Borago officinalis Oil: Fatty Acid Fractionation by Immobilized *Candida rugosa* Lipase

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ABSTRACT: γ-Linolenic acid (*Z,Z,Z*-6,9,12-octadecatrienoic acid), a very important polyunsaturated fatty acid is found in the free fatty acid fraction prepared by the hydrolysis of borage oil. Our aim was to enrich this fraction in γ-linolenic acid using selective esterification. *Candida rugosa* lipase was used as catalyst after immobilization on the following ion-exchange resins: Amberlite IRC50, IRA35, IRA93, and Duolite A7, A368, A568. In every case, immobilization modified the lipase's specificity: palmitic, stearic, oleic, and linoleic acids were preferentially esterified compared to γ-linolenic acid, thus allowing a γ-linolenic acid enrichment of 3.0.

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KEY WORDS: *Borago officinalis, Candida rugosa,* enzyme immobilization, fatty acid enrichment, γ-linolenic acid, lipase.

Candida rugosa lipase (ex-*Candida cylindracea*, E.C. 3.1.1.3) has been widely used for different reactions such as hydrolysis in aqueous (1,2) and organic (3) media, interesterification, transesterification, and ester synthesis (4–6). Lipases from *C. rugosa* (4,6,7) and *Rhizomucor miehei* (6–8) have the same selectivity toward unsaturated fatty acids having the first double bond in the δ-4, 6, or 8 position.

Many authors report the advantages of lipase immobilization: increased stability of the enzyme in organic solvent (9,10) and its possible reuse (11–13).

γ-Linolenic acid is important biologically, being an indispensable precursor for the synthesis of long-chain unsaturated fatty acids having structural functions in cellular membranes and of eicosanoid hormones (14–16). It also affects cholesterol metabolism by reducing total cholesterol (more efficiently than linoleic acid) and low-density lipoprotein cholesterol as well as increasing high-density lipoprotein cholesterol. Like other n-6 fatty acids, γ-linolenic acid has an antiaggregating effect on platelets.

In some pathological conditions (alcohol abuse, stress, aging, insulin-dependent diabetes), the activity of Δ6 desaturase, required in γ-linolenic acid synthesis from linoleic acid, can decrease significantly (16–18). It is then necessary to give the patient exogenous γ-linolenic acid since intakes through food are usually low.

Among the plants screened for γ-linolenic acid, three families are rich sources: Onagracea, Saxiferagacea, and Boraginacea. Three species gave birth to industrial developments: onagre (*Œnothera biennis*), blackcurrant pip (*Ribes nigrum*), and borage (*Borago officinalis*) (19–21). γ-Linolenic acid as a proportion of the total fatty acids ranges from 8 to 11% in onagre, 14 to 19% in blackcurrant pips, and 18 to 26% in borage.

Recent research has been conducted in two areas: finding new natural sources of this acid and developing processes to enrich the existing ones. Urea crystallization is the most used method to separate saturated and unsaturated fatty acids. Some authors (22–24) extracted γ-linolenic acid from blackcurrant pip oil (containing 18% of this acid) with an enrichment approaching 80%. With an additional purification step by preparative high-performance liquid chromatography on reversed-phase C18, the acid was obtained with a purity higher than 95%. The production of this fatty acid can be achieved by bioconversion of rapeseed oil (25).

Enzymatic enrichment of oils containing γ-linolenic acid can be carried out either by selective hydrolysis (26) or by selective esterification (27). We report here γ-linolenic acid enrichment of borage oil using the second method with immobilized and nonimmobilized *C. rugosa* lipase.

EXPERIMENTAL PROCEDURES

Materials. Borage seeds were given by the Bertin Company (Courbevoie, France). *Candida rugosa* MY lipase is from Meito Sangyo Co. Ltd. (Tokyo, Japan). Cation exchange resin Amberlite IRC50 was from Aldrich (Saint Quentin Fallavier, France); anion exchange resins Duolite A7, A368, Amberlite IRA35, and IRA93 were from Supelco (Saint Quentin Fallavier, France). Duolite A568 was a generous gift from Rohm and Haas (Chauny, France). The anion exchange resins differ in their functional groups**,** which are either secondary or tertiary amino groups. 1-Butanol rectapur was from Prolabo (Fontenay-sous-bois, France).

