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**Characterization of all the lipolytic activities in pancreatin
and comparison with porcine and human pancreatic juices**

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Abstract

Porcine pancreatic extracts (PPE), also named pancreatin, are commonly used as a global source of pancreatic enzymes for enzyme replacement therapy in patients with exocrine pancreatic insufficiency. They are considered as a good substitute of human pancreatic enzymes and they have become a material of choice for *in vitro* models of digestion. Nevertheless, while the global PPE contents in lipase, protease and amylase activities are well characterized, little is known about individual enzymes. Here we characterized the lipase, phospholipase, cholesterol esterase and galactolipase activities of PPE and compared them with those of porcine (PPJ) and human (HPJ) pancreatic juices. The phospholipase to lipase activity ratio was similar in PPJ and HPJ, but was 4-fold lower in PPE. The galactolipase and cholesterol esterase activities were found at lower levels in PPJ compared to HPJ, and they were further reduced in PPE. The enzymes known to display these activities in HPJ, pancreatic lipase-related protein 2 (PLRP2) and carboxylester hydrolase/bile salt-stimulated lipase (CEH/BSSL), were identified in PPJ using gel filtration experiments, SDS-PAGE and LC-MS/MS analysis. The galactolipase and cholesterol esterase activities of PPE indicated that PLRP2 and CEH/BSSL are still present at low levels in this enzyme preparation, but they were not detected by mass spectrometry. Besides differences between porcine and human enzymes, the lower levels of phospholipase, galactolipase and cholesterol esterase activities in PPE are probably due to some proteolysis occurring during the production process. In conclusion, PPE do not provide a full substitution of the lipolytic enzymes present in HPJ.

Keywords: galactolipase; lipase; lipid digestion; pancreas; phospholipase;

1. Introduction

Pancreatin is an enzyme preparation containing amylase, protease and lipase activities isolated from the pancreas. It is the active principle ingredient (API) of various drug products for pancreatic enzyme replacement therapy (PERT) in patients with exocrine pancreatic insufficiency (EPI) caused by cystic fibrosis, chronic pancreatitis and other pathological conditions characterized by a deficiency in the secretion of pancreatic enzymes [1-3]. It allows improving food digestion and absorption in these patients [4]. Besides this medical application, pancreatin has become a convenient source of pancreatic enzymes for *in vitro* digestion studies [5, 6]. Indeed, it provides a global source of the most relevant pancreatic enzyme activities and, as such, it is considered as a good substitute of human pancreatic enzymes.

The first observations and experimental demonstrations that pancreatin could be used to improve protein and fat digestion in animals and human patients are from the 19th century, soon after Claude Bernard had revealed the essential role of pancreatic secretion in the digestion of foods [7]. In 1859, the dutch physician Joseph Alexander Fles showed that the daily intake of calf pancreas extract could restore the digestion of fat in a diabetic patient who suffered from diarrhea and had large amounts of fat in the stools [8]. In 1890, Abelman showed that the consumption of fresh pig pancreas could improve protein and fat digestion in dogs after surgical removal of the pancreas [9]. This is considered as the first experimental demonstration of enzyme replacement [10, 11].

Today, porcine pancreatic extracts (PPE) are the main source of pancreatin. They have been manufactured and used in PERT for more than one century [10], with various improvements in their formulation such as enteric-coated granules that prevent their release and degradation in the gastric environment [1]. They are produced from porcine pancreas

glands collected in slaughterhouses as a by-product of meat production. Details on the manufacturing, safety and sourcing of pancreatin raw materials can be found in the Pancreatin chapter <1025> of the United States Pharmacopeia (USP) [12]. An important step in the manufacturing process is the conversion of the inactive pancreatic zymogens into active enzymes, which is ensured by addition of proteases as activators. This critical step of limited proteolysis is performed under controlled conditions and the zymogen activation is stopped by the addition of solvents, such as acetone, isopropanol or diethyl ether [12]. Conditions have been established to preserve the main enzyme activities and the global contents in lipase, protease and amylase activities of PPE are usually well characterized. Nevertheless, little is known or reported about individual enzymes present in PPE [13-15] and some of them, as well as non-enzymatic proteins from the pancreas, might be partly degraded during the production process. In 2006, the US Food and Drug Administration, Center for Drug Evaluation and Research, issued a guidance requiring that PPE as a drug substance should be adequately characterized using appropriate chemical, physical, and biological testing [16]. This includes chemical identity and biological activity of different classes of enzymes. While the main PPE manufacturers have performed these characterizations, scientific publications reporting these data are still missing. A patent from Abbot Laboratories reported however an identification method based on 2D gel electrophoresis coupled to MALDI-TOF mass spectrometry and reverse phase HPLC, and provided a list of the various enzymes identified in PPE [15]. Whereas most pancreatic enzymes were well identified, including pancreatic lipase, colipase and phospholipase A2, carboxyl ester hydrolase/bile salt stimulated lipase (CEH/BSSL) and pancreatic lipase-related protein 2 (PLRP2) were not indicated [15]. These lipolytic enzymes are however present in the pancreatic secretion of several species including humans [17, 18]. PLRP2 has not been found so far in porcine pancreas pancreatic juice (PPJ) [17, 18] although the PLRP2 gene is well present in the pig genome and is expressed in the

pancreas [19]. A better characterization of individual enzymes and enzyme activity levels present in PPE is therefore required for a better comparison with pancreatic juices and evaluation of human digestive enzyme replacement by this preparation from porcine origin.

Here we chose pancreatin from Sigma-Aldrich (P7545; 8×USP) as reference material for PPE because it is widely used for *in vitro* digestion studies and described in highly cited and consensual protocols [5, 6]. As a reference for porcine and human pancreatic enzymes, respectively, we analyzed PPJ collected in pigs with pancreatic fistula [20] and human pancreatic juice (HPJ) collected by endoscopic retrograde catheterization on the main pancreatic duct [21]. We focused on the lipolytic enzymes present in PPE, PPJ and HPJ and measured lipase, phospholipase, cholesterol esterase and galactolipase activities using specific assay conditions and substrates. These activities were further associated with the pancreatic proteins separated by gel filtration chromatography and SDS-PAGE, and identified by N-terminal sequencing and mass spectrometry analysis.

We used a single technique (titration of free fatty acid released upon lipolysis) and experimental device (pHstat) for all lipolytic enzyme assays. Besides existing assay conditions and substrates for measuring lipase, phospholipase and galactolipases activities, we developed a new and simple assay for measuring cholesterol esterase activity using a short chain cholesterol ester mixed with bile salts. Prior to the comparison of lipolytic enzyme activities in PPE, PPJ and HPJ, we checked the suitability of assay conditions for both human and porcine enzymes, the levels of colipase and the complete activation of phospholipase A2 zymogen.

2. Materials and methods

2.1 Reagents

Cholesteryl acetate (CholA; Ref. 151114), tributyrin (TC4; T8626), gum arabic (GA; G9752), sodium taurodeoxycholate (NaTDC; T0557), sodium taurocholate (NaTC; T4009) and sodium deoxycholate (NaDC; D6750) were obtained from Sigma-Aldrich. USP Bile Salts RS was purchased from USP (Rockville, MD, USA). Crude olive oil was purchased from Lesieur (Aix-en-Provence, France). Egg yolk phosphatidylcholine (PC; Lipoid E PC S, ~99.2 % pure) was obtained from Lipoid GmbH (Ludwigshafen, Germany). The monogalactosyl diglyceride substrate (3-*O*- β -D-Galactopyranosyl-1,2-di-*O*-octanoyl-*sn*-glycerol; C8-MGDG) was synthesized as previously described [22].

2.2. Enzyme sources

Porcine pancreatic extracts (PPE) or pancreatin [P7545; 8 \times USP specifications activity] and trypsin (T4799) were purchased from Sigma-Aldrich. Human pancreatic juice (HPJ) was provided by Prof. R. Laugier, MD (La Timone University Hospital, Marseille, France) and was obtained in 1987 from a patient (36-years old woman) by performing endoscopic retrograde catheterization on the main pancreatic duct. HPJ contained 4 mg/mL proteins and a lipase activity of 4000 U/mL (tributyrin as substrate), which corresponds to a pancreatic lipase concentration of 500 μ g per mL of HPJ and 125 μ g lipase per mg of proteins. This concentration is in the upper range established for healthy subjects using an ELISA test for classical pancreatic lipase [21]. Porcine pancreatic juice (PPJ) was a generous gift of Prof. T. Corring (INRA, Jouy-en-Josas, France). It was collected and pooled from growing castrated male Large White pigs (mean weight of 45 kg), under stimulation by secretin (36 pmol/kg/h). All pancreatic juices were collected on ice and immediately mixed

with a solution of protease inhibitors (phenylmethylsulfonyl fluoride (PMSF) and benzamidine), each at a final concentration of 2 mM. Samples were then lyophilized and stored at -20°C before use. In the case of HPJ, 17.4 mg of powder per mL of juice were obtained.

Recombinant human pancreatic lipase-related protein 2 (rHPLRP2), guinea pig pancreatic lipase-related protein 2 (rGPLRP2) and *Yarrowia lipolytica* LIP2 lipase (YLLIP2) were produced in the yeast *Pichia pastoris* and purified as described previously in [23, 24], [25] and [26], respectively. Native porcine pancreatic lipase (PPL) and colipase were purified to homogeneity from porcine pancreas according to methods previously described [27, 28]. Human pancreatic carboxyl ester hydrolase/bile salt stimulated lipase (HCEH/BSSL) was purified to homogeneity from human pancreatic juice according to [29]. Recombinant dog gastric lipase (rDGL) was produced in transgenic maize by Meristem Therapeutics (Clermont-Ferrand, France) and was purified according to [30]. All purified lipases were prepared as 1 mg/mL solutions before use.

2.3. Preparation of pancreatic juice and pancreatin solutions for enzyme assays

All solutions at 10 and 100 mg/mL were prepared by dissolving pancreatin (PPE) or lyophilized pancreatic juice (HPJ or PPJ) in 10 mM Tris-HCl buffer, 150 mM NaCl, pH 8.0 and stored on ice before use. A cocktail of protease inhibitors (Complete™ mix, Roche) was added to these solutions (40 µL/mL of a solution prepared by dissolving a Complete™ tablet in 2 mL MilliQ water) when required to avoid proteolysis of lipolytic enzymes and measure optimal lipase, cholesterol esterase, galactolipase and phospholipase activities.

