Further biochemical characterization of human pancreatic lipase-related protein 2 expressed in yeast cells

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Abstract **Recombinant human pancreatic lipase-related protein 2 (rHPLRP2) was produced in the protease A-deficient yeast *Pichia pastoris*. A major protein with a molecular mass of 50 kDa was purified from the culture medium using SP-Sepharose and Mono Q chromatography. The protein was found to be highly sensitive to the proteolytic cleavage of a peptide bond in the lid domain. The proteolytic cleavage process occurring in the lid affected both the lipase and phospholipase activities of rHPLRP2. The substrate specificity of the nonproteolyzed rHPLRP2 was investigated using pH-stat and monomolecular film techniques and various substrates (glycerides, phospholipids, and galactolipids). All of the enzyme activities were maximum at alkaline pH values and decreased in the pH 5–7 range corresponding to the physiological conditions occurring in the duodenum. rHPLRP2 was found to act preferentially on substrates forming small aggregates in solution (monoglycerides, egg phosphatidylcholine, and galactolipids) rather than on emulsified substrates such as triolein and diolein. The activity of rHPLRP2 on monogalactosyldiglyceride and digalactosyldiglyceride monomolecular films was determined and compared with that of guinea pig pancreatic lipase-related protein 2, which shows a large deletion in the lid domain. The presence of a full-length lid domain in rHPLRP2 makes it possible for enzyme activity to occur at higher surface pressures. The finding that the inhibition of nonproteolyzed rHPLRP2 by tetrahydrolipstatin and diethyl-*p*-nitrophenyl phosphate does not involve any bile salt requirements suggests that the rHPLRP2 lid adopts an open conformation in aqueous media.**—Eydoux, C., J. De Caro, F. Ferrato, P. Boullanger, D. Lafont, R. Laugier, F. Carriére, and A. De Caro. **Further biochemical characterization of human pancreatic lipase-related protein 2 expressed in yeast cells. *J. Lipid Res.* 2007.** 48: **1539–1549.**

Supplementary key words constitutive expression • *Pichia pastoris* • inhibition • galactolipid • lid domain • galactolipase • digestion

In 1992, Giller et al. (1) identified three different mRNAs encoding pancreatic lipases (PLs) in the human exocrine pancreas and proposed that PLs should be divided into three subgroups: classical PLs and pancreatic lipase-related proteins 1 and 2 (PLRP1 and PLRP2). PLRPs have been isolated from exocrine pancreas using protein purification procedures (2–5) and identified by isolating mRNAs from pancreas (2–4, 6) and other tissues (7–9). Although there is a high level of sequence identity (65%) between these proteins and classical PL, and both PLRPs contain an identical catalytic triad (serine-aspartic acid-histidine) and have superimposable three-dimensional structures (10–12), PLRPs were found to have different biochemical properties from those of classical PL. PLRP1 shows no lipase activity on any of the substrates tested to date, whereas PLRP2 shows both lipase and phospholipase A1 activities as well as galactolipase activity (13, 14), and human PL hydrolyzes only triglycerides (TGs).

Unlike the classical PL, PLRP2 can be expressed not only in the pancreas but also in various tissues and cell types, depending on the species. For this reason, various physiological functions have been proposed for PLRP2. Rat PLRP2 was identified as GP-3, a zymogen granule membrane-associated protein (6), which may be involved in granule trafficking, fusion of zymogen granule membrane with the cytoplasmic membrane, and exocytosis of the secretory granule contents. Because of the temporal pattern of mouse PLRP2 mRNA expression (15) and because PLRP2-deficient suckling mice had fat malabsorption deficits (16), it was suggested that PLRP2 might play a crucial role in dietary fat digestion in suckling mammals. Other functions for this lipase have been proposed in the same species. The gene expression of mouse PLRP2 was found to be interleukin-4-inducible in CD8+ T-cells (7), which led to the hypothesis that PLRP2 might be involved in immune defense mechanisms. This hypothesis was fur-

Manuscript received 14 November 2006 and in revised form 15 February 2007 and in re-revised form 30 March 2007.

Published, JLR Papers in Press, March 30, 2007.
DOI 10.1194/jlr.M600486-JLR200

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ther supported by the decrease in T-cell cytotoxicity observed in PLRP2-deficient mice (16). Mouse PLRP2 mRNA was also found to be expressed throughout the small intestine in both enterocytes and paneth cells (8). Immunohistochemical studies on the small intestine confirmed the presence of PLRP2 in the enterocytes and possibly in the paneth cells (17). In view of the latter possible location, it was suggested that PLRP2 may contribute to the antimicrobial activity of the paneth cells, because it seems unlikely that it might have a digestive function in the distal intestine. Lastly, a PLRP2 was cloned from total mRNA isolated from the goat bulbourethral gland (also named Cowper's gland) (9). The goat PLRP2 might be involved in the cleansing of the genital tract, where it might play an antimicrobial role.

The native human pancreatic lipase-related proteins (HPLRPs) secreted into pancreatic juice were isolated and characterized in our laboratory. Because PLRP1 is inactive and no enzymatic assays are available to specifically measure the enzymatic activity of PLRP2 without interference by other lipolytic enzymes present in the gastrointestinal tract, we developed an immunological approach using antipeptide antibodies specifically recognizing HPLRPs (HPLRP1 and HPLRP2) to discriminate between these two proteins and the more abundant classical human pancreatic lipase (HPL). This procedure made it possible to identify the native enzymes and to monitor their chromatographic behavior during the purification procedure (5, 18). The native and recombinant forms of human PLRP2 were purified from pure pancreatic juice (5) and from insect cells and *Pichia pastoris* cultures (19), respectively. Some of the biochemical properties of both native and recombinant HPLRP2 have been investigated using various lipid substrates and found to be identical (5, 19). From these results, it seems likely that the main physiological role of HPLRP2 as a digestive enzyme is that of a galactolipase. The presence of an immunoreactive form of HPLRP2 was also detected in the colon, based on Western blot analysis of proteins extracted from various biopsies along the digestive tract. The role of HPLRP2 in the colon still remains to be elucidated, however (20).

Using specific ELISAs, we recently quantified the HPL and HPLRP2 secretory levels in pancreatic juice from patients with and without pancreatic disorders (20). Control subjects had significantly higher levels of both HPL and HPLRP2 (7.7% and 1.7% of the total proteins, respectively) than patients with chronic calcifying pancreatitis (3.7% and 0.63% of the total proteins, respectively). Because only low levels of HPLRP2 are present in normal pancreatic juice (1.7% of the total proteins), it was not possible to isolate the native protein in large amounts. Therefore, a recombinant HPLRP2 was produced in insect cells and the yeast *P. pastoris* (19). The biochemical characterization performed with recombinant HPLRP2 suggested that the main physiological function of this protein is that of a galactolipase. To study the molecular properties and the physiological role of HPLRP2 more closely, the constitutive level of expression of recombinant human pancreatic lipase-related protein 2 (rHPLRP2) in *P. pastoris* was improved in this study, and a modified procedure for purifying rHPLRP2 was also developed to produce an enzyme showing greater purity and stability. Further characterization of the molecular properties of rHPLRP2 was also undertaken using various substrates, including monomolecular films of monogalactosyldiglycerides and digalactosyldiglycerides. The experiments performed with the latter substrates showed that the structure of the lid domain can influence galactolipase activity. The sensitivity of the rHPLRP2 lid to proteolysis and the results of inhibition studies performed with diethyl-*p*-nitrophenyl phosphate (E600) and tetrahydrolipstatin (THL) suggest that the lid of rHPLRP2 adopts an open conformation in solution.

