Production of a Very Low Saturate Oil Based on the Specificity of *Geotrichum candidum* Lipase

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ABSTRACT: Lipase B (GCB) produced by the fungus *Geotrichum candidum* CMICC 335426 is known for its high specificity towards *cis*-Δ9 unsaturated fatty acids. The wild-type lipase (not genetically modified) as well as the lipase obtained by heterologous expression of the corresponding gene in *Pichia pastoris* (genetically modified) were studied in a process aiming to produce an oil containing very little saturated fatty acids (SAFA). The approach described in this paper is based on the selective hydrolysis of sunflower oil (12% SAFA) using the *G. candidum* type B (GCB) lipases. Depending on the lipase input, up to 60% w/w degree of hydrolysis was obtained within 6–8 h. Because of the high specificity of the GCB lipases (specificity factor ~30), the level of unsaturates in the free fatty acid fraction was >99% w/w. In contrast with literature data, no loss of specificity was observed, even at the highest degree of hydrolysis obtained. Though both GCB lipases are stable at 30°C, the rate of hydrolysis decreased considerably during the process. Product inhibition as well as time-dependent deactivation (half-life ≈ 2 h) were shown to be involved. After separation of the oil phase, the unsaturated free fatty acids were recovered from the mixture by evaporation and reconverted to triglycerides by enzymatic esterification with glycerol. Because the GCB lipases have a very low efficiency for esterification, this reaction was carried out with immobilized *Rhizomucor miehei* lipase. Under continuous removal of the water generated during the process, >95% triglycerides were obtained in less than 24 h. Standard deodorization resulted in an odorless, colorless, and tasteless oil with less than 1% SAFA.

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KEY WORDS: Esterification, Lipozyme, *Rhizomucor miehei*, specific hydrolysis, *cis*-Δ9-unsaturated fatty acids.

In recent decades the effects of fat in the diet on human health have received much attention. Recognition that overconsumption of fat is related to obesity, which in turn is associated with cardiovascular and other diseases (1), has led to recommendations to limit the daily intake of calories from oils and fats, especially saturated fatty acids (2,3), and to a growing interest in low-fat products, preferably low in saturated fatty acid content (2). However, oils and fats are important carriers of essential nutrients, among which are fat-soluble vitamins (A, D, E, and K) as well as essential fatty acids. The health benefits of various fatty acids in the diet have been studied extensively. For example, a recent study shows that replacement of saturated fatty acids by mono- and polyunsaturated fatty acids is more effective in preventing coronary heart disease in women than is a reduction of the overall fat intake (4).

Commercially available oils such as sunflower oil and soybean oil generally contain 11–15% w/w saturated fatty acids (SAFA), whereas the SAFA content in canola oil can be as low as 5–8% w/w (5). It should be noted that Montmorency cherry pits have been reported to contain a real zero-SAFA oil, containing only oleic acid (63.8% w/w) and linoleic acid (31.5%, w/w) in the form of OLO triglycerides. However, total oil production from these cherry pits, if any, is estimated to be only 300 metric tons per year (tpa) (6).

One way of modifying the oil fatty acid profile is by enzymatic processing, using lipases that exhibit a distinct fatty acid selectivity. In the last decade much literature has been published on the specificity of extracellular lipases from *Geotrichum* toward *cis*-Δ9 unsaturated fatty acids (7–19). The *Geotrichum* fungus produces two major types of lipases, generally indicated as A and B (7,8), or sometimes II and I (9). The B (or type I) lipase is highly specific, whereas the A (or type II) lipase is rather nonspecific.

The major objective of the current work was to evaluate the selective hydrolysis of sunflower oil using *G. candidum* B lipase (GCB) from the strain CMICC 335426 (7,8) as a means to produce a very low-saturate fatty acid concentrate. After separation from the partial glyceride fraction these free fatty acids were converted into triglycerides by a lipase-catalyzed esterification.

MATERIALS AND METHODS

Fermentation and purification. The wild-type lipase B from strain CMICC 335426 was obtained by fermentation of *G. candidum* CMICC 335426, as reported earlier (10). The inoculum for this fermentation was kindly supplied by Dr A.R. Macrae, Unilever Research Colworth House (Bedford, United Kingdom). The fermentation broth, containing a crude mixture of lipases A and B, was concentrated on an anion exchange column (Q-Sepharose, 50 mM Bis Tris pH 6.0, gradi-

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ent: 0–0.5 M NaCl) as described previously (10). Further purification of lipase activity was achieved with a cation exchange column (S-Sepharose, 50 mM citric acid pH 4.3, gradient: pH 4.3–5.3), as reported earlier (7), and then by hydrophobic interaction chromatography (butyl-Sepharose, 50 mM citric acid, pH 5.3, gradient: 2–0 M ammonium sulfate, followed by 50 mM citric acid in 75% vol/vol ethylene glycol). Protein elution was detected by absorbance at 280 nm, and fractions were assayed for lipase activity. From three separate fermentations, three batches of purified lipase were obtained, which will be referred to as batches GCB1 to GCB3.

