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Lipase-catalysed hydrolysis of short-chain substrates in solution and in emulsion: a kinetic study

Lylia Nini [a], Louis Sarda [b,*], Louis-Claude Comeau [a], Elisabeth Boitard [c], Jean-Paul Dubès [c], Henri Chahinian [d]

[a] *Laboratory of Applied Biochemistry, Faculty of Sciences and Technology, St-Jérôme, 13397 Marseille Cedex 20, France*
[b] *Laboratory of Biochemistry, Faculty of Sciences St-Charles, University of Provence, 13331 Marseille Cedex 3, France*
[c] *Thermochimie MADIREL/UMR 6121, Faculty of Sciences St-Charles, University of Provence, 13331 Marseille Cedex 3, France*
[d] *Laboratory of Enzymatic Lipolysis, Centre National de la Recherche Scientifique, 13402 Marseille Cedex 20, France*

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Abstract

We have studied the enzymatic hydrolysis of solutions and emulsions of vinyl propionate, vinyl butyrate and tripropionin by lipases of various origin and specificity. Kinetic studies of the hydrolysis of short-chain substrates by microbial triacylglycerol lipases from *Rhizopus oryzae*, *Mucor miehei*, *Candida rugosa, Candida antarctica* A and by (phospho)lipase from guinea-pig pancreas show that these lipolytic enzymes follow the Michaelis–Menten model. Surprisingly, the activity against solutions of tripropionin and vinyl esters ranges from 70% to 90% of that determined against emulsions. In contrast, a non-hyperbolic (sigmoidal) dependence of enzyme activity on ester concentration is found with human pancreatic lipase, triacylglycerol lipase from *Humicola lanuginosa* (*Thermomyces lanuginosa*) and partial acylglycerol lipase from *Penicillium camembertii* and the same substrates. In all cases, no abrupt jump in activity (interfacial activation) is observed at substrate concentration corresponding to the solubility limit of the esters. Maximal lipolytic activity is always obtained in the presence of emulsified ester. Despite progress in the understanding of structure–function of lipases, interpretation of the mode of action of lipases active against solutions of short-chain substrates remains difficult. Actually, it is not known whether these enzymes, which possess a lid structure, are in open or/and closed conformation in the bulk phase and whether the opening of the lid that gives access to the catalytic triad is triggered by interaction of the enzyme molecule with monomeric substrates or/and multimolecular aggregates (micelles) both present in the bulk phase. From the comparison of the behaviour of lipases used in this study which, in some cases, follow the Michaelis–Menten model and, in others, deviate from classical kinetics, it appears that the activity of classical lipases against soluble short-chain vinyl esters and tripropionin depends not only on specific interaction with single substrate molecules at the catalytic site of the enzyme but also on physico-chemical parameters related to the state of association of the substrate dispersed in the aqueous phase. It is assumed that the interaction of lipase with soluble multimolecular aggregates of tripropionin or short-chain vinyl esters or the formation of enzyme–substrate mixed micelles with ester bound to lipase, might represent a crucial step that triggers the structural transition to the open enzyme conformation by displacement of the lid.

Keywords: Carboxylesterase; Lipase; Vinyl ester; Tripropionin; Solution; Emulsion; Kinetics; Interfacial activation; Substrate specificity.

* Corresponding author. Fax: +33-4-9110-6350. x_it;E-mail address: sarda@newsup.univ-mrs.fr (L. Sarda).

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1. Introduction

Living organisms produce several classes of lipolytic enzymes which include carboxylesterases (EC 3.1.1.1.) and lipases (EC 3.1.1.3.). Originally, the differentiation between esterase and lipase activity was founded on the comparison of the kinetics of hydrolysis of solution and emulsion of methyl butyrate or triacetin by horse liver esterase and porcine pancreatic lipase [1]. It was observed that, under the particular conditions used for measuring activity, the rate of hydrolysis of the partially water-soluble esters by liver esterase was maximal at substrate concentration well below the solubility limit of the substrate and did not increase in the presence of emulsified particles. Conversely, the activity of pancreatic lipase was low against soluble ester and abruptly increased as soon as an emulsion was formed in oversaturated solution. Then, the increase in activity observed with porcine pancreatic lipase in the presence of emulsified substrate (interfacial activation) was considered as a characteristic property of the lipolytic enzyme. Moreover, it was speculated that pancreatic lipase was activated at a lipid–water interface as the result of a conformational change of the enzyme bound to its water-insoluble substrate [2]. Later, this hypothesis received support from the determination of the crystal structure of the lipase and lipase–inhibitor complex from *Mucor miehei* [3–5], lipase from *Geotrichum candidum* [6,7] and human pancreatic lipase and its complex formed with its protein cofactor, colipase, in the absence and presence of mixed micelles of phosphatidylcholine and bile salts [8–10]. It was clearly established that the catalytic triad Ser-His-Asp/Glu, found in serine hydrolases, was buried under a short helical peptide segment of a surface loop (flap or lid) which had to move away to give access to the active site. It was proposed that the movement of the lid domain which leads to an open active conformation of the enzyme could result from specific hydrophobic lipase-substrate interaction at a lipid–water interface.

