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Enzymes involved in lipid digestion

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Lipid digestion is a complex process that takes place at the lipid-water interface and involves various lipolytic enzymes present predominantly in the stomach and the small intestine (Carey, Small, & Bliss, 1983). These enzymes catalyse the hydrolysis of a variety of dietary lipids from animal and plant sources, such as triacylglycerols (TAGs), phospholipids, galactolipids, cholesterol and vitamin esters. They include gastric lipase, colipase-dependent pancreatic lipase, pancreatic lipase-related proteins 2 (PLRP2), carboxyl ester hydrolase or bile salt-stimulated lipase (CEH, BSSL), and pancreatic phospholipase A2. A debate still exist about the existence of a lingual lipase in human (Stewart et al., 2010; Kulkarni & Mattes, 2014; Brignot & Feron, 2019), an enzyme that has been demonstrated to be present and active in rat and mice tongue only and which is the product of a gene ortholog (Docherty et al., 1985) to the gene of gastric lipase (Bodmer et al., 1987) in humans and many other species. Bakala N’Goma et al. (Bakala N’Goma, Amara, Dridi, Jannin, & Carriere, 2012) have reviewed the key findings that support the existence of lingual or gastric lipases in several species in term of gene expression, enzyme immunocytolocalization and lipase activity. So far, no supporter of the existence of a lingual lipase in humans has been able to provide similar data.

Contrary to the other major digestive enzymes, i.e. proteases and amylases, lipases act at the lipid-water interface because their substrate is insoluble in water (Aloulou et al., 2006; Reis, Holmberg, Watzke, Leser, & Miller, 2009; Reis, Watzke, Leser, Holmberg, & Miller, 2010). This characteristic makes the development of kinetic models challenging and the usual Michaelis-Menten model no longer applies (Panaiotov & Verger, 2000). As for any enzyme, the rate of lipase activity depends on the initial quantity and type of substrate, the temperature, the pH, but a key parameter is the accessible surface available for lipase adsorption and activity. Indeed, activity increases with the rate of emulsification and the decrease in lipid droplet size, which corresponds to a larger specific surface (Benzonana & Desnuelle, 1965). The accumulation of lipolysis products and/or surfactants at the interface can however interfere with lipase activity in various ways, including enzyme inhibition or activation. In other words, lipolysis can be affected by environmental conditions and compounds that can alter the structure and/or the integrity of the interface (e.g. surface-active molecules such as proteins, phospholipids and bile salts) (Carriere et al., 1991; Embleton & Pouton, 1997; Reis, Watzke, Leser, Holmberg, & Miller, 2010). Besides these parameters, the form in which the lipids are delivered to the GI tract, including the food matrix, also impact the rate and extent

of lipolysis. This particular topic is reviewed in chapters 3.1, 3.2 and 3.3. One important point to consider is that gastrointestinal lipolysis is a dynamic process that starts at the lipid-water interface and progressively proceeds through enzyme reactions on substrates with higher polarity and interaction with water (some lipolysis products like monoacylglycerols (MAGs), phospholipids). The enzymes involved in the steps subsequent to TAG and diacylglycerol (DAG) hydrolysis (carried out by enzymes with high affinity for lipid-water interfaces, the so-called true lipases) have usually a higher affinity for substrates forming mixed micelles and dispersed in the water phase.

This chapter gives a brief overview of the main dietary lipids from both animal and plant origins. Then, the lipolytic enzymes present in the human gastrointestinal (GI) tract and their structure/function relationship are described. Finally, the kinetic models and the sources of enzymes used in vitro models of digestion are discussed.

1- Dietary lipids

Lipids, and in particular TAGs, represent the most condensed form of energy available in biological tissues (Gibbons, Islam, & Pease, 2000). Outside of cell membranes, lipids are stored in organelles called lipid bodies, oil bodies or oleosomes in plant seeds, that are present in different cell types. These lipid bodies have a spherical shape and are composed of a core of neutral lipids (TAGs and cholesterol esters) surrounded by a monolayer of phospholipids, cholesterol and proteins (Huang, 1994), as well as other polar lipids in plants and algae. In all organisms, the metabolic pathways responsible for the formation of lipid bodies and their mobilization through hydrolytic reactions have strong similarities. Detailed reviews of lipid bodies in mammals, plants and microorganisms have been published (Murphy & Vance, 1999; Zweytick, Athenstaedt, & Daum, 2000; Alvarez & Steinbüchel, 2002).

Membrane lipids

Eukaryotic cells are compartmentalized into organelles (e.g. endoplasmic reticulum, Golgi apparatus, and mitochondria) that are delimited by membranes with different biological functions.

These membranes are essentially made up of phosphoglycerolipids, with the exception of plant photosynthetic membranes that contain large amounts of galactoglycerolipids. This is the case, for instance, in the plasma membrane which delimits a cell and the endomembrane

system which consists of different organelles (e.g. lysosomes and endosomes) dispersed in the cytoplasm of a eukaryotic cell (Lipowsky, 1995). Phosphoglycerolipids are consisting of two fatty acid residues esterifying a glycerol residue which is itself esterified by a phosphate residue. They are lipids organized around a glycerol-3-phosphate residue, unlike triglycerides which do not contain a phosphate group but are instead formed of three fatty acid residues esterifying a glycerol residue. In contrast to triglycerides, phosphoglycerides are highly amphiphilic molecules, i.e. consist of a hydrophilic polar head (the phosphate group and its substituents, such as choline, ethanolamine, glycerol or inositol) and hydrophobic aliphatic tails (the fatty acid residues).

Other than phosphoglycerides, membranes also contain other lipids such as sphingolipids and sterols, the latter are mainly represented in animals by cholesterol (McMullen, Lewis, & McElhaney, 2004). Biological membranes are organized around a lipid bilayer consisting of two sheets of lipid molecules in which the phosphoglycerides are oriented to present their hydrophilic heads in contact with water and their hydrophobic tails in contact with the other sheet.

Finally, in plants and photosynthetic organisms in general, membranes, such as those of thylakoids present in chloroplast, are mainly composed of galactolipids (Amara et al., 2010). The main galactolipids are monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG) and sulfoquinovosyldiacylglycerol (SQDG).

In almost all living organisms, the cell membrane consists of a lipid bilayer, as do the membranes surrounding the cell nucleus and organelles (MacCallum & Tieleman, 2008). Enveloped viruses also have a membrane around which they surround themselves during exocytosis. The lipid bilayers are impermeable to ions and hydrophilic molecules, allowing the cells to regulate the pH and salinity of their cytosol with the help of transmembrane proteins that act as membrane transporters capable of generating and maintaining a concentration gradient of various chemical species between the cytoplasm and the extracellular medium. These bilayers also tend to close on themselves (budding) to form vesicles. Depending on the nature and composition of the lipids, their interactions can lead to the formation of a distinct phase that separates from water (e.g. oil droplet and emulsions), micelles, and lamellar phases including liposomes or membrane surfaces (Le Maire, Champeil, & Moller, 2000).