MATERIALS AND METHODS

Lipids

Pure trioleoylglycerol (triolein) (T-7140) and purified egg L-α-phosphatidylcholine (P-5394) were purchased from Sigma-Aldrich Chemical (St. Louis, MO). Pure monoolein and diolein were purified from a commercial low-grade DL-α-monoolein from Fluka. The Intralipid emulsion (20%, w/w) soybean oil emulsified with egg phosphatidylcholine (1.2%, w/w) used here, which is generally used for parenteral nutrition purposes, was from Kabi France.

DNA sources and strains

HPLRP2 cDNA was cloned at Hoffmann-La Roche, Ltd. (Basel, Switzerland), and was a generous gift from Dr. Thomas Giller (1). As described previously by Sias et al. (19), to induce HPLRP2 expression in the wild-type *P. pastoris* yeast (X-33 strain; Invitrogen), the cDNA of HPLRP2 was cloned into the pGAPZB vector and the recombinant plasmid was integrated by electroporation into the protease A-deficient *P. pastoris* yeast SMD 1168 strain (Invitrogen). This transformation resulted in the constitutive expression of HPLRP2 under the control of the glyceraldehyde-3-phosphate dehydrogenase promoter and its secretion into the culture medium.

Production of rHPLRP2 in *P. pastoris*

After transforming the yeast by electroporation, the cells were plated onto Yeast Peptone Dextrose (YPD) 1% agar medium containing Zeocin (100 μg/ml) and grown for 3 days at 30°C. The clones selected were then left in 5 ml of YPD medium with Zeocin for 1 night without agitation. Before protein production started, a preculture step was performed for 24 h in a 250 ml Erlenmeyer flask containing 50 ml of YPD medium. This cell culture was used to inoculate larger culture media at an optical density (600 nm) of 1 to start the cell culture directly in the exponential growth phase. The yeast was then grown at 30°C with orbital agitation at a rate of 100 rpm in a 1 liter Erlenmeyer flask containing 200 ml of YPD medium devoid of Zeocin. Cell growth was stopped after 40 h, and dithiothreitol and phenylmethylsulfonyl fluoride were added to the culture medium at a final concentration of 1 mM to limit the oxidation of sulfhydryl groups and the proteolysis of rHPLRP2, respectively. The secretion of rHPLRP2 into the culture medium was monitored by measuring the lipase activity on tributyrin after the cells had been pelleted by centrifugation at 5,000 rpm for 5 min.

Purification of rHPLRP2

The culture medium (1.5 liters) was collected after 40 h of yeast growth to limit the proteolysis of the recombinant li-

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pase. Cells were removed by centrifuging the medium twice at 5,000 rpm for 20 min at 4°C in a Beckman J-25 centrifuge. After adjusting the pH to 5.0, the culture supernatant was incubated under batch conditions with SP-Sepharose High Performance (Pharmacia) for 1 h at 4°C under gentle stirring. The gel was first equilibrated with 10 mM MES buffer, pH 5.0. More than 95% of the lipase activity was found to be bound to the gel when a 10 ml gel per 1 liter of culture supernatant was used. The gel was washed extensively with 10 mM MES buffer, pH 5.5, and packed into a chromatographic column (Pharmacia; 1.6 × 7.5 cm) connected to an ÄKTA explorer™ purification device (Amersham Pharmacia Biotech). After column washing with the same buffer described above, two successive linear NaCl concentration gradients (0–0.3 and 0.3–0.5 M NaCl) were applied for 120 and 15 min, respectively. The active lipase fractions were pooled, dialyzed against 20 mM Tris-HCl buffer, pH 8.0, containing 1 mM benzamidine, and concentrated using an Amicon PM 30 ultrafiltration membrane. The resulting solution was applied to a Mono Q HR 5/5 column equilibrated with the dialysis buffer. rHPLRP2 was eluted from the column at 0.25 M NaCl using a linear concentration gradient from 0 to 0.5 M NaCl for 40 min. The flow rate was adjusted to 0.5 ml/min, and 0.5 ml fractions were collected. The fractions containing lipase activity were pooled, concentrated to ~3–4 mg/ml, divided into aliquots, and stored at −20°C. The concentration of rHPLRP2 was determined by performing quantitative amino acid analysis using a Beckman 6300 amino acid analyzer.

Lipase and phospholipase activity determination

The lipase activity was measured potentiometrically at 37°C using a pH-stat (718 STAT Titrino; Metrohm). The reaction was carried out under vigorous stirring in a vessel containing 50 μl of monoolein, diolein, or triolein, or 0.5 ml of tributyrin or trioctanoin, or 5 ml of olive oil emulsified (10%, w/w) in gum arabic (GA; 10%, w/w) and a solution of 1 mM Tris-HCl buffer, pH 8.0, containing 0.1 M NaCl and 5 mM $CaCl_2$, giving a final volume of 15 ml. Under standard assays at pH 8.0, the fatty acids released were titrated directly. In the assays performed at pH values down to pH 8.0, the free fatty acids released were back-titrated at the end of the incubation period (5 min) by shifting the pH value up to pH 9.0 (21). Porcine pancreatic colipase was purified from pancreatic tissue as described previously (22). Phospholipase activities of rHPLRP2 and porcine pancreatic phospholipase A_2 (P-0861; Sigma) were measured potentiometrically using purified egg L-α-phosphatidylcholine (23) as the substrate. Specific activities were expressed as international lipase units (1 unit = 1 μmol of fatty acid released per minute) per milligram of enzyme.

Monomolecular film experiments for measuring galactolipase activities

The galactolipase activity was measured using monomolecular films of monogalactosyldiglyceride [1,2-di-*O*-dodecanoyl-3-*O*-β-D-galactopyranosyl-*sn*-glycerol (MGDG *sn*-3) and 2,3-di-*O*-dodecanoyl-1-*O*-β-D-galactopyranosyl-*sn*-glycerol (MGDG *sn*-1)] and digalactosyldiglyceride [3-*O*-(6-*O*-α-D-galactopyranosyl-β-D-galactopyranosyl)-1,2-di-*O*-dodecanoyl-*sn*-glycerol (DGDG)] as substrates. MGDG and DGDG were synthesized using chemical procedures, as described in Refs. 19 and 24, respectively.

The kinetics of monomolecular film hydrolysis were determined using a KSV-2200 barostat (KSV, Helsinki, Finland) and a "zero order" Teflon trough (25). Because the products of the lipolysis (monododecanoyl-galactopyranosyl-glycerol, dodecanoic acid, monolauroyl-galactopyranosyl-glycerol, and lauric acid) are soluble in water, the trough was equipped with a mobile Teflon barrier to keep the surface pressure constant during enzymatic hydrolysis of the substrate film and desorption of the lipolysis products. Surface pressure was measured using a Wilhelmy plate (perimeter, 3.94 cm) attached to an electromicrobalance connected to a microprocessor controlling the movements of the mobile barrier. The reactions were performed at room temperature. The aqueous subphase was composed of 10 mM Tris-HCl, 100 mM NaCl, 21 mM $CaCl_2$, and 1 mM EDTA, pH 8.0. The enzyme (0.1 μg; 0.1 nM final concentration) was injected through the film. The surface area of the reaction compartment was 100 cm^2, and the volume was 100 ml. The reservoir used was 27.9 cm long and 14.8 cm wide. Enzyme activity was estimated from the surface of the trough covered by the mobile barrier and the known molecular area of the substrate molecule. The molecular areas of the MGDG and DGDG substrates were determined versus the surface pressure by performing a compression isotherm (**Fig. 1**). The rHPLRP2 activity was expressed in moles of substrate hydrolyzed per surface unit (cm^2) per minute and referred to the overall molarity of the enzyme initially injected into the aqueous subphase ($mol \cdot cm^{-2} \cdot min^{-1} \cdot M^{-1}$).