In the last years, kinetic and structural studies carried out with carboxyl ester hydrolysing enzymes as cutinase from *Fusarium solani* [11], lipases from *Candida antarctica* [12], *Pseudomonas aeruginosa* and *Bacillus subtilis* [13] and with the (phospho)lipase (pancreatic lipase related protein 2) from guinea-pig pancreas [14] have challenged the original distinction between esterases and lipases. It was observed that these lipases were active towards partially water-soluble esters as tributyrin and *p*-nitrophenyl butyrate and were not interfacially activated. In some of these enzymes, the α-helical peptide structure covering the active site is absent or partially deleted. It was suggested, then, that the observed lack of interfacial activation could be in direct relationship with the absence or partial deletion of the lid domain. As a consequence of their particular behaviour against soluble substrates, these lipolytic enzymes could be classified as esterases although they are active against water-insoluble long-chain triacylglycerols.

Recently, we have isolated two extracellular lipolytic enzymes (lipases I and II) produced by the filamentous fungus *Penicillium cyclopium* and we have studied their substrate specificity and kinetic properties [15,16]. Lipase I is a triacylglycerol lipase structurally homologous to triacylglycerol lipases from *Penicillium expansum* [17] and *Penicillium solitum* [18]. It is active on emulsions of short, medium and long-chain triacylglycerols but weakly hydrolyses mono- and diacylglycerols. Lipase II shows biochemical and structural properties similar to those of the partial acylglycerol lipase from *Penicillium camembertii* [19,20]. It hydrolyses mono- and diacylglycerols with preference for medium chains and shows no activity towards triacylglycerols. To directly compare the hydrolysing properties of *P. cyclopium* lipases I and II, we have used short and long-chain vinyl esters as substrates [21]. As shown earlier, vinyl esters are good substrates for lipases including porcine pancreatic lipase [22,23] and lipases from *P. camembertii* [19] and *Humicola lanuginosa* [24]. Moreover, vinyl propionate and vinyl butyrate, which are partially soluble in water, can be used for studying the dependence of activity on substrate concentration and the effect of the physical state of the substrate on activity by comparing the rates of hydrolysis of soluble and emulsified esters within a rather large range of concentration. Unexpectedly, it was found that, in the absence of surface-active agent, *P. cyclopium* lipases I and II hydrolysed solution of vinyl propionate or vinyl butyrate at relatively high rates compared to maximal rate measured against emulsion. Similar observation was made with human pancreatic lipase. The substrate concentration dependency of the rate

of hydrolysis of short-chain vinyl esters (v/S curves) by *P. cyclopium* lipase I was hyperbolic and followed the classical Michaelis–Menten model whereas in the case of *P. cyclopium* lipase II and human pancreatic lipase, the v/S curves were non-symmetrical sigmoids. In all cases, no abrupt jump in enzyme activity occurred at the saturation point of the solution of ester.

With the view to further investigate the capacity of lipases to hydrolyse solutions of carboxyl esters, we have studied kinetics of hydrolysis of solutions and emulsions of vinyl propionate, vinyl butyrate and tripropionin by well-characterised acylglycerol-hydrolysing enzymes of known tridimensional structure. Enzymes used in these studies include lipases from *Rhizopus oryzae*, *M. miehei*, *Humicola* (*Thermomyces*) *lanuginosa*, *P. camembertii*, *Candida rugosa*, *C. antarctica* A and the (phospho)lipase from guinea-pig pancreas GPLRP2). The results reported in this communication, together with those previously reported obtained with lipases from *P. cyclopium* and human pancreas, show that all classical lipases have the capacity to hydrolyse optically clear solutions of tripropionin and short-chain vinyl esters.

2. Materials and methods

2.1. Chemicals

Vinyl propionate, vinyl butyrate, vinyl laurate, bovine serum albumin and benzopurpurin 4B were purchased from Sigma-Aldrich-Fluka Chimie (St-Quentin-Fallavier, France). Tripropionin, tributyrin and trioctanoin (tricaprylin) were from Acros Organics (Noisy-Le-Grand, France). The determination of the limit of solubility of vinyl propionate, vinyl butyrate and tripropionin has been reported previously [21].

