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Lipase reaction at interfaces as self-limiting processes

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Abstract

Lipases are lipolytic enzymes that play a key role in fat metabolism. They are catalysts for the hydrolysis of triacylglycerides, which contribute for major portion of calories to our daily diets. Due to the apolar nature of oils and fats, the oil−water interface is where the control of lipolytic conversion and finally digestion takes place. It might come as a surprise that despite past efforts the interfacial behaviour of lipases is not completely understood. We have undertaken a detailed study of the interfacial behaviour of lipases, their substrates and the products. Our results demonstrate that lipase activity is a function of interfacial composition and changes concurrently with lipolytic conversion. In these cases lipase "inhibition" should be attributed to substrate depletion and not to lipase desorption or denaturation as previously hypothesized. This self-limiting effect through the feedback of interfacial composition to the reaction conditions of the enzyme may open a new way to control lipase catalysis through the interface. To prove our point, we appreciably reduced oil hydrolysis in a model gastro-intestinal system by interfacial engineering of the oil. We anticipate that our findings can contribute in finding new approaches for controlling fat metabolism, which is central to health threats like obesity and diabetes mellitus II and important for the regulation of energy metabolism in general. *To cite this article: P. Reis et al., C. R. Chimie 12 (2009).*

Résumé

Les lipases sont des enzymes lypolytiques jouant un rôle clé dans le métabolisme des graisses [R. Schmid, R. Verger, Angew. Chem., Int. Ed. 37 (1998) 1608]. Ce sont des catalyseurs de l'hydrolyse des triglycerides, lesquels contribuent pour une grande part à notre apport calorique quotidien [J. Walker, S. Roberts, K. Halmi, S. Goldberg, Am. J. Clin. Nutr. 32 (1979) 1396]. En raison de la nature apolaire des graisses et des huiles, c'est à l'interface eau-huile qu'a lieu la conversion lipolytique et donc la digestion. Il apparaît surprenant que, malgré les efforts passés, le comportement interfacial des lipases ne soit complètement élucidé. Nous avons entrepris une étude détaillée du comportement interfacial des lipases, de leurs substrats et de leurs produits. Nos résultats démontrent que l'activité des lipases est en relation directe avec la composition de l'interface qui va évoluer au cours de la

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conversion lipolytique. Ainsi, la perte d'activités des lipases pourrait être due à un appauvrissement en substrat dans le milieu et non à une désorption ou à une dénaturation des enzymes, comme cela a pu être suggéré précédemment [R. Verger, Trends Biotechnol. 15 (1997) 32]. Cet effet d'autorégulation du fonctionnement de l'enzyme, grâce à la modification de la composition de l'interface, ouvre de nouvelles perspectives de contrôle de l'activité des lipases. Pour prouver notre hypothèse, nous avons sensiblement réduit l'hydrolyse d'huiles dans un système gastro-intestinal modèle dont la composition de l'interface était contrôlée. Nos conclusions pourraient fournir une nouvelle approche pour le contrôle du métabolisme des graisses, qui joue un rôle central dans des problèmes de santé tels que l'obésité, et les diabètes mellitus II (type II) [M. Furuhashi, G. Tuncman, C. Görgün, L. Makowski, G. Atsumi, E. Vaillancourt, K. Kono, V. Babaev, S. Fazio, M. Linton, R. Sulsky, J. Robl, R. Parker, G. Hotamisligil, Nature 447 (2007) 959], et est de première importance dans la régulation du métabolisme énergétique en général [H. Stam, W.C. Hülsmann, Eur. Heart J. 6 (1985) 158]. ***Pour citer cet article : P. Reis et al., C. R. Chimie 12 (2009).***

Fat metabolism plays a major part in the energy balance of our body. Deregulation of metabolic processes has been closely linked to metabolic disorders observed in obesity, a major public health issue [1–5]. Classical strategies to tackle obesity are increasing physical activity, reducing energy intake or inhibiting fat absorption during digestion [6,7]. Inhibitory molecules interact with the active site of lipases reducing the lipolysis of dietary fats. While this classical and pharmacological approach is valid, the phenomenon of surface activation of lipases opens another way to control their enzymatic reactions. As a consequence of the fact that the majority of lipase substrates are apolar and water insoluble, lipolytic reactions have to take place at the interface between the immiscible phases [8]. Recent studies have confirmed that lipases become activated through conformational rearrangements of parts of the molecule which opens the active site upon adsorption to an apolar interface [9–11]. Furthermore, while the majority of proteins, as surface active macromolecules, adsorb to interfaces by unfolding and denaturation, lipases adsorb in a highly active state [12]. Adsorption studies of lipases have revealed that the enzymes are also able to adsorb and to be activated at a variety of hydrophobic and hydrophilic functionalized liquid-crystalline solid surfaces (SAMs) [13].