All results are the mean value of three experiments.

Methods. Borage oil was extracted from crushed seeds, using *n*-hexane in a Soxhlet extractor for 6 h. After extraction, *n*-hexane was removed in a Büchi (Flawil, Switzerland)

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rotary evaporator. The mixture of fatty acids used for the esterification experiments arises from the complete chemical hydrolysis of the oil, carried out using boiling 1 N KOH/EtOH for 1 h under a nitrogen atmosphere to reduce the risk of double bond oxidation. After acidification with 1 N HCl and hexane extraction, the fatty acids contained no trace of partial glycerides as shown by thin-layer chromatography. The methylated fatty acid mixture was analyzed by gas chromatography, as described below, and showed the following composition: linoleic acid (C18:2n-6), 40.0%; γ-linolenic acid (C18:3n-6), 22.8%; oleic acid (C18:1n-9), 18.5%; palmitic acid (C16:0), 11.0%; stearic acid (C18:0), 3.9%; and arachidic acid (C20:0), 3.8%.

Enzyme activity was measured using a Radiometer (Copenhagen, Denmark) pH-stat, equipped with an ABU 12 automatic burette, a TTT2 titrator, a thermostated glass vessel, and a Titrigraph recorder. Measurements were made at pH 7, 37°C under a nitrogen flow. In the thermostated glass vessel were placed 40 mL of pH 7 buffer (35 mM Tris/HCl, 0.1 M NaCl, 5 mM $CaCl_2$), 250 µL of tributyrin (Fluka, Villeneuve Saint Georges, France), and 30 µL of the enzyme solution or 10 mg of immobilized enzyme preparation. The butyric acid liberated during hydrolysis was quantified by titration using a 35 mM sodium carbonate solution.

Esterified fatty acids were analyzed using a CR 4A Shimadzu (Tokyo, Japan) gas chromatograph equipped with a split injector, a flame-ionization detector, and a CR 3A Shimadzu integrator. The methyl and butyl esters were separated on a BPX 70 (0.22 mm i.d. × 25 m × 0.25 µm) column (SGE, Saint Quentin Fallavier, France). Nitrogen was used as carrier gas. System temperatures were 185°C for the column oven, 220°C for the injector, and 260°C for the detector. Separations were made isothermally.

Immobilization of *C. rugosa* lipase on Amberlite IRC 50 was performed by dissolving 2 g of *C. rugosa* lipase powder in 20 mL of 50 mM sodium acetate buffer at pH 3.5. This solution was centrifuged at 8000 rpm for 15 min at 4°C. The supernatant was desalted on a G25 Sephadex column (Pharmacia, Uppsala, Sweden) using the same buffer. The active fractions were pooled (volume, 30 mL) and passed through 5 g of IRC 50 Amberlite resin previously soaked in the buffer. The decrease in enzyme activity in the solution allowed monitoring of the immobilization process, which was stopped when activity reached a constant value. The enzyme-resin complex was rinsed with buffer and air-dried on filter paper. Immobilization on the anion exchange resins (Duolite A7, A568, A368 and Amberlite IRA35, IRA93) used the same procedure except for the buffer which was 10 mM potassium phosphate at pH 7.

The percentage enzyme immobilization was calculated as follows:

$$\%\ \text{immobilization} = \frac{C}{A-B} \times 100 \qquad [1]$$

where A is the specific activity of the initial solution, B is the specific activity of the residual solution, and C is the specific activity of the enzyme-resin complex. Enzyme activity in international units (I.U.) is the microequivalents of butyric acid liberated per minute, and the specific activity is the microequivalents of butyric acid liberated per minute, per gram of immobilized enzyme preparation.

The percentage of fixed enzyme is given by the formula

$$\%\ \text{immobilization} = \frac{A-B}{A} \times 100 \qquad [2]$$

In this case, the activity of the enzyme-resin complex is not considered because it is not proportional to the quantity of enzyme fixed.

