ELSEVIER

Journal of Molecular Catalysis B: Enzymatic 11 (2001) 165–171

JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC

www.elsevier.com/locate/molcatb

Interfacial catalysis by lipases ☆

N. Miled, F. Beisson, J. de Caro, A. de Caro, V. Arondel, R. Verger *

Centre National de la Recherche Scientifique, Laboratoire de Lipolyse Enzymatique, 31, Chemin Joseph-Aiguier, 13402 Marseille cedex 20, France

Abstract

We designed a convenient, specific, sensitive and continuous lipase activity assay using natural long-chain triacylglycerols (TAGs). Oil was extracted from *Parinari glaberrimum* seed kernels and the purified TAGs used as a substrate for detecting low levels of lipase activities. The purified TAGs are naturally fluorescent. The presence of detergents above their critical micellar concentration dramatically increases the fluorescence of the parinaric acid released by various lipases. This increase is linear with time and proportional to the amount of lipase added. Quantities as low as 0.1 ng of pure pancreatic lipase could be detected under standard conditions (pH 8).

The interfacial activation of human pancreatic lipase (HPL) probably involves the motion of a lid covering the active site of the enzyme. We observed that the presence of either bile salts or a small proportion of water-miscible organic solvents (called activator compounds) considerably enhances the enzymatic activity of HPL on a monomeric solution of tripropionin. This finding suggests that the activator compounds may favor the opening of the lid. This hypothesis was checked by comparing the immunoreactivity of HPL and HPL with a mini-lid (HPL(-lid)) towards anti-HPL monoclonal antibodies (mAbs), in the presence and absence of the activator compounds.

Keywords: Lipase assay; Interfacial catalysis; Conformational changes

1. A lipase assay using as substrate the naturally fluorescent triacylglycerols from *Parinari glaberrimum*

1.1. Introduction

Some lipases, such as digestive lipases, are fairly abundant in animal organs or their exocrine secretions, and their catalytic activity can easily be detected using titrimetric methods. Many other lipases are present in very small amounts in biological samples (for example in mammalian cells and plant seed homogenates). To detect them, for both purification,

☆ The key concepts summarized in this overview have been presented by Robert Verger during the 4th International Symposium on Biocatalysis and Biotransformations held at Taormina (Italy), September 26–October 1, 1999.

Abbreviations: BHT: butylhydroxytoluene; CMC: critical micellar concentration; ELISA: enzyme-linked immunosorbent assay; HPL: human pancreatic lipase; NaTDC: sodium taurodeoxycholate; SEM: standard error of the mean; TAG: triacylglycerol; THL: tetrahydrolipstatin

* Corresponding author. Tel.: +33-4-91-16-40-93; fax: +33-4-91-71-58-57.

E-mail address: verger@ibsm.cnrs-mrs.fr (R. Verger).

1381-1177/01/$ - see front matter
PII: S1381-1177(00)00041-2

medical diagnosis, and food quality analysis purposes, more sensitive methods are required. A review of some of the lipase detection and assay methods is available on our laboratory's web site (http://lle-sg.cnrs-mrs.fr).

The methods involving the use of radiolabelled substrates [1] are very sensitive but cannot be monitored continuously and need time-consuming chromatographic or organic solvent separation steps to isolate the released fatty acids. Easy and continuous assays, compatible with a high sample throughput, have been developed using chromogenic or fluorogenic substrates. However, almost all these molecules are chemically unrelated to triacylglycerols (TAGs) and can be hydrolysed by the non-specific esterases often present in crude biological media. The use of a TAG containing fatty acyl chains in which a fluorescent pyrene residue is linked to the ω position, was first described by Nègre et al. [2]. However, this sensitive assay requires the isolation of the released pyrene–fatty acid. A quencher residue (trinitrophenylamine residue) was introduced by Duque et al. [3] as a means of lowering the basal fluorescence of this TAG containing acyl–pyrene (1-*O*-hexadecyl-2-pyrenedecanoyl-3-trinitrophenylaminododecanoyl-*sn*-glycerol and its enantiomer). This intramolecularly quenched TAG containing acyl–pyrene can therefore be used in a continuous fluorescent assay. Unfortunately, as can be seen from the data reported in Duque et al. [3], this kind of chemically modified TAG is poorly hydrolysed by lipases, probably for steric reasons.

