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In vitro lipolysis of dairy and soy based infant formula

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ABSTRACT

Hydrolysed and non-hydrolysed dairy and soy proteins with vegetable oils are commonly used in infant formulations. Lipid digestion of these infant formula emulsions was studied under an *in vitro* gastrointestinal condition for 60 min in the stomach and 120 min in the small intestine (with and without the presence of enzyme proteases). The distribution of oil droplets, the released free fatty acids, and micro-structure changes of the digesta were monitored over the digestion period. Oil droplet coalescence was observed during gastric phase but not in the intestinal phase for most of formulations. The emulsion structure and the oil-water interface of the oil droplets play an important part in lipolysis kinetics. Higher rate of lipolysis in infant formula emulsion stabilized by hydrolysed proteins was noted. The obtained results suggest that digestive proteases had a limited impact on lipolysis of infant formulations.

1. Introduction

Infant formula is considered as mother's milk substitution for the infants who cannot access mother's milk due to various reasons. Lipids in mother's milk and formulas provide nearly half of the total energy to infants. The lipids contain *n-6* and *n-3* fatty acids such as linoleic acid (C18:2, *n-6*) and α-linolenic acid (C18:3, *n-3*) that are essential for infant brain and eye development (Hermoso et al., 2010; Joeckel & Phillips, 2009). The activity of digestive lipases in infants is lower compared to adults, but infants have three to five times higher lipid intake per kilogram of bodyweight (Andersson, Hernell, Bläckberg, Fält, & Lindquist, 2011). Lipid digestion in infants takes place both in the stomach and small intestine.

Gastric lipolysis plays a more important role in fat digestion in infants than in adults (Carey, Small, & Bliss, 1983; Hamosh et al., 1981) as the pH in the infant stomach (4.0–5.0) is less acidic than in adults which is closer to the optimum pH of gastric lipase (3.5–6.0) (Liao, Hamosh, & Hamosh, 1984). Also because gastric lipase does not require bio-surfactants (bile salts), and it is not inhibited by milk fat globule membranes (Hamosh, 1996; Hernell, Blackberg, & Bernback, 1988; Ville, Carriere, Renou, & Laugier, 2002), these properties allow gastric lipase to hydrolyse triglycerides in the infant stomach much easier than in small intestine. The level of gastric lipase in infants is similar or even higher to the level found in adults and that possibly compensates for the low activity of pancreatic lipases and explains why infants can consume a high dietary fat (Armand et al., 1995; Armand et al., 1996; Sarles, Moreau, & Verger, 1992). It has been reported that gastric lipase in animal infants can digest up to 25–60% of total lipids as compared to 10–25% in human adults (Abrahamse et al., 2012; Hamosh, 2006). There is limited data about physiological lipid digestion in human infants due to the restricted access to clinically invasive procedures (Abrahamse et al., 2012).

The duodenal digestion of lipids in infants is predominantly by pancreatic lipase-related to protein 2 (PLRP2) and bile salt-stimulated lipase (BSSL), while pancreatic lipase (PTL) is the key lipase involved with the duodenal lipid digestion in adults (Andersson et al., 2011; Lindquist & Hernell, 2010). However, the exact activity of PLRP2 and BSSL in infants is still not clear (Andersson et al., 2011). PTL and BSSL cannot hydrolyse the core of triglycerides because of their inability to penetrate into milk fat globules (Cohen, Morgan, & Hofmann, 1971; Roman et al., 2007). Therefore, bile salts play an important role in emulsifying the lipid in the duodenum before being hydrolysed by pancreatic lipases, but pancreatic lipase activity and bile salt concentration in infants are very low compared to adults (Lindquist & Hernell, 2010). In term infants, the activity of pancreatic lipase and bile salt concentration are approximately 5–10% and 50% of adults' figures, respectively (Lebenthal, Lee, & Heitlinger, 1983).

