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Thermomyces lanuginosus lipase-catalyzed hydrolysis of the lipid cubic liquid crystalline nanoparticles

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ABSTRACT

In this study well-ordered glycerol monooleate (GMO)-based cubic liquid crystalline nanoparticles (LCNPs) have been used as substrates for *Thermomyces lanuginosus* lipase in order to establish the relation between the catalytic activity, measured by pH-stat titration, and the change in morphology and nanostructure determined by cryogenic transmission electron microscopy and synchrotron small angle X-ray diffraction. The initial lipase catalyzed LCNP hydrolysis rate is approximately 25% higher for large 350 nm nanoparticles compared to the small 190 nm particles, which is attributed to the increased number of structural defects on the particle surface. At pH 8.0 and 8.4 bicontinuous *Im3m* cubic LCNPs transform into "sponge"-like assemblies and disordered multilamellar onion-like structures upon exposure to lipase. At pH 6.5 and 7.5 lipolysis induced phase transitions of the inner core of the particles, following the sequence *Im3m* cubic → reversed hexagonal → reversed micellar *Fd3m* cubic → reversed micelles. These transitions to the liquid crystalline phases with higher negative curvature of the lipid/water interface were found to trigger protonation of the oleic acid produced during lipase catalyzed reaction. The increase curvature of the reversed discrete micellar cubic phase was suggested to cause an increase in the oleic acid pK_a to a larger value observed by pH-stat titration.

1. Introduction

Lipolytic enzymes or lipases have important biological functions in the lipid metabolism, but are also used in numerous applications [1]. The substrates for lipolytic enzymes are self-assembled structures or aggregates of different lipid and fat molecules. Most natural substrates have low aqueous solubility and are dispersed in or exposed to an aqueous solution containing the enzyme. The enzymes therefore act at the oil–water interface and hence the term "interfacial activation" has been used to describe lipase action, which implies that the interfacial structure of the substrate is important [2]. The surface properties of the substrate are dependent on the lipid composition as well as the conditions in the aqueous phase. Another important factor to consider is that the lipolytic enzyme is generally small in size in comparison to the composite substrate assembly.

In the pioneering *in vitro* study of lipolysis of triglyceride droplets in an intestine-like environment, Patton and Carey observed a sequence of liquid crystalline phases depending on the solution conditions, among them a viscous isotropic phase composed of monoglycerides and fatty acids, which is identical to the one formed in monoglyceride systems [3]. The lipolysis products formed transiently what was later defined as the cubic phase, after which they rapidly solubilized in mixed micelles of fatty acids and bile salts if present in excess. However, after a fat rich meal, the bile acid amounts *in vivo* are not always sufficient to solubilize all lipids, and therefore it has been argued that the cubic liquid crystalline phases participate during *in vivo* digestion [4]. This study is therefore focused on the glycerol monooleate (GMO) cubic liquid crystalline phases as a substrate for the lipolytic activity. Such a substrate can also be prepared as nanoparticles with narrow size distribution and with a well-defined liquid crystalline internal structure. Here, the bicontinuity as well as the ability of the cubic monoglyceride phases to solubilize hydrophobic and amphiphilic compounds are thought to be important features for the lipolysis process [5]. These structural features will make it

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possible for *e.g.* lipase and water to freely diffuse through the phases formed by the lipolysis products, surrounding the diminishing fat droplet.

Although the study of lipases has increased very sharply during the last years, most studies concern either simple bulk studies, using less-defined substrates, or only concern the interfacial reactions, using monolayers [6,7]. Recent interfacial studies of lipase activities on monolayers have provided some leads on how to control the lipase activity by modulating the lipid composition [7]. In this study we will demonstrate that lipase activity can also be controlled by the substrate assembly structure.

In early studies, we investigated effects of lipase action on the GMO liquid crystalline phase as well as other self-assemble structures such as vesicles and dispersions of cubic phases [8–10]. We showed that the observed changes in self-assembled structures could be mapped by following either the (i) GMO/oleic acid aqueous ternary phase diagram (low pH), where the lipolysis gives rise to a transition of cubic → reversed hexagonal → micellar cubic → reversed micellar phase + dispersion or (ii) GMO/sodium oleate aqueous ternary phase diagram (high pH), where the corresponding sequence is lamellar → normal hexagonal. These differences in structural changes could be related to the degree of protonation of the fatty acids [8]. In these studies we found a similar specific activity of *Thermomyces lanuginosus* lipase (TLL) on the cubic as well as on the reversed hexagonal GMO-based liquid crystalline phases [8].

Using small-angle X-ray diffraction, cryogenic transmission electron microscopy, and dynamic light scattering, Salentinig et al. looked at the impact of lipolysis products, *i.e.* oleic acid [11]. They found that the system undergoes structural transitions from dispersions of bicontinuous cubic phases (cubosomes) through dispersions of reversed hexagonal phases (hexosomes) and micellar cubic phases (*Fd3m* symmetry) to emulsified microemulsions occurring with increasing oleic acid concentration. As expected and previously reported by Borne et al., the internal structure of the dispersed particles depended strongly on the pH, where at a high pH it tends to favor vesicles instead of reversed phases [9]. They discuss their findings in terms of an apparent pK_a for oleic acid in the cubic phase, which could be estimated from the change in structure with pH. In a follow-up study by the same group they followed the *in vitro* digestion of β-lactoglobulin and β-casein stabilized triglyceride emulsions, by pancreatic lipase [12]. The structural analysis showed a transition from oil emulsion to emulsified microemulsion, micellar cubic, inverse hexagonal, and bicontinuous cubic liquid crystalline droplets as the lipolysis progresses. They also observed strong effects on the lipolysis reaction of solution properties such as bile-juice concentration and pH as well as of hydrophobic additives.

