Immobilised lipase for *in vitro* lipolysis experiments

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Abbreviations:

BS, bile salt

CALB, lipase B from *Candida antarctica*

FA, fatty acid

SAXS, small angle X-ray scattering

TBU, tributyrin units

Abstract

In vitro lipolysis experiments are used to assess digestion of lipid-based formulations, and probe solubilisation by colloidal phases during digestion. ~~—h~~ However proteins and other biological components in the pancreatin often used as the lipase result in high background scattering when interrogating structures using scattering approaches, complicating the resolution of colloidal structures. In this study, to circumvent this problem, a modified *in vitro* digestion model employing lipase immobilized on polymer beads, which allows for separation of the lipid digestion components during lipolysis, was investigated. Titration of the fatty acids released during digestion of medium chain triglycerides using pancreatin compared to immobilized lipase, combined with HPLC was used to follow the digestion, and small angle X-ray scattering was used to determine colloidal structure formation. Digestion of medium chain triglycerides at the same nominal activity revealed that for the immobilized lipase, a longer digestion time was required to achieve the same extent of digestion. However, the same structural endpoint was observed, indicating that structure formation was not affected by the choice of lipase used. Lipolysis with immobilized lipase led to the reduction of parasitic scattering, resulting in clearer and more defined scattering from the structures generated by the lipolysis products.

Introduction

Lipid based formulations (LBF) are of interest for the delivery of poorly water soluble drugs. During the lipolysis of triglycerides, monoglycerides (MG) and fatty acids (FA) are produced. These combine with endogenous amphiphilic molecules in the gastrointestinal tract to form bile salt/phospholipid mixed micelles and other liquid crystalline phases. These structures are important in maintaining drug solubilisation in the gastrointestinal environment, leading to enhanced absorption and bioavailability of co-administered drugs [1, 2].

In vitro lipolysis experiments using a pH stat apparatus are frequently utilised to evaluate digestion of LBF to examine drug disposition, solubilisation and precipitation, with the aim to achieve *in vitro-in vivo* correlations. The study of lipolysis from a compositional aspect remains largely reliant on the titration profile. The pH stat follows the progress of triglyceride digestion, by titrating against the fatty acids liberated.

Understanding structure formation during lipolysis remains a major hurdle, due to lack of real time methods to elucidate structural changes during the dynamic process, and this has recently been reviewed [3]. Until recently, studies have been limited to microscopy, scattering and spectroscopic

techniques to investigate equilibrium structures that are assembled from the individual components representing the end point of lipid digestion. The field has since progressed to the use of *in vitro* lipolysis models coupled to synchrotron small angle X-ray scattering [4, 5], where the digest medium is flowed through a capillary placed in the X-ray beam for probing of nanostructure formation in the millisecond time frame.

The current protocol for *in* vitro digestion studies often utilizes porcine pancreatin extract [4, 6-8] as the lipase source to simulate digestion in the small intestine. However there are many proteins and enzymes in the crude extract which contribute to high undesired scattering from pancreatic components, when using techniques such as small angle X-ray scattering (SAXS) and dynamic light scattering (DLS) to understand the structural evolution caused by lipolysis products in these systems. These may obscure scattering from colloidal species produced on digestion, and complicate the resolution of structures that are present.

Novozym® 435 is a commercially available recombinant lipase B from *Candida antarctica* (CALB) manufactured by Novozymes. It is immobilized by physical adsorption to macroporous polyacrylate resin, which has an average particle size of 315-1000 µm, surface area of 130 $m^{2/}$g and pore diameter 150 Å [9, 10]. Immobilized lipases are widely used as biocatalysts in the food, detergent, textile, cosmetic and pharmaceutical industries, for example, hydrolysis of triglycerides to produce fatty acids and esterification, transesterification, aminolysis in organic solvents. Immobilized lipases have been successfully manufactured by ionic binding, covalent binding, cross-linking, entrapment and encapsulation. Advantages of immobilized lipase over free lipase are that they are cost effective and reusable, for continuous large-scale commercial processes, has enhanced chemical and thermal stability and exhibit high enantioselectivity [11]. In the context of *in vitro* lipolysis experiments, having enzyme immobilized on beads enables separation from the digesting medium which may improve the ability to study structural aspects using scattering methods.

Consequently the aim of this study was to investigate the possibility of using lipase immobilised to polymer beads as an alternative to powdered pancreatin to improve the current method for studying structures during lipolysis. To the best of our knowledge immobilized lipase has not been applied to *in vitro* lipolysis experiments. Digestion of medium chain triglycerides (tricaprylin and Captex 355, a commonly used formulation lipid consisting of a mixture of tricaprylin and tricaprin, were assessed to compare the activity, effect of pH, temperature, and buffer type and resulting structural detail after digestion with immobilized lipase compared to pancreatin extract.