In order to mimic the composition of fat in mothers' milk with a high content of long chain polyunsaturated fatty acids (LCPUFAs), vegetable oils are currently used as a lipid source in manufacturing infant formula (Bourlieu et al., 2015; Nguyen, Bhandari, Cichero, & Prakash, 2015a). However, the structure of fat globules in infant formula is different from mothers' milk in terms of droplet size and membrane components. The oil droplet size distribution in infant formula is in the range of 0.1–1 μm

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which is smaller than in mothers' milk 0.1–10 μm (Michalski, Briard, Michel, Tasson, & Poulain, 2005). Also, due to the way the fat globules in mothers' milk are made and secreted from the mammary gland cells, the fat droplets in mothers' milk are covered by a phospholipid trilayered membrane composed of phospholipids, proteins and cholesterol (Gallier et al., 2015). However, oil droplets in infant formula are stabilized by an absorbed proteins layer based on caseins, whey proteins, whey protein aggregates, and even soy proteins on the surface of oil droplets (Bourlieu et al., 2015; Reis, Holmberg, Watzke, Leser, & Miller, 2009). Because enzyme lipases act on the insoluble emulsified substrates, lipid digestion process may depend on the lipase adsorbed on to the surface of the emulsified droplets (Armand et al., 1997; Porter, Trevaskis, & Charman, 2007). It is well documented that the rate and extent of lipolysis is controlled not by the enzymes' level but their ability to access the interface of the oil/water (Golding et al., 2011). This means that the digestibility of lipids in infant formula could be affected by the structure of the emulsion such as the surface area, and the composition of their interfacial layer surrounding the oil droplets (Bourlieu et al., 2015; Bourlieu et al., 2015; Reis et al., 2009). One would therefore expect that if the absorbed protein layer of the oil droplet surface is hydrolysed by digestive proteases, the lipase could access the droplet core easier, resulting in an increase in the rate of the lipolysis process. Bourlieu et al. (2015) suggested the presence of pepsin destabilised the emulsions due to proteolysis during the *in vitro* gastric phase. This study used infant formula emulsions from standardized cow's milk with different homogenization pressure and pasteurization treatment conditions. It remains unclear how infant formulation emulsions with vegetable oils stabilized by dairy proteins and plant proteins behave under the simulated infant gastrointestinal digestion. Therefore, the objective of this study is to estimate the effect of protease hydrolysis on lipid digestion in an *in vitro* infant GI tract using hydrolysed and non-hydrolysed proteins (dairy and soy proteins) in infant formulations. This was achieved by determining the oil droplet size and distribution, released free fatty acids, and micro-structural changes during an *in vitro* infant gastrointestinal digestion.

2. Materials and method

2.1. Materials

2.1.1. Enzymes and chemicals

The following enzymes and chemicals were used for the experiment: Lipase DF 15 (180 units/mg, stable pH 4.0–7.0, optimum pH 6.0–7.0, obtained from Amano Enzyme Inc., Japan) was used as analogue gastric lipase. One unit of Lipase DF 15 activity is defined as that quantity of a standard lipase preparation (Fungi Lipase-International FIP Standard) that liberates the equivalent of 1 μmol of fatty acid per minute from the substrate emulsion under the described assay conditions (pH 7.0 and 37 °C). Other enzymes were obtained from Sigma-Aldrich, Castle Hill, New South Wales, Australia and stored at the recommended temperature of −20 °C. Other enzymes included: Lipase from porcine pancreas (EC 232.619.9, 59,578 units/mg). Pepsin from porcine gastric mucosa (EC 3.4.23.1, 3840 units/mg protein, one unit will produce a change in A280 of 0.001 per min at pH 2.0 at 37 °C, measured as TCA-soluble products using haemoglobin as substrate). Trypsin from bovine pancreas (EC 3.4.21.4, 13,165 units/mg protein, one unit will produce a change in A253 of 0.001 per minute at pH 7.6 at 25 °C using Nα-Benzoyl-L-arginine Ethyl Ester (BAEE) as a substrate). Chymotrypsin from bovine pancreas (EC 3.4.21.1, 54.49 units/mg protein, one unit will hydrolyze 1.0 μmol of N-Benzoyl-L-Tyrosine Ethyl Ester (BTEE) per min at pH 7.8 at 25 °C as stated by manufacturer).

Porcine pancreatic colipase, sodium taurocholate, pepstatin, trypsin-chymotrypsin inhibitor, and orlistat were obtained from Sigma-Aldrich, Castle Hill, New South Wales, Australia. They were stored at recommended temperature (−20 °C). The other ingredients used in the study such as lactose, sodium chloride, hydrochloric acid, sodium hydroxide, and sodium azide were of analytical grade.