Storage lipids

In mammals, lipids are found in various forms: constitutional lipids which are the primordial cellular constituents of membranes, represent 10% of the dry weight of tissues, serve to protect vital organs (heart, liver, kidneys, spleen, brain and spinal cord), circulating lipids which appear in the blood in the form of lipoproteins, and then disappear at the end of digestion to settle in the liver and other tissues, and reserve lipids which accumulate in adipose tissue and are very important in the production of energy.

Adipose tissue is made up of fat cells called adipocytes, which are animal cells that specialize in energy storage. They contain more than 95% of the body's TAGs (Coppack, Jensen, & Miles, 1994). They store lipids up to a critical cell size between 70 and 120 μm . Beyond this maximum size, the adipocyte, is no longer able to store fat, and divides, leading to the formation of a new adipocyte (mitosis process). Once mobilized by lipolysis, the TAGs represent an energy supply for the other organs and in particular the muscle tissue (Coelho, Oliveira, & Fernandes, 2013). Adipose tissue is the most important site for storing metabolic energy. In mammals, there are 2 types of adipocytes: white and brown (Tiraby & Langin, 2003).

White adipose tissue represents one of the body's most important energy reserves, it is the main tissue responsible for the storage of TAGs, while brown adipose tissue is involved in the conversion of TAGs into heat (thermogenesis). When the carbohydrate reserves are depleted (in case of fasting, physical exertion, fighting the cold, etc.), or unusable (in case of severe diabetes), the body calls upon the reserves of white adipose tissue to provide the organs with the necessary energy substrates.

Brown fat is involved in thermogenesis through lipolysis of the TAGs of the adipocytes. Unlike white adipocytes, which contain a single lipid droplet per cell, brown adipocytes contain many smaller droplets and a much higher number of mitochondria that contain iron, giving the tissue its brown colour. Brown adipose tissue also contains more capillaries than white adipose tissue because it has a higher oxygen requirement than most other tissues (Murphy & Vance, 1999).

Small lipid bodies are also detected in different types of tissues, such as liver, muscles, heart, kidneys, small intestine, mammary glands, cultured fibroblasts and macrophages. Lipid droplets rich in cholesterol esters are also detected in steroidogenic cells of the adrenal cortex and in the testes and ovaries (Freeman & Ascoli, 1982; Gwynne & Strauss, 1982).

These lipid bodies, rich in cholesterol esters, provide a source of cholesterol that can be used, for example, for the synthesis of steroid hormones (Freeman & Ascoli, 1982; Gwynne & Strauss, 1982; Bisgaier, Chanderbhan, Hinds, & Vahouny, 1985) or membrane biogenesis (Nagy & Freeman, 1990). In specific cells such as liver stellate cells, which are rich in retinol esters, lipid bodies contain significant amounts of vitamin A (Azais-Braesco et al., 1995).

Lipid globules in breast milk are another form of transient storage and energy transport. The lipid content of breast milk can vary from 0.2% to 61% (w/w). These lipid globules (0.2 to 2 μm in diameter) are formed mainly by a hydrophobic core (triglycerides, esterified cholesterol and fat-soluble vitamins) surrounded by a triple layer membrane made of proteins and phospholipids, and resulting from the exocytosis of lipid droplets during native milk production by the cells of the mammary gland (Bourlieu & Michalski, 2015). After processing (pasteurization and homogenization), the phospholipids surrounding milk droplets can be re-organized in monolayers (Patton & Keenan, 1975; Michalski, 2009).

In vertebrates, lipoproteins are essential for lipid transport to the different tissues of the body. These include chylomicrons (CM) synthesized by the enterocyte during digestion and very low density lipoproteins (VLDL) synthesized by the liver (Cardin et al., 1984; Hobbs, Brown, Russell, Davignon, & Goldstein, 1987; Leitersdorf, Tobin, Davignon, & Hobbs, 1990; Hobbs, Brown, & Goldstein, 1992; Simard et al., 1994). Lipoproteins are large complexes of proteins and lipids, the outer membrane of which is a monolayer of phospholipids and free cholesterol with one or more proteins called apolipoproteins (e.g., Apo-A, Apo-B, etc.); the core of lipoproteins contains TAGs, cholesterol esters and small amounts of other hydrophobic substances, such as fat-soluble vitamins. CM and VLDL are primarily hydrolyzed by lipoprotein lipase (LPL), while the other lipoproteins, low density lipoproteins (LDL) and high density lipoproteins (HDL), are preferentially hydrolyzed by hepatic lipase (HL) and endothelial lipase (EL) (Nykjaer et al., 1993; Rader & Jaye, 2000; Jansen, 2004).

2- Lipolytic enzymes in the human GI tract

True lipases acting at the lipid-water interface

These water-soluble enzymes show a high affinity for lipid-water interfaces and are essential for initiation the lipolysis of dietary TAG. In the GI tract, gastric and pancreatic lipases belong to this category of lipolytic enzymes.

Gastric lipase

Human gastric lipase (HGL) is produced in the fundic mucosa of the stomach (Moreau, Laugier, Gargouri, Ferrato, & Verger, 1988) and is co-located with pepsinogen in the chief cells of the fundic glands (Moreau et al., 1989). HGL is a highly glycosylated, 50 kDa serine enzyme with an α/β hydrolase fold (Carriere et al., 1994; Roussel et al., 1999). Several signals trigger the secretion of gastric lipase such as stomach movements, cholinergic stimuli (Carriere et al., 1992), test meals (Carriere, Barrowman, Verger, & Laugier, 1993) and the gastrin gastrointestinal hormone gastrin, which also stimulates the secretion of pepsinogen and gastric acid (Szafran, Szafran, Popiela, & Trompeter, 1978; Ville, Carriere, Renou, & Laugier, 2002). Gastric lipase is active and stable in the acidic environment of the stomach, where the gastrointestinal lipolysis of dietary fat is initiated (Carriere, Barrowman, Verger, & Laugier, 1993; Ville, Carriere, Renou, & Laugier, 2002; Aloulou & Carriere, 2008), although HGL can potentially hydrolyze all esters bonds in a TAG molecule in vitro (Carriere et al., 1991). Nevertheless, in vivo, MAGs are rarely observed in gastric contents because the gastric lipolysis reaction by HGL is rapidly inhibited by reaction products such as FFA (Gargouri et al., 1986b; Pafumi et al., 2002). Gastric lipase has high levels of activity on TAG (**Figure 1**), to a lesser extent on DAG, and its activity on MAG is generally very low compared to its activity on TAG (Fernandez et al., 2007; Fernandez et al., 2008). Gastric lipase also hydrolyzes polyethylene glycol (PEG) mono- and di-esters and is one of the most active lipases on mixtures of acylglycerols and PEG esters such as the pharmaceutical lipid-based excipients Gelucire[®] 44/14 and Labrasol[®] (Fernandez et al., 2007; Fernandez et al., 2008; Fernandez et al., 2009; Fernandez et al., 2013). However, HGL does not hydrolyze cholesterol esters or phospholipids (**Figure 2**) (Carriere et al., 1991).