Experimental

Materials

Captex 355 (MCT composed of 59% caprylic acid (C_8), 40% capric acid (C_{10}), < 1% lauric acid (C_{12}) as stated in the product information) was obtained from Abitec Corporation (Janesville, WI, USA) and used without further purification. Tributyrin (>98%) was obtained from TCI Co. Ltd (Kawaguchi City, Saitama, Japan). Tricaprylin (> 99%), Tris maleate (reagent grade), bile salt (sodium taurodeoxycholate, > 95%) and 4-bromophenylboronic acid (4-BPBA, > 95%) were purchased from Sigma Aldrich (St. Louis, MO, USA). Sodium azide was purchased from Merck Schuchardt OHG (Eduard-Buchner-Straße, Hohenbrunn, Germany). Phospholipid (1,2-dioleoyl-sn-glycero-3-phosphocholine, DOPC) was from Trapeze Association Pty Ltd (Clayton, Victoria, Australia). USP grade pancreatin extract was purchased from Southern Biologicals (Nunawading, Victoria, Australia). Novozym® 435 was obtained from Novozymes (Bagsvaerd, Denmark). Calcium chloride (> 99%) was obtained from Ajax Finechem (Seven Hills, NSW, Australia). Sodium chloride (> 99%) was purchased from Chem Supply (Gillman, SA, Australia). HPLC grade methanol (MeOH) was purchased from Merck. Trifluoroacetic acid (TFA) was obtained from Pierce (Rockford, Illinois, USA). Water used was sourced from a Millipore water purification system using a Quantum™ EX Ultrapure Organex cartridge (Millipore, Sydney, Australia).

Determination of lipolytic activity

The lipolytic activity was determined using the pH stat method, which was used to construct a calibration curve. Digestion buffer ~~typically~~ was prepared with 50 mM Tris maleate, 5 mM $CaCl_2.2H_2O$, 150 mM NaCl, 6 mM NaN_3 as anti-microbial agent, and adjusted to pH 6.5. The substrate used was 6 g tributyrin [6, 12] dispersed in 10 mL of digestion buffer for immobilized lipase or 9 mL digestion buffer for pancreatic lipase respectively, which itself was dispersed in 1 mL digestion buffer as below. The quantity of digestion buffer used differed depending on the lipase source to maintain a constant buffer:tributyrin ratio.

Immobilized lipase was added to the test system at different masses; whereas the pancreatic lipase was prepared by weighing the required mass of the pancreatic lipase in a centrifuge tube, adding 5 mL digestion buffer to form a suspension, which was adjusted to pH 6.5 by the addition of ~~30 µL 0.5 M~~ NaOH ~~and 5 mL buffer to form a suspension~~. The suspension was magnetically stirred for 15 min, and centrifuged for 15 min at 3500 rpm at 25 °C as a compromise to prevent a denaturation of the lipase and a decline in activity, and minimize the temperature difference between the lipase

suspension and the digestion medium. Fresh pancreatin extracts were prepared each day and the supernatant was kept at room temperature prior to use.

The reaction was typically performed at 37 °C and in Tris buffer pH 7.5 and lipase (mg immobilized or 1 mL pancreatic lipase suspension, containing 10 000 TBU units) was added to initiate digestion and the reaction was titrated with 0.6 M NaOH. In the case where the influence of buffer type, temperature or pH on activity ~~were~~ was studied, these variables were changed accordingly. The activity was defined in Tributyrin Units (TBU) where 1 TBU is the amount of enzyme used to liberate 1 µmol of titratable free fatty acid per minute. A plot of µmol FA titrated per minute yields an initial linear portion, where the slope gives the activity in TBU per mL of digest under the assay conditions.

***In vitro* lipolysis**

~~Digestion buffer typically was prepared with 50 mM Tris maleate, 5 mM $CaCl_2.2H_2O$, 150 mM NaCl, 6 mM NaN_3 as anti-microbial agent, and adjusted to pH 6.5.~~ A micellar solution was prepared in digestion buffer with bile salt (sodium taurodeoxycholate) and phospholipid (DOPC) at concentrations of 5 mM:1.25 mM ~~to~~ as simulated fasted intestinal fluid [13, 14]. The phospholipid was dissolved in chloroform in a round bottom flask and the choloroform was evaporated off under vacuum to leave a thin film. Bile salt and digestion buffer were added and the solution was placed in a sonicator bath for 30 min, before it was stored at 4 °C to equilibrate overnight. Pancreatin extracts were prepared by adding 4 g of porcine pancreatin powder to 5 mL of digestion buffer to achieve an activity of 10 000 TBU/mL. The suspension was magnetically stirred for 15 min, and centrifuged for 15 min at 3500 rpm at 25 °C. ~~Fresh pancreatin extracts were prepared each day and the supernatant was kept at room temperature prior to use.~~

