Factors Affecting the Acyltransfer Activity of the Lipase from *Candida parapsilosis* in Aqueous Media

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ABSTRACT: This study describes the influence of various factors on the hydrolysis and alcoholysis activities of the lipase from *Candida parapsilosis* (Ashford), Langeron and Talice in aqueous media. Optimal activities were obtained at 45°C and pH 6–6.5. The influence of the nature of the substrate on the temperature activity profiles was observed. Total or partial recovery of the activities was obtained when methanol was added to the enzyme extract after thermal denaturation. A tyrosin residue appeared to be necessary for lipase function. Magnesium was a required metal cofactor. These activities were optimal in the presence of high amounts of water (water activity > 0.9).
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KEY WORDS: Acyltransferase, alcoholysis, *Candida parapsilosis,* hydrolysis, lipase.

The lipase from *Candida parapsilosis* (CBS 604) has been described previously by Riaublanc *et al.* (1). This enzyme displays an acyltransfer activity in aqueous media (2). These reactions were carried out in biphasic liquid/liquid media, consisting of a rapeseed oil emulsion in an aqueous medium containing free enzyme and methanol in solution. In the presence of methanol in the aqueous phase, methyl esters were produced by alcoholysis of triacylglycerols and, under optimal conditions, hydrolytic activity was almost completely inhibited. Esters also were produced from other alcohols, such as ethanol, propanol-1, propanol-2, butanol-1, and butanol-2. The substrates for these reactions exhibit opposite polarities—the fatty ester used as an acyl donor is nonpolar and the acyl acceptor is polar (alcohol) or very polar (water). In such a system, any physicochemical change in the reactant medium could influence access of the substrates to the active site. This study showed the influence of lipase hydration and some other factors affecting the interface between the lipid substrate and the aqueous phase upon the acyltransfer activity of this lipase in aqueous media.

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MATERIALS AND METHODS

The yeast strain used was *C. parapsilosis* (Ashford) Langeron and Talice, CBS 604, obtained from the Centraalbureau voor Schimmelcultures (Yeast Division, Delft, Holland). All cultures were performed in Erlenmeyer flasks filled to 10% of their volume, incubated at 28°C, and shaken at 80 oscillations min^{-1} (amplitude 7 cm). The basal medium was medium G (3), adjusted to pH 6.5 with a 50-mM NaH_2PO_4/Na_2HPO_4 phosphate buffer. The carbon source was rapeseed oil (0.5% wt/vol) sterilized by autoclaving at 110°C for 30 min. The culture was defatted with three volumes of hexane at the end of the exponential growth phase. The cells were then harvested by centrifugation (7000 × *g*, 15 min). The supernatant contained the enzyme, which was then purified according to Riaublanc *et al.* (1). The lipid substrates were emulsified (5% wt/vol) in an aqueous poly(vinyl alcohol) solution (2% wt/vol) as described by Biehn and Ernsberger (4).

High-performance liquid chromatography (HPLC) was performed with a Spherisorb column ODS2 (250 × 4.6 mm, particule size 5 μm; Alltech Associates, Inc., Deerfield, IL). The chromatographic system was equipped with an ultraviolet detector, and absorbance was read at 210 nm. The solvent was acetonitrile/acetone (vol/vol). The flow rate was maintained at 0.8 mL/min for 6 min then increased from 0.8 to 2 mL/min in 4 min, maintained at 2 mL/min for 22 min, and was finally decreased to 0.8 mL/min in 4 min. The analysis was carried out at 30°C.

Enzyme assays were performed as follows: 200 μL of an emulsion of lipid substrate were added to 2 mL of 50 mM phosphate buffer (pH 6.5) containing the purified enzyme and, eventually, methanol. Unless otherwise specified, methanol concentration was 2.2 M, the reaction was carried out at 45°C for 15 min and was stopped by the addition of an ethanol/acetone/concentrated sulfuric acid (50:50:0.1, vol/vol/vol) mixture. The products were extracted with hexane. One enzyme unit (U) will hydrolyze 1 μmol fatty acids from rapeseed oil in one minute in the standard reaction conditions, in the absence of methanol. Determination of total free fatty acids was performed according to Van Autryve *et al.* (5). Methyl esters and diolein, dissolved in acetone after removal of the hexane under vacuum, were analyzed by HPLC.