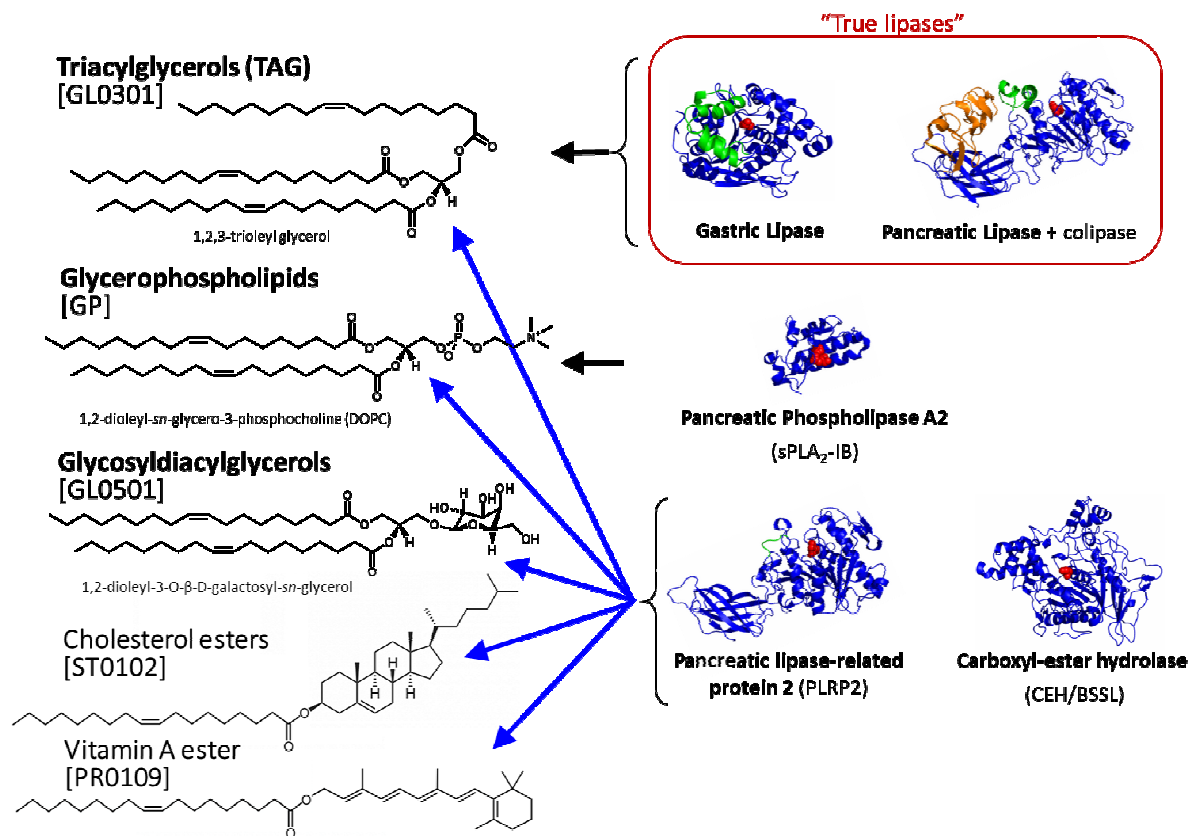


Figure 1: Chemical structures of the main lipid classes found in human diet and gastrointestinal lipolytic acting on these lipids. Numbers below the substrate types correspond to the Lipid Maps Classification system (see <https://www.lipidmaps.org/>)(Fahy et al., 2005; Fahy et al., 2009)

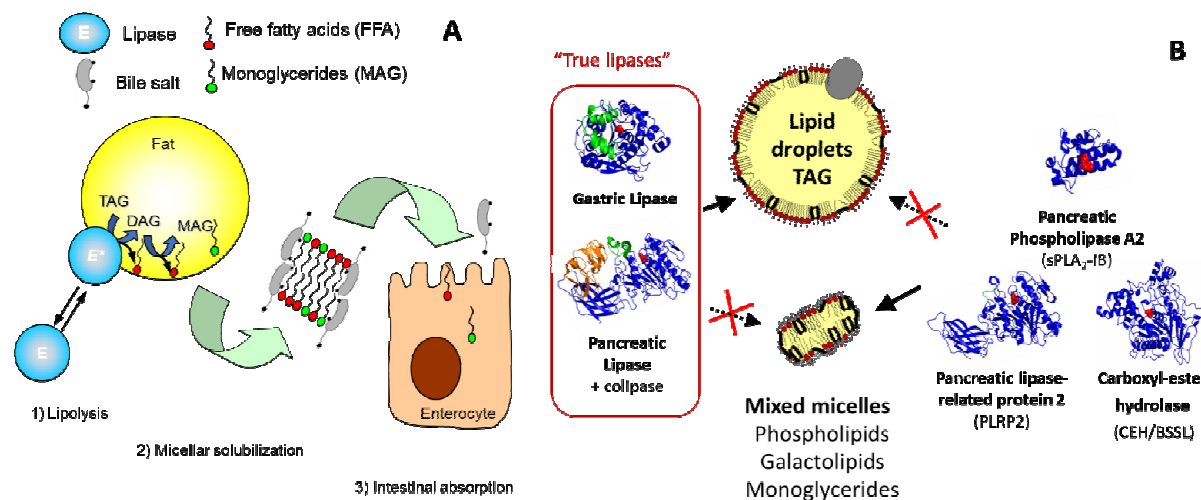


Figure 2: Schematic representation of the essential steps of gastrointestinal lipolysis and intestinal fat absorption (A), including 1) the lipolysis reaction after lipase adsorption at the oil-water interface, 2) the micellar solubilisation of lipolysis products, 3) the intestinal absorption of lipolysis products by enterocytes), and substrate specificity of the various lipolytic enzymes found in the GI tract (B). Two main categories of lipolytic enzymes can be defined, with the so-called “true lipases” showing a preference for water-insoluble substrates (TAG, DAG) and acting preferentially at the oil-water interface and the enzymes showing a preference for substrates forming mixed micelles with bile salts and dispersed in the aqueous phase like phospholipids, galactolipids and monoglycerides. Adapted from (Carriere, Thirstrup, Hjorth, & Boel, 1994; Mateos-Diaz et al., 2018a).

Gastric lipase shows stereopreference for the hydrolysis of the ester bond at the sn-3 position of TAG, that has been demonstrated both in vitro and in vivo (Carriere et al., 1997). This enzyme is therefore tailored to release the short and medium chain fatty acids found at this position in milk TAG (Roman et al., 2007).

In vivo, the level of intragastric lipolysis of TAG ranges from 10% of total TAG acyl chains (normal solid-liquid meal) to 25% (emulsified liquid meal), and DAG and FFAs are primarily generated in the stomach by gastric lipase (Carriere, Barrowman, Verger, & Laugier, 1993; Carriere et al., 2001; Carriere et al., 2005). During a meal, HGL is always present and stable

in the duodenal contents, and can therefore contribute to intestinal lipolysis (Carriere, Barrowman, Verger, & Laugier, 1993) (**Figure 3**). Overall, it has been estimated that HGL can release 1 acyl chain out of the four that have to be released for the intestinal absorption of 2 TAG molecules in the form of MAG (Carriere, Barrowman, Verger, & Laugier, 1993). This is the reason why it is often mentioned in literature that HGL can contribute to as much as 25% of fat digestion. Studies in patients with severe exocrine pancreatic insufficiency and no significant levels of pancreatic lipase have confirmed that HGL alone can achieve around 30% of dietary TAG digestion (Carriere et al., 2005).