Since human and porcine pancreatic juices were collected in the presence of PMSF and benzamidine, pro-phospholipase A₂ present in these juices had to be activated by

additional trypsin in order to measure full phospholipase activity after zymogen cleavage. This was achieved by adding trypsin (5 or 50 μL of a 10 mg/mL solution) to 1 mL of HPJ or PPJ solution at 100 mg/mL. Bovine serum albumin (BSA) at 10 mg/mL and bile salts (5 mM NaTDC) were added in some reactions to limit proteolysis and preserve phospholipase activity after cleavage of the propeptide.

2.4. Generalities on lipolytic enzyme assays

All lipolytic enzyme assays were performed using the pHstat technique using a TTT80 Radiometer™ pH-Stat, a 50-mL thermostated (37°C) reaction vessel, mechanical stirring with a propeller and an automated delivery of 0.1 N NaOH for the titration of the free fatty acids (FFA) released upon substrate hydrolysis by the enzyme at a constant pH. All enzyme activities were expressed in international units (U) with 1 U corresponding to 1 μmole of FFA released per minute, and specific activities were expressed in U per mg of enzyme.

2.5. Assays of lipase activity

As a routine assay, lipase activity was measured using a mechanically stirred emulsion of the short chain tributyrin substrate (TC4), at a constant pH of 8.0. The reaction mixture was prepared by dispersing 0.5 mL TC4 in 14.5 mL of 0.3 mM Tris, 150 mM NaCl, 2 mM CaCl_2 , and 4 mM NaTDC. When required, porcine colipase (10 to 100 μL) was added in the reaction vessel to ensure full measurement of pancreatic lipase activity in the presence of bile salts. Colipase was prepared from pancreatin as previously described [31]. This lipase assay was validated according to Tuvignon et al. [32] using both purified human and porcine pancreatic lipases and PEE, mixed or not with duodenal contents, and therefore with other pancreatic enzymes including proteases.

Lipase activity was also assayed with long chain triglycerides from olive oil according to USP pancreatin monograph, at a constant pH value of 9.0 and at 37°C. A 10 % w/v olive oil emulsion in 10% w/v gum arabic was prepared using a warring blender. The reaction mixture (30 mL final volume) was prepared by mixing 10 mL of the 10 % w/v olive oil emulsion with 8.0 mL of 5 mM Tris (hydroxymethyl) aminomethane and 40 mM NaCl buffer, 2.0 mL of bile salts solution (80 mg of USP Bile Salts RS in each mL), 9.0 mL of water and 1 mL of the pancreatin solution (8 to 16 USP Units of lipase per mL) or sample to be tested.

2.6. Assays of phospholipase activity

Phospholipase activity was measured using mixed egg yolk phosphatidylcholine (PC)-bile salts (NaDC) micelles as substrate. Each assay was performed in a thermostated (37°C) vessel containing 15 mL of 1 mM Tris, 150 mM NaCl, 5 mM CaCl₂, 17 mM PC and 13 mM NaDC. Prior to the assay, PC was dissolved in chloroform/methanol 2:1 (v/v) and evaporated under nitrogen flux in a glass flask in order to obtain a thin film. PC was then re-suspended in the assay solution containing Tris, NaCl, CaCl₂ and NaDC using vigorous stirring. Assays were performed at various pH values and back-titration at pH 9 was performed to avoid underestimating enzymatic activity due to partial ionization of long-chain fatty acids.

2.7. Assay of galactolipase activity

The galactolipase activity was measured using mixed micelles of medium chain monogalactosyl diglyceride (C8-MGDG) and bile salts (NaTDC), at pH 8.0. To prepare the galactolipid dispersion, 25 mg of C8-MGDG were mixed with 5 mL of 0.33 mM Tris-HCl buffer, containing 0.1 M NaCl and 13.3 mM sodium taurodeoxycholate (NaTDC) and then subjected to ultrasonic treatment for 6-8 min in a water bath (HF-Frequ 35 kHz). This assas

was previously developed and validated by Amara et al. [22] using both purified enzymes and human pancreatic juice. Coefficient of variations for galactolipase activity measurements were 8.7 % for HPJ and 5.6 % for both purified HPLRP2 and HCEH/BSSL,

2.8. Assay of cholesterol esterase activity

The cholesterol esterase activity was measured using mixed micelles of a short chain cholesterol ester (cholesteryl acetate; CholA; 428.701 g/mol) and bile salts (NaTC; 537.688 g/mol), at pH 4 to 8. To prepare the substrate dispersion, 43 mg of CholA were dissolved in 100 μ L chloroform; 71.5 mg of NaTC were solubilized in 2.5 mL ethanol and mixed with the CholA chloroform solution in a 20-mL glass vial. The solvent was then evaporated under a N₂ stream. The dried lipid mixture was resuspended in 10 mL of 0.25 mM Tris-HCl buffer, pH 8 containing 150 mM NaCl and 2 mM CaCl₂. Final concentrations in CholA and NaTC were 10 mM and 13.3 mM, respectively

2.9. Gel filtration chromatography

Gel filtration chromatography of PPE, HPJ and PPJ solutions was performed using an ÄKTA explorer chromatographic device (GE Healthcare) and a Sephadex S200 26/60 gel filtration column (GE Healthcare). The column was equilibrated in 10 mM Tris buffer, 150 mM NaCl, pH 8.0, at 4°C. Solutions of PPE, HPJ and PPJ at 100 mg/mL were prepared in the same buffer containing protease inhibitors (see section 2.3). After a solubilisation step of 30 min at 4°C, the solutions were centrifuged at 4000 \times g for 15 min at 4°C. After separation of the pellet and for each sample, 2 mL of the clear supernatant were loaded onto the Sephadex S200 column. The flow rate was adjusted to 1 mL/min and fractions of 1 mL were collected. Protein elution was detected by measuring the optical density at 280 nm.

2.10. N-terminal protein sequencing

Following SDS-PAGE separation of proteins, the gel was left unstained and placed in a transfer buffer (50 mM Tris, 50 mM Boric acid, 20 % Ethanol, pH 8.3) for 15 min under slow shaking. Proteins were then transferred on a polyvinylidene difluoride (PVDF) membrane using a Trans-Blot® Turbo™ Transfer System at 25 V and 100 mA for 30 minutes. The membrane was then washed in water for 10 min, stained with 2 g/L Ponceau red in 30 g/L TCA, 30 g/L sulfosalicylic acid solution and washed with pure water until red protein bands appeared. Protein bands were excised from the PVDF membrane and N-terminal sequencing analysis by Edman degradation was performed using a PPSQ-1B sequencer (Shimadzu, Japan).

2.11. Protein identification by LC-MS/MS

Samples containing around 5 µg total proteins were mixed with 5 µL of SDS-PAGE loading buffer and were incubated at 100°C for 5 min to denature the proteins. Bands colored by Coomassie blue were cut either in the stacking or after separation by SDS-PAGE (voltage of 100 V was applied for 20 min) and analyzed for protein identification by mass spectrometry. Bands were digested by trypsin and peptides were analyzed by LC-MS/MS on an ESI-Q-Exactive Plus mass spectrometer (ThermoFisher, San Diego) coupled to a nano liquid chromatography (Ultimate 3000, Dionex) as previously described by Zhang et al. [33]. For protein identification, spectra were processed by Proteome Discoverer software (Thermo Fisher, version: 2.1.0.81) using the *Sus scrofa domesticus* (pig) NCBI database (Taxonomy ID 9823, 57526 sequence entries) uploaded from NCBI on February 27th, 2019.

2.12. Dynamic light scattering on cholesterol ester-bile salt micelles

Dynamic light scattering (DLS) experiments on CholA-NaTC dispersion in 0.25 mM Tris-HCl buffer, pH 7.5, 150 mM NaCl and 2 mM CaCl₂ were carried out using a Zetasizer Nano S (Malvern Instruments) at 37°C. Each measurement with mixtures of cholesterol ester and bile salts was performed in triplicate and consisted in 10-15 runs of 10 seconds at a scattering angle of 173°. The determination of the hydrodynamic diameter (DH) was based on the Einstein-Stokes relation to obtain the intensity-averaged size distribution. A viscosity of 0,736 cP and a refractive index of 1.3335 (at 37°C) were used for the dispersion medium, while a value of 1.49 was used as an approximation of the refractive index for micelles [34]. Collected data were analyzed by applying a customized method using 70 classes with a size-range analysis of 0.6 to 10000 nm.

3. Results and Discussion

3.1 Pancreatic lipase and colipase levels in HPJ, PPJ and PPE based on lipase assay with tributyrin as substrate

As a routine assay for measuring lipase activities, we used tributyrin as substrate [35]. To ensure the robustness of this assay and lower the main matrix effect (presence of proteases), samples of HPJ, PPJ and PPE were prepared in the presence of protease inhibitors (see section 2.3 and Table S1 in Supplementary Data section). It is already known that purified human (HPL) and porcine (PPL) pancreatic lipases show a similar specific activity of 8,000 U/mg on this substrate at pH 8, in the presence of bile salts (NaTDC) and colipase [32]. Colipase is the specific cofactor of pancreatic lipase present in exocrine pancreatic secretion that allows lipase adsorption and activity at the oil-water interface in the presence of bile salts [36, 37]. We performed assays with and without additional colipase to check whether juices and pancreatin contained enough endogeneous colipase for measuring optimal activities. We used a preparation of porcine colipase that is known to restore the activity of both PPL and HPL [38]. While the lipase activities of HPJ and PPJ were not significantly changed upon addition of colipase, the activity of PPE on tributyrin increased by around 20% (**Table 1**), which suggests a lower level of endogeneous colipase in PPE compared to PPJ. This may be due to some degradation of colipase during PPE production. Indeed, colipase can be partly cleaved by proteases and less efficient in restoring the activity of pancreatic lipase in the presence of bile salts [39]. We estimated the levels of functional colipase in PPE and PPJ by using the ratio of the lipase activities with and without colipase in excess (**Table 1**) and a reference curve of PPL activity as a function of the colipase to lipase molar ratio established with purified PPL and colipase (**Figure 1**). According to this variation curve, a colipase to lipase molar ratio of 2 is required for measuring the optimal activity of purified PPL on tributyrin, as previously reported by Borgström and Erlanson [40]. Therefore, the colipase to

lipase molar ratio in PPJ is probably close to 2 or higher since no additional colipase is required for measuring the maximal lipase activity (**Table 1**). One can assume that HPJ also contains a similar colipase to lipase molar ratio ≥ 2 . With PPE, however, only 83 % of the maximal activity was measured in the absence of additional colipase (**Table 1**), which corresponds to a colipase to lipase molar ratio of around 1.5 (**Figure 1**). Colipase therefore appears as a limiting factor for measuring the maximal lipase activity of PPE on tributyrin at pH 8. This may seem surprising since colipase interacts with pancreatic lipase in the form of an equimolecular complex [41-43] and is in slight excess in PPE. Nevertheless, it is worth noticing that the colipase and pancreatic lipase concentrations in the pHstat vessel during the assay are in the nM range (**Figure 1**), which is the order of magnitude of the apparent dissociation constant ($K_D \approx 1.5 \pm 0.4$ nM) of the lipase-colipase complex in the presence of tributyrin and bile salts [37]. These conditions are not optimal for ensuring the full association of the lipase with colipase and this is the reason why pancreatic lipase assay with tributyrin as substrate is usually performed in the presence of a large excess of additional colipase.