Parinaric acid is a naturally fluorescent fatty acid. Since Wolf et al. [4] used a phospholipid containing parinaric acid to monitor a phospholipase A_2 activity, we decided to investigate whether TAG containing parinaric acid might constitute a convenient and specific lipase substrate.

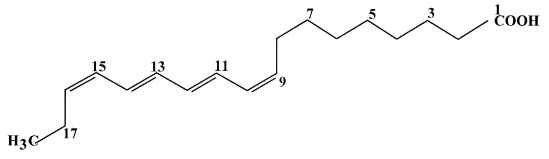

Fig. 1. Chemical structure of α-parinaric acid = 9,11,13,15-octadecatetraenoic acid.

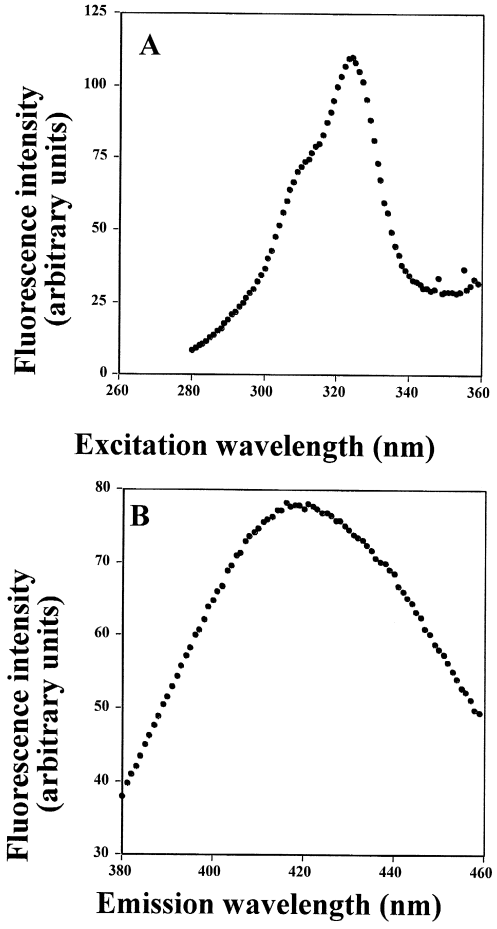

Fig. 2. Fluorescence excitation (A) and emission (B) spectra of purified *P. glaberrimum* TAGs dispersed by injection of the ethanolic solution in a standard buffer: 50 mM Tris–HCl (pH 8), 4 mM NaTDC, 100 mM NaCl, 6 mM $CaCl_2$, 0.001% (w/v) BHT. The final TAGs concentration was 18 μg/ml and the temperature was 25°C. Excitation wavelength was adjusted at 324 nm in the case of the emission spectrum, and emission wavelength was adjusted at 420 nm in the case of the excitation spectrum.

1.2. Results

1.2.1. Lipolysis of purified TAG from P. glaberrimum can be monitored, in the presence of detergent, by recording the increase in the fluorescence intensity at 420 nm

More than half of the fatty acids extracted from *P. glaberrimum* oil are known to be parinaric acid (9,11,13,15-octadecatetraenoic acid) [5], which is the *cis*, *trans*, *trans*, *cis* isomer, as shown in Fig. 1.

The fluorescent oil is commercially available from Molecular Probes on request (Eugene, OR). We purified the TAG of *P. glaberrimum* oil by preparative thin layer chromatography. Comparisons between the unique spectroscopic characteristics [6] of pure parinaric acid and the absorption spectrum of our purified TAG diluted in ethanol (data not shown) confirmed that this fluorescent fatty acid was indeed present in the TAG.

Fig. 2A shows the excitation spectrum of purified *P. glaberrimum* TAG dispersed in an aqueous phase (pH 8). At the maximum excitation wavelength (324 nm), the emission spectrum displayed a broad bell-shaped curve centered around 420 nm (Fig. 2B).

An increase in the fluorescence intensity of the emission spectrum was observed after adding human pancreatic lipase (HPL) in the presence of 4 mM sodium taurodeoxycholate (NaTDC), which was not accompanied by any wavelength shift (data not shown). To monitor the lipolysis, we therefore selected 324 nm as the excitation wavelength and 420 nm as the emission wavelength.