Inhibition of PLs by E600 and THL

Inhibition experiments were performed with E600 in 50 mM sodium acetate buffer (pH 6.0) containing 50 mM NaCl and 5 mM $CaCl_2$ (26). HPLRP2 (5.2×10^{-5} M) and HPL (2.0×10^{-5} M) were incubated at 25°C with E600 [stock solution at 4.16 M (D-9286, Sigma)] at a lipase-inhibitor molar ratio of 1:85 in the absence or presence of 4 mM sodium taurodeoxycholate and colipase at a molar ratio of 1:1.

Inhibition experiments were also performed with THL in 20 mM Tris-HCl buffer (pH 8.0) containing 10 mM NaCl. HPLRP2 (5.2×10^{-5} M) and HPL (2.0×10^{-5} M) were incubated at 25°C with THL (stock solutions at 100 and 10 mM in an ethanolic solution) at a lipase-inhibitor molar ratio of 1:100 in the absence or presence of 4 mM NaTDC and colipase at a molar ratio of 1:1.

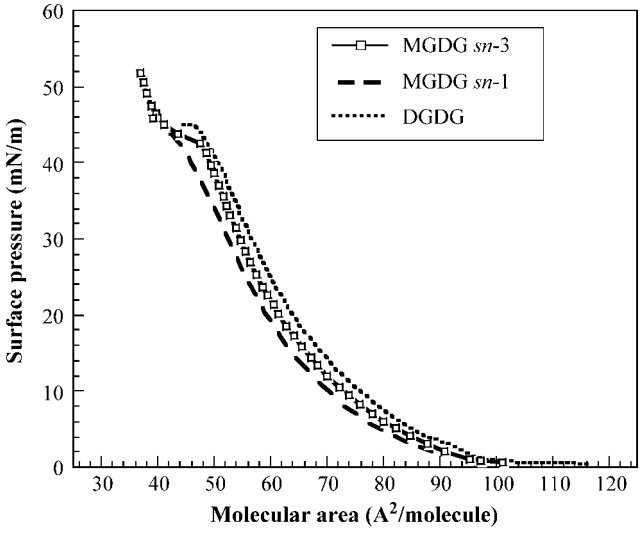

Fig. 1. Compression isotherms of the galactolipid substrates used for monomolecular film experiments. Galactolipids solubilized in chloroform (2.4 mg/ml) were spread individually at the air-water interface, and their compression isotherms were determined. Their collapse points occurred at ~50 mN/m. DGDG, digalactosyldiglyceride 3-*O*-(6-*O*-α-D-galactopyranosyl-β-D-galactopyranosyl)-1,2-di-*O*-dodecanoyl-*sn*-glycerol; MGDG *sn*-1, 2,3-di-*O*-dodecanoyl-1-*O*-β-D-galactopyranosyl-*sn*-glycerol; MGDG *sn*-3, 1,2-di-*O*-dodecanoyl-3-*O*-β-D-galactopyranosyl-*sn*-glycerol.

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Residual lipase activity was measured at various times using a trioctanoin emulsion substrate in the presence of 0.1 mM NaTDC and a 2-fold molar excess of colipase to optimize the lipase activity measurements.

Gel electrophoresis and Western blotting analysis

Electrophoresis was carried out on 7.5% polyacrylamide gels in the presence of SDS as described by Laemmli (27). Western blotting was performed as described by Gershoni and Palade (28). Membranes were incubated with anti-HPLRP2 rabbit serum diluted 1:1,000 and then with goat anti-rabbit immunoglobulins conjugated to alkaline phosphatase (diluted 1:2,000; A-0418; Sigma) and the alkaline phosphatase substrate solution. Quantitative Western blot analysis was carried out using the Scion Image software program (Scion Corp.). The presence of sugars in rHPLRP2 was determined by performing immunological detection on blots after labeling the protein with digoxigenin (DIG Glycan Detection Kit, 1 142 372; Roche Diagnostics).

N-terminal sequencing and mass determination

To determine the N-terminal sequence of the rHPLRP2 and those of the fragments generated by partial proteolysis, the protein material was first separated by means of the SDS-PAGE procedure and then electroblotted onto polyvinylidene difluoride membranes (Bio-Rad). The peptide bands identified by Ponceau S staining were cut and subjected to protein sequence analysis on an Applied Biosystem 476 A sequencer. Matrix-assisted laser-desorption ionization time of flight (MALDI-TOF) mass spectrometry of samples was carried out on a Perspective Biosystems Voyager DE-RP spectrometer.

RESULTS

Production of rHPLRP2

rHPLRP2 was found to be expressed in the *P. pastoris* SMD 1168 strain lacking protease A and secreted into the culture medium, which also contained small peptides. The lipase, which was the main protein observed in the culture medium, was detected 24 h after cell growth was initiated. The lipase activity was 17 U/ml at that time and reached 25 U/ml after 40 h. To obtain the maximum level of expression of rHPLRP2 combined with a low proteolysis, the yeast culture was stopped after 40 h of growth. At this stage, the biomass of the yeast reached the stationary phase and the lipase activity remained fairly constant. As shown by Western blot analysis using anti-HPLRP2 polyclonal antibodies (data not shown), a protein with an apparent molecular mass of 50 kDa was detected in the yeast culture medium, without the occurrence of any proteolytic degradation. The rHPLRP2 production rate reached 15 mg/l.

Purification and limited proteolysis of rHPLRP2

The rHPLRP2 was purified to homogeneity from the yeast culture medium using a two-step procedure as described in Materials and Methods. The elution profile obtained for the cationic exchange purification step is given in **Fig. 2A**. rHPLRP2 was eluted with 0.25 M NaCl. The pooled fractions containing lipase activity showed a pale yellow color, and the high optical density values measured were probably attributable to the peptone present in the medium. This color was removed by performing anion-exchange chromatography on a Mono Q column (Fig. 2B), and a final yield of 11 mg of pure rHPLRP2 per liter of culture medium was obtained. The protein N-terminal sequence (KEV-YGQLG) was found to be identical to that of the mature native protein. Therefore, rHPLRP2 was secreted in its mature form after its signal peptide was correctly cleaved. SDS-polyacrylamide gels stained with Coomassie blue and Western blotting analysis with an anti-HPLRP2 peptide serum showed that the purified rHPLRP2 migrated in the form of a doublet with a molecular mass of ~50 kDa (data not shown). When rHPLRP2 was incubated with digoxigenin and then with anti-digoxigenin-alkaline phosphatase, a single positive band indicating the presence of sugars in rHPLRP2 was detected on the blots (Fig. 2B, inset, lane 5). MALDI-TOF mass spectrometry yielded two different average masses corresponding to 52,343 ± 600 Da and 49,946 ± 200 Da. The mass of the minor form (49,946 Da) was very similar to the theoretical mass calculated from the protein sequence of the mature HPLRP2 (50,081 Da), which showed that no glycosylation had occurred. The mean mass difference between the two forms was 2,397 Da. However, the major form showed a large peak width (Δm = 1,200 Da), consistent with a heterogeneous glycosylation. It still remains to be established, however, whether the two theoretical *N*-glycosylation sites (N336 and N411) observed in the protein sequence are occupied.