After performing these controls and measuring the maximal lipase activities in the presence of additional colipase, we could estimate the mass concentrations of active pancreatic lipase in the dried powders of HPJ, PPJ and PPE based on the known specific activity of 8000 U/mg of the pure enzyme. HPJ, PPJ and PPE contain respectively 28.9 ± 0.3 , 29.0 ± 0.4 and 10.2 ± 0.3 $\mu\text{g}/\text{mg}$ of pancreatic lipase. We then deduced the colipase mass concentrations from the colipase to lipase molar ratio and molar masses of 50kDa and 10kDa for lipase and colipase, respectively. HPJ, PPJ and PPE contain respectively ≥ 11.6 , ≥ 11.6 and 3.5 $\mu\text{g}/\text{mg}$ of colipase.

In the case of HPJ, we could also estimate a pancreatic lipase concentration of 502.9 ± 5.2 $\mu\text{g}/\text{mL}$ in the original juice before it was lyophilized, knowing that 17.4 mg of powder were obtained per mL of HPJ. Since pancreatic lipase output during a meal ranges from 88 to

442 mg/3 hours [44], one can estimate that 200 to 900 mL of HPJ have to be secreted to reach these values, which is consistent with the volume of pancreatic juice secreted after a lunch in humans [45] and overall with the daily output of pancreatic juice (1000 to 2000 mL; [46]). For comparison, 8.6 to 43.3 g of PPE would be necessary to deliver the same amounts of pancreatic lipase, which would represent a very high number of PPE-containing drug capsules (28 to 144 assuming a PPE content of 300 mg per capsule). Current daily recommendations for EPI patients are still far below this estimation, based on both an underestimation of lipase amounts required for normal digestion [47] and the fact that such a huge amount of capsules can be hardly ingested by patients. Pancreatic extracts enriched in their enzyme contents would certainly represent a significant progress in the treatment of EPI and patient comfort.

3.2 Pancreatic lipase and colipase levels in HPJ, PPJ and PPE based on USP lipase assay with olive oil as substrate

Since the lipase activity present in pancreatin and derived drug products is usually characterized in USP (or FIP) units, we also performed lipase assays according to USP Pancreatin monograph [12], in which a gum arabic (or acacia)-stabilized emulsion of olive oil is used as substrate and the FFAs released upon lipolysis are titrated at a constant pH value of 9, in the presence of bile salts. The USP Pancreatin monograph does not recommend the use of additional colipase, nor protease inhibitors. However, we show here that the measurement of PPL activity with the USP assay is also dependent on the colipase to lipase molar ratio and the maximal activity is reached at a ratio higher than 1.5 (**Figure 1**). We therefore checked the effects of additional colipase on the lipase activities of PPE, PPJ and HPJ measured with the USP assay. While the lipase activities of HPJ and PPJ were not significantly changed upon addition of colipase, the activity of PPE in the absence of additional colipase was 92 % of the

maximal activity measured after addition of colipase (**Table 1**). These results confirm the estimation of the colipase to lipase molar ratio in HPJ, PPJ and PPE deduced from lipase activity measurement with tributyrin. We also checked that the used of protease inhibitors allowed a better precision of the USP lipase assay for all the samples tested over a period of 2 hours (see Table S1 in Supplementary Data section).

The USP assay is based on conditions that have been initially developed and optimized for measuring the activity of porcine pancreatic lipase [48]. Using a pH of 9 is not physiologically relevant since the mean pH of intestinal contents during test meal in humans is close to 6 [49, 50]. Nevertheless, it is convenient because it ensures full ionization, and therefore titration, of long chain FFA, without the need to perform back-titration. By varying the pH of the assay, we showed that optimal activities of PPE and PPJ *in vitro* are measured at pH 9, in the absence of additional colipase (**Figure 2**). In both cases, however, the optimal activity can be shifted to a lower pH of 7.5 and slightly increased when an excess of colipase is added (**Figure 2**). Similarly, the maximal activity of HPJ shifted from pH 8.5 to pH 7.5 by addition of colipase (**Figure 2**). These findings indicate that the USP assay conditions are not optimized for measuring the maximal activity of pancreatic lipase on olive oil emulsified with gum arabic. Nevertheless, the choice of pH 9 allows measuring lipase activities that are poorly changed by additional colipase as shown in **Figure 2**. These data may be useful for a future revision of USP pancreatin monograph since they support the absence of additional colipase in the lipase assay, a point that has been often questioned.

3.3 Phospholipase levels in HPJ, PPJ and PPE

The activity of pancreatic phospholipase A2 (PLA2) was first measured at pH 8 using egg yolk phosphatidylcholine as substrate. Since HPJ and PPJ were collected and stored in the

presence of protease inhibitors to avoid zymogen activation, their PLA2 activity could not be measured without prior activation of phospholipase A2 by additional trypsin. Conditions to obtain maximal activation by exogenous trypsin and stable phospholipase activity had to be optimized. Using a 100 mg/mL solution of PPJ powder, the addition of 50 and 500 μ g/mL trypsin allowed measuring the maximal activity after 100 and 60 minutes, respectively (**Figure 3A**). Such large amounts of trypsin were required to overcome the presence of protease inhibitors added during PPJ collection and still present in the PPJ solution. A drawback was that phospholipase activity was not stable with time, probably due to extended proteolysis and activation of other pancreatic proteases, and the activity decreased with a half-life of around 1 hour in the presence of 500 μ g/mL trypsin (**Figure 3A**). Since proteins and bile are known to preserve enzyme activities in duodenal contents [51], we tested the addition of bovine serum albumin (BSA) and bile salts (BS) and found that 10 mg/mL BSA combined with 5 mM BS allowed preserving the phospholipase activity over 1 hour (**Figure 3A**). The coefficient of variation (CV%) of the phospholipase assay was 3.8 % during that period (see Table S1 in Supplementary Data). Similar results were obtained with HPJ (**Figure 3B** and **Table S1**). Human and porcine pancreatic phospholipases A2 differ however in their pH optima for measuring phospholipase activity [52]. Porcine PLA2 shows a maximal activity at pH 8 and a similar activity (>90%) at pH 6, while human PLA2 shows its maximal activity at pH 6 and only 23% of maximal activity at pH 8 [52]. We confirmed here that the phospholipase activity of HPJ was 2.6-fold higher at pH 6 than at pH 8 (**Figure 3B**).

After optimization of sample activation and assay conditions, we compared the phospholipase activities of HPJ, PPJ and PPE (**Table 1**). There was no need however to activate PPE in which maximal phospholipase activity was measurable immediately after dissolution of the powder (data not shown). As for the assays of other lipolytic enzymes, the addition of protease inhibitors to PPE was required to avoid further proteolysis after

dissolution, measure optimal activity and obtain an acceptable precision of the phospholipase assay (CV=3.8%; see Table S1 in Supplementary Data). As seen before with lipase activity, HPJ and PPJ had similar levels of phospholipase activity, but it was around 10-fold lower in PPE (**Table 1**). The phospholipase to lipase (tributyryl assay) activity ratios were 0.18, 0.2 and 0.05 for HPJ, PPJ and PPE, respectively, which indicates some loss of phospholipase activity upon PPE preparation. We could estimate the mass concentrations of active pancreatic PLA₂ in PPJ and PPE based on the known specific activity of 2800 U/mg of the pure porcine pancreatic PLA₂ under these assay conditions. PPJ and PPE contain respectively 16.4 ± 0.3 and 1.5 ± 0.1 $\mu\text{g/mg}$ of pancreatic PLA₂. We could not estimate the mass concentrations of PLA₂ in HPJ because the specific activity of pure human PLA₂ under the present assay conditions is unknown.

3.4 Cholesterol esterase levels in HPJ, PPJ and PPE

Prior to measuring cholesterol esterase activities, we searched assay conditions that were specific of cholesterol ester hydrolysis. Indeed, the preparation of substrate for measuring cholesterol esterase activity is not obvious since cholesterol esters are highly apolar and preferentially solubilized in oil. Other lipids such as triacylglycerols (TAG) and phospholipids have usually to be used to allow the dispersion in water of small lipid aggregates containing cholesterol esters [53]. Since most enzymes with cholesterol esterase activity also display lipase and phospholipase A₁ activities, they can also act on TAG or phospholipids present in the reaction mixture and one cannot estimate cholesterol esterase activity only from the release of FFAs. Cholesterol esters with long acyl chains have also a poor affinity for bile salt micelles and cannot be dispersed in this form. Here we tested the possibility to use a short chain cholesterol ester that could present a higher affinity for bile salt

micelles and found that cholesteryl acetate (CholA)-sodium taurocholate (NaTC) dispersions were a good substrate for pancreatic cholesterol esterase using an optimal NaTC to CholA molar ratio of 1.33 (**Figure 4A**). This optimal ratio was independent of the pH of the assay (6 or 8; data not shown).

The CholA-NaTC dispersions obtained under these conditions were characterized by DLS. At 37°C, micelles were visible with an average size of 4.28 ± 0.47 nm. Some larger aggregates could be detected, but in negligible amounts, and they tended to disappear with increased time at 37°C as judged from the z-average variations (Data not shown). CholA-NaTC dispersions were therefore kept at 37°C after preparation until they were used as substrate for cholesterol esterase activity measurements.