In the absence of lipolysis (control experiment without HPL (○)) bile salts have a negligible effect on the fluorescence intensity measured at 420 nm (Fig. 3A). In the assay experiment (◆), free fatty acids were released by HPL from the purified *P. glaberrimum* TAG and the lipolysis was stopped by addition of tetrahydrolipstatin (THL), a potent HPL inhibitor. The fluorescence intensity was read after each NaTDC addition, corresponding to an increase in the concentration in 0.4 mM steps. A sigmoid-shaped curve was obtained with the half maximum intensity at around 2.5 mM NaTDC (Fig. 3A). It should be noticed that this effect of NaTDC on parinaric acid fluorescence is totally unrelated to the activation of HPL on a monomeric tripropionin solution (see Section 2, third paragraph).

The above results clearly show that a concentration of 4 mM NaTDC is required to be able to properly detect the fluorescent fatty acids released by

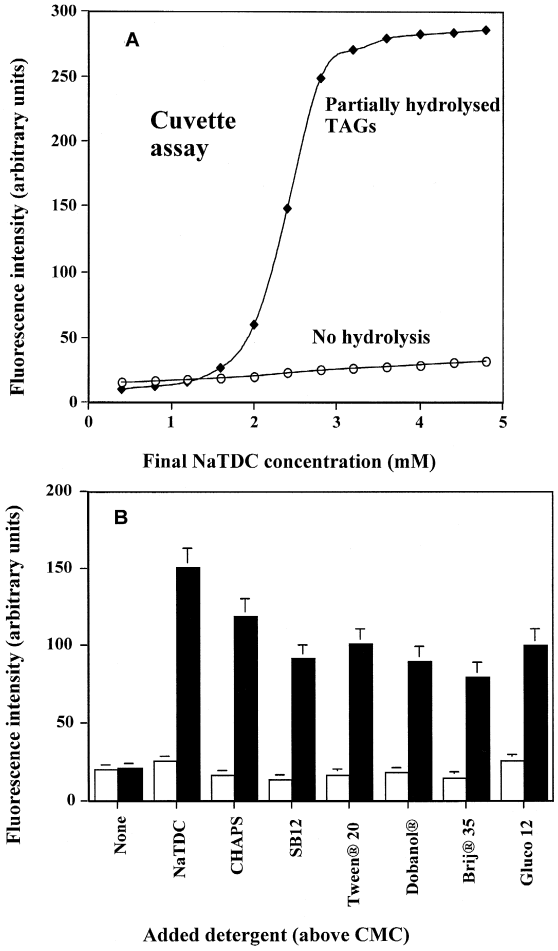

Fig. 3. (A) Effect of NaTDC concentration on the fluorescence intensity of hydrolysed TAGs. The fluorescent TAGs (18 μg) dispersed in 1 ml standard buffer (pH 8) containing 0.4 mM NaTDC were incubated for 5 min with (◆) 0.2 nM HPL and 2 nM colipase (corresponding to 17% of acyl chain hydrolysed) or without lipase (○). THL 200 nM was then added to stop the lipolysis process (enzyme/inhibitor molar ratio: 1/1000). The effect of THL on the lipolysis under our assay conditions is demonstrated in Fig. 4. The fluorescence intensity was read (excitation 324 nm and emission 420 nm) after each NaTDC addition (1 μl), corresponding to an increase in the concentration in 0.4 mM steps. (B) Effect of various detergents (see abbreviations) above their CMC on the fluorescence intensity of the hydrolysed TAGs. Filled columns: partial hydrolysis was performed under the conditions described in Fig. 2A. Empty columns: controls with no lipase added. Ten microliters of the detergent solution was added to the 1-ml cuvette before the fluorescence intensity was read (excitation 324 nm, emission 420 nm). The final concentrations of the added detergents were: NaTDC: 4 mM; CHAPS: 8 mM; Sulfobetaine SB 12: 4 mM; Tween 20: 0.2 mM; Brij 35: 0.4 mM; Dobanol: 0.4 mM; Gluco 12 (*n*-Dodecylglucoside): 0.2 mM. Each bar gives the mean ± SEM, based on triplicate experiments.

HPL. It is worth noting that NaTDC, an ionic detergent, is not unique and can be replaced by other detergents. Fig. 3B shows that NaTDC gives the most satisfactory fluorescence signal but that other neutral or zwitterionic detergents (NaTDC, CHAPS, Sulfobetaine SB 12, Tween 20, Brij 35, Dobanol, *n*-Dodecylglucoside) can be used as well.

