Peculiar Properties of Lipase from *Candida parapsilosis* (Ashford) Langeron and Talice

A. Riaublanc[a], R. Ratomahenina[a,*], P. Galzy[a] and M. Nicolas[b]
[a]Chaire de Microbiologie Industrielle et de Génétique des Microorganismes, INRA-ENSA, 34060 Montpellier Cedex 1, France and [b]INSERM-U65, Immunologie, Institut de Biologie, 34000 Montpellier, France

A new *Candida parapsilosis* lipase was isolated and studied. This enzyme was purified by hydrophobic chromatography on a phenyl-sepharose CL4B column followed by gel permeation on a Sephacryl S300 HR column. It was a 160 kg•mol^{-1} molecular-weight oligomeric enzyme. Optimal activity was obtained at 45°C and pH 6.5. The lipase activity toward various acylglycerols and esters was studied. The hydrolysis rate was greater for secondary acylesters than for primary acylesters. This lipase showed a high specificity for long-chain fatty acids and particularly for polyunsaturated fatty acids. This enzyme was able to catalyze the synthesis of various oleoylesters in aqueous medium.

KEY WORDS: *Candida parapsilosis*, 1,3 diglycerides, esterase, esters, lipase, lipids, monoglycerides, specificity, synthesis.

Lipases (EC 3.1.1.3.) and esterases (EC 3.1.1.1.) are recognized as being important industrial enzymes (1). These enzymes are able to hydrolyze acylglycerides in aqueous medium (2) and catalyze the synthesis of esters in organic medium (3). The numerous products obtained with these enzymes could have many applications in the food, chemical, pharmaceutical and medical sectors. However, most current applications involve commercial enzymes (4–9), and research on lipases with peculiar properties has been reduced. In the present study, we investigated the purification of *Candida parapsilosis* lipase, its specificity toward triacylglycerides and monoesters and its synthesis qualities in aqueous medium.

MATERIALS AND METHODS

Organism. The yeast strain used was *C. parapsilosis* (Ashford) Langeron and Talice, CBS 604, obtained from the Centraalbureau of Schimmelcultures, Yeast Division (Delft, The Netherlands).

Culture techniques. All cultures were performed in Erlenmeyer flasks filled to 10% of their volume, incubated at 28°C and shaken (80 oscillations min^{-1}; amplitude 7.5 cm). The basal medium was Difco Yeast Nitrogen Base (Difco Labs, Detroit, MI) adjusted to pH 6.5 with 0.05 M phosphate buffer. The carbon substrate was glucose (0.5%) or rapeseed oil (0.5%) sterilized by autoclaving at 110°C for 30 min.

Enzyme extract preparation. The cells were harvested at the end of the log phase by centrifugation (16,000 × *g* × 30 min). The supernatant was twice defatted with hexane (1:5, vol/vol). Traces of hexane were removed under vacuum. Hexane has no inhibitory effect on enzyme activity.

Growth determination. Growth was monitored by turbidity measurements at 500 nm. Cell suspensions were defatted by filtration (0.45 μm) and washed with ethanol and hexane.

*To whom correspondence should be addressed at Chaire de Microbiologie Industrielle et de Génétique des Microorganismes, INRA-ENSA, Place Viala, 34060 Montpellier Cedex 1, France.

Enzyme assay. As none of the substrates were water-miscible, we used the method described by Montet *et al.* (10). The different substrates were emulsified (5% wt/vol) in an aqueous polyvinylalcohol solution (2% wt/vol); 1 mL of this emulsion was added to 7 mL buffer at the desired pH and to 2 mL enzyme extract; the enzyme reaction was carried out under agitation at the desired temperature. The reaction was stopped by the addition of an ethanol/acetone mixture (50:50).

Analysis of enzyme reaction products. The enzyme reaction products were extracted with hexane. Free fatty acids are determined spectrophotometrically with Rhodamine 6G (11). The esters were determined by thin-layer chromatography (TLC) (silica G60). Migration was carried out with a hexane/diethyl ether/acetic acid mixture (75:25:1). Spots were revealed after carbonization at 180°C for 4 min with a copper acetate/orthophosphoric acid mixture (50:50); a photodensitometer GS 300 (Hoefer/Scientific Instruments, San Francisco, CA) was used for quantitative determinations. Fatty alcohol and cholesterol were determined by TLC. A dichloromethane/acetone mixture (100:5) was used for fatty alcohol migration, and dichloromethane/diethyl ether (100:20) for cholesterol.