In vitro digestion studies were performed using a pH-stat auto titrator (Metrohm, Switzerland), similar to previous reports [4, 6, 8, 15, 16]. Lipid was added to the fasted simulated intestinal fluid in the thermostatted digestion vessel at 37 °C, and magnetically stirred for 5 min for complete mixing and thermal equilibration. The pH was adjusted to 6.5 ± 0.003, chosen as a compromise between the optimum for pancreatic lipase activity pH (6 – 10) [17] and duodenal pH (5.9 – ~~5~~6.5) [18, 19]. On addition of lipase (~1000 TBU/mL digest) the pH-stat titrated the digestion with 0.6 M NaOH in order to maintain pH 6.5 ± 0.003. Digestion was allowed to proceed for up to 8 hr, to ensure a similar extent of digestion, in which the degree of enzymatic digestion of the lipid was reflected in the volume of NaOH used to neutralize the fatty acids liberated during the digestion process.

A blank digestion without lipid but with bile salt micelles present was performed as a background experiment, to account for fatty acids that were produced from phospholipids and was subtracted from the profiles for the lipolysis experiments.

Lipid composition in digestion medium

A few techniques have been used to separate and analyze lipid digestion products including HPTLC, GC and HPLC-MS. However these have shortcomings such as long run times, labour intensive, high cost, requirement of specialized equipment and use of volatile solvents. Reversed phase HPLC using refractive index detection has recently been shown to be a useful tool to determine lipid composition following *in vivo* digestion that it is simple and non-destructive [20]. Thus this method was employed to separate and quantify fatty acid analytically during digestion for correlation with total fatty acid produced as indicated by the pH stat titration. Samples (200 µL) were taken during digestion for analysis of fatty acid content by HPLC. To halt digestion in the sample lipase inhibitor (20 µL of 0.05 M 4-BPBA in methanol) was added to eppendorf tubes, where the methanol was evaporated off prior to addition of samples.

Reversed-phase high performance liquid chromatography (HPLC) - Samples were analysed for fatty acid content and separated by an isocratic reversed-phase HPLC method using a 4.6 × 150 mm Phenomenex Luna C_8 (2) (5 µm, 100 Å) analytical column, with a 15×3 mm Brownlee RP-18 (7 µm) guard column. The HPLC system consisted of a Shimadzu CBM-20A system controller, LC-20AD solvent delivery module,SIL-20A auto sampler and a CTO-20A column oven set at 40°C, coupled to a RID-10A differential refractometric detector (Shimadzu Corp., Kyoto, Japan). An injection volume of 40 µL was used to separate caprylic acid and capric acid using a mobile phase consisting of MeOH/water (75:25 v/v) with 0.1% TFA (v/v of total mobile phase) at a flow rate of 1 mL/min. Assay parameters are shown in Table SI-1.

Preparation of standards and samples - A stock solution of caprylic and capric acid was prepared at a concentration of 10 mg/mL in methanol. A set of standards containing 0.1, 0.2, 0.5, 1.0 and 2.0 mg/mL of both lipids was prepared by mixing and dilution of the stock solution in the mobile phase. All stock solutions and standards were stored at 4 °C before analysis. Calibration curves were prepared by plotting the area under the curve against known concentration of standard solutions. Lipolysis samples were diluted in mobile phase prior to HPLC analysis. Unknown sample

concentrations were calculated from the standard equation $y = mx + c$, as determined by the linear regression of the unweighted standard curve.

Assay validation - Validation of the HPLC assay was run over three days and the results are shown in Table SI-2. Intra-assay accuracy was determined by replicate analysis (n=5) of standard solutions of lipids at three concentrations (0.1, 1.0 and 2.0 mg/mL). Inter-assay accuracy was determined on three separate days. The data were expressed as a percentage of the measured concentration over the theoretical concentration, where mean accuracy was within ± 15% of the theoretical concentration. Intra-assay precision (repeatability) and inter-assay precision (reproducibility) were calculated in all three runs for each lipid at all three concentrations and expressed as the coefficient of variation (% CV) of replicate assays. Linearity was performed on standard curves for each run and linearity was fulfilled when the correlation coefficient (r^2) of the regression line was > 0.99.