Aliquots of enzyme solution were frozen in Eppendorf tubes at −80°C and freeze-dried for 12 h using a Christ Beta 1-8 apparatus (Christ, Osterode and Harz, Germany).

Water activity (a_w) was measured with a JEL20 (Novasina AG, Zürich, Switzerland) equipped with an enBSK4 cell. Trioleoylglycerol and oleic acid ethyl ester (99% pure) were purchased from Sigma Chemical Co. (St. Louis, MO). All reagents used were at least of analytical grade. Water was purified using a Milli-Q Plus apparatus (Millipore Co., Bedford, MA).

RESULTS

Three enzymatic reactions were studied—rapeseed oil hydrolysis, oleic acid ethyl ester hydrolysis, and rapeseed oil alcoholysis by methanol (methanolysis). The latter reaction was performed in the presence of 2.2 M methanol in the reactant medium. The pH of the reactant medium was adjusted with NaOH or HCl in order to avoid the use of various buffers which could influence the activities. The fatty acids released by the reaction (less than 2 μmol mL^{-1}) had no significant influence on the pH of the medium. Figure 1 shows that the enzyme catalyzed methanolysis over the same range as hydrolysis (pH 3–7) with a pH optimum at pH 6–6.5.

Hydrolysis activity after 15 min was maximal between 40 and 50°C, and decreased substantially when exposed to temperatures above 50°C (Fig. 2). The shape of the curve was unusual for temperatures lower than the optimum when the substrate was oleic acid ethyl ester; oleic acid ethyl ester hydrolysis activity between 25 and 50°C was higher than rapeseed oil hydrolysis activity under the same conditions. The peak was sharper for methanolysis, but the optimum was in the same range.

Residual enzymatic activity was studied in the standard reaction conditions when the extract was exposed to 45–65°C

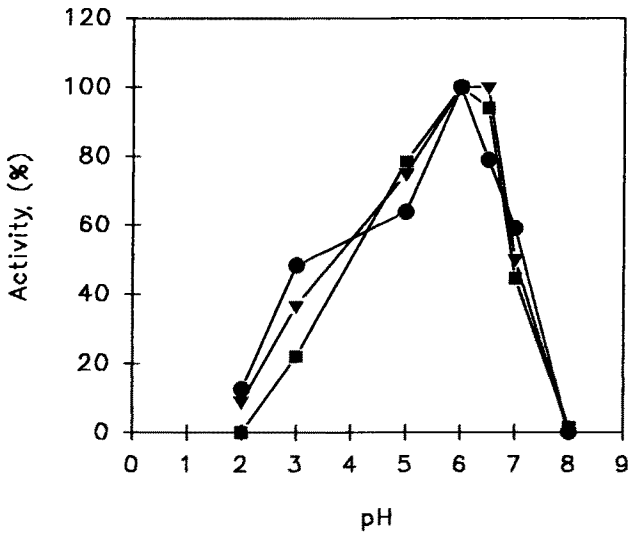

FIG. 1. Effect of pH on the acyltransfer activity. Results are expressed as percentages of the maximal activity for each reaction studied. ●, Rapeseed oil hydrolysis; ■, rapeseed oil methanolysis; and ▼, oleic acid ethyl ester hydrolysis.

