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Relation between lipase structures and their catalytic ability to hydrolyse triglycerides and phospholipids

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Abstract

Lipases with different structures were investigated for their catalytic ability to hydrolyse sunflower oil, soybean lecithin and their mixtures in heptane at 60 °C in a biphasic mixture heptane-buffer pH 7.0. Besides, the substrate adsorption mechanism was studied theoretically with the Chem 3D 5.0 Ultra program and the MM2 (Cambridge Soft) method by using trilinolein and phosphatidylcholine as system models of triglycerides and phospholipids, respectively. Lipolase 100T, a granulated silica immobilised commercial preparation of Thermomyces (formerly Humicola) lanuginosa lipase, evidenced the highest conversion to fatty acids when sunflower oil (85.3% conversion to fatty acids) and its mixture with lecithin (100 % conversion to fatty acids) were tested. The free lipase from *Rhizomucor meiheir* (RML) evidenced the highest activity with lecithin as substrate (35.6% conversion to fatty acids). The lipases with their active sites on the surface presented the highest strength of substrate coordination – as a decrease of steric energy. In the case of RML and the artificial mix sunflower oil–lecithin, the product distribution between MG, DG and FA was: 29.70% MG, 32.96% DG and 37.34% of fatty acids (FA). When Lipolase 100T was used, the distribution was: 14.91% MG, 15.46% DG and 69.63% FA. Besides the FA, monoglycerides (MG) and diglycerides (DG) a new product was detected when lecithin was present (alone or in the mix) specially when RML was used as biocatalyst. No evidence of this peak was found after the reaction using Lipolase 100T. This new product can be related to the presence of phosphate charged group in lecithin and to the possibility of cracking of one of the hydrocarbon chains of the FA in TG. A possible reaction with the exposed active site of RML is discussed. The assignation of this new compound as a MG of a shorter fatty acid chain – with 9 carbon atoms – is presented and supported with further analytical analysis.

Keywords: Lipase; Hydrolytic activity; Gas chromatography; Lecithin; Sunflower oil

1. Introduction

Lipases (glycerol ester hydrolases EC 3.1.1.3) constitute a special group of enzymes whose biological function is to catalyse the hydrolysis of triglycerides to give diglycerides, monoglycerides, free fatty acids (FFA) and glycerol. These enzymes may also catalyse the hydrolysis of phospholipids such as phosphatidylcholine, which is one of the major components of commercial lecithin. At present, there is a worldwide increasing interest in the development of new applications of lipases to products and processes, particularly oils and fats, detergents, and oleochemistry industries [1].

Experimental studies have been carried out in order to analyse the ability of lipases to hydrolyse vegetable oils and phosphatides under different conditions [2,3]. Marked differences were observed in lipase hydrolytic activity in terms of source, degree of purity, state (free or immobilised), substrate, and reaction medium (solvent-free or biphasic). In view of the different structural characteristics of commercially available lipases, it is reasonable to assume that under fixed-reaction conditions there is a relation between the lipase structure and its catalytic ability.

Lipase immobilisation onto solid materials not only enhances the operational lifetime and stability of biocatalysts but also facilitates the recovery, reuse and continuous operation of lipases. It also restricts product contamination. However, the high cost of the materials that are commonly used for lipase immobilisation (namely, silica-based carriers, acrylic resins, synthetic polymers, active membranes, exchange resins, etc.) as well as the technology necessary to apply fixation methods greatly increase the costs of biocatalysts. Such high costs are therefore a barrier to the industrial use of enzymatic processing. In the last years, cheaper support substitutes such as wood

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cellulignin [4], rice husk, rice straw [5], chitin [6], Amberlite [7], and chitosan [8–13], have become the centre of attention of researchers.

One of the principal goals of immobilisation procedures is to fix the maximum amount of enzymes by keeping maximum activity at the lowest cost. The quantification of the amount of immobilised lipase is therefore very important. Accurel PP, a commercial product that includes binders and stabilisers, is, in general, the most widely selected support. Lipase desorption from Accurel PP has been reported after usage in organic media [7].

In view of the above, a thorough understanding of support–lipase interactions will greatly contribute to designing more efficient catalysts. In this respect, silica in different forms – including silica-gel – is currently available as a suitable support [1,2]. Researchers claim that the activity measurements of supernatant solution before and after the immobilisation period are useless for the quantification of immobilised lipase. Differences in the structure of lipases in solution upon contact with the support have been reported [12]. On the other hand, it has been observed that the application of different methods based on protein tests in the supernatant solution after enzyme immobilisation have yielded a higher immobilisation efficiency when hydrophobic polymers, such as polypropylene (PP), are used as supports.

Taking into account the reaction medium, the volumetric production in solvent-free systems (SFS) was higher than that achieved in biphasic systems. Still, the mixing and mass transfer problems in SFS associated with viscosity of the medium require thorough attention [8]. The practical application of lipases requires their immobilisation on an inert support. Commercial lipases with no additional purification because of the high costs of purification, are available for the synthesis of supported catalysts. In view of this, impure lipases from Amano Inc. have been used for the synthesis of pharmaceutical compounds [9].

The commercial lipases used in the present study were immobilised either on acrylic macroporous resin (for Novozyme 435) or on an exchanged-anionic resin (for Lipozyme RM IM) or on a granulated silica (for Lipolase). Due to the commercial nature of these enzymatic catalysts and because of patent protection regulations, the availability of literature regarding preparation procedures for lipase immobilisation is poor.

In view of the above, the aim of the present study is to analyse the hydrolytic activity of several commercial lipases in sunflower oil and soybean lecithin in a biphasic medium. To this end, both lipase structures and the experimental data collected were thoroughly analysed taking into account other researchers' publications [2,3]. In addition, the theoretical information regarding lipase structure and active site conformations was obtained using computational tools as well as our previously published data [14].