Synchrotron small angle X-ray scattering (SAXS)

SAXS measurements were performed at the SAXS/WAXS beamline [21] at the Australian Synchrotron. An X-ray beam with a wavelength of 1.1271 Å (11 keV) was used. A sample to detector distance of 1588 mm covered the q-range $0.01 < q < 0.7$ Å^{-1} with q being the magnitude of the scattering vector defined as $q = (4\pi/\lambda)\sin\vartheta/2$, λ being the wavelength and ϑ the scattering angle.

Samples were drawn into a capillary which was fixed in a thermostatted metal heating block controlled by a Peltier system accurate to ± 0.1 °C, for measurements taken at 37 °C. The 2D SAXS patterns were acquired over 1 s using a Pilatus 1M detector with active area 169 x 179 mm^2 and with a pixel size of 172 µm. 2D scattering patterns were integrated into the 1D scattering function I(q) using the in-house developed software package scatterBrain. Scattering curves are plotted as a function of relative X-ray intensity, $I(q)$, versus q.

Results

Lipolytic activity

Activity of the immobilized lipase was very different to that of the pancreatin extract (Figure 1). The immobilized lipase displayed consistent lipolytic activity towards tributyrin where it increased linearly with concentration, with approximately 37 TBU/mg immobilized lipase. In contrast, the activity of pancreatic lipase reached a plateau with increasing concentration, approximately 12 TBU/mg dry pancreatin powder at saturation.

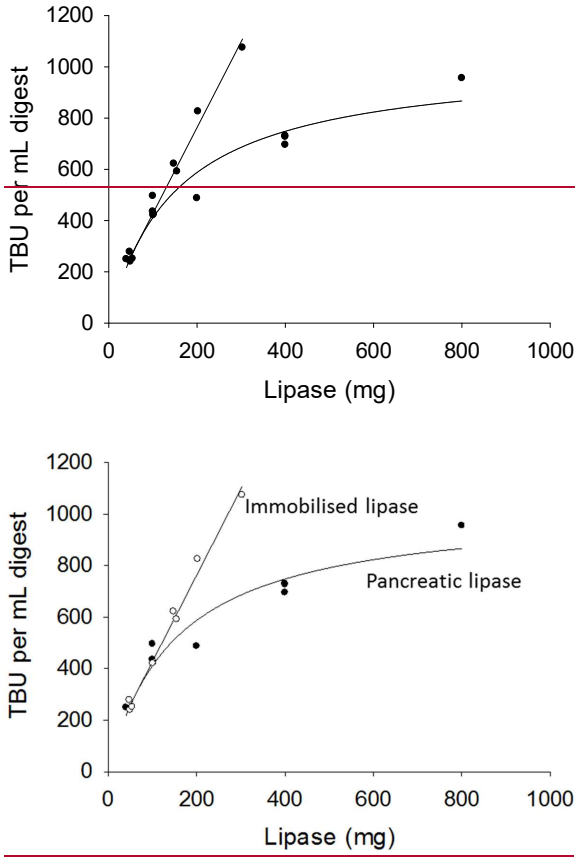

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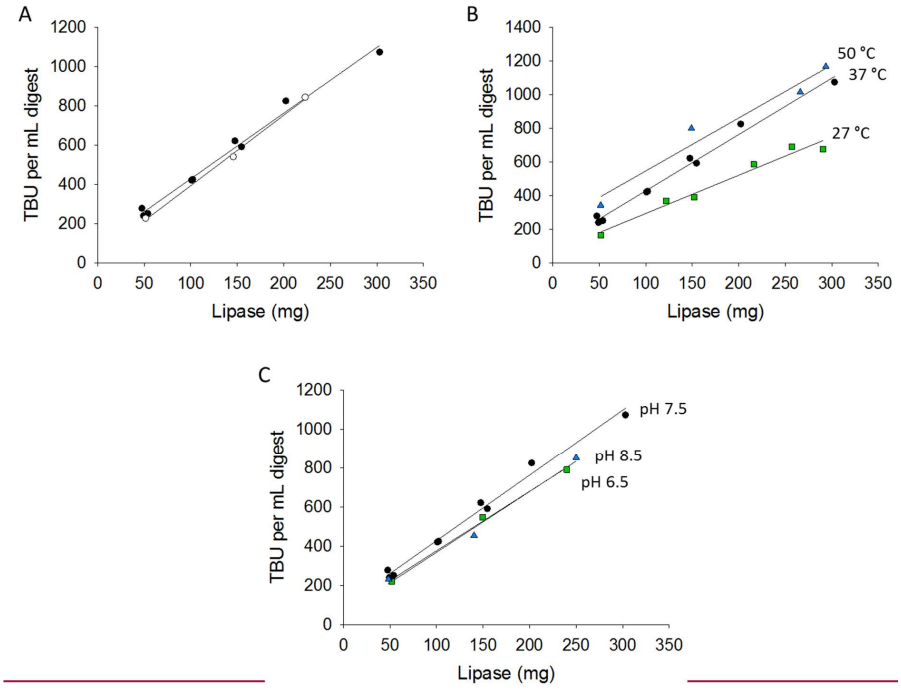
Figure 1: Comparison of activity (TBU) of immobilized (open circles) and pancreatic (closed circles) lipase with increasing mass in Tris buffer pH 7.5 at 37 °C

