Biochimie xxx (2014) 1–9

Contents lists available at ScienceDirect

Biochimie

journal homepage: www.elsevier.com/locate/biochi

Research paper

An interfacial and comparative *in vitro* study of gastrointestinal lipases and *Yarrowia lipolytica* LIP2 lipase, a candidate for enzyme replacement therapy

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ARTICLE INFO

Article history:
Received 3 February 2014
Accepted 5 March 2014
Available online xxx

Keywords:
Monomolecular film
Enzyme adsorption
Enzyme kinetics
Interfacial enzymology
Lipase

ABSTRACT

Lipolytic activities of *Yarrowia lipolytica* LIP2 lipase (YLLIP2), human pancreatic (HPL) and dog gastric (DGL) lipases were first compared using lecithin-stabilized triacylglycerol (TAG) emulsions (Intralipid) at various pH and bile salt concentrations. Like DGL, YLLIP2 was able to hydrolyze TAG droplets covered by a lecithin monolayer, while HPL was not directly active on that substrate. These results were in good agreement with the respective kinetics of adsorption on phosphatidylcholine (PC) monomolecular films of the same three lipases, YLLIP2 being the most tensioactive lipase. YLLIP2 adsorption onto a PC monolayer spread at the air/water interface was influenced by pH-dependent changes in the enzyme/lipid interfacial association constant (K_{Ads}) which was optimum at pH 6.0 on long-chain egg PC monolayer, and at pH 5.0 on medium chain dilauroylphosphatidylcholine film. Using substrate monolayers (1,2-dicaprin, trioctanoin), YLLIP2 displayed the highest lipolytic activities on both substrates in the 25–35 mN m^{-1} surface pressure range. YLLIP2 was active in a large pH range and displayed a pH-dependent activity profile combining DGL and HPL features at pH values found in the stomach (pH 3–5) and in the intestine (pH 6–7), respectively. The apparent maximum activity of YLLIP2 was observed at acidic pH 4–6 and was therefore well correlated with an efficient interfacial binding at these pH levels, whatever the type of interfaces (Intralipid emulsions, substrate or PC monolayers). All these findings support the use of YLLIP2 in enzyme replacement therapy for the treatment of pancreatic exocrine insufficiency, a pathological situation in which an acidification of intestinal contents occurs.

1. Introduction

In healthy humans, the main lipolytic enzymes involved in the digestion of dietary triacylglycerols (TAG) within the gastrointestinal (GI) tract are human gastric (HGL) and pancreatic (HPL) lipases [1,2], two enzymes well characterized biochemically and structurally [3–5]. These lipases exhibit however different contributions during the digestion process of a meal as observed in healthy human volunteers [2]. HGL is secreted by the chief cells located in the fundic mucosa of the stomach [6]. Although its maximum activity is found around pH 5.4 [7,8], HGL remains active and stable at pH values as low pH 2.0 in gastric environment [9]. HPL and its specific cofactor, colipase, are secreted by pancreatic acinar cells and act in the small intestine [10]. The HPL-colipase complex shows a maximum activity in the 6.5–7.5 pH range in the presence of bile salts [11,12]. This enzyme is not significantly active below pH 5.0 and is denatured below pH 3.0 [13]. In the duodenum, the acidic contents of the stomach are neutralized by the bicarbonate secretion of the exocrine pancreas [14], leading to an increase of duodenal content pH reaching an average value of 6.25 during a liquid test meal [2].

Pancreatic exocrine insufficiency (PEI) is mainly related to chronic pancreatitis and cystic fibrosis. In these pathologies, both pancreatic enzyme and bicarbonate secretions are drastically decreased [15,16] leading to nutrient malabsorption and malnutrition. In particular, this lack of bicarbonate leads to very acidic pH values in the small intestine [16]. To date, the current treatment of PEI consists in the oral administration of exogenous pancreatic

Abbreviations: DAG, diacylglycerol; DGL, dog gastric lipase; FFA, free fatty acid; GI, gastrointestinal; HGL, human gastric lipase; HPL, human pancreatic lipase; MAG, monoacylglycerol; PC, phosphatidylcholine; PEI, pancreatic exocrine insufficiency; PERT, pancreatic enzyme replacement therapy; TAG, triacylglycerol; TC8, trioctanoin; YLLIP2, *Yarrowia lipolytica* lipase.