1.1. Lipase structures

In most lipases there is a mobile element, which is known as "lid", consisting of either one or two short α-helices linked to the body of the lipase by flexible structural elements. In the open active site of lipases, the lid moves away making the binding site accessible to the substrate [15]. On the other hand, the lid needs an interface to be opened [16–20].

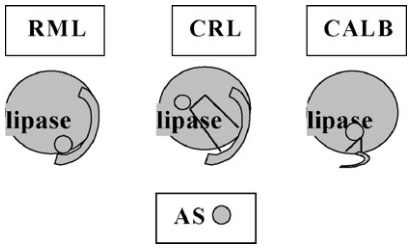

Fig. 1. Scheme of the active site (AS) and the lid in several lipases [21]: (a) *Rhizomucor miehei* lipase (RML), (b) *Candida antarctica* lipase B (CALB), and (c) *Candida rugosa* lipase (CRL).

Three types of lipases could be identified according to their coordination-substrate site. One of these types of lipases corresponds to Rhizomucor family including *Thermomyces* (formerly *Humicola*) *lanuginosa*, which has an active site and a lid on the surface of the enzyme. Another type of lipases corresponds to *Pseudomonas* and *Candida antarctica* family, which has an active site and a funnel-like lid. *Candida antarctica* lipase B exhibits a very small lid and a funnel-like binding site. The last type corresponds to *Candida rugosa* family and it is characterised by the presence of an active site at the end of a tunnel containing the lid in its external part [21]. This peculiarity affects the coordination of the substrate because *R. miehei*'s reaction is stimulated in position either 1 or 3 – rather than in position 2 – of the triglyceride whereas in *C. rugosa* the serine, which is part of the catalytic triad of the lipase, attacks all positions of the triglyceride (1 = 3 and 2) [19]. A simplified view [21] of both the active site and the lid in *R. miehei* lipase (RML), *C. antarctica* lipase B (CALB) and *C. rugosa* lipase (CRL) is shown in Fig. 1.

2. Materials and methods

2.1. Substrates

A granulated soybean lecithin with 98% purity and sunflower oil were purchased in a local market.

2.2. Enzymes

The following commercial lipases were supplied by Novo Nordisk (Bagsvaerd, Denmark): Novozym 435 (immobilised lipase from *C. antarctica* B, 5000 U/g), Lipozyme RM IM (immobilised lipase from *R. miehei*, 5000 U/g), Lipolase 100T (immobilised lipase from *T. lanuginosa*, 5000 U/g), and free (non-immobilised) lipases from *R. miehei* (RML) (5000 U/mL) and *C. antarctica* B (5000 U/mL). Furthermore, the following three lipases were provided by Amano (Nagoya, Japan): Lipase AY 30 (from *C. rugosa*, 30,000 U/g), Lipase PS (from *Pseudomonas cepacia*, 30,000 U/g) and Lipase AK 20 (from *Pseudomonas fluorescens*, 20,000 U/g). In addition, a porcine-pancreas lipase grade B (3000 U/g) was purchased from Calbiochem (brand of EMD Biosciences, an affiliate of Merck, Darmstadt, Germany). A Lipase type II (high purity lipase from porcine-pancreas) from Sigma (St. Louis, MO, U.S.A.) and Lecitase 10 L (a Phospholipase A2, 10,580 U/mL) from Novo Nordisk (Bagsvaerd, Denmark) were also tested for comparative purposes. The former was assayed with an oil–lecithin mixture whereas the latter was assayed only with lecithin.

2.3. Chemicals

All reagents were of analytical reagent grade, except *n*-hexane, which was of chromatographic grade. Standards of palmitic acid, stearic acid, oleic acid (*cis*-9-octadenoic acid), linoleic acid, 1-monopalmitoyl-rac-glycerol, 1-monostearoyl-rac-glycerol, 1-oleoyl-rac-glycerol, 1-linoleoyl-rac-glycerol, 1,3-dipalmitin, 1,3-diolein, tricaprin, tripalmitin, triolein, *n*-tetradecane and glycerol with purities higher than 98% were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.) for the chromatographic analysis. *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA) and pyridine from J.T. Baker Inc. (Phillipsburg, NJ, U.S.A.) were used as silylating agents.

2.4. Substrate characterisation

Sunflower-oil FA composition was determined by gas chromatography of methyl esters following IUPAC standard methods (2.301–2.302) [22]. The quantitative determination of lecithin phospholipids was carried out by HPLC-UV using the AOCS official method (Ja 7b-91) [23]. Acetone-insoluble matter, hexane-insoluble matter and humidity of lecithin were determined following AOCS standard methods (Ja 4-46, Ja 3-87, Ja 2b-87) [23]. On the other hand, acidity of free fatty acids (FFA) was determined following IUPAC standard methods (IUPAC 2.201) [22]. Analyses were performed by triplicate.

2.5. Hydrolysis reaction

The reaction mixtures were:

- 1 g oil, 2.5 g buffer (pH 7), 1.4 mL heptane and 0.050 g lipase (Mix 1);
- 0.2 g lecithin, 2.5 g buffer (pH 7), 1.4 mL heptane, 0.005 g enzyme (Mix 2);
- 0.15 g oil, 0.2 g lecithin, 0.35g buffer (pH 7), 1.4 mL heptane, 0.050 g lipase (Mix 3).

The purpose of our work was to study, with excess of water, triglyceride (TG) hydrolysis in Mix 1, phospholipid (PLs) hydrolysis in Mix 2 and hydrolysis of an oil–lecithin mixture resembling sunflower oil gum composition (30% phospholipids, 20% sunflower oil and 50% water) in Mix 3.

