RESEARCH ARTICLE

Individual and combined action of pancreatic lipase and pancreatic lipase-related proteins 1 and 2 on native *versus* homogenized milk fat globules

Amélie Berton[1], *Corinne Sebban-Kreuzer*[2], *Stéphanie Rouvellac*[1], *Christelle Lopez*[3] *and Isabelle Crenon*[1]

[1] UMR INRA 1260, INSERM U476, Univ Aix-Marseille, Faculté de Médecine, Nutriments Lipidiques et Prévention des Maladies Métaboliques, Marseille, France
[2] IBSM-CNRS FRE3083, Chemin Joseph Aiguier, Marseille, France
[3] INRA, AGROCAMPUS OUEST, UMR 1253 Science et Technologie du Lait et de L'œuf, Rennes, France

Received: December 9, 2008
Revised: March 13, 2009
Accepted: April 6, 2009

Pancreatic lipase (PL) and pancreatic lipase-related proteins 1 and 2 (PLRP1 and PLRP2) display different functional properties, despite close structures. The aim of the study was to compare the kinetic properties of recombinant human PLRP1, PLRP2, and PL on a physiological substrate: the milk fat under native and homogenized structures. No lipolytic activity is measured for PLRP1. PLRP2 hydrolyses milk fat with a lower catalytic efficiency than that of PL. PLRP2 activity, higher on homogenized than on native milk fat, is differently influenced by fatty acids (FA) and colipase depending on a proteolytic cleavage in the lid domain. FA enhance the activity on both milks. A colipase positive effect on the non-proteolyzed PLRP2 is observed on homogenized milk and with FA on native milk fat. Bile salts are necessary. An original observation is a synergic effect between PL and PLRP2 on the two milks. An inhibitory effect of PLRP1 on PL activity is also demonstrated. The combined action of pancreatic lipases on milk fat digestion proposes PLRPs as modulators of PL. Our study supports the hypothesis of a major role of PLRP2 in fat digestion in newborns and brings new insights to understand the physiological role of PLRPs.

Keywords:
Colipase / Digestion / Milk fat globules / Pancreatic lipase-related protein 1 / Pancreatic lipase-related protein 2

1 Introduction

In human, digestion of dietary triacylglycerols (TG) begins in the stomach with the action of the gastric lipase [1] and continues in the duodenum, by the concerted action of pancreatic and biliary secretions. The pancreatic lipase (PL) is the main enzyme involved in intestinal digestion. In physiological conditions, PL needs a pancreatic cofactor, the colipase, to overcome the inhibitory effect of bile salts on its activity [2, 3]. The three-dimensional structure of PL reveals that the protein has two domains: the N-terminal domain with the catalytic triad and the C-terminal domain involved in colipase interaction. The active site is covered by a surface loop called the lid domain that must move to allow the access to the catalytic triad by the substrate [4, 5].

Correspondence: Dr. Isabelle Crenon, UMR 1260 INRA/476 INSERM, Faculté de Médecine la Timone, 27 Boulevard Jean-Moulin 13385 Marseille Cedex 5, France
E-mail: Isabelle.Crenon@univmed.fr
Fax: +33-4-91-78-21-01

Abbreviations: BSSL, bile salt-stimulated lipase; **FA,** fatty acids; **HM,** homogenized milk; **MFGM,** milk fat globule membrane; **NaTDC,** sodium taurodeoxycholate; **NM,** native milk; **NP-rHPLRP2,** non-proteolyzed form of recombinant human pancreatic lipase-related protein 2; **PhL,** phospholipids; **PL,** pancreatic lipase; **PLRP1 and PLRP2,** pancreatic lipase-related proteins 1 and 2; **P-rHPLRP2,** proteolyzed form of recombinant human pancreatic lipase-related protein 2; **rHCol,** recombinant human colipase; **rHPL,** recombinant human pancreatic lipase; **rHPLRP,** recombinant human pancreatic lipase-related protein; **TG,** triacylglycerols

Pancreatic lipase-related proteins 1 and 2 (PLRP1 and PLRP2) showing a high level of identity with PL were identified in the pancreas of several species [6, 7]. Although structurally close, PL, PLRP1, and PLRP2 display very different functional properties [7]. No significant lipolytic activity has been shown for PLRP1. This particular feature is because of two amino acid substitutions in the catalytic cavity of PLRP1 [8].

PLRP2 presents a broader substrate specificity compared with PL and hydrolyses TG, phospholipids (PhL), and galactolipids [7, 9, 10]. The TG lipase activity of PLRP2 is lower than that of PL. The effects of bile salts and colipase on the PLRP2 activity are less certain and may diverge among various species, depending on the nature of the substrate [7].

The kinetic characteristics of PLRPs raise important questions about their role in lipids digestion in human. One of the physiological functions proposed for PLRP2 is a role in the digestion of lipids in newborns [11]. This hypothesis is supported by the high expression level of PLRP2 in human and rat around the neonatal period as compared with PL expression that is low or undetectable until the suckling–weaning transition [12, 13]. Decreased neonatal dietary fat absorption observed in PLRP2-deficient mice strengthened this hypothesis [14].

Most of the PLRP studies were performed on non-physiological substrates or artificial emulsions. The composition and the structural organization are important factors for lipids digestion [15, 16]. Therefore, according to the role of PLRP2 in newborns, it is essential to study the action of this enzyme on a physiological complex substrate, the milk fat that is the only source of lipids in the early life stage. We have focused our interest on cow milk for several reasons: (i) it is largely consumed in our Western diet, (ii) it is easily available, (iii) the structural organization of cow and human milks fat is similar [17], and (iv) dairy processes of cow milk allow to obtain milk fat differently structurally organized.

TG are the major constituents of milk fat (97–99%). With more than 400 different fatty acids (FA), which were identified [18], milk fat is the most complex fat found in nature. In milk, fat is organized as droplets called fat globules, the size distribution of which ranges from 1 μm to about 10 μm with a mean diameter around 4 μm [18]. These fat globules are constituted by a core rich in TG surrounded by a biological membrane, called the milk fat globule membrane (MFGM). The MFGM resulting from the mechanism of fat globule secretion [19] is composed mainly of PhL, proteins, glycoproteins, cholesterol, and vitamins [20, 21]. The MFGM acts as a natural emulsifier, prevents fat globules aggregation and coalescence, and also protects the globules against lipolysis. The inefficient hydrolysis by PL, the influence of lipolysis partners, and the contribution of other lipases on native milk (NM) fat globules were extensively investigated [22–26].

The processing of milk to manufacture dairy products can greatly affect the structure of fat globules. Homogenization (high-pressure mechanical treatment) leads to a partial disruption of the MFGM, to an adsorption of casein micelles at the interface and greatly reduces the globules size [27, 28]. This newly formed globule membrane, called synthetic fat globule membrane, is composed of casein micelles, other milk proteins, and also MFGM fragments [27]. The reduction of the globules size leads to an increase of the specific surface area, which are with the composition of the globules surface, two parameters very important for lipid digestion with interfacial enzymes [15]. No information is available either about the hydrolysis efficiency by PL, *in vitro*, of homogenized milk (HM) fat globules as compared with NM fat globules or about the hydrolysis by PLRPs on both structural organizations of milk fat.