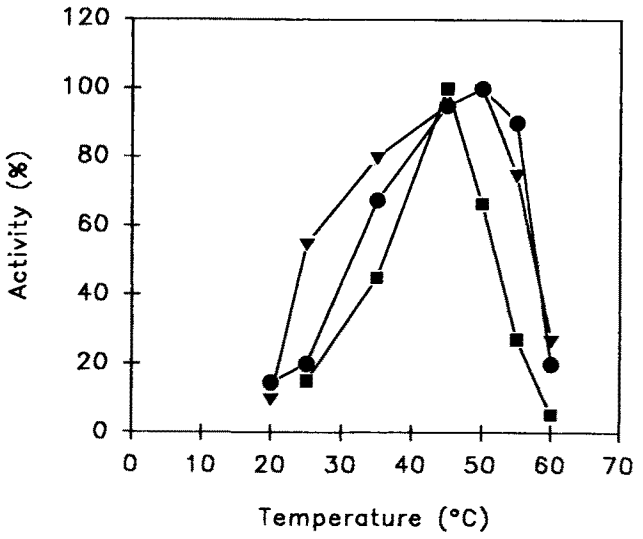

FIG. 2. Effect of temperature on the acyltransfer activity. Results are expressed as percentages of the maximal activity for each reaction studied. ●, Rapeseed oil hydrolysis; ■, rapeseed oil methanolysis; and ▼, oleic acid ethyl ester hydrolysis.

for different times and then cooled to 20°C for 15 min. Residual activities after thermal treatment are shown in Figure 3. The denaturation of the lipase was apparently rapid at temperatures higher than 50°C when the activity studied was rapeseed oil hydrolysis (Fig. 3A). Similar results were obtained using oleic acid ethyl ester. Denaturation profiles of both hydrolysis in the presence of methanol and methanolysis reactions were unusual (Fig. 3B and 3C). There was a total or partial recovery of the activities when methanol was added to the reactant medium. For example, the enzyme extract incubated for 40 min at 55°C lost 80% of its hydrolysis activity measured without methanol, but only 10 and 25% of its initial hydrolysis and alcoholysis activities, respectively, in the presence of methanol. Furthermore, the reactions performed in the presence of methanol appeared to be activated after being incubated for 30 to 40 min at 45°C.

In order to test the involvement of peculiar amino acids in the structure–function of the enzyme, the lipase from *C. parapsilosis* was incubated for 10 min at room temperature in the presence of various amino acid reactive reagents (Table 1). Hydrolysis and methanolysis were sensitive to the same effectors. *N*-Bromosuccinimide and I_2 were strong inhibitors, indicating a tyrosin residue was necessary to activity. No significant effect of the specific inhibitor of serine was observed. On the other hand, it has been shown that the inactivation of several lipases by serine-reactive reagents was only effective when the enzymes were activated by the presence of an interface or in organic solvents (6–8). In aqueous media, these reagents do not act on the enzymes. The presence of a serine residue in the active site of the lipase from *C. parapsilosis* therefore cannot be excluded.

The effect of ethylenediaminetetraacetic acid (EDTA) and various cations on the activities was the same as that de-