During the work described here the genetically modified organism (GMO) lipase B, obtained by heterologous expression of the corresponding gene in *Pichia pastoris* (GCB-GMO), became available. Details of the fermentation are described in (11). The inoculum was kindly supplied by Professor R.D. Schmid from Stuttgart University.

Materials. Q-Sepharose FF, S-Sepharose, and butyl-Sepharose were obtained from Pharmacia (Uppsala, Sweden). Olive oil substrate, 50% vol/vol, was obtained from Sigma (St. Louis, MO; code 800-1). All other chemicals were of high quality and were from Merck (Darmstadt, Germany) or Sigma. Regular sunflower oil was used in all experiments. Free fatty acids from sunflower oil were obtained by enzymatic hydrolysis using *Candida rugosa* lipase (ex Sigma). *Rhizomucor miehei* lipase immobilized on Duolite ion exchange resin, (i.e., Lipozyme) was obtained from Novo Nordisk A/S (Bagsvaerd, Denmark). Commercial Amano Pharmaceutical Co. Ltd. (Nagoya, Japan) *G. candidum* GC4 powder was used in tests comparing the performance of crude and purified *Geotrichum* lipases.

Electrophoresis. Pooled fractions eluting from the hydrophobic interaction column were analyzed using a Pharmacia LKB PhastSystem by isoelectric focusing polyacrylamide gel electrophoresis (IEF–PAGE) on a precast IEF PhastGel, pI 4–6.5. *Geotrichum* lipases A and B have pI of 4.71 and 4.50, respectively (10), although a certain degree of microheterogeneity has also been reported because of the different degrees of glycosylation (12,13) of *Geotrichum* lipases. Focusing was carried out according to the manufacturer's instructions.

Activity assay. The activity of *G. candidum* lipase solutions was determined using the olive oil assay at 30°C and pH 9 in a pH-stat (Mettler-Toledo).

Specificity assay. Selective hydrolysis of sunflower oil was carried out in 100-mL round-bottom flasks, which were placed in a thermostatic bath. The temperature was maintained at 30°C, unless stated otherwise, and an oil/water ratio of 1:1 w/w was applied. In a typical experiment, 30 g oil and 30 g demineralized water were thoroughly mixed with a blade stirrer (~500 rpm) for 30 min, after which 0.5–5 mL of a concentrated GCB-lipase solution (600–800 kLU/L) was added. The course of the hydrolysis reaction was followed by withdrawing samples at appropriate time intervals and measuring the FFA content in the oil phase by titration against 0.1 M NaOH. Before titration the emulsion samples were extracted twice with diethyl ether (1:1 vol/vol), after which the solvent was removed by evaporation. The composition of the fatty acids released upon selective enzymatic hydrolysis was determined by gas–liquid chromatographic (GLC) analysis.

From these results the specificity was determined using the following procedure. The specificity factor Σ describing the selectivity of a lipase for one fatty acid over the other is described by (15)

$$\Sigma = \frac{\left(\frac{V_{\max}}{K_m}\right)_U}{\left(\frac{V_{\max}}{K_m}\right)_S} = \frac{\ln\left(\frac{U}{U_0}\right)}{\ln\left(\frac{S}{S_0}\right)} \quad [1]$$

in which U_0 and S_0 are, respectively, the concentrations of unsaturated (UFA) and saturated fatty acids (SAFA) in the oil at time $t = 0$, whereas U and S reflect the UFA and SAFA concentrations of the partial glycerides remaining after specific hydrolysis.

For a small time increment (i.e., a small change in the overall degree of hydrolysis), Equation 1 can be simplified to

$$\Sigma = \frac{\left(\frac{U}{S}\right)_{\text{product}}}{\left(\frac{U}{S}\right)_{\text{start}}} = \frac{\left(\frac{U}{S}\right)_{\text{FFA}}}{\left(\frac{U}{S}\right)_{\text{PG}}} \quad [2]$$

in which U and S reflect the UFA and SAFA content of the free fatty acid (subscript FFA) and residual fraction of (partial) glycerides (subscript PG), respectively. At small degrees of hydrolysis, the specificity factor can be determined using Equations 1 or 2.

If the hydrolysis process is seen as a sequence of small steps, e.g., a change in hydrolysis of 1% w/w FFA, it can be derived from Equation 2 that the UFA content of those free fatty acids produced in the single step (i) is equal to

$$P_{\text{FFA}_i} = \frac{R \cdot \Sigma}{R \cdot \Sigma + 1} \quad [3]$$

in which R is the ratio of polyunsaturated fatty acid (PUFA) to SAFA in the residual partial glycerides. Using Equation 3, the total content of unsaturated fatty acids in the free fatty acid fraction as well as in the residual partial glyceride fraction can be calculated from a mass balance over the system after each step. By repeating this calculation, the composition of the free fatty acid fraction is obtained as a function of the degree of hydrolysis. The resulting curve was fitted to the experimental data, and an average specificity factor was determined.