The increase in the fluorescence intensity observed upon lipolysis was probably due to the change from a nonpolar medium (TAG) to a more polar environment (mixed bile salt micelles). We further investigated the changes in the fluorescence intensity during the course of lipolysis.

Upon subsequently adding a solution of HPL and colipase (+), the fluorescence intensity measured at 420 nm increased sharply (Fig. 4). This increase can be stopped readily by the addition of THL (♦). No changes in fluorescence intensity were detected when either heat-treated HPL and colipase (●), or HPL in the absence of colipase (○), were used.

Lipase activity could also be assayed in acidic media (pH 5) using human gastric lipase (data not shown). Furthermore, this new method could be scaled down to microtiter plate measurements.

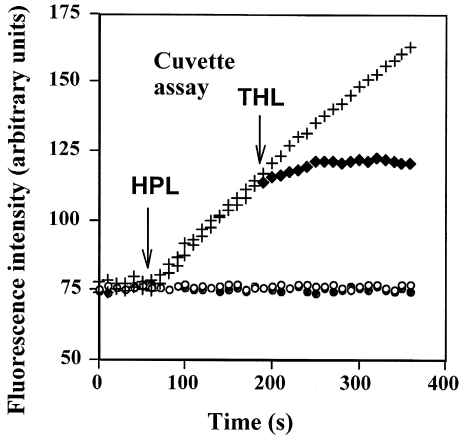

Fig. 4. Kinetics of TAGs hydrolysis followed by fluorescence intensity changes in a spectrofluorimetric cuvette (1 ml). Excitation at 324 nm and emission at 420 nm. In 1 ml of the standard incubation medium, 1 μl of a mixture of HPL (1 ng) and colipase (2 ng) was added at time 60 s (first arrow). The final HPL and colipase concentrations were 20 and 200 pM, respectively. Symbols: (+): HPL without THL; (○): No HPL; (●): heat-treated HPL. Lipolysis was stopped by adding 1 μl of an ethanolic solution (2 μM) of THL (♦) at time 190 s (second arrow), so that HPL/THL molar ratio was 1/100 (final concentration of THL: 2 nM). Control assay: 1 μl of ethanol.

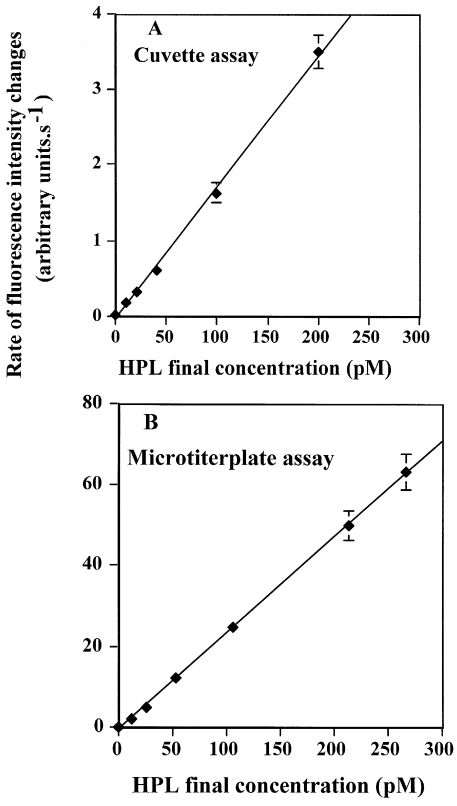

Fig. 5. (A) Rate of fluorescence intensity changes (measured during 5 min) versus HPL concentrations in a spectrofluorimetric cuvette. Each point gives the mean ± SEM of four replicates (the standard errors for most of the data points were smaller than the symbol size). Variable amounts of an HPL/colipase solution (10 μl) were added to 1 ml of the standard reaction medium in the cuvette, and the fluorescence was recorded for 5 min. The increase in the fluorescence intensity was linear with time for at least 2 min. Excitation at 324 nm and emission at 420 nm. (B) Rate of fluorescence intensity changes (measured during 5 min) versus HPL concentrations in microtiterplate wells. Each point gives the mean ± SEM of four replicates (the standard errors for most of the data points were smaller than the symbol size). Variable amounts of an HPL/colipase solution (10 μl) were added to 140 μl of the standard reaction medium in the microwells. The fluorescence intensity was read every minute during 30 min. Increase in fluorescence intensity was linear with time for at least 10 min. The excitation filter was at 325 nm and emission filter at 400 nm.