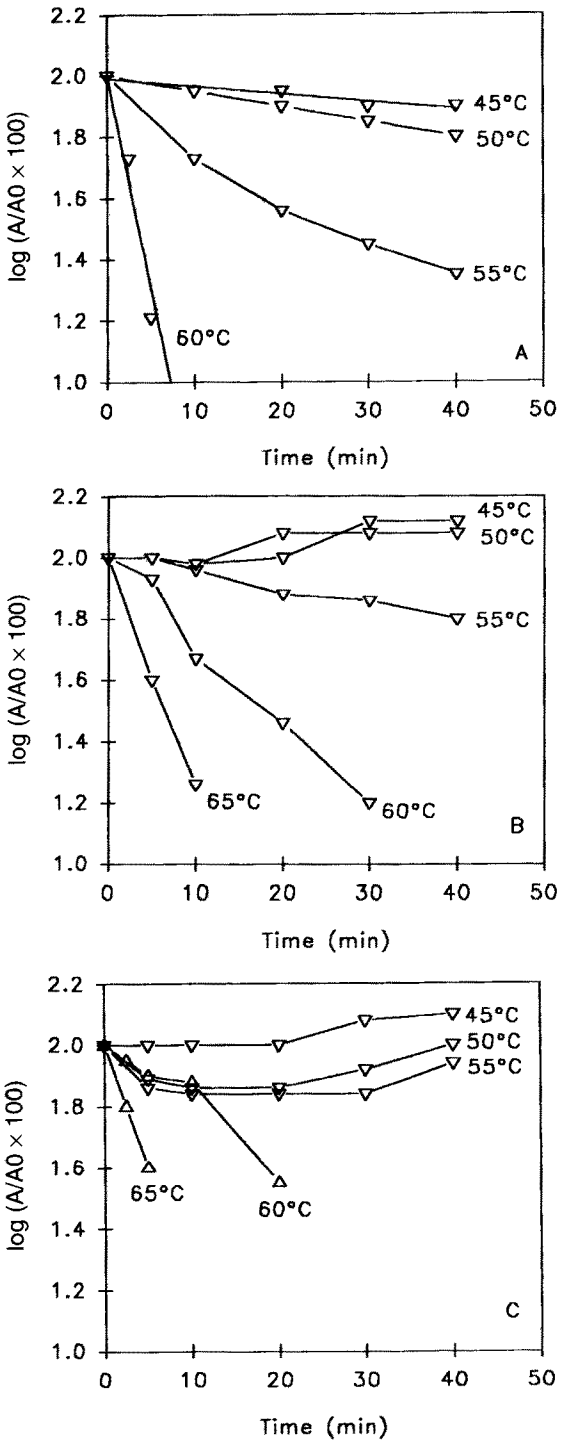

FIG. 3. Thermal denaturation: A, rapeseed oil hydrolysis; B, rapeseed oil methanolysis; and C, rapeseed oil residual hydrolysis in the presence of methanol. For (A/A0 × 100): A, activity after thermal treatment; A0, activity in the standard conditions.

scribed by Riaublanc *et al.* (1) concerning hydrolysis. Mg^{2+} was the only cation tested that effected the activities. Mg^{2+} enhanced the activities by 17%. Activities reduced by EDTA could be partially recovered by adding Mg^{2+}.

Dried enzyme extracts were prepared by lyophilization to determine the influence of water on catalytic activity. In order to preserve the activity of the enzyme, which was otherwise lost, the addition of various protecting substances was tested. The lyophilized enzyme extracts were rehydrated prior to the activity determination. Table 2 gives the initial kinetics of both hydrolysis and methanolysis reactions before and after lyophilization. The best lyoprotectants were glycerol (20% wt/vol), sucrose (20% wt/vol), a mixture of bovine serum albumin (BSA) (0.4% wt/vol) and rapeseed oil or trioleoylglycerol (1% wt/vol). BSA alone was a poor lyoprotectant. As glycerol and sucrose may interfere during the reactions catalyzed by the lipase, and rapeseed oil contains a complex mixture of triacylglycerols, the mixture of BSA and trioleoylglycerol was preferred for this study. Increasing amounts of water were added to the lyophilized extract. The a_w was determined for each experimental condition. For methanolysis reactions, a volume of methanol equal to one-tenth of the volume of water was added. Under these conditions, the effect of water on the activity of the enzyme could be distinguished, to some extent, from the competition between water and methanol for the acyltransfer reaction (the results are given in Fig. 4). The total transfer activity, corresponding to the sum of hydrolysis and methanolysis, was higher in the presence of methanol than the hydrolysis activity in the absence of this alcohol, whatever the amount of water or water + methanol added. Both hydrolysis and methanolysis reactions required water, and maximal activities were obtained for high amounts of water when a_w was higher than 0.9. The optimum was reached when 500 μL were added (a_w = 0.95 and 0.98 in the presence or not of methanol, respectively). When higher amounts of water or water + methanol were added (500–2000 μL), the activities decreased slowly, although a_w did not vary significantly. With between 500 and 2000 μL of water or water + methanol added, the enzyme was diluted about four times; however, the activities were only reduced by 25%. This could be explained by an activation of the enzyme by water or that the enzyme accumulates at the oil/water interface. Experiments were performed in which a mixture of 1 g of rapeseed oil and 3000 μL of an aqueous solution containing the enzyme, with or without methanol, were shaken. It was shown that 30–50% of the activities were removed from the aqueous phase. Thus, the poor sensitivity of the system to dilution probably was due to an accumulation of the enzyme at the interface.