The whole rHPLRP2 purification procedure was initially performed at room temperature. As shown in the inset in Fig. 2B (lanes 1, 2), partial proteolysis of the protein occurred during the purification process, generating two fragments with molecular masses of 27 and 22 kDa observed in the presence of a reducing agent (Fig. 2B, inset, lane 2). The relative proportions of the proteolyzed rHPLRP2 versus total rHPLRP2 were found to be 40%, based on Western blot analysis. N-terminal sequencing yielded two sequences (KEV-YGQL and DIDGIWEG) corresponding to the 27 and 22 kDa fragments, respectively. The 27 kDa fragment corresponds to the N-terminal sequence of the protein extending from residue 1 to residue 248, whereas the 22 kDa fragment corresponds to the sequence extending from residue 249 to the C terminus of the polypeptide chain. Therefore, the rHPLRP2 was partly and specifically cleaved by a *P. pastoris* protease at the T248-D249 peptide bond present in the lid domain controlling access to the active site of PLs. By contrast, no partial proteolysis of the protein was detected when the purification step was performed at 4°C (Fig. 2B, inset, lanes 3, 4). These data suggested that proteases from the culture medium were still present during the first step of the purification process and could act on rHPLRP2, although rHPLRP2 was not found to be degraded when the complete culture medium was collected.

Effects of bile salts and colipase

The effects of bile salts and colipase on the activity of rHPLRP2 of the nonproteolyzed and proteolyzed forms were determined at NaTDC concentrations ranging from

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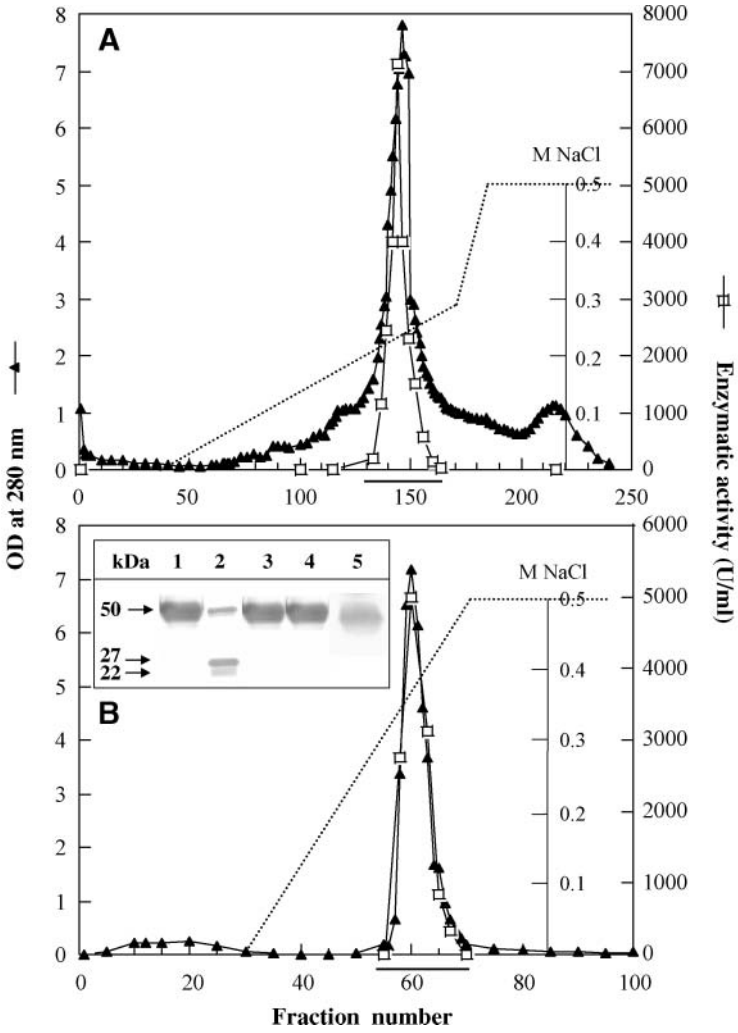

Fig. 2. Purification of recombinant human pancreatic lipase-related protein 2 (rHPLRP2) expressed in the yeast *P. pastoris*. A: Chromatography on SP-Sepharose High Performance. The proteins were eluted by performing two successive linear NaCl concentration gradients (0–0.3 and 0.3–0.5 M). Fraction volume was 1 ml, and flow rate was 1 ml/min. B: Chromatography on a Mono Q HR 5/5 column. rHPLRP2 was eluted using a linear NaCl concentration gradient (0–0.5 M). Fraction volume was 0.5 ml, and flow rate was 0.5 ml/min. Absorbances were measured at 280 nm (closed triangles), and lipase activity was measured using tributyrin as substrate (open squares). Pooled fractions are indicated by the horizontal bars. Inset: Immunoblot analysis of the purified rHPLRP2 using anti-HPLRP2 rabbit serum. The lipase sample was obtained by performing the purification procedure at room temperature (lanes 1, 2) and at 4°C (lanes 3, 4). Protein samples were obtained in the absence (lanes 1, 3) and presence (lanes 2, 4) of DTT. Sugars were detected by performing Western blotting with the DIG Glycan Detection Kit (lane 5). OD, optical density.

0 to 4 mM using short-, medium-, and long-chain TGs. The activity of rHPLRP2 was inhibited by increasing the concentrations of bile salts and poorly restored by adding colipase. Using tributyrin, the maximum specific activity was found to be 900 U/mg in the presence of 0.1 mM NaTDC and then slowly decreased at bile salt concentrations ranging from 0.5 to 4 mM (**Fig. 3A**). Adding colipase increased the activity slightly at NaTDC concentrations ranging from 2 to 4 mM. As reported previously for PLRP2 (29), the enzyme may remain active on the soluble fraction of tributyrin when it is desorbed from the oil-water interface by NaTDC. Using trioctanoin, the maximum specific activity was found to be 1,300 U/mg in the presence of 0.1–0.5 mM NaTDC and then decreased strongly at bile salt concentrations ranging from 0.5 to 2 mM (Fig. 3B). Adding colipase was found to slightly enhance the activity of both forms of rHPLRP2. Using olive oil emulsified with GA, the maximum specific activity was found to be 800 U/mg with the nonproteolyzed rHPLRP2 in the presence of 1 mM NaTDC and colipase. The activity was lower in the absence of colipase, but overall, bile salts had a weak inhibitory effect on rHPLRP2 from 1 to 4 mM NaTDC (Fig. 3C). Adding colipase did not significantly affect the activity of the proteolyzed rHPLRP2. At high NaTDC concentrations, the proteolyzed form of rHPLRP2 always showed lower levels of lipase activity than the nonproteolyzed form with all of the substrates tested.