2.1.2. Dairy and soy proteins

Whey protein isolate (WPI 85.15% protein, 1.0% fat, 1.2% carbohydrate, all w/w) and calcium caseinate (CC, 86.7% protein, 1.01% fat, 0.15% carbohydrate, all w/w) were purchased from Total Foodtec Pty Ltd. (Australia).

Hydrolysed whey protein (HWP, 81.9% protein, 0.5% fat, 2.67% carbohydrate all w/w) and hydrolysed casein protein (HCP, 86.7% protein, 0.9% fat, 0.2% carbohydrate, all w/w) were purchased from Total Foodtec Pty Ltd. and Myopure Pty, Australia.

Soy protein isolate (SPI, 82.4% protein, 4.5% fat, < 1.0% carbohydrate) was purchased from Food Manufacturers Pty., Australia. Hydrolysed SPI (HSPI) was made from SPI by pepsin at a concentration of 22.75 U/mg of total protein, at 37 °C for 1 h. The hydrolysed mixture was alkalised to pH 8.0–8.5 by 0.1 N NaOH then neutralised by 0.01 N HCl followed by freeze-drying for 72 h (Pasupuleti & Braun, 2008).

Sunflower vegetable oil was obtained from the local supermarket.

2.2. Method

2.2.1. Preparation of infant formulations

The procedure for making infant formulations was as described by Nguyen, Bhandari, Cichero, and Prakash (2016). 100 mL of liquid formulation containing 1.5 g of protein, 4.0 g of lipid and 6.5 g of lactose was chosen based on the recommendation for infant formula by the European Union (Koletzko et al., 2005). Preliminary screening of the commercial infant formula available in Australia that contained hydrolysed proteins was conducted and it was found that the hydrolysed whey, hydrolysed casein, and hydrolysed soy protein were the commonly used hydrolysed proteins in the formulas. This study was focused on 4 formulations: WPI:CC = 1:1, HWP:HCP = 1:1, 100% SPI, and 100% HSPI.

2.2.2. In vitro infant lipid digestion

The lipase from *Rhizopus oryzae* was chosen to substitute gastric lipase in this study. Some previous studies used gastric lipase from human and animals such as dogs and rabbits (Amara et al., 2014; Bourlieu et al., 2015; Carrière et al., 2000). Animal gastric lipase has similar properties to human gastric lipase. However, due to ethical issues and clinically invasive procedures, access to human and animals enzymes is limited. Although fungal lipase has high specificity to the sn-1 and sn-3 position of triglyceride while human gastric lipase only cleaves at sn-3, there is no better analogue gastric lipase than fungal lipase so far (Ménard et al., 2014).

A static *in vitro* digestion unit equipped with water bath, and overhead stirrer carried out the digestion trials. Two water-jacketed reaction vessels in the unit were connected to a water bath that provided a constant circulation of warm water in and out of the reaction vessel and maintained a constant temperature of 37 °C. A glass stirrer connected to an overhead stirrer continuously mixed the *in vitro* digesta at 250 rpm. The activity of analogue gastric lipase (21 units/mL), pancreatic lipase (200 units/mL), molar ration between colipase and pancreatic lipase was 2:1, bile salts (4 mM) were chosen based on the data published by Minekus et al. (2014) and Carrière et al. (2000). Levels of proteases and bile salts are described in Nguyen, Bhandari, Cichero, and Prakash (2015b). In this study, the gastric pH 4.5 was used that is closer to the optimum pH of analogue gastric lipase which is in the appropriate range for the infant gastric conditions. The stomach digestion lasted for 60 min and digesta samples were collected at the start (S0), after 30 and 60 min of digestion (S30, S60). The intestinal pH is 6.5. The intestinal digested samples were collected at the start (0 min) and after 30, 60, and 120 min of intestinal digestion (S30, S60, S120). Digested samples were inhibited by adding 0.85 μM of pepstatin to inhibit the equivalent amount of pepsin in the sample (Rich & Sun, 1980). Trypsin-chymotrypsin inhibitor and orlistat were added to inhibit twice