Nine lipases were selected for the present study and they were individually tested in Mix 1 and Mix 2. In addition, Lecitase 10 L was tested only with lecithin as substrate (Mix 2), in which case, a pH 8 buffer was used. The reaction systems using Mix 3 were prepared with a high-purity porcine-pancreas lipase type II as well as with lipases showing the highest conversion with oil or lecithin.

The reaction systems were magnetically stirred in 10 mL glass flasks with septum using a thermostated bath for 6 h at 60 °C, a temperature at which most enzymes have an optimal performance without being denaturated. The 6-h reaction time was also suitable for a comparative study on the response of each enzyme on account of the fact that the released FFAs could be detected efficiently and conversions were high. All reactions were carried out by duplicate. 200 μL aliquots were withdrawn from the stirred reaction mixture both at the beginning ($t = 0$) and at the end of the reaction ($t = 6$ h). These aliquots constituted the samples to be analysed by titration and chromatography [24,25].

2.6. Hydrolysis analysis by titration

Hydrolysis was analysed through the FA concentration increase. Acidity analysis was carried out by titration following the standard IUPAC method (IUPAC 2.201) [22]. Samples were mixed with 10 mL diethyl ether:ethanol (1:1, v/v) and titrated with 0.02N KOH using phenolphthalein as indicator. The average values corresponding to four independent determinations were reported in tables.

2.7. Hydrolysis analysis by gas capillary chromatography (GC)

Two standard solutions containing either tetradecane (20 mg/mL) or tricaprin (30 mg/mL) in pyridine were prepared as internal standards. In addition, a reference solution (RS) in pyridine containing 10 mg/mL of each standard of FAs, monoglycerides, diglycerides, triglycerides, tetradecane, glycerol and tricaprine was used to determine response factors.

The samples selected for chromatographic analysis were evaporated to dryness under nitrogen flow; 4 mg of samples were weighted in a 1 mL screw-cap vial; 10 μL of tetradecane standard solution, 30 μL of tricaprin standard solution and 120 μL of MSTFA were added to the mixture. After 15 min at room temperature, the silylated mixtures were dissolved in *n*-hexane in a 1:20 volume ratio. In addition, 10 μL of the reference solution (RS) was mixed with 30 μL of MSTFA, and the same treatment as that to which samples were exposed, was followed in order to carry out the qualitative analysis and calculate response factors.

The GC assay was carried out following the standard AOCS method Cd 11b-91 [23] with a VARIAN 3700 chromatographer equipped with a split-splitless injector, a flame ionisation detector, a data acquisition system RIAC – PROCESAR V 2.0, and a MXT-65TG, 15 m × 0.25 mm (0.1 μm) column (crossbond 35% dimethyl–65% diphenyl polysiloxane) supplied by RESTEX (Bellefonte, PA, U.S.A.). Hydrogen was used as carrier gas at 3 mL/min. Oven temperature programme was: 40 °C (3 min)–25 °C/min–365 °C (15 min). The temperatures of the flame ionisation detector and split-splitless injector were 370 and 320 °C, respectively. Samples (1 μL) were manually injected in split-off mode for 1 min.

Only the samples belonging to Mix 1, 2 and 3 and treated with Lipolase 100T and RML were analysed chromatographically. All response factors were higher than 0.5, thus indicating either the absence of decomposition or the loss of components in the chromatographic system.

2.8. Conversion calculation

Conversion (X) was calculated taking into account the total triglycerides (TG) conversion to FAs depending on specificity (1,3 or non-specific) for the total possible amount of FAs to be obtained in both cases. If lipase is non-specific, then the final products with total conversion will be glycerol and FA. If lipase is 1,3-specific and has maximum activity, then the total conversion to FA will be 66% when it is calculated as a non-specific enzyme and it will produce FAs and monoglycerides as final products.

PL conversions were calculated taking into account that only one position (sn-1) is attacked and considering either the attack of positions 1 and 2 or the isomerisation from position 2 to 1.

The following formula were used in this study to calculate conversions:

$$X_{\mathrm{TGs}}\,(\%) = \left[\frac{\text{moles of FA produced}}{\text{initial TG moles} \times F_{\mathrm{TG}}}\right] \times 100$$

$$X_{\mathrm{PLs}}\,(\%) = \left[\frac{\text{moles of FA produced}}{\text{initial PLs moles} \times F_{\mathrm{PL}}}\right] \times 100$$

$$X_{\mathrm{TGs+PLs}}\,(\%) = \left[\frac{\text{moles of FA produced}}{(\text{initial TG moles} \times F_{\mathrm{TG}}) + (\text{initial PLs moles} \times F_{\mathrm{PL}})}\right] \times 100$$

where $F_{\mathrm{TG}} = 3$ for non-specific lipases, $F_{\mathrm{TG}} = 2$ for 1,3 specific lipases, $F_{\mathrm{PL}} = 1$ considering the attack of position 1 for PLs, $F_{\mathrm{PL}} = 2$ considering the attack of positions 1 and 2 or isomerisation from position 2 to 1. The conversion in percentage reveals either the degree or percentage of hydrolysis.