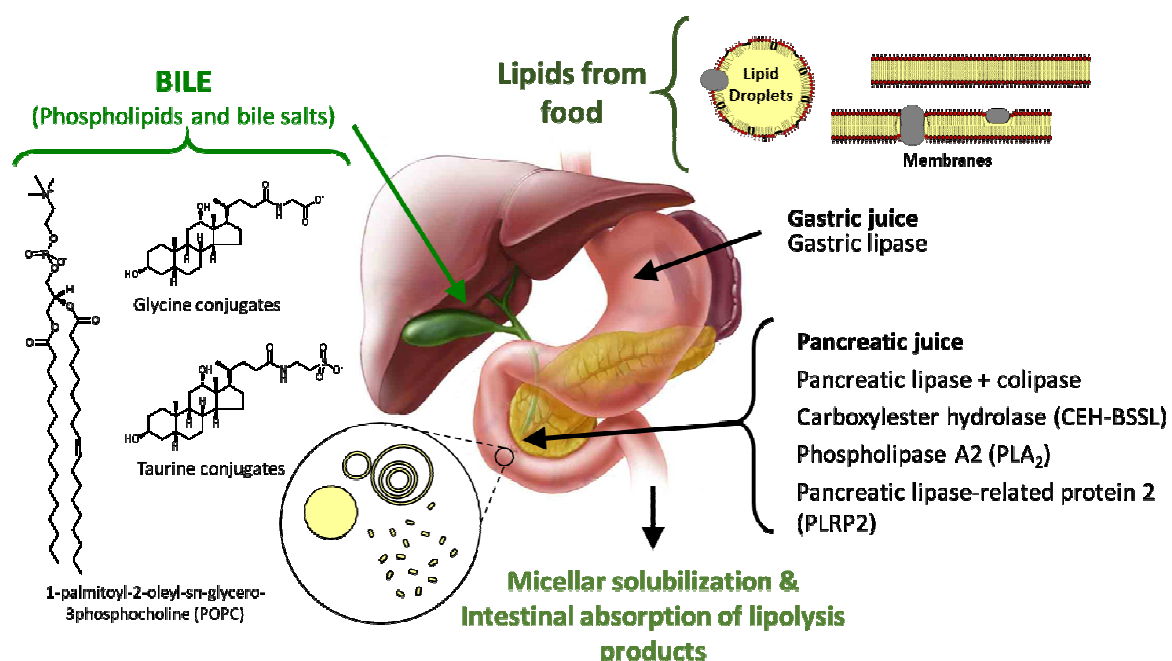


Figure 3: *Physiology of gastrointestinal lipolysis.*

Pancreatic lipase and colipase

Quantitatively, human pancreatic lipase (HPL) is the major lipase involved in the lipolysis of dietary fat. In the small intestine, HPL acts in the presence of bile with its specific protein cofactor colipase acting as an anchor for the enzyme at the oil-water interface and counteracting the competitive inhibitory effects of bile salts (**Figure 3**). The biochemical, structural and physiological properties of pancreatic lipase have been extensively reviewed (Verger, 1984; Carriere et al., 1998). HPL is a 50.5 kDa glycosylated serine hydrolase, it is synthesized by the acinar cells of the pancreas in the form of an active enzyme and not as an

inactive zymogen like most pancreatic enzymes. It is delivered into the intestinal lumen via the pancreatic duct.

In vitro, HPL alone can act on TAG emulsions (**Figure 1**), but hydrolysis of TAG is inhibited in the presence of bile salts that compete with lipase for adsorption at the lipid-water interface (Bezzine, Carriere, De Caro, Verger, & De Caro, 1998). In vivo, HPL therefore requires its specific 10 kDa colipase cofactor, also produced by the pancreas, to counteract the effects of the bile salts and hydrolyze TAGs. Pancreatic lipase also acts on DAG, which actually could be its major substrate after the action of gastric lipase and ultimately produces 2-MAG together with FFA. When DAG serves as an initial substrate for HPL in vitro, the activity measured is usually lower than the activity on TAG (Eydoux et al., 2007; Fernandez et al., 2008). However, when the relative hydrolysis rates of TAG and DAG are estimated in the course of TAG hydrolysis followed by the subsequent hydrolysis of the generated DAG, the rate of DAG hydrolysis was found to be higher than that of TAG (Mitchell, Carriere, & Krieger, 2008). HPL is 1,3-regioselective lipase tailored for efficiently producing 2-MAG from TAG. It is not active on 2-MAG, is weakly active on medium-chain 1(3)-MAGs and is inactive on long-chain 1(3)-MAGs. HPL is not active on phospholipids (**Figure 2B**) (Thirstrup, Verger, & Carriere, 1994; Carriere et al., 1998), while phospholipase A1 activity has been reported for porcine pancreatic lipase (de Haas GH et al. 1965). HPL has very low levels of activity on PEG mono- and di-esters, and is completely inactive on mixtures of these esters and acylglycerols, such as Labrasol[®] and Gelucire[®] 44/14 (Fernandez et al., 2007; Fernandez et al., 2008). HPL has no activity on cholesterol esters (Ali et al., 2005) or galactolipids (Andersson, Carriere, Lowe, Nilsson, & Verger, 1996; Amara et al., 2009). Pancreatic lipase has been extensively studied for many years, however, some basic data such as the amount of HPL produced during a meal and its specific activity on test meal TAG had to wait for clinical studies to be obtained (Carriere, Barrowman, Verger, & Laugier, 1993; Carriere et al., 2000).

Other lipolytic enzymes present in pancreatic secretion

Pancreatic lipase-related protein 2 (PLRP2)

Two pancreatic lipase related proteins (PLRP1 and PLRP2) are produced by the pancreas. Human PLRP1 and PLRP2 have 65% and 68% identity respectively with classical pancreatic lipase HPL (Giller, Buchwald, Blum-Kaelin, & Hunziker, 1992) and a highly conserved catalytic triad (Ser, Asp, His). The three-dimensional structures of these three proteins are

superimposable in their structural domains, but differ at the level of the lid that controls access to the active site for PLRP2 and at the level of steric hindrance in the vicinity of the active site for PLRP1 (Winkler, Darcy, & Hunziker, 1990; Roussel et al., 1998; Eydoux et al., 2008). As a consequence, both native and recombinant HPLRP1 do not exhibit lipolytic activity and its eventual contribution to lipid digestion is still unknown.

