Human Pancreatic Lipase-Related Protein 2 Is a Galactolipase†

Barbara Sias,‡,|| Francine Ferrato,‡ Philippe Grandval,‡ Dominique Lafont,§ Paul Boullanger,§ Alain De Caro,‡ Bernard Leboeuf,|| Robert Verger,‡ and Frédéric Carrière*,‡

Laboratoire d'Enzymologie Interfaciale et de Physiologie de la Lipolyse, CNRS UPR 9025, IBSM, Marseille, France, Université de Lyon 1, Laboratoire de Chimie organique II-Glycochimie, UMR 5181. 43, boulevard du 11 Novembre 1918, 69622 Villeurbanne Cedex, France, and Station Expérimentale d'Insémination Artificielle, INRA, 86600 Rouillé, France

Received January 25, 2004; Revised Manuscript Received May 4, 2004

ABSTRACT: Human pancreatic lipase-related protein 2 (HPLRP2) was found to be expressed in the pancreas, but its biochemical properties were not investigated in detail. A recombinant HPLRP2 was produced in insect cells and the yeast *Pichia pastoris* and purified by cation exchange chromatography. Its substrate specificity was investigated using pH-stat and monomolecular film techniques and various lipid substrates (triglycerides, diglycerides, phospholipids, and galactolipids). Lipase activity of HPLRP2 on trioctanoin was inhibited by bile salts and poorly restored by adding colipase. In vivo, HPLRP2 therefore seems unlikely to show any lipase activity on dietary fat. In human pancreatic lipase (HPL), residues R256, D257, Y267, and K268 are involved in the stabilization of the open conformation of the lid domain, which interacts with colipase. These residues are not conserved in HPLRP2. When the corresponding mutations (R256G, D257G, Y267F, and K268E) are introduced into HPL, the effects of colipase are drastically reduced in the presence of bile salts. This may explain why colipase has such weak effects on HPLRP2. HPLRP2 displayed a very low level of activity on phospholipid micelles and monomolecular films. Its activity on monogalactosyldiglyceride monomolecular film, which was much higher, was similar to the activity of guinea pig pancreatic lipase related-protein 2, which shows the highest galactolipase activity ever measured. The physiological role of HPLRP2 suggested by the present results is the digestion of galactolipids, the most abundant lipids occurring in plant cells, and therefore, in the vegetables that are part of the human diet.

Three different mRNAs encoding pancreatic lipases have been found in the human pancreas (*1*). They result from the expression of the genes of classical pancreatic lipase (HPL),[1] pancreatic lipase related-protein 1 (HPLRP1) and pancreatic lipase related-protein 2 (HPLRP2), which have also been characterized in various species (*2*−*5*). The deduced amino acid sequences of HPLRP1 and HPLRP2 show 65 and 68% identity, respectively, with HPL, and the catalytic triad (S152, D176, and H263 in HPL) and major determinants of the tertiary structure are conserved (*6*, *7*). These enzymes, or putative enzyme in the case of HPLRP1, differ in their kinetic properties.

PLRP1 displays no significant activity on any of the substrates tested up to now, and its physiological role is still unknown. From the 3D structure of dog PLRP1 sequence comparisons and mutagenesis experiments, it has been established that two amino acid residues (V178 and A180) conserved in PLRP1 of various species are responsible for the lack of activity on triglycerides. Upon replacing these residues of HPLRP1 by those found in HPL (A178, P180), a high level of lipase activity was restored (*8*). A178 and P180 residues are conserved in all the active enzymes belonging to the pancreatic lipase gene family.

PLRP2, which hydrolyses triglycerides, phospholipids, and galactolipids (*9*, *10*), shows a broader range of substrate specificities than PL, which acts only on triglycerides. In hystricomorph rodents such as guinea pig and coypu, there is no pancreatic phospholipase A2 (*11*, *12*), and the physiological role of guinea pig and coypu pancreatic lipase-related proteins 2 (GPLRP2 and CoPLRP2, respectively) is thought to be the digestion of dietary phospholipids (*9*). Moreover, GPLRP2 and CoPLRP2 lipase activities are inhibited by high bile salt concentrations, even in the presence of colipase, but classical pancreatic lipases are produced in large amounts in both guinea pig and coypu pancreas (*9*, *12*, *13*). These findings indicate that dietary triglycerides cannot be the physiological substrate of PLRP2

† The Ph.D. work of Mrs Barbara Sias was supported by a grant from Institut National de la Recherche Agronomique and Region Poitou-Charentes.

* To whom correspondence should be addressed. Frédéric Carrière, PhD, Laboratoire d'Enzymologie Interfaciale et de Physiologie de la Lipolyse, CNRS UPR 9025, 31 chemin Joseph Aiguier, 13402 Marseille cedex 20, France. Tel.: (33) 4 91 16 41 34. Fax: (33) 4 91 71 58 57. E-mail: carriere@ibsm.cnrs-mrs.fr.

‡ Laboratoire d'Enzymologie Interfaciale et de Physiologie de la Lipolyse.

§ Université de Lyon 1.

|| Station Expérimentale d'Insémination Artificielle.

[1] Abbreviations: AcMNPV, *Autographa californica* multiple nuclear polyhedrosis virus; CEL, carboxyl ester lipase; CoPLRP2, coypu pancreatic lipase-related protein 2; DGDG, digalactosyldiglyceride; DGMG, digalactosylmonoglyceride; HPL, human pancreatic lipase; HPLRP1, human pancreatic lipase-related protein 1; HPLRP2, human pancreatic lipase-related protein 2; GPLRP2, guinea pig pancreatic lipase-related protein 2; MGDG, monogalactosyldiglyceride; NaTDC, sodium taurodeoxycholate; PLRP1, pancreatic lipase-related protein 1; PLRP2, pancreatic lipase-related protein 2; Sf9, *Spodoptera frugiperda* insect cells; TC4, tributyrin; TC8, trioctanoin; YPD, Yeast Peptone Dextrose culture medium for yeast.

10.1021/bi049818d CCC: $27.50
Published on Web 07/16/2004

in these species. It has also been established that GPLRP2 and rat PLRP2 hydrolyze galactolipids (*10*). PLRP2 might therefore play a dual role in the digestion of galactolipids and phospholipids, especially in herbivores such as hystricomorph rodents. This hypothesis is supported by the recent finding that a PLRP2 is present in horse pancreas (*14*).

Gastrointestinal lipolysis of galactolipids does not seem to be restricted to herbivores, as Anderson et al. have reported that human pancreatic juice and duodenal contents both hydrolyze digalactosyldiacylglycerol (DGDG), generating digalactosyl monoacylglycerol (DGMG), non polar-lipids and fatty acid (*15*). Gel filtration of pancreatic juice led to the separation of two protein fractions showing galactolipase activity. The first fraction (consisting of 100 kDa proteins) contained carboxyl ester lipase (CEL), and the second one (consisting of 50 kDa proteins) contained HPL. The highest activity occurred, however, in the second fraction. It was therefore suggested that this activity might be displayed by HPLRP2, other PLRP2 being active on galactolipids (*10*).

The aim of this study was to examine the substrate specificity of a purified HPLRP2. Since it has been very difficult to isolate the native protein from human pancreatic juice up to now, we produced recombinant HPLRP2 in yeast and in baculovirus-infected cells. Both recombinant enzymes were purified and tested to determine their lipase, phospholipase, and galactolipase enzymatic activities. Using the monomolecular film technique, we measured the activity of HPLRP2 on a monogalactosyldiglyceride (MGDG/1,2-di-*O*-dodecanoyl-3-*O*-β−D-galactopyranosyl-*sn*-glycerol), various phospholipids and diglycerides. We also investigated the lipase activity of HPLRP2 on short and medium chain triglycerides, as well as the effects of increasing the bile salt concentration in the presence and absence of colipase.

EXPERIMENTAL PROCEDURES

DNA Sources and Preparation/Procedures. HPLRP2 cDNA was cloned at Hoffmann-La Roche Ltd. (Basel, Switzerland) and was a generous gift from Dr. Thomas Giller (*1*). A 1482 bp DNA fragment including the complete coding sequence was inserted into the EcoR I restriction site of pBluescript SK II vector (Stratagene). For the expression in the baculovirus-infected cells, HPLRP2 cDNA was subcloned into the EcoR I restriction site of pVL1393 transfer vector (Invitrogen) prior to further introduction into the AcMNPV baculovirus genome. HPLRP2 cDNA was also subcloned into the EcoR I restriction site of pGAPZ B vector (Invitrogen) for further insertion into the *Pichia Pastoris* genome and constituve expression of HPLRP2.