Esterification reactions were conducted at different temperatures (30, 40, 50°C) in an agitated bath (250 rev/min) in 5 mL flasks. The reaction mixture was composed of 0.5 g (1.80 mmoles) of fatty acids obtained by alkaline hydrolysis of borage oil and 0.135 g (1.80 mmoles) of 1-butanol. When this mixture reached the desired temperature, 520 I.U. of nonimmobilized *C. rugosa* lipase or of enzyme-resin complex was added. Following incubation, the nonesterified fatty acids were then esterified by diazomethane prepared in the laboratory by a literature method (28) in order to allow their analysis. The methyl and butyl esters were analyzed by gas–liquid chromatography (GLC).

Quantitation was by area integration of the butyl and methyl ester peaks. The reaction kinetics were followed by monitoring the formation of fatty acid butyl esters as described by Rangheard *et al.* (6). The following equation was used :

$$C_n = \left(A_b/PM_b\right) \Big/ \left(A_b/PM_b + A_m/PM_m\right) \qquad [3]$$

where C_n is the conversion rate of a chosen fatty acid, A_b and A_m are the peak areas of corresponding butyl and methyl esters, and PM_b and PM_m are the molecular mass of these esters.

Reactions catalyzed by lipases in organic solvent, such as ester synthesis, follow Michaelis-Menten kinetics with the formation of an intermediate acyl-enzyme complex (29). When two substrates are present in the reaction mixture, they compete for the active site of the enzyme. A simple competition factor (α) has been proposed by Deleuze *et al.* (29) as defined in Equation 4:

$$\alpha = \log\left(1 - C_{\text{ref}}\right) \Big/ \log\left(1 - C_n\right) \qquad [4]$$

where C_{ref} is the conversion rate of the substrate chosen as reference and C_n is the conversion rate of the other substrate. By definition, the competition factor of the reference substrate is 1, and all other competition factors are greater than 1. Rangheard *et al.* (6) defined 1/α as a convenient measure for an enzyme's specificity for a given substrate.

RESULTS

Immobilization of C. rugosa *lipase*. Resins suspended in a buffer solution (pH 7 or 3.5) were put in contact with a solu-

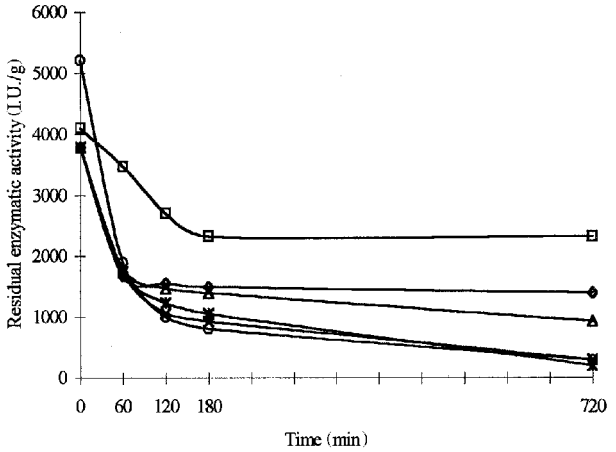

FIG. 1. Free enzymatic activity decrease during immobilization of *Candida rugosa* lipase on different ion exchange resins: Amberlite IRC50 (○; Aldrich); Duolite A7 (◇), A368 (△), A568 (×), IRA35 (✱), and IRA93 (□; Supelco).

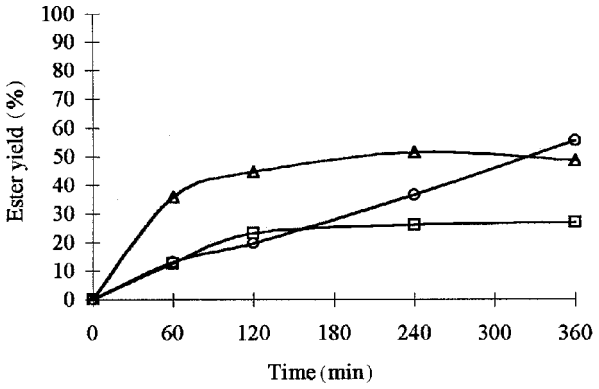

FIG. 2. Temperature dependence of the percentage of butyl esters formed during esterification of fatty acids from borage oil by 1-butanol, with 520 I.U. of nonimmobilized *Candida rugosa* lipase. 30°C (○); 40°C (△); 50°C (□).

tion of previously desalted *C. rugosa* lipase. Immobilization was followed by measuring the activity of the enzyme solution (Fig. 1). Enzymatic activity of the lipase solution decreased quickly for the Amberlite IRC50, IRA35, and Duolite A7, A568, A368 resins. For the Amberlite IRA93 resin, immobilization was slow, and little enzyme was bound.