The pH optima for the cholesterol esterase activities of porcine and human pancreas were different. Indeed, the maximal activities of both PPJ and PPE were recorded at pH 8, while that of HPJ was measured at pH 6 (**Figure 4B and Table 1**). Moreover, human pancreatic carboxyl ester hydrolase/bile salt stimulated lipase (HCEH/BSSL) purified from HPJ displayed a specific activity of 513 ± 12 U/mg at pH 6, that was 10-fold higher than at pH 8 (**Table 2**). To our knowledge, this is the highest specific activity of HCEH/BSSL ever measured on cholesterol esters and other substrates [22, 54, 55] and therefore, these assay conditions are the most sensitive ones for detecting this enzyme in biological samples. We also checked the specificity of this assay by comparing HCEH/BSSL with other purified enzymes. Among enzymes well-known for their high specificity towards TAG, rDGL and YLLIP2 showed no activity while PPL had a very low activity on cholesterol esters (**Table 2**). However, a significant cholesterol esterase activity was measured with rHPLRP2 and to a lower extent with rGPLRP2 (**Table 2**). It confirms previous observations that PLRP2 can hydrolyze cholesterol esters with a lower activity than HCEH/BSSL [56]. It is therefore worth

noting that cholesterol esterase activity in HPJ, and potentially in PPJ and PPE, can result from both CEH/BSSL and PLRP2.

The comparison of cholesterol esterase activities of HPJ, PPJ and PPE at pH 6 and pH 8 is shown in **Table 1**. As for the measurements of other lipolytic activities, the addition of protease inhibitors to all samples was required to measure optimal activity and obtain an acceptable precision of the cholesterol esterase assay (see Table S1 in Supplementary Data). Taking into account the maximal activities recorded, cholesterol esterase activities are 2.3-fold and 7.3-fold lower in PPJ and PPE compared to HPJ. The lipase (tributyryl assay) to cholesterol esterase activity ratios are 82, 191 and 210 for HPJ, PPJ and PPE, respectively, which indicates lower levels of cholesterol esterase activity in the pig pancreas and some further loss of activity upon PPE preparation.

3.5 Galactolipase levels in HPJ, PPJ and PPE

The galactolipase activities of HPJ, PPJ and PPE were measured using the medium chain monogalactosyl diglyceride C8-MGDG as substrate, after addition of protease inhibitors to ensure an optimal activity and obtain an acceptable precision of the galactolipase assay (see Table S1 in Supplementary Data). No significant differences were observed between the assays performed at pH 6 and 8 (**Table 1**). At pH 8, PPJ and PPE galactolipase activities were 4.3-fold and 24.9-fold lower compared to HPJ. The lipase (tributyryl assay) to galactolipase activity ratios were 30, 130 and 264 for HPJ, PPJ and PPE, respectively, which indicates lower levels of galactolipase activity in the pig pancreas and some further loss of activity upon PPE preparation.

3.6 Stability of lipase, phospholipase, galactolipase and cholesterol esterase activities in HPJ, PPJ and PPE

Since some lipolytic activities appeared lower in PPJ and PPE compared to HPJ, we tested their stability as a function of time in the presence and absence of protease inhibitors. This was not possible with the phospholipase activities of HPJ and PPJ that required activation by trypsin. It was first shown that protease inhibitors were required to ensure the measurement of maximal activities at time 0 (**Figure 5B, D and E**). The presence of inhibitors allowed preserving lipase (tributylin assay), galactolipase and cholesterol esterase activities for at least 2 hours without any significant loss of enzyme activity (**Figure 5A to 5I**). Similar results were obtained with the phospholipase activity of PPE (see Table S1 in Supplementary Data). In the absence of protease inhibitors, all activities decreased with time (**Figure 5A to 5I**), which reveals the degradation of lipolytic enzymes by endogeneous proteases present in HPJ, PPJ and PPE, and indicates that the proteases initially present in HPJ and PPJ were not fully inactivated by addition of PMSF and benzamidine upon juice collection. Galactolipase activities in PPJ (**Figure 5E**) and PPE (**Figure 5F**) were the less stable ones. There was no apparent correlation between the losses of galactolipase and cholesterol esterase activities, which suggests that these activities are displayed by several enzymes with distinct specificity and stability.

3.7 Comparison of all lipolytic activities in HPJ, PPJ and PPE

Table 1 shows relative activity levels for better comparison of HPJ, PPJ and PPE, using the lipase activity measured on tributyrin (TC4) as the reference. For both pancreatic juices and PPE, the lipase activity measured with the USP assay conditions was 31-34.6 % of that measured with TC4, which confirms that pancreatic lipase specific activity on long chain

TAG (olive oil) is about one third of the activity measured with TC4. HPJ and PPJ contained similar levels of phospholipase activity compared to lipase activity (around 19%), but PPE had a 3-fold lower relative level of phospholipase activity (6.4 %). Both galactolipase and cholesterase activities were represented at low levels in all samples, but their relative levels were lower in both PPJ and PPE than in HPJ. Galactolipase was the most reduced with a 4.5 (PPJ) to 7-fold (PPE) decrease, while cholesterol esterase activity decreased 2-fold in both PPJ and PPE versus HPJ. Since galactolipase and cholesterol esterase activities are associated with PLRP2 and CEH/BSSL in humans, our findings suggest that these two enzymes are less represented in pig than in humans. Moreover, some galactolipase activity seems to be lost upon PPE preparation which would fit with the lower stability of this activity (**Figure 5**).

Since a typical dosage of PPE in PERT drug products is 25,000 USP lipase units per capsule, we also compared all the lipolytic activities of PPE and juices for a common basis of 25,000 USP lipase units (**Table 4**). This comparison may be useful for future improvements of PPE for the treatment of EPI.

3.8 Separation by gel filtration of proteins and lipolytic activities present in pancreatic juices and pancreatin

The proteins from HPJ, PPJ and PPE were separated in various fractions using gel filtration on a Sephadex S200 column and these fractions were tested for the lipase (TC4 as substrate), galactolipase, cholesterol esterase and phospholipase activities (**Figure 6**). Phospholipase activity was tested after zymogen activation by trypsin as described in section 3.3. In all cases, a first peak containing lipase, galactolipase and cholesterol esterase activities was eluted after the void volume of the column and SDS-PAGE analysis revealed the presence of a protein band of around 100 kDa in the corresponding fractions (see F2 for HPJ

in **Figure 6A**; F1 for PPJ and PPE in **Figures 6B** and **6C**, respectively), that could correspond to CEH/BSSL. The following peaks, containing also a mixture of lipase, galactolipase and cholesterol esterase activities, were eluted similarly to proteins of around 50 kDa. Nevertheless, SDS-PAGE analysis also revealed the presence of several protein bands of lower molecular masses (see F4 for HPJ in **Figure 6A**; F2 for PPJ and PPE in **Figures 6B** and **6C**, respectively). These fractions contained the highest lipase activity, what could be expected from the known molecular mass of pancreatic lipase (50 kDa). The detection of galactolipase and cholesterol activities also suggest the presence of PLRP2 (50 kDa) in these fractions. Finally, in all cases, the phospholipase activity was detected in fractions eluted later and corresponding to lower molecular mass proteins (see F5 for HPJ in **Figure 6A**; F3 for PPJ **Figure 6B** and F4 for PPE in **Figure 6C**, respectively), as expected for pancreatic phospholipase A2 (14 kDa).

3.9 Identification of lipases present in HPJ, PPJ and PPE

Pancreatic lipases and phospholipases A2 from human and porcine pancreas have been already well characterized [27, 52, 57-60], as well as human CEH/BSSL [54, 61-63] and PLRP2 [17]. Our main focus was therefore to search for CEH/BSSL and PLRP2 in PPJ and PPE, and to compare them to the human enzymes. For this purpose we analyzed the fractions obtained from gel filtration by SDS-PAGE, N-terminal sequencing of specific bands transferred onto PVDF membrane and LC-MS/MS after digestion by trypsin.

We first confirmed by N-terminal sequencing (AKLGA-) that the 100-kDa protein band observed by SDS-PAGE in fraction F2 of HPJ (**Figure 6A**) corresponds to HCEH/BSSL (**Figure 7**). Mature HCEH/BSSL (733 amino acid residues) is a polypeptide of 79.3 kDa (UniProtKB/Swiss-Prot: P19835.3) and it is highly glycosylated [29], which explains its

electrophoretic migration with proteins of around 100 kDa. It contains 17 C-terminal proline-rich tandem repeats of 11 amino acids [64]. The N-terminal sequence (KEVXYG-) of mature HPLRP2 (**Figure 8**) was identified from the analysis of the 50-kDa band of fraction F4 from HPJ (**Figure 6A**). Minor N-terminal sequences were also determined that corresponds to proteolytic cleavage of HCEH/BSSL in its C-terminal part (534-DQEA-) and of HPLRP2 in the lid domain (250-DIDGIW-).

Similar analysis on the 100-kDa and 50-kDa protein bands isolated from fractions F1 and F2 of PPJ (**Figure 6B**) were performed, as well as peptide map fingerprint (PMF) by LC-MS/MS on all fractions of PPJ gel filtration. While N-terminal sequencing of the 100-kDa band failed to determine the expected sequence of the mature porcine CEH/BSSL (AKLGS), probably because its quantity was not high enough, PMF allowed the identification of 16 peptides covering 37% of the predicted sequence for porcine CEH/BSSL (**Table 3** and **Figure 7**; **Table S6** in Supplementary data). The complete sequence of porcine CEH/BSSL (NCBI Accession ID: XP_003353742.2) was deduced from the pig genome sequencing but it was not described previously in the literature. With 590 amino acid residues in the mature protein, it is shorter than HCEH/BSSL and it is due to a lower number (n=4) of C-terminal proline-rich tandem repeats of 11 amino acids (**Figure 7**). In their conserved parts, porcine and human CEH/BSSL share 82% amino acid identity. Since the polypeptide of porcine CEH/BSSL has a molecular mass of 64.2 kDa, the apparent molecular mass of around 100 kDa estimated from SDS-PAGE indicates that this enzyme is highly glycosylated. We were not able however to determine a more precise mass from the 100-kDa band using MALDI-TOF mass spectrometry. The protein identified here corresponds to the porcine CEH/BSSL previously purified and N-terminally sequenced (AKLG(A/L)VYTEG) by Labow et al. [65], except for residue 5 that is a serine in the sequence available in NCBI databank. Labow et al. described two forms of 90 and 45 kDa with the same N-terminal sequence for porcine

CEH/BSSL and suggested a dimer-monomer relationship, while the smaller form was probably a degradation product lacking a part of its C-terminal region. Rudd et al. later reported the existence of two forms with the same N-terminal sequence, but of 76 and 167 kDa [66]. From crude PPE preparations, Birner-Grünberger et al. detected by SDS-PAGE a band of 98 kDa corresponding to porcine cholesterol esterase, but only after addition of the protease inhibitor PMSF [67]. Thus, there were large discrepancies in the molecular mass estimated for porcine CEH/BSSL and this can be attributed to various levels of proteolytic degradation in these preparations. Before the sequence of the pig genome was available, it was therefore difficult to conclude on the precise size of porcine CEH/BSSL, especially because this enzyme has different size in various species due to the occurrence of a series of proline-rich tandem repeat units at its C-terminal end [64].