The FA composition of sunflower oil (TGs) includes $C_{14:0} = 0.08\%$, $C_{16:0} = 5.95\%$, $C_{16:1} = 0.14\%$, $C_{18:0} = 3.16\%$, $C_{18:1} = 36.78\%$, $C_{18:2} = 52.65\%$, $C_{18:3} = 0.17\%$, $C_{20:0} = 0.11\%$, $C_{20:1} = 0.10\%$, $C_{22:0} = 0.62\%$, and $C_{24:0} = 0.24\%$. In turn, the FA composition of commercial lecithin includes $C_{16:0} = 19.28\%$, $C_{16:1} = 0.09\%$, $C_{18:0} = 4.34\%$, $C_{18:1} = 10.05\%$, $C_{18:2} = 56.97\%$, $C_{18:3} = 8.20\%$, $C_{20:0} = 0.13\%$, $C_{22:0} = 0.48\%$, and $C_{22:1} = 0.17\%$, $C_{24:0} = 0.29\%$. These FAs percentages with their respective molecular weight (MW) were used to estimate the average FA–MW of sunflower oil and soybean lecithin. The analysis of lecithin yielded the following mean values with its confidence interval at 95%: acetone-insoluble matter (%) = 93.5 ± 0.4, hexane-insoluble matter (%) = 0.29 ± 0.02 and humidity (%) = 2.85 ± 0.11. The acetone-insoluble matter value was in agreement with the total phospholipid content obtained by high pressure liquid chromatography (HPLC). Soybean-lecithin (PLs) phospholipid content was 87.73% and its phospholipid profile was PE = 23.64%, PA = 25.98%, PI = 11.40%, PC = 38.98%. Acidity (as a percentage of FAs) of oil and lecithin was 0.07 and 26%, respectively.The average phospholipid MW (M_{PL}) can be

Table 1
Structure of the selected lipases and type of catalytic triad

Lipase	Position of the catalytic triad				Type of coordination-site location
	Ser	His	Asp. Ac.	Glut. Ac.	
Lipase AY 30	209	449	–	341	Tunnel
R. miehei	144	257	203	–	Surface
Lipase PS	87	286	264	–	Funnel
C. antarctica B	105	224	187	–	Funnel
Lipolase 100T	146	258	201	–	Surface
Lipase AK 20	114	199	168	–	Funnel

estimated from the average FA–MW (M_{FA}) and the phospholipid composition of lecithin according to Eq. (1):

$$M_{PL} = \left[\frac{W_{PC}}{221.2 + 2M_{FA}} + \frac{W_{PI}}{217.2 + 2M_{FA}} + \frac{W_{PE}}{179.1 + 2M_{FA}} + \frac{W_{PA}}{136.0 + 2M_{FA}}\right]^{-1} \quad (1)$$

with $M_{FA} = [\sum W_i/M_i]$, where W_i and M_i represent the mass fraction and the MW of the *i*th FA, respectively. By means of this formula, the estimated average MW of lecithin was 746.34 g/mol. The average MW of triglycerides (M_{TG}) can be estimated from the average MW (M_{FA}) by means of

$$M_{TG} = 3M_{FA} + 37.04 \quad (2)$$

according to which, the estimated average MW of sunflower oil was 861.08 g/mol.

2.9. Theoretical study

The substrate adsorption mechanisms were studied using the Chem 3D 5.0 Ultra program and the MM2 (Cambridge Soft) method with trilinolein and phosphatidylcholine as model systems of TGs and PLs, respectively. By means of this program, steric energy changes upon adsorption on the lipase active site in its "open" form were modelled. The PM3 method and a short model including only the catalytic triad and the stabilisers close to it (oxyanion hole, for example) were used to evaluate the electronic local changes during the reaction with triacetin.

3. Results

3.1. Lipase hydrolytic activities

The positions of amino acids in the primary structure of the different lipases are shown in Table 1 together with the structure of the substrate coordination site [19]. Table 2 shows the degree of hydrolysis corresponding to 10 lipases and one phospholipase for TGs and PLs after 6 h of reaction. The relative standard deviation (R.S.D.) was less than 10% for conversions higher than 20% whereas it was lower than 22% for conversions lower than 20%.

3.2. Free lipases

Considering that lipases attack all TG positions, Lipase PS and Lipase AK 20, respectively, showed 69.0 and 55.1% percentages of total conversion to FAs. If 1,3 specificity is considered for Lipase PS, conversion is higher than 100% (103.5%). Free RML has a low hydrolytic activity (11.9%). For TGs hydrolysis Lipase AY (from *Candida rugosa*—14.4%) and in CALB (from *Candida antarctica* B—5.8%) have very low activity. For lecithin, the free RML was very efficient (35.6% conversion to FAs). The remaining free lipases showed an 11% conversion

Table 2
Percentages of hydrolysis calculated by titration

Lipase	Sunflower oil		Soybean lecithin	
	1,2,3[a]	1,3[b]	1[c]	1,2[d]
Rhizomucor miehei (*RML*)	11.9	17.8	35.6	17.8
Lipase from porcine pancreas grade B	7.9	11.9	19.7	9.8
Lipolase 100T (immobilised *Thermomyces lanuginosa*)	85.3	127.9	19.5	9.8
Lipase PS (from *Pseudomonas cepacia)*	69.0	103.5	11.9	5.9
Lipase AK 20 (from *Pseudomonas fluorescens*)	55.1	82.6	21.2	10.6
Lipozyme RM IM (immobilised *Rhizomucor miehei*)	47.6	71.3	6.0	3.0
Lipase AY 30 (from *Candida rugosa*)	14.4	21.6	–[e]	–[e]
Candida antarctica B (CALB)	5.8	8.6	–[e]	–[e]
Novozym 435 (immobilised *Candida antarctica B*)	2.5	3.8	–[e]	–[e]
Lecitase 10 L pH 7.0 (Phospholipase A2 from porcine pancreas)	–	–	85.4	42.7
Lecitase 10 L pH 8.0 (Phospholipase A2 from porcine pancreas)	–	–	56.7	28.3

[a] Taking into account all the positions of hydrolysed TG including isomerisation from position 2 to 1 in the case of reported 1,3-specific lipases.
[b] Taking into account specificity 1,3 for TG hydrolysis with lipases.
[c] Taking into account the attack of position 1 for PLs.
[d] Taking into account either the attack of positions 1 and 2 or isomerisation from position 2 to 1.
[e] These lipases were reported as non-active in the hydrolysis of lecithin.