Plasmid DNAs were isolated from *Escherichia coli* cell cultures using the alkaline lysis procedure (*16*) and purified using the Wizard Plus Midipreps DNA Purification System (Promega). Restriction enzyme digestion and ligation steps were performed with T4 DNA ligase as recommended by the enzyme suppliers. Plasmid DNAs were introduced into *E. coli* (ElectroMAX DH10B cells, Life Technologies, Gaithersburg, MD) by electroporation using a gene pulser (Bio-Rad). DNA sequencing was carried out by Genome Express (Grenoble, France).

Prior to the yeast transformation procedure, pGAPZB vector containing HPLRP2 cDNA (pGAPZB−HPLRP2, 4.38 kbp) was linearized using BspH I endonuclease to increase the probability of genetic recombination with *Pichia pastoris* genome at the GAP promoter locus. pGAPZB−HPLRP2 vector was introduced into the wild-type yeast (X33 strain, Invitrogen) by electroporation using a gene pulser (Bio-Rad, 1500 V, 200 ohms, 25 μF). The recombinant yeast clones were selected using the Zeocin resistance introduced by the genetic recombination of pGAPZB−HLRP2 with the yeast genome. In addition, the selected clones were screened by PCR to confirm the integration of HPLRP2 DNA into the yeast genome. Each clone was grown overnight at 30 °C in a 5-mL suspension medium containing Zeocin, with orbital agitation at a rate of 100rpm. A sample of yeast suspension (1 μL) was then mixed with 5 μL of Zymoliase (1 mg/mL; Invitrogen) and incubated for 10 min at 37 °C. The sample was then immersed in liquid nitrogen for 1 min. The crude yeast extract containing yeast genomic DNA was subjected to a hot Start PCR using pFu DNA polymerase (Promega), as well as forward (pGAP) and reverse (3′AOX1) primers from the Invitrogen *Pichia* kit. The PCR reaction included 30 cycles of denaturation at 95 °C for 1 min, hydridization at 54 °C for 1 min, and elongation at 72 °C for 1 min and was performed in a Crocodile II thermocycler (Appligene). The last cycle at 72 °C was extended for an additional 5 min.

Production of HPLRP2 using the Baculovirus Expression System. Recombinant enzyme production in insect cells was performed as previously described (*17, 18*). The pVL1393 transfer vector containing HPLRP2 DNA was used for the cotransfection of SF9 cells (*Spodoptera frugiperda*) with the linearized genomic DNA from *Autographa californica* virus (AcMNPV DNA from the BaculoGold transfection kit, Pharmingen). The SF9 cells were grown in monolayers at 27 °C in tissue culture flasks, using TNM-FH medium (Sigma) supplemented with 10% fetal calf serum (Bio-Wittaker) and 1% of an antibiotic-antimycotic solution (Gibco BRL-Life Technologies). Recombinant viruses were collected after 6 days of growth in transfected insect cells and were amplified by two additional SF9 cell infection cycles. The titers of the viral stocks were assessed from the formation of plaques on the insect cell monolayers and expressed as plaque-forming units (pfu). The titer of the viral stock used for the production of HPLRP2 was 10^7pfu/ml. To ensure that the recombinant virus contained HPLPR2 DNA, the virus genomic DNA was purified and subjected to PCR with reverse and forward primer specific to HPLRP2 DNA (data not shown).

Recombinant HPLRP2 was produced in High-Five cells forming monolayers in 175 cm^2 culture flasks containing Excell 400 serum-free culture medium (JRH−Bioscience). The recombinant virus produced in SF9 cells was added to the High-five cells at a multiplicity of infection of 2 pfu per cell. After 6 h of incubation, the culture supernatant was removed and replaced by fresh Excell 400 medium. The culture medium was then sampled every day for 5 days to measure the lipase production levels (see Results section).

Production of HPLRP2 in Pichia 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 growing in the presence of Zeocin were then subcultured in 10-mL tubes containing 5 mL of YPD medium with Zeocin and screened to determine their lipolytic activity,

using the pH-stat method and tributyrin as the substrate. A clone of the recombinant yeast producing the maximum lipase activity was selected for the production of recombinant HPLRP2. After a second round of isolation, the recombinant *Pichia pastoris* clone was grown in Erlenmeyer flasks containing YPD medium without any Zeocin. For the production of HPLRP2, a pre-culture step was performed for 24 h in a 250-mL Erlenmeyer flask containing 50 mL of YPD medium. This cell culture was further used to inoculate larger cultures of the yeast at an optical density (600 nm) of 1, to be able to start the cell growth directly in the exponential growth phase, as well as to establish reproducible cell culture conditions. The yeast was grown for 3 days at 30 °C, with orbital agitation at a rate of 100 rpm in a 1-L Erlenmeyer flask containing 200 mL YPD medium. The optimum YPD medium to flask volume ratio for lipase production was found to be 1/5. The culture was stopped after 3 days, and benzamidine was added to the culture medium at a final concentration of 2 mM to limit the proteolysis of HPLRP2. The secretion of HPLRP2 into the culture medium was monitored at various times by measuring the lipase activity on tributyrin, after the cells had been pelleted by centrifuging them at 3000 rpm for 5 min.

Purification of HPLRP2 Produced in Insect Cells and Pichia pastoris. The insect cell culture medium was harvested after 3 days of infection to prevent the degradation of recombinant HPLRP2 by intracellular proteases released upon cell lysis between the third and fourth day of culture. The cells were pelleted by centrifuging them at 10000 rpm for 10 min, and the supernatant was lyophilised for 24 h. The dried material was dissolved in a few milliliters of distilled water and dialyzed overnight against 10 mM MES, pH 5.5 buffer.

In the case of the recombinant HPLRP2 produced in yeast, the culture medium (2.6 L) was collected after 3 days of yeast growth to limit the proteolysis of the recombinant enzyme. Yeast was pelleted by centrifuging it at 12000 rpm for 15 min at 4 °C. The supernatant was concentrated 10-fold by ultrafiltration with a 10 kDa cutoff, using a Vivaflow200 filtration cassette, Vivascience. The concentrate (250 mL) was dialyzed overnight against 10 mM MES, pH 5.5 buffer.

The subsequent purification procedure was the same with the two recombinant HPLRP2 produced in yeast and insect cells. HPLRP2 samples in MES buffer were centrifuged at 10000 rpm for 20 min, and the supernatant was filtered through a 0.45-μm filter (Millipore). Cation exchange chromatography was performed on a Mono S HR 5/5 column equilibrated in 10 mM MES, buffer, pH 5.5, using FPLC (Pharmacia). The flow rate was adjusted to 1 mL/min. After sample injection and column washing, a linear NaCl gradient was applied (0−0.1 M NaCl, for 60 min). The protein elution was recorded spectrophotometrically at 280 nm, and the lipase activity was measured in all the fractions collected using tributyrin as the substrate. HPLRP2 was eluted at around 0.03M NaCl.

Electrophoresis and Western Blotting Procedures. The recombinant HPLRP2 present in the culture medium and purification fractions was analyzed by electrophoresis on 12% polyacrylamide gels in the presence of SDS as described by Laemmli (*19*). Western Blotting was performed as described by Gershoni and Palade (*20*) using rabbit polyclonal antibodies raised against recombinant HPLRP2 produced in insect cells. The anti-HPLRP2 rabbit serum was used at a 1:1000 dilution and the reacting antibodies were detected with goat anti-rabbit immunoglobulins conjugated to alkaline phosphatase.

Analysis of Recombinant HPLRP2. The amino acid composition was analyzed as previously described (*17*). To check that the cleavage of the HPLRP2 signal peptide had occurred appropriately, and to ensure that no proteolytic degradation had taken place, the purified protein was subjected to N-terminal sequence analysis using an Applied Biosystem Model 473A gas-phase sequencer. MALDI-TOF mass spectrometry was performed on samples using Voyager DE-RP equipment (Perspective Biosystems Inc.).

Lipase and Phospholipase Activity Measurements using a pHstat. The lipase activity of HPLRP2 was assayed by measuring the free fatty acids released from a mechanically stirred emulsion of tributyrin (TC4) or trioctanoin (TC8) (puriss grade from Fluka). By use of a pH-stat (TTT 80 Radiometer, Copenhagen, Denmark), the free fatty acids were automatically titrated with 0.1N NaOH at a constant pH value of 7.5. Each reaction was performed in a thermostated vessel (37 °C) containing 0.5 mL of substrate (TC4 or TC8), 14.5 mL of 0.28 mM Tris-HCL buffer, 150 mM NaCl, 1.4 mM $CaCl_2$, and various concentrations of taurodeoxycholate sodium salt (from 0 to 4 mM NaTDC). When required, pure colipase from porcine pancreas was added at a molar excess of 5. The standard assay used to estimate the HPLRP2 activity in the culture medium and chromatography fractions was performed using TC4 as the substrate, 0.5 mM NaTDC, and colipase in molar excess. HPLRP2 displays its maximum specific activity under these conditions (see results). The lipolytic activities are expressed here in international units (U). One U corresponds to 1 μmol of fatty acid released per minute.