Fatty acid separation. Separation of the free fatty acids produced by specific enzymatic hydrolysis was carried out in a 2-L deodorizer at 0.15 mbar and 225°C (steam dosing 0.2–0.25% w/w per hour).

Esterification. Nonselective esterification was carried out in a 1-L jacketed reactor with a six-blade turbine impeller ($d_{\text{impeller}} = 1/3\ d_{\text{reactor}}$). FFA was mixed with glycerol in a 3:1 stoichiometric molar ratio at 600–1200 rpm. The water generated during the reaction was removed by sparging with dry nitrogen through the oil under reduced pressure. During the reaction the vacuum was released to allow intermittent sampling of the mixture. Samples for measurement of the total

FFA content were treated as described above. The reaction was started by the addition of immobilized lipase (Lipozyme). Typically 1–5% w/w of catalyst was applied on oil basis. Experimental data were fitted to a pseudo-second-order kinetic model, given by

$$\left(\frac{1}{\text{FFA}}\right)_{t=t} - \left(\frac{1}{\text{FFA}}\right)_{t=0} = \frac{k_r \cdot \rho^2 \cdot 3600 \cdot C_{\text{cat}}\, t}{M_w} \qquad [4]$$

in which k_r is the specific catalyst activity in $m^6/(mol \cdot kg_{cat} \cdot s)$; C_{cat} is the weight concentration of catalyst on oil basis (kg/kg); ρ is the oil density (kg/m^3); M_w is the fatty acid molar weight (kg/mol).

Determination of the free fatty acid content and composition by means of GLC (free fatty acid total, FFAT). The oils and fats were dissolved in a mixture of diethyl ether/heptane (1:1). A known amount of a pentanoic acid (C_5), heptanoic acid (C_7), and tridecanoic acid (C_{13}) mixture, dissolved in heptane, was used as an internal standard and added to the oil mixture. The solution was then applied to an aminopropyl column, which allows the separation of free fatty acids from the mono-, di-, and triglycerides. The composition and quantity of the fatty acids were determined by gas chromatographic (GC) analysis. Detection was carried out by means of a flame-ionization detector.

Peak identification was based on the comparison of retention times with reference compounds. Quantification was based on the internal fatty acid standards, namely C_5 (for C_3 and C_4), C_7 (for C_6, C_8, and C_9) and C_{13} (for C_{10} to C_{12}, C_{14} to C_{22}). The detector response varied with the fatty acid chain length, and therefore all values were corrected according to response factors obtained from reference standards containing C_3 to C_{22} fatty acids.

Triglyceride fatty acid composition (TFC) by capillary GLC. Fatty acid methyl esters were prepared from the oils and fats after alkaline hydrolysis, followed by methylation in methanol with BF_3 as catalyst. The fatty acid methyl esters were analyzed as described previously (20).

RESULTS AND DISCUSSION

Purification of the lipase isozymes. The cation-exchange step using S-Sepharose media considerably improved the purity of the lipase preparation by removing much of the nonlipase protein together with a large amount of colored material, but it did not separate lipases A and B, and the lipase activity eluted as a single peak (data not shown). This result contrasts with the complete separation of lipases A and B obtained originally using this medium (7). It was necessary to add an additional butyl-Sepharose step to obtain a complete separation of the two lipases. Three well-separated peaks of protein were eluted from this column, the final one eluting only with 75% ethylene glycol in 50 mM citric acid subsequent to completion of the salt gradient. According to isoelectric focusing, the first peak appeared to be a relatively complex mixture of proteins (perhaps representing a single lipase protein with different levels of glycosylation), but it contained only lipase A. The latter was checked by determining the specificity factor ($\Sigma \approx 0.6$). The second peak consisted chiefly of lipase B and had a specificity value comparable with the GMO-obtained lipase B ($\Sigma \approx 30$), indicating that it was not contaminated with significant quantities of lipase A; after isoelectric focusing there was only one strongly staining band of protein corresponding to this peak. The third peak was eluted with 75% ethylene glycol in citric acid (pH 5.3) and it contained a mixture of proteins with no detectable lipase activity. The purified lipase was largely free of contaminating proteases, as was checked with casein–resorufin as a substrate (data not shown). The lipase preparation was kept refrigerated, and was stable for several months.

It appears from Table 1 that the purification yields are excellent (>90% after three chromatography steps). However, some lipase activity (50–100 kLU) did not bind to the butyl-Sepharose column even under high salt conditions, so it is possible that the activity measured in the original culture supernatant does not equate with the total amount of lipase present, perhaps due to the presence of lipase inhibitors or modulators. An alternative suggestion is that the lipase activity did not increase through the removal of inhibitors during the purification procedure but through the action of proteases on prolipases present in the original supernatant.