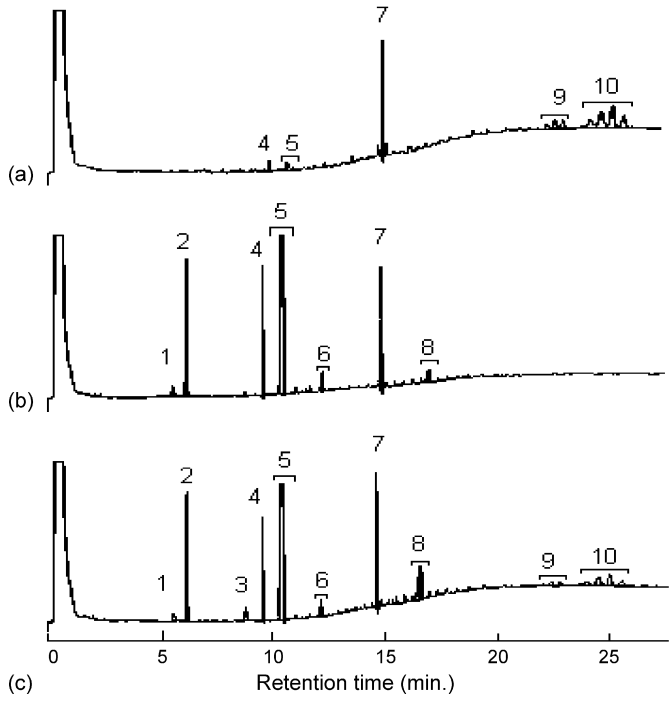

Fig. 2. Gas chromatogram of sunflower oil. Tetradecane and tricaprin as internal standard. (a) Before hydrolysis. (b) After hydrolysis with Lipolase 100T. (c) After hydrolysis with RML. Peak assignment: 1 = glycerol; 2 = tetradecane; 3 = new compound; 4 = palmitic acid; 5 = stearic, oleic and linoleic acids; 6 = monoglycerides; 7 = tricaprin; 8 = diglycerides; 9 = triglycerides with carbon numbers of 52; 10 = triglycerides with carbon numbers of 54.

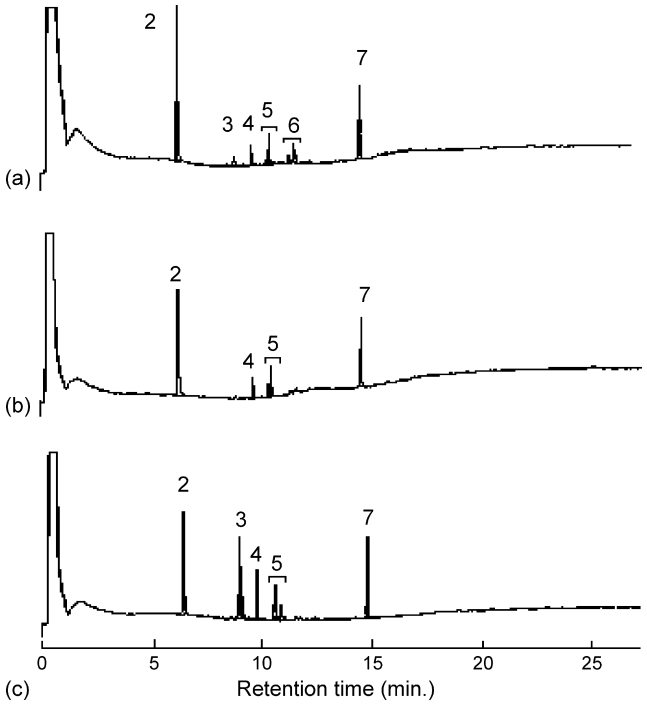

Fig. 3. Gas chromatogram of soybean lecithin. Tetradecane and tricaprin as internal standard. (a) Before hydrolysis. (b) After hydrolysis with Lipolase 100T. (c) After hydrolysis with RML. Peak assignment: 2 = tetradecane; 3 = new compound; 4 = palmitic acid; 5 = stearic, oleic and linoleic acids; 6 = monoglycerides; 7 = tricaprin.

of soybean lecithin and only position 1 of PL was considered hydrolysable. Mustranta et al. demonstrated that lipases from Lipase AY and *Candida antarctica B* have no activity in the hydrolysis of lecithin [3]. These catalysts were not tested in the hydrolysis of lecithin. Lecitase 10L (a phospholipase C) showed the highest percentage of conversion of lecithin to FAs (85%) at pH 7.0 (Table 2).

Hara et al. [2] reported high conversions in phosphatidylcholine hydrolysis with lipases from *Mucor* family, lower conversions with *Thermomyces* and none with *Candida*, which is in agreement with our results.

3.3. Immobilised lipases

Lipolase 100T (from *Thermomyces lanuginosa*) showed the highest conversion to FAs from TGs hydrolysis (85.3%). Table 2 reveals that Lipozyme RM IM is better for hydrolysis of sunflower oil instead of lecithin (47.6% versus 6.0%). In contrast, Novozyme 435 showed almost zero activity in TGs hydrolysis (Table 2).