The aim of this study was to compare the kinetic properties of human PLRP1 and PLRP2 and PL on milk fat either as native or as homogenized fat globules. PLRP2 being sensitive to a proteolytic cleavage in the lid domain, both non-proteolyzed and proteolyzed forms were used. Influence of the lipolysis partners (bile salts and colipase) and free FA was investigated. Because of the potential concomitant presence of pancreatic lipases in the duodenum, we studied the concerted action of PL and PLRPs on the digestion of both milk fat globules.

2 Materials and methods

2.1 Protein expression and purification

Human PL, PLRP1, PLRP2, and colipase recombinant proteins (recombinant human pancreatic lipase (rHPL), recombinant human pancreatic lipase-related proteins 1 and 2 (rHPLRP1 and rHPLRP2), and recombinant human colipase (rHCol), respectively) were expressed in *Pichia pastoris* yeast as secreted proteins, as previously described for rHPL [29] and rHPLRP2 [30] or according to the manufacturer's indications (Invitrogen, San Diego, CA, USA) for rHPLRP1 and rHCol. The recombinant proteins were produced in buffered methanol-complex medium, with methanol being supplemented to a final concentration of 1% every 24 h. The cultures were maintained at 30°C for 4 days, then after centrifugation, the cell-free supernatants were dialyzed overnight against 20 mM sodium acetate buffer, pH 5. rHPL, rHPLRP1, and rHPLRP2 purifications were performed on an anion-exchange column diethylaminoethyl cellulose [30]. A second step on cation-exchange sulfopropyl sepharose column (Amersham Biosciences, Uppsala, Sweden) was added for rHCol purification. Bound proteins were eluted by applying a linear NaCl salt gradient. Purified proteins were analyzed by SDS-PAGE on 12% polyacrylamide gels as described by Laemmli [31], by Western blot using purified anti-HPLRP2 peptide antibodies as described by Burnette [32], and by activity measurement. Protein concentrations were determined with the bicinchoninic acid protein assay reagent (Pierce, USA).

2.2 Samples

Three batches of NM and the respective HM (prepared from NM) were used in this work. Raw cow whole milk was purchased from a local dairy plant (Entremont, Montauban de Bretagne, France). The milk was heated to 50°C and skimmed to obtain the skimmed milk used for dilutions. Raw whole milk was heated to 60°C and homogenized at 300×10^5 Pa using a two-stage high-pressure homogenizer (Rannie slow model LAB 16/15, APV France, Evreux, France). The native whole milk, skimmed milk, and homogenized whole milk were thermized (73°C for 20 min) to inactivate milk lipase. The samples were kept at room temperature to avoid fat crystallization that may lead to a physical destabilization of fat droplets and analyzed as detailed below.

2.3 Particle size measurements

The size distributions of fat globules in the natural milk and the HM were measured by laser light scattering using a Mastersizer 2000 (Malvern, UK), with two laser sources. The refractive indexes used were 1.458 and 1.460 for milk fat at 633 and 466 nm, respectively, and 1.33 for water. The samples of milk (about 0.2 mL) were diluted in 100 mL water directly in the measurement cell of the apparatus in order to reach 10% obscuration. The casein micelles were dissociated by adding 1 mL of 35 mM EDTA/NaOH pH 7 buffer (>98%, disodium salt, $2H_2O$, Prolabo, France) to the milks, in the apparatus.

From the size distribution, the volume/surface average diameter of fat globules is defined as $D(3,2) = \Sigma n_i d_i^3 / \Sigma n_i d_i^2$, the volume-weighted diameter $D(4,3) = \Sigma n_i d_i^4 / \Sigma n_i d_i^3$, where n_i is the number of fat globules of diameter d_i, and the specific surface area $S = 6 \cdot \varphi / d_{32}$, where φ is the volume fraction of milk fat were calculated.

2.4 Lipase activity assays

Tributyrin and sodium taurodeoxycholate (NaTDC) were purchased from Sigma-Aldrich, Saint Quentin Fallavier, France.

Lipases activities were titrimetrically measured at 20°C under magnetic gentle stirring in a final volume of 15 mL. For characterization of purified proteins, the assay mixtures consisted of 0.11 M emulsified tributyrin in the buffer A (1 mM Tris–HCl pH7.5, 0.1 M NaCl, 5 mM $CaCl_2$) with 0–4 mM NaTDC and colipase (0- to 5-fold molar). For hydrolysis of milk fat globules, the assay mixtures contained different volumes of NM or HM completed up to 2 or 4 mL with skimmed milk, different concentrations of NaTDC and colipase as indicated in the legends, in buffer A. At the beginning of the assays, different concentrations of rHPL, rHPLRP1, or rHPLRP2, alone or together, were added. Samples were taken at different times of the kinetic, immediately frozen in liquid nitrogen and stored at −80°C until lipids extraction and free FA measurements or TLC analysis. One unit of lipase corresponds to the release of 1 μmol of FA *per* minute. One unit of colipase corresponds to the amount of colipase required to restore one lipase unit.

2.5 Lipids extraction

Total lipids were extracted from milks or lipolysis media by a chloroform/methanol method according to the Bligh and Dyer procedure with minor modifications [33]. Briefly, during the procedure, a solution containing 150 mM NaCl and 2% acetic acid was added instead of water. The methanol phase was extracted twice. The chloroform phase was evaporated under nitrogen flux. Total lipid extracts were stored at −20°C until further analysis.

2.6 Lipids analysis

Free FA determination was performed by enzymatic assay using the commercial kit NEFA RANDOX (Crumlin, UK) on total lipids extract dissolved in isopropyl alcohol. Analysis of lipid classes released during lipolysis assays was performed by TLC. The total lipid extracts, dissolved in hexane, were spotted on silica gel plates (VWR, Fontenay sous bois, France) developed in hexane/diethyl ether/acetic acid (80/20/1 *v*/*v*/*v*). The spots corresponding to different classes of lipids were visualized by heating plates, after staining by 10% phosphomolybdic acid in ethanol.

3 Results

3.1 Recombinant proteins production and milks characteristics

To avoid any contamination between them, human pancreatic lipases and colipase proteins were expressed in yeast *P. pastoris*. The four recombinant proteins were secreted into the culture medium with yields reaching 20–40 mg of recombinant proteins *per* liter after 4 days of culture. Recombinant proteins were purified from culture supernatants with a recovery yield of 50–70%. SDS-PAGE analysis (Fig. 1) showed that the purified proteins had an expected molecular weight around 50 kDa for rHPL, rHPLRP1, and rHPLRP2 and 10 kDa for rHCol. The molecular weight of rHPLRP2 was slightly higher than those of the other lipases due to the protein glycosylation [30]. The specific activity of the rHPL, rHPLRP2, and rHCol, measured on emulsified tributyrin, were 8000, 800, and 35 000 U/mg, respectively. As expected, no significant lipolytic activity was detected for rHPLRP1. Our previous works revealed that rHPLRP2 was sensitive to a proteolytic cleavage of the thr248-asp249 peptide bond in the lid domain generating two fragments (27 and

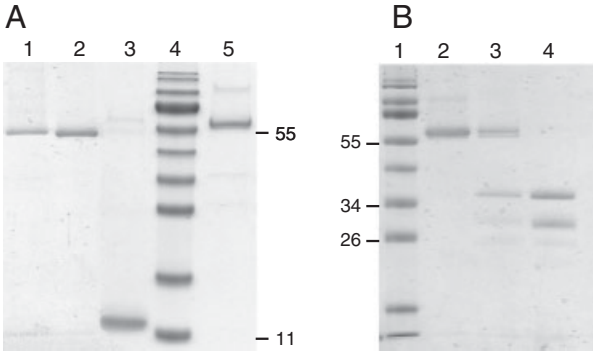

Figure 1. SDS-PAGE analysis of purified PL, colipase, and PLRP proteins. Electrophoresis was performed using 12% PAGE. Gel A: Lane 1: rHPLRP1, Lane 2: rHPL, Lane 3: colipase, Lane 4: molecular mass markers (170, 130, 95, 72, 55, 43, 34, 26, 17, and 11 kDa), Lane 5: NP-rHPLRP2. Gel B: Lane 1: molecular mass markers (170, 130, 95, 72, 55, 43, 34, 26, 17, and 11 kDa), Lane 2: NP-rHPLRP2, Lane 3: rHPLRP2 partially proteolyzed form, Lane 4: rHPLRP2 totally proteolyzed form.