Esterase activity was determined photometrically (400 nm) by measuring liberated *p*-nitrophenol. One enzyme unit (U) corresponded to the liberation of one μmol of product per minute. Protein concentrations were measured according to Smith *et al.* (12) with bicinchoninic acid or by absorbance determination at 280 nm. The glucose content was determined according to Somogyi (13) with dinitrosalycilic acid.

PURIFICATION

The crude enzyme extract was concentrated in an Amicon cell (YM:10 membrane) (Amicon, Epernon, France) under nitrogen atmosphere. The resulting concentrated solution had a specific activity of 3.7 μmol free fatty acids mg^{-1} min^{-1}. The enzyme was then purified on two chromatography columns: A Sepharose CL4B hydrophobic column (Pharmacia France, Saint-Quentin les Yvelines, France) was previously equilibrated with phosphate buffer (50 mM, pH 6.5). Elution was performed with a linear gradient of ethyleneglycol (0 to 100%). A Sephacryl S 300 HR gel permeation column (Pharmacia, France) was previously equilibrated with ethyleneglycol (75%) and phosphate buffer (50 mM, pH 6.5). Proteins were eluted with 75% ethylene glycol.

The column was calibrated with molecular weight protein standards for molecular weight determination. The molecular weight of the *C. parapsilosis* lipase was estimated at about 160 ± 25 kg mol^{-1}. The molecular weight estimated by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was about 67 ± 3 kg mol^{-1}, suggesting that the native enzyme was an oligomeric enzyme constituted of two to three monomers of similar molecular weight.

PROPERTIES OF THE LIPASE

Hydrolysis tests of rapeseed oil with purified enzyme, at various pH and temperature levels, showed optimal activity at pH 6.5 and about 45°C. Enzyme incubation of 10 to 60 min from 35 to 60°C induced rapid loss of activity at temperatures higher than 50°C. The activation energy and thermal inactivation constant determined according to Arrhenius were 41.8 kJ mol^{-1} and 83.6 kJ mol^{-1}, respectively.

Actions of some cations and effectors were studied at 3 mM concentration. The reactions were carried out for 1 h at pH 6.5 and 45°C. The cation Hg^{2+} completely inhibited enzyme activity; Ca^{2+}, Mn^{2+}, Zn^{2+}, Fe^{2+} and Cu^{2+} strongly inhibited the activity, while Mg^{2+} and Na^{2+} had no effect on the action of the enzyme.

N-ethylmaleimid and β-mercaptoethanol did not affect the *C. parapsilosis* lipase, so this enzyme probably does not possess thiol groups at its active site. This is identical to results obtained by Montet *et al.* (10) for *Candida curvata* lipase and by Muraoka *et al.* (14) for *Staphylococcus aureus* 226 lipase. *N*-bromosuccinimide strongly inhibited the activity, which is similar to data obtained by Muderhwa *et al.* (15) for *Rhodotorula pilimanae* lipase. Thus, the enzyme must contain tyrosin or tryptophan amino acids at its active site. SDS completely inhibited the activity. In the presence of ethylenediaminetetraacetic acid, this lipase lost 50% of its activity, suggesting the presence of a metal cofactor in its structure. The nature of this ion will be determined in further investigations.

STUDY OF ENZYME SPECIFICITY

Position specificity. The specificity of the lipase was examined by monitoring the hydrolysis of rapeseed oil. The nature and amount of different hydrolysis products were determined by TLC. The retardation factors for triglycerides, 1,2 diglycerides, 1,3 diglycerides, mixed monoglycerides and fatty acids are 0.72, 0.106, 0.164, 0.02 and 0.27, respectively. Results are given in Figure 1. The production rates of diacylglycerides 1,2 and 1,3 were similar. Because the glycerol molecule has two external positions and only one internal position, we observed two 1,2 diglycerides for every single 1,3 diglyceride when they were hydrolyzed at the same rate. Clearly, the internal position (No. 2) was more rapidly hydrolyzed than the external positions (No. 1 and No. 3).

The production rate of diacylglycerides (1,2 and 1,3) was lower than that of free fatty acids, and monoglycerides were never detected among the hydrolysis products. They were therefore rapidly hydrolyzed and not present in detectable quantities in the mixture.

The results may have been influenced by the nature and position of the fatty acids on the glycerol molecule. We thus compared the hydrolysis rates of triolein, diolein (1,2 + 1,3) mixture, diolein (1,3) and mixed monooleins under the same conditions. Results are given in Figure 2. The hydrolysis rate of the diolein mixture (1,2 + 1,3) was greater than the rate of diolein 1,3. This confirms the specificity of this lipase for the internal position. Hydrolysis of monoacylglyceride mixtures is extremely rapid in spite of the difficulties occurring during emulsification. Therefore, during the hydrolysis of rapeseed oil, monoacylglycerides are hydrolyzed as soon as they are produced.