Phospholipase activity of HPLRP2 was assayed with the pHstat technique using lecithin from egg yolk as the substrate (*21*).

Monomolecular Film Experiments. The lipase activity was assayed using monomolecular films of 1,2-rac-didecanoyl glycerol (1,2-dicaprin, 1,2 $DiC_{10}DG$). Phospholipase activity was assayed using monomolecular films of 1,2-didodecanoyl-*sn*-glyceryl-3-phosphatidylglycerol (1,2 $DiC_{12}PG$), 1,2-didodecanoyl-*sn*-glyceryl-3-phosphatidylserine (1,2 $DiC_{12}PS$), 1,2-didodecanoyl-*sn*-glyceryl-3-phosphatidylethanolamine (1,2 $DiC_{12}PE$), and 1,2-didodecanoyl-*sn*-glyceryl-3-phosphatidylcholine (1,2 $DiC_{12}PC$) as substrates. Dicaprin was from Sigma Aldrich, and phospholipids were from Avanti Polar Lipids. Galactolipase activity was assayed using 1,2-di-*O*-dodecanoyl-3-*O*-β−D-galactopyranosyl-*sn*-glycerol (MGDG) as the substrate (see the synthesis procedure). The kinetics of monomolecular film hydrolysis were determined on a KSV-2200 barostat (KSV, Helsinki) using a "zero order" Teflon trough (*22*). The trough was equipped with a mobile Teflon barrier, which was used to keep the surface pressure constant upon enzyme hydrolysis of the substrate film because the products of the lipolysis (monodecanoyl glycerol, decanoic acid, lyso-dodecanoyl phospholipids, monododecanoyl-galactopyranosyl-glycerol, and dodecanoic acid) are soluble in water and desorbed from the interface. Surface pressure was measured using a Wilhelmy plate (perimeter 3.94 cm) attached to an electromicrobalance connected to a

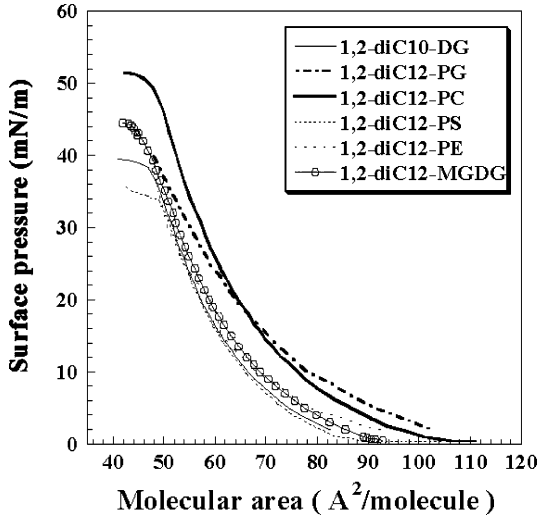

FIGURE 1: Compression isotherms of the lipid substrates used for monomolecular film experiments. Lipids solubilized in chloroform (5 μg/mL−5 mg/mL) were spread individually at the air−water interface, and their compression isotherms were determined. The collapse point of MGDG was found to be around 50 mN/m, and a phase transition was observed at around 45 mN/m.

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 buffer was prepared with Milli-Q water. Any residual active surface impurities were removed before each assay by sweeping and suction of the surface. The subphase of the reaction compartment was continuously stirred with a 2.0-cm magnetic stirrer set at 250 rpm. The enzyme (final concentration 0.1 μg; 0.1 nM) was injected through the film with a Hamilton syringe. The surface area of the reaction compartment was 100 cm^2, and the volume was 100 mL. The length of the reservoir was 27.9 cm, and the width was 14.8 cm. Enzyme activity was estimated from the area of the trough covered by the mobile barrier and the known molecular area of the substrate molecule. The molecular area of each substrate was determined as a function of the surface pressure by performing compression isotherms (Figure 1). The enzyme activity was expressed in moles of substrate hydrolyzed per min per surface unit (cm^2) and referred to the overall molarity of the enzyme initially injected into the aqueous subphase (mole $\cdot$ min^{-1} $\cdot$ m^{-2} $\cdot$ M^{-1}). Before each measurement, the Teflon trough used to form the monomolecular film was washed with water, gently brushed in the presence of distilled ethanol, washed again with tap water, and rinsed with Milli-Q water.

Synthesis of Monogalactosyl Diglyceride. General Methods. Pyridine was dried by boiling it with CaH_2 prior to distillation. Dichloromethane was washed twice with water, dried with $CaCl_2$, and distilled from CaH_2. Methanol was distilled from magnesium. Pyridine and CH_2Cl_2 were stored over 4-Å molecular sieves and MeOH over 3-Å molecular sieves. Melting points were determined on a Büchi apparatus and were uncorrected. Thin-layer chromatography was performed on aluminum sheets coated with Silica gel 60 F_{254} (E. Merck). Compounds were detected by spraying the TLC plates with dilute 15% aq H_2SO_4 and charring them at 150 °C for a few minutes. Column chromatography was performed on Silica gel Geduran Si 60 (Merck). Optical rotations were recorded on a Perkin-Elmer 241 polarimeter in a 1-dm cell at 21 °C. ^1H and ^{13}C NMR spectra were recorded with a Bruker AC-200 spectrometer working at 200 and 50 MHz, respectively, with Me_4Si as the internal standard. Element analyses were performed by the "Laboratoire Central d'Analyses du CNRS" (Vernaison, France).

A monogalactosyldiglyceride substrate (MGDG; 1,2-di-*O*-dodecanoyl-3-*O*-β-D-galactopyranosyl-*sn*-glycerol **1**) was synthesized in six steps, as shown in Figure 2 and using procedures adapted from refs *23* and *24*.Glycosylation of 1,2-*O*-isopropylidene-*sn*-glycerol (**2**) (*25*) with 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl trichloroacetimidate (**3**) (*26*) in the presence of trimethylsilyl trifluoromethanesulfonate resulted in the expected derivative **4** with an 83% yield. Compound **4** was de-*O*-acetylated using Zemplén's method (catalytic sodium methylate in methanol), and the crude product was directly perbenzylated to **5** with benzyl chloride in dimethyl sulfoxide, in the presence of potassium hydroxide. The isopropylidene group of **5** was removed by performing mild hydrolysis (80% aqueous acetic acid, room temperature), resulting in the diol **6** with a high yield. The fatty ester functions were then introduced by reacting the diol **6** with an excess of dodecanoyl chloride in methylene chloride, in the presence of pyridine (product **7**); the benzyl protecting groups were removed by performing hydrogen transfer by treating **7** with cyclohexene in refluxing ethanol in the presence of 20% palladium hydroxide on carbon, giving compound **1** with a high yield.

3-O-(2,3,4,6-Tetra-O-acetyl-β-D-galactopyranosyl)-1,2-O-isopropylidene-sn-glycerol (**4**). 1,2-*O*-Isopropylidene-*sn*-glycerol (**2**) (*25*) (0.495 g, 3.47 mmol, 1.50 equiv), 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl trichloroacetimidate (**3**) (*26*) (1.231 g, 2.50 mmol), and activated 4-Å molecular sieves (0.5 g) were added to alcohol-free methylene chloride (15 mL). The reaction mixture was flushed with argon while cooling it to −10 °C. A solution of trimethylsilyl trifluoromethanesulfonate (0.015 mL, 0.07 mmol) in methylene chloride (0.5 mL) was introduced via a syringe during a period of 1 h. The mixture was stirred for 6 h at −10 °C before it was filtered over Celite and diluted with methylene chloride (50 mL). The organic phase was washed with a saturated aqueous solution of sodium hydrogen carbonate and dried (Na_2SO_4). Concentration of the solution gave a residue, which was purified by column chromatography (4:3 petroleum ether−EtOAc) to obtain the pure derivative **4** in the form of a solid (0.960 g, 83% yield); mp 88−89 °C (EtOH), $[\alpha]_D$ -6.1 (*c* 1.0, $CHCl_3$); R_f 0.45. ^1H NMR ($CDCl_3$): δ 5.39 (dd, 1 H, $J_{3,4}$ 3.3, $J_{4,5}$ 0.9 Hz, H-4), 5.21 (dd, 1 H, $J_{1,2}$ 7.9, $J_{2,3}$ 10.5 Hz, H-2), 5.00 (dd, 1 H, H-3), 4.58 (d, 1 H, H-1), 4.26 (m, 1 H, H-2′), 4.19 (dd, 1 H, $J_{5,6a}$ 5.8, $J_{6a,6b}$ 11.5 Hz, H-6a), 4.12 (dd, 1 H, $J_{5,6b}$ 6.3 Hz, H-6b), 4.02 (dd, 1 H, $J_{1'a,2'}$ 6.4, $J_{1'a,1'b}$ 8.2 Hz, H-1′a), 3.91 (ddd, 1 H, H-5), 3.90 (dd, 1 H, $J_{2',3'a}$ 4.2, $J_{3'a,3'b}$ 10.6 Hz, H-3′a), 3.80 (dd, 1 H, $J_{1'b,2'}$ 4.2 Hz, H-1′b), 3.65 (dd, 1 H, $J_{2',3'b}$ 6.0 Hz, H-3′b), 2.15, 2.07, 2.05, 1.98 (4s, 12 H, 4 CH_3CO), 1.42, 1.34 (2s, 6 H, (CH_3)$_2$C). ^{13}C NMR ($CDCl_3$): δ 170.27, 170.16, 170.01, 169.32 (4 C, CH_3CO), 109.23 ($C(CH_3)_2$), 101.31 (C-1), 74.21 (C-2′), 70.82 (C-5), 70.65 (C-3), 69.02 (C-3′), 68.69 (C-2), 67.02 (C-4), 66.12 (C-1′), 61.25 (C-6), 62.00 (C-6), 26.52, 25.09 (2 C, (CH_3)$_2$C), 20.65, 20.56, 20.56, 20.47 (4 C, CH_3-COO). Anal. Calcd for $C_{20}H_{30}O_{12}$ (462.44): C, 51.94; H, 6.54. Found: C, 51.65; H, 6.44.