Hydrophobic interaction chromatography of various sorts has been applied previously to the separation of lipases from various microbial sources, including *G. candidum* and *Penicillium camembertii*. Butyl-Toyopearl (16–18), methyl-Bio-Rad (19), phenyl-Sepharose (12), and octyl-Sepharose (14) have been used to purify *G. candidum* lipases within a three-step procedure. Those lipases that included either butylated or methylated media appear best able to resolve the key lipase specificities, and it is possible that this step as used here could be optimized further through alterations in pH or choice of ligand. Such optimization might also obviate the need for one or both of the ion exchange chromatography steps; as in this process, removal of nonlipase protein and colored material is relatively unimportant. In any upscaled process this optimization would reduce costs. However, through heterologous expression in *P. pastoris*, pure lipase B can now be obtained without cross-contamination with lipase A. Thus,

TABLE 1
Activities of the Various Fractions Obtained from the Three Purification Steps

Separation step	Total lipase activity (kLU)	Yield (%)
Culture supernatant	245	100
Q-Sepharose	239	97.7
S-Sepharose	222	90.4
Butyl-sepharose	223	91.1
of which		
1st peak: lipase A	47 (47[a])	19.3
2nd peak: lipase B	176 (70[a])	71.8

[a]The amount of lipase in mg, assuming a specific lipase activity of 1000 and 2500 LU/mg for lipase A and B, respectively, as reported earlier (Ref. 7).

only lipase concentration and diafiltration is required prior to use (11).

Lipase specificity. As described above, three batches of non-GMO GCB lipase (GCB1 to GCB3) and one batch of GMO lipase (GMO-GCB) were obtained by "in-house" fermentation and purification. Each of these lipases was tested for its specific hydrolysis of sunflower oil. In these experiments the composition of the free fatty acids released by the enzyme was determined as a function of the degree of hydrolysis. A summary of all results thus obtained is plotted in Figure 1. This figure shows that the SAFA content of the free fatty acids is significantly reduced compared to the original sunflower oil. Using either the GMO or non-GMO GCB lipases and with 12% w/w in the starting material, a SAFA content of 0.5–1% w/w is obtained in the free fatty acid fraction (Table 2).

The specificity factor for both GCB lipases was determined by fitting the experimental data to the fatty acid mass balances as described in the Materials and Methods section. From this analysis the GCB specificity factor was found to be approximately 30–35, which is in agreement with data reported previously (21). The dotted line in Figure 1 reflects the theoretical SAFA content of free fatty acids obtained from SF hydrolysis for an enzyme specificity factor of 33. It can be concluded from the figure that this specificity is true for GCB2 and GCB3 as well as the GMO lipase. Batch GCB1 shows a noticeably lower specificity, probably due to contamination of the preparation with the nonspecific lipase A.

Figure 1 also shows that the high specificity factor is maintained up to degrees of hydrolysis as high as 70% w/w. This result is in contrast to literature data (12,15) that showed that the specificity factor of a number of GC lipases rapidly decreases with an increase in the degree of hydrolysis. The purity of the lipase preparations used by Baillargeon *et al.* (15) is rather unclear, the Amano GC-4 lipase described by Hedrich *et al.* (12) is claimed to consist of pure lipase B. Analysis by electrophoresis revealed the presence of four isozymes that differ only in their degree of glycosylation (12,13), which should not affect the specificity (7). A comparison of their results with the current work (Fig. 1) suggests that either the GC-4 lipase preparation reported by Hedrich *et al.* (12,13) was impure (i.e., containing both the A and B isozyme), or the degree of glycosylation does affect the lipase B specificity and stability. A comparison of lipases from *G. candidum* CMICC 335426 indicates that lipase B has a lower stability than the A isozyme (Fellinger, A.J., E. Cationi, and R.M.M. Diks, unpublished data). Consequently, the presence of both lipases in previous reports (12,15) could explain the decrease of the overall specificity factor as a function of the degree of hydrolysis. Due to a more rapid loss of lipase B, the A isozyme becomes the predominant lipase in the mixture, displaying an average specificity factor of approximately 4. A similar specificity factor is shown in Figure 1 for all crude lipase preparations, which contain high proportions of lipase A (an enzyme specificity factor of 3–4 results in a SAFA content of 3–5% w/w in the free fatty acids, with up to 50% hydrolysis).

