lipase powder. Stirring took place in a stoppered, flat-bottom glass tube, 3 cm × 5 cm, which was placed in a glass mantle. The reaction temperature was controlled by circulating water from a constant temperature water bath through the mantle. Samples of approximately 5 mg were removed periodically, and the partial glycerides and the free fatty acids (FFA) were converted to trimethylsilyl derivatives by heating the sample for 15 min at 100°C in the presence of 50 μL dry pyridine and 100 μL BSTFA.

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Gas chromatography. Samples which were derivatized with BSTFA as described above were separated by class [FFA, monoglyceride (MG), diglyceride (DG) and TG] and within each class according to carbon number using gas chromatography. A 15-m long, nonpolar high-temperature capillary column, i.d. 0.32 mm, film thickness 0.1 micron, was used (DB1-HT; J&W Scientific, Folsom, CA). On-column injection was carried out using a Hewlett-Packard 5610 gas chromatograph, helium carrier gas flow rate of 5.5 mL/min and with flame-ionization detection. Initial oven temperature was 110°C followed by a temperature program of 20°/min to a final temperature of 350°C, which was held for 18 min. Standards (Sigma Chemical Co., St. Louis, MO) were used to determine the retention times of the following components—FFA: oleic acid (C18), erucic acid (C22); MG: monoolein (C18), monoerucin (C22); DG: diolein (C36), dierucin (C44); TG: triolein (C54), trierucin (C66). Other carbon numbers were determined by inference. Due to the large number of components in chromatograms, it was not possible to determine response factors and results are reported as area%.

RESULTS

Time course of hydrolysis. Figure 1 shows the change in concentration of MG, DG, TG and FFA during the course of hydrolysis of HEAR oil by three different lipases at 35°C. The nonspecific lipase of *P. cepacia* almost completely hydrolyzed the TG to FFA in 48 h (Fig. 1A). Both MG and DG appeared as intermediates, reaching a maximum of 35 and 10%, respectively, after 2 h and were almost completely hydrolyzed after 48 h.

Hydrolysis using *C. rugosa* lipase occurred in two phases (Fig. 1B): a fast release of FFA up to 6 h reaction time, reaching a level of 60%, and a slow release between 6 and 48 h, reaching a final concentration of 75%. Decrease in the concentration of DG was slow compared to the reaction with *P. cepacia* lipase, and after 48 h reaction time, hydrolysis of DG was not complete.

In Figure 1c the hydrolysis products of HEAR oil by lipase from *G. candidum* are shown. With this lipase, DG concentration reached 45% after 4 h reaction time, and in contrast to the other lipases no hydrolysis of DG occurred beyond this time. A maximum FFA level of 50% was reached, and MG levels were almost zero throughout the reaction.

Composition of the reaction mixture. Table 1 lists the detailed composition of the reaction mixture during hydrolysis of HEAR oil (as shown in Fig. 1) after 4 and 24 h reaction time at 35°C. After 4 h hydrolysis with *P. cepacia* lipase, the major DG species was C40, corresponding to one molecule of erucic acid and one molecule of C18 acid bound to glycerol. TG which contain erucic acid (e.g., C60) were hydrolyzed extensively, and the major FFA was erucic acid (C22). At 24 h, release of more than 85% fatty acids had occurred. The relative proportions of the individual FFA are consistent with a nonspecific hydrolysis.