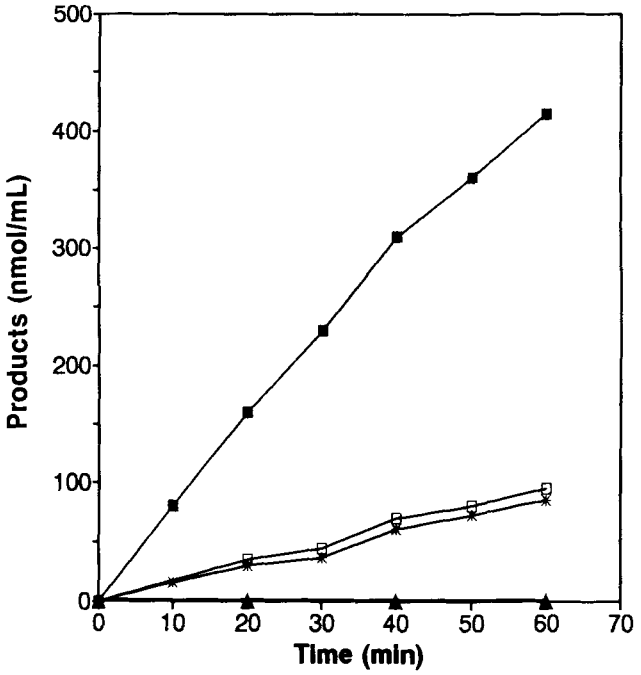

FIG. 1. Hydrolysis products of triacylglycerides: free fatty acids (■), 1,3 diacylglycerides (□), 1,2 diacylglycerides (*), monoacylglycerides (▲).

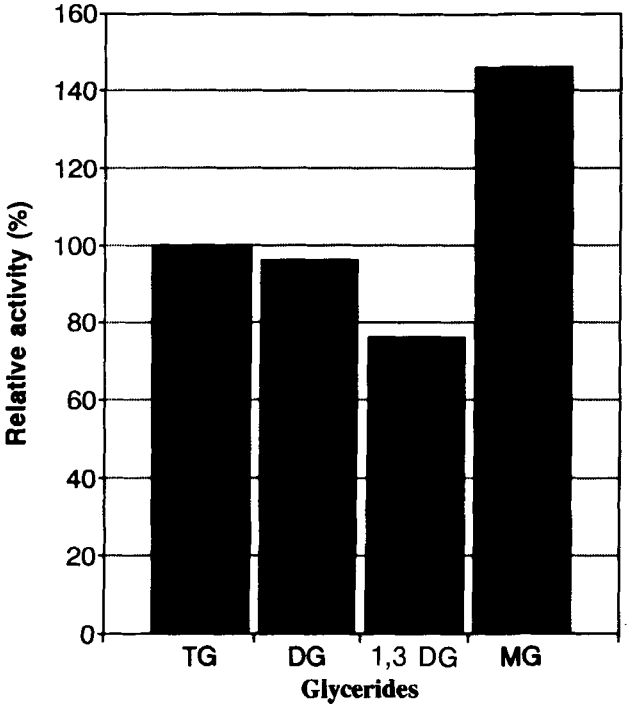

FIG. 2. Relative activity of *Candida parapsilosis* lipase toward various acylglycerides: triacylglycerides (TG), mixed diglycerides (DG), 1,3 diglycerides (DG 1,3), mixed monoacylglycerides (MG).

Specificity for fatty acids. We tested the influence of long fatty acid carbon chains by using homogeneous triacylglycerides obtained from Nu-Chek-Prep Inc. (Elysian, MN) (Fig. 3). For lengths ranging from 12 to 18 car-