Effect of the lipase concentration. To reduce process costs, maximum lipase efficiency is required. Therefore the effect of the lipase input was investigated in a series of hydrolysis experiments, using various batches of GCB lipase. A typical plot of the FFA content vs. the reaction time at various lipase concentrations is shown in Figure 2 for batch GCB3. Obviously the initial rate of hydrolysis increases once the lipase concentration (expressed as LU/g oil) is increased. However, hydrolysis seems to stop after approximately 6–8 h of reaction, independent of the amount of lipase added. As a result

TABLE 2
Fatty Acid Composition of the Sunflower Oil (SF) and the Fatty Acid Fraction Obtained by Selective Hydrolysis Using GCB Lipase

	SF[a]	FFA[b]
12:0	0.09	0.00
14:0	0.07	0.00
16:0	6.27	0.63
16:1	0.10	0.09
18:0	4.04	0.13
18:1	22.30	27.90
18:2	64.90	71.20
18:3	0.07	0.00
20:0	0.25	0.00
20:1	0.12	0.00
22:0	0.69	0.00
24:0	0.23	0.00
SAFA[c]	11.74	0.80

[a]Triglyceride fatty acid composition (TFC) analysis.
[b]Free fatty acid total (FFAT) analysis.
[c]Saturated fatty acids.

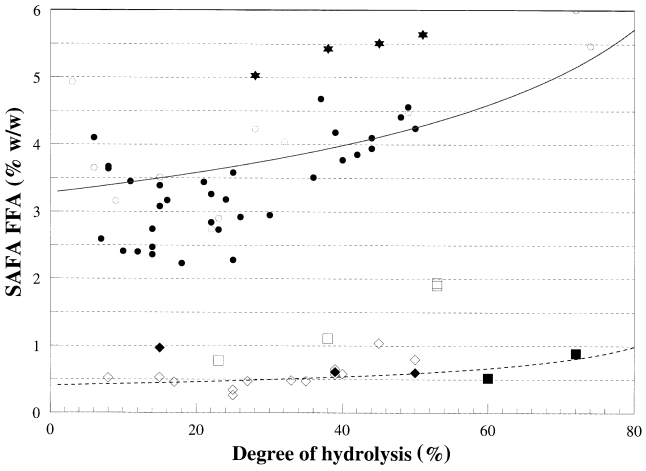

FIG. 1. The saturated fatty acid (SAFA) content of the free fatty acid (FFA) fraction obtained by specific hydrolysis of sunflower oil (SF) using *Geotrichum candidum* lipase B (GCB) batches of various origins and purity. GC4: crude preparation ex Amano (✶); GCA/B: lipase A and B from strain CMICC 335426 immobilized on Accurel (○); GCB 1–3: purified lipase obtained from the fermentation of strain CMICC 335426 (□, ◆, ◇, respectively); GCB1-crude: the lipase mixture before purification (●); GCB-genetically modified (GMO): the pure lipase B obtained by heterologous expression of the corresponding gene in *Pichia pastoris* (■). The drawn lines represent the model curves using a specificity factor of 4 (solid line) and 33 (dashed line).

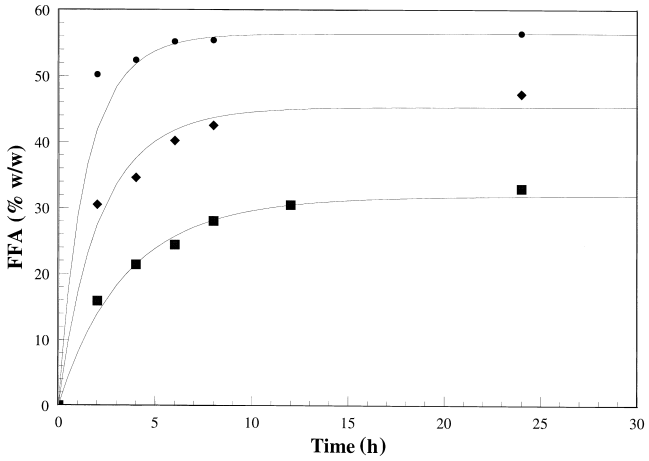

FIG. 2. Fatty acid content vs. time during the hydrolysis of SF using GCB3 lipase at three different lipase concentrations (concentration in olive oil units per gram of oil); SF/water ratio = 1:1 v/v. Symbols represent experimental data (■, 30 LU/g; ◆, 60 LU/g; ●, 120 LU/g); solid lines model the data according to Equation 6 in text. See Figure 1 for abbreviations.

TABLE 3
Kinetic Data Describing the Hydrolysis of Sunflower Oil by *Geotrichum candidum* Lipase (batch GCB3) According to a Two-Parameter Model

Lipase concentration[a] (LU/g_{oil})	Initial FFA concentration[b] (% w/w)	k_0[c] (g_{FFA}/g_{oil}·h)	k_d[c] (1/h)
30	0	0.13	0.30
60	0	0.23	0.38
120	0	0.45	0.53
30	0	0.21	0.39
30	5	0.16	0.30
30	15	0.11	0.29
30	30	0.09	0.32
30	35	0.02	0.40
Average[d]		—	0.3 (33%)[e]

[a]Effect of the lipase concentration as shown in Figure 2.
[b]Effect of the initial FFA content as shown in Figure 4.
[c]Initial rate of hydrolysis (k_0) and lipase deactivation constant (k_d) according to Equation 4 in text; half-life time ($t_{0.5}$) relates to the deactivation constant as $k_d = \ln 2/t_{0.5}$.
[d]Average deactivation constant is based on 40 individual determinations.
[e]95% confidence interval.

of this rapid deactivation process, the final degree of hydrolysis also becomes a function of the lipase input.