We have shown in a previous paper that methanol inhibits hydrolysis (2). Hydrophilic molecules, like alcohols, could occupy the reactant interface and prevent access of hydrophobic substrates to the active site (9). In such a case, enzymatic reactions are inhibited by dilution of the lipid substrate at the reactant interface, and not by competition between the alcohol and the lipid substrate at the active site. However, this does not seem to apply to the enzyme studied here. Overall acyltransfer activity was not affected by 0–2.2 M methanol, alcoholysis being enhanced to the detriment of hydrolysis. To determine the influence of increasing methanol concentra-

TABLE 1
Effects of Various Effectors on Rapeseed Oil Hydrolysis and Methanolysis by the Lipase from *Candida parapsilosis* in Aqueous Medium[a]

Effector (3 mM)	Catalytic center	Hydrolysis activity (%)	Methanolysis activity (%)
Reference (no effector)		100	100
Iodoacetic acid	Cys-His (10)	107	100
N-Bromosuccinimide	Tyr-Trp-His (11)	10	0
Dithiothreitol	S-S bonds (10)	100	100
N-Ethyl-5-Phenylisoxazodium 3'-Sulfonate	Carboxyl groups (12)	100	100
Acetylacetone	Arg (10)	71	100
Dimethylaminobenzaldehyde	Trp (13)	96	84
Trifluoroacetic anhydride	Lys (11)	77	70
2,5-Dimethoxytetrahydrofurane	Asp-Glu (11)	77	70
2-Mercaptoethanol	S-S bonds (10)	77	70
Phenylmethylsulfonyl fluoride	Ser (7)	102	100
Iodin	Tyr (10)	0	0

[a]Results are expressed relative to the reference activity determined in the standard conditions. The numbers in parentheses are reference numbers.

tions in the reactant medium, the purified enzyme extract (0.1 U) was dissolved in 1.2 mL of phosphate buffer and trioleoylglycerol emulsion, to which 1 mL of a mixture of the same buffer and methanol was added (Fig. 5). There was an optimal methanol concentration for methyl esters formation (1.1–2.2 M); a_w was then about 0.95. The preeminence of alcoholysis over hydrolysis in the presence of methanol thus did not seem to be related to the depressing effect of methanol on a_w, but more directly to a competition between methanol and water for the acyltransfer reaction, favorable to the alcohol.

Additional factors affecting the amount or the properties of the reactant interface, and thus likely to modify the observed reaction rate, were studied. The effects of sodium dodecyl sulfate (SDS) are given in Table 3. The effects of this detergent depended on the concentrations tested. High concentrations (≥1 mM) inhibited both enzymatic activities. Concentrations lower than 0.5 mM inhibited hydrolysis, but not methanolysis, which was slightly activated. Figure 6 shows the influence of increasing poly(vinyl alcohol) (PVA) concentrations on the enzymatic activity. The optimal concentration was different for a same substrate (rapeseed oil), according to

TABLE 2
Effect of the Lyophilization in the Presence of Various Lyoprotecting Agents [BSA: bovin serum albumin; PVA: poly(vinyl alcohol)]

Lyoprotectant agents (% wt/vol)	Activity (μmol/min/0.25 U)			
	Before lyophilization		After lyophilization and rehydration	
	Hydrolysis	Methanolysis	Hydrolysis	Methanolysis
No lyoprotectant	0.25	0.22	0.00	0.00
Glycerol 5%[a]	0.25	0.22	0.00	0.00
Glycerol 10%[a]	0.23	0.22	0.07	0.07
Glycerol 20%[a]	0.21	0.22	0.25	0.26
Sucrose 10%[a]	0.21	0.18	0.16	0.25
Sucrose 20%[a]	0.21	0.20	0.24	0.26
BSA 0.1%[a]	0.18	0.15	0.08	0.06
BSA 0.4%[a]	0.25	0.22	0.14	0.14
BSA 1%[a]	0.13	0.13	0.09	0.11
Rapeseed oil 1%[b]	0.00	0.00	0.00	0.00
Emulsion of rapeseed oil in PVA 1%[b]	0.25	0.22	0.19	0.23
Rapeseed oil 1% + BSA 0.4%[b]	0.25	0.22	0.25	0.25
Trioleoylglycerol 1% +BSA 0.4%[b]	0.25	0.22	0.25	0.25

[a]Reactions were performed in the standard conditions in the presence of the lyoprotecting agents.
[b]The initial rapeseed oil or trioleylglycerol was used as substrate.