2.2. Enzymes

Lipases from *M. miehei* and *C. rugosa* were purchased from Sigma-Aldrich-Fluka Chimie and used without further purification. Lipase from *R. oryzae* was prepared at the laboratory as described previously [25]. Crude lipase from *P. camembertii* was obtained from Amano (Amano Enzyme Europe, Milton Keynes, UK) and partially purified by gel filtration through a column of Sephadex G75 in 25 mM Tris buffer, pH 7.5. Pure samples of lipases from *H. lanuginosa*, *C. antarctica* A and (phospho)lipase from guinea-pig pancreas were gifts from Dr R. Verger (CNRS, Marseille, France) and Dr S. Patkar (Novo Nordisk, Copenhagen, Denmark). Protein concentration was estimated by the colorimetric method of Lowry et al. using bovine serum protein as standard protein [26].

2.3. Lipase activity measurements

The course of enzyme-catalysed hydrolysis of tripropionin, tributyrin and trioctanoin at 25°C and pH 7.0 was monitored using a pH-stat (TTT 80, Radiometer, Copenhagen, Denmark) as described in a recent report [21]. Lipase activity on olive oil emulsified with gum arabic was determined at pH 8.5 [27]. Hydrolysis of the vinyl esters was followed potentiometrically at pH 7.0 and 25°C with the pH-stat under conditions previously described [21]. Lipase activity was expressed as units. One enzyme unit corresponds to the release of one microequivalent acid per minute. Assays were performed in duplicate with less than 5% deviation. The amount of enzyme used in assays corresponded to 5–10 lipase units measured at substrate concentration ensuring maximal enzyme activity (V_m). The relative rate of enzymatic hydrolysis (100 v/V_m) of solutions and emulsions was plotted against substrate concentration expressed arbitrarily as mol l^{-1} both below (solution) and above (emulsion) the limit of solubility of the ester.

2.4. Study of the physical state of solutions of tripropionin and vinyl esters with a spectral dye method

The water-soluble dye benzopurpurin 4B is currently used for the detection of multimolecular aggregates (micelles) in aqueous solutions of non-ionic surface active agents [28]. Interaction between the dye and micellar aggregates induces spectral differential absorbance at a wavelength of 510 nm. The procedure followed to detect aggregates in the solutions of vinyl propionate, vinyl butyrate and tripropionin, was adapted from that described earlier by Entressangles and Desnuelle [29]. A solution of benzopurpurin (50 mg l^{-1}) was prepared in 25 mM Tris

buffer, pH 7.0. Five-ml aliquots of the solution of benzopurpurin were mixed with increasing volumes of ester in stoppered vials. After 30 min, absorbance was read at a wavelength of 510 nm against the solution of benzopurpurin, using a 1-cm cell, and plotted against ester concentration. All assays were carried out in duplicate.

3. Results

3.1. Kinetic behaviour of lipases from R. oryzae, M. mieihei and H. lanuginosa studied with vinyl esters and tripropionin

Lipases of the filamentous fungi *R. oryzae*, *M. miehei* and *H. lanuginosa* are triacylglycerol hydrolases showing partial sequence homology. The sequence identity of *R. oryzae* vs. *M. miehei* lipases is 55% [30,31] whereas that of *H. lanuginosa* vs. *M. miehei* lipases is 26% [32]. Crystallographic studies have shown that, in spite of their relatively low level of amino acid identity, lipases from *M. miehei* and *H. lanuginosa* have similar molecular architecture including the presence of a lid domain formed by a single loop which is around 15 residues long [33]. The three enzymes hydrolyse emulsions of tributyrin, trioctanoin and olive oil (Table 1). Here, we have studied their capacity to hydrolyse solutions and emulsions of vinyl esters and tripropionin. Fig. 1A shows the effect of increasing concentration of substrate on the rate of hydrolysis of vinyl propionate by *R. oryzae* lipase in the absence and presence of 0.1 M NaCl. It can be observed from the *v*/*S* curves that

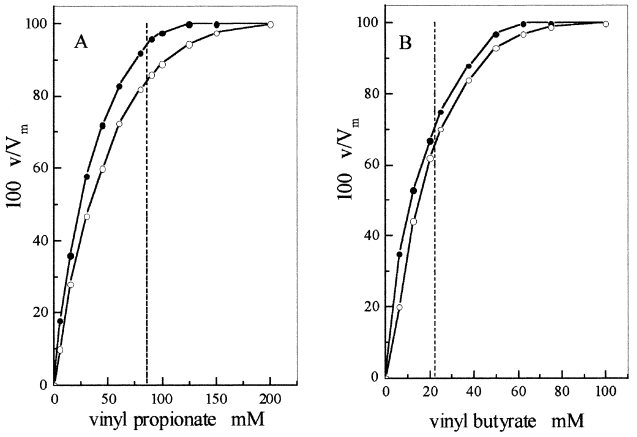

Fig. 1. Effect of ester concentration on the rates of hydrolysis of vinyl propionate (A) and vinyl butyrate (B) by lipase from *Rhizopus oryzae*. Activity is expressed as percentage of maximal activity (100 v/V_m) measured at optimal substrate concentration. The vertical dotted lines indicate the limit of solubility of the esters. Activity is determined at pH 7.0 and 25°C in 2.5 mM Tris–HCl buffer, in the absence (○) and presence (●) of 0.1 M NaCl.