A pseudo-first-order kinetic model was used to describe the hydrolysis process. However, in contrast with literature data (22), this model could not describe the FFA concentration profiles as a function of time. Therefore, the model was extended by incorporation of a first-order time-dependent lipase deactivation process according to:

$$k = k_0\, e^{-k_d \cdot t} \qquad [5]$$

in which k_0 is the initial rate of hydrolysis (initial activity) [$g_{FFA}/(g_{oil} \cdot h)$] and k_d is the lipase deactivation constant (1/h).

Solving the resulting differential equation shows that the degree of hydrolysis with time can be described by

$$FFA_t = 1 - \exp\left[\frac{k_0}{k_d}\left(e^{-k_d \cdot t} - 1\right)\right] \qquad [6]$$

By fitting this model to the FFA production curves, using SAS software (SAS Inst. Inc., Cary, USA) the initial rate of hydrolysis (k_0) and the rate constant reflecting lipase activity decay (k_d) were determined. As illustrated in Figure 2 this model could adequately describe the time course of fatty acid production. Table 3 lists some results obtained according to this model.

In Figure 3 the k_0 data are summarized for various batches of GCB lipase. This plot shows a linear correlation between the initial rate of hydrolysis and the lipase input. Apparently interfacial mass transfer is not limiting in the system (23), even though the oil/water emulsion was not initially stabilized by any emulsifier or detergent.

The lipase deactivation constant, as defined above, appears rather independent of the reaction conditions, as will be described below. An average value of 0.3–0.4 h^{-1} was obtained, which reflects an apparent half-life time of the lipase of only 2 h (Table 3).

Product inhibition. So far in this paper, the loss of lipase activity has been described as a function of time only. However, product inhibition (i.e., free fatty acid inhibition) may also be involved here (24). In order to quantify this effect, experiments were carried out in which the initial FFA concentration was varied between 0 and 35% w/w.

Figure 4 shows the net production of free fatty acids with time. These results indeed confirm that product inhibition does occur. The effect of the FFA concentration on the initial rate of hydrolysis was determined by fitting the kinetic model to these net FFA-production curves (Fig. 4). The results are shown in Figure 5, which shows the specific lipase activity for both the GMO and the non-GMO lipases. No difference seems to exist between the inhibitory effect of free fatty acids for both lipases. By fitting these data to a general expression

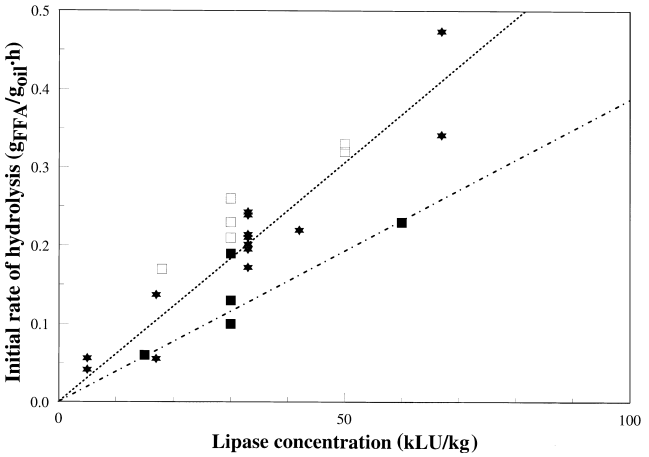

FIG. 3. Initial rate of SF hydrolysis vs. the input of the various batches of GCB lipase tested. Specific rate constants: 6.1 $g_{FFA}/(kLU \cdot h)$ (GCB1, □; GMO, ✶) and 3.9 $g_{FFA}/(kLU \cdot h)$ (GCB3, ■). See Figure 1 for abbreviations.

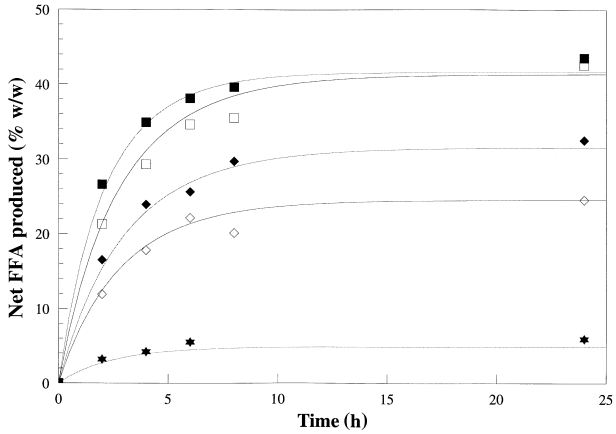

FIG. 4. Net FFA production during SF hydrolysis in the presence of various amounts of FFA added to the starting oil. Symbols represent experimental data (■, 0%; □, 5%; ◆, 15%; ◇, 30%; ✶, 35%); solid lines represent the model described by Equation 6 in text. See Figure 1 for abbreviations.

for product inhibition ($r/r_0 = K_i/(K_i + \text{FFA})$, in which K_i is the inhibition constant and FFA the free fatty acid fraction), an inhibition constant of approximately 0.2 is obtained. It should be noted that addition of calcium salts during the hydrolysis may reduce lipase inactivation through soap formation with the free fatty acids and subsequent precipitation.