PLRP2 exhibits lipolytic activity on various substrates including TAGs, phospholipids (phospholipase A1 activity), galactolipids and cholesterol esters (**Figure 1**). The lipase activity of PLRP2 is inhibited by micellar concentrations of bile salts and is not reactivated by colipase, suggesting that PLRP2s does not have lipase activity in vivo but may preferentially act as a phospholipase (Thirstrup, Verger, & Carriere, 1994) or a galactolipase (Sias et al., 2004) (**Figure 2B**). Indeed, PLRP2 also has a high level of galactolipase activity on both MGDG and DGDG (Andersson, Carriere, Lowe, Nilsson, & Verger, 1996). It has been established that human pancreatic juice contains at least two enzymes that effectively hydrolyze DGDG, HPLRP2 and CEH have been identified as these galactolipases (Andersson et al., 1995). In addition, HPLRP2 is also active on lipophilic vitamin esters (Reboul et al., 2006), as well as on cholesterol esters (Andersson, Hernell, Bläckberg, Falı́, & Lindquist, 2011; Salhi et al., 2020) and is one of the most active monoacylglycerol lipase identified so far (Eydoux et al., 2007; Eydoux et al., 2008). The broad substrate specificity of HPLRP2 is also illustrated by the fact that this enzyme has significant activities on mixtures of acylglycerols and PEG esters such as Gelucire[®] 44/14 and Labrasol[®] (Fernandez et al., 2007; Fernandez et al., 2008). Cholesterol esterase activity is the most recent activity attributed to both recombinant human (rHPLRP2) and guinea pig lipase (rGPLRP2) PLRP2 (Salhi et al., 2020) because HPLRP2 has been described as having cholesterol esterase activity (Bakala-N'Goma, Amara, Dridi, Jannin, & Carriere, 2012) (**Figure 1**). They confirmed a low cholesterol esterase activity for the latter (18.7 ± 1.1 U/mg at pH 6) and a lower activity for GPLRP2 (3.7 ± 0.6 U/mg at pH 6).

Carboxyl ester hydrolase - bile salt-stimulated lipase (CEH/BSSL)

Carboxyl ester hydrolase (CEH, EC 3.1.1.1) is a non-specific esterase with different names: bile salt-stimulated lipase (BSSL) (Hernell & Olivecrona, 1974; Lombardo, Fauvel, & Guy, 1980), cholesterol esterase (Mueller, 1915; 1916), carboxyl ester lipase (CEL) (Rudd, 1984) and lysophospholipase (van den Bosch, Aarsman, De Jong, & Van Deenen, 1973). In humans CEH/BSSL, which is secreted by acinar cells of the exocrine pancreas, is present in pancreatic juice (Figarella, 1966; Guy & Figarella, 1981). It is also produced by the mammary glands,

present in breast milk and is often referred to as BSSL in this context (Hernell & Olivecrona, 1974; Blackberg, Angquist, & Hemell, 1987). Like PLRP2, CEH-BSSL hydrolyzes various substrates *in vitro* like cholesterol esters, TAG, MAG, vitamin (A, E) esters, and phospholipids (Hyun et al., 1969; Erlanson, 1975; Lombardo, Fauvel, & Guy, 1980; Lombardo & Guy, 1980; Lynn, Chuaqui, & Clevette-Radford, 1982).

CEH-BSSL also hydrolyses carotenoid esters (lutein and capsanthin diesters, esters of β -cryptoxanthin)(Breithaupt, Bamedi, & Wirt, 2002), galactolipids (monogalactosyldiglycerides) (Amara et al., 2009) and PEG mono- and di-esters (Fernandez et al., 2007; Fernandez et al., 2008; Fernandez et al., 2010). Recently, a new potentiometric test has been developed to measure cholesterol esterase activity using cholesterol acetate as substrate (Salhi et al., 2020). To measure cholesterol esterase activity, the preparation of the substrate is not straightforward as cholesterol esters are highly apolar and oil-soluble. Other lipids, such as TAGs and phospholipids, have generally been used to allow the dispersion in water of small lipid aggregates containing cholesterol esters. Since most cholesterol esterase enzymes also have lipase and phospholipase A1 (PLA1) activities, they can also act on the TAGs or phospholipids present in the reaction mixture, releasing fatty acids from these substrates and it is therefore impossible to estimate the cholesterol esterase activity by titration of the released fatty acids alone. Long-chain cholesterol esters also have a low affinity for bile salt micelles and cannot be dispersed in mixed micelles like phospholipids and galactolipids. This problem was solved by a short-chain cholesterol ester which can be dispersed in water using bile salt micelles. Dispersions of cholesterol acetate (CholA) and sodium taurocholate (NaTC) were found to be good substrate for both pancreatic CEH and PLRP2. (Salhi et al., 2020). The specific activity of human CEH/BSSL measured under these conditions was found to be the highest recorded with cholesterol esters (Borgstrom, Dahlqvist, Lundh, & Sjovall, 1957; Benzonana & Desnuelle, 1965; Nunes & Corring, 1979). CEH-BSSL has no regiospecificity on TAG and can hydrolyse all three ester bonds, whatever their position on the glycerol backbone (Wang et al., 1983). The physiological role of CEH is still subject to debate (Howles, Carter, & Hui, 1996), but it seems likely that CEH plays a major role in the digestion of cholesterol esters and lipophilic vitamins in adults, and probably in that of acylglycerols in newborn children (Hernell & Bläckberg, 1994).

Its contribution to the lipolysis of 2-MAG seems to be physiologically relevant only during the lactation period in humans (Hernell & Bläckberg, 1982; Bernback, Blackberg, & Hernell, 1990). *In vitro* bile salt-stimulated lipase alone cannot hydrolyze native milk fat globule TAG,

but low rates of hydrolysis by gastric lipase trigger the hydrolysis of milk by pancreatic lipase and bile salt-stimulated lipase. HGL and HPL hydrolyze about two thirds of the total ester bonds present in milk, generating MAG and FFA, and adding bile salt-stimulated lipase results in the hydrolysis of MAG. The concerted action of these three lipases results in the complete digestion of milk TAG, generating free glycerol and FFA as the end-products.

Pancreatic phospholipase A2

Pancreatic phospholipase A2 (PLA2; phosphatide 2-acylhydrolase, EC 3.1.1.4) is involved in the digestion of dietary phospholipids. PLA2 is a stereospecific enzyme which catalyzes the hydrolysis of the sn-2 fatty acyl ester bond of 3-sn-glycerophospholipids, generating FFA and 1-sn-lysophospholipids (de Haas, Sarda, & Roger, 1965). Pancreatic PLA2 belongs to Group IB of secretory low molecular weight phospholipases A2 (sPLA2-IB) (Dennis, 1994). It is mainly present in pancreatic secretion, but also in the smooth muscle cells of the lungs and vessels. sPLA2 are involved in many biological functions other than digestion, such as cell proliferation, contraction of smooth muscle in the airways and vessels, and in various inflammatory diseases such as rheumatoid arthritis, endotoxic shock, respiratory distress syndrome and certain types of cancer (Vadas & Pruzanski, 1986; Kishino et al., 1995). On the contrary to HGL, HPL, HPLRP2 and CEH-BSSL that are serine hydrolases with a Ser-His-Asp catalytic triad (Winkler, Darcy, & Hunziker, 1990; Roussel et al., 1999; Eydoux et al., 2008), sPLA2 has no catalytic triad, and the nucleophile required for cleavage of the ester bond is an activated water molecule which is hydrogen-bonded to a His residue, which in turn is hydrogen-bonded to an Asp residue (Thunnissen et al., 1990).