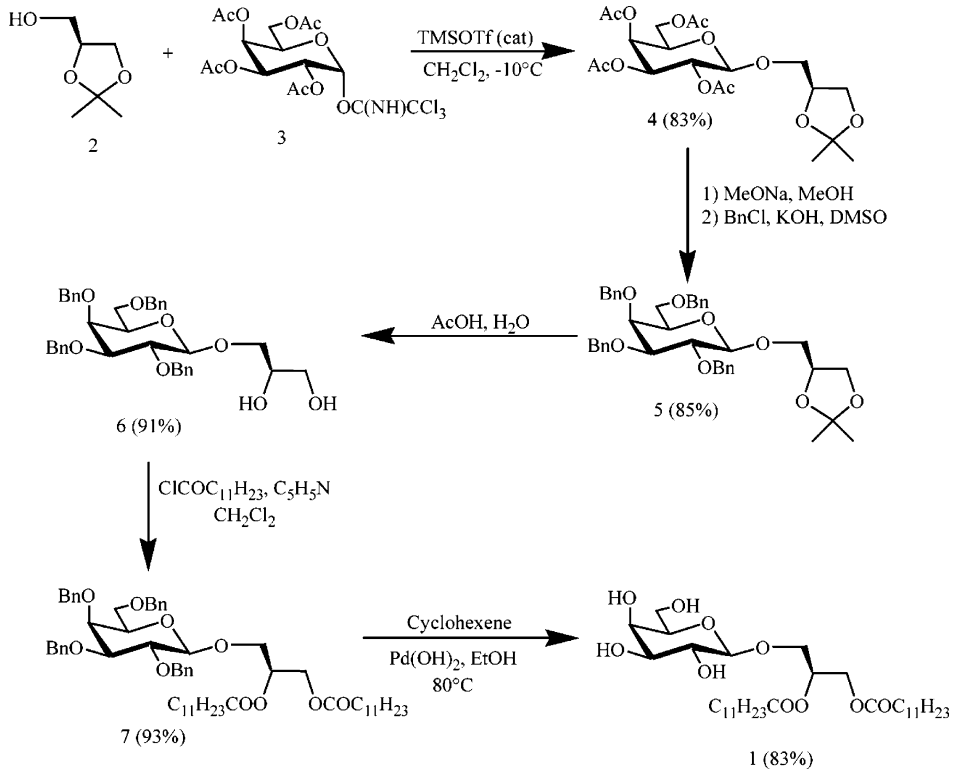

FIGURE 2: Synthesis scheme of 1,2-di-*O*-dodecanoyl-3-*O*-*β*-D-galactopyranosyl-*sn*-glycerol.

3-O-(2,3,4,6-Tetra-O-benzyl-β-D-galactopyranosyl)-1,2-O-isopropylidene-sn-glycerol (**5**). Glycoside **4** (0.647 g, 1.40 mmol) was added to a solution of catalytic sodium methylate in dry methanol (25 mL). The mixture was stirred for 16 h, the solvent was then evaporated, and the residue was concentrated twice from ethyl acetate (10 mL) and chloroform (10 mL). The residue was dissolved in dimethyl sulfoxide (2 mL) and added to a precooled (0 °C) mixture of powdered potassium hydroxide (0.730 g, 13.05 mmol) and benzyl chloride (0.942 g, 4.45 mmol) in dimethyl sulfoxide (5 mL). The mixture was allowed to reach room temperature, stirred for 16 h, and poured into icy water (50 mL). The product was extracted with a 4:1 petroleum-ether−EtOAc mixture (3 × 40 mL); the combined organic phases were washed with water (20 mL), dried, and concentrated to a residue, which was purified by performing column chromatography (3:1 petroleum-ether−EtOAc) to obtain the pure derivative **5** in the form of an oil (0.778 g, 85% yield). $[\alpha]_D$ +1.0 (*c* 2.0, $CHCl_3$); R_f 0.55. ^{1}H NMR ($CDCl_3$): δ 7.35−7.31 (m, 20 H, 4 C_6H_5), 4.96 and 4.64 (m, 2 H, *J* 11.6 Hz, $CH_2C_6H_5$), 4.92 and 4.78 (m, 2 H, *J* 10.7 Hz, $CH_2C_6H_5$), 4.78 and 4.72 (m, 2 H, *J* 12.4 Hz, $CH_2C_6H_5$), 4.48 and 4.42 (m, 2 H, *J* 12.5 Hz, $CH_2C_6H_5$), 4.40 (d, 1 H, $J_{1,2}$ 7.7 Hz, H-1), 4.33 (m, 1 H, H-2′), 4.07 (dd, 1 H, $J_{1'a,2'}$ 5.6, $J_{1'a,1'b}$ 8.5 Hz, H-1′a), 4.02 (dd, 1 H, $J_{2',3'a}$ 4.7, $J_{3'a,3'b}$ 9.5 Hz, H-3′a), 3.91 (m, 1 H, H-4), 3.88 (dd, 1 H, $J_{1'b,2'}$ 5.8 Hz, H-1′b), 3.84 (dd, 1 H, $J_{2,3}$ 9.1 Hz, H-2), 3.62−3.50 (m, 5 H, H-3,5,6a,6b,3′b), 1.42, 1.37 (2s, 6 H, $(CH_3)_2C$). ^{13}C NMR ($CDCl_3$): δ 138.87, 138.75, 138.63, 138.07, 128.59−127.70 (24 C, C_6H_5), 109.36 ($C(CH_3)_2$), 104.34 (C-1), 82.35 (C-3), 79.54 (C-2), 75.34, 74.70 (2 C, $CH_2C_6H_5$), 74.40 (C-2′), 73.68 ($CH_2C_6H_5$), 73.65, 73.60 (2 C, C-4,5), 73.20 ($CH_2C_6H_5$), 70.34 (C-6), 69.94 (C-3′), 67.23 (C-1′), 27.05, 25.55 (2 C, $(CH_3)_2C$). Anal. Calcd for $C_{40}H_{46}O_8$ (654.77): C, 73.37; H, 7.08. Found: C, 73.26; H, 7.12.