NaCl has almost no effect on the kinetic behaviour of the enzyme which follows the classical Michaelis–Menten model. The rate of hydrolysis of vinyl propionate, at substrate concentration corresponding to the point of saturation of the solution of substrate, represents around 90% of the maximal rate measured against emulsion. The substrate concentration that corresponds to half maximal activity ($K_{0.5}$) is 25 mM. The kinetic behaviour of *R. oryzae* lipase against vinyl butyrate (not shown) is similar to that against vinyl propionate. The relative activity against soluble ester amounts to 70% of that measured against emulsion, with a $K_{0.5}$ of 12 mM (Fig. 1B).

Table 1
Rates of hydrolysis of triacylglycerols by lipases from *Rhizopus oryzae*, *Mucor miehei*, *Humicola lanuginosa*, *Penicillium camembertii*, *Candida rugosa*, *Candida antarctica* (lipase A) and guinea-pig pancreas

	Tripropionin	Tributyrin	Trioctanoin	Olive oil
R. oryzae	110	900	7000	1000
M. miehei	400	2200	9200	3300
H. lanuginosa	225	2250	9250	2900
P. camembertii	0	0	0	0
C. rugosa	30	70	280	25
C. antarctica A	350	310	320	145
Guinea-pig pancreas	250	550	175	35
Human pancreas	4000	9500	6500	4000

Specific activities are expressed as unit mg^{-1}.

Table 2
Rates of hydrolysis of vinyl esters by lipases from *R. oryzae*, *M. miehei*, *H. lanuginosa*, *P. camembertii*, *C. rugosa*, *C. antarctica* (lipase A) and guinea-pig pancreas

	Vinyl propionate	Vinyl butyrate	Vinyl laurate
R. oryzae	1600	3300	2700
M. miehei	7500	11000	11000
H. lanuginosa	3000	3700	12000
P. camembertii	5400	7250	3500
C. rugosa	60	100	20
C. antarctica A	320	240	355
Guinea-pig pancreas	360	270	160
Human pancreas	960	750	450

Specific activities are expressed as units mg^{-1}.

Specific activities of *R. oryzae* lipase, calculated from the maximal rates of hydrolysis of emulsions of vinyl propionate and vinyl butyrate, are 1600 U mg^{-1} and 3300 U mg^{-1}, respectively, both in the absence and presence of 0.1 M NaCl (Table 2).

The kinetic behaviour of *R. oryzae* lipase against tripropionin is shown in Fig. 2A. The enzyme is active against a solution of tripropionin and displays about 90% of its maximal activity at ester concentration below the solubility limit with a $K_{0.5}$ of 4 mM. The specific activity of the enzyme, measured against emulsified tripropionin, is 110 U mg^{-1}, in the absence and presence of 0.1 M NaCl. Kinetic studies with vinyl laurate as substrate (Fig. 2B) show that the enzyme has a higher affinity for the water-insoluble long-chain substrate than for short-chain vinyl esters. The value of $K_{0.5}$, estimated from the activity substrate concentration curve, is 1.25 mM. The specific activity of *R. oryzae* lipase, measured against vinyl laurate, is 2700 U mg^{-1} (Table 2).

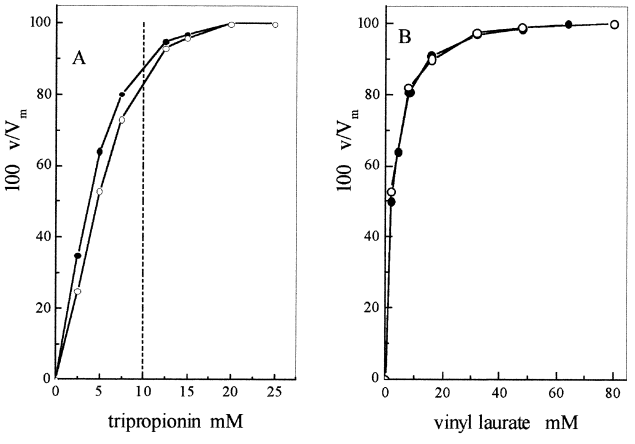

Fig. 2. Effect of ester concentration on the rates of hydrolysis of tripropionin (A) and vinyl laurate (B) by lipase from *Rhizopus oryzae*. Assays were performed at pH 7.0 and 25°C, and activity was expressed as in Fig. 1. Symbols are the same as in Fig. 1.