3- Main structural features of gastrointestinal lipases

Gastric lipase: lipase with a single domain

HGL is a globular protein consisting of a single polypeptide chain of 379 amino acids (Bodmer et al., 1987). The molecular weight of native HGL, purified from gastric juices, is approximately 50 kDa. This mass corresponds to the mass of the mature 379-amino acid polypeptide plus significant N-glycosylation. There are 4 major isoforms of human gastric lipase with isoelectric points between 6.8 and 7.4 (Moreau et al., 1992). In 1999, the three-dimensional structure of HGL in its closed form was resolved at 3.0 Å, following the production of the recombinant protein in insect cells using the baculovirus expression system. It revealed several structural elements (Roussel et al., 1999):

- A main globular domain with an α/β hydrolase fold, organized around a central structure composed of eight β -stands surrounded by six α -helices.
- A flap or lid (residues 210 to 267) composed of 58 amino acids, articulated around two α -helices (Roussel, et al., 1999) and controlling the accessibility to the active site. The flap reveals near the active site a large hydrophobic patch of 29 amino acids (residues 215-244), which may be involved in the binding of substrate (Miled et al., 2000).

Pancreatic lipases: lipases with two domains

Both HPL and HPLRP2 are composed of two domains: a N-terminal and globular catalytic domain, with an α/β hydrolase fold and a lid controlling the access to the active site as in HGL (van Tilbeurgh, Sarda, Verger, & Cambillau, 1992; van Tilbeurgh et al., 1993a; Eydoux et al., 2008; Mateos-Diaz et al., 2017), and a C-terminal domain involved in interactions with colipase and lipids. The C-terminal domain of pancreatic lipases has a sandwich structure containing an exposed hydrophobic loop (loop $\beta 5'$; residues 405-414) inserted between two strands $\beta 5$ and $\beta 6$. This structural domain has an important role in the interaction of lipase with its cofactor, colipase (van Tilbeurgh, Sarda, Verger, & Cambillau, 1992; van Tilbeurgh et al., 1993b). Structure-function studies have shown that the C-terminal domain also has an important role in the interaction with the lipid interface and has some homologies with C2 lipid binding domains (Bezzine, Carriere, De Caro, Verger, & De Caro, 1998; Chahinian et al., 2002). The $\beta 5'$ loop has a large accessible apolar surface area which is of the order of 877 \AA^2 . The hydrophobic (H), charged (C) and semi-polar (S) residues of the $\beta 5'$ loop have accessible surfaces of 488 \AA^2 , 105 \AA^2 and 284 \AA^2 respectively. The loop $\beta 5'$ is located on the same side of the enzyme as the hydrophobic loops surrounding the active site (lid and $\beta 9$ loop). In addition, the hydrophobic surface of the $\beta 5'$ loop is not masked after colipase fixation. In HPL, the $\beta 5'$ loop contributes with the open lid, the $\beta 9$ loop and the hydrophobic tips of colipase to the formation of a large hydrophobic plateau that may parallel the lipid-water interface (Mateos-Diaz et al., 2017).

HPLRP2 is organized in two domains similar to those found in HPL, except that the lid is found in an open conformation in the absence of surfactants and inhibitors (Eydoux et al., 2008). This conformation is different from the one observed in the open HPL, which probably explains differences in kinetic properties between HPLRP2 and HPL. Moreover, most of the amino acid residues of HPL involved in the interaction with colipase, both within the lid and the C-terminal domain, are mutated in HPLRP2 (Thirstrup, Verger, & Carriere, 1994), which explains why HPLRP2 has only a weak interaction with colipase (Sias et al., 2004). Indeed,

the lipase activity of HPLRP2 on TAG is inhibited by bile salts and is not restored by addition of colipase.

4- Important parameters governing the activity of gastrointestinal lipases

The role of bile salts

The influence of bile salts on the activity of lipases of different origins has been extensively studied *in vitro*. It has been established that, at low concentrations, bile salts have an activating effect on the hydrolysis of emulsified long-chain TAG by pancreatic (Borgstrom & Erlanson, 1973) and gastric (Gargouri et al., 1986a) lipases. In interpreting these results, it is generally accepted that bile salts, by lowering the tension at the water-substrate interface, prevent the interfacial denaturation of the enzyme. In the lipolysis reaction, the enzyme partitions between the aqueous phase and the interface (Verger & de Haas, 1976) (**Figure 2A**). It has been assumed that the enzyme adsorbed at the interface would exist in a conformational state with an open lid, in equilibrium with an inactive form with the closed lid remaining in the water phase (**Figure 2A**). It has been shown however that the lid can open in solution in the presence of bile salts with a maximum aperture in the presence of supramicellar concentrations of bile salts (Belle et al., 2007). Under physiological conditions, it is therefore expected that the lid opens in solution prior to HPL adsorption at the interface, which is supported by the exposition of a large hydrophobic plateau upon lid opening. The adsorption and activity of pancreatic lipase is however dependent on the presence of colipase, which anchors the lipase at the interface when the concentration of bile salts exceed its micellar concentration (Borgstrom & Erlanson, 1973; Bezzine et al., 1999). Contrary to HPL, HGL is not inhibited by micellar concentrations of bile salts and does require a cofactor (Lengsfeld et al., 2004). This is due to a higher tensioactivity of HGL, that has a high penetration capacity at the lipid-water interface, even in the presence of phospholipids (De La Fournière, Ivanova, Blond, Carrière, & Verger, 1994; Sams et al., 2017).

Bile salts have a dual impact on the activity of true lipases. On the one hand, they can impair lipase adsorption and activity. On the other hand, they can ensure the continuity of lipolysis by removing the reaction products from the oil-water interface by a process of micellar solubilisation. Concerning the other lipolytic enzymes acting on more polar lipids (phospholipids, galactolipids, MAG), bile salts have an essential role in the dispersion of these substrates in the aqueous phase in the form of mixed micelles (**Figure 2**) and the bile salt to substrate molar ratio is critical for the activities of HPLRP2, CEH-BSSL and PLA2

(Mateos-Diaz et al., 2018a; Mateos-Diaz et al., 2018b; Mateos-Diaz, Sutto-Ortiz, Sahaka, Rodriguez, & Carriere, 2018).

Gastric and duodenal pH variations

The variations in the gastric and duodenal pH which occur during digestion have been studied in several test meal experiments on healthy humans and patients with exocrine pancreatic insufficiency (Carriere, Barrowman, Verger, & Laugier, 1993; Carriere et al., 2001; Renou et al., 2001; Carriere et al., 2005). After the ingestion of a meal, the pH of the gastric content ranges between 5.5 and 7, depending on the composition of the meal ingested. Then, the gastric content is diluted by the gastric acid secretion and the pH decreases. Gastric emptying also contributes to this pH decrease by removing the meal components from the gastric contents and thus reducing their buffering effects. The intragastric pH value is found in the 4 to 5 pH range at half gastric emptying time (approx. 60 min) in healthy volunteers, which corresponds to the optimum pH conditions for HGL activity on TAG (Gargouri et al., 1986a; Lengsfeld et al., 2004).

The duodenal pH variations are less pronounced and are usually restricted to the 5 to 7 pH range, giving a mean value of 6.25 in healthy volunteers. These pH values also correspond to the optimum pH conditions for HPL activity on TAG in the presence of bile salts (Borgstrom, 1975; Lengsfeld et al., 2004). Thus, both HGL and HPL are tailored for acting optimally on TAG at the pH values found in the stomach and small intestine, respectively. In the case of HPLRP2 and CEH-BSSL, the optimum pH of activity depends on the type of substrate hydrolyzed and it is usually found in the neutral to slightly alkaline pH range (Eydoux et al., 2007). Both enzymes show however an optimum activity on cholesterol esters at pH 6 (Salhi et al., 2020).