The physical state of the interface has long been recognized as important in enzymatic interfacial reactions influencing the fate of lipase catalysis [4]. However, the still unsatisfying situation in the understanding of the interfacial impacts on lipase catalysis needs a novel line of attack to reveal the determining interfacial physics. We chose to work with a well-defined interfacial approach using a biphasic system comprising an apolar mineral oil (e.g. decane) and an aqueous phosphate buffer containing the lipase at physiological pH. Physical measurements were performed with the pendant drop technique allowing measurements at a precisely predetermined and constant surface area. Moreover, the apolar phase is inert against lipolytic attack providing a defined and controllable interface during the enzymatic reactions.

This set up allowed us to study the interface through precise control of the surface pressure (interfacial tension) by independently dosing lipases and interacting molecules into the two separate phases. Both the mineral oil and the aqueous phase serve as source and sink for molecules partitioning to the interface. Moreover, sensitive measurements of the interfacial tensions permit us to model interfacial composition and relate this knowledge to the interfacial behaviour of the enzyme.

In the past, the interfacial influence has been described through the "quality of the interface" which determines the behaviour of the lipases and the outcome of the enzymatic reactions [4]. However, defining the quality of the interface is ambiguous and has proven difficult to define [14]. Additionally, a variety of interfacial phenomena were reported to have detrimental influence on both enzymes and reactions [15]. For example, enzymes irreversibly denature at interfaces under high interfacial tension conditions (low surface pressure) or are excluded from the interface under low interfacial tension conditions, respectively [16]. On the other side, a large number of studies of lipases in microemulsions show that lipases can be part of the interface and stay fully active at even very low interfacial tension [17]. Lipase action at the interface can also be inhibited by the interaction with amphiphiles such as bile salts or fatty acid salts. Electrostatic interactions

leading to conformational changes or complexation of small surface active molecules were reported to lead to enzyme inhibition [18,19]. Furthermore, the enzyme reaction might stall through competitive inhibition from small molecules [20]. An important source of inhibition was found to be the interaction of detergents with lipases [21]. However, no direct relationships were established between lipase activity and the changes of interfacial tensions due to the presence of detergents. Despite intensive studies of interfacial properties of lipases and their behaviour in self-assembly surfactant systems, no conclusive understanding could be established on the interfacial physics ruling lipase biocatalysis [22–24]. Although large differences in the interfacial behaviour of lipases can be observed, they have not been subjected to a systematic interfacial study together with their substrates, intermediates and product molecules.

A large group of lipases expresses Sn-1,3 regiospecificity by cleaving the ester bonds of fatty acids in the Sn-1 and Sn-3 positions of triacylglycerides. Lipase from *Rhizomucor miehei* is a typical and well-studied example of this group. Metabolic lipases also fall into this class. We used lipases from *R. miehei* throughout this study. Additionally, we employed a non-regiospecific lipase from *Candida rugosa* to present a different interfacial behaviour. A third type of lipase used in our study is represented by pancreatic lipase which needs a colipase to function at the interface.

To gain new insight into interfacial behaviour of lipases, it is not enough to study the protein alone. On the contrary, it has to be studied in the presence of its substrate and products. The lipase might start with a substrate saturated interface but, due to its enzymatic action, it constantly changes the composition by generating molecules of different interfacial activity. The changing composition, on the other hand, will influence the behaviour of the lipase creating a feedback loop for its activation.

Our approach focuses on the behaviour of lipase in the presence of substrate, intermediates and products as a model of lipase catalysis. To gain insight into the interfacial behaviour of individual molecules and their mixtures, we measured the equilibrium interfacial tension by the well established pendant drop technique at varying concentrations at a given total interfacial area between decane and an aqueous phosphate buffer at physiological pH. We used the triacylglyceride tricaprylin (TC8) as model substrate. Our measurements showed that interfacial tension of an individual molecule varied greatly with concentration.