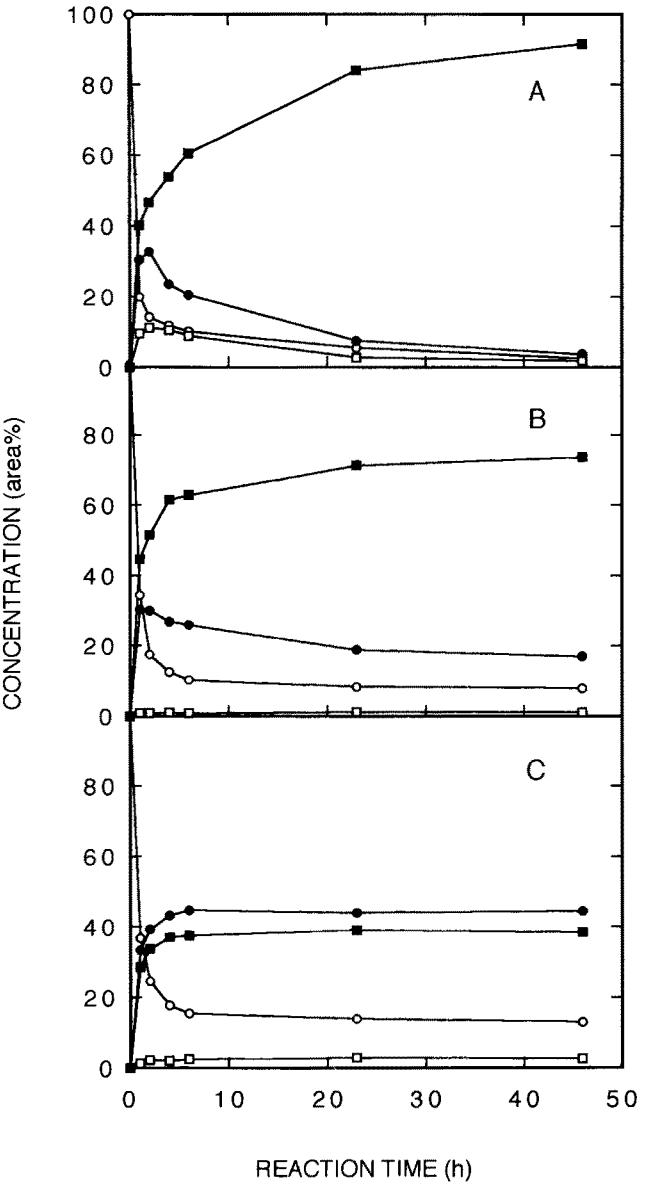

FIG. 1. Change in composition of glycerides and free fatty acid (FFA) during lipase-catalyzed hydrolysis of high erucic acid rapeseed oil at 35°C: A = *Pseudomonas cepacia*, B = *Candida rugosa*, C = *Geotrichum candidum*. Triglyceride = ○, diglyceride = ●, monoglyceride = □, FFA = ■.

The composition of the reaction mixture after 4 h hydrolysis using *C. rugosa* lipase is clearly different from that of *P. cepacia* lipase. The major DG was dierucin (C44), and the concentration of C18 FFA was almost three times greater than C22 FFA. The C22 FFA concentration had increased to approximately 23% after 24 h reaction time with a corresponding decrease in C44 DG.

After 4 h hydrolysis using lipase from *G. candidum*, DG containing C20 or C22 fatty acids (C40, C42 and C44) had reached a higher level than occurred in the reaction with *P. cepacia* or *C. rugosa* lipases. At 24 h reaction time, the concentration of these DG had increased slightly, and approxi-

TABLE 1
Composition of the Reaction Mixture (area %) After 4-h and 24-h Reaction Time During Lipase-Catalyzed Hydrolysis of HEAR Oil at 35°C[a]