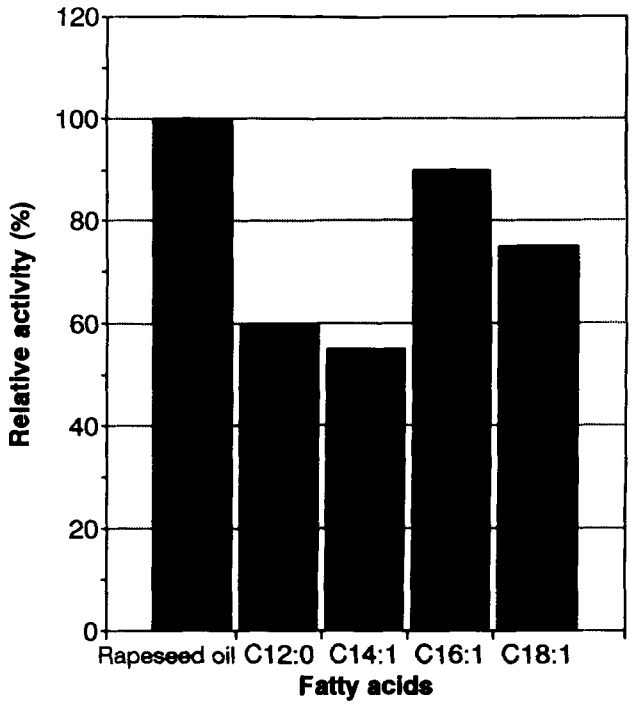

FIG. 3. Relative activity of *Candida parapsilosis* lipase toward long-chain fatty acids on homogeneous triacylglycerols.

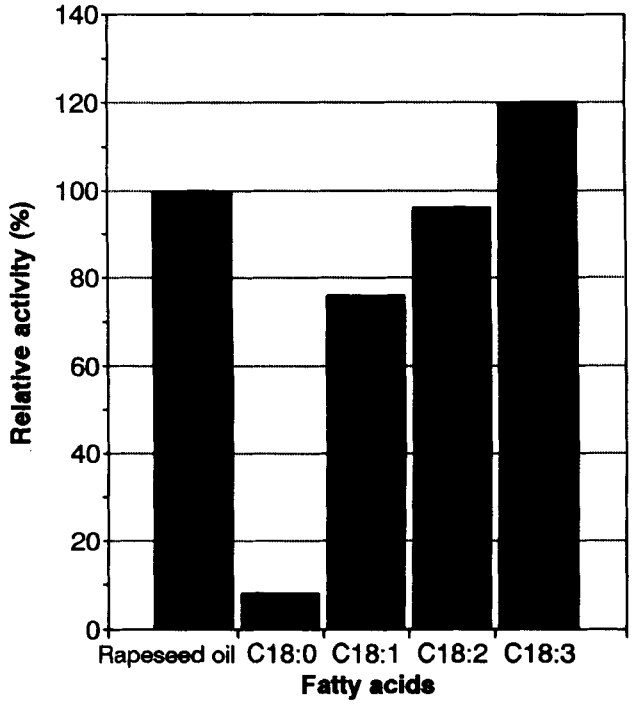

FIG. 4. Relative activity of *Candida parapsilosis* lipase at different degrees of fatty acid unsaturation on homogeneous triacylglycerols.

bon atoms, the hydrolysis rates were slightly greater for longer chains, but the specificity toward this parameter was probably not significant. Figure 4 shows results obtained when the degree of unsaturation was investigated. The specificity of this enzyme increases with the degree of fatty acid unsaturation. However, the results with tristearin must be interpreted with caution because of its high fusion temperature.

TABLE 1

Relative Hydrolysis of Various Monoesters by *Candida parapsilosis* Lipase

Esters	Relative activity (%)
Rapeseed oil	100
Ethyl laurate	428
Ethyl oleate	100
Cholesteryl butyrate	0
Cholesteryl laurate	18.6
Cholesteryl oleate	50.5
p-Nitrophenyl butyrate	0
p-Nitrophenyl laurate	366
p-Nitrophenyl oleate	0
Lauryl acetate	0
Oleyl acetate	0

Monoesterase activity of the enzyme. Because the enzyme was able to hydrolyze monoacylglycerides more rapidly than triacylglycerides, we tested its activity on various monoesters formed from different alcohols and acids (Table 1). The enzyme showed high activity for fatty acid monoesters, but no activity was detected for short-chain fatty acids regardless of the alcohol content. This confirms a tendency of increased activity with large-size acids as shown in Figure 3.

Synthetase activity of the enzyme. During purification, we noticed that in the presence of ethylene glycol two new products appeared on the TLC plates: ethyleneglycol monoesters and diesters. We studied this synthetase activity in aqueous media with various alcohols and oleic acid. In our experiments, there was a great excess of alcohol (50-fold). We have calculated the percentage of esterified oleic acid for each product (Fig. 5). When ethanol was used, the synthesis was rapid and yield transformation was about 35% in 24 h. When ethyleneglycol was used, the monoester formed slowly while the diester reached 10% in 8 h. Fixation of a second fatty acid seemed to be facilitated by the presence of the first fixed fatty acid. When a primary alcohol (*n*-propanol) and a secondary alcohol (isopropanol) were used, the synthesis rates were quite different. The *n*-propyl oleate formed at a slow rate whereas the isopropyl oleate formed more rapidly and reached 10% after 3 h. This lipase seems to show specificity for the secondary alcohol. This confirms our results concerning specificity for the internal position on glycerol.

