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The effect of lactoferrin on physical changes in phospholipid stabilised emulsions during neonatal *in vitro* gastric digestion: Does synergism of pepsin and lipase promote lipolysis in protein-stabilised emulsions?

C. Lueamsaisuk [a], R.G. Lentle [a], A.K.H. MacGibbon [b], L. Matia-Merino [a], M. Golding [a, *]

[a] Massey University, Private Bag 11222, Palmerston North, New Zealand
[b] Nutrition and Health, Fonterra Research and Development Centre, Palmerston North, New Zealand

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ABSTRACT

The effect of the interaction and alignment of protein at the oil/water interface in stable emulsions on *in vitro* simulated gastric lipolysis by *Rhizopus oryzae* lipase was explored using Intralipid®, a commercial soybean oil fine emulsion stabilised by phospholipid, before and after electrostatic binding with lactoferrin in the pH range 2–5.5.

For 3.6% (v/v) untreated Intralipid® emulsion droplet coalescence, as observed by laser light scattering and confocal microscopy, occurred at pH levels between 3.5 and 5.5, and was evident both with lipase alone and with mixtures of pepsin and lipase. Conversely, for Intralipid® treated with lactoferrin, no coalescence was evident on digestion with lipase alone at pH 2–4.5. However, coalescence of droplets in lactoferrin treated Intralipid® did take place at pH 3.5–5.5 when both pepsin and lipase were present.

The authors conclude that, dependent on pH conditions and in the absence of pepsin, electrostatic binding of lactoferrin to the phospholipid interface of the Intralipid emulsion stabilises droplets against coalescence during gastric incubation.

1. Introduction

Early workers in the field of enzymatic digestion detailed the kinetics of various gastrointestinal enzymes during the digestion of substrates in aqueous solution. However evidence suggests that both natural and synthetic food substrates comprise complex organised matrices of different macronutrients both in solid and in liquid form, that rather than being dissipated by simple solution of the constituents, persist in some form throughout the digestive tract (Lentle & Janssen, 2011; Lundin, Golding, & Wooster, 2008). Hence, consortia of enzymes must work in concert to digest these structures and liberate oligomeric and monomeric macronutrients. In liquids such as milk, the surface constituent droplets of fat that are dispersed as an emulsion provide an oil/water interface across which amphoteric moieties such as proteins may orientate with their hydrophobic and hydrophilic domains thermodynamically positioned (Zhai, Day, Aguilar, & Wooster, 2013). Human milk contains a number of proteinous and polar lipid components that are specifically adapted to occupy and stabilise the oil/water interfaces of such emulsions during the process of secretion and lactation. Again it is likely that disruption and fragmentation of the secondary and tertiary structures of dietary proteins during proteolysis will liberate oligomeric species that may similarly partition across such interfaces (Singh & Ye, 2013). Given that lipolytic digestion depends upon the location of the lipolytic enzyme on the oil/water interface (Golding & Wooster, 2010), either alone as in the case of gastric lipases, or with the aid of other elements such as colipase in the case of pancreatic lipase it is likely that oligomeric fractions and other dietary proteins with significant hydrophobic regions that partition across the oil/water interface may sterically hinder their action (Chu et al., 2009; van Aken, Bomhof, Zoet, Verbeek, & Oosterveld, 2011). Such complexities may be particularly important in neonatal nutrition where milk is the principal diet and fat the principal energostatic metabolite. Hence we may expect to see particular adaptation to maximise lipolytic efficiency and minimise competitive proteinous steric exclusion of lipases (Armand et al., 1996; Gargouri, Ransac, & Verger, 1997).

* Corresponding author. Institute of Food, Nutrition and Human Health, Massey University, Private Bag 11 222, Palmerston North 4442, New Zealand. Tel.: +64 6 3569099x81428, +64 21 804175.
E-mail address: m.golding@massey.ac.nz (M. Golding).

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An understanding of such adaptation is particularly important in devising breast milk substitutes. However variation in the composition of breast milk between individuals and with time (Khan et al., 2013; Malbon, 2006) along with difficulties in repeated sampling of the gastric contents of neonates compromise repeatability. Hence it is necessary to simulate the physical and chemical environment *in vitro* (Shani-Levi, Levi-Tal, & Lesmes, 2013).

In a previous study (Lueamsaisuk, Lentle, MacGibbon, Matia-Merino, & Golding, 2014), we examined changes in the coalescence stability of formula emulsions *in vitro*. The findings from this earlier study confirmed that the coalescence of emulsion droplets, which appeared to be symptomatic of the onset of enzymatic lipolysis, was most likely to occur at pH levels between 3.5 and 4.5, in the presence of both fungal lipase and pepsin. Further, when incubation was carried out with either lipase or pepsin alone, coalescence was either slowed or did not occur. It was also shown that propensity to coalescence increased with rate of shear indicating that it was necessary to limit shear rate to those which would normally be encountered in the gastric lumen.

These findings fitted with a hypothesis that destabilisation of the droplet interface was caused by the adsorption of free fatty acids arising from lipolysis, which in turn lead to coalescence. However, this process was inhibited by a layer of adsorbed protein that acted as a barrier for lipase adsorption and required synchronous action of gastric protease to provide initial disruption of the adsorbed protein membrane, thereby providing more favourable binding sites for lipase adsorption.

To further explore this hypothesis and ascertain how this apparent enzymatic co-dependency facilitates the digestion of protein-stabilised emulsions, we utilised a pH dependent layer-on-layer technique to coat a phospholipid stabilised emulsion system with a protein coat, and examine how this change in interfacial composition influenced the stability of the emulsion under *in vitro* gastric conditions. This paper reports the results of this work.