Acyl carbon number		HEAR oil	*Pseudomonas cepacia* 4 h	*Pseudomonas cepacia* 24 h	*Candida rugosa* 4 h	*Candida rugosa* 24 h	*Geotrichum candidum* 4 h	*Geotrichum candidum* 24 h
FFA	C16	0.0	2.5	3.1	3.7	3.5	3.3	3.2
	C18	0.0	16.9	32.6	39.0	37.5	35.2	34.1
	C20	0.0	6.1	8.5	5.0	7.8	0.0	0.8
	C22	0.5	28.4	39.9	13.8	23.3	0.7	0.8
MG	C18	0.0	5.1	1.6	0.6	0.8	0.5	0.0
	C20	0.0	0.8	0.0	0.1	0.2	0.0	0.0
	C22	0.0	4.1	1.2	0.3	0.3	2.2	2.4
DG	C34	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	C36	0.0	4.1	1.5	0.0	0.2	0.0	0.0
	C38	0.0	2.7	0.8	0.0	0.2	2.0	1.5
	C40	0.6	10.7	3.4	1.4	0.8	6.3	6.4
	C42	0.0	1.7	0.6	6.1	1.9	9.6	9.9
	C44	0.0	3.5	1.3	18.2	13.9	24.4	26.2
	C46	0.0	0.0	0.0	1.1	0.8	0.8	1.0
TG	C52	2.7	0.4	0.0	0.0	0.0	0.0	0.0
	C54	8.8	1.3	0.8	0.0	0.2	0.0	0.0
	C56	9.3	1.6	0.7	0.0	0.2	0.9	0.7
	C58	10.4	3.9	2.0	1.4	0.7	2.3	2.0
	C60	19.4	1.5	0.8	3.7	1.7	4.5	3.7
	C62	46.3	3.1	1.3	7.3	5.2	10.1	7.3
	C64	1.9	0.0	0.0	0.1	0.8	0.6	0.0
	C64	0.0	0.0	0.2	0.0	0.0	0.0	0.0

[a]HEAR, high erucic acid rapeseed oil; FFA, free fatty acids; MG, monoglycerides; DG, diglycerides; TG, triglycerides.

mately 13% of TG remained unhydrolyzed. These TG were mainly C58, C60 and C62, each of which must contain two or three molecules of C20 or C22 fatty acids (except for C58 which could contain only one molecule of C22 and two of C18). In the FFA fraction, the concentration of C20 and C22 fatty acids never exceeded 1% of total reaction mixture throughout the reaction period.

Effect of temperature on hydrolysis of HEAR oil. In order to optimize the partial fatty acid hydrolysis selectivity toward C22 fatty acids, which was observed with *C. rugosa* lipase, the effect of various external parameters was investigated. Hydrolysis of HEAR oil was carried out at 35, 20, 15 and 10°C. At 15 and 10°C, the reaction mixture became cloudy after 20 min reaction time and after 2 h was a soft solid. The reaction proceeded although stirring was no longer possible. At 35°C no cloudiness or solidification occurred, but at 20°C the reaction mixture was cloudy and viscous after 2 h.

In Figure 2, the appearance of selected products and disappearance of selected TG during the course of the reaction at different temperatures are compared. The decrease in concentration of the C62 TG (Fig. 2A) was similar at all temperatures, but complete hydrolysis did not occur at 35°C.

The concentration of C44 DG (dierucin), as shown in Figure 2B, increased at all temperatures during the first hour of reaction but thereafter exhibited a strong temperature-dependent variation. At 10 and 15°C, a level of approximately 30% was reached after 6 h. At 10°C this level remained constant throughout the reaction, but at 15°C it decreased slightly. At 35°C the C44 concentration peaked at 20% after 2 h reaction; then it steadily decreased to 5% after 48 h. The results at 20°C were intermediate, with a maximum C44 DG concentration of 27% after 6 h which gradually decreased to 14% after 48 h.

The production and final concentration of free erucic acid (C22) were also strongly temperature-dependent (Fig. 2C). The final concentration of C22 decreased with decreasing temperatures. At 20 and 35°C, the relatively large increase in C22 between 6 and 48 h reaction time corresponds to the decrease in concentration of C44 DG. A small increase in C22 concentration at 10°C during this period may be caused by the decrease in the C62 TG concentration (Fig. 2A).