The intermediate product of TC8 lipolysis, the monoacylglyceride monocaprylin (MC8), is the most

interfacially active component: it reduces the interfacial tension to the very low values of $5 \, \mathrm{mN \, m^{-1}}$ ($1.8 \times 10^{-6} \, \mathrm{mol \, L^{-1}}$). The lipase reduced the original interfacial tension of the decane–aqueous buffer interface from $51 \, \mathrm{mN \, m^{-1}}$ to $15 \, \mathrm{mN \, m^{-1}}$ at interfacial saturation concentrations of $3.5 \times 10^{-5} \, \mathrm{mol \, L^{-1}}$, exhibiting strong surface active properties. The triacylglyceride TC8 has a small interfacial activity, producing a value of $32 \, \mathrm{mN \, m^{-1}}$ ($1.0 \times 10^{-2} \, \mathrm{mol \, L^{-1}}$). Diacylglyceride (DC8) exhibited similar decreases at lower concentrations as the lipase and free caprylic acid (C8). However, it is slightly more interfacially active at higher concentrations ($22 \, \mathrm{mN \, m^{-1}}$ at $3.0 \times 10^{-5} \, \mathrm{mol \, L^{-1}}$) than free caprylic acid ($27 \, \mathrm{mN \, m^{-1}}$ at $3.0 \times 10^{-5} \, \mathrm{mol \, L^{-1}}$). The interfacial tension variation versus concentration of the individual components points to strong interfacial effects when all components are simultaneously present during the catalytic reaction.

These strong interfacial effects are born out by the behaviour of the binary mixture of substrate and reaction products. Fig. 1 summarizes the measured interfacial tension at constant total bulk concentration of the component mixture ($5.0 \times 10^{-3} \, \mathrm{mol \, L^{-1}}$) under variation of the molar ratio in the mixture. The parameter ϕ defines the mol% of the bulk mixture (e.g. $\phi = \mathrm{DC8}/(\mathrm{DC8} + \mathrm{C8})$). While the mixtures of diacylglyceride DC8 with caprylic acid C8 lead to a small, almost linear decrease of the interfacial tension ($27 \, \mathrm{mN \, m^{-1}}$ to $22 \, \mathrm{mN \, m^{-1}}$), mixtures of monoacylglyceride MC8 with both DC8 and C8 result in almost identical curves exhibiting a strong reduction of the measured interfacial tension. Already at 50 mol% of MC8 we reach the individual molecule interfacial
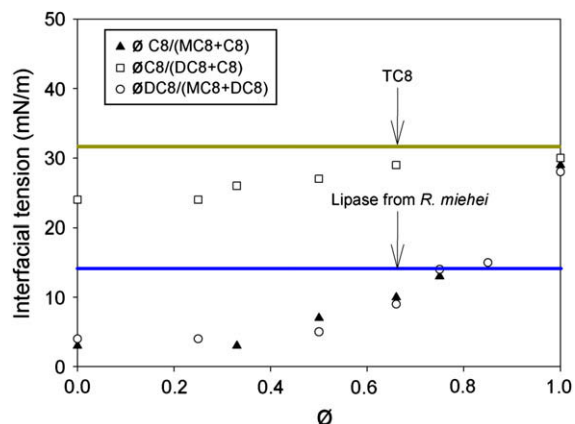

Fig. 1. Characterization of the interfacial tension at room temperature of binary systems containing lipase generated products at phosphate buffer/decane interface for a fixed total molar concentration of 5.0 mM.

tension of MC8 (5 mN m^{-1}). The strong effect on the interfacial tension will have consequences for the changes in the interfacial composition of the two components. Monoacylglyceride MC8 is expected to dominate the interfacial composition already at low bulk concentrations in both mixtures. The higher interfacial activity of monoacylglycerides leaves only trace amounts of the other components at the interface.

Comparing the equilibrium interfacial tension of lipase alone (blue parallel line) (For interpretation of the references to colour in this text, the reader is referred to the web version of this article.), we can see that already 20 mol% of monoacylglyceride would lower the interfacial tension below that of the lipase. Lipases, as any other surface active macromolecules, can undergo competitive desorption caused by molecules with higher interfacial activity [21]. The consequence of competitive desorption would be a halting of the catalytic reaction. For the enzymatic reaction to proceed to completion, the accumulation of highly interfacially active molecules like monoacylglycerides at the interface has to be neutralized by removing, solubilisation or hydrolysis of the molecules.