3-O-(2,3,4,6-Tetra-O-benzyl-β-D-galactopyranosyl)-sn-glycerol (**6**). A solution of glycoside **5** (0.760 g, 1.19 mmol) in 80% aqueous acetic acid (25 mL) was stirred at room temperature for 24 h. After the residue was concentrated and coevaporated from toluene (2 × 15 mL), it was purified on a short column of silica gel (1:2 petroleum-ether−EtOAc) to obtain the pure derivative **6** in the form of a solid (0.665 g, 91% yield); mp 73−74 °C (EtOH); $[\alpha]_D$ -7.5 (*c* 1.5, $CHCl_3$), litt. (*24*) −5, *c* 7, $CHCl_3$; R_f 0.50. ^{1}H NMR ($CDCl_3$): δ 7.40−7.25 (m, 20 H, 4 C_6H_5), 4.96 and 4.63 (2d, 2 H, *J* 11.6 Hz, $CH_2C_6H_5$), 4.89 and 4.82 (m, 2 H, *J* 11.1 Hz, $CH_2C_6H_5$), 4.79 and 4.73 (m, 2 H, *J* 12.1 Hz, $CH_2C_6H_5$), 4.50 and 4.41 (m, 2 H, *J* 11.8 Hz, $CH_2C_6H_5$), 4.38 (d, 1 H, $J_{1,2}$ 7.7 Hz, H-1), 3.92 (dd, 1 H, $J_{5,6a}$ 2.3, $J_{6a,6b}$ 12.4 Hz, H-6a), 3.82 (m, 2 H, H-2,4), 3.80 (d, 1 H, $J_{5,6b}$ 4.6 Hz, H-6b), 3.75−3.40 (m, 7 H, H-3,5,1′a,1′b,2′,3′a,3′b). ^{13}C NMR ($CDCl_3$): δ 138.70−137.86, 128.62−127.74 (24 C, C_6H_5), 104.74 (C-1), 82.36 (C-3), 79.58 (C-2), 75.48, 74.77, 73.75 (3 C, $CH_2C_6H_5$), 73.67, 73.56 (2 C, C-4,5), 73.22 (2 C, $CH_2C_6H_5$, C-3′), 71.15 (C-2′), 68.97 (C-6), 63.57 (C-1′). Anal. Calcd for $C_{37}H_{42}O_8$ (614.71): C, 72.29; H, 6.89. Found: C, 72.57; H, 6.94.

3-O-(2,3,4,6-Tetra-O-benzyl-β-D-galactopyranosyl)-1,2-di-O-dodecanoyl-sn-*glycerol* (**7**). A solution of dodecanoyl chloride (0.594 mL, 2.57 mmol) in dry methylene chloride (3.0 mL) was added dropwise for 1 h to a solution of diol, product **6** (0.450 g, 0.732 mmol) and pyridine (1.2 mL) in dry methylene chloride (6 mL), and the mixture was stirred overnight. The excess dodecanoyl chloride was destroyed by the addition of water (50 μL) and the reaction mixture was stirred for a further 3 h. After adding methylene chloride (50 mL), the organic phase was washed with a saturated aqueous solution of sodium hydrogen carbonate (50 mL) and then with water (25 mL), dried (Na_2SO_4), and concentrated to a residue, which was purified by column chromatography

(4:1 petroleum-ether−EtOAc) to obtain the pure product **7** in the form of a liquid (0.667 g, 93% yield). $[\alpha]_D$ +4.2 (*c* 3.5, $CHCl_3$); R_f 0.55 (5:1 petroleum-ether−EtOAc). ^{1}H NMR ($CDCl_3$): δ 7.33−7.27 (m, 20 H, 4 C_6H_5), 5.26 (ddd, 1 H, $J_{1'a,2'}$ 2.1, $J_{1'b,2'}$ 6.6, $J_{2',3'a}$ 4.4, $J_{2',3'b}$ 5.1 Hz, H-2′), 4.95 and 4.63 (2d, 2 H, *J* 11.8 Hz, $CH_2C_6H_5$), 4.92 and 4.76 (m, 2 H, *J* 10.8 Hz, $CH_2C_6H_5$), 4.77 and 4.70 (m, 2 H, *J* 10.8 Hz, $CH_2C_6H_5$), 4.47 and 4.40 (m, 2 H, *J* 11.8 Hz, $CH_2C_6H_5$), 4.40 (dd, 1 H, $J_{1'a,1'b}$ 12.0 Hz, H-1′a), 4.35 (d, 1 H, $J_{1,2}$ 7.7 Hz, H-1), 4.22 (dd, 1 H, H-1′b), 4.05 (dd, 1 H, $J_{3'a,3'b}$ 10.8 Hz, H-3′a), 3.90 (bd, 1 H, $J_{3,4}$ 3.4, $J_{4,5}$ 0.5 Hz, H-4), 3.82 (dd, 1 H, $J_{2,3}$ 9.7 Hz, H-2), 3.67 (dd, 1 H, H-3′b), 3.60−3.49 (m, 4 H, H-3,5,6a,6b), 2.27 (q, 4 H, *J* 6.8 Hz, 2 CH_2-CO), 1.58−1.26 (m, 36 H, 18 CH_2), 0.89 (t, 6 H, 2 CH_3CH_2). ^{13}C NMR ($CDCl_3$): δ 173.37, 173.05 (2 C, $COCH_2$), 138.77, 138.65, 138.56, 138.01, 128.52−127.63 (24 C, C_6H_5), 104.39 (C-1), 82.19 (C-3), 79.33 (C-2), 75.22, 74.66, 73.64 (3 C, $CH_2C_6H_5$), 73.50 (2 C, C-4,5), 73.19 (2 C, $CH_2C_6H_5$), 70.15 (C-2′), 68.85 (C-6), 67.96 (C-3′), 62.80 (C-1′), 34.36, 34.22, 32.03, 29.74−29.19, 25.00, 22.80 (alkyl CH_2), 14.24 (2 C, CH_3CH_2). Anal. Calcd for $C_{61}H_{86}O_{10}$ (979.30): C, 74.81; H, 8.85. Found: C, 74.85; H, 9.03.

1,2-Di-O-dodecanoyl-3-O-β-D-galactopyranosyl-sn-*glycerol* (**1**). A mixture of the perbenzylated derivative **7** (0.330 g, 0.337 mmol), fresly distilled cyclohexene (5 mL), and Pd-$(OH)_2$/C (0.125 g) in absolute ethanol was heated for 6 h at 80 °C. After cooling and filtering the solution on Celite, it was concentrated, and the residue was purified by column chromatography (8:1 CH_2Cl_2−MeOH) to obtain the pure compound **1**, which crystallized from EtOAc (0.173 g, 83% yield); mp 135 °C, litt. (*24*) 135−137 °C; $[\alpha]_D$ -3.5 (*c* 1.0, $CHCl_3$), litt. (*24*) −5, *c* 7, $CHCl_3$ −4.5, *c* 1, $CHCl_3$; R_f 0.57 (9:1 CH_2Cl_2-MeOH). ^{1}H NMR ($CDCl_3$): δ 5.30 (m, 1 H, H-2′), 4.41 (dd, 1 H, $J_{1'a,2'}$ 3.3, $J_{1'a,1'b}$ 11.9 Hz, H-1′a), 4.29 (d, 1 H, $J_{1,2}$ 7.7 Hz, H-1), 4.22 (dd, 1 H, $J_{1'b,2'}$ 3.3 Hz, H-1′b), 4.03 (m, 1 H, H-4), 3.93 (dd, 1 H, $J_{2',3'a}$ 5.2, $J_{3'a,3'b}$ 11.2 Hz, H-3′a), 3.90−3.83 (m, 2 H, H-2,5), 3.72 (dd, 1 H, $J_{2',3'b}$ 6.3 Hz, H-3′b), 3.67−3.52 (m, 3 H, H-3,6′a,6′b), 3.30, 3.22, 3.12, 2.92 (4 m, 4 H, 4 O*H*), 2.33 (m, 4 H, 2 CH_2CO), 1.70−1.27 (m, 36 H, 18 CH_2), 0.89 (t, 6 H, 2 CH_3CH_2). ^{13}C NMR ($CDCl_3$): δ 173.86, 173.46 (2 C, $COCH_2$), 104.09 (C-1), 74.57 (C-5), 73.49 (C-3), 71.13 (C-2), 70.16 (C-2′), 68.92 (C-4), 68.08 (C3′), 62.96 (C-1′), 61.43 (C-6), 34.32, 34.19, 31.95, 29.70−29.23, 24.94, 22.21 (alkyl CH_2), 14.12 (2 C, CH_3CH_2). Anal. Calcd for $C_{33}H_{62}O_{10}$,H_2O (618.83): C, 62.23; H, 10.13. Found: C, 62.13; H, 10.06.

RESULTS

Production and Purification of Recombinant HPLRP2 Expressed in Insect Cells. HPLRP2 was secreted into the culture medium of insect cells with yields ranging from 5 to 10 mg/L of recombinant protein and was found to be the main protein present in the serum-free culture medium. Lipase activity was detected in the culture medium after 24 h of infection of the cells by the baculovirus, and Western blot analysis showed the presence of a protein with a molecular mass of around 50 kDa recognized by anti-HPL antibodies (see Supporting Information). The secretion of the recombinant protein increased up to day 4. To prevent the occurrence of proteolytic degradation, we stopped the culture after post-infection day 3. HPLRP2 was further purified to homogeneity by performing a single cationic exchange chromatography step (see Supporting Information).