22 kDa) connected by disulfide bridge as shown in Fig. 1B [30]. Since the influence of this proteolytic cleavage on the rHPLRP2 kinetic pattern is different depending on the substrate [34], its non-proteolyzed (NP-rHPLRP2) and proteolyzed (P-rHPLRP2) forms were used to test its activity on milk fat.

The physico-chemical parameters for each kind of milk are presented in Table 1. Homogenization resulted in the most profound changes in the physical structure of milk. It reduced fat droplet size and altered interface composition but the fat content was maintained [27, 28]. As expected, the particle size parameters D(4,3) and D(3,2) were 13- and 20-fold smaller, respectively, for homogenized fat globules than for native fat globules. Thus, the specific surface area of fat globules was 20-fold larger for HM than for NM. Both kinds of milk were used to test the effect of fat droplet organizations that were fundamentally different on the activity of human pancreatic lipases.

3.2 PL activity on milk fat globules

A first set of experiments was performed to determine the required lipolysis partners for rHPL activity on NM and HM fat globules.

A similar amount of milk fat (about 160 mg) under either native or homogenized globule structure was hydrolyzed by rHPL with or without bile salts and/or rHCol. The pattern of lipids, extracted from different samples collected after 1 h of hydrolysis (checked by titrimetry), was analyzed by TLC and compared with the pattern of lipids of non-hydrolyzed milks (Fig. 2). No free FA was visualized before hydrolysis for milk (lanes 1 and 6) in agreement with what is described for fresh milk [18]. Increasing amounts of free FA and 1,2-diacylglycerols were mainly observed in the presence of both bile salts and colipase (lanes 5 and 9). When NaTDC was omitted, in presence of colipase, a weak activity was observed only on the NM (lanes 3 and 7). These results are in agreement to the hydrolysis kinetics measured by titrimetry. The modification of the monoacylglycerol/PhL spot, observed in absence of hydrolysis is an artifact due to the presence of NaTDC. The assay conditions for the milk fat hydrolysis by rHPL were defined from these experiments: 10 M excess of rHCol and 6 mM NaTDC.

Table 1. Characteristics of the different milks

Milk	Fat content (g/kg ± 0.5)	Specific surface area (m^2/g)	D(4,3) (μm)	D(3,2) (μm)
NM 1	44	1.87	4.16	3.48
HM 1	43.5	41.7	0.29	0.16
NM 2	47.5	1.84	4.25	3.54
HM 2	47	38.10	0.32	0.17
NM 3	41.5	1.83	4.25	3.57
HM 3	41.5	37.5	0.33	0.17

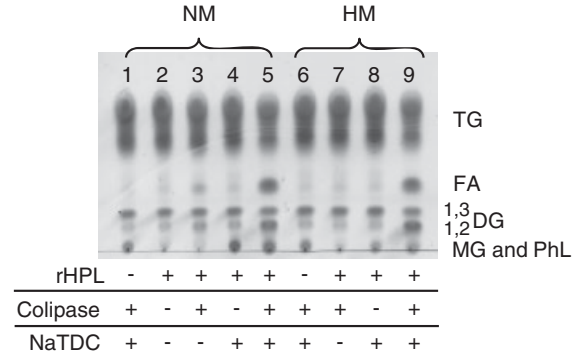

Figure 2. Effect of lipolysis partners on milk lipids hydrolysis by PL. About 4 mL of NM or HM was used. The pH was controlled and maintained constant titrimetrically for 1 h as described in Section 2. After lipids extraction, samples were analyzed on TLC. DG: diacylglycerols, MG: monoacylglycerols. Lane 1 and 6: non-hydrolyzed milk. Lane 2: without NaTDC and colipase. Lane 3 and 7: without NaTDC with colipase. Lane 4 and 8: with NaTDC without colipase. Lane 5 and 9: with NaTDC and with colipase.

Hydrolysis kinetics were performed with various concentration of milk fat using different volumes of milk (Fig. 3). The hydrolysis kinetics of rHPL on NM fat globules are presented in Fig. 3A. We observed neither rHPL activity on skimmed milk nor endogenous lipolytic activity on NM. As previously described [23], a lag phase of about 250 s was observed on NM hydrolysis by rHPL. After this delay, the rate of hydrolysis increased rapidly and became constant. The length of this lag phase was reproducible for the same NM but varied between NMs.

The activity of rHPL on NM fat globules, determined after the lag time, was reported as a function of the fat concentration in mg/mL of assay mixture (Fig. 3B).