Inhibition by monoglycerides did not occur, and the hydrolysis process was not affected even at initial monoglyceride concentrations of 25% w/w (data not shown). Apparently these intermediates are hydrolyzed as rapidly as triglycerides.

It should be noted that the two-parameter model for hydrolysis presented earlier does not explicitly take into account FFA inhibition. However, a three-parameter model incorporating both FFA inhibition and lipase deactivation could not be fitted, due to insufficient experimental data.

Lipase stability. During hydrolysis the free fatty acid content varies between zero and approximately 60% w/w. Though the resulting FFA inhibition (Fig. 5) can account for a considerable reduction in the rate of hydrolysis (approximately a factor of 4), it cannot fully explain the rapid loss in activity observed after 6–8 h. Furthermore, experiments in which the lipase was added in sequential doses at time intervals of 6 h showed that on addition of fresh lipase the rate of hydrolysis is initially restored (data not shown). This implies that the original lipase has been deactivated rather than inhibited. Moreover, after 6–8 h no residual lipase activity was measured in the water phase as such, nor was any activity found in the interface layer. The latter was checked by replacing the hydrolyzed oil phase after batch centrifugation and decanting (thereby leaving the interface layer in the water phase containing the lipase). Displacement of the lipase from the interface (25) was therefore not considered a major contributor to the loss of lipase activity during hydrolysis. All the foregoing results indicate that, apart from product inhibition, rapid lipase deactivation takes place, although the mechanism by which this occurs is unknown.

Incubation of the non-GMO lipase in potassium phosphate buffer (50 mM) at pH 5, 6, and 8 did not result in any significant loss of lipase activity below 40°C over a period of 6 d (data not shown). Similar results were reported for the GMO lipase (11). Moreover, as shown in Figure 6, pH did not affect the rate of oil hydrolysis, either. The latter is in contrast to literature data that indicate an activity optimum around pH 9, with no activity being found below pH 6 (11). Nevertheless, at each pH, rapid lipase deactivation is observed. Effects of temperature or pH on the lipase stability were not anticipated within the operating window applied.

The effect of mixing was also addressed. It was hypothesized that in the biphasic hydrolysis system the continuous coalescence and breakup of oil droplets could lead to enhanced lipase deactivation. However, this was not supported by the experimental data. Varying the stirring speed in a 100-mL crystallizer equipped with gate stirrer between 66 rpm

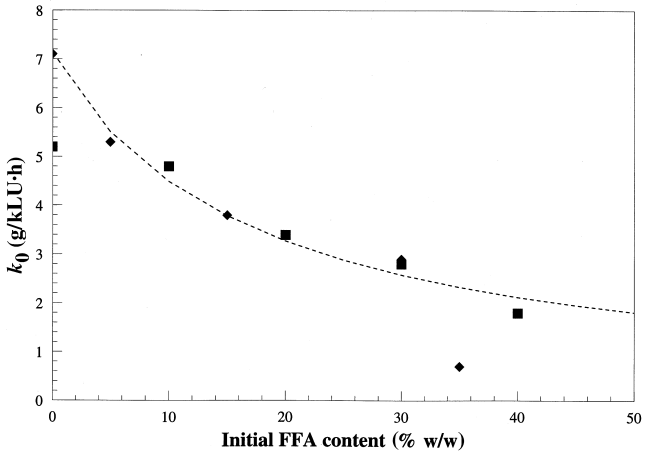

FIG. 5. The specific activity vs. the initial FFA content of the oil, using the GCB3 (non-GMO, ◆) as well as the GMO (■) GCB lipase. Dotted line represents the inhibition model as described for K_i = 0. 2 (see text). See Figure 1 for abbreviations.