Hydrolysis of HEAR oil by *C. rugosa* lipase at 10°C was repeated four times, and the composition of the reaction mixture after 48 h was used to calculate the mean and standard deviation for the major glycerides and FFA (area% ± SD): FFA: C16 = 3.8 ± 0.2; C18 = 39.3 ± 0.6; C20 = 7.7 ± 0.5; C22 = 12.6 ± 0.5; MG: C18 = 0.8 ± 0.1; C20 = 0.2 ± 0.1; C22 = 0.1 ± 0.1; DG: C38 = 0.3 ± 0.1; C40 = 0.4 ± 0.1; C42 = 3.6 ± 0.3; C44 = 29.7 ± 0.7; C46 = 1.0 ± 0.1; TG: C58 = 0.1 ± 0.1; C60 = 0.3 ± 0.1; C62 = 0.5 ± 0.1.

Hydrolysis of HEAR oil by *P. cepacia* lipase was also carried out at 10°C, but no difference in the composition of the reaction mixture compared with reaction at 35°C was observed after 48 h (data not shown).

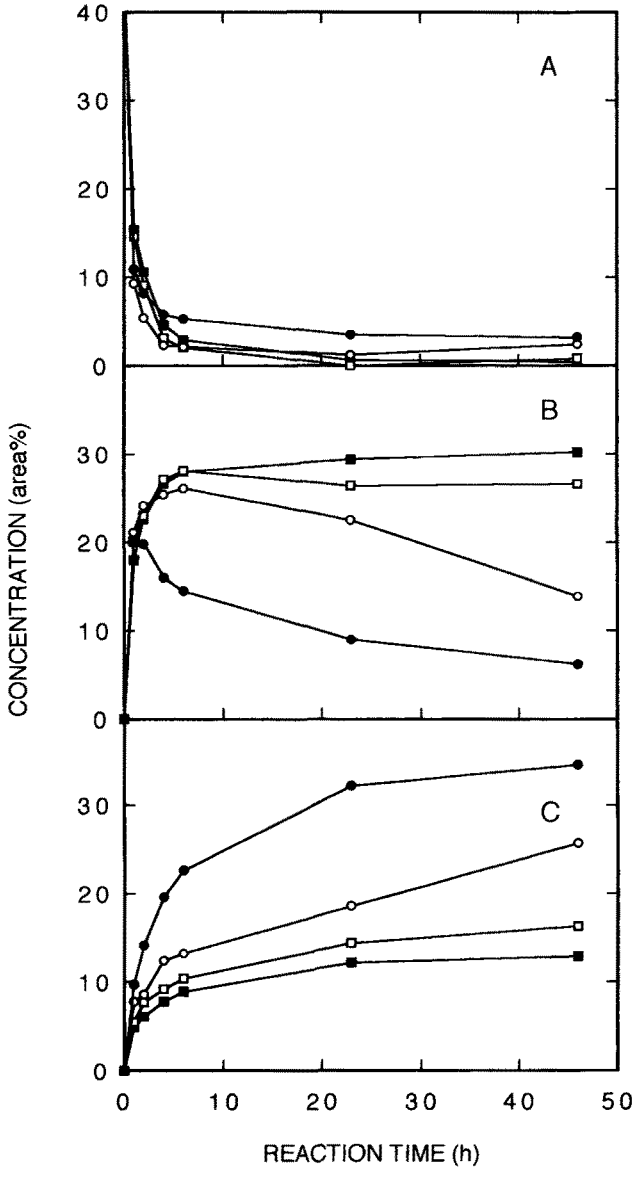

FIG. 2. Change in concentration of glycerides and free fatty acid (FFA) during hydrolysis of high erucic acid rapeseed oil using *Candida rugosa* lipase at different temperatures : A = C62 triglyceride, B = C44 diglyceride, C = C22 FFA. Temperatures : ● = 35°C, ○ = 20°C, □ = 15°C, ■ = 10°C.