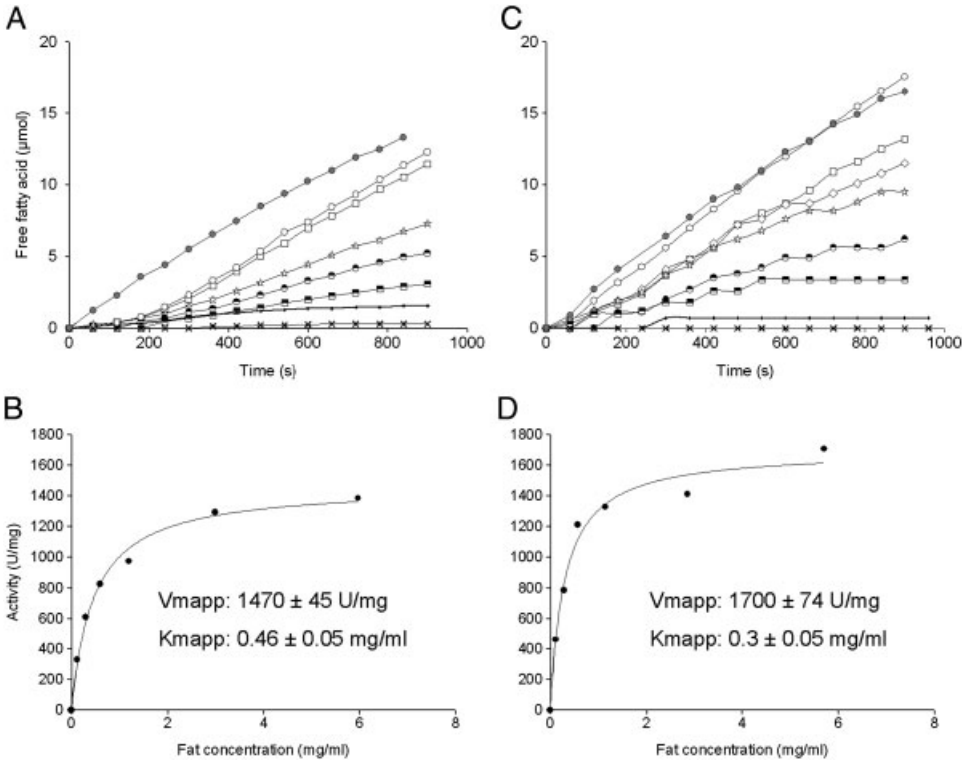

Figure 3. PL activity on milk fat globules. Kinetics were performed on NM (A and B) or HM (C and D). (A and C) Kinetics of PL activity for different milk fat concentrations (open symbols). Kinetics performed in the presence of exogenous FA: oleic acid 2 mM final (gray symbols). (B and D) rHPL activity in U/mg (micromoles of FA released/min/mg of enzyme) on milk reported in function of fat concentration. Enzyme concentration: rHPL: 1×10^{-9} M, rHCol: 1×10^{-8} M. —○— 5.83 mg/ml, —□— 2.92 mg/ml, —◇— 1.17 mg/ml, —☆— 0.58 mg/ml, —●— 0.29 mg/ml, —■— 0.12 mg/ml, —•— Skimmed milk, —×— 5.83 mg/ml without enzyme.

Assuming that the enzyme had a Michaelien behavior, the apparent kinetic parameters were determined: Vmapp = 1470 ± 45 U/mg; Kmapp = 0.46 ± 0.05 mg/mL.

As previously reported, the addition of free FA resulted in a complete disappearance of the lag phase (Fig. 3A). The addition of exogenous free FA did not seem to affect the apparent kinetic parameter of rHPL on NM (data not shown).

The same experiments were performed using HM fat globules as substrate (Figs. 3C and D). No lag phase was observed (Fig. 3C). The apparent kinetic parameters of rHPL were measured on HM fat globules: Vmapp = 1700 ± 74 U/mg; Kmapp = 0.3 ± 0.05 mg/mL (Fig. 3D). A prior addition of exogenous free FA seemed to have no effect on rHPL activity on HM fat globules (Fig. 3C). The released FA content determined in samples removed during the kinetic were similar to titrimetry measurement. In conclusion, the rHPL behavior on bovine NM is similar to that of native PL on human NM as previously reported [24, 25]. Furthermore, if we consider that the ratio Vmapp/Kmapp could characterize the apparent catalytic efficiency of rHPL, we conclude that the rHPL catalytic efficiency was almost two-fold higher on HM than on NM fat globules (5667 *versus* 3196 U × mL/mg × mg).

3.3 Kinetic characterization of PLRPs on milk fat globules

The aim of this set of experiments was to compare the catalytic behavior of PLRP1 and PLRP2 (proteolyzed and non-proteolyzed forms) on NM and HM fat globules with that of classical PL. First experiments performed in the same conditions than for rHPL (similar enzyme concentration, *i.e.* 10^{-9} M, in the presence of bile salts and colipase) did not show any release of free FA whatever the structural organization of milk fat. Thus, in the following experiments, the enzyme concentration used was 30-fold higher (3×10^{-8} M). Concerning rHPLRP1, no activity could be detected on NM or HM fat globules.

Figure 4 presents the hydrolysis kinetics of milk fat globules by rHPLRP2. In the absence of free FA, no difference was observed between P-rHPLRP2 and NP-rHPLRP2. The rHPLRP2 activity on maximum concentration of NM fat globules was very low (1.36 U/mg), even for a time period up to 1 h (Fig. 4A and Table 2). Because of the low activity of rHPLRP2, the kinetic parameters were not determined.

The rHPLRP2 activity was about 10- to 15-fold higher on HM than on NM fat (Fig. 4B and Table 2). The rHPLRP2 activity (in U/mg) on HM fat globules was reported as a function of the milk fat concentration (in mg/mL) (Fig. 4C). The apparent kinetic parameters Vmapp and Kmapp could not be determined. Only an apparent catalytic efficiency (ratio Vmapp/Kmapp corresponding to the slope of the linear part of the curve) could be estimated to about 4.5 U × mL/mg × mg. Thus, on HM fat globules, the apparent catalytic efficiency of rHPLRP2 was about 1000-fold lower compared with rHPL.

The effect of exogenous FA and colipase on rHPLRP2 activity on milk fat was also studied (Table 2). The addition

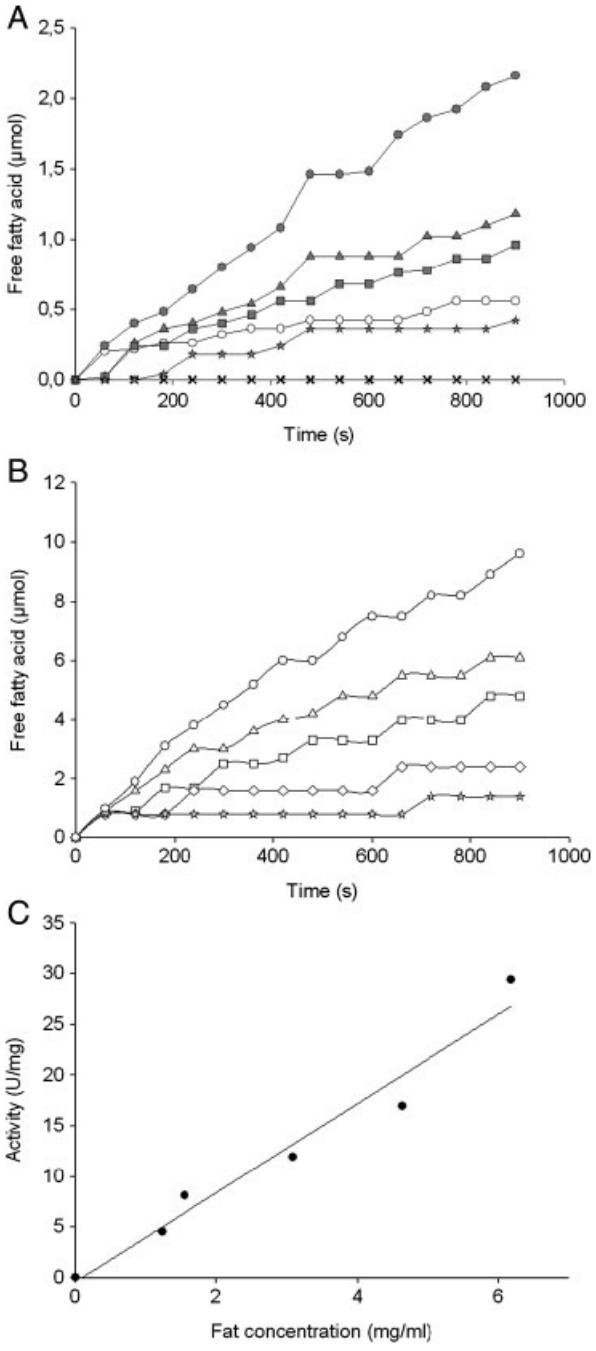

Figure 4. PLRP2 activity on milk fat globules. (A) Kinetics activity on NM with and without exogenous FA. (B) Kinetics activity on HM for different fat volume. (C) rHPLRP2 activity in U/mg on HM reported in function of fat concentration. Symbols of white color correspond to the experiment without FA and symbols of gray color correspond to the kinetics with exogenous addition of free FA. Enzyme concentration: rHPLRP2: 3×10^{-8} M, rHCol: 1×10^{-8} M. —○—6.3 mg/ml, —△— 4.73 mg/ml, —□— 3.2 mg/ml, —◇—1.26 mg/ml, —☆— 0.63 mg/ml, —×— 6.3 mg/ml without enzyme.

of free FA enhanced the rHPLRP2 activity on milk fat but this effect depends on the proteolytic state of the enzyme, the type of the milk, and the presence of colipase (Fig. 4 and Table 2). The activity of P-rHPLRP2 on NM was enhanced about four-fold in the presence of exogenous free FA but was not significantly modified on HM fat. Colipase has no significant effect.