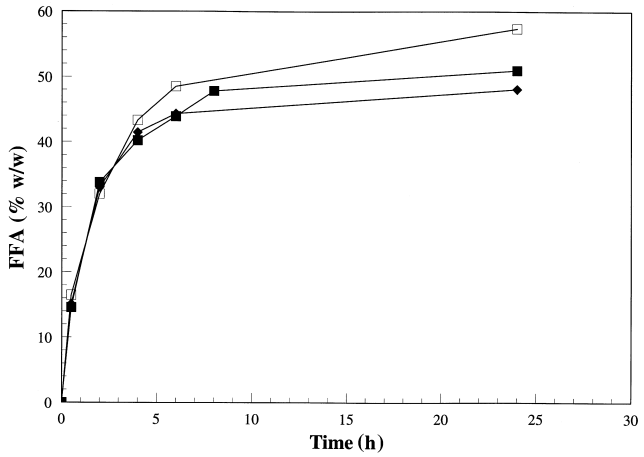

FIG. 6. Effect of pH on the hydrolysis of SF. ■, pH = 6; □, pH = 8; ◆, unbuffered. See Table 1 for abbreviation.

("mild"; two phases) and 750 rpm ("vigorous"; homogeneous dispersion) had no effect on the rate of hydrolysis observed.

As none of the system parameters described above appeared to be involved in the rapid deactivation of the GCB lipase, it is hypothesized that this phenomenon apparently is an unavoidable and intrinsic property of this enzyme, requiring increased amounts of lipase to obtain a high degree of oil hydrolysis.

Enzymatic reesterification. After separation of the free fatty acid fraction by batch distillation, the FFA were esterified with glycerol using *R. miehei* lipase immobilized on Duolite. A 3:1 FFA/glycerol molar ratio was used in all experiments.

As the immobilized catalyst is known to be fairly stable at high temperatures, the reesterification experiments were carried out in the range of 30–70°C. As shown in Figure 7, the rate of reesterification was maximal at 60°C. Above this temperature the catalyst activity (i.e., the specific rate constant) decreases again. By fitting the experimental data to the pseudo-second order kinetic model as described in the Materials and Methods section, a rate constant under optimal conditions of 3×10^{-9} $m^6/(mol \cdot kg \cdot s)$ was obtained.

The results plotted in Figure 7 show that a high degree of conversion can be obtained in approximately 24 h at 60°C. These reaction conditions were therefore selected for a series of experiments aiming to assess the stability of the catalyst. In each experiment the FFA content was followed during reesterification. Once 5% w/w FFA was achieved, stirring was stopped, and the oil was removed by decanting after the catalyst had settled; then a new batch of FFA and glycerol were added. The results plotted in Figure 8 show that the activity of the catalyst slowly decreases over the time course of the repeated process. After 9 cycles, the degree of conversion obtained after 24 h had dropped from >95 to 84% w/w. These results indicate that a significant improvement of process economics can be expected from reuse of the biocatalyst.

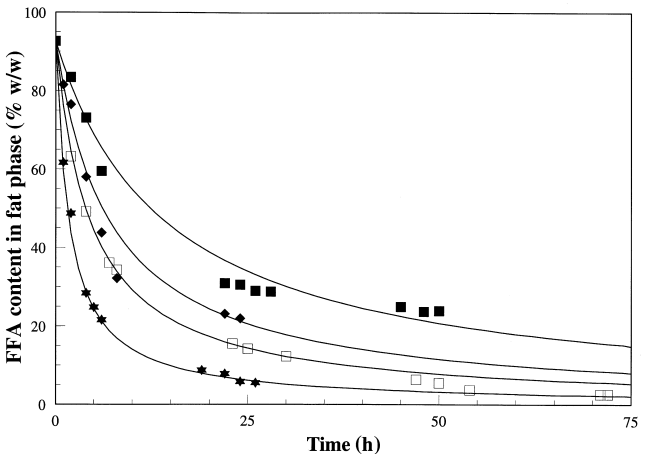

FIG. 7. Effect of temperature on the reesterification of FFA and glycerol. The symbols represent experimental data (■, 30°C; ◆, 40°C; ✶, 60°C; □, 70°C). The solid lines are drawn according to a pseudo-second-order kinetic model. Molar ratio FFA/glycerol 3:1. See Figure 1 for abbreviation.

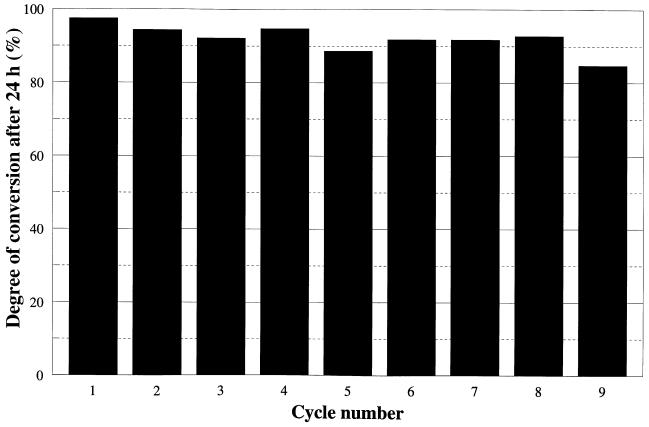

FIG. 8. Reuse of the Lipozyme biocatalyst in a series of nine batch reesterification experiments at 60°C.

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