Purified HPLRP2 was subjected to N-terminal amino acid analysis, which confirmed that the protein was produced in its mature form [KEV(C)YGQ...] and that its signal peptide was correctly cleaved. The molecular mass of recombinant HPLRP2 (51179 Da) was determined experimentally by MALDI-TOF mass spectrometry and found to be 1098 Da greater than the theorical mass of the polypeptide (50081 Da). A similar result was previously obtained with the recombinant HPL produced in insect cells (*17*). There is only one *N*-glycosylation site in HPL (N166), and carbohydrate analysis showed that the single glycan chain consisted of two *N*-acetylglucosamine, three mannose, and one fucose residues. Short glycan chains of this kind are classically observed in glycosylated recombinant proteins secreted by baculovirus-infected insect cells (*27*). The protein sequence of HPLRP2 shows the presence of two theoritical *N*-glycosylation sites (N336, N411), and the mass of recombinant HPLRP2 determined experimentally therefore suggests that only one of the two sites is glycosylated.

Production and Purification of Recombinant HPLRP2 Expressed in Pichia pastoris. The cDNA of HPLRP2 was cloned in the pGAPZ B vector, downstream of the pGAP promoter. The recombinant plasmid was integrated into the yeast genome by recombination after electroporation of the yeast cells. The aim of this transformation was to induce the constitute secretion of HPLRP2, the expression of which is under the control of the strong GAP (Glyceraldehyde-3-Phosphate dehydrogenase) promoter. HPLRP2 was found to be secreted into the culture medium of yeast, with yields ranging from 4 to 6 mg/L of recombinant protein. The YPD yeast culture medium contained only small peptides, and HPLRP2 was the main protein observed in the culture medium. The secretion of the enzyme was detected 24 h after the culture was initiated with a lipase activity of 4 U/mL and reached 10U/ml at 48 h (see Supporting Information). After 48 h of culture, the biomass of the yeast reached the stationary phase and the lipase activity remained fairly constant. A protein with an apparent molecular mass of 50−60 kDa was detected by Western blot analysis in the yeast culture medium after 24 h of culture. The intensity of the single immunoreactive band increased up to 30 h of culture, after which some immunoreactive bands with lower molecular masses were also observed (see Supporting Information). To avoid severe proteolytic degradation of the secreted HPLRP2, the yeast culture was stopped after 30 h of culture.

The recombinant HPLRP2 produced in yeast was purified to homogeneity by performing cation exchange chromatography on a Mono S HR 5/5 column using FPLC (see Supporting Information). It was further subjected to N-terminal sequence analysis, MALDI-TOF mass determination and amino acid analysis. The experimentally determined mass of the recombinant HPLRP2 produced in *Pichia pastoris* (53195 Da) was higher than the theoretical mass of the polypeptide (50081 Da). This result suggests the existence of a 3114-Da glycan chain in the recombinant HPLRP2 produced in *Pichia pastoris*, whereas only a short 1098-Da glycan chain was present in the recombinant HPLRP2 produced in insect cells. The two *N*-glycosylation sites of HPLRP2 might also be occupied, but this was not checked in the present study. In *Pichia pastoris*, HPLRP2 was also

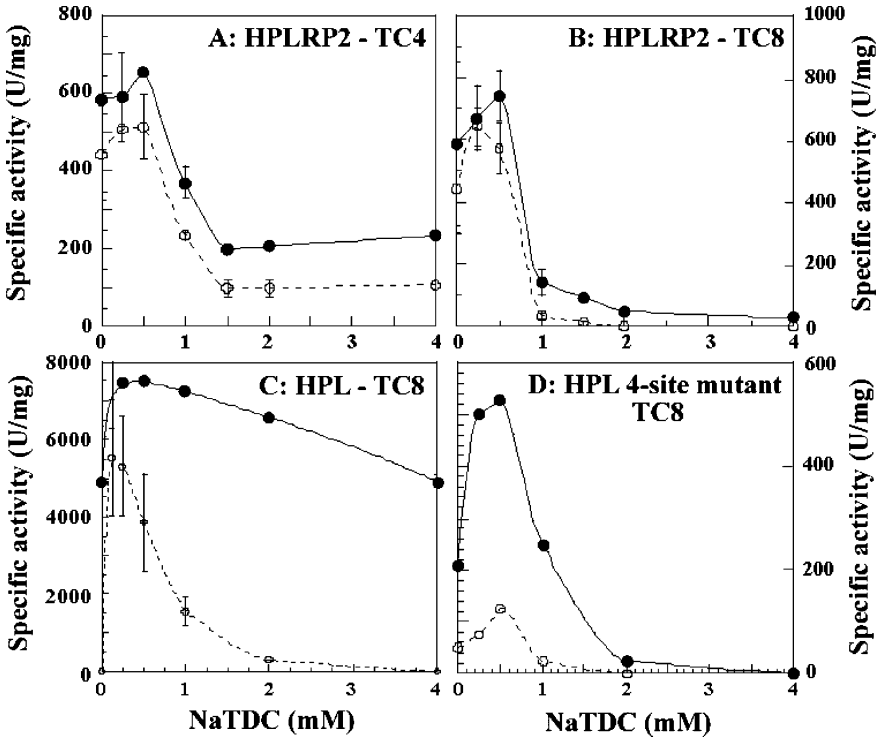

FIGURE 3: Effects of NaTDC and colipase on the lipase activities of HPLRP2, HPL, and HPL 4-site mutant. Lipase activities were measured using either tributyrin (panel A) or trioctanoin (panels B, C, and D), in the presence (●) and absence (○) of colipase (molar excess of 2 vs lipase). Recombinant HPLRP2 was produced in the yeast *Pichia pastoris*. Similar data were obtained with recombinant HPLRP2 produced in insect cells. Each data point (mean ± SD) is the result of triplicate experiments.

secreted in its mature form, and its signal peptide was correctly cleaved. The first 10 amino acids [KEV(C)-YGQLGC] were found to be identical to those of the mature protein expressed in insect cells.

Lipase Activity of HPLRP2 and Effects of Bile Salts and Colipase. The lipase activity of recombinant HPLRP2 was measured using the pH-stat technique, with tributyrin (TC4) and trioctanoin (TC8) as substrates, in the presence of various concentrations of sodium taurodeoxycholate (NaTDC), with and without colipase. Using TC4, the maximum specific activity of the HPLRP2 produced in insect cells and *Pichia pastoris* was found to be 640 and 660 U/mg, respectively, in the presence of 0.5 mM NaTDC and colipase in a 2-fold molar excess versus HPLRP2. Using TC8, the maximum specific activities were found to be 1020 and 740 U/mg, respectively, in the presence of 0.5 mM NaTDC and colipase in a 2-fold molar excess.

HLRP2 activity on TC4 was not completely inhibited by supramicellar concentrations of NaTDC in the absence of colipase (Figure 3A), contrary to what usually occurs with classical pancreatic lipases. Similar results were previously published on the coypu and guinea pig PLRP2, and it was suggested that these enzymes may remain active on the soluble fraction of tributyrin when they are desorbed from the oil−water interface by NaTDC (*9*). In the presence of colipase and high NaTDC concentrations, the HPLRP2 activity on TC4 increased slightly but was still 3-fold lower than the maximum activity recorded in the presence of 0.5 mM of NaTDC. Using the completely insoluble TC8 (puriss grade) as the substrate, HPLRP2 lipase activity was completely inhibited by supramicellar concentrations of NaTDC (Figure 3B), like the classical HPL (Figure 3C). Contrary to what is observed with HPL (Figure 3C), the lipase activity of HPLRP2 on TC8 was not restored by adding colipase at high NaTDC concentrations (Figure 3B).

We previously produced an HPL mutant in which four residues involved in the open lid stabilization process were replaced by the residues present in HPLRP2 in the homologous positions (R256G, D257G, Y267F, K268E mutations) (*28*). In mature HPLRP2, the corresponding amino acid numbers are G258, G259, F269 and E270. As shown in Figure 3D, the HPL 4-site mutant displayed similar behavior to that of HPLRP2 when TC8 was used as the substrate, in the presence of colipase. The maximum activity observed at a concentration of 0.5 mM NaTDC in the presence of colipase decreased drastically in comparison with that of HPL (525 U/mg vs 7500 U/mg). This specific activity was similar, however, to the maximum specific activity of HPLRP2 (743 U/mg). Like HPLRP2, the mutant was inhibited by supramicellar concentrations of NaTDC, and the activity was not restored by adding colipase. The only significant difference with respect to HPLRP2 was observed at low NaTDC concentrations, where colipase significantly affected the HPL 4-site mutant, whereas the HPLRP2 activity was only slightly affected by colipase.