The presence of exogenous FA, without colipase, increased the activity of NP-rHPLRP2 about three-fold on NM and only a slight effect was observed on HM. In the absence of FA, increasing concentration of colipase resulted in an enhancement of NP-rHPLRP2 activity on HM fat. In the presence of both colipase, and exogenous free FA, the activity of NP-rHPLRP2 was dramatically enhanced about 30-fold on NM and only about five-fold on HM. Finally, in presence of bile salts, colipase, and exogenous free FA, the activity of NP-rHPLRP2 was approximately the same on both milks.

In the absence of colipase, a high concentration of NaTDC was absolutely required for NP-rHPLRP2 and P-rHPLRP2 activity on native as well as homogenized globules (data not shown).

3.4 Combined actions of rHPL, rHPLRP1, and rHPLR2 on milk fat hydrolysis

In this set of experiments, we explored the effects of the combined action of both PLRPs and PL on the milk fat hydrolysis in the presence of colipase (ratio PL/colipase = 1/10) and bile salts (Fig. 5). In this experiment, only NP-rHPLRP2 was used. The lipolysis rate in micromoles of FA released/min/mL of assay mixture was measured on NM and HM. The results are expressed in percent; the amount of FA released by rHPL was arbitrary set to 100%. The activity of rHPLRP1 on both milks was null. The lipolysis rate of NP-rHPLRP2 represented 5.8 and 100% of hydrolysis rate of rHPL on NM and HM fat globules, respectively. In the absence of colipase, the hydrolysis rate of rHPLRP2 was 5.8 and 47.5% of hydrolysis rate of rHPL on NM and HM fat globules, respectively (data not shown). Interestingly, the lipolytic rate decreased when rHPLRP1 was added simultaneously to rHPL (about 48.9 and 81.9% of rHPL lipolysis rate on NM and HM fat, respectively). Another very original observation concerned the increase of the hydrolysis rate when rHPL and rHPLRP2 were simultaneously added (about 310.7 and 265.5% of rHPL lipolytic rate on NM and HM fat, respectively). When rHPL and rHPLRP2 were incubated together, the hydrolysis rate for both milks was higher than the sum of the hydrolysis rates measured for each enzyme individually. The synergic effect is particularly important on the NM. The inhibitory effect of the rHPLRP1 addition was also observed on the hydrolysis rate obtained with the concomitant presence of both rHPL and rHPLRP2, on NM and HM fat globules. It is noteworthy that the rHPLRP1 inhibitory effect was circumvented by the addition

Table 2. Influence of colipase and exogenous free FA on PLRP2 activity on milk fat

		NM		HM	
		−FA	+FA	−FA	+FA
NP-rHPLRP2	−rHCol	1.9±0.3	5.8	22±7.3	29±7
NP-rHPLRP2	+rHCol 1×10^{-8} M	2.5±0.8	59.1±8.4	55±7.4	96±19.6
	+rHCol 3×10^{-8} M			110	
	+rHCol 6×10^{-8} M	1.98±0.15		136±16	
P-rHPLRP2	−rHCol	1.34±0.03	5.8	21±7.5	27±5.3
P-rHPLRP2	+rHCol 1×10^{-8} M	1.38±0.02	7.6±2.5	24±7	32±5.8
	+rHCol 3×10^{-8} M			27±1.3	
	+rHCol 6×10^{-8} M			37	

Lipase activities in U/mg were determined for a milk fat concentration of ~5 mg/mL. When indicated, each value is the average±SD of three to six determinations.

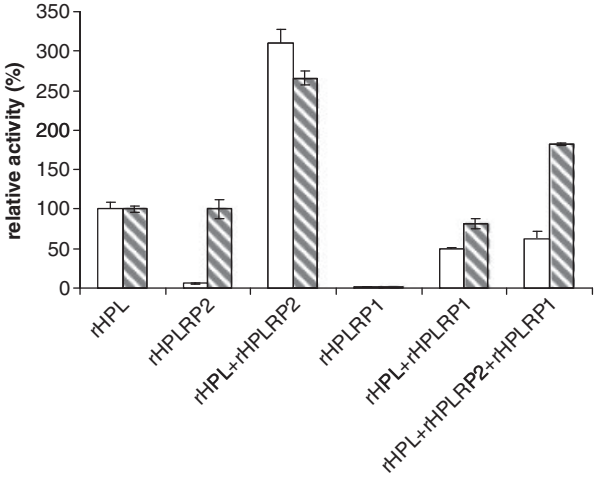

Figure 5. Combined action of PLRP1 and PLRP2 with PL. The activities of rHPL, rHPLRP2, rHPLRP1 alone or together on NM (white bar □), and HM (striped bar ▨) (4 mL) were compared. Enzyme concentration: rHPL: 1×10^{-9} M, rHPLRP1: 3×10^{-8} M, NP-rHPLRP2: 3×10^{-8} M, and rHCol: 1×10^{-8} M. The activities were determined in kinetics experiment as described in Section 2 in the presence of NaTDC and colipase and expressed as a percentage of PL activity. These experiments were carried out in triplicate.

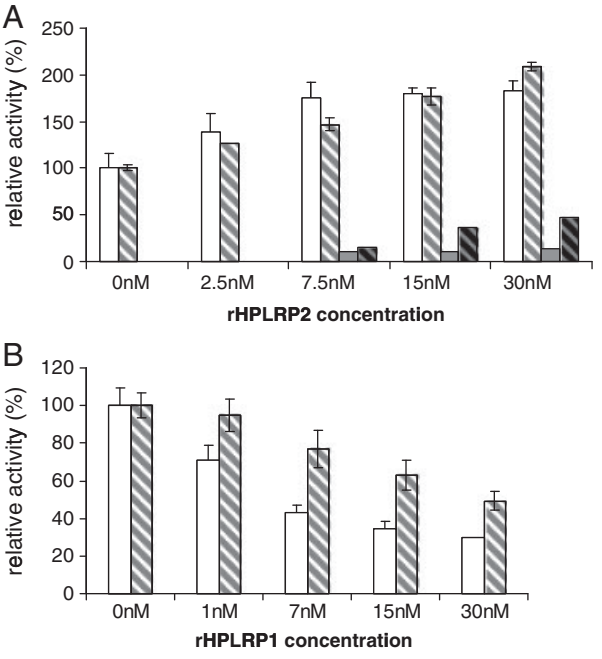

Figure 6. Dose–response relationship between the PLRPs and PL. The activity of rHPL in the presence of different concentrations of rHPLRP2 (A) or rHPLRP1 (B) on NM (white bar □) and HM (striped bar ▨) (2 mL) were measured. The activities were determined in kinetics experiments as described in Section 2 in the presence of NaTDC and colipase and expressed as a percentage of PL lipolysis rate. Enzyme concentration: rHPL: 1×10^{-9} M, rHCol: 1×10^{-9} M. The rHPLRP2 activity on NM (gray bar ■), and HM (stripped gray bar ▨) was also reported (A) and expressed as a percentage of PL lipolysis rate. These experiments were carried out in triplicate.

of rHCol (data not shown). We observed that the addition of rHPLRP1 had no effect on rHPLRP2 activity on both milks in the absence of colipase. On HM, in the presence of colipase, we observed an inhibition of rHPLRP2 activity after addition of rHPLRP1 (data not shown). All the effects observed were independent of the addition sequence of enzymes. Indeed, the same final activity is obtained when the two enzymes are added together or when an incubation period is performed with one lipase before adding the other.