1.2.2. The kinetics of fluorescence intensity changes are proportional to the amount of lipase used

Using purified TAG from *P. glaberrimum* as a substrate, the fluorescence intensity was recorded versus time after adding variable amounts of

HPL/colipase in the cuvette or the microtiter plate assay. The initial rates of kinetic curves were found to be linearly proportional to the amount of HPL used as shown in Fig. 5.

One has to note that the fluorescence intensity units are not identical in the two assays. In the microtiter plate and cuvette assays, it is possible to detect quantities of pure HPL as low as 0.1 and 0.5 ng, respectively.

No oxidation-related problems were noted during a 30-min period of incubation in the microtiter plate assay, using butylhydroxytoluene (BHT)-containing buffer.

1.3. Conclusions

This fluorescence assay is relatively easy to perform from the technical point of view and does not require an emulsification of the TAG with gum arabic. Moreover, since this assay can be performed in microtiter plates, it can be used conveniently to perform extensive screening of lipase activity in numerous samples. The tendency of parinaric acid to oxidize during the cuvette assay is likely to decrease the emitted fluorescence intensity and this drawback can be overcome by adding an antioxidant (BHT 0.001% w/v) to the buffers and by keeping the cuvettes closed under a nitrogen atmosphere. The assay needs the presence of a selected detergent in order to solubilize in mixed micelles the released parinaric acid. Of course, this detergent should not inhibit the lipase activity.

In the method described here, the fluorophore is a naturally conjugated polyene fatty acid that does not contain any chemical substituents likely to create steric hindrance. The natural TAG extracted from *P. glaberrimum* seeds are therefore specific, convenient and sensitive lipase substrates [7].

2. A conformational transition between an open and closed form of human pancreatic lipase revealed by a monoclonal antibody (mAb)

The interfacial activation of HPL probably involves the motion of a lid covering the active site of the enzyme. Here we observed that the presence of either bile salts or a small proportion of water-miscible organic solvents (called activator compounds) considerably enhances the enzymatic activity of HPL on a monomeric solution of tripropionin. This finding suggests that the activator compounds may favor the opening of the lid. This hypothesis was checked by comparing the immunoreactivity of HPL and HPL possessing a deletion within the lid (HPL(-lid)) towards anti-HPL mAbs, in the presence and absence of the activator compounds.

Lipase assays were carried out with a monomeric tripropionin solution (8 mM), in the presence of a 5-fold molar colipase excess. We observed that HPL was maximally activated 8-fold by *tert*-butanol (5% v/v) and 6-fold by dioxane (5% v/v), reaching 63% and 40%, respectively, of the rates measured with emulsified tripropionin; whereas acetonitrile and formamide did not significantly affect the hydrolysis of the monomeric tripropionin used as substrate. These results may be attributable to the fact that *tert*-butanol and dioxane are rather protic solvents characterized by low dielectric constants (10.9 and 2.2, respectively) and high octanol–water partition coefficients (K_{ow}) (2.238 and 0.537, respectively); acetonitrile and formamide, on the contrary have high dielectric constants (50 and 109, respectively) and low K_{ow} values (0.457 and 0.03, respectively). These data suggest that the presence of water-miscible organic solvents with a low dielectric constant is responsible for a conformational change in HPL

Like *tert*-butanol and dioxane, NaTDC at a molar concentration of 3 mM was found to enhance hydrolysis of monomeric tripropionin about 4-fold This bile salts concentration is approximately equal to the critical micellar concentration (CMC).

Three anti-HPL mAbs (81-23, 146-40 and 248-31) were used with the immunoprecipitation procedure (IP). It was previously established by Aoubala et al [8] that in the native and sodium dodecyl sulfate-denaturated HPL, mAbs 81-23 and 146-40 recognize the C- and the N-terminal domains, respectively, whereas, mAb 248-31 recognizes only native HPL. In addition, the latter mAb did not react with HPL in a simple sandwich enzyme-linked immunosorbent assay (ELISA), but did so in a double sandwich ELISA. To explain these results, it was assumed by these authors [8] that the epitope recognized by mAb 248-31 is conformational and located in a hydropho-