Using the experimental data, we modelled the interfacial distributions of the binary mixtures in equilibrium using the approach of Fainerman et al. [25–27]. Fainerman's model has the advantage over previous interfacial models that it takes directly into account that a protein molecule can occupy a variable interfacial area in the surface layer depending on surface coverage. Furthermore, it also takes into account the non-ideality of enthalpy in the formation of mixed surface layers through molecular interactions. Surface areas of the molecules obtained from fitting experimental data to adsorption isotherms (Langmuir isotherms) and their adsorption constants were used as input parameters. The protein molecule surface area is added in the form of three parameters determining the minimum, normal, and maximum surface area a protein can adopt at the interface. These values were obtained from comparable and independent protein measurements. We used beta-lactoglobulin as our reference.

Fig. 2 summarizes the distributions obtained from titrating triacylglyceride (TC8) and lipase from *R. miehei* with Sn-2 monoacylglyceride. The distribution is expressed in mol% at the interface with increase in the bulk concentration of Sn-2 monoacylglyceride (Sn-2 MC16) in the decane phase. The results reveal that the bulk concentrations of the Sn-2 MC16 in decane needed to reach 50 mol% of surface coverage by Sn-2 MC16 for the mixture with triacylglyceride TC8 and with lipase,
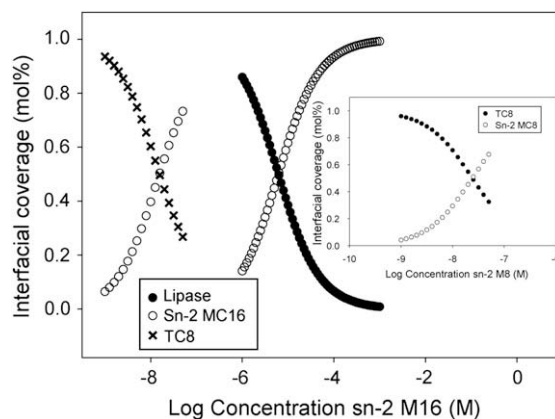

Fig. 2. Interfacial coverage as a function of bulk concentration of Sn-2 monopalmitin (Sn-2 M16) for a fixed concentration of lipase from *Rhizomucor miehei* (3.5×10^{-5} mol L^{-1}) or for a fixed concentration of tricaprylin (5.0×10^{-3} mol L^{-1}). The experiments were run at room temperature.

respectively, are two orders of magnitude different (2.0×10^{-8} mol L^{-1} and $8.0. \times 10^{-6}$ mol L^{-1}). The inset gives the results for the mixture of Sn-2 monoacylglyceride Sn-2 MC8 with TC8. The 50 mol% coverage is again reached at almost the same bulk concentration as the Sn-2 monoacylglyceride. This high sensitivity of the interfacial composition against the amount of Sn-2 monoacylglyceride proofs that the substrate TC8 is very easily excluded from the interface, even by the short-chain monoacylglyceride Sn-2 monocaprylin (Sn-2 MC8).

Consequently, the substrate (TC8) will be completely replaced by Sn-2 MC16 long before desorption of the lipase begins to remove the enzyme from the interface (competitive desorption) in the case of simultaneous presence of monoacylglycerides, triacylglycerides and lipase during the reaction. This sensitivity of the interfacial composition to the presence of molecules with large interfacial activity differences also explains why a conclusive understanding of the interfacial behaviour of lipases has so far not been established beyond doubt. The behaviour of the lipase depends on the relative interfacial excess concentrations of the surface active components. Furthermore, the interfacial composition will also determine whether the enzyme will appear active or inhibited having access to substrates or not.

To test the hypothesis, that the accumulation of the Sn-2 monoacylglyceride stalls the enzymatic reaction because the Sn-1,3 regiospecificity of lipase from *R. miehei* does not allow it to catalyze hydrolysis of the monoacylglyceride and consequently lets it accumulate at the interface, we set up an experiment employing the lipase from *R. miehei* and then continuing with the

non-regiospecific lipase from *C. rugosa*. Using both enzymes consecutively allows us to modify the interfacial composition and to measure the resulting changes in interfacial tensions. Fig. 3 shows the experimental data for this experiment. After equilibration of the interface with lipase from *R. miehei* ($\gamma = 15$ mN m^{-1}), the addition of triacylglyceride TC8 quickly lowers the interfacial tension to 6 mN m^{-1} due to the biocatalytic conversion. The value remained there for the full duration of phase 1 of the experiment is of 25 h. Repetition of this experiment with pancreatic lipase in the presence of colipase or with human lipoprotein lipase led to similar results suggesting that the observed phenomenon is general (results not shown here).