To understand the effects of the combined action of pancreatic lipases on the milk fat hydrolysis, dose–response relationship studies were performed, using a lipase/colipase ratio of 1/1 and with increasing concentrations of rHPLRP1 or rHPLRP2 (non-proteolyzed form) (Fig. 6). In these conditions, colipase (10 nM) has no significant effect on the NP-rHPLRP2 activity. As shown in Fig. 6A, the hydrolysis rate by rHPLRP2 on both milks was not detectable with concentrations under 7.5 nM. When rHPL and rHPLRP2 were simultaneously added, the hydrolysis rate increased with increasing concentrations of rHPLRP2. A plateau value corresponding to 180–200% was obtained with rHPLRP2 plus rHPL when rHPLRP2 concentration

was above 7.5 nM up to 30 nM. With the rHPL/rHPLRP2 ratio of 1/30, the synergic effect was higher with an rHPL/colipase ratio of 1/10 since the hydrolysis rate was 310.7 and 265.5% on NM and HM fat, respectively (Fig. 5). The lipolytic activity observed when rHPL and rHPLRP1 were simultaneously added decreased with increasing concentration of rHPLRP1 on the two milks (Fig. 6B). With an rHPLRP1 concentration of 30 nM, the lipolysis rate was 30 and 50% of lipolysis rate with rHPL on NM and HM fat, respectively. With the rHPL/rHPLR1 ratio of 1/30, the inhibitory effect is less important with an rHPL/colipase ratio of 1/10, since the lipolysis rate was about 48.9 and 81.9% on NM and HM fat, respectively.

4 Discussion

Up to now, the physiological role of the PLRPs is not well established. Two main hypotheses have been proposed concerning the role of PLRP2. First, PLRP2 may be involved in the digestion of plant lipids in some adult species [9, 35]. Second, PLRP2 may be one of the predominant enzymes involved in duodenal lipids digestion in suckling newborns [11, 13, 14, 36]. The PLRP1 has no known substrates [7]. The present study concerns the action of PLRPs on a physiological complex substrate, the milk fat, by comparison with PL, which was extensively studied on milk fat [7, 22–25]. Furthermore, to investigate the influence of milk fat organization on its digestion, NM and HM fat globules were used as substrates.

First, we investigated the individual action of pancreatic lipases on both milk fat globule structures. Our study not only confirms that classical PL is able to hydrolyze milk fat but also shows, for the first time, that PLRP2 is able to hydrolyze milk fat. The lack of lipolytic activity of PLRP1 again raises the question whether this protein is really involved in lipids digestion.

As previously described, the lag phase observed for the hydrolysis of native fat globules by PL is due to the presence of PhL that cover the triglycerides core. It was extensively demonstrated, with artificial emulsions or human NM globules, that PhL prevent the PL access to the interface [22, 24, 25, 37]. The length of this lag phase is dependent on factors such as lipase, colipase, calcium and bile salts concentration, or pH [37]. During this lag phase, some substrate hydrolysis occurs [3]. Thus, the end of the lag phase is correlated with the release by PL of a small amount of lipolysis products (FA and diacylglycerols) that modified the interface composition. As previously reported, we observed that the addition of free FA suppressed the lag phase of native fat globules hydrolysis by PL [22, 24, 25]. It is proposed that in PhL-rich interfaces, colipase laterally concentrates substrates and lipolysis products in its vicinity creating lipid-colipase nanodomains, which would favor the binding of lipase [37, 38].

Homogenization of milk causes a reduction of fat globules size and an increase in the fat surface area. It alters the native MFGM and modifies the interface composition. We observe a hydrolysis of HM by PL without lag time indicating that the interface is directly accessible. If we compare the hydrolysis of NM and HM, we can conclude that HM is more rapidly hydrolyzed by PL than the NM (taking into account the lag phase and the catalytic efficiency). Nevertheless, in the presence of FA, mimicking the gastric phase, the lag is suppressed and the difference between the two milks is reduced. This raises the following question: is it possible that in the physiological context, both milk fat globules were approximately hydrolyzed with the same efficiency? This result is surprising taking into account the surface area that is larger for HM than for NM fat globules at the same fat concentration. From these results, two hypotheses could be drawn. The positive effect of the available surface area of the homogenized fat globules is compensated by a negative effect of the interface composition. The second hypothesis is that, during the lag phase, structural modifications of the native globules would occur allowing a better accessibility by PL.

For PLRP2, the weak constant lipolytic activity observed on NM could be due to a low efficiency of the enzyme or to a very long lag phase. In absence of colipase and FA, the PLRP2 activity is about 10- to 20-fold higher on HM than on NM for a same fat concentration. Even if the kinetics parameters have not been measured on NM, we can suppose that the catalytic efficiency (expressed as a function of milk fat concentration) of PLRP2 is clearly higher on HM than on NM fat globules.

Our experiment reveals several interesting observations about the effects of colipase and FA on the PLRP2 activity. A colipase positive effect was observed, in the absence of FA, only on the non-proteolyzed form of PLRP2 and this effect was different depending on milks. These results confirm that the lipids structural organization is a key factor for PLRP2 activity as for PL. Indeed, when using artificial substrate, the sensitivity of NP-rHPLRP2 and P-rHPLRP2 for colipase is less straight [30, 34]. In the presence of FA and colipase, the PLRP2 activity was enhanced and the difference between the hydrolysis of both milks was reduced. This result emphasizes the possible impact of the gastric phase on the digestion of milk fat by PLRP2. Recent works conclude that PLRP2 and colipase do not interact [39]. In the context of milk fat hydrolysis, whether the effects of colipase/FA involve PLRP2/colipase interactions or/and a redistribution of lipids at the interface is still questionable. In conclusion, as previously suggested [11, 14, 36, 40], PLRP2 may be the major colipase-dependent lipase dominating with bile salt-stimulated lipase (BSSL) in milk fat digestion in newborns, a physiological context where the expression of PL is low. Nevertheless, because lipase proteolysis will occur in the duodenum, it is difficult to arrive at a conclusion about the exact contribution of PLRP2.

Furthermore, our study reveals that the activity of PLRP2 on milk fat is completely dependent on the presence of bile salts in absence of colipase. Although it has been shown that

bile salt micelles play a role in the opening of the flap (activation of the enzyme) [41], it is the first time that a positive effect on PLRP2 activity is reported. Nevertheless, it is clear that the nature of biliary salts is an important factor that influences the PL activity. Therefore, it will be interesting to check the PLRP2 activity in presence of different concentrations of various bile salts as regard to the hypothetical role of this protein during the neonatal period. Indeed in human newborns, the composition and the concentration of bile is different as compared with adults.