Table 1
Immunoreactivity of mAb 248-31 with HPL, DP-HPL and HPL(-lid) in the presence or in the absence of *tert*-butanol or NaTDC (activator compounds)

Activator compound	HPL	DP.HPL	HPL(-lid)
NaTDC	−	−	+
Tert-butanol (5% v/v)	−	−	+
None	+	−	+

bic region. We first checked that mAb 248-31 recognized the N- and not the C-terminal domain of HPL. We then checked whether the three anti-HPL mAbs (81-23; 146-40 and 248-31) might immunoprecipitate HPL in the absence or presence of *tert*-butanol (5% v/v) or bile salts (3 mM). The IP procedure was used to estimate the amount of immunoprecipitated lipase present in the pellet (IP yield). In order to check our measurements, the total lipase activity was estimated in the supernatant by performing the standard tributyrin assay.

Similar immunoreactivities of mAbs 81-23 and 146-40 with HPL were obtained in the presence or absence of water-miscible organic solvents or bile salts. The fact that similar immunoreactivities were observed in all these cases indicates that the presence of *tert*-butanol or bile salts in the medium does not significantly affect the dissociation constant of the antigen–antibody complex. Identical results were obtained with HPL(-lid).

We observed striking differences when mAb 248-31 was incubated in the presence of either water-miscible organic solvents or bile salts (Table 1). The presence of bile salts led to a greater loss of immunoreactivity with mAb 248-31 (IP yield 10%) than that observed with *tert*-butanol (IP yield 50%) (data not shown). It is worth noting that these HPL immunoreactivity losses observed with mAb 248-31