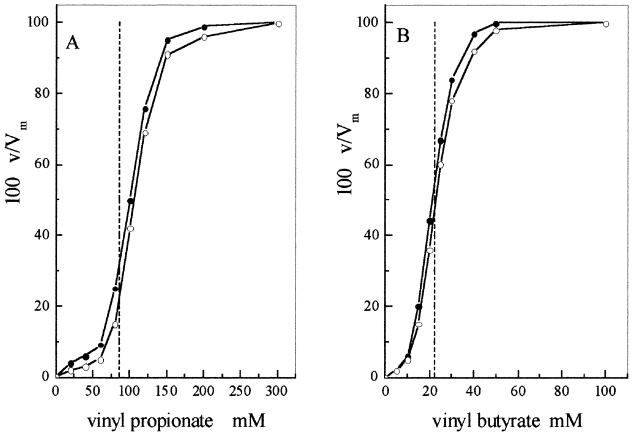

Fig. 3. Effect of ester concentration on the rates of hydrolysis of vinyl propionate (A) and vinyl butyrate (B) by lipase from *Humicola lanuginosa*. Symbols are the same as in Fig. 1.

Parallel experiments were carried out with lipases from *M. miehei* and *H. lanuginosa*. The *v*/*S* curves representative of the hydrolysis of the vinyl esters and tripropionin by *M. miehei* lipase are hyperbolic and almost identical to those obtained with lipase of *R. oryzae* (not shown). Conversely, it can be observed from Figs. 3 and 4, that the kinetic behaviour of *H. lanuginosa* lipase against vinyl propionate, vinyl butyrate and tripropionin markedly differs from

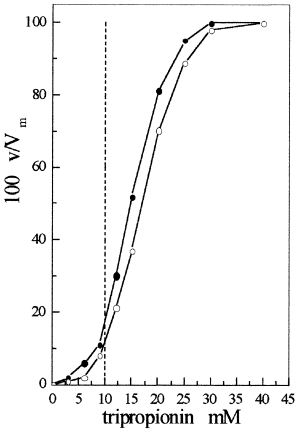

Fig. 4. Effect of ester concentration on the rate of hydrolysis of tripropionin by lipase from *Humicola lanuginosa*. Symbols are the same as in Fig. 1.

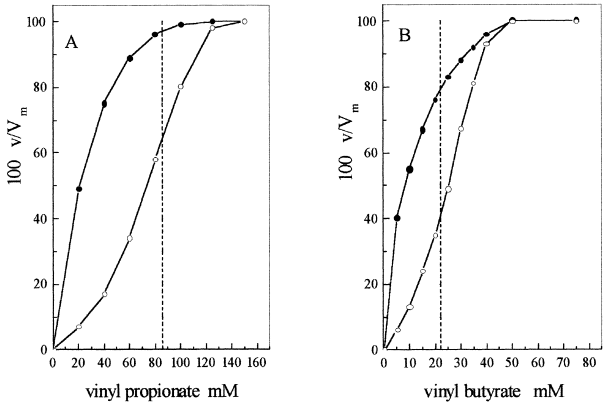

Fig. 5. Effect of ester concentration on the rates of hydrolysis of vinyl propionate (A) and vinyl butyrate (B) by lipase from *Penicillium camembertii*. Symbols are the same as in Fig. 1.

that of lipases of *R. oryzae* and *M. miehei*. Kinetics of hydrolysis of short-chain vinyl esters and tripropionin by *H. lanuginosa* lipase deviate from the classical Michaelis–Menten model as observed previously with human pancreatic lipase [21]. Activity against soluble substrates rapidly increases as the ester concentration approaches the limit of solubility. No abrupt jump in activity occurs in the presence of emulsion. It can be noticed that the *v*/*S* curve representative of the hydrolysis of emulsified vinyl laurate by *H. lanuginosa* is hyperbolic, as found with lipases from *R. oryzae* and *M. miehei* and human pancreatic lipase [21], with a $K_{0.5}$ value around 1 mM (not shown). Specific activities of the fungal lipases, measured against vinyl esters and tripropionin are listed in Tables 1 and 2.

3.2. Kinetic studies with lipase from P. camembertii

The lipase from *P. camembertii* has a level of amino acid identity of 23% with *M. miehei* lipase and of 40% with lipase from *H. lanuginosa* [33,34]. Comparison of the three-dimensional structures of lipases from the *M. miehei* family, namely lipases from *H. lanuginosa*, *P. camembertii* and *Rhizopus delemar*, reveals that these enzymes share common features including the presence of a lid domain covering the active site [31,32]. In spite of this homology, the lipase from *P. camembertii* specifically hydrolyses mono- and diacylglycerols and not triacylglycerols. The *v*/*S* curves obtained with *P. camembertii* lipase and vinyl propionate or vinyl butyrate are shown in Fig. 5A and B, respectively. One can observe that the enzyme hydrolyses solutions of short-chain vinyl esters at high relative rates compared to emulsions. In the absence of NaCl, the *v*/*S* curves deviate from the Michaelis–Menten model while in the presence of 0.1 M NaCl, the curves are hyperbolic showing comparable kinetic behaviour of the lipase with lipases from *R. oryzae* and *M. miehei*. It is remarkable that, in this particular case, the rate of hydrolysis of soluble esters is affected by the addition of salt the reaction mixture. The values of $K_{0.5}$ for the hydrolysis of vinyl propionate (20 mM) and vinyl butyrate (7 mM) in the presence of NaCl are similar to those found above with fungal triacylglycerol lipases. The kinetic behaviour of *P. camembertii* lipase against emulsified vinyl laurate (not shown) is hyperbolic and almost identical to that found with triacylglycerol lipases from *R. oryzae*, *M. miehei* and *H. lanuginosa*, with a $K_{0.5}$ value of 1.5 mM.