We have previously studied lipase-catalyzed hydrolysis of cubic nanoparticles formed from GMO, which is the final step in the lipolysis of glycerol trioleate, leads to drastic changes in the liquid crystalline structure [13]. For that study we used lipase, TLL, conjugated to gold nanoparticles to visualize the enzyme location and the enzymatic digestion of lipid aggregates by means of cryogenic transmission electron microscopy. We showed that the use of lipase–gold nanoparticle conjugates provide a handle on single lipase molecules, but compared to lipase alone the manner in which enzymatic digestion occurs at the single molecule level is affected in terms of the lipid nanostructure. In the present studies we used well-defined GMO-based cubic phase nanoparticles as substrates for TLL with an aim to directly correlate the structural and morphological changes to the lipase activity and progression of the lipolysis. The main object of the present study is not to mimic the physiological conditions in the gastrointestinal tract, but rather to present and discuss in detail an assay for testing technologically relevant lipolytic enzymes that we previously outlined in 2008 [14].

2. Materials and methods

Chemicals. RYLO™ MG19 Glycerol Monooleate (GMO) was produced and provided by Danisco Ingredients (Brabrand, Denmark) with the following fatty acid composition (Lot No. 2119/65-1): 89.3% oleic, 4.6% linoleic, 3.4% stearic, and 2.7% palmitic acid. The poly(ethylene oxide) (PEO)–poly(propylene oxide) (PPO)–poly(ethylene oxide) triblock copolymer with the trade name Lutrol® F127 and an approximate formula of $PEO_{98}PPO_{57}PEO_{98}$ (average molecular weight of 12,600 g/mol) was obtained from BASF Svenska AB (Helsingborg, Sweden). Wild-type TLL and its inactive mutant (iTLL, in which the catalytic Ser146 is changed to Gly) were kindly supplied by M. Skjøt (Novozymes A/S, Denmark). Protein concentration was determined spectrophotometrically at 280 nm. A molar extinction coefficient of 43,000 M^{-1} cm^{-1} with Mw of 32 kDa was used [15]. Milli-Q purified water was used for all experiments. All other solvents and reagents were of analytical grade and were used as received.

Preparation of lipid nanoparticles. Nanoparticle dispersions were prepared by adding appropriate amounts of melted GMO (40 °C) into an aqueous F127 solution. In all experiments the GMO/F127 ratio was fixed to 9/1 (w/w) and the total amphiphile (GMO + F127) concentration in water was either 2 or 5 wt%. The total sample volume was usually 200–300 mL. The samples were immediately sealed, shaken, and mixed for 24–48 h on a mechanical mixing table at 350 rpm and room temperature. The resulting coarse dispersions were homogenized by passing 5 times through a Microfluidizer 110S (Microfluidics Corp., Newton, USA) at 345 bar and 25 °C. Homogenized dispersions were then exposed to heat treatment in order to improve dispersion properties in terms of reducing the amount of metastable vesicular aggregates. Heat treatment was performed using a bench-type autoclave (CertoClav CV-EL, Certoclav Sterilizer GmbH, Traun, Austria) operated at 125 °C and 1.4 bar. The samples were subjected to heat treatment for 20 min at 125 °C and allowed to cool to room temperature before analysis. In all cases this nanoparticle preparation procedure resulted in homogenous milky dispersions with narrow and monomodal size distributions (polydispersity index of 0.2) with the mean particle size of 190 and 350 nm for dispersions containing 2 and 5 wt% of amphiphile, respectively.

Particle size measurements. Particle size distributions were measured using a Coulter LS230 laser diffraction particle size analyzer (Beckman-Coulter, Inc., Miami, U.S.A.), which operates on the principles of Fraunhofer diffraction for large particles (0.4–2000 μm) and uses the polarization intensity differential scattering (PIDS) method for small particles (0.04–0.5 μm). The instrument was fitted with a 125 mL volume module. Data were collected during 90 s. A standard model based on homogenous oil spheres with a refractive index (RI) of 1.46 was used for the particle size calculations. The change in RI to either side only shifts the obtained particle size distributions within a few percents. Note that the model is based on spherical particles and the measured mean particle size is calculated based on this assumption.

pH-stat titration. pH-stat titration experiments were performed on a computer-controlled TitraLab 856 titration workstation (Radiometer Analytical SAS, France) equipped with an automatic 10 ml burrete (volume controlled down to 0.6 μl for each injection step) and a general purpose Red Rod-combined pH electrode. During the lipolytic reaction the GMO within the cubic liquid crystalline nanoparticles is hydrolyzed and oleic acid is formed, which lowers the pH of the reaction mixture. The aim of the pH-stat titration method is therefore to follow the lipolytic reaction kinetics by continuously titrating generated oleic acid as a function of time while keeping the pH constant. In all experiments the reaction was carried out in a Teflon vessel (total reaction volume 20 ml) and reaction media contained 1.5 mM potassium phosphate buffer

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