Factors affecting the activity of immobilized lipase

The influence of buffer, temperature and pH on lipolytic activity of immobilized lipase was assessed to further understand the physic-chemical understanding of digestion, as these factors may affect lipase activity. No significant buffer- or pH-dependency was observed on the activity (Figure 2A and C). Lipase activity was however observed to vary with temperature as reported in the product information (Figure 2B). An increase in activity was observed with increasing temperature as anticipated due to an increase in kinetic energy in the system and lowering of the activation energy required for the reaction, and a decrease in temperature hampered the reaction.


A
TBU per mL digest
Lipase (mg)
B
50 °C
37 °C
27 °C
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Lipase (mg)
C
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pH 8.5
pH 6.5
TBU per mL digest
Lipase (mg)

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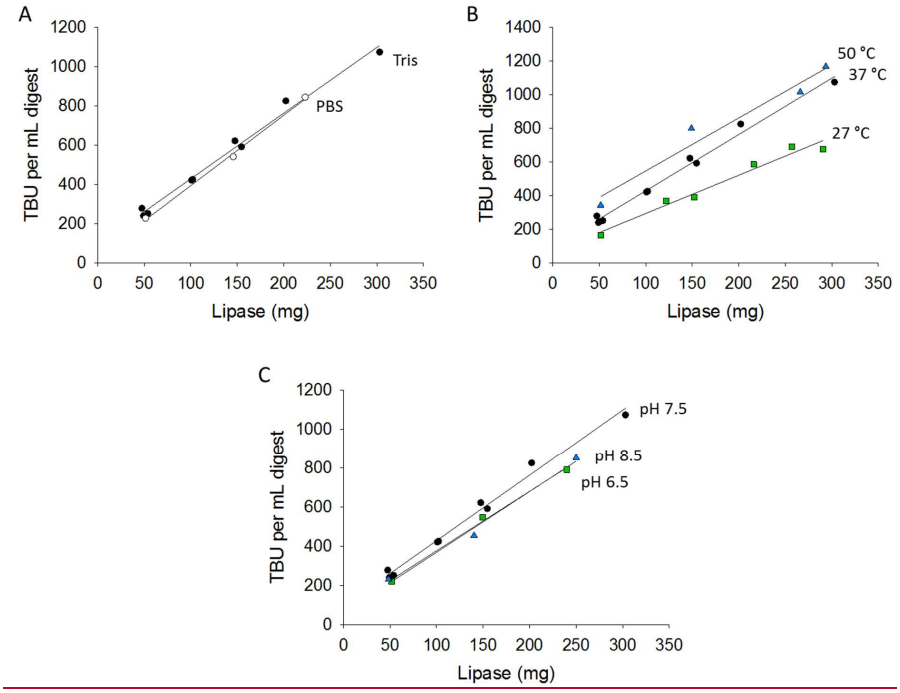

Figure 2: Effect of reaction variables on activity of immobilized lipase in A) Buffer type (Tris (closed circles) and PBS (open circles) pH 7.5 at 37 °C). B) Temperature (Tris buffer pH 7.5). C) Buffer pH (Tris buffer pH 6.5, 7.5 and 8.5 at 37 °C).

Digestion kinetics and quantitation of fatty acid released during *in vitro* lipolysis of medium chain triglycerides

The kinetics of digestion when the immobilized lipase was added to initiate digestion of tricaprylin and Captex 355 in the fasted state was slower compared to pancreatic lipase. A longer digestion time was required to achieve a plateau in the titration curve, indicative of the end of lipolysis of ~50 mM medium chain triglyceride, Captex 355 (Figure ~~2A~~3A). The % digested was calculated to be 69.3 and 84.1% for pancreatic and immobilized lipase respectively. This was calculated from the number of moles of NaOH added during the reaction, on the basis that one mole of triglyceride is hydrolysed to produce 1 mole of 2-monoglyceride and 2 moles of fatty acid [22, 23]. Notably, the digestion profiles of Captex 355 for both the lipases had a sigmoidal shape but covered different time scales.

A strong correlation between the moles of fatty acid determined by pH stat titration and HPLC with refractive index detection during lipolysis was observed (Figure 3). However, at the endpoint of

digestion, the higher fatty acid content determined by HPLC was higher at 92.7 and 90.6% for pancreatic and immobilized lipase respectively.