These lipase complexes were tested with respect to hydrolysis and esterification. Table 1 summarizes the activity affixed to the resin with the observed activity in the hydrolysis and esterification reactions, together with immobilization yields.

The Amberlite resin IRC50 appeared to be the best support since it fixed the largest quantity of enzyme (immobilization yield was 85.4%), while displaying the highest activities for hydrolysis and for esterification. The Duolite A568 and A35 resins also gave good results. The enzyme activity was very variable, from 2000 to 5800 I.U./g. The activity of the complexes was not proportional to the amount of enzyme fixed except for Duolite A568 and Amberlite IRC50, IRA35. This result arises probably from a partial denaturation of the enzyme on some resins.

Esterification of borage fatty acids. The nonimmobilized *C. rugosa* lipase and the six enzymatic complexes were tested in an esterification of a mixture of fatty acids obtained from alkaline hydrolysis of borage oil with 1-butanol. The enzyme-resin complexes contained an average 15 wt% water (measured with a DL18 Mettler autotitrator according to Karl Fischer's method).

Selective esterification, using nonimmobilized lipase and a fatty acid/1-butanol equimolar ratio, was performed at different temperatures to learn the optimal temperature for lipase reaction (Fig. 2). The same number of enzyme units was used in each experiment. Above 40°C, the amount of fatty acids esterified leveled off after 90 min, probably owing to lipase inactivation above 40°C. Nevertheless, the reaction's initial speed at 40°C was greater than at 30°C, yielding 51.4% of esters formed in 240 min. But after 330 min, about 50% of the initial fatty acids were esterified at both temperatures. Furthermore, the greater difference in $1/\alpha$ between γ-linolenic and other fatty acids was obtained at 30°C, after a 360-min reaction (Fig. 3), giving the best γ-linolenic enrichment value with nonimmobilized *C. rugosa* lipase (Table 2).

Linoleic acid was the most esterified fatty acid and was therefore taken as reference for the $1/\alpha$ calculation. When this

TABLE 1
Specific Activities of Enzyme-Resin Complexes and Immobilization Yields

Resins	Theoretical activity immobilized,[a] $A - B$ (I.U./g)	Complex hydrolysis activity,[a] C (I.U./g)	Complex esterification activity (I.U./g)[b]	Immobilization yield (%)
IRA35	4270 ± 214	3200 ± 160	18.8 ± 0.9	75.0 ± 7.5
IRA93	2120 ± 106	1300 ± 65	15.8 ± 0.8	61.0 ± 6.1
A7	2850 ± 143	500 ± 25	3.1 ± 0.2	17.5 ± 1.8
A368	3400 ± 170	500 ± 25	11.7 ± 0.6	14.7 ± 1.5
A568	4160 ± 208	3000 ± 150	20.8 ± 1.0	72.1 ± 7.2
IRC50	5850 ± 293	5000 ± 250	30.1 ± 1.5	85.4 ± 8.5

[a]*A*, specific activity of the initial solution; *B*, specific activity of the residual solution; *C*, specific activity of the enzyme-resin complex.
[b]In this case, enzymatic activity is the number of ester microequivalents formed per minute and per gram of biocatalyst.