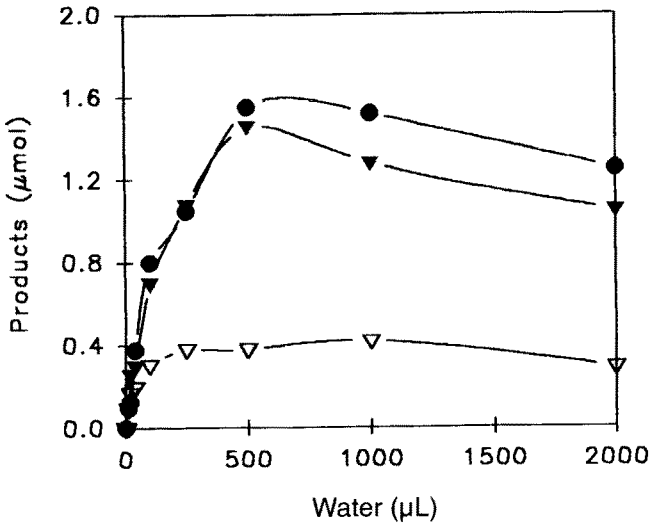

FIG. 4. Effect of the progressive rehydration of the lyophilized lipase [0.1 U, 4 mg total protein including bovine serum albumin (BSA)] on the bioconversion of trioleoylglycerol. Lyophilization was performed in the presence of BSA 0.4% (wt/vol) and trioleoylglycerol 1% (wt/vol). ●, Free fatty acids released during hydrolysis without methanol; ▼, methyl esters formed in the presence of methanol; and ▽, free fatty acids released in the presence of methanol.

the acyl acceptor (water or methanol), and for a same reaction, according to the acyl donor (hydrolysis of rapeseed oil or oleic acid ethyl ester). Table 4 shows that enzymatic activities were inhibited in the presence of free exogenous oleic acid in the reactant mixture. The strongest inhibitory effects were obtained with hydrolysis reactions.

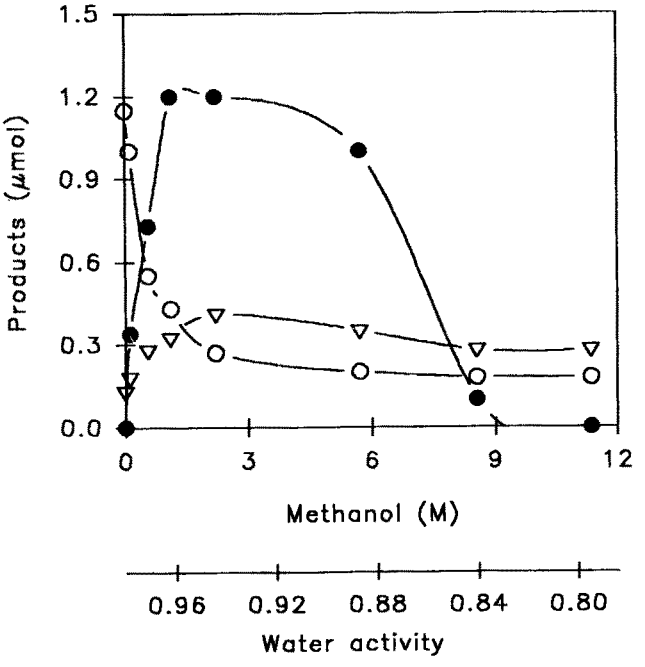

FIG. 5. Bioconversion of trioleoylglycerol in the presence of increasing methanol concentrations. ○, Free fatty acids released; ●, methyl esters formed; and ▽, diolein formed.

TABLE 3
Effect of Sodium Dodecyl Sulfate (SDS) on Rapeseed Oil Hydrolysis and Methanolysis by the Lipase from *Candida parapsilosis* in Aqueous Medium[a]

SDS concentration (mM)	Hydrolysis activity (%)	Methanolysis activity (%)
0.00	100	100
0.01	95	120
0.10	76	108
0.50	9	100
1.00	7	0
3.00	4	0

[a]Results are expressed as percentages of the reference activity determined in the standard conditions.