Previous works report the concomitant presence of at least two of the three pancreatic lipases – PL, PLRP1, and PLRP2 – in the pancreas of various species or in a particular physiological context as, for example, the period around the birth in human [13]. So, the combined action of PL, PLRP1, and PLRP2 was investigated on the digestion of NM and HM fat globules. A decrease of the lipolysis rate of both milk fat globules by PL is observed when PLRP1 is present. The inhibitory effect was also observed on hydrolysis by PLRP2, only when colipase-sensitive NP-PLRP2 form was used. PLRP1 was reported to bind colipase *in vitro* [42, 43]. We conclude that the effect of PLRP1 on milk fat globule hydrolysis by PL or NP-PLRP2 is due to a competition for colipase binding. Indeed, this inhibitory effect is suppressed by addition of colipase, and is dependent on the relative concentration of the three proteins PL, PLRP1, and colipase. The hydrolysis rate on both NM and HM fat globules obtained in the simultaneous presence of PL and PLRP2 reveals a synergic effect. Such synergic phenomenon between PL and PLRP2, in the presence of colipase, was reported for the hydrolysis of retinyl palmitate [44]. In this case, it has been proposed that PL produces lipid structures that can be substrate for PLRP2 [44]. Other cooperative effects between different lipases and PL were reported for the hydrolysis of milk fat. These lipases release FA that promote PL activity on milk fat globules [22–26]. The synergic phenomenon observed between PLRP2 and PL raises the question of the mechanism involved. Does one of the two lipases release lipolysis products that promote the action of the other lipase? This hypothesis seems unlikely, since the synergic effect is independent of the order of addition of PL and PLRP2. In this synergic effect, the molecular mechanisms could involve the formation of specific complex proteins–lipids.

Lowe and co-workers suggest that BSSL and PLRP2 are the dominating lipases in neonatal fat digestion in mice, rats, and probably in human, when the milk is the unique source of lipids and the expression of PL is low [11, 13, 14, 40]. During this period, a high expression of PLRP1 is also reported. We showed that PLRP1 exhibit an inhibitory effect on PL and PLRP2 activity. The role of PLRP1, at the suckling period, is unknown when PL levels are low. Is it possible to have combined effects between other enzymes like PLRPs/BSSL? Further studies on the combined action of PLRPs, BSSL, and PL on the digestion of milk fat globules in neonatal condition are necessary to understand the role of the PLRPs at this period of life. The observation of different effects of PLRP1 and PLRP2 on PL activity is very interesting from a biochemical and mechanistic point of view and raises the question of the physiological relevance of these combined effects. Furthermore, not enough information is available on the expression level of these proteins in human, particularly during the postprandial period. Several studies show a discoordinate regulation of the PLRPs and PL by dietary fat [45] and a high level of expression of PLRPs in several adult species has been reported [10]. The action of PLRPs as modulator of the PL activity could exist in some different physiological contexts other than neonatal period.

In conclusion, our study gets new insights concerning the digestion of milk fat by pancreatic lipases and consequently about the physiological role of PLRPs. In particular, we demonstrate that catalytic behavior and efficiency of pancreatic lipases were different when milk fat is organized as NM or HM fat globules. In a physiological context, the conclusions concerning the contribution of pancreatic lipases in milk fat globules hydrolysis under different structural organization have to be modulated. Actually, the impact of gastric phase, generating FA, and other parameters as acid pH, peristaltic movement, and proteases activities may affect the pancreatic lipases efficiency. Furthermore, the study of the combined action of pancreatic lipases on the digestion of milk fat reveals an inhibitory effect of PLRP1 and a synergic effect between PL and PLRP2. These new observations raise the question whether PLRPs have to be considered as modulators of the PL activity rather than lipases *per se*.

This research was carried out with the financial support of the "Agence Nationale de la Recherche" under the "Programme National de Recherche en Alimentation et nutrition humaine", project "ANR-06-PNRA-012, AGILAIT" and the financial support of ARILAIT Recherches Industry, France. The Ph.D work of Miss Amélie Berton was supported by a grant from the "Institut National de la Recherche Agronomique" and ARILAIT Recherches Industry, France. The authors thank B. Robert, F. Rousseau, E. Beaucher, and V. Briard-Bion for milk sample preparation and physico-chemical characterization. They also thank P. Deprez-Beauclair and C. Serveau for helpful experimental assistance, Dr. D. Lairon for helpful advices and Dr. B. Kerfelec for fruitful discussions. The authors are very grateful to Dr. M. Armand for critical reading of the manuscript.

The authors have declared no conflict of interest.

5 References

[1] Armand, M., Lipases and lipolysis in the human digestive tract: where do we stand? *Curr. Opin. Clin. Nutr. Metab. Care.* 2007, *10*, 156–164.

[2] Borgstrom, B., On the interactions between pancreatic lipase and colipase and the substrate, and the importance of bile salts. *J. Lipid Res.* 1975, *16*, 411–417.

[3] Brockman, H. L., Kinetic behavior of the pancreatic lipase-colipase-lipid system. *Biochimie* 2000, *82*, 987–995.

[4] van Tilbeurgh, H., Egloff, M. P., Martinez, C., Rugani, N. *et al.*, Interfacial activation of the lipase-procolipase complex by mixed micelles revealed by X-ray crystallography. *Nature* 1993, *362*, 814–820.

[5] Winkler, F. K., D'Arcy, A., Hunziker, W., Structure of human pancreatic lipase. *Nature* 1990, *343*, 771–774.

[6] Giller, T., Buchwald, P., Blum-Kaelin, D., Hunziker, W., Two novel human pancreatic lipase related proteins, hPLRP1 and hPLRP2. Differences in colipase dependence and in lipase activity. *J. Biol. Chem.* 1992, *267*, 16509–16516.

[7] Lowe, M. E., The triglyceride lipases of the pancreas. *J. Lipid Res.* 2002, *43*, 2007–2016.

[8] Crenon, I., Jayne, S., Kerfelec, B., Hermoso, J. *et al.*, Pancreatic lipase-related protein type 1: a double mutation restores a significant lipase activity. *Biochem. Biophys. Res. Commun.* 1998, *246*, 513–517.

[9] Sias, B., Ferrato, F., Grandval, P., Lafont, D. *et al.*, Human pancreatic lipase-related protein 2 is a galactolipase. *Biochemistry* 2004, *43*, 10138–10148.

[10] Jayne, S., Kerfelec, B., Foglizzo, E., Chapus, C., Crenon, I., High expression in adult horse of PLRP2 displaying a low phospholipase activity. *Biochim. Biophys. Acta* 2002, *1594*, 255–265.

[11] Li, X., Lindquist, S., Lowe, M., Noppa, L., Hernell, O., Bile salt-stimulated lipase and pancreatic lipase-related protein 2 are the dominating lipases in neonatal fat digestion in mice and rats. *Pediatr. Res.* 2007, *62*, 537–541.

[12] Payne, R. M., Sims, H. F., Jennens, M. L., Lowe, M. E., Rat pancreatic lipase and two related proteins: enzymatic properties and mRNA expression during development. *Am. J. Physiol.* 1994, *266*, G914–G921.

[13] Yang, Y., Sanchez, D., Figarella, C., Lowe, M. E., Discoordinate expression of pancreatic lipase and two related proteins in the human fetal pancreas. *Pediatr. Res.* 2000, *47*, 184–188.