Catalytic properties of rHPLRP2 on long-chain glycerides

Because it was rather surprising that the activity of rHPLRP2 on olive oil emulsified with GA was high and only poorly affected by bile salts and colipase, the catalytic properties of rHPLRP2 were investigated titrimetrically using a variety of long-chain glyceride substrates (**Table 1**). rHPLRP2 showed a much higher specific activity on monoolein at pH 6.5 and 8.0 than on pure diolein and triolein. The weak effects of colipase observed at both pH 6.5 and 8.0 support the idea that rHPLRP2 acts more efficiently on a substrate dispersed in solution (micelles) than at an oil-water interface. Other substrates containing long-chain TGs, such as Intralipid and bovine milk, were also tested. The activity of rHPLRP2 on Intralipid 20%, at pH 8.0, was found to be very low and of the same order of magnitude as that occurring on pure triolein. rHPLRP2 showed no activity on Intralipid at pH 6.5. With bovine milk, a complex physiological substrate containing fat globules, no activity was detected at pH 8.0, as a result of the great instability of the emulsion. At pH 6.5, the activity

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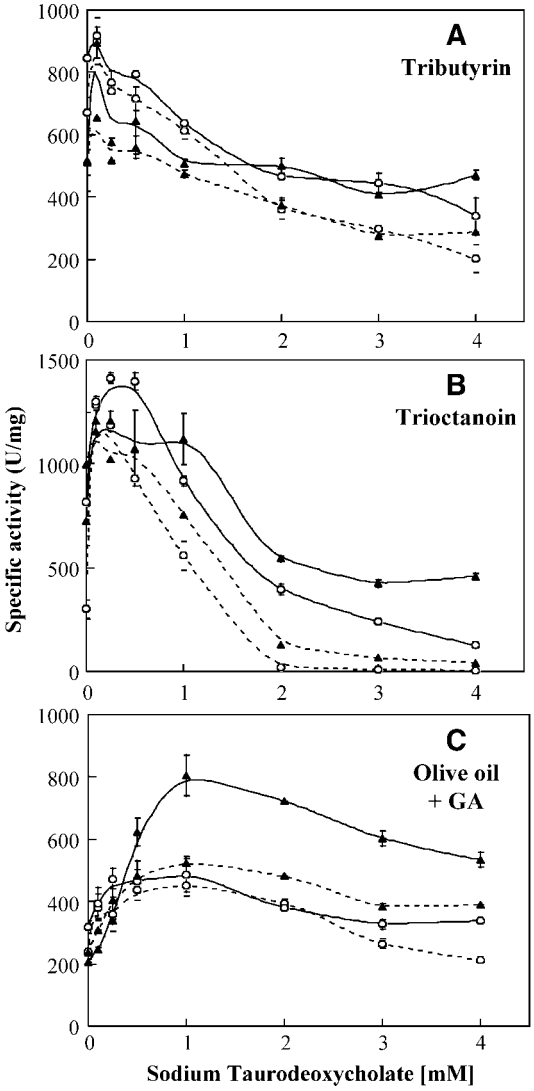

Fig. 3. Effects of sodium taurodeoxycholate and colipase on the activity of rHPLRP2. Lipolytic activities of proteolyzed (open circles) and nonproteolyzed (closed triangles) forms of rHPLRP2 were measured on emulsified tributyrin (A), trioctanoin (B), and olive oil (C) in the presence (solid lines) and absence (dashed lines) of colipase. Each data point (mean ± SD) is the result of triplicate experiments. GA, gum arabic.

of rHPLRP2 was found to be low and of the same order of magnitude as that observed with pure triolein. rHPLRP2 only showed a high specific activity on the long-chain TGs from olive oil emulsified with GA at pH 6.5 and 8.0 in both the presence and absence of colipase. Without GA, the activity of rHPLRP2 decreased greatly from 210 to 20 U/mg at pH 6.5 and from 534 to 32 U/mg at pH 8.0 in the presence of colipase. These low values are of the same order of magnitude as those obtained with pure triolein, Intralipid, and milk.

Phospholipase activity of rHPLRP2

The phospholipase activity of rHPLRP2 was measured using the pH-stat technique and a purified egg L-α-phosphatidylcholine substrate. The effect of calcium chloride on phospholipase activity was determined at calcium chloride concentrations ranging from 0 to 40 mM and compared with that of porcine pancreatic phospholipase A_2, which is calcium-dependent (**Fig. 4**). A significant effect of calcium was observed on the nonproteolyzed rHPLRP2, with a maximum specific activity of 50 ± 10 U/mg at 24 mM calcium chloride and no activity at 0 and 40 mM $CaCl_2$. The maximum activity of porcine pancreatic phospholipase A_2 (500 U/mg) was also recorded with a calcium chloride concentration of 24 mM, but phospholipase A_2 activity remained high at 40 mM $CaCl_2$.

Galactolipase activity of rHPLRP2

The galactolipase activity of rHPLRP2 and recombinant guinea pig pancreatic lipase-related protein 2 (rGPLRP2) was measured on galactolipid monomolecular films at various surface pressures using DGDG and two MGDG diastereoisomers having the galactosyl group at the *sn*-1 and *sn*-3 position on the glycerol backbone (**Fig. 5**). The activity of rHPLRP2 on DGDG reached a maximum (3.2 $mol \cdot cm^{-2} \cdot min^{-1} \cdot M^{-1}$) at 12 mN/m. This maximum value was of a similar order of magnitude to that reached with GPLRP2 (2.5 $mol \cdot cm^{-2} \cdot min^{-1} \cdot M^{-1}$) at a lower surface pressure (5 mN/m; Fig. 5A) using the same DGDG substrate. rHPLRP2 showed a lower activity on both MGDG diastereoisomers, reaching a maximum value (1.4 $mol \cdot cm^{-2} \cdot min^{-1} \cdot M^{-1}$ at 15–20 mN/m) that was approximately half the maximum activity measured with rGPLRP2 (3.5 $mol \cdot cm^{-2} \cdot min^{-1} \cdot M^{-1}$ at 10–12 mN/m) using the same MGDG substrate (Fig. 5B, C). Here again, rHPLRP2 showed its maximum activity at higher surface pressures than rGPLRP2. No significant difference was observed between the hydrolysis of MGDG *sn*-1 and that of MGDG *sn*-3 by the two enzymes.

rHPLRP2 activity as a function of pH

Using an olive oil substrate emulsified with GA, the maximum lipase activity of rHPLRP2 occurred at basic pH values (8.0–9.0) (**Fig. 6A**). Maximum activities of ~530 and 440 U/mg were recorded in the presence and absence of colipase, respectively, at pH 8.8. A strong decrease in the rHPLRP2 activity was observed under acidic pH conditions. At pH values of ~6.0–6.5, similar to those recorded in the small intestine, rHPLRP2 showed a low lipase activity (125 U/mg). Unlike the activity of rHPLRP2, that of rHPL was maintained at a high level from pH 5.5 to 7.5, reaching a maximum value at pH 7.0 (3,424 U/mg) (Fig. 6B). At pH 6.0, rHPL showed a specific activity of 2,671 U/mg on olive oil, which was >20 times higher than that of rHPLRP2.

The phospholipase A_1 activity of rHPLRP2 on egg L-α-phosphatidylcholine was measured in the same pH range (Fig. 6C). Here again, the maximum specific activity (74 U/mg) was found to occur at basic pH (pH 8.5). At pH 6.0, rHPLRP2 showed very low phospholipase A_1 activity (5 U/mg). Similar experiments were performed with galactolipids at various surface pressures (10, 12, and 15 mN). The maximum enzymatic activity occurring on DGDG was observed at pH 8.0 at all of the surface pres-

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TABLE 1. Activities of rHPLRP2 and rHPL on various long-chain glycerides

	rHPLRP2				
	pH 6.5		pH 8.0		
Glyceride	−Colipase	+Colipase	−Colipase	+Colipase	rHPL (pH 8.0) + Colipase
1-3 Monoolein	170 ± 3	192 ± 7	317 ± 11	317 ± 12	0
1,2-2,3 Diolein	0	2 ± 1	1.3 ± 0.6	5 ± 2	2,300
Triolein	18.5 ± 0	31 ± 3	5 ± 0	27.5 ± 13	3,200
Olive oil + GA	165 ± 3	210 ± 3	390 ± 0	534 ± 24	4,200
Olive oil − GA	8 ± 2	20 ± 7	19 ± 1	32 ± 7	nd
Intralipid 20%	0	1 ± 0.5	10 ± 0	31 ± 6	0
Bovine milk	7 ± 3	25 ± 7	nd	nd	0

nd, not determined; rHPLRP2, recombinant human pancreatic lipase-related protein 2. Lipolytic activities of the nonproteolyzed form of rHPLRP2 were measured at pH 6.5 and 8.0 using the pH-stat technique. These experiments were carried out in triplicate in the presence of 4 mM NaTDC in the absence or presence of a 2-fold molar excess of colipase. The data for rHPL activities on long-chain glycerides measured at pH 8.0 in the presence of colipase and bile salts (4 mM) were adapted from Y. Ben Ali's thesis (2005).

sures tested (Fig. 6D). A decrease in rHPLRP2 activity was observed at acidic pH, regardless of the surface pressure. Similar results were obtained with MGDG (data not shown).