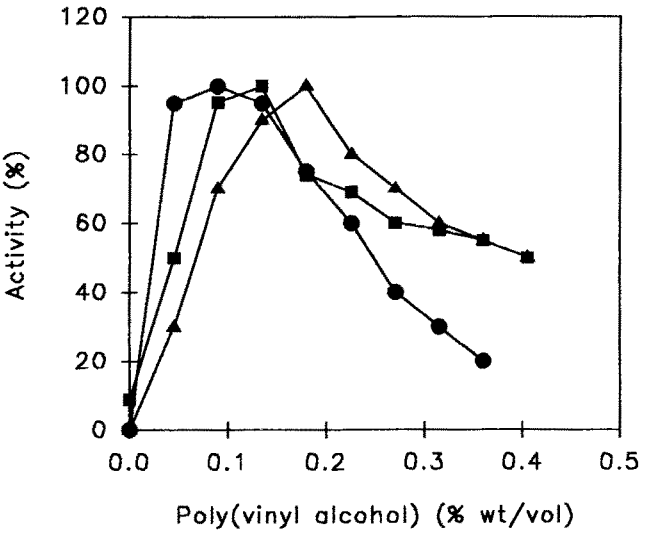

FIG. 6. Effect of the emulsifier concentration on the acyltransfer activity. Results are expressed as percentages of the maximal activity for each reaction studied. ●, Rapeseed oil hydrolysis; ■, rapeseed oil methanolysis; and ▲, oleic acid ethyl ester hydrolysis.

TABLE 4
Effect of the Addition of Oleic Acid on the Activities of the Lipase from *Candida parapsilosis* in Aqueous Medium[a]

Oleic acid added (μmol)	Rapeseed oil hydrolysis activity (%)	Oleic acid ethyl ester hydrolysis activity (%)	Rapeseed oil methanolysis activity (%)
0.0	100	100	100
1.0	63	74	89
2.0	58	67	76
2.5	44	62	67
4.0	0	0	55

[a]Results are expressed as percentages of the reference activity determined in the standard conditions.

DISCUSSION

Most extracellular yeast lipases have a broad pH activity profile, showing high activity between pH 5 and 9 with a maximum between pH 6 and 8 (14). The acyltransferase from *C. parapsilosis* reacted in a slightly more acidic pH range (3–7),

with an optimum at pH 6–6.5. The optimal temperature was 45–50°C with our conditions, but the shape of the activity vs. temperature profile depended on the substrate (rapeseed oil or oleic acid ethyl ester) and on the reaction concerned (hydrolysis or methanolysis). Notably, the peak was broader when the substrate was oleic acid ethyl ester. The activity vs. temperature profiles resulted from both the thermodynamic properties of the lipase (activation and denaturation) and the physicochemical properties of the substrates used. By acting on the viscosity of the lipid substrates, temperature may modify their access to the active site of the enzyme. The interpretation of the profiles observed is thus complicated. Moreover, after thermal treatment of the enzyme (in the absence of methanol), the denaturation was apparently lower when the activities were measured in the presence of methanol than in the absence of this alcohol. Hydrolysis, especially, which could be measured in both conditions, appeared less affected when methanol was present. This suggests that methanol changed the conformation of the protein. Some investigators consider that the presence of alcohols could indirectly modify the native conformation state of enzymes by favoring the location of the hydrophobic groups of amino acids at the periphery of the protein (15). This seems to apply to the acyltransferase from *C. parapsilosis.*

Water widely influences the structure and the functioning of enzymes. It is also a substrate of the hydrolysis reaction, but it is theoretically not involved in the alcoholysis reaction. At the lowest a_w values, increasing a_w enhanced both reactions. Water requirement could be attributed to the lubricant properties of water, which would release the enzyme structure by forming hydrogen bonds with functional groups of the protein previously bound together (16). It is generally considered that acyltransfer reactions catalyzed by lipases (such as methanolysis) should usually be carried out at the lowest possible a_w values in order to minimize hydrolytic side reactions (17). In this case, lipases which can function at low a_w are attractive. Our study showed that high amounts of water ($a_w > 0.9$) are required for full function of the acyltransferase from *C. parapsilosis* for both hydrolysis and methanolysis reactions. In the presence of methanol in the aqueous medium, methyl esters were formed and hydrolysis was inhibited.