Effect of water content. The initial water concentration in the reaction mixture was varied, and the hydrolysis of HEAR oil was carried out at 10°C using *C. rugosa* lipase as catalyst. The composition of the reaction mixture after 48 h is shown in Table 2. At the lowest water content investigated (2% water), hydrolysis of TG was incomplete, and the concentration of C44 DG was relatively low (20%). At all other water contents, almost total hydrolysis of TG was attained, and a concentration of approximately 30% C44 DG was observed. When the water content was less than 30%, the content of C36C42 DG and C18 MG was higher while the content of C18 and C22 FFA was correspondingly lower. During the reaction at all water contents, the reaction mixture solidified.

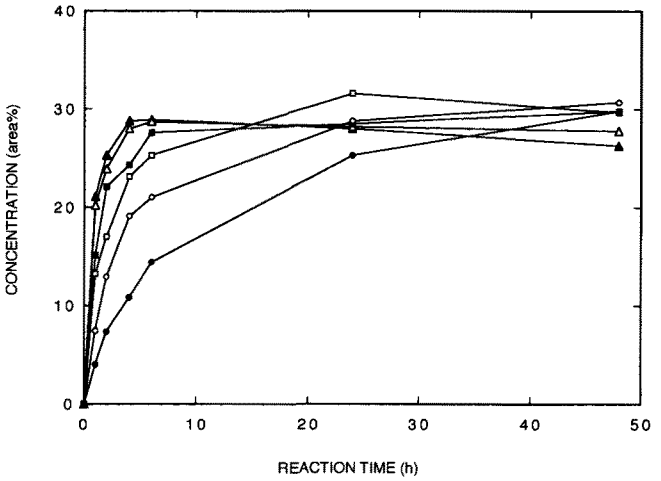

FIG. 3. Effect of concentration of *Candida rugosa* lipase on production C44 (dierucin) during hydrolysis of 5 g of high erucic acid rapeseed oil at 10°C : ● = 10 mg, ○ = 30 mg, □ = 50 mg, ■ = 100 mg, △ = 200 mg, ▲ = 400 mg.

The reaction mixture at 60% water content was not homogeneous, with free aqueous phase visible in pockets distributed around a semisolid emulsion of oil and buffer.

Effect of enzyme concentration. The rate of production of C44 DG increased with increasing concentration of *C. rugosa* lipase (from 10 to 400 mg) during the hydrolysis of HEAR oil at 10°C as shown in Figure 3. However, the rates for 200 and 400 mg were almost identical under the conditions used here. At the higher enzyme concentrations, a small decrease in the C44 DG concentration occurred between 24 and 48 h reaction time. At all enzyme concentrations, a level of approximately 30% C44 DG was reached within the 48-h reaction period investigated.

DISCUSSION

Previous studies on the relative reactivity of fatty acids ranging from C16–C22 toward *P. cepacia* lipase showed no selectivity against any particular acid (5). Detailed analysis of the reaction mixture during hydrolysis of HEAR oil, as described in this paper, confirms this finding and shows that the major intermediate is a DG containing one erucic acid molecule and one molecule of C18 fatty acid. Because altering the reaction conditions had no effect on this breakdown pattern, this enzyme shows no promise as a means to purify erucic acid from HEAR oil.

On the other hand, lipase from *G. candidum* is a useful candidate for this purpose. Although this lipase is well known for its ability to preferentially hydrolyze esters of *cis* δ9 unsaturated C18 fatty acids, compared to their saturated counterparts, it has only recently been shown (5) that esters of unsaturated fatty acids longer than C18 are hydrolyzed extremely slowly. This chainlength selectivity was observed with *G. candidum* lipase from different sources, indicating that all isozymes of this lipase are selective against long-chain fatty acids. In the present work, HEAR oil hydrolysis using

TABLE 2
Effect of Water Content on the Composition of the Reaction Mixture (area %) After 24-h Hydrolysis of HEAR Oil (5 g) Using *Candida rugosa* Lipase (100 mg) at 10°C[a]