Inhibition of PLs

The inhibition of rHPLRP2 was performed with E600 and THL in the presence and absence of 4 mM NaTDC and colipase. Experiments in which rHPL was inhibited by E600 and THL were run in parallel, under similar conditions.

In the absence of NaTDC and colipase, the time required to obtain 50% inactivation of rHPLRP2 with E600 was 3 min, whereas rHPL was not significantly inhibited under the same conditions after a 7 h period of incubation (**Fig. 7A**). In the presence of 4 mM NaTDC and colipase, the rate of inhibition of rHPLRP2 by E600 increased significantly, and 50% inhibition was reached within 1 min (Fig. 7A). Under the same assay conditions, the rate of rHPL inhibition was higher than in the absence of NaTDC; however, 1 h was required to obtain 50% inhibition.

The inhibition of rHPLRP2 by THL was also studied and compared with that of rHPL. In the absence of NaTDC and colipase in the incubation medium, THL only partially inhibited rHPL and the residual activity reached a plateau at ~60%, whereas rHPLRP2 was rapidly inhibited by THL and 50% inhibition was reached within only 5 min (Fig. 7B). In the presence of 4 mM NaTDC and colipase, fast and nearly complete inhibition of rHPLRP2 and rHPL by THL occurred after incubation times of 1 and 8 min, respectively.

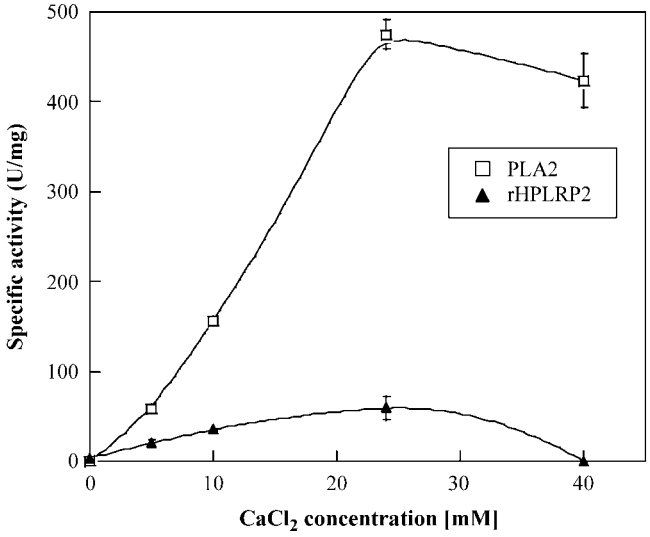

Fig. 4. Variations with calcium concentration in the phospholipase activity of porcine pancreatic phospholipase A_2 (PLA2; open squares) and nonproteolyzed rHPLRP2 (closed triangles) on purified egg L-α-phosphatidylcholine. Values shown are means ± SD.

DISCUSSION

In this study, we reinvestigated the production, purification, and characterization of rHPLRP2. rHPLRP2 was produced here by a mutant strain of *P. pastoris* lacking protease A to obtain an enzyme showing greater stability in the culture medium. rHPLRP2 was purified from the culture medium using a new purification process involving the use of SP-Sepharose and Mono Q chromatography. This modified procedure gives a purer and particularly stable rHPLRP2 within ~5 days with better productivity (15 mg/l) than that obtained previously with insect cells (5 mg/l) (19) and with wild-type *P. pastoris* (4–6 mg/l) (19), and it gives a reproducible yield of 75%.

We previously identified a single protein band corresponding to native HPLRP2 in the case of the pancreas (5). We recently reported the presence of an immunoreactive doublet protein band in a human colon homogenate (20) using Western blotting with a purified antipeptide HPLRP2 antibody. This was the first time two forms of recombinant HPLRP2 have been identified from their different molecular masses determined by MALDI-TOF, the difference in the mass being attributable to glycosylation. Sebban-Kreuzer et al. (30) then established that the rHPLRP2 produced in *P. pastoris* migrates as a single band in SDS-PAGE analysis with an apparent molecular mass of 55 kDa. In vitro deglycosylation experiments showed that the protein was *N*-glycosylated and

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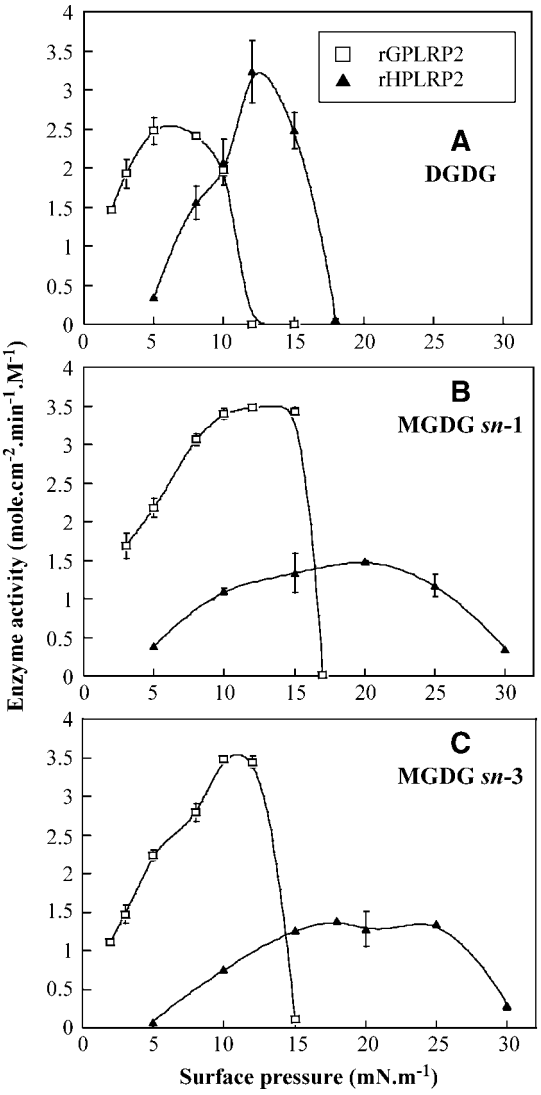

Fig. 5. Variations with surface pressure in the galactolipase activities of guinea pig pancreatic lipase-related protein 2 (rGPLRP2; open squares) and nonproteolyzed rHPLRP2 (closed triangles) on MGDG and DGDG (A) monomolecular films. The data for rGPLRP2 activity on MGDG *sn*-1 (B) and MGDG *sn*-3 (C) are adapted from Refs. 5 and 19, respectively. Values shown are means ± SD.

that the rHPLRP2 produced in the presence of tunicamycin, which blocks glycosylation, yielded two recombinant protein forms. The first form, which had a higher molecular mass, corresponded to the glycosylated form, and the second form corresponded to the nonglycosylated form.