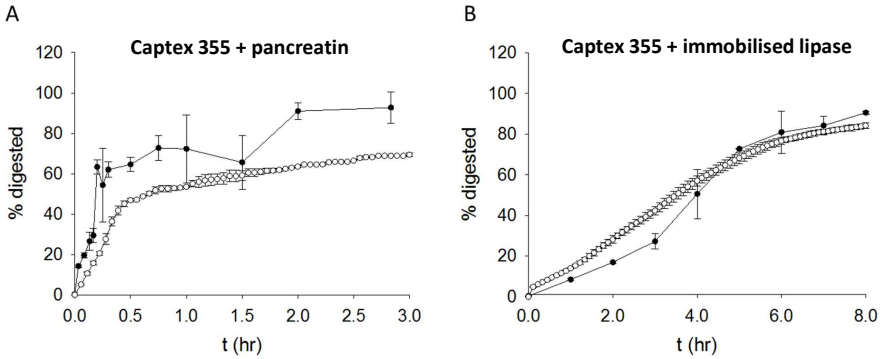

Figure 3: Kinetics of digestion, during lipolysis of ~50 mM Captex 355 in the fasted state at 37 °C with A) pancreatic and B) immobilised lipase, showing fatty acid by HPLC (closed circles) and titration (open circles). Data are mean ± range (n=2).

In the case of lipolysis of 5 mM tricaprylin, digestion proceeded past completion based on the aforementioned assumption of two fatty acids and one monoglyceride (Figure 4). For pancreatic lipase, the extent of digestion was calculated to be 178.4 % by pH stat titration and 195.6% by HPLC compared to 128.2% by pH stat titration and 116.9% by HPLC for the immobilized lipase.

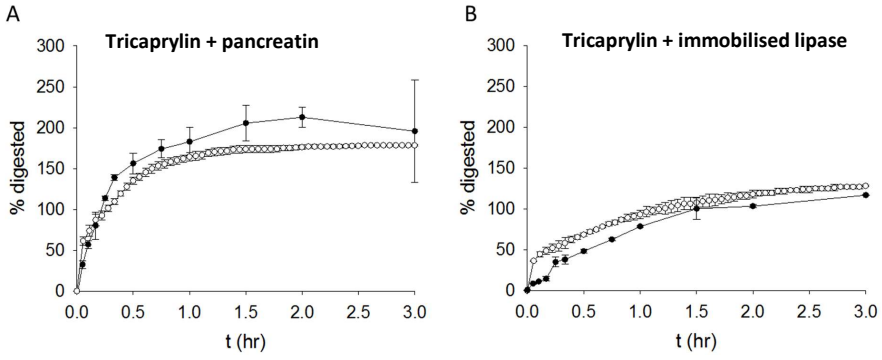

Figure 4: Kinetics of digestion, during lipolysis of 5 mM tricaprylin in the fasted state at 37 °C with A) pancreatic and B) immobilised lipase showing fatty acid by HPLC (closed circles) and pH stat titration (open circles). Data are mean ± range (n=2).

To determine whether separation of immobilised lipase from the digestion medium would be sufficient to halt digestion, samples were removed at predetermined timepoints, without addition of

the commonly used inhibitor, 4-BPBA. Correlation of fatty acid content by HPLC and pH stat titration again was observed, confirming that the lipase remains adsorbed to the polymer beads rather than leaching into the digestion medium (Figure SI-1).

Structure formation during *in vitro* lipolysis

Synchrotron SAXS was used to determine the structures formed at the completion of digestion to test the hypothesis of reduced scattering from the immobilized lipase, and to confirm independence of structures formed. After subtraction of the buffer as the background, a significant order of magnitude reduction in the scattering was observed in the dispersion of immobilized lipase in digestion buffer compared to pancreatic lipase in digestion buffer (Figure ~~6~~5).

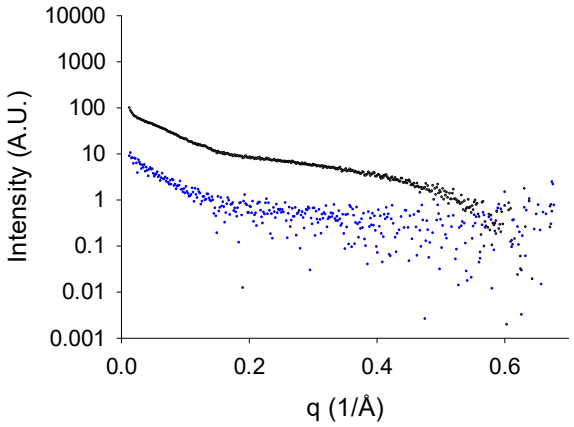

Figure ~~6~~5: SAXS profiles of pancreatic (black) and immobilized (blue) lipase dispersed in digestion buffer pH 6.5 measured at 37 °C