[14] Lowe, M. E., Kaplan, M. H., Jackson-Grusby, L., D'Agostino, D., Grusby, M. J., Decreased neonatal dietary fat absorption and T cell cytotoxicity in pancreatic lipase-related protein 2-deficient mice. *J. Biol. Chem.* 1998, *273*, 31215–31221.

[15] Armand, M., Borel, P., Ythier, P., Dutot, G., Effects of droplet size, triacylglycerol composition, and calcium on the hydrolysis of complex emulsions by pancreatic lipase - an in vitro study. *J. Nutr. Biochem.* 1992, *3*, 333–341.

[16] Borel, P., Armand, M., Ythier, P., Dutot, G. *et al.*, Hydrolysis of emulsions with different triglycerides and droplet sizes by gastric lipase in-vitro – effect on pancreatic lipase activity. *J. Nutr. Biochem.* 1994, *5*, 124–133.

[17] Jensen, R. G., Ferris, A. M., Lammi-Keefe, C. J., Henderson, R. A., Lipids of bovine and human milks: a comparison. *J. Dairy Sci.* 1990, *73*, 223–240.

[18] Jensen, R. G., The composition of bovine milk lipids: January 1995 to December 2000. *J. Dairy Sci.* 2002, *85*, 295–350.

[19] Heid, H. W., Keenan, T. W., Intracellular origin and secretion of milk fat globules. *Eur. J. Cell Biol.* 2005, *84*, 245–258.

[20] Keenan, T. W., Patton, S., The Milk Lipid Globules Membrane, in: Jensen, R. G. (Ed.), *Handbook of Milk Composition*, Academic Press, San Diego 1995.

[21] Lopez, C., Briard-Bion, V., Menard, O., Rousseau, F. *et al.*, Phospholipid, sphingolipid, and fatty acid compositions of the milk fat globule membrane are modified by diet. *J. Agric. Food Chem.* 2008, *56*, 5226–5236.

[22] Bernback, S., Blackberg, L., Hernell, O., Fatty acids generated by gastric lipase promote human milk triacylglycerol digestion by pancreatic colipase-dependent lipase. *Biochim. Biophys. Acta* 1989, *1001*, 286–293.

[23] Bernback, S., Blackberg, L., Hernell, O., The complete digestion of human milk triacylglycerol in vitro requires gastric lipase, pancreatic colipase-dependent lipase, and bile salt-stimulated lipase. *J. Clin. Invest.* 1990, *85*, 1221–1226.

[24] Blackberg, L., Hernell, O., Olivecrona, T., Hydrolysis of human milk fat globules by pancreatic lipase: role of colipase, phospholipase A2, and bile salts. *J. Clin. Invest.* 1981, *67*, 1748–1752.

[25] Borgstrom, B., Erlanson-Albertsson, C., Hydrolysis of milk fat globules by pancreatic lipase. Role of colipase, phospholipase A2, and bile salts. *J. Clin. Invest.* 1982, *70*, 30–32.

[26] Iverson, S. J., Kirk, C. L., Hamosh, M., Newsome, J., Milk lipid digestion in the neonatal dog: the combined actions of gastric and bile salt stimulated lipases. *Biochim. Biophys. Acta* 1991, *1083*, 109–119.

[27] Lopez, C., Focus on the supramolecular structure of milk fat in dairy products. *Reprod. Nutr. Dev.* 2005, *45*, 497–511.

[28] Michalski, M. C., Januel, C., Does homogenization affect the human health properties of cow's milk? *Trends Food Sci. Technol.* 2006, *17*, 423–437.

[29] Colin, D. Y., Deprez-Beauclair, P., Allouche, M., Brasseur, R., Kerfelec, B., Exploring the active site cavity of human pancreatic lipase. *Biochem. Biophys. Res. Commun.* 2008, *370*, 394–398.

[30] Sebban-Kreuzer, C., Deprez-Beauclair, P., Berton, A., Crenon, I., High-level expression of nonglycosylated human pancreatic lipase-related protein 2 in Pichia pastoris. *Protein Expr. Purif.* 2006, *49*, 284–291.

[31] Laemmli, U. K., Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970, *227*, 680–685.

[32] Burnette, W. N., ''Western blotting'': electrophoretic transfer of proteins from sodium dodecyl sulfate--polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. *Anal. Biochem.* 1981, *112*, 195–203.

[33] Bligh, E. G., Dyer, W. J., A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* 1959, *37*, 911–917.

[34] Eydoux, C., De Caro, J., Ferrato, F., Boullanger, P. *et al.*, Further biochemical characterization of human pancreatic

lipase-related protein 2 expressed in yeast cells. *J. Lipid Res.* 2007, *48*, 1539–1549.

[35] De Caro, J., Eydoux, C., Cherif, S., Lebrun, R. *et al.*, Occurrence of pancreatic lipase-related protein-2 in various species and its relationship with herbivore diet. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* 2008, *150*, 1–9.

[36] d'Agostino, D., Lowe, M. E., Pancreatic lipase-related protein 2 is the major colipase-dependent pancreatic lipase in suckling mice. *J. Nutr.* 2004, *134*, 132–134.

[37] Borgstrom, B., Importance of phospholipids, pancreatic phospholipase A2, and fatty acid for the digestion of dietary fat: in vitro experiments with the porcine enzymes. *Gastroenterology* 1980, *78*, 954–962.

[38] Momsen, M. M., Dahim, M., Brockman, H. L., Lateral packing of the pancreatic lipase cofactor, colipase, with phosphatidylcholine and substrates. *Biochemistry* 1997, *36*, 10073–10081.

[39] Berton, A., Sebban-Kreuzer, C., Crenon, I., Role of the structural domains in the functional properties of pancreatic lipase-related protein 2. *FEBS J.* 2007, *274*, 6011–6023.

[40] Miller, R., Lowe, M. E., Carboxyl ester lipase from either mother's milk or the pancreas is required for efficient dietary triglyceride digestion in suckling mice. *J. Nutr.* 2008, *138*, 927–930.

[41] Jayne, S., Kerfelec, B., Foglizzo, E., Granon, S. *et al.*, Activation of horse PLRP2 by bile salts does not require colipase. *Biochemistry* 2002, *41*, 8422–8428.

[42] Miled, N., Berti-Dupuis, L., Riviere, M., Carriere, F., Verger, R., In vitro lipolysis by human pancreatic lipase is specifically abolished by its inactive forms. *Biochim. Biophys. Acta* 2003, *1645*, 241–246.

[43] Crenon, I., Foglizzo, E., Kerfelec, B., Verine, A. *et al.*, Pancreatic lipase-related protein type I: a specialized lipase or an inactive enzyme. *Protein Eng.* 1998, *11*, 135–142.

[44] Reboul, E., Berton, A., Moussa, M., Kreuzer, C. *et al.*, Pancreatic lipase and pancreatic lipase-related protein 2, but not pancreatic lipase-related protein 1, hydrolyze retinyl palmitate in physiological conditions. *Biochim. Biophys. Acta* 2006, *1761*, 4–10.

[45] Birk, R. Z., Regan, K. S., Boyle-Roden, E., Brannon, P. M., Pancreatic lipase and its related protein 2 are regulated by dietary polyunsaturated fat during the postnatal development of rats. *Pediatr. Res.* 2004, *56*, 256–262.