3.3. Hydrolysis of vinyl esters and tripropionin by lipases from the yeasts C. rugosa and C. antarctica A

The lipase from the yeast *C. rugosa* is a protein of larger molecular mass than lipases of the *Rhizomucor miehei* family. Crystallographic studies of *C. rugosa* lipase have also revealed the existence of two conformations of the enzyme, a closed conformation with the active site shielded from the solvent by a lid domain [35] and an open conformation with a sol-

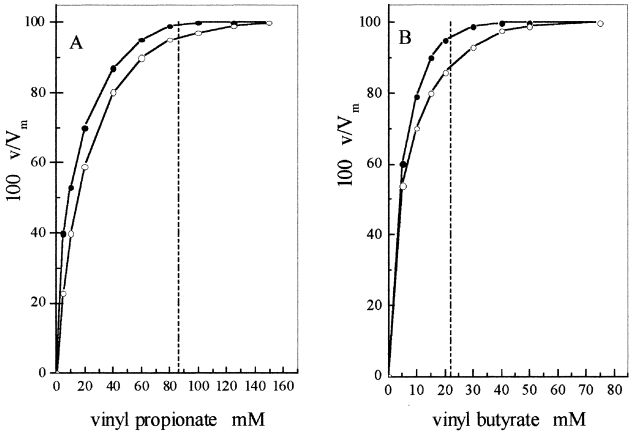

Fig. 6. Effect of ester concentration on the rates of hydrolysis of vinyl propionate (A) and vinyl butyrate (B) by lipase from *Candida rugosa*. Symbols are the same as in Fig. 1.

Table 3
Relative rates of hydrolysis of trioctanoin and short-chain vinyl esters by lipases from *R. oryzae*, *M. miehei*, *H. lanuginosa*, *P. camembertii*, *C. rugosa*, *C. antarctica* (lipase A) and from guinea-pig pancreas

	Trioctanoin/vinyl propionate	Trioctanoin/vinyl butyrate
R. oryzae	4.3	2.1
M. miehei	1.2	0.8
H. lanuginosa	3.1	2.5
P. camembertii	–	–
C. rugosa	4.6	2.8
Candida antarctica A	1.0	1.3
Guinea-pig pancreas	0.5	0.6
Human pancreas	6.7	8.6

vent-accessible active site [36]. It has been proposed that the two lipase conformations correspond to the inactive and active forms of the enzyme molecule that predominate in aqueous solution and at lipid–water interface, respectively. As demonstrated with other microbial lipases and human pancreatic lipase, it is assumed that the movement of the lid is associated with the transition to the active form of the lipase.

The substrate concentration dependency of the rates of hydrolysis of vinyl propionate and vinyl butyrate by lipase from *C. rugosa* is shown in Fig. 6A and B, respectively. In both cases, the *v*/*S* curves are hyperbolic as in the case of lipases from *R. oryzae* (Fig. 1). The activity against solutions of short-chain vinyl esters reaches 90% of the maximal activity measured against emulsions. Very similar results were found with lipase from *C. antarctica* A. The kinetic behaviour of *C. rugosa* and *C. antarctica* A lipases against soluble and emulsified tripropionin and emulsified vinyl laurate (data not shown) is similar to that of lipase from *R. oryzae* shown in Fig. 2. In all cases, the enzymes follow Michaelis–Menten kinetics. Values of $K_{0.5}$, estimated from the hyperbolic *v*/*S* curves, are comparable to those found with the fungal lipase.