3.4. Comparison of lipases for TGs and lecithin hydrolysis

Fig. 2 shows one chromatogram for sunflower oil before the hydrolysis reaction (a) and another one corresponding to sunflower oil hydrolysed with Lipolase 100T (b). The chromatographic studies on oil showed the following fatty acids as reaction products: palmitic acid (5.7%); stearic, linoleic and oleic acids (85.2%). Fig. 2 also shows results after sunflower oil hydrolysis with RML (c). The amount of FAs produced in this case was evidently lower (13.1%) than in the case of Lipolase 100T. FA composition after the hydrolysis reaction included palmitic acid (0.8%), stearic, oleic and linoleic acids (12.3%). Fig. 3 shows the initial soybean lecithin chromatogram and the comparison of different lipases in PL hydrolysis using RML and Lipolase 100T. Phospholipids did not appear in the chromatogram-probably retained in the column. When RML was the biocatalyst, an additional peak was observed at 8.80 min. This peak was located between the tetradecane standard and the FA region. Several compounds were injected for the determination of their retention times to compare with the unknown (glycerophosphorylcholine, methyl nonanoate, methyl hexadecanoate, methyl dodecanoate, methyl tetradecanoate and 10-undecenoic acid). No match with 8.80 min was found.

3.5. Lipase theoretical study

Lipase theoretical study was focused on the adsorption step of TGs and PLs at the "open" structure of different lipases, which were obtained from the Protein Data Bank (PDB), where the lid leaves available the active site [19,20].

TGs and PLs were modelled as trilinolein (LLL) and phosphatidylcholine (PC), respectively. These model compounds were adsorbed at a distance lower than 3 Å on the active site.

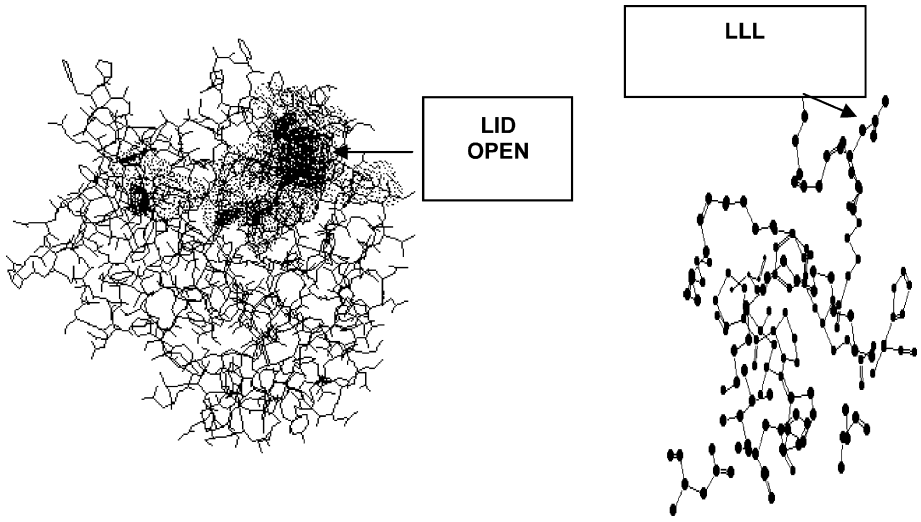

Fig. 4. Coordination of TGs on the active site of RML and open conformation of RML.

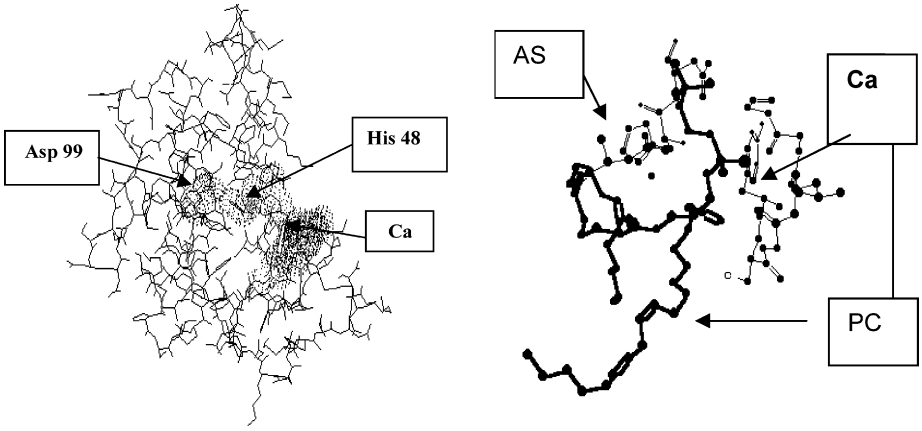

Fig. 5. Coordination of PLs on phospholipase Bovine A2.

3.5.1. Candida rugosa lipase (CRL)

It was observed that due to the structure of the tunnel, LLL had no restrictions when approaching the triad. Based on TG outside the tunnel, its adsorption on CRL showed a steric energy change of +0.5 kcal/mol. PC adsorption had a barrier of almost 40 kcal/mol when it approached towards the catalytic triad.

3.5.2. Rhizomucor meihei lipase (RML)

LLL adsorption in the active site of RML was favoured by −32.2 kcal/mol. On the other hand, PC adsorption was stimulated by −47 kcal/mol. Fig. 4 shows the TG coordination on the RML active site and the RML open conformation.

3.5.3. Pseudomonas cepacia lipase (PCL)

LLL adsorption was favoured by −18 kcal/mol whereas PC adsorption by −27 kcal/mol.

3.5.4. Phospholipase Bovine A2

PC adsorption on phospholipase Bovine A2 was stimulated by −18 kcal/mol. The presence of Ca^{2+} in a pocket-like structure, where the phosphate group is located, was particularly important to stabilise this structure (that has no serine in the active site). PC adsorption on phospholipase Bovine A2 is shown in Fig. 5.

4. Discussion

In all instances, Lipolase 100T (immobilised) evidenced the highest percentage of hydrolysis of the artificial mixture (100%) whereas Lipase PS and Lipase AK (both free lipases) revealed lower – though very good – conversions, particularly in relation to the attack of lecithin in position 1 (Table 3). Isomerisation of position 2 from TG and lecithin must also be taken into account to explain these results. Hara et al. [2] claimed that lipase from porcine pancreas has 1,3-specificity. Lipase from porcine pancreas grade B was contaminated with phospholipase while lipase from porcine pancreas Type II was purer (see Table 2). A higher conversion occurred in Mix 3 with respect to Mix 1 and Mix 2 as a result of a more favourable substrate to catalyst mass ratio (Fig. 6).