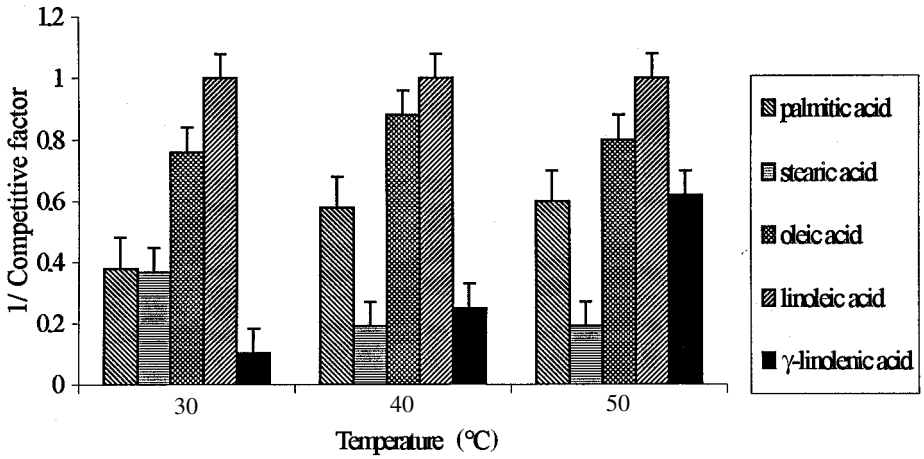

FIG. 3. Competition factors of borage oil fatty acids at different esterification temperatures using 520 I.U. of nonimmobilized *Candida rugosa* lipase, 360-min reaction time. Error bars represent error ratio.

ratio is high, the corresponding fatty acid is preferentially esterified.

Selective esterifications were carried out under the same conditions with the enzyme-resin complexes. Figure 4 shows that the percentage of esters formed varied from resin to resin and ranged from 50 to greater than 80%. The Duolite A7 complex yielded less than 5% of esters and can be considered as inactive. The Amberlite IRC50, with 88% conversion to esters, gave the best result.

As for the nonimmobilized lipase, linoleic acid had the highest conversion rate and was chosen as reference for the $1/\alpha$ calculation. γ-Linolenic acid was little esterified compared with the other fatty acids (Fig. 5), especially with Duolite A568 and Amberlite IRC 50 complexes ($1/\alpha = 0.04$ and 0.03, respectively; and $1/\alpha = 0.4$ to 1 for the other fatty acids). For these two complexes, enrichment of γ-linolenic acid was 2.7 and 3.0, respectively (Table 3), with a loss of 3.5 and 3.8% in weight. The maximal enrichment obtainable, considering that the free fatty acid fraction of borage oil contains 22.8% of γ-linolenic acid, is 4.4 (100/22.8 = 4.38).

DISCUSSION

The hydrophilic or hydrophobic character, the adsorption site density, and diffusion phenomena arising from molecular structure differ according to resin type. However, a resin's pore size is a key factor for enzyme adsorption. Immobilization can induce conformational changes in a lipase's tertiary structure and can reduce its catalytic activity. Reduction also can be explained by enzyme denaturation, which depends on immobilization temperature or pH. During synthesis or hydrolysis, partition effects cause different substrate concentrations at the complex surface and in the reaction mixture. These effects either increase or decrease the reaction speed, depending on the complexes involved.

These different effects may explain the enzymatic activities obtained in this study. Hydrolytic activity was always lower than the theoretically calculated value; this difference was even greater for esterification.

The lipase-resin complexes were tested with regard to fatty acid selectivity. Esterification reaction kinetics were followed by GLC. This method allowed the calculation of competition factors independent of physical and chemical conditions, es-

TABLE 2
γ-Linolenic Acid Enrichment by Nonimmobilized Lipase, Depending on Reaction Temperature

Temperature (°C)	Enrichment[a]	γ-Linolenic acid in the residual acid fraction (wt%)	Butyl γ-linolenate in ester fraction (wt%)
30	1.7 ± 0.1	39.0 ± 1.2	5.4 ± 0.2
40	1.2 ± 0.1	26.7 ± 0.8	9.0 ± 0.3
50	1.0 ± 0.1	23.9 ± 0.7	21.2 ± 0.6

[a]Percentage of γ-linolenic acid in the residual acid fraction over the percentage of γ-linolenic acid in the initial acid fraction.