3.4. Hydrolysis of solutions and emulsions of vinyl esters and tripropionin by guinea-pig pancreatic (phospho)lipase

The exocrine secretion of guinea-pig pancreas does not contain the classical phospholipase A_2 found in higher mammals but a related protein (GPL-RP2) which hydrolyses both olive oil and egg yolk lecithin at comparable rate [37,38]. This enzyme exhibits phospholipase A_1 activity and is insensitive to colipase effect. It shows structural homology with human pancreatic lipase except that the loop covering the active site (lid) is made of five amino acid residues instead of 23 in the human and higher mammals lipases [14]. It has been hypothesised that this particular structure might account, at least in part, for the unusual substrate specificity of the enzyme. As reported some years ago, the guinea-pig lipase is active against a solution of tributyrin and is not interfacially activated although the very low solubility of tributyrin in water (0.4 mM) does not allow to carry out kinetic studies in homogeneous system within a large range of substrate concentration [14]. In the present study, we have found that guinea-pig pancreatic lipase hydrolyses solutions and emulsions of short-chain vinyl esters and tripropionin. The *v*/*S* curves are hyperbolic (not shown). Values of $K_{0.5}$ are very similar to those found with lipases of *R. oryzae*, *M. miehei*, *C. rugosa* or *C. antarctica* A.

3.5. Comparison of the activity of lipases against triacylglycerols and vinyl esters

In Tables 1 and 2 are reported the specific activities of the lipases measured against short, medium and long-chain triacylglycerols and against vinyl esters, respectively. It can be observed that lipases from *M. miehei*, *H. lanuginosa*, *P. camembertii* and *R. oryzae* which belong to the same lipase family on the base of their sequence homology [39], are highly active on triacylglycerols and on vinyl esters whereas lipases from *C. rugosa* and *C. antarctica* A and (phospho)lipase from guinea-pig pancreas show lower activity against the same substrates. However, ra-

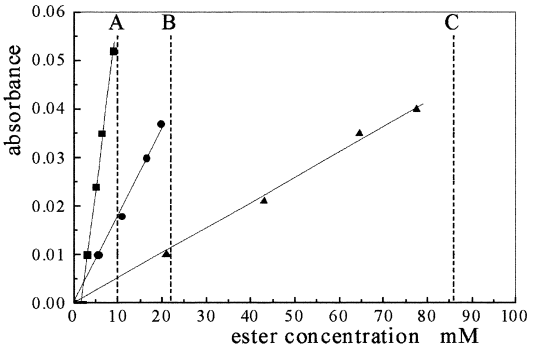

Fig. 7. Differential absorbance of solutions of benzopurpurin 4B at 510 nm as a function of the concentration of (A) tripropionin, (B) vinyl butyrate and (C) vinyl propionate. The dye was made up as a 0.005% solution in 25 mM Tris–HCl buffer, pH 7.0. The dotted lines indicate the limit of solubility of the ester.

tios of the rates of hydrolysis of trioctanoin and short-chain vinyl esters are not significantly different among the lipases (Table 3). Although it is difficult to rationalise the pronounced differences observed in the absolute values of the specific activities of the two families of lipases measured against triacylglycerols and vinyl esters, it is likely that they are not related to fatty acyl chain specificity but, more likely, reflect structural differences at the level of the portion of enzyme surface interacting with the substrate molecules.

3.6. *Detection of molecular aggregates in solutions of vinyl propionate, vinyl butyrate and tripropionin with a spectral dye method*

The dye method described in the Materials and Methods section was used to detect the formation of molecular aggregates in the solutions of tripropionin and short-chain vinyl esters at concentration below the solubility limit. The results shown in Fig. 7 are indicative of the formation of aggregates in solutions of vinyl esters, even at low concentration, and confirm their presence in solution of tripropionin. The critical concentration (CMC) above which aggregates are detected in the solution of tripropionin is 2 mM.

4. Discussion

Lipases (EC 3.1.1.3.) are carboxyl ester hydrolases produced by all living organisms. Their natural substrates are long-chain triacylglycerols but most of them are active on emulsions of short- and medium-chain triacylglycerols such as tripropionin, tributyrin and trioctanoin. Some lipases, as the enzyme produced by *P. camembertii*, specifically hydrolyse mono- and diacylglycerols but not triacylglycerols. We have recently shown that *P. cyclopium* produces two extracellular lipases acting on triacylglycerols and partial acylglycerols, respectively, and we have directly compared their hydrolysing properties using short and long-chain vinyl esters which are good substrates for porcine and human pancreatic lipase. Moreover, the water solubility of short-chain vinyl esters as vinyl propionate (86 mM) and vinyl butyrate (22 mM) which is higher than that of tripropionin (10 mM) and largely exceeds that of tributyrin (0.4 mM) and *p*-nitrophenyl butyrate (1.2 mM) allows, then, to evaluate the capacity of lipolytic enzymes to hydrolyse substrates both in solution and emulsion. These studies have shown that, unexpectedly, solutions of short-chain vinyl esters were hydrolysed by human pancreatic lipase and lipases 1 and 2 from *P. cyclopium* at high relative rate compared to maximal rate measured against emulsions [21].