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http://dx.doi.org/10.1016/j.biochi.2014.03.004
0300-9084/

enzymes, such as porcine pancreatic extracts (Pancreatin or Pancrelipase) and it is known as pancreatic enzyme replacement therapy (PERT) [17]. Most pancreatic enzyme preparations are delivered in the form of gastro-protected enteric coated microgranules resistant to stomach acidity and are dissolved in the small intestine at pH values above 5.0 to 5.5 [18,19]. However, duodenal hyperacidity, such as the one observed in patients with cystic fibrosis [20,21] and severe chronic pancreatitis [22], is likely to delay dissolution of the enteric coating and thus the release of active enzyme at the appropriate time and site of digestion. This can reduce the time during which enzymatic digestion occurs before the meal passes into the colon. Drug products of animal origin also present some potential risks of viral transmission to humans as well as important variability in their pharmaceutical quality [23]. In that context, the current challenges are focusing on identifying new highly active and stable recombinant enzyme sources showing a high activity and an improved stability in the conditions of the GI tract. Among them, lipases are important targets because they are essential for fat digestion and thus contribute to a major part of energy uptake from the diet. Developing the oral administration of a lipase resistance to digestive proteases; such as pepsin in the stomach, and trypsin and chymotrypsin in the small intestine; is currently an important challenge.

Previous biochemical studies on the LIP2 lipase (YLLIP2) from the yeast *Yarrowia lipolytica* have suggested that this lipase is a good candidate for PERT in the treatment of PEI [19,24–27]. Comparison of wild-type *N*-glycosylated and non-glycosylated YLLIP2 showed that the *N*-glycosylation clearly contributes to a high stability of YLLIP2 in the presence of pepsin *in vitro*, and to a lower extent in the presence of chymotrypsin [27]. Glycosylated YLLIP2 could indeed be taken orally and retain its activity after crossing the stomach without the need for a gastro-resistant formulation, as currently used for pancreatic enzymes. YLLIP2 also displays one of the highest lipase activities on long-chain TAGs ever characterized, and retains a high activity at low pH levels such as those observed in the stomach but also in the small intestine of PEI patients. It was shown that YLLIP2 is more active at pH 4.0 [24] than the acid-stable dog gastric lipase (DGL) [28], an enzyme also selected as a good candidate for PERT [29], and which shares 85.7% amino acid sequence identity with HGL. Among all lipases, YLLIP2 is also one of the few enzymes that hydrolyze long-chain TAG faster than tributyrin [30,31] and it therefore shows a chain length specificity similar to that of DGL [28]. Moreover, the adsorption of YLLIP2 at the oil/water interface is not inhibited by bile salts, contrary to what is observed with some other microbial lipases [24]. YLLIP2 could thus be active in the conditions of the human GI tract like gastric and pancreatic lipases [12].

Although YLLIP2 is tailored for acting efficiently on pure TAG substrates *in vitro*, these substrates can be found in various forms in the GI tract, including emulsions stabilized by dietary phospholipids and proteins. Phospholipids thus play an important role in the stabilization of fat emulsions by forming a monolayer at the surface of TAG droplets [32,33]. Such an interface is often preformed in industrial food emulsions. The presence of phospholipids at the lipid–water interface is known to impair the activity of pancreatic lipase on TAG whereas it has no such effect on gastric lipase. It has been clearly established that the release of fatty acids by gastric lipase promote the action of pancreatic lipase on TAG droplets covered by phospholipids such as Intralipid (a soybean TAG emulsion stabilized with egg-yolk lecithins and used for parenteral intravenous infusions) or native milk fat globules [34,35]. From these observations, it is crucial to investigate the effects of phospholipids on novel lipases developed for acting in the GI tract. Since the lipase adsorption at the lipid–water interface must occur before the insoluble substrate is hydrolyzed, non-hydrolysable (by lipases) phospholipid monolayers spread at the air–water interface can be useful biomimetic models for studying lipase adsorption under controlled conditions of lipid composition and density at the interface [36–40].