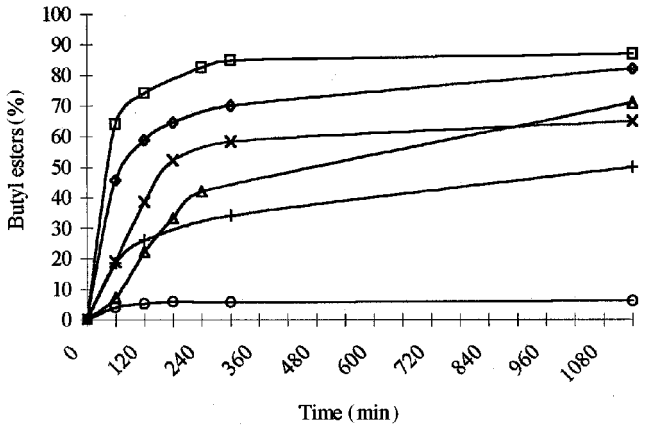

FIG. 4. Percentage of butyl esters formed at 30°C during esterification of fatty acids from borage oil by 1-butanol depending on the kind of resin used to immobilize *Candida rugosa* lipase. Resin IRA93 (△), A568 (◇), A7 (○), A368 (+), IRA35 (×), and IRC50 (□). For manufacturers see Figure 1.

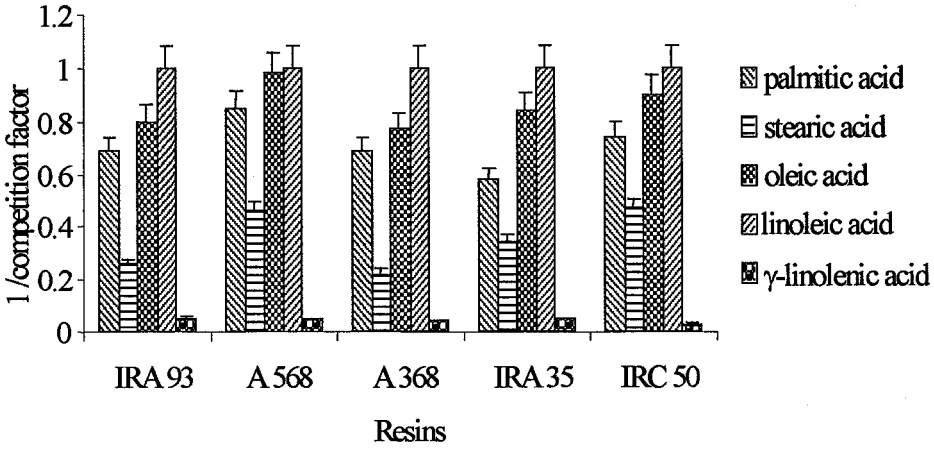

FIG. 5. Esterification competition factors of borage oil fatty acids at 30°C depending on the kind of resin used to immobilize *Candida rugosa* lipase. For manufacturers see Figure 1.

TABLE 3
γ-Linolenic Acid Enrichment at 30°C Depending on Resin Type Used for *Candida rugosa* Lipase Immobilization

Resin[a]	Enrichment[b]	γ-Linolenic acid in the residual acid fraction (wt%)	Butyl γ-linolenate in ester fraction (wt%)
IRA 35	1.9 ± 0.2	43.3 ± 1.3	2.6 ± 0.1
IRA 93	2.4 ± 0.2	54.7 ± 1.6	2.5 ± 0.1
A7	1.0 ± 0.1	22.8 ± 0.7	19.3 ± 0.6
A 368	1.4 ± 0.1	32.0 ± 1.0	1.5 ± 0.1
A568	2.7 ± 0.2	61.6 ± 1.9	5.0 ± 0.2
IRC 50	3.0 ± 0.3	68.4 ± 2.0	6.0 ± 0.2

[a]For manufacturers see Table 1.
[b]Percentage of γ-linolenic acid in the residual acid fraction over the percentage of γ-linolenic acid in the initial acid fraction.

pecially from the alcohol used, according to Rangheard *et al.* (6). This factor only reflects the enzyme's specificity toward a fatty acid during acyl-enzyme formation in organic media.

The results obtained show that nonimmobilized *C. rugosa* lipase prefers linoleic acid (C18:2n-3) but that palmitic, stearic, oleic, and γ-linolenic acids are also esterified. Therefore, γ-linolenic acid enrichment is very low. Yet lipase immobilization, perhaps by inducing conformational changes in the lipase, modifies its specificity toward a fatty acid, allowing efficient enrichment.

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