Acyl carbon number		Added water (wt% in total reaction mixture)						
		2%	5%	9%	17%	30%	40%	60%
FFA	C16	2.5	3.3	3.2	3.5	3.7	3.7	3.8
	C18	22.5	34.8	34.8	37.0	38.9	40.1	38.7
	C20	1.4	4.1	6.2	6.9	7.1	7.6	6.8
	C22	2.3	6.3	9.0	10.6	10.9	12.2	10.6
MG	C18	1.2	1.3	1.7	1.2	1.6	0.6	0.4
	C20	0.2	0.0	0.2	0.1	0.1	0.2	0.1
	C22	0.6	0.4	0.3	0.2	0.0	0.0	0.2
DG	C34	0.5	0.0	0.3	0.3	0.0	0.0	0.0
	C36	2.2	2.1	1.1	0.7	0.2	0.2	0.0
	C38	2.4	1.4	1.0	0.8	0.4	0.4	0.4
	C40	3.6	1.4	1.2	1.0	0.5	0.5	0.5
	C42	5.7	7.7	5.0	4.2	4.2	4.2	4.4
	C44	17.5	31.3	32.2	30.6	30.5	29.4	32.3
	C46	0.1	1.1	1.1	1.5	2.2	0.7	1.7
TG	C52	0.5	0.0	0.0	0.0	0.0	0.0	0.0
	C54	1.7	0.0	0.4	0.1	0.0	0.0	0.0
	C56	2.6	0.0	0.3	0.1	0.0	0.0	0.0
	C58	3.9	0.0	0.4	0.2	0.1	0.0	0.0
	C60	8.3	1.5	0.6	0.4	0.2	0.4	0.2
	C62	20.1	3.0	0.8	0.6	0.0	0.6	0.0
	C64	0.0	0.0	0.2	0.0	0.0	0.0	0.0

[a]See Table 1 for abbreviations.

this enzyme resulted in almost no release of erucic acid with extensive release of C18 fatty acids, which in HEAR oil are mainly *cis* δ9 unsaturated. Most of the erucic acid was concentrated in the DG fraction as dierucin (C44). However, almost no release of C20 fatty acids was observed resulting in accumulation of these fatty acids in the DG fraction as non-C44 DG. This means that in a short period of time, the concentration of erucic acid in the DG fraction reaches a maximum of approximately 85%.

The similarity in the specificities of the lipases from *C. rugosa* and *G. candidum* is not surprising, considering the close homology which has recently been demonstrated between these enzymes on the molecular level (11). Due to the relatively poor selectivity of the *C. rugosa* enzyme, erucic acid is poorly concentrated into the DG fraction, and a large amount of erucic acid is lost to the FFA fraction at reaction temperatures of 35 and 20°C. Below 20°C, the accumulation of dierucin with the simultaneous solidification of the reaction mixture suggests that dierucin crystallizes as it is being synthesized. The solid is not available as a substrate to the enzyme, therefore preventing further hydrolysis to free erucic acid. A similar phenomenon was previously observed during low-temperature enzymatic glycerolysis of fats and oils: solidification of the reaction mixture with accumulation of MG was demonstrated to be caused by preferential crystallization of MG containing a saturated fatty acid (12). The result of dierucin crystallization is an accumulation of erucic acid in the DG fraction at a higher purity than found with the more fatty-acid selective *G. candidum* lipase. In spite of the crystallization of dierucin, release of free erucic acid does occur at the early stages of the reaction, causing a loss of about 20% of total erucic acid to the FFA fraction. Both *G. candidum* and *C. rugosa* lipases are suitable catalysts for the concentration of erucic acid in specific glyceride fractions during hydrolysis of HEAR oil. The choice of enzyme in a practical situation will depend on the required purity of erucic acid, the desired recovery of erucic acid from HEAR oil and the relative costs of the enzymes. Clearly, an additional processing step, for example crystallization from acetone (10), is needed for the separation of dierucin from the FA.

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