N-terminal sequencing of the 50-kDa band isolated from fraction F2 of PPJ failed to identify porcine PLRP2. We were not able however to determine a more precise mass from the 50-kDa band using MALDI-TOF mass spectrometry. Nevertheless, PMF allowed the identification of 12 peptides covering 39% of porcine PLRP2 sequence (**Table 3** and **Figure 8**; **Table S5** in Supplementary Data). The complete sequence of porcine PLRP2 (NCBI Reference Sequence: NP_001177220) was deduced from the mRNA isolated from pig pancreas (NCBI Reference Sequence: NM_001190291.1) [19, 68]. With 454 amino acid residues in the mature protein, porcine PLRP2 shares 78% amino acid identity with human PLRP2 (**Figure 8**).

Several other pancreatic enzymes and proteins were also detected by PMF of PPJ (**Tables S2-S7** in Supplementary Data), including porcine pancreatic lipase (PPL), pancreatic lipase-related protein 1 (PLRP1) and colipase (**Table 3**). It is worth noting however that peptides corresponding to porcine pancreatic PLA2 were not detected, although

phospholipase activity of PPJ is high and the PLA2 to PPL mass ratio is estimated to be 0.6 (16.4 and 29 $\mu\text{g}/\text{mg}$, respectively; see sections 3.1 and 3.3).

PPL and colipase were also identified in PPE by PMF (**Table 3**), as well as other pancreatic enzymes and proteins (**Tables S8-S10** in Supplementary Data). Our attempts to identify porcine CEH-BSSL and PLRP2 in PPE were however unsuccessful, including from N-terminal sequencing of the 100-kDa and 50-kDa protein bands separated by SDS-PAGE (**Figure 6C**). The detection of cholesterol esterase and galactolipase activities in the corresponding fractions F1 and F2 of PPE strongly suggests the presence of these two enzymes in PPE (**Figure 6B**), but their lower level compared to PPJ (**Table 1**) probably impairs the detection of the corresponding peptides. This may explain why porcine CEH/BSSL and PLRP2 have not been identified in PPE by 2D gel electrophoresis coupled to mass spectrometry [15].

3.10 Estimation of PLRP2 and CEH/BSSL mass concentrations in HPJ

Since PLRP2 and CEH/BSSL share a broad substrate selectivity and are active on the same substrates, it is not possible to deduce their mass concentration in pancreatic juices from their specific activities on a given substrate and single activity measurements, as done with pancreatic lipase (see section 3.1). Nevertheless, since PLRP2 and CEH/BSSL are the only pancreatic enzymes acting on both galactolipids and cholesterol esters, it was possible to estimate their respective mass concentrations in HPJ ([HPLRP2], [HCEH/BSSL]; $\mu\text{g}/\text{mg}$) using the following system of two equations with two unknowns:

$$\text{HPJ cholesterol esterase activity at pH 6 (2.3 U/mg)} = A1.[\text{HPLRP2}] + B1.[\text{HCEH/BSSL}]$$

HPJ galactolipase activity at pH 8 (7.73 U/mg) = A2.[HPLRP2] + B2.[HCEH/BSSL]

in which A1 is the specific activity of pure HPLRP2 on cholesterol acetate at pH 6 (18.7 U/mg; **Table 2**), B1 is the specific activity of pure HCEH/BSSL on cholesterol acetate at pH 6 (513 U/mg; **Table 2**), A2 is the specific activity of pure HPLRP2 on C8-MGDG at pH 8 (1786 U/mg [22]) and B2 is the specific activity of pure HCEH/BSSL on C8-MGDG at pH 8 (230 U/mg [22]).

[HPLRP2] and [HCEH/BSSL] were found to be 3.6 and 5.4 μg per mg of lyophilized HPJ, and their mass ratios compared to HPL ($28.9 \pm 0.3 \mu\text{g}$) were 0.12 and 0.19, respectively. A HPLRP2 to HPL mass ratio of 0.28 was previously reported based on ELISA assays of both HPLRP2 and HPL in HPJ [21].

A similar estimation of PLRP2 and CEH/BSSL mass concentrations in PPJ and PPE was not possible because the specific activities of purified porcine PLRP2 and CEH/BSSL are not known yet.

4. Conclusion

PPE contain the same lipolytic activities as HPJ and PPJ, but phospholipase, galactolipase and cholesterol esterase activities are much reduced compared to lipase activity taken as a common reference (**Tables 1 and 3**). The fact that PPE from various manufacturers can differ considerably in their lipase and phospholipase activities was previously reported and the activity levels of CEH/BSSL (also named carboxyl ester lipase in the cited work) were found to be particularly low [13]. Besides differences in the respective levels of porcine and human pancreatic enzymes, the comparison of PPJ and PPE reveals a significant loss of

phospholipase and galactolipase activities in PPE, which is probably due to some proteolysis occurring during the process of PPE production. Indeed, the lipolytic enzymes present in PPE, as well as in PPJ and HPJ, are highly sensitive to proteolytic degradation and inactivation by the endogenous proteases present in these materials (**Figure 5**). The enzyme inactivation during the PPE manufacturing procedure was also supported by the finding that CEH/BSSL activity varies considerably more than the amounts of immunoreactive CEH/BSSL [13].

The galactolipase activity in PPE was the lowest (**Table 1**) and the least stable one (**Figure 5F**), which may explain why it was not detected previously [22]. This may also explain why PLRP2, the enzyme showing the highest galactolipase activity [69], was not identified previously among porcine pancreatic enzymes [18]. To our knowledge, we identified here for the first time porcine PLRP2 in PPJ, which is in agreement with the presence of PLRP2 gene in the pig genome and its expression in the pancreas in pigs [19, 68]. The characterization of recombinant porcine PLRP2 expressed in mammalian cell cultures has confirmed that this enzyme displays galactolipase and phospholipase activities, as well as a lipase activity on triolein that is poorly dependent on the presence of colipase [19]. Porcine PLRP2 may therefore contribute to all lipolytic activities of PPJ and PPE, which renders more complex their characterization.

Similarly, porcine CEH/BSSL is another enzyme with several lipolytic activities that was identified here in PPJ. Besides lipase, phospholipase A1 and galactolipase activities, CEH/BSSL is assumed to be the most important pancreatic enzyme for the digestion of cholesterol esters, as well as vitamin A (retinol) and E (tocopherols and tocotrienols) esters [54, 63]. We did not study specifically the lipolytic activity of PPE, PPJ and HPJ on these later esters, assuming that it is tightly associated with cholesterol esterase activity. The low levels of cholesterol esterase activity in PPE compared to PPJ and HPJ also suggest that PPE will be less active on lipophilic vitamin esters than pancreatic juices.

In conclusion, PPE do not provide a full substitution of the enzymes and lipolytic activities present in HPJ. Although their lipase activity on olive oil is well characterized and lipase units are used as a reference for adapting PERT in patients with EPI, PERT with current PPE may not ensure an optimal digestion and intestinal absorption of phospholipids, vitamin esters and galactolipids. This may not impact significantly the overall uptake of fatty acids, most of them being contained in dietary triglycerides, but the bioavailability of essential lipophilic micronutrients may be impaired. The analysis performed here with Sigma PPE, a preparation widely used for *in vitro* digestion studies, should be extended however to other PPE preparations entering in the composition of drugs for PERT since variations in the manufacturing process of PPEs are very likely. The present work and quantitative data on the respective levels of all lipolytic activities in HPJ may provide a rational basis for improving PPE for PERT, either at the manufacturing process level or by adding exogenous enzymes. Alternatively, it may lead to the development of novel enzyme preparations with relevant recombinant enzymes.

Our findings have also a direct impact on the current use of PPE for *in vitro* digestion studies. The low levels of phospholipase, cholesterol esterase and galactolipase activities in PPE are not adapted for testing the bioavailability of the corresponding dietary lipids and vitamin esters. These activities are probably not the most important for the release of dietary fatty acids and energy uptake, but they may be critical for the whole digestion process. For instance, galactolipase activity is important because it allows the release of the essential α -linolenic acid from the main lipids present in vegetables [70]. The lipolysis of phospholipids, often used as emulsifiers, has a direct impact on the lipolysis of dietary triglycerides [71]. Finally, PLRP2 and CEH/BSSL have been shown to be important enzymes for the *in vitro* digestion of lipid-based formulations (LBF) for the oral delivery of lipophilic drugs [31, 50, 72, 73]. Their low levels in PPE may limit the validity of *in vitro* models for predicting the

fate of LBF in the gastrointestinal tract and the impact of digestion on drug solubilisation and intestinal absorption.

Conflicts of Interest

We declare no conflict of interest.

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Contributors

Amal Salhi and Sawsan Amara performed all the enzyme activity measurements, separation of proteins by gel filtration and SDS-PAGE and contributed to the writing of the article. Pascal Mansuelle, Rémy Puppo and Régine Lebrun performed N-terminal sequencing and LC-MS/MS analysis. Brigitte Gontero and Ahmed Aloulou contributed to the design of the study and made a critical revision of the manuscript. Frédéric Carrière designed the study and wrote the article.