Lipolytic Activities of HPLRP2 on Various Phospholipids. The phospholipase activity of HPLRP2 was investigated using the pH-stat technique with egg yolk as the substrate. The phospholipase activities measured with the HPLRP2 produced in insect cells (21 ± 0.6 U/mg) and in *Pichia pastoris* (29 ± 8.1 U/mg) were 20-fold lower than that of GPLRP2 (570 U/mg; (*29*)) and 7-fold lower than that of CoPLRP2 (180 U/mg; (*9*)). The phospholipase-to-lipase (TC8) activity ratio of HPLRP2 (0.02) was much lower than that of GPLRP2 (0.8), but was similar to that of CoPLRP2 (0.04). Contrary to GPLRP2, HPLRP2 and CoPLRP2 have

Table 1: Maximum HPLRP2 and GPLRP2 Activities Measured with the Monomolecular Film Technique on Various Phospholipids, Monogalactosyldiglycerides, and Diglycerides[a]

substrate	HPLRP2 maximum activity ($mol \cdot min^{-1} \cdot m^{-2} \cdot M^{-1}$)	HPLRP2 surf pressure ($mN \cdot m^{-1}$)	GPLRP2 maximum activity ($mol \cdot min^{-1} \cdot m^{-2} \cdot M^{-1}$)	GPLRP2 surf pressure ($mN \cdot m^{-1}$)	HPLRP2 to GPLRP2 activity ratio (%)
1,2-diC_{12}-PC	0.04*	10	1.3	3−5	3.08
1,2-diC_{12}-PE	0.05*	10−18	3.6	10	1.39
1,2-diC_{12}-PG	0.045*	20−22	3.3	10−15	1.36
1,2-diC_{12}-PS	0.019*	17	2.3	5−12	0.82
1,2-diC_{12}-MGDG	1.4	10−17	3.4	10−12	41
1,2-diC_{10}-DG	2	35−37	4	10−30	50

[a]All experiments were performed with 100 ng of enzyme injected below the lipid film, except the experiments performed with HPLRP2 and phospholipids (*), in which 2 μg of enzyme were injected below the lipid film.

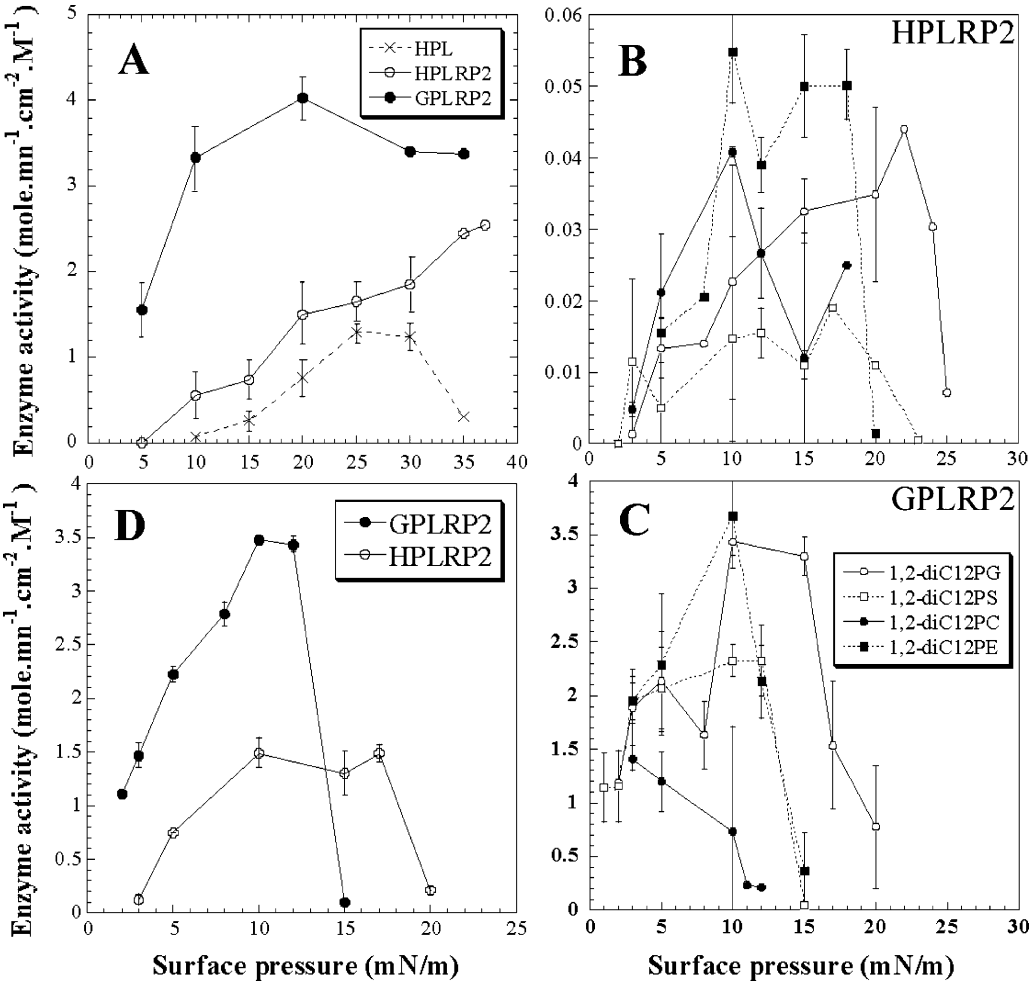

FIGURE 4: HPLRP2 lipolytic activities on various monomolecular lipid films. (A) Variations in the lipase activities of HPLRP2, GPLRP2, and HPL with the surface pressure on dicaprin. (B) Variations in the phospholipase activities of HPLRP2 with the surface pressure on various phospholipids. (C) Variations in the phospholipase activities of GPLRP2 with the surface pressure on various phospholipids. (D) Variations in the galactolipase activities of GPLRP2 and HPLRP2 with surface pressure on various MGDG substrates. Each data point (mean ± SD) is the result of triplicate experiments.

a full length lid that controls the access of the substrate to the active site. It has been previously reported that adding a full-length lid domain to GPLRP2 depressed the phospholipase activity versus the lipase activity (*30*).

The phospholipase and lipase activities of HPLRP2 and GPLRP2 were also investigated using monomolecular films of pure phospholipids and 1,2-dicaprin, respectively. Maximum lipase activities on 1,2-dicaprine of 2 $mol \cdot min^{-1} \cdot m^{-2} \cdot M^{-1}$ and 4 $mol \cdot min^{-1} \cdot m^{-2} \cdot M^{-1}$ were measured with HPLRP2 and GPLRP2, respectively (Table 1 and Figure 4A). A significant lipase activity of GPLRP2 was observed at low surface pressures (5 mN/m), whereas HPLRP2 was inactive. The HPLRP2 activity was found to increase continuously with the surface pressure, and the maximum activities were reached at 35−37 mN/m. In the case of GPLRP2, the maximum activities were recorded in a larger range of surface pressures (10−30 mN/m; Figure 4).

The phospholipase activities of HPLRP2 on 1,2diC_{12}PC, 1,2diC_{12}PE, 1,2diC_{12}PS, 1,2diC_{12}PG (Figure 4B) were found to be 100-fold lower than those measured with GPLRP2 (Figure 4C). While the phospholipase activity of GPLRP2 was found to be in the range of 1.3 to 3.6 mol ·

$min^{-1} \cdot m^{-2} \cdot M^{-1}$, the phospholipase activity of HPLRP2 did not exceed 0.05 mol $\cdot$ $min^{-1} \cdot m^{-2} \cdot M^{-1}$, and the maximum activities were measured on 1,2DiC_{12}PE with both enzymes (Table 1).

Galactolipase Activity of HPLRP2 Determined using the Monomolecular Film Technique. A monogalactosyldiglyceride substrate (MGDG; 1,2-di-*O*-dodecanoyl-3-*O*-*β*-galactopyranosyl-*sn*-glycerol; Figure 2) was used to measure the galactolipase activities of HPLRP2, GPLRP2, and HPL by means of the monomolecular film technique. HPLRP2 produced in insect cells displayed a high level of galactolipase activity on MGDG and its maximum activity (1.4 mol $\cdot$ $in^{-1} \cdot m^{-2} \cdot M^{-1}$ at 10−17 mN/m) was approximatively half the maximum activity (3.4 $\cdot$ $in^{-1} \cdot m^{-2} \cdot M^{-1}$ at 10−12 mN/m) measured with GPLRP2 (Figure 4D). HPLRP2 was found to act on MGDG monomolecular films at higher surface pressures than did GPLRP2 (Figure 4D). HPL showed no activity on MGDG.