In the present article, the lipolytic activity of YLLIP2 was first explored using Intralipid and compared with those of digestive lipases (HPL and DGL) on the same substrate under pH conditions found in healthy and PEI patients. The monomolecular film technique was then used for comparing the kinetics of adsorption on phospholipid monolayers and substrate (1,2-dicaprin, trioctanoin) hydrolysis of the same three lipases as a function of pH and surface pressure.

2. Materials and methods

2.1. Materials

L-α-phosphatidylcholine (egg PC), 1,2-dilauroyl-*sn*-glycero-3-phosphocholine (DLPC) and 1,2-didecanoyl-*sn*-glycerol (1,2-dicaprin) from Avanti Polar Lipids Inc. were purchased from COGER (Paris, France) and were >99% purity. All other chemicals (trioctanoin (TC8), MES, Glycine, NaCl, $CaCl_2$, Tris, EDTA and sodium acetate) were purchased from Sigma-Fluka-Aldrich (St-Quentin-Fallavier, France) and were BioXtra grade (≥99.0% purity). Chloroform (anhydrous for analysis, stabilized with amylene) was purchased from Carlo Erba Reactifs-SDS (Val de Reuil, France). Intralipid 20% from Fresenius Kabi (Sèvres, France) was purchased in a local drugstore.

2.2. Lipases and colipase

YLLIP2 was overproduced by a genetically modified *Yarrowia lipolytica* strain and purified according to [27]. Crude recombinant dog gastric lipase (DGL) was provided by Meristem Therapeutics (Clermont-Ferrand, France) [41] and purified as previously reported [3]. Recombinant Human pancreatic lipase (HPL) was produced in the yeast *Pichia pastoris* and purified as previously described [42]. Porcine pancreatic colipase (*i.e.* colipase) was purified according to [43]. The following enzyme stock solutions were prepared: 0.8 mg mL^{-1} YLLIP2 or 1.1 mg mL^{-1} DGL in 10 mM MES (pH 6.0) containing 150 mM NaCl; 0.4 mg mL^{-1} HPL in 10 mM MES (pH 6.5) containing 150 mM NaCl; and 1.2 mg mL^{-1} colipase in 50 mM Tris (pH 4.0) containing 150 mM NaCl and 5 mM $CaCl_2$. HPL-colipase complex was prepared at a 1:2 molar ratio.

2.3. Monomolecular film experiments

2.3.1. General methodology

All experiments were performed at room temperature (25 °C) using home-made Teflon troughs and the KSV5000 system (KSV, Helsinki, Finland) equipped with a temperature sensor probe, a mobile-barrier for compression isotherm experiments and a Langmuir film balance to measure the surface pressure (Π). Temperature, barrier movement and surface pressure were monitored by the KSV Device Server Software v.3.50 installed on a computer running under Windows 7®. Before each experiment, the Teflon trough was cleaned with tap water, and then gently brushed in the presence of distilled ethanol, before being washed again with tap water and abundantly rinsed with Milli-Q™ water. Residual surface-active impurities were removed before each experiment by simultaneous sweeping and suction of the surface [44]. The aqueous subphase containing 100 mN NaCl, 21 mM $CaCl_2$ and 1 mM EDTA was prepared with either 50 mM Glycine-HCl for a pH value adjusted either at 2.0 or 3.0; 10 mM sodium acetate buffer for a pH value adjusted either at 4.0, 5.0 or 6.0; or 10 mM Tris buffer for a pH

Please cite this article in press as: A. Bénarouche, et al., An interfacial and comparative *in vitro* study of gastrointestinal lipases and *Yarrowia lipolytica* LIP2 lipase, a candidate for enzyme replacement therapy, Biochimie (2014), http://dx.doi.org/10.1016/j.biochi.2014.03.004