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Figure legends

Figure 1. Effects of colipase on the specific activity of purified porcine pancreatic lipase (PPL) measured with the pHstat technique. The lipase activity of PPL was measured at 37°C in the presence of various amounts of purified porcine colipase, using either tributyrin (pH 8) or olive oil (pH 9; USP lipase assay) emulsions as substrates, as described in the Materials and Methods section. In both cases, the presence of bile salts at concentrations above the critical micellar concentration (4 mM NaTDC in the tributyrin assay and around 10 mM bile salts in the USP assay) impairs lipase adsorption and activity at the oil-water interface and colipase is required to anchor the lipase at the interface and counteracts the inhibitory effects of bile salts. PPL concentration in the pHstat vessel was 2 µg/mL or 40 nM based on a molar mass of 50,000 g/mol for PPL. The lipase specific activity is expressed in units (U) per mg of enzyme, with 1 U = 1 µmole of FFA released per min. Values are mean ± SD (n=3).

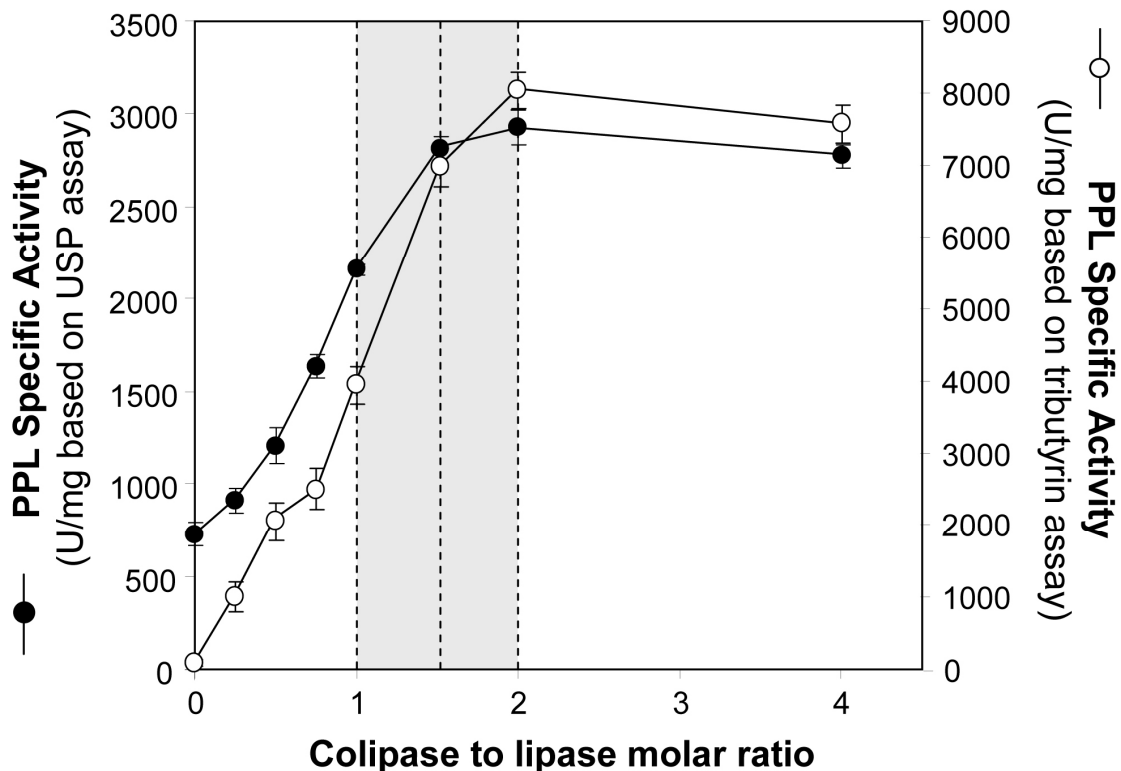


Figure 2. Variations with pH in the lipase activity of HPJ, PPJ and PPE measured with olive oil as substrate. Assay conditions were based on the standard conditions of USP Pancreatin monograph using a gum arabic-stabilized emulsion of olive oil, except for the pH of titration of FFA released upon lipolysis (pH 9 under USP standard conditions; dotted box). Assays were performed in the absence and presence of additional colipase. The lipase specific activity is expressed in units (U) per mg of powder, with 1 U = 1 μ mole of FFA released per min. Values are means \pm SD (n=3).

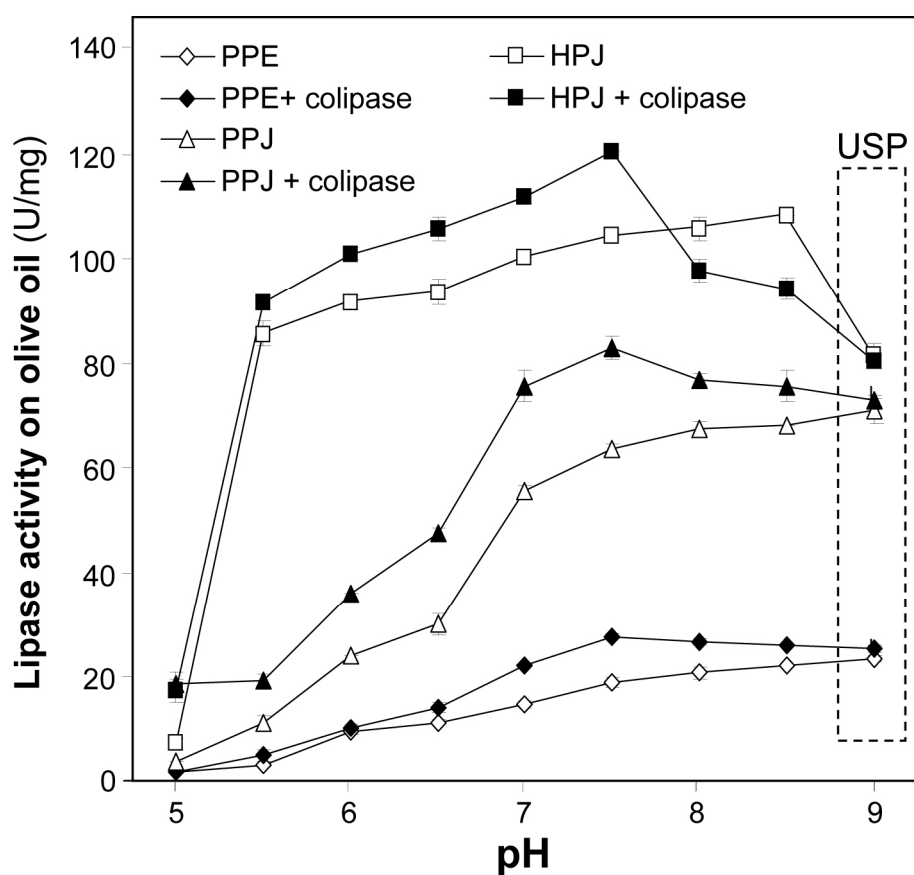


Figure 3. Activation of pro-phospholipase A2 present in pancreatic juices. Pro-phospholipase A2 of porcine (panel A; PPJ) and human (panel B; HPJ) pancreatic juices was activated using 50 or 500 μg trypsin per mL of solutions at 100 mg/mL pancreatic juice lyophilized powder in 10 mM Tris buffer, 150 mM NaCl, pH 8.0, with and without BSA (1 or 10 mg/mL) and bile salts (0.5 or 5 mM). Phospholipase activity was measured at 37°C using the pHStat technique and egg PC-NaDC micelles as substrate, at pH 8 (PPJ and HPJ) and 6 (HPJ). Values are means \pm SD (n=3).

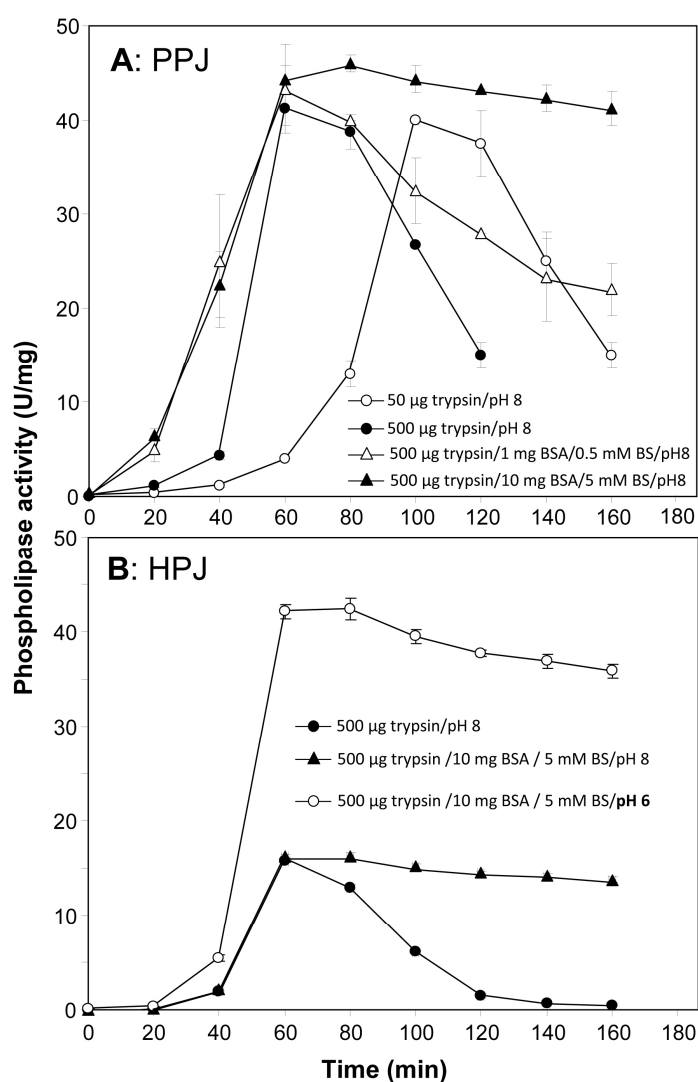


Figure 4. Optimization of the cholesterol esterase assay using the pHstat technique and cholesterol acetate-sodium taurocholate micelles. Panel A: Effects of the sodium taurocholate (NaTC) to cholesterol acetate (CholA) molar ratio on the cholesterol esterase activity of HPJ and PPE. Panel B: variation with pH in the cholesterol esterase activity of HPJ, PPJ and PPE. The CholA concentration in all assays was 10 mM. The NaTC concentration in panel B experiments was 13.3 mM. All assays were performed at 37°C. Values are means \pm SD (n=3).