The two lipases were then used to initiate lipolysis in *in vitro* digestion of formulation lipids and a sample was removed at the endpoint of digestion for structural characterization by SAXS. After 8 hr lipolysis by immobilized lipase, the peaks indicative of a lamellar phase were observed to be more prominent in the scattering profiles after buffer subtraction compared to the sample digested for 3 hr with pancreatic lipase (Figure ~~7A~~6A). The lamellar phase had a repeat distance of 29.6 Å. Similarly, better structural resolution of the micellar structure was achieved using immobilised lipase following lipolysis of 5 mM tricaprylin under the same digestion conditions (Figure ~~7B~~6B).

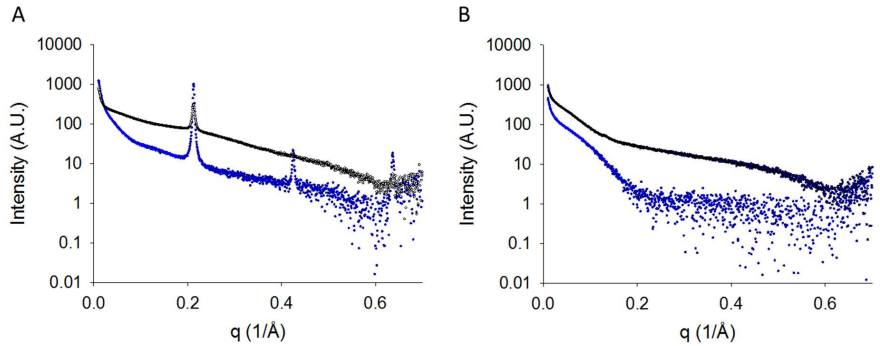

Figure ~~7~~6: SAXS profiles of end point of lipolysis of A) ~50 mM Captex 355 and B) 5 mM tricaprylin in the fasted state at 37 °C reveal formation of lamellar phase and micelles respectively. Lipolysis was performed with pancreatic (upper) and immobilized (lower) lipase.

Discussion

Lipases have been employed in the food, dairy, pharmaceutical, detergent, textile, pulp and paper, animal feed, leather and cosmetics industry. The application of immobilised lipases is increasing due to advantages over the free form such as enantioselectivity, increased chemical and thermal stability, cost efficiency and ability for reuse in continuous large-scale commercial processes. Significant resources have been deployed in recent times to attempt to improve and standardize *in vitro* digestion experiments to assist the development of LBFs [8]. It is therefore surprising that immobilized lipases have not been investigated in these activities.

Factors affecting lipase activity

The lipolytic activity of the immobilised lipase was characterized to determine the influence of buffer, pH and temperature. The independence of lipase activity on pH and buffer type opens new avenues for improved and more versatile versions of the 'accepted' in vitro digestion models. Specifically, more intestinally-relevant buffer systems, at pH values closer to those expected in the duodenum, rather than those traditionally employed in such test methods, would be expected to not deviate from the enzyme activity and structure formation while adding versatility to the choice of model variables. The adherence to past protocols may be claimed to have to a degree actually held back the research field through restricted access to components such as pancreatic lipase and specific bile components. Challenging these accepted norms through alternative approaches to the models will help to broaden our understanding of the lipid based formulation field.

Immobilised lipase displays differen~~t~~ces in digestion kinetics

This is the first description of immobilised lipases used for *in vitro* lipolysis. Formerly, the definition of a "true" lipase included the presence of a lid and activation by the presence of an interface, such that increased activity is observed when the substrate is present as an emulsion [24]. However this definition has been revised to carboxylesterases that catalyse the hydrolysis and synthesis of acylglycerols of chain length of 10 carbon atoms or more [23-25].

Pancreatic lipase is a typical lipase in that the active site is shielded from solvent, but when it is exposed to a hydrophobic interface~~, it~~ it undergoes a conformational change ~~where the~~to expose the active site ~~is exposed~~. This is known as ~~, resulting in~~ interfacial activation [26-29]. Co-lipase is also required to bind to pancreatic lipase to allow it to act at the oil/water interface to hydrolyse triglycerides, and remove bile acids from the interface which could inhibit further digestion [30]. In the current study, the immobilized lipase used, CALB, has a typical lipase α/β hydrolase fold, and the active site consists of a serine, aspartic acid and histidine catalytic triad and no typical lid domain. The amino acids are the same as those found in human and porcine pancreatic lipase [31]. Despite this difference, the current work confirms the lipolytic activity of CALB, and is in agreement with literature reports of little or no interfacial activation [24, 26]. It was found to display enhanced lipolytic activity against tributyrin compared to pancreatic lipase. This is potentially due to differences in conformation; immobilized lipase is adsorbed onto hydrophobic support, which resembles the substrate, thus rendering the lipase in a fixed "on" conformation [32, 33].