Table 4 shows the activities of lipases in different units. The comparison of these activities is useful to analyse costs in terms of desirable product per gram of immobilised, commercial catalyst. It is clear that Lipolase (immobilised lipase) is

Table 3
Percentages of hydrolysis of the oil–lecithin mixture calculated by titration

Lipase	Oil–lecithin mixture		
	Sunflower oil		Lecithin
	1,2,3[a]	1,3[b]	
Rhizomucor miehei	29.3	35.5	1,2[c]
	38.6	50.1	1[d]
Lipase from porcine pancreas grade B	17.2	20.8	1,2
	22.6	29.3	1
Lipase from porcine pancreas Type II	24.0	29.2	1,2
	31.3	40.7	1
Lipolase 100T (from *Thermomyces lanuginosa*)	100.0	100.0	1,2
	100.0	100.0	1
Lipase PS (from *Pseudomonas cepacia*)	43.3	52.2	1,2
	57.1	73.8	1
Lipase AK 30 (from *Pseudomonas fluorescens*)	43.4	52.6	1,2
	58.3	75.0	1

[a] Taking into account all the positions of hydrolysed TG including isomerisation from position 2 to 1 in the case of reported 1,3-specific lipases.
[b] Taking into account specificity 1,3 for TG hydrolysis with lipases.
[c] Taking into account the attack of positions 1 and 2 of PL or isomerisation from position 2 to 1.
[d] Taking into account the attack of position 1 for PLs.

a good catalyst for hydrolysis of TGs and oil–lecithin mixture whereas RML (free lipase) is a good catalyst for hydrolysis of PLs.

Previous studies from our group addressed the problem of inhibition using CALB and Novozyme 435 in ester synthesis using solvent-free systems [14,24]. Alcohols have been proposed to act as inhibitors [19]. Previous research [28–31] concluded that short-chain FAs are lipase inhibitors. The possibility of an undesirable reaction between lipase and hydrolysis products should not therefore be discarded although this issue is beyond the scope of the present work.

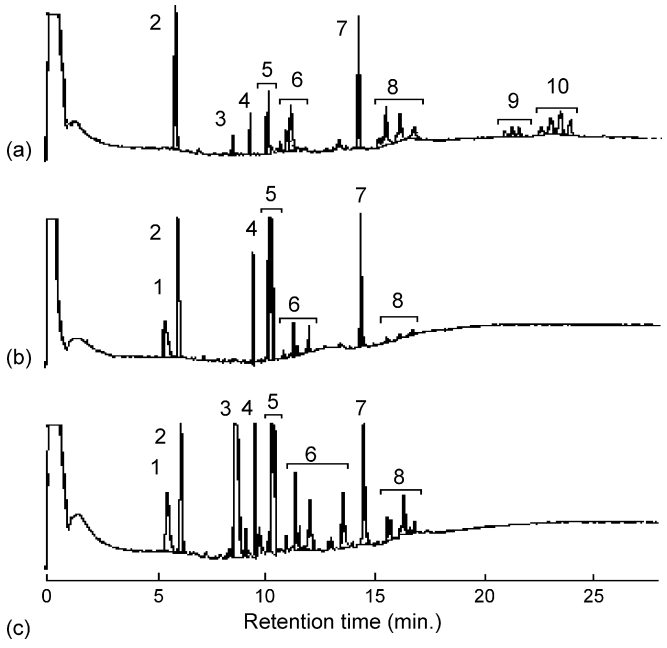

Fig. 6. Gas chromatogram of the artificial mixture. Tetradecane and tricaprin as internal standard. (a) Before hydrolysis. (b) After hydrolysis with Lipolase 100T. (c) After hydrolysis with. RML. Peak assignment: 1 = glycerol; 2 = tetradecane; 3 = new compound; 4 = palmitic acid; 5 = stearic, oleic and linoleic acids; 6 = monoglycerides; 7 = tricaprin; 8 = diglycerides; 9 = triglycerides with carbon numbers of 52; 10 = triglycerides with carbon numbers of 54.

The use of other lipases in lecithin and TGs hydrolysis showed that the compound appearing at 8.80 min was found as a very small peak after very long reaction times. However, in the case of RML the peak at 8.80 min was the main one after the hydrolysis of lecithin. This new compound (assignable to the 8.80 min peak) may belong to a product formed from FA hydrolysis in position 1 of the phospholipid molecule. We consider that the compound in the case of RML results from lecithin-derivatives reaction with the lipase (see fig. 7). Phosphate/choline polar groups may behave as isomerisation/cracking catalysts in the presence of lipase. In the absence of evidence regarding contamination produced by phospholipase C or D and isomerases in the lipase preparation – private communication from the supplier upon request – the products related to the activity of these enzymes were discarded. Isomerisation from position 2 to 1 has been reported for RML [3].

We have analysed the following formation routes for this compound:

(a) After hydrolysis of the P–O group and the first OH from position 1 of lecithin, isomerisation from position 2 to posi-

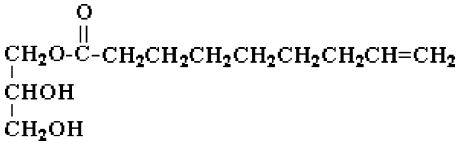

Fig. 7. Postulated formula of the unknown compound.