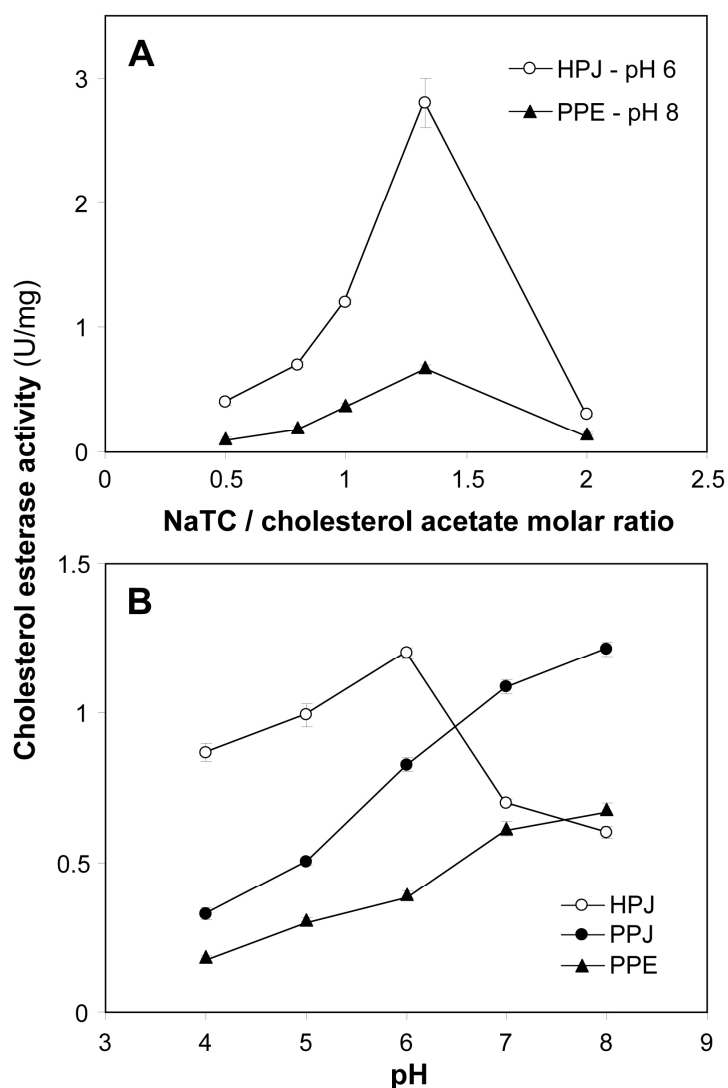


Figure 5. Stability of lipase (panels A to C), galactolipase (panels D to F) and cholesterol esterase (panels G to I) activities in preparations of HPJ, PPJ and PPE. Enzyme activities were measured immediately after preparation (time 0) and after 2 hours of incubation at room temperature, in the absence (white bars) and presence (grey bars) of protease inhibitors.

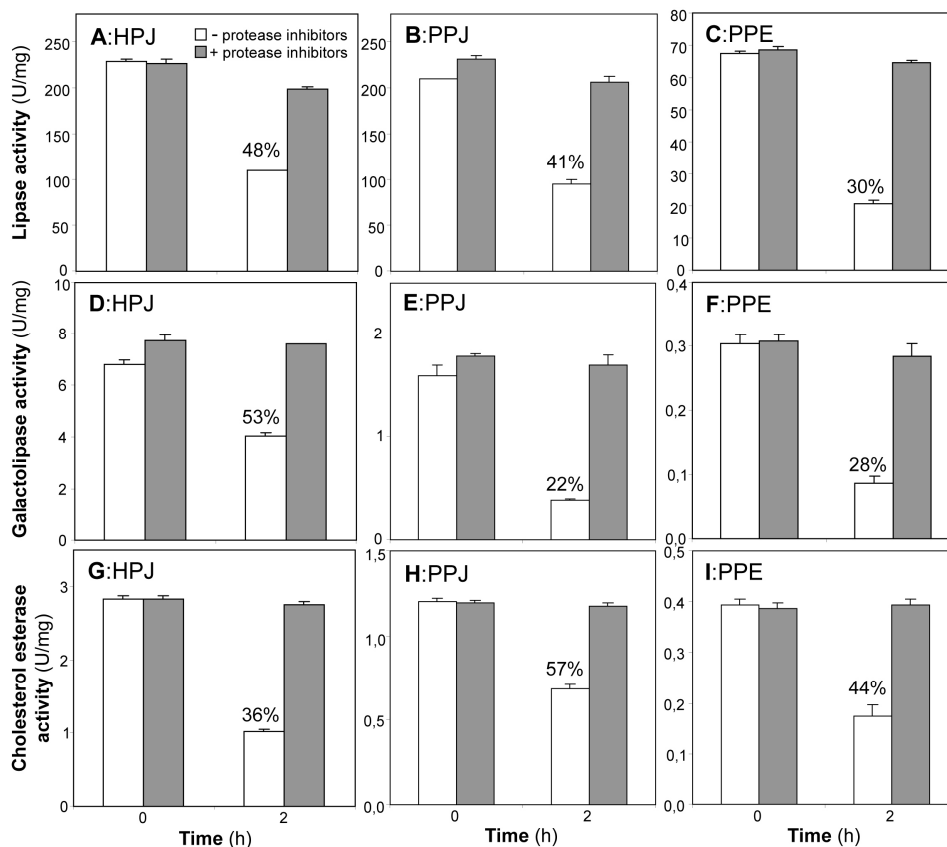


Figure 6. Separation by gel filtration of proteins from HPJ (panel A), PPJ (panel B) and PPE (panel C) and corresponding SDS-PAGE of fractions showing lipase, galactolipase, cholesterol esterase and phospholipase activities. Gel filtration was performed on Sephadex S200 column and protein elution profile was recorded at 280 nm. The calibration of the column with 100, 50 and 14kDa Mw markers is indicated above panel A. Lipolytic enzyme activities, expressed in U/mL, were measured as described in the Materials and Methods section after fractions were either mixed with protease inhibitors (preservation of lipase, galactolipase and cholesterol esterase activities) or incubated with trypsin for full activation of phospholipase A2. Protein bands of interest on SDS-PAGE gels are indicated by arrows.

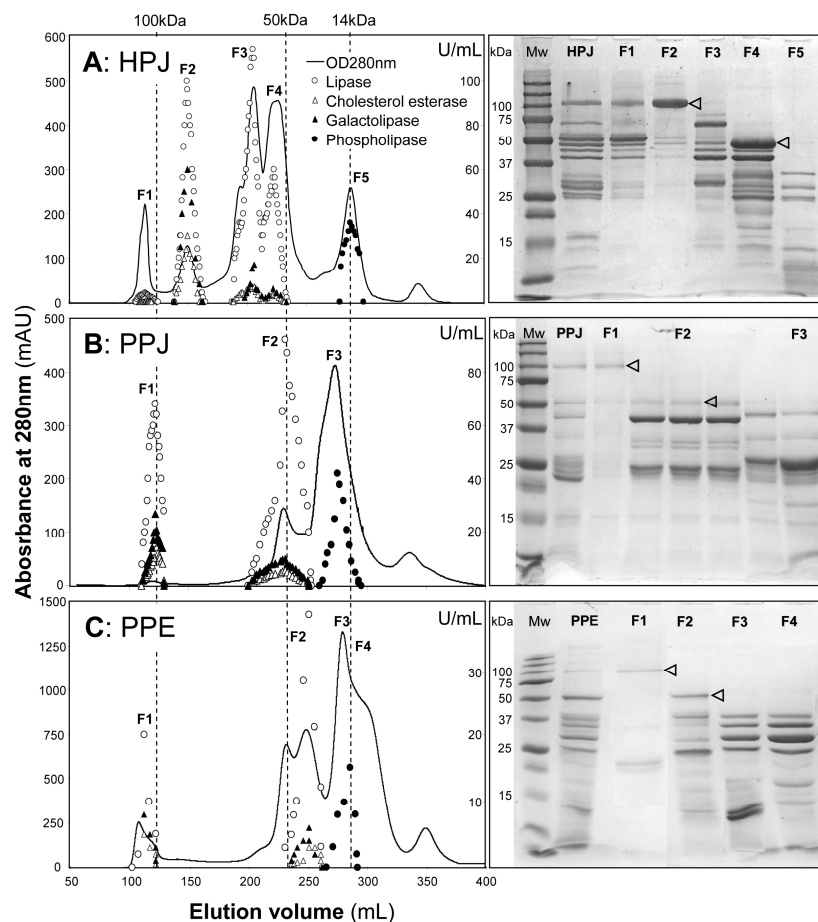


Figure 7: Protein sequence alignment of human (UniProtKB/Swiss-Prot: P19835.3) and porcine CEH/BSSL (NCBI Accession ID: XP_003353742.2). Sequence alignment was performed using the Clustal Omega web server (<https://www.ebi.ac.uk/Tools/msa/clustalo/>) [74]. Secondary structure elements shown above the sequence alignment were obtained from the known 3D structure of human CEH/BSSL (PDB: 1JMY; [75]) and the DSSP software [76] accessible via the web server: <http://www.cmbi.ru.nl/xssp/>. The presentations of sequence alignments were made using ESPrpt 3.0 (<http://esprpt.ibcp.fr/>) [77]. The peptides of porcine CEH/BSSL identified by PMF are underlined by a grey line.