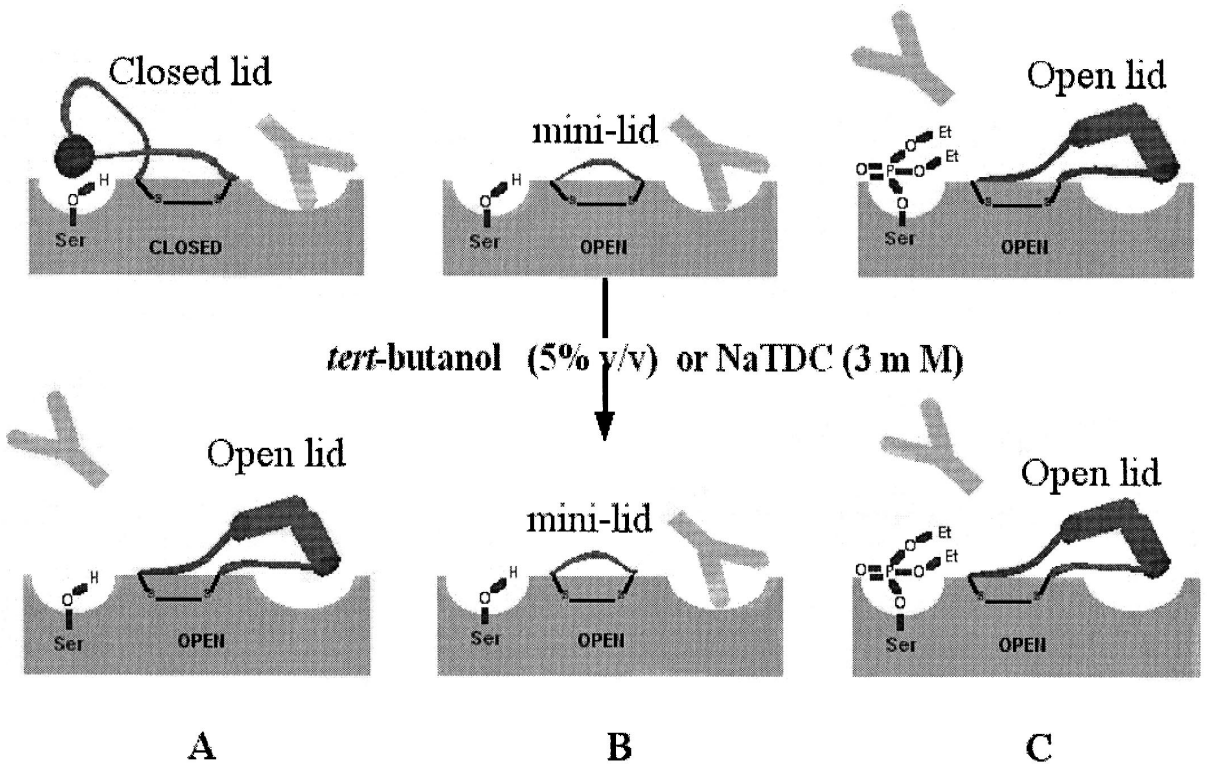

Fig. 6. Hypothetical scheme illustrating a conformational transition of HPL (A), HPL(-lid) (B) and DP.HPL (C) as revealed by mAb 248-31. Only the N-terminal domain of the HPL is represented with the catalytic serine 152 (Ser–OH). The epitope of mAb 248-31 may be masked by the lid opening in the presence of *tert*-butanol or bile salts.

depend on the presence of the lid domain. The HPL(-lid) reacts in exactly the same way in the presence and absence of *tert*-butanol or bile salts (Table 1). These findings suggest that the mAb 248-31 does not immunoreact with HPL under its open conformation.

HPL inhibition by tetrahydrolipstatin [9] or phosphonate inhibitors [10] as well as *Rhizomucor miehei* lipase inhibition by diethyl *p*-nitrophenyl phosphate (E600) [11] leads to a conformational transition from the closed to an open conformation. Based on these data, we can also assume that the HPL inhibited by E600 (DP.HPL) in solution used in our study was present in its open conformation. As was to be expected, a great loss of reactivity of DP.HPL was observed with the mAb 248-31 (Table 1) but not with the mAbs 81-23 and 146-40 (data not shown), in the presence or absence of water-miscible organic solvents, which confirmed our initial hypothesis. Fig. 6 illustrates the reactivity of HPL, HPL(-lid) and DP.HPL towards the mAb 248-31 in the presence or absence of activator compounds.

One key point here was the need to check whether or not the loss of immunoreactivity observed in mAb 248-31 with the open form of HPL depends on the presence of colipase. Interestingly, in the absence of colipase, bile salts but not *tert*-butanol induced a loss of immunoreactivity. In the absence of colipase, bile salts but not *tert*-butanol can therefore be said to be able to trigger the opening of the lid.

The present data obtained using enzyme kinetic as well as epitope mapping procedures are consistent with the conformational changes in HPL previously described on the basis of X-ray crystallography data [12]. This approach could be generalized to other types of enzymes acting on biomembranes or present therein.

References

[1] V. Briquet-Laugier, O. Ben-Zeev, M.H. Doolittle, Methods Mol. Biol. 109 (1998) 81–94.
[2] A. Nègre, R. Salvayre, A. Dagan, S. Gatt, Clin. Chim. Acta 149 (1985) 81–88.
[3] M. Duque, M. Graupner, H. Stütz, I. Wicher, R. Zechner, F. Paltauf, A. Hermetter, J. Lipid Res. 37 (1996) 868–876.
[4] C. Wolf, L. Sagaert, G. Bereziat, Biochem. Biophys. Res. Commun. 99 (1981) 275–283.
[5] J.P. Riley, J. Chem. Soc. (1950) 12–18.
[6] L.A. Sklar, B.S. Hudson, R.D. Simoni, Methods Enzymol. 72 (1981) 479–482.
[7] F. Beisson, N. Ferté, J. Nari, G. Noat, V. Arondel, R. Verger, J. Lipid Res. 40 (1999) 2313–2321.
[8] M. Aoubala, L. de la Fournière, I. Douchet, A. Abousalham, C. Daniel, M. Hirn, Y. Gargouri, R. Verger, A. de Caro, J. Biol. Chem. 270 (1995) 3932–3937.
[9] Q. Lüthi-Peng, F.K. Winkler, Eur. J. Biochem. 205 (1992) 383–390.
[10] M.P. Egloff, F. Marguet, G. Buono, R. Verger, C. Cambillau, H. van Tilbeurgh, Biochemistry 34 (1995) 2751–2762.
[11] A.M. Brzozowski, U. Derewenda, Z.S. Derewenda, G.G. Dodson, D.M. Lawson, J.P. Turkenburg, P. Bjorkling, B. Huge-Jensen, S.A. Patkar, L. Thim, Nature 351 (1991) 491–494.
[12] H. van Tilbeurgh, M.P. Egloff, C. Martinez, N. Rugani, R. Verger, C. Cambillau, Nature 362 (1993) 814–820.