2. Materials and methods

2.1. Materials and solutions

Intralipid®, a phospholipid stabilised emulsion of soybean oil suitable for intravenous administration to human subjects was purchased from Fresenius Kabi Australia Pty Limited, NSW Australia. The solution contained 20 vol% triglyceride, 1.2 vol% phospholipid and 2.2 vol% glycerol. Hence 500 ml Intralipid® contained 100 ml soybean oil, 6 ml egg lecithin, 11 ml glycerol, titrated with sodium hydroxide q.s. to pH 6.0 to 9.0, made up to 500 ml with water for injection BP. Osmolality was 350 mOsm/kg. Energy content was 4200 kJ (1000 kcal) per 500 ml. The material was stable when stored below 25 °C.

A 3.6% (v/v) solution of Intralipid® was prepared by dilution with MilliQ water (3.6 ml of 20% (v/v) Intralipid® added to 16.4 ml MilliQ water).

Bovine lactoferrin was a gift from Fonterra Research and Development Centre, Palmerston North, New Zealand. A 1% (w/v) of lactoferrin powder in 3.6% (v/v) Intralipid® was prepared by first dissolving 0.2 g of lactoferrin powder in 16.4 ml MilliQ water, adjusting pH to 6.8 and stirring for one hour to allow complete dissolution. 16.4 ml of this solution were then added 3.6 ml of 20% (v/v) Intralipid® the mixture stirred at room temperature for a further 2 h before use.

Porcine pepsin (porcine, 800–2500 IU/mg) was supplied by Sigma–Aldrich Pty Ltd., St. Louis, MO. Fungal lipase extracted from *Rhizopus oryzae* (80 IU/mg) was supplied by Valley Research Corp. Austin, Texas. Other analytical grade chemicals were supplied by BDH Chemicals (BDH Ltd, Poole, England) or Sigma Chemical Co. (St. Louis, MO, USA).

Simulated gastric fluid was prepared by dissolving 450 mg of dry powdered porcine pepsin (porcine, 800–2500 IU/mg) and/or 20 mg of dry powdered *R. oryzae* lipase equivalent to 1600 IU (80 U/mg) in 80 ml of solution of 200 mg of NaCl and 1.3 ml of 1 M HCl in 80 ml of MilliQ water and dissolved by constant stirring at 37 °C, for 10 min (Lueamsaisuk et al., 2014).

The dissociating solution used in the determination of emulsion particle size in subsamples of digestate, comprised 0.375% (w/v) of disodium ethylenediamine tetra-acetate and 0.125% (v/v). Tween 20 from BDH Chemicals (BDH Ltd., Poole, England) made up in MilliQ water at pH 10. Tween 20 is able to displace the proteins from the interface of the oil droplet and hence disperse any proteinous flocs. The alkaline pH arrests pepsin and lipase activity and hence prevents any further change in particle size following sampling.

2.2. Methods

2.2.1. In vitro digestion

Either 20 ml of the 3.6% (v/v) Intralipid® solution alone, or the similarly diluted Intralipid® with 0.2 g of lactoferrin solution per 20 ml were each sheared at a speed of 100 rpm for two hours in a 400 ml beaker at 37 °C prior to the commencement of *in vitro* digestion.

Simulated gastric fluid (Intralipid® solution: SGF ratio = 20:50 v/v) was then added (Lueamsaisuk et al., 2014). This ratio was based on the reported volume of human gastric secretion (Thiagarajah & Verkman, 2006) in relation to that of a meal. The pH of the mixture was maintained at either 2, 3.5, 4.5 or 5.5 using 1 M HCl or 1 M $NaHCO_3$. The mixture was incubated at 37 °C and stirred with a 7 cm wide and 1 cm depth bar at 10 rpm in a 400 ml beaker that was 7.5 cm diameter for a total of 2 h. One ml aliquots of digestate were taken every 15 min for subsequent determination of oil droplet size distribution, confocal microscopy and determination of droplet charge.

The length of the stirrer bar was close to the internal diameter of container so as to mix the entire contents. The low stirring rate was chosen to match that likely in the infant stomach. Hence strain rates in the smooth muscle of the gastric antrum of adult humans are low (average strain velocity generally between <15 and 20% s^{-1} (Ahmed, Gilja, Hausken, Gregersen, & Matre, 2009) as is the maximal shear generated within the gastric contents (around 0.7 s^{-1}). Whilst strain rates in the infant stomach have not been determined, the contractile activity in preterm infants is reported to be lower than that in the stomach of term infants and adults (Berseth, 1996; Dumont & Rudolph, 1994).

Fungal lipase is well characterised (Hiol et al. 2000). It exhibits similar region-specific hydrolysis of triglyceride substrates to that of HGL and is acid stable. Like human and fungal lipases it possesses a α/β hydrolase fold covered by an amphiphilic lid that becomes accessible only after binding of the enzyme to the oil water interface (Brzozowski et al., 1991). Suitably standardised supplies are more readily available and have been used in other *in vitro* models (Golding & Wooster, 2010; Golding et al., 2011; van Aken et al., 2011). However the 'optimal' pH of hydrolysis by *R. oryzae* lipase is 7.5 and the enzyme is said to be stable in the range pH 4.5–7.5 (Hiol et al., 2000). These values are some distance from the lower of the two pK_a's of porcine gastric lipase which is stated to be 3.5 (Campos & Sancho, 2003) and are outside of the reported pH spectrum of activity of human gastric lipase (Aloulou & Carrière, 2008). However there has been considerable debate in regard to other fungal lipases as to whether the reported pK_a's and range of optimal activity reflect the local pH at the active site rather than