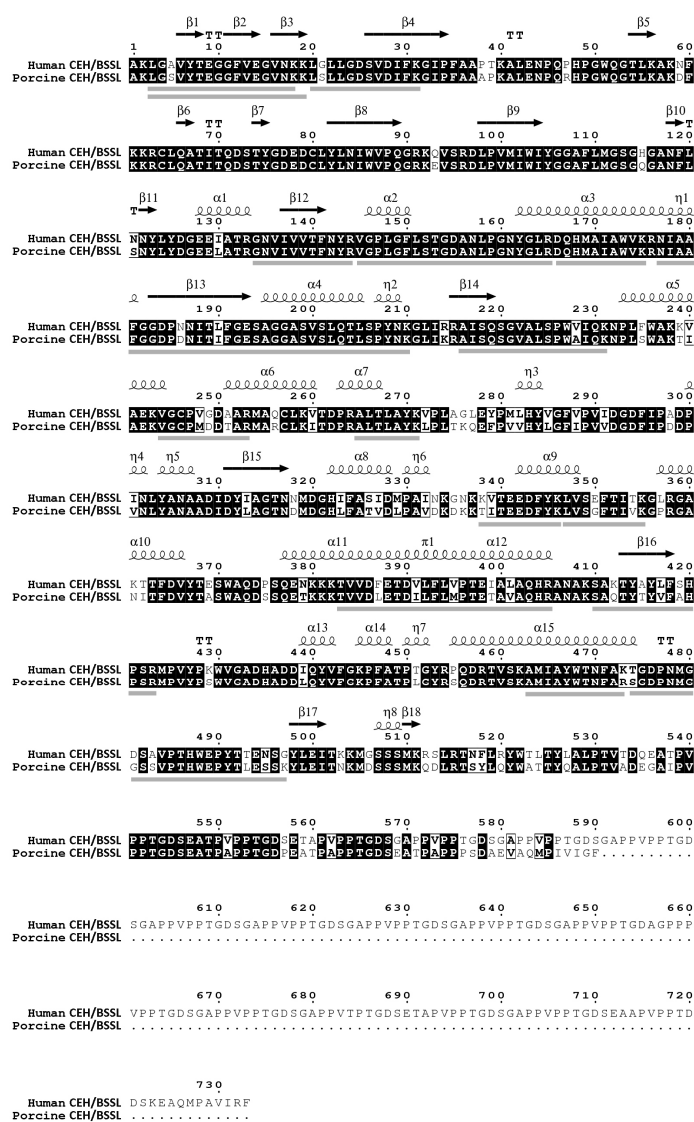


Figure 8: Protein sequence alignment of human (UniProtKB/Swiss-Prot: P54317) and porcine PLRP2 (NCBI Accession ID: NP_001177220). Sequence alignment and presentation were performed as indicated in Figure 7 legend. Secondary structure elements shown above the sequence alignment were obtained from the known 3D structure of human PLRP2 (PDB: 2OXE; [78]). The peptides of porcine PLRP2 identified by PMF are underlined by a grey line.

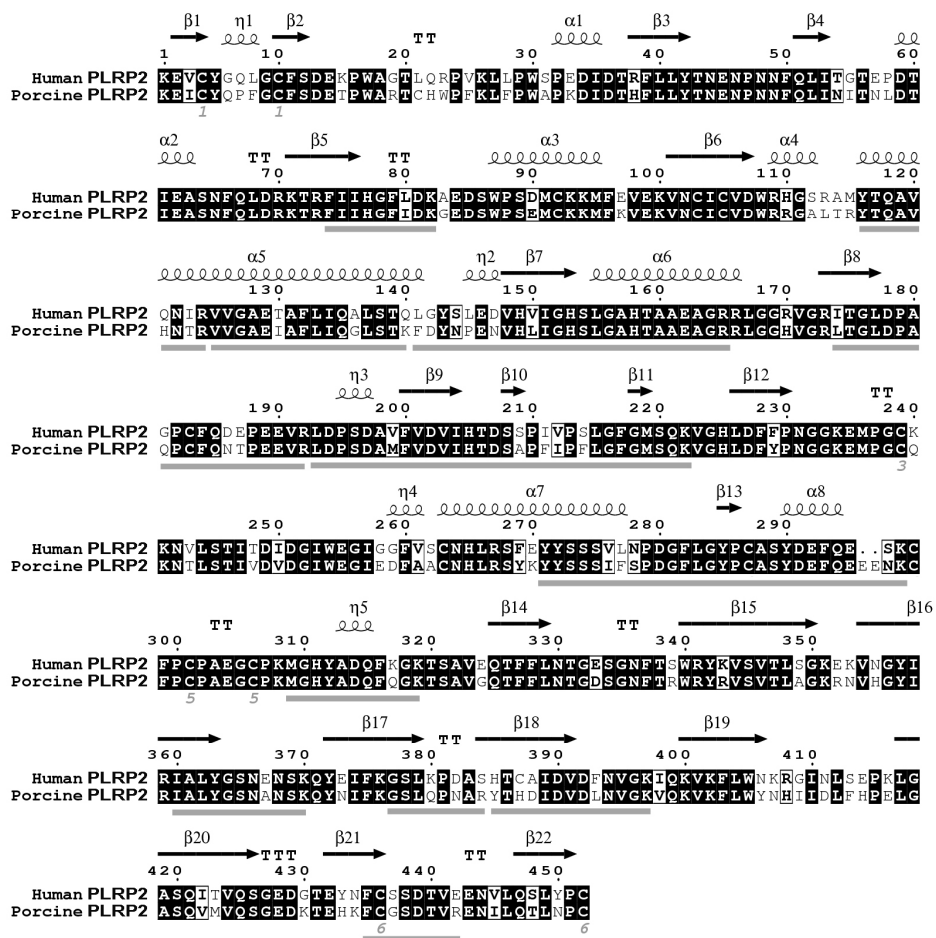


Table 1: Lipolytic activities (U/mg) of HPJ, PPJ and PPE. Lipase activities on TC4 and olive oil (USP assay) were performed at pH 8 and 9, respectively, in the absence and presence of additional colipase. Phospholipase activity on egg PC, galactolipase activity on C8-MGDG and cholesterol esterase activity on cholesterol acetate were measured at both pH 6 and 8. All enzyme solutions were prepared in the presence of protease inhibitors, except for measuring the phospholipase activities of HPJ and PPJ that required an activation by addition of trypsin (see section 2.3). Values are means \pm SD (n=3). 1 U = 1 μ mole FFA released per min. nd, not determined. Lipase activity on olive oil, phospholipase, cholesterol esterase and galactolipase activities are also expressed in percentage of lipase activity on tributyrin (values in brackets).

Assay	pH	colipase	HPJ	PPJ	PPE
Lipase activity on tributyrin	8	- col	228.3 \pm 2.9	231.7 \pm 2.9	67.7 \pm 0.6
	8	+ col	231.0 \pm 2.7	231.7 \pm 2.9	81.7 \pm 2.1
Lipase activity on olive oil (USP)	9	- col	81.3 \pm 2.0	70.7 \pm 2.3	23.3 \pm 0.6
	9	+ col	80.0 \pm 1.2 (34.6 %)	72.7 \pm 1.2 (31.4 %)	25.3 \pm 0.6 (31.0 %)
Phospholipase activity	6		42.4 \pm 1.1 (18.4 %)	nd	nd
	8		16.2 \pm 0.3 (7.0 %)	46.0 \pm 0.9 (19.9 %)	4.3 \pm 0.2 (6.4 %)
Cholesterol esterase activity	6		2.83 \pm 0.02 (1.2 %)	0.82 \pm 0.02 (0.3 %)	0.23 \pm 0.01 (0.3 %)
	8		1.40 \pm 0.04 (0.6 %)	1.21 \pm 0.02 (0.5 %)	0.39 \pm 0.01 (0.5 %)
Galactolipase activity	6		8.40 \pm 0.01 (3.6 %)	1.42 \pm 2.89 (0.6 %)	0.28 \pm 0.01 (0.3 %)
	8		7.73 \pm 0.20 (3.3 %)	1.78 \pm 0.02 (0.8 %)	0.31 \pm 0.01 (0.4 %)

Table 2: Cholesterol esterase activities of purified lipases measured with the pHstat technique and cholesterol acetate-sodium taurocholate micelles as substrate. HCEH/BSSL, native human pancreatic carboxyl ester hydrolase (or bile salt-stimulated lipase); PPL, native porcine pancreatic lipase; rDGL, recombinant dog gastric lipase; YLLIP2, LIP2 lipase from *Yarrowia lipolytica*; rHPLRP2, recombinant human pancreatic lipase-related protein 2; rGPLRP2, recombinant guinea pig pancreatic lipase-related protein 2. All assays were performed at 37°C. Values are means \pm SD (n=3).

Enzyme	pH	Specific activity (U/mg)
HCEH / BSSL	6	513 \pm 12
	8	53 \pm 11
PPL	6	0.7 \pm 1.1
	8	1.3 \pm 2.3
rDGL	5	0
	6	0
	8	0
YLLIP2	6	0
	8	0
rHPLRP2	6	18.7 \pm 1.1
	8	3.7 \pm 0.6
rGPLRP2	6	3.7 \pm 0.6
	8	0

Table 3: Identification by LC-MS/MS of lipases and colipase present in PPJ and PPE. All data obtained for individual gel filtration fractions were merged to display the maximal number of peptides and sequence coverage for each protein identified in PPJ and PPE. For protein identification, MS spectra were processed using *Sus scrofa domesticus* (pig) NCBI database (Taxonomy ID 9823, 57526 sequence entries). PSM, peptide spectral matched; AA and MW, number of amino acids and molecular masses deduced from the complete protein sequence in database.

Source	NCBI accession number	Name	Coverage (%)	Number of peptides	PSM	Unique Peptides	AA	MW [Da]
PPJ	295444923	pancreatic triacylglycerol lipase precursor (PPL)	75	29	732	6	465	51,648
	218847752	pancreatic lipase-related protein 1 precursor (PLRP1)	56	23	607	21	467	51,660
	204307484	pancreatic lipase-related protein 2 (PLRP2)	39	12	33	11	471	53,165
	1191811834	carboxyl ester hydrolase - bile salt-stimulated lipase (CEH-BSSL)	37	16	56	16	610	66,214
	47523482	colipase precursor (COL)	48	3	6	3	112	12,132
PPE	295444923	pancreatic triacylglycerol lipase precursor (PPL)	22	8	9	2	465	51,648
	47523482	colipase precursor (COL)	50	5	18	5	112	12,132

Table 4: Comparison of HPJ, PPJ and PPE for a common basis of 25,000 USP lipase units. Values are enzyme units (1 U = 1 μ mole FFA released per min), estimated from the data in **Table 1** and the following assay conditions: Lipase activity on olive oil (USP assay) measured at pH 9, in the absence of colipase; Lipase activity on tributyrin measured at pH 8, in the presence of additional colipase; Phospholipase, galactolipase and cholesterol esterase activities measured at pH 8 for PPE and PPJ and pH 6 for HPJ.

Assay	HPJ	PPJ	PPE
Lipase activity on olive oil (USP)	25000	25000	25000
Lipase activity on tributyrin	71033	81931	87661
Phospholipase activity	13038	16266	4614
Cholesterol esterase activity	870	428	418
Galactolipase activity	2583	629	333