Table 4
Enzymatic activities of lipases over the three substrates

Enzyme	Units	TG	PLs	TG + PLs
Lipolase 100T (*H. lanuginosa*)	mmol FFA/g cat h	10.2598	1.6715	2.8472
	g FFA/g cat h	2.8933	0.4714	0.8029
	g FFA/g cat	17.3596	2.8282	4.8175
	g FFA/h	0.1447	0.0024	0.0401
	μmol FFA/min	8.5	0.1	2.4
Lipase PS (*P. cepacia*)	mmol FFA/g cat h	8.3086	1.1249	1.1271
	g FFA/g cat h	2.3430	0.3172	0.3178
	g FFA/g cat	14.0582	1.9033	1.9071
	g FFA/h	0.1172	0.0016	0.0159
	μmol FFA/min	6.9	0.1	0.9
Lipase AK (*P. fluorescens*)	mmol FFA/g cat h	6.6265	1.1246	1.1415
	g FFA/g cat h	1.8687	0.3171	0.3219
	g FFA/g cat	11.2120	1.9028	1.9314
	g FFA/h	0.0934	0.0016	0.0161
	μmol FFA/min	5.5	0.1	1.0
RML (*Rhizomucor miehei*)	mmol FFA/g cat h	1.3766	3.0825	0.7384
	g FFA/g cat h	0.3882	0.8693	0.2082
	g FFA/g cat	2.3292	5.2156	1.2494
	g FFA/h	0.0194	0.0043	0.0104
	μmol FFA/min	1.1	0.3	0.6
Lipase grade B (porcine pancreas)	mmol FFA/g cat h	0.9333	1.6806	0.4636
	g FFA/g cat h	0.2632	0.4739	0.1307
	g FFA/g cat	1.5791	2.8436	0.7844
	g FFA/h	0.0132	0.0024	0.0065
	μmol FFA/min	0.8	0.1	0.4

Results are expressed in different units to show the diversity of activity expression.

tion 1 may occur. Under a certain degree of constraint, RML may have some phospholipase C activity.

(b) After isomerisation to position 1, cracking may occur because of: (i) the exposed nature of the active site in RML and the close neighbourhood of phosphatide/choline charged to the double bond, and (ii) the presence of one/two double bonds in FAs.

(c) Hydrolysis of P–O bond from lecithin is a probable – though unexpected – ability of RML.

Taking into account that cracking takes place in the double bond, we have $C_{12}H_{23}O_4$ (MW = 231 g/mol) corresponding to this peak with the ester group in position 1 or 2 (Fig. 6).

The peak at 8.80 min (indicated as peak 3 in Figs. 2 and 3) could be observed: (i) in the gas chromatogram of sunflower oil hydrolysis with RML (Fig. 2c); (ii) in the gas chromatogram of soybean lecithin with RML (Fig. 3c) and (iii) in the initial gas chromatogram of soybean lecithin and the initial artificial mixture (Figs. 3a and 6a); and (iv) in the artificial mixture treated with RML (Fig. 6c).

In view of the chemical structure of lecithin, hydrolysis reaction may occur either inter- or intra-molecularly in lecithin without lipases. Peak 3 was absent in Fig. 2a (sunflower oil) while it was present in Fig. 3a (lecithin) and Fig. 6a (artificial mixture). After using Lipolase 100T, the peak was absent (Figs. 2b, 3b and 6b). In contrast, it was always present when RML was used (Figs. 2c, 3c and 6c). In view of the above, the compound at 8.80 min may thus be formed even from sunflower oil but at a much lower proportion than in the case of either lecithin or the artificial mixture, indicating a significantly important contribution of the chemical composition of lecithin and the lipase. This reaction in lecithin may occur spontaneously with time, thus producing the peaks shown in Figs. 3a and 6a. In view of this, and due to the presence of phosphate groups and the probabilities of acid–base catalysis to occur, chemical catalysis is possible independently of enzymatic catalysis.

Acyl migration can be catalysed by supports with surface charges [19,26,27]. In the absence of information regarding the support of *T. lanuginosa* in Lipolase 100T, the factibility of acyl migration cannot be discarded as well as unknown stabilisation conditions [18,20].

The steric energy changes upon adsorption are the reason why lecithin is more strongly adsorbed in *R. meihei* than in *P. cepacia* while it is not adsorbed in *C. rugosa*. This trend was not observed in TGs, such as sunflower oil. The different stages of the substrate accessibility to the active site account for the activity differences of the tested lipases.

5. Conclusions

Lipolase 100T showed the highest conversion to fatty acids when sunflower oil (85.3% conversion to fatty acids) and its mixture with lecithin (100% conversion to fatty acids) were tested. The biocatalyst from RML presented the highest activity with lecithin as substrate (35.6% conversion to fatty acids). The lipases with their active sites on the surface were the most active

in the hydrolysis of lecithin, sunflower oil and their mixtures. Lipases with active sites at the end of a tunnel or a funnel were by far less active, specially considering the size of TG and PLs as substrates (and this was confirmed by the theoretical study in terms of changes of steric energies on adsorption).

Besides the FA, monoglycerides (MG) and diglycerides (DG) a new product was detected when lecithin was present (alone or in the mix) mainly when RML was used as biocatalyst. No evidence of this peak is found after the reaction using Lipolase 100T. This new product can be related to the presence of phosphate charged group in lecithin and to the possibility of cracking of one of the hydrocarbon chains of the FA in TG, involving also the lipase. This new compound was assigned to a MG of a shorter fatty acid chain – with 9 carbon atoms – product of the chemical reaction of the lecithin with the exposed active site of RML.

Lipases have potential in the hydrolysis of oil gums and lecithin, with high conversion to FA, mainly Lipolase 100T and RML This is the first report – at the author's knowledge-of the involvement of the lipase RML in the lecithin hydrolysis with the generation of a short fatty acid monoglyceride.

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