In the present communication we report studies of the dependence of the activity of several lipases of known three dimensional structure on substrate concentration in the hydrolysis of vinyl propionate, vinyl butyrate and tripropionin. Enzymes used in these studies were from the fungi *R. oryzae*, *M. miehei*, *H. lanuginosa* (*Thermomyces lanuginosus*) and *P. camembertii*, from the yeasts *C. rugosa* and *C. antarctica* (lipase A) and from guinea-pig pancreas. Kinetic studies show that solutions of short-chain substrates are hydrolysed by all lipases at varying relative rates compared to maximal rates (V_m) measured against emulsions. The dependence of the hydrolysing activity of the lipases on ester concentration is either hyperbolic or deviates from the classical Michaelis–Menten model thus indicating that the rate of enzymatic hydrolysis of soluble substrates does not merely depends on substrate concentration. Of importance is the observation that no abrupt jump in activity (interfacial activation) occurs at substrate concentration beyond the saturation point of the solution. In some cases, addition of NaCl to the reaction system increases the rate of enzymatic hy-

drolysis of substrate in solution but addition of salt never affects the value of V_m measured against emulsion.

To interpret these observations, one must take in account the fact that, in the case of the lipases used in this study, the substrate molecules are prevented from reaching the catalytic triad by a flexible loop covering the active site (lid) of the enzyme. Lipases exist in two forms, a closed (inactive) and an open (active) forms, as demonstrated by crystallography and, more recently, by immunological studies with specific monoclonal antibody [40]. Several hypotheses can be put forward to account for their capacity to hydrolyse solutions of short-chain substrates. On the one hand, it can be hypothesised that the closed and open conformations are in equilibrium in the aqueous phase and that transition to the active conformation corresponding to the displacement of the lid domain, is triggered by monomeric substrate having direct access to the active site in the open form of the lipase molecule. On the other hand, lipases, in closed or/and open forms, might interact, through hydrophobic forces, with small multimolecular aggregates of substrate present in the bulk phase or with substrate molecules bound to the surface of the enzyme (mixed micelles). According to this latter hypothesis, the structural transition to the active conformation of the lipolytic enzyme would be triggered in the bulk phase by substrate molecules in the form of organised structures of small size. Several observations, made in earlier studies, are in accordance with this conclusion. For example, the activity of porcine pancreatic lipase against solutions of triacetin and tripropionin, observed in early studies with porcine pancreatic lipase, was accounted for by the presence of aggregates of triacylglycerol molecules detected with a colorimetric method and by light scattering [29]. Also, it was found that the reaction of porcine pancreatic lipase with diethyl-*p*-nitrophenyl phosphate (E600), a specific inhibitor of serine hydrolases, requires the presence of bile salts at concentration above their critical micelle concentration [41]. More recently, the same observation was made with cutinase, a fungal carboxyl ester hydrolase having functional properties between esterases and classical lipases. Cutinase is inhibited by E600 in the presence of micelles of sodium dodecyl sulfate and the lipolytic enzymes requires the presence of Triton X-100 and/or bile salt to express activity [42]. Cutinase hydrolyses both soluble and emulsified tributyrin without displaying significant interfacial activation [14]. However, it is now recognised, from nuclear magnetic resonance studies of the protein in solution, that the enzyme shows large flexibility at the level of the oxyanion hole and undergoes discrete conformational changes at this level following substrate binding [43]. In the work reported here, we have obtained evidence, with the colorimetric method, that molecules of vinyl propionate and vinyl butyrate, as tripropionin, spontaneously associate in solution. The presence of a lipid–water interface due to the formation of aggregates in the bulk phase might account for the activity of lipases against soluble substrates. As mentioned above, kinetic curves show that the activity of lipases against short-chain substrates continuously increases at substrate concentration exceeding the solubility limit of the aqueous phase and, consequently, enzymes do not show the phenomenon of interfacial activation under these assay conditions. There is no doubt that beyond the saturation point, the concentration of monomers and micellar aggregates remains constant and that hydrolysis takes place at the surface of emulsified particles. Although it is not yet possible to definitely rule out the idea that the activity of lipases against soluble substrates is directed to monomeric substrate, the observed lack of interfacial activation argues in favour of the conclusion that lipases interact at the lipid–water interface not only of the emulsified particles but also, in solution, with small multimolecular aggregates. It can be further speculated that changes in size and properties of these micellar aggregates with increasing ester concentration might affect the affinity of the enzyme for soluble substrates which could explain the non-hyperbolic dependence of activity on substrate concentration observed with some lipases. Taken together, the results reported in this communication point to the fact that the solubility limit of partially water-soluble esters is not a critical parameter that governs the kinetic behaviour of lipases in systems containing no surface-active agents as SDS, bile salt or gum arabic. A better knowledge of the physical state of ester molecules in solution and conformation of the enzyme in the bulk phase should help for understanding the mode of action of lipases against soluble short-chain substrates. Fur-

ther achievements in this domain will certainly contribute to answer the controversial issue of what distinguishes true lipases from esterases [44–47].

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