The study of the action of effectors showed that a tyrosin residue was necessary to the activity of the acyltransferase and that Mg^{2+} was required. Cofactors are generally not required for the expression of lipase activity. Although properties of the interface may have an effect on the reaction rate (14), bivalent cations, like Ca^{2+} or Mg^{2+}, may enhance lipase activity by removing the fatty acids released during the reaction. In our case, Ca^{2+} had only a slight effect.

The acyltransferase activity from *C. parapsilosis* was resistant to lyophilization in the presence of an emulsion of triacylglycerols in PVA. Other studies have shown that a lipase from *Rhizomucor miehei*, lyophilized in the presence of substrates, was activated compared to its initial form when used in poorly hydrated organic media. This activation disappeared when the lipase was used in aqueous medium (18). We did not observe any activation phenomenon in the conditions tested.

Substrates also are influenced by reactant conditions. We have shown that the activity vs. temperature profile depended on the lipid substrate studied. It is well known that the physical properties of lipids, notably their viscosity, are influenced by temperature in a range compatible with lipase activity. This, added to the influence of other factors, like the presence of ions, emulsifiers, or solvents (including methanol), may cause variations in the ability of lipids to be emulsified in water and to access to the active site of the enzyme. Because of its lower polarity, methanol may preferentially access hydrophobic areas where the enzymatic reaction takes place, as compared to water.

Emulsifying agents prevent the contact between the enzyme and its substrates. At low PVA concentrations, we observed that the lack of interface induced very low reaction rates. We showed that the optimal PVA concentration depended on the reaction concerned. Besides its lyoprotecting effect, BSA also played the role of an emulsifier when used with lipid substrates without PVA. Moreover, by acting as a fatty acid acceptor, albumin theoretically suppresses enzyme inhibition by the free fatty acids released during the hydrolysis reaction and enhances activity (9). We did not observe this activation. The synergetic effect of low BSA concentrations and rapeseed oil may then be related to the fact that BSA permitted an intimate mixture of the enzyme and lipid substrate. The emulsifying properties of BSA and its protective effects as a protein (19) thus added to the protecting effect of oil. Low SDS concentrations, by increasing the hydrophobicity of the enzyme, might favor the access of methanol to the active reactant interface, which might explain the activation of alcoholysis.

At high concentrations, albumin is also known to defavor lipase-catalyzed reactions by occupying the reactant interface, which prevents the mutual approach of enzyme and substrate (9). At high concentrations, SDS surrounds enzymes. The free fatty acids formed during lipase-catalyzed reactions, more polar than the residual triacylglycerols, also prevent access of the latter to the active site. Proteins, surfactants, and free fatty acids tend to accumulate at the interface and, thus, to exclude the substrate from the active site. We have observed that the depressing effect of high BSA, SDS, or free fatty acid concentrations was effective in the case of the acyltransferase from *C. parapsilosis.* Although methanol was favored compared to water in the presence of free fatty acids, both hydrolysis and alcoholysis reactions were inhibited.

Because of the various interactions between the substrates and the enzyme, the interpretation of the variations induced by physicochemical factors is difficult. Methanol plays an important role because of its multilevel effects. As a substrate of the alcoholysis reaction, it competes with water and influences the conformation of the acyltransferase. As a solvent, it modifies the polarity of the reactant medium and the access of other substrates at the reactant interface. Because it has a lower polarity than water, methanol may preferentially access

the hydrophobic reactant interface. It is thus difficult to distinguish between a real enzymatic preference for methanol and a favored access of this acyl acceptor to the reactant area. In any case, this makes the acyltransferase from *C. parapsilosis* an interesting enzyme for the catalysis of alcoholysis reactions at high a_w values.

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