The effects of calcium on lipolysis

Calcium contributes decisively to the catalytic activity of sPLA2-IB, since a calcium ion interacts with the phosphate group of the phospholipid substrate, with the catalytic water molecule and with the tetrahedral intermediate generated during the deacylation reaction (stabilization of the negative charge in the oxyanion hole) (Verheij & Dijkstra, 1994). Calcium may also contribute to the interactions of sPLA2 with lipid aggregates. A second calcium ion has been observed near the N-terminal part of the enzyme and the interfacial binding surface (i-face) in the 3D structure of bovine sPLA2-IB (Rajakannan et al., 2002), as well as in other sPLA2 (Scott et al., 1990). Finally, calcium may also contribute to the

interfacial organization of the phospholipid substrate itself by condensing the polar heads of negatively charged phospholipids (Sovago, Wurpel, Smits, Müller, & Bonn, 2007).

Therefore, when studying or using PLA₂, it is essential to monitor calcium concentration, whereas lipases do not have absolute calcium requirements. A calcium ion is found in the 3D structures of pancreatic lipases, but it is remote from the active site and interfacial binding sites and has probably only structural functions. However, some non-specific effects of calcium may be observed in lipase assays, as calcium can compensate for the electrostatic repulsion that occurs between the enzyme and a charged lipid-water interface. Calcium and FFAs may also form soaps during the lipolysis reaction, and lipase kinetics may be affected by the removal of FFAs from the lipid-water interface (Benzonana, 1968; Benzonana & Desnuelle, 1968; Verger, 1984).

5- Kinetic models of lipolysis and enzyme assays

When we speak of the Michaelis-Menten model, we are talking about homogeneous enzymology where the enzymatic reaction takes place in a homogeneous medium, i.e. in a single phase in which the enzyme and the substrate are soluble. For lipolytic enzymes acting essentially at the lipid-water interface (in a heterogeneous medium), we speak of heterogeneous enzymology and the classical Michaelian model of enzyme kinetics can no longer be applied. One then passes from a three-dimensional or volumetric system to a two-dimensional or surface system. In 1973, an interfacial kinetic model was proposed by Verger and de Haas (Verger & de Haas, 1973). According to this model, the enzymatic reaction takes place in two elementary stages:

- An adsorption step of the enzyme at the lipid interface (adsorption or penetration) (E: water soluble enzyme, E*: adsorbed enzyme). This step is governed by two rate constants: a constant of penetration (k_p) or adsorption of the enzyme at the lipid-water interface and a constant of desorption (k_d) of the enzyme. During this step, the enzyme may or may not undergo a conformational change.
- An actual catalytic step analogous to a Michaelis Menten pseudo-reaction that takes place on the surface and no longer in solution. All concentrations of the species involved in the reaction such as the enzyme (E and E*), the lipid substrate (S), the reaction product (P) and the enzyme/substrate complex (E*S) are expressed in surface density (mole.m⁻²) instead of volume concentration (mole.m⁻³). In order to differentiate soluble enzyme forms in the

aqueous phase (E) from those present at the interface, the latter are denoted with an asterisk (E*, E*S).

Kinetic phenomenon of interfacial activation

Lipases are water-soluble enzymes, which however have very low activity on water-soluble esters. Their activity becomes very high when the concentration of the substrate exceeds solubility and allows the formation of aggregates such as lipid droplets and thus the formation of a water-lipid interface (Sarda & Desnuelle, 1958; Ferrato, Carriere, Sarda, & Verger, 1997). This phenomenon is called interfacial activation, which distinguishes lipases from esterases acting only on water-soluble carboxylic esters. Its observation requires the use of non-natural short chain TAG with some solubility in water and its relevance for natural long chain TAG has often been questioned. Nevertheless, it has triggered numerous researches to explain this phenomenon at the enzyme molecular level, first with speculations on conformational changes occurring in the presence of a lipid-water interface, and finally with the obtention of lipase 3D structures showing indeed the presence of a lid controlling the access to the active site through conformational changes in both HPL (van Tilbeurgh et al., 1993a) and gastric lipase (Roussel et al., 2002).

Methods to measure lipolytic enzymes activity

There are numerous methods for measuring lipase activities and their principles have been reviewed by Beisson et al. (Beisson, Tiss, Rivière, & Verger, 2000). We briefly describe here the methods commonly used for the characterization of digestive lipolytic enzymes.

Measurement of lipase activity by the pH-stat technique

The pH-stat is a potentiometric method for measuring lipolytic activities at constant pH using emulsified substrates or substrates dispersed in water in the form of mixed micelles (Beisson, Tiss, Riviere, & Verger, 2000; Pinsiroadom & Parkin, 2001; Li, Hu, & McClements, 2011). It is convenient for testing all the lipolytic enzymes of the GI tract using specific substrates and conditions. The principle of this assay is the continuous titration of the FFAs release during substrate hydrolysis by the lipase. The released FFAs are usually titrated by adding 0.1 N sodium hydroxide solution to the reaction medium using an automated burette coupled to a pH-meter and a regulation system in which a pH endpoint can be selected (usually the optimum pH of activity on the selected substrate). The reaction is performed in a thermostated reaction vessel equipped with a mechanical or a magnetic stirrer to ensure fine emulsion and dispersion of the substrate with a large accessible surface. The amount of NaOH

added (or fatty acids released) as a function of time corresponds to the enzyme activity expressed in International Units, with 1 IU corresponding to the release of 1 μ mole of fatty acid released per minute).

Measurement of lipase activity by the monomolecular film technique

The monolayer technique using Langmuir films was adapted to the study lipolytic enzymes and lipid/protein interactions at the air-water interface (Verger & de Haas, 1973; 1976; Verger, 1984; Ransac, Moreau, Riviere, & Verger, 1991; Gargouri, Bensalah, Douchet, & Verger, 1995). By analogy with the pH-stat technique, the parameter that is kept constant here is the surface pressure, which characterizes at minima the physical-chemical parameters of the interface. The lipase is acting on a monolayer of substrate (TAG, DAG, phospholipid or galactolipid) spread at the air-water interface, the water phase being used to control the pH and other parameters like salt concentrations. This technique consists of the use of a "zero-order Teflon trough", consisting of two compartments, a reaction compartment, and a reservoir compartment, connected to each other by a small sintered glass channel. Once the two compartments are filled with the reaction solution, the lipid is spread at the surface using few microliters of a solution in chloroform. A monomolecular film of lipids is formed over the entire surface of the trough through surface channels. However, the diffusion of compounds present in the aqueous phase from the reaction compartment to the reservoir compartment is negligible. When the lipolytic enzyme is injected into the reaction compartment, it can partition between the aqueous phase and the monomolecular film and hydrolyze the lipid molecule substrate present in the film. Prior to enzyme injection, surface pressure is measured using a Wilhemy platinum blade connected to an electromicrobalance and can be adjusted using a Teflon mobile barrier to compress the film. The lipids chosen as substrates for these assays are usually medium chain TAG (trioctanoin, (Benarouche, Point, Carriere, & Cavalier, 2014)), DAG (1,2-dicaprin, (De La Fournière, Ivanova, Blond, Carrière, & Verger, 1994)), phospholipids (di-lauroyl-PC, (Hjorth et al., 1993)) and galactolipids (dilauroyl-MGDG, (Sias et al., 2004)). While these lipids are insoluble in water and form stable monolayers, their lipolysis products are soluble in water and are transferred from the interface to the bulk, which results in a decrease in molecular packing and surface pressure at the air-water interface. The use of the zero-order trough together with the Teflon mobile barrier controlled by surface pressure changes allows keeping the surface pressure constant as a function of time ("barostat" mode) by compressing the film after injecting the lipase below the monolayer. The enzyme activity is estimated from the surface covered by the mobile barrier as a function of time and its conversion to a number of substrate molecule hydrolyzed