The results of our kinetic studies on rHPLRP2 using various TGs (apart from olive oil emulsified with GA) show that the lipase activity of rHPLRP2 is inhibited by bile salts and only weakly reactivated by colipase. It turned out that the high activity recorded on olive oil was artificially induced by GA. Our findings suggest that GA might partly solubilize olive oil TGs in the form of small aggregates, thus providing a suitable substrate for rHPLRP2. This was speculation until now, but we have no other explanation because HPLRP2 is poorly active on pure triolein and acts preferentially on substrates forming small aggregates in solution. It has been shown that GA is not only an emulsifier but also an amphiphilic mixture that can affect lipase activity, probably by modifying the lipid-water interface (31). However, these experimental conditions are very different from those occurring in vivo, because no GA is present in the human duodenum. The activities of rHPLRP2 on triolein, diolein, and monoolein were then compared with those recorded with the classical HPL (Table 1). The data obtained clearly show that these enzymes have a reversed specificity for glycerides, because HPLRP2 shows a preference for substrates forming small aggregates in solution such as monoolein, whereas HPL does not act on this substrate and prefers emulsified substrates such as triolein and diolein, which do not interact with water. rHPLRP2 showed significant phospholipase A_1 activity on phospholipids, which depended on the calcium concentration, whereas HPL does not act on this substrate. Glycerophospholipids and monoglycerides, which are lipids of class II according to Small's classification (32), form liquid crystals (vesicles and multilamellar liposomes) when they are taken alone, but they can form micelles when they are mixed with amphiphiles such as bile salts (sodium taurodeoxycholate and deoxycholate), as observed in the gut. Galactolipids were only tested in the form of monolayers here, but their physicochemical properties are rather similar to those of phosphatidylcholine, and they can also form mixed micelles with bile salts and be substrates for PLRP2, as already shown (14).

The activity of rHPLRP2 on monogalactosyldiglyceride and digalactosyldiglyceride monomolecular films was found to be of the same order of magnitude as the activity of rGPLRP2, which shows the highest galactolipase activity ever measured (14). Andersson et al. (13) previously established that human pancreatic juice contains two enzymes able to hydrolyze the galactolipids. The first is carboxyl ester lipase (13) and the second is HPLRP2 (19). However, HPLRP2 was found to display the major galactolipase activity. Rat PLRP2 has also been found to display galactolipase activity (12, 14). Our results suggested that HPLRP2 may play the role of a galactolipase in the small intestine and, to a lesser extent, that of a monoglyceride lipase and a phospholipase A_1. In this study, the galactolipase activities of rHPLRP2 and rGPLRP2 were investigated more closely. It was established here that the presence of an additional galactose residue in DGDG compared with MGDG decreases the interactions between both rHPLRP2 and rGPLRP2 and the lipid-water interface when the molecular packing (i.e., surface pressure) of the substrate molecules increases. One possible explanation for this finding is that the increased surface density of the hydrophilic moiety of DGDG may impair the adsorption of the enzyme, which is driven mainly by hydrophobic interactions. To confirm this hypothesis, however, it will be necessary to simultaneously measure the interfacial excess of lipase and its catalytic activity. We also established that the position of the galactosyl group on the glycerol backbone (*sn*-1 or *sn*-3) does not significantly affect the activity of both enzymes and the variations of this activity with surface pressure.

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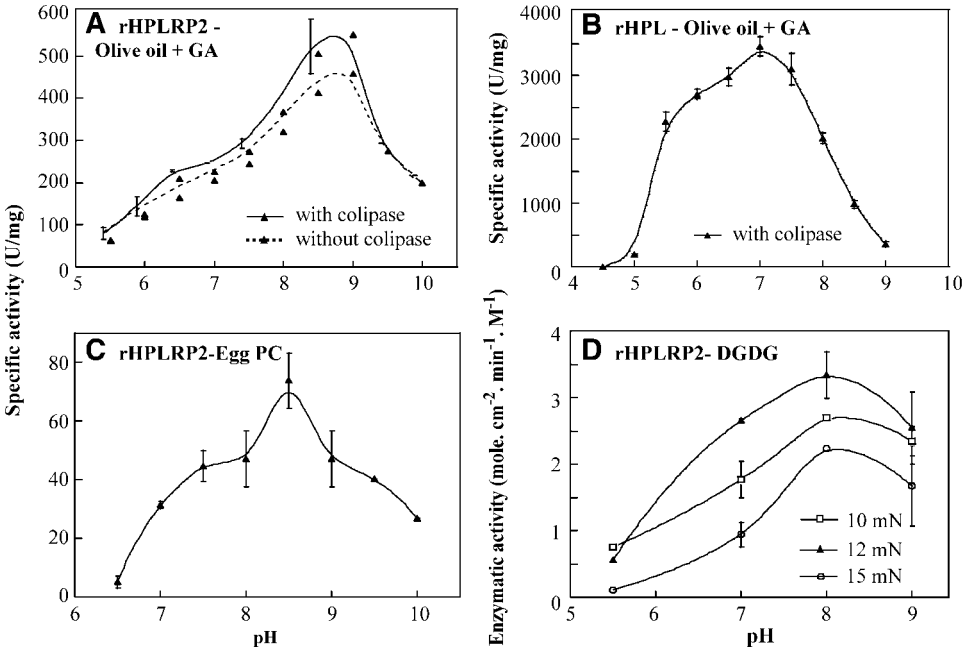

Fig. 6. Variations with pH in the activities of rHPLRP2 and rHPL. A, B: Specific activities of rHPLRP2 (A) and rHPL (B) on olive oil emulsified with GA (3%, w/v). Lipolytic activities were determined in the presence and absence of colipase, with 0.1 and 0.5 mM NaTDC for rHPLRP2 and rHPL, respectively. C: Specific activity of rHPLRP2 on purified egg phosphatidylcholine (PC). D: Enzymatic activity of rHPLRP2 on DGDG monomolecular films. Activities were measured at surface pressures of 10, 12, and 15 mN. Values shown are means ± SD.

Although rHPLRP2 has a full-length lid domain consisting of 23 amino acid residues, its kinetic properties, such as its activity on substrates forming small aggregates in solution, suggest that the lid might be in an open conformation in solution, contrary to what is observed with the classical HPL. Therefore, the active site of rHPLRP2 might be directly accessible to a substrate. To test this hypothesis, we used lipase inhibitors and compared the inhibition rates of rHPLRP2 and rHPL. The experimental conditions used in this study for the inhibition of rHPLRP2 and rHPL were the same as those used previously by Moreau et al. (26) for E600 and by Tiss et al. (33) with THL. THL, which is a potent inhibitor of gastrointestinal lipases (i.e., human gastric lipase and HPL), is now being used as a weight-loss drug for the treatment of obesity under the trademark Xenical®. The present results obtained with HPL confirm those obtained previously (33) with the same enzyme: bile salts (with or without colipase) are required for lipase inhibition to occur, because bile salts induce the lid-opening process. On the contrary, rHPLRP2 is rapidly inhibited by both THL and E600 in the absence of bile salts and colipase. In the presence of bile salts, the rate of inhibition is much faster.