STUDY OF LIPASE BIOSYNTHESIS

Assays of *C. parapsilosis* cultures with glucose or ethanol as the sole carbon source did not reveal any lipase production. Biosynthesis of this enzyme was induced in the presence of oil in the culture medium. When a glucose/rapeseed oil (2.5:2.5 g/L) substrate was used, biosynthesis was not repressed by glucose (Fig. 6). Maximum activity obtained for the two cultures was the same, although the final cell density was higher when only rapeseed oil was used.

C. parapsilosis lipase presents specificity for long-chain polyunsaturated fatty acids. It is more active on fatty acid

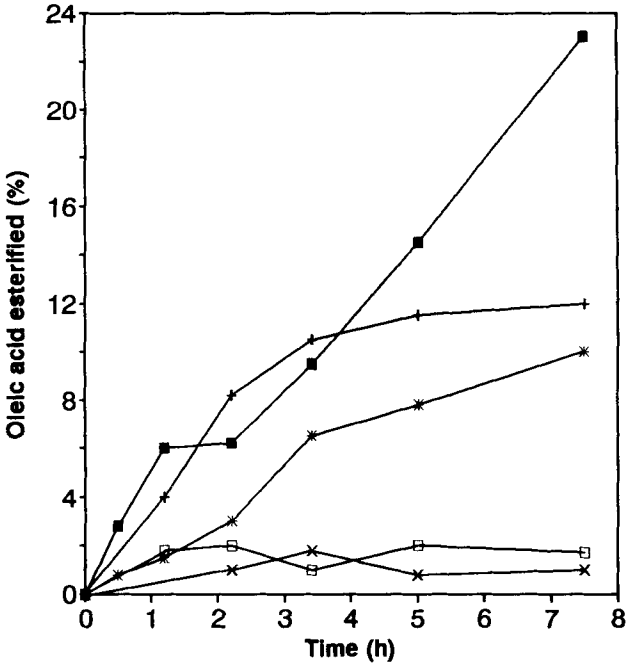

FIG. 5. Synthesis kinetics of oleylesters by *Candida parapsilosis* lipase in aqueous media. Ethyl oleate (■), *n*-propyl oleate (×), isopropyl oleate (+), ethyleneglycol monooleate (□), ethylene glycol dioleate (*).

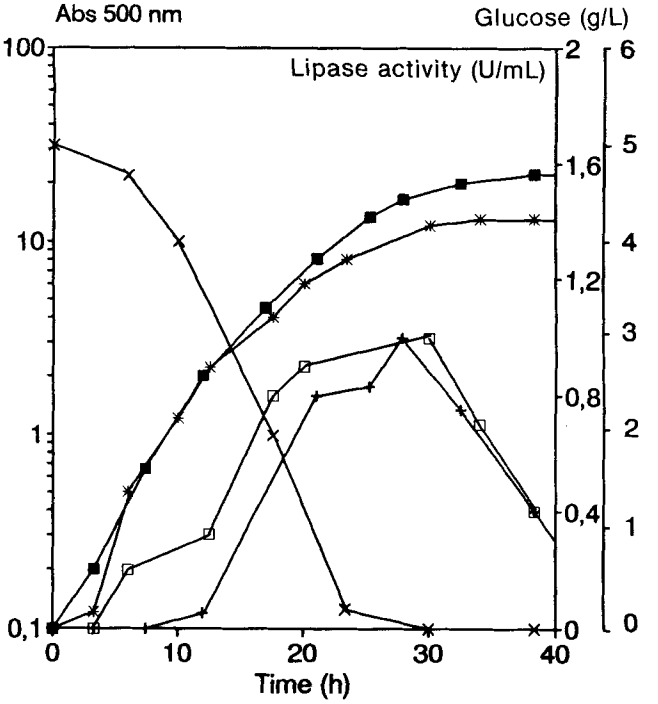

FIG. 6. Biosynthesis of *Candida parapsilosis* lipase during growth on rapeseed oil (0.5%) and a glucose/rapeseed oil (0.25:0.25%) mixture. Oil growth curve (■), mixture growth curve (*), residual glucose (×), extracellular lipase activity in the presence of oil (+) and in the presence of the mixture (□). Abbreviations: U, unit; Abs, absorbance.

monoesters than triglycerides and shows specificity for secondary alcohol esters. This enzyme is thus able to catalyze the synthesis of these esters in aqueous media. This property could be used to produce polyalcohol fatty acid esters to be used as emulsifiers in the food industry.

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