DISCUSSION

Anderson et al. have established that the galactolipase activity observed in human pancreatic juice can be separated into two protein fractions by gel filtration (*15*). In the first fraction, consisting of high molecular mass proteins, the enzyme acting on galactolipids was found to be carboxyl ester lipase (CEL). The second fraction contained proteins with similar molecular masses to that of HPL (50 kDa). Later on, the same authors demonstrated that CEL displays a low specific activity of 1.6 U/mg on DGDG, whereas GPLRP2 displays a 200-fold higher activity on the same substrate (260 U/mg). Except for patatin-like enzymes (*31*, *32*) and the fungal lipase from *Rhizopus delemar (arrhizus)*, which is employed as a galactolipase by plant biochemists (*33*, *34*) enzymes with galactolipase activity have not yet been studied in detail. On the basis of the data available in the litterature, it seems likely that GPLRP2 is the galactolipase with the highest specific activity (*10*). We observed in the present study that HPLRP2 displays a galactolipase activity, which is of the same order of magnitude as GPLRP2 (Figure 4D). Our results support the hypothesis put forward by Anderson et al. that an enzyme present in human pancreatic juice and having a similar mass to that of HPL may be responsible for most of the galactolipase activity found in the juice (*10*).

A preliminary report on native HPLRP2 purification from human pancreatic juice indicated that HPLRP2 is probably present in the exocrine secretion of human pancreas (*35*). A very low phospholipase activity on egg-PC was reported to occur in native HPLRP2: this activity was 200-fold lower than that of GPLRP2 (570 U/mg). We have established in this study that recombinant HPLRP2 displays a low phospholipase activity on egg yolk phospholipids (21−29 U/mg), as well as on various monomolecular films of pure phospholipids, including 1,2 DiC_{12}PC (Figure 4B); the HPLRP2 activity on phospholipids monomolecular films is about 100-fold lower than the GPLRP2 phospholipase activity (Table 1; Figure 4, parts B and C). Since a phospholipase A2 is present in human pancreatic juice, it is unlikely that HPLRP2 plays an important role in the digestion of phospholipids in the gastrointestinal tract of humans. It is also unlikely that dietary triglycerides may constitute the physiological substrate of HPLRP2, because HPLRP2 lipase activity is drastically reduced by bile salts and poorly reactivated by colipase (Figure 3).

To explain the weak effect of colipase on HPLRP2, we compared the lipase activities of HPLRP2 and HPL with that of an HPL mutant, in which four substitutions observed in HPLRP2 vs HPL (R256G, D257G, Y267F, K268E) were introduced by site-directed mutagenesis (*28*). These four amino acid residues are involved in a salt bridge (D257-K268) and a hydrogen bond (R256−Y267) stabilizing the open conformation of the lid domain in HPL, and this domain interacts with colipase in its open form (*36*). In the HPL 4-site mutant, the maximum lipase activity on TC8 is 10-fold lower than in HPL, but it is in the same range as the HPLRP2 lipase activity (Figure 3). The lipase activity of HPL 4-site mutant is inhibited by supramicellar concentrations of NaTDC and is not restored by colipase. These results suggest that residues R256, D257, Y267 and K268 are essential for a stable lipase-colipase interaction to occur, leading to the adsorption of the lipase-colipase complex at the oil−water interface in the presence of bile salts. Since the essential lipase residues involved in the HPL-colipase interactions are conserved in HPLRP2, both in the lid and the C-terminal domain (*37*), it is possible that a different conformation of the lid domain in HPLRP2 may induce a weak HPLRP2-colipase interaction (as can be seen to occur at low bile salt concentrations in Figure 3), resulting in the inhibition of HPLRP2 lipase activity at high bile salt concentrations. It is, however, difficult from the present experiments to estimate whether the weaker colipase effects observed with HPLRP2 and HPL 4-site mutant are due to weaker lipase−colipase interactions or a weaker lipase binding at the oil−water interface. It might be possible that the amino acid substitutions present in the lid of HPLRP2 and HPL 4-site mutant induce a weaker lipase binding at the interface, although it was shown in a previous work that the complete deletion of the lid domain in HPL does affect the apparent lipase-colipase affinity, as well as lipase binding at the oil−water interface (*28*).

The expression of PLRP2 in the pancreas of various species and its identification in the pancreatic secretion of rats also support the idea that PLRP2 plays a physiological role in the gastrointestinal lipolysis of dietary lipids (*38*). Rat PLRP2 is also secreted into the culture medium from rat acinar cells and the AR42-J acinar cell line (*39*). Studies on the cellular localization as well as on the temporal patterns of expression of the mRNA encoding PLRP2 suggest, however, that the enzyme may have other functions. The expression of human PLRP2 observed at birth when classical PL is not yet expressed suggests that PLRP2 may play an important role in dietary fat digestion in suckling mammals (*40*). Since the HPLRP2 activity on triglycerides is inhibited by bile salts and not restored by colipase, this function would have to be operative prior to the full development of bile secretion. The PLRP2 gene disruption in transgenic mice supports the idea that PLRP2 may play a role of this kind just after birth and during the lactation period, since a decrease in the neonatal dietary fat absorption rates has been observed in the suckling transgenic mice (*41*). Alternatively, the intracellular location of PLRP2 in rats suggests another function for this enzyme in the same species. The cDNA sequence encoding GP-3, a glycoprotein from zymogen granule membrane, was found to match the PLRP2 cDNA.

GP-3 or PLRP2 is closely associated with the zymogen granule membrane (ZGM) (*42*). Despite its tight membrane association, PLRP2 is also present in the pancreatic juice and undergoes a regulated process of secretion (*38*). The PLRP2 present on the zymogen granule membrane might be involved in granule trafficking, fusion of ZGM with cytoplasmic membrane, and exocytosis of the secretory granule contents. In mice, PLRP2 mRNA was isolated by differential display from cytotoxic T cell culture treated or not by Interleukin-4, and the level of PLRP2 mRNA increased significantly in response to Interleukin-4 treatment (*43*). No such effect of interleukin-4 was observed with human cytotoxic T lymphocytes (*1*). Since a decrease in T cell cytotoxicity was observed in PLRP2-deficient mice (*41*), PLRP2 might also be involved in immunological defense, together with other hydrolytic enzymes produced by T cells, at least in mice. PLRP2 mRNA was also detected later on in the mouse small intestine. Both enterocytes and paneth cells express PLRP2 mRNA with the strongest signal observed in paneth cells. Immunohistochemical data have confirmed the localization of PLRP2 in enterocytes, but reproducible staining of paneth cells was not obtained, indicating that paneth cells may synthesize little or no PLRP2 protein (*44*).

A question remains concerning the fact that PLRP2 has the ability to hydrolyze galactolipids and phospholipids whereas classical HPL does not. This question was previously investigated by Withers-Martinez et al., who modeled phospholipid and galactolipid molecules lying in the catalytic crevice of GPLRP2 (*45*). The steric hindrance due to the lid domain present in HPL is absent in GPLRP2 because of the large deletion of the lid. As a result, the polar head of either a phospholipid molecule or a digalactosyldiacylglycerol molecule can be easily accommodated in the active site. It was proposed that the phospholipase A1 and galactolipase activities of GPLRP2 may result from the lid deletion. This hypothesis was supported by the fact that the addition of a full-length lid in GPLRP2 impaired the phospholipase activity (*30*). No phospholipase activity was introduced, however, into an HPL mutant with a lid deletion. Therefore, the ability of PLRP2 to hydrolyze glycerolipids with a large polar head such as phospholipids is not due only to a deletion of the lid. In PLRP2 with a lid, as occurs with HPLRP2, CoPLRP2, RPLRP2, and horse PLRP2, the phospholipase A1 activities are much lower than in GPLRP2. HPLRP2 is able, however, to hydrolyze a substrate with a polar head such as monogalactosyldiacylglycerol to the same extent as GPLRP2. The lid domain therefore plays a significant role in substrate selectivity toward phospholipids, but not toward galactolipids. In conclusion, further structure−function investigations are now required to explain the substrate specificity toward galactolipids within the pancreatic lipase gene family.

All the results presented here suggest that the main physiological role of HPLRP2 is that of a galactolipase. Up to now, HPLRP2 and CEL are the only enzymes found to be present in pancreatic juice displaying galactolipase activity. The human pancreas therefore seems to be well equipped for the digestion of galactolipids, the main plant lipids that mainly occur in membranes of the photosynthetic machinery (chloroplasts, thylakoides) and are therefore present in the vegetables which are part of our diet. The native HPLRP2 has been identified in human pancreatic juice, and its purification is now under progress at our laboratory (Dr. J. De Caro, to be published): further investigations will therefore soon be possible.

ACKNOWLEDGMENT

We are grateful to Dr Thomas Giller (Hoffmann-La Roche Ltd., Basel, Switzerland) for his generous gift of HPLRP2 cDNA. We wish to thank Jacques Bonicel, Regine Lebrun, and Angela Guevara for performing mass spectrometry, *N*-terminal sequence and amino acid analysis.

SUPPORTING INFORMATION AVAILABLE

Time-course expression, Western blotting, and SDS-PAGE analyses of HPLRP2. This material is available free of charge via the Internet at http://pubs.acs.org.

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BI049818D