The difference in extent of digestion by the two lipases for tricaprylin could be due to the specificity of the lipase. Novozym® 435 can show positional specificity, according to the product specifications, whereas the porcine pancreatic extract contains other enzymes such as Pancreatic Lipase-Related Protein 2, Pancreatic Phospholipase A2, co-lipase and cholesterol esterase so it is possible that further digestion occurs as a result. The lipid composition during digestion of Captex 355 with pancreatic lipase is in agreement with quantification of lipid content during medium chain digestion by HPTLC [34]. It has also been previously reported that after digestion, the MG:FA ratio could be higher ~~than 1:2~~as high as 1:6 [35-37].

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Captex 355 is a mixture of medium chain triglycerides commonly used in lipid based formulations [38, 39]. Digestion of Captex 355 with immobilised lipase was slower than with pancreatin. This could be attributed to the lack of access of the lipase active site to the substrate due to confinement to the polymer beads compared to the pancreatic lipase dispersed freely in solution. The likely

mechanism of triglyceride digestion by immobilized lipase is at the droplet interface, as the majority of the triglyceride is in the emulsion droplets due to their low solubility in water ~~by collision of the beads with lipid droplets~~. If this is the case, it would be reasonable to expect that if the bead size was reduced and the surface area was increased at a nominal enzyme activity, then the rate of digestion with longer chain triglycerides would increase.

Interestingly, the immobilized lipase was found to be very effective in digesting the short chain lipid, tributyrin, compared to tricaprylin. It has been proposed in literature that lipolysis is dependent on solubility of substrate in the solvent [40]. The difference in activity can be explained by the solubility of the lipid in the solvent; tributyrin is more water soluble than tricaprylin [41]. This is in accordance with previous studies that demonstrated a higher specificity constant of CALB [40, 42], and other lipase [43] for a short chain substrates compared to long chain counterparts.

The product information states that the method of lipase immobilization is by physical adsorption to a macroporous polyacrylic resin, but does not give specific information about their immobilization process. The adsorption technique has weaker linkages between the enzyme and support than expected for covalent linkage [11, 33], thus it was of importance to determine whether desorption of lipase could occur. It has previously been demonstrated that physical desorption or leaching of CALB can occur in the presence of detergent such as Triton-X and organic solvents, leading to the conclusion that CALB is most likely physisorbed onto the support by hydrophobic interactions [10, 44]. Absence of leaching into the medium was confirmed due to correlation of fatty acid by HPLC and pH stat titration during lipolysis, indicating that the removal of immobilised lipase halts digestion. Consequently, unlike pancreatic lipase, inhibition with a molecular inhibitor such as 4-BPBA is not required [5].

Immobilized lipase provides reduced background scattering during *in vitro* lipolysis

SAXS measurements indicated a clear reduction in parasitic scattering in a dispersion of immobilised lipase compared to porcine pancreatin extract, which contains other digestive enzymes such as amylase, trypsin, ribonuclease, deoxyribonuclease, gelatinase and elastase. Interestingly, in the case of the immobilized lipase sample, an upturn was observed at low *q*. This indicates the presence of some larger particles in the scattering volume which may originate from fragments of the polymer particles.

The SAXS results following *in vitro* lipolysis of Captex 355 and tricaprylin demonstrated that the structural change that occurs at the "kink" in the sigmoidal profile previously attributed to vesicle formation is retained regardless of lipase used [5]. The decreased parasitic scattering observed during lipolysis with immobilized lipase renders it a better lipase for structural studies. It is not straightforward to simply subtract the buffer and lipase curve from the overall scattering data, as the composition of the bulk phase changes due to the formation of new interfaces and adsorption of these materials during the digestion. Application to time-resolved structural determination through improved quality of scattering data is expected to now be enabled by this discovery.

Conclusion

This study provides insight into the use of immobilised lipase for *in vitro* lipolysis experiments. The digestion kinetics of the immobilised lipase depended on the chain length of the substrate, lipid loading and temperature. The structural and compositional changes during *in vitro* lipolysis were maintained regardless of lipase used, however the decreased background scattering observed with immobilised lipase renders it advantageous over lipase sourced from porcine pancreatic extract for understanding structure formation during lipolysis.

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