In a previous study, recombinant rat PLRP2 (rRPLRP2) (12) was found to be rapidly inhibited by THL in the presence of NaTDC, which also occurs with rHPLRP2, but no data are available on the effects of THL in the absence of NaTDC. With E600 in the absence of NaTDC, the rate of rRPLRP2 inhibition was found to be low, suggesting that the inhibition of rRPLRP2 is similar to that of rHPL and requires a conformational change in the lid to occur. The effects of NaTDC were not documented, however, in that study (12). For comparison, the inhibition of rGPLRP2 by THL has been studied in the absence and presence of NaTDC (4 mM) (34). The fast rate of inhibition recorded was similar to that observed with rHPLRP2 in the absence of bile salts, and these effects were enhanced by adding bile salts. No data on the inhibitory effects of E600 on rGPLRP2 are available to date in the literature. Because GPLRP2 shows a large deletion in the lid domain and a freely accessible active site, our data suggest that the rHPLRP2 lid may have an open conformation in solution in the absence of bile salts. Bile salts are known to induce the opening of the HPL lid and the subsequent access of inhibitors to the active site. In the absence of bile salts, the rate of inhibition of rHPL is extremely slow. The fast inhibition of rHPLRP2 observed here under all of the conditions tested suggests that the rHPLRP2 lid is already open in solution and therefore that it does not require the presence of amphiphiles to become open. This might explain why rHPLRP2 shows high activity on monoglycerides and other substrates forming small aggregates in solution, whereas rHPL is not active on these substrates (Table 1). Based on the rHPLRP2 inhibition experiments performed in this study, the possibility cannot be ruled out that ethanol at a final concentration of 10% (v/v) may affect the lid-opening process, because it was always present in the inhibition experiments. It was not possible to check the inhibitory effects of THL and E600 in the absence of ethanol, because ethanol is required to solubilize the

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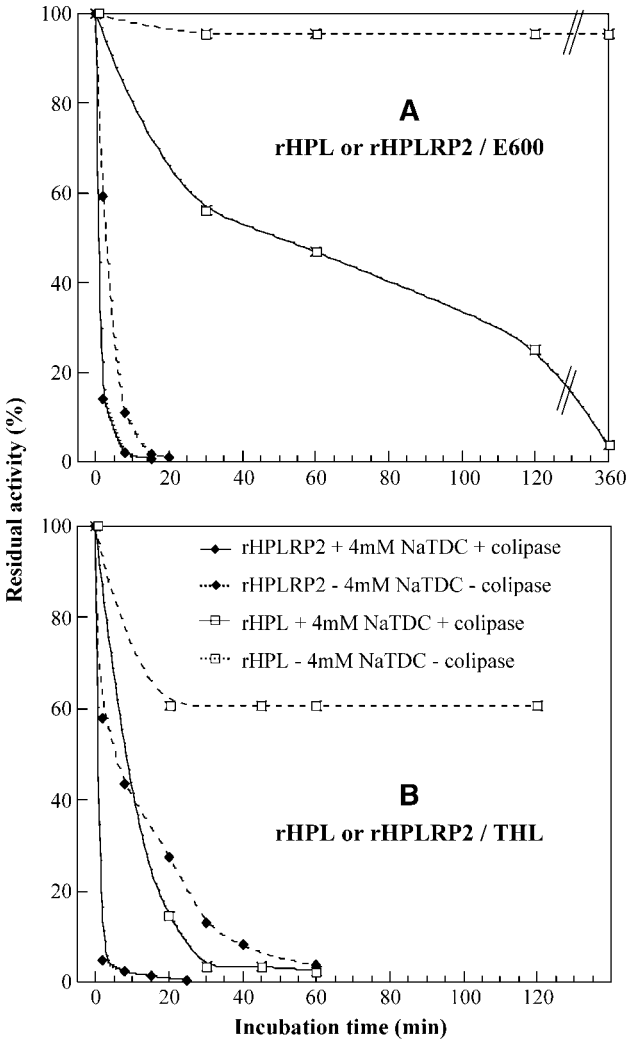

Fig. 7. Residual activity of rHPLRP2 and rHPL on a trioctanoin emulsion, versus the incubation time with diethyl *p*-nitrophenyl phosphate (E600) (A) and tetrahydrolipstatin (THL) (B), as determined using the pH-stat technique. The experiments on HPLRP2 (closed diamonds) and HPL (open squares) were performed in the absence (dashed lines) or presence (solid lines) of both 4 mM NaTDC and colipase at a molar ratio of 1:1 in the incubation medium.

inhibitor. We have observed, however, that similar ethanol concentrations to those used here do not induce the opening of the lid in HPL, which cannot be inhibited under these conditions.

During the purification procedure, the rHPLRP2 was found to be highly sensitive to the proteolytic cleavage of a peptide bond (T248-D249) present in the lid domain. Proteolytic cleavage of this peptide bond was also observed by Sebban-Kreuzer et al. (30) during the purification of rHPLRP2 expressed in *P. pastoris* strain KM71. Sensitivity of the lid domain to proteolytic cleavage has been observed in a HPL mutant (D177E) (35), native HPLRP2 (5), horse PLRP2 (36), and a rGPLRP2 chimera with a HPL lid produced in *Aspergillus oryzae* (37). The proteolytic cleavage in the lid domain of native HPLRP2 was found to occur at the I253-W254 peptide bond, generating two 27 and 22 kDa fragments after the reduction of the disulfide bridges with DTT (J. De Caro, unpublished data). With both the native and recombinant horse PLRP2, the cleavage of the lid domain was found to occur at the S244-T245 bond and resulted from an elastase attack (36). The partial proteolysis of the protein also generated two 27 and 22 kDa fragments. Lowe (35) reported that site-directed mutagenesis of D177 to E177 resulted in a HPL mutant that was less stable than wild-type HPL because of its increased susceptibility to the proteolytic cleavage occurring in the lid at the D248-I249 peptide bond. The increased sensitivity of the lid domain in the E177 mutant to proteases suggested that the mutation may have affected the conformation of the lid domain, although residue 177 was not found to be present in the lid but was buried in the protein core. An identical finding was made by Carrière et al. (37) during the purification of a GPLRP2 mutant with a full-length HPL lid domain. This mutant was specifically cleaved by an *A. oryzae* protease at a T255-R256 peptide bond present in the lid domain. A single protein peak was observed during the chromatographic separation procedure, and a single protein band was obtained upon performing Western blotting in the absence of DTT, which suggests that the two protein peptides were still connected by the disulfide bridge linking the two cysteine residues C237 and C261 present at both extremities of the lid.

All of the results presented here suggest that, in vivo, *1*) the main physiological role of HPLRP2 is that of a galactolipase, and *2*) long-chain monoglycerides seem to be the only acylglyceride substrate available for rHPLRP2 under physiological conditions. The experimental data obtained using lipase inhibitors are consistent with the existence of an accessible active site in HPLRP2 in solution. The opposite situation was found to occur with HPL, which is inactive on substrates forming small aggregates in solution (monoglycerides) and shows a high specificity for TGs. To determine the conformational state of the HPLRP2 lid more precisely, structural studies are now in progress, including experiments with and without the use of lipase inhibitors.

The authors are grateful to Dr. T. Giller and Dr. H. Lengsfeld (Hoffmann-La Roche, Ltd., Basel, Switzerland) for providing us with HPLRP2 cDNA and THL, respectively. Our thanks also to Régine Lebrun (Réseau de Plateformes Marseille-Nice Génopole) for performing mass spectrometry and N-terminal sequence analysis. The help of Jorge Rodriguez (PhD student) in performing the rHPL activity assays is acknowledged. The authors thank Dr. Yassine Ben Ali and Dr. Abdelkarim Abousalham for performing the rHPL activity assays on various glycerides. The authors also thank Dr. Jessica Blanc for revising the English manuscript.

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