based on the molecular area of these molecules deduced from compression isotherms (Ransac, Moreau, Riviere, & Verger, 1991). Thanks to the film compression and the replacement of the hydrolyzed substrate molecules, the reaction can be carried out at a set constant surface pressure and a constant surface concentration of substrate, hence the name “zero order trough” given to this device (Verger & de Haas, 1973; 1976).

6- Enzymes used in vitro models of digestion

In vitro digestion models reproducing the physiological conditions of the digestive tract find nowadays many applications, whether in food (Minekus et al., 2014) or in the field of galenic pharmacy (Carriere, 2016). In particular, they are used to test the digestibility of dietary lipids or the lipolysis of lipid formulations intended for the oral administration of hydrophobic active ingredients (Williams et al., 2012). The implementation of these models requires digestive tract enzymes or analogous enzymes obtained from various sources or recombinant. Human digestive lipases are however not commercially available for these applications and the use of digestive juices collected from volunteers are raising ethical concerns. As a result, human enzymes have been replaced by similar enzymes from animal origin. This was easily achieved in the case of pancreatic enzymes since porcine pancreatic extracts (PPEs) or pancreatin are commonly produced as an active pharmaceutical ingredient for the treatment of exocrine pancreatic insufficiency and are commercially available at a reasonable price for this type of application. There are several commercial sources of pepsin for gastric digestion of proteins but it is more difficult to obtain a reliable source of gastric lipase, which resulted in the absence of gastric lipolysis in many protocols or the use of non-relevant microbial lipases (Sams, Paume, Giallo, & Carriere, 2016). Rabbit gastric extract (RGE) however appeared as a relevant source of both gastric lipase and pepsin (Sams et al., 2018) and has been used in combination with PPE to replace human enzymes in a two-step in vitro digestion model with a gastric and a duodenal steps (Capolino et al., 2011). By analogy with in vitro studies on intestinal digestion using porcine pancreatin as a global source of pancreatic enzymes (Williams et al., 2012; Brodkorb et al., 2019), it is important to have access to a global source of gastric enzymes. RGE is such a mixture produced from rabbit stomach as a side product from slaughterhouses. RGE has recently become commercially available and was introduced in the standard INFOGEST static method of in vitro digestion (Brodkorb et al., 2019).

As a result of these developments, PPEs are increasingly used for in vitro digestion assays. Although the overall content of PPE in lipase, protease and amylase activities are well characterized, the individual enzymes present in these extracts are not well characterized and

a limited number of activity assays have been developed. PPEs contain the same lipolytic activities as human pancreatic juice (HPJ) and porcine pancreas pancreatic juice (PPJ), but it was recently shown that their phospholipase, galactolipase and cholesterol esterase activities, relative to the lipase activity, are reduced compared to those found in HPJ (Salhi et al., 2020). The fact that PPEs from different manufacturers may differ significantly in their lipolytic activities has already been reported. The levels of activity of CEH/BSSL have been shown to be particularly low in some preparations (Sternby & Nilsson, 1997). In addition to differences in the respective levels of porcine and human pancreatic enzymes, comparison of the activity levels of PPL and PPE reveals a significant loss of phospholipase and galactolipase activities in PPE, which is probably due to some proteolysis occurring during the production process of PPE. Indeed, the lipolytic enzymes present in PPE, as well as in PPJ and HPJ, are very sensitive to proteolytic degradation and inactivation by the endogenous proteases present in these enzyme mixtures. The inactivation of the enzymes during the manufacturing process of PPE was also supported by the finding that the activity of CEH/BSSL varies much more than the amount of immunoreactive CEH/BSSL (Sternby & Nilsson, 1997). Galactolipase activity in PPE was the lowest and least stable lipolytic activity, which may explain why it had not been detected previously (Amara et al., 2009). This may also explain why PLRP2, the pancreatic enzyme with the highest galactolipase activity (Amara et al., 2013), has been identified among porcine pancreatic enzymes only recently (Salhi et al., 2020). The native enzyme has not been isolated yet (De Caro et al., 2008) but characterization of recombinant porcine PLRP2 expressed in mammalian cells has confirmed that this enzyme exhibits galactolipase and phospholipase activities, like HPLRP2, as well as a lipase activity on triolein that is not strongly dependent on the presence of colipase (Xiao, Ross, Sevilla, Wang, & Lowe, 2013). Thus, porcine PLRP2 may contribute to all lipolytic activities of PPE, making their characterization more complex. Similarly, porcine CEH/BSSL has been identified here in PPE. In addition to lipase, phospholipase A1 and galactolipase activities, CEH/BSSL is believed to be the most important pancreatic enzyme for the digestion of cholesterol esters, as well as esters of vitamins A (retinol) and E (tocopherols and tocotrienols) (Lombardo, Fauvel, & Guy, 1980; Lombardo & Guy, 1980). The lipolytic activity of PPE on these latter esters, has not been explored in detail yet but it is assumed that it is closely associated with cholesterol esterase activity. The low levels of cholesterol esterase activity in PPE compared to PPJ and HPJ suggest that PPE will be less active on lipophilic vitamin esters than pancreatic juices.

In conclusion, PPE do not allow a complete substitution of the enzymes and lipolytic activities present in human pancreatic juices and one has to be cautious with their use for in vitro digestion, especially for studying the digestibility of cholesterol and vitamin esters.

Conclusion

Our understanding of the activities of the lipases present in the human GI tract has been growing following decades of study. Thanks to this knowledge, scientists are now able to improve the enzymes they use, either recombinants or extracts obtained from animals. In vitro digestion models are widely employed especially in the pharmaceutical and food areas, for research as well as for the industry. However, in order to acquire accurate and representative lipolysis data, those models ought to include the range of lipases found in vivo.

The digestion models are sometimes coupled with human trials and biophysical methods to validate the results and assess the bioavailability of food compounds, such as for instance carotenoids. Moreover, in vitro models can be used to test the dispersion and solubilization of drugs, and to establish screening tools to design and optimize lipid-based formulations. In the literature, different types of in vitro gastrointestinal digestion models have been developed, ranging from very simple experiments with a mixture of pancreatic enzymes (porcine pancreatin) to complex experimental devices mimicking the entire GI tract. The reader is referred to chapter 1.3 to obtain more information on the subject.

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