Chemical analysis of the system at the end of phase 1 found that only 5% of the TC8 was converted into products. Phase 2 started at the indicated time in Fig. 3 by addition of the non-regiospecific lipase from *C. rugosa*, which is able to catalyze hydrolysis of the accumulated Sn-2 monoacylglycerides producing fatty acids and glycerol. The interfacial tension increased immediately, eventually reaching that of the lipase from *C. rugosa* with free fatty acids ($\gamma = 10.5$ mN m^{-1}). Chemical analysis at the end of phase 2 (an additional 21 h) revealed that a further 24% of TC8 was converted to product.

Changes in the interfacial composition due to lipase catalysis determine not only the presence of molecular species at the interface, but also influence the physical properties of the interface. The interface may be converted from being purely hydrophobic to being hydrophilic and may also contain charged groups from
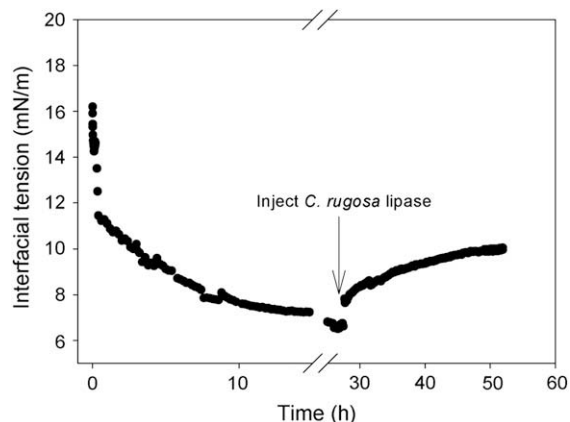

Fig. 3. Interfacial tension plot of tricaprylin hydrolysis by the Sn-1,3 regiospecific lipase from *Rhizomucor miehei* at a buffer/decane–tricaprylin system, followed by injection of the non-regiospecific lipase from *Candida rugosa* after 25 h reaction time at room temperature.

adsorbed deprotonated fatty acids. As our modelling has indicated, only high bulk amounts of monoacylglyceride displace the protein from the interface. We infer from our results that the lipase molecules remain active and adsorbed at the interface. To strengthen this conclusion, we can cite our previously published studies on lipase interactions with functionalized SAM layers on gold chips [13]. We conclusively showed that lipase from *R. miehei* not only adsorbs onto hydrophobic interfaces but also onto hydrophilic functionalized surfaces (SAM molecules had hydroxyl groups as end groups). Moreover, the lipases remained active and exhibited both hydrolysis and esterification in the presence of fatty acids and glycerol [13].

To investigate whether the adsorbed enzyme remains active after achieving a low interfacial tension, we undertook a complete chemical analysis including the fate of our marker, palmitic acid (C16). Chemical analysis revealed that the enzyme was still active after stopping to convert TC8 at very low interfacial tension ($\gamma = 5$ mN m^{-1}). It produced new diacylglycerides by esterifying the dominating monoacylglyceride Sn-2 MC8 with the palmitic acid marker, C16. Fig. 4 summarizes the analysis of three trial runs with lipases of *R. miehei* and TC8 in the presence of C16. Fig. 4A represents the control experiment without C16.

To see the influence of bile salt micelles, Fig. 4B and C presents two experiments in the presence of the marker C16. The presence of newly synthesized diacylglycerides is revealed by peaks more hydrophobic than TC8 triacylglyceride in runs of Fig. 4B and C. Quantitative LS-MS analysis confirmed that the molecules are diacylglycerides. Bile salt micelles present in physiological concentrations cannot hinder but only reduce the extent of the formation of diacylglycerides (chromatographic traces in Fig. 4C versus Fig. 4B). Apparently, some of the interfacially active markers were cleared from the interface by solubilisation into the bile salt micelles.

The experiments and modelling presented so far clearly show that qualitative description of the interface is insufficient to understand the physical phenomena occurring during lipase catalysis. It is important to realize that the reaction undergoes a sort of self-limitation by which a given interface controls its own reaction environment and determines what reactive component remains at the interface. As long as the original substrate has access to the interface the reaction will yield the expected hydrolysis products. However, the reaction products will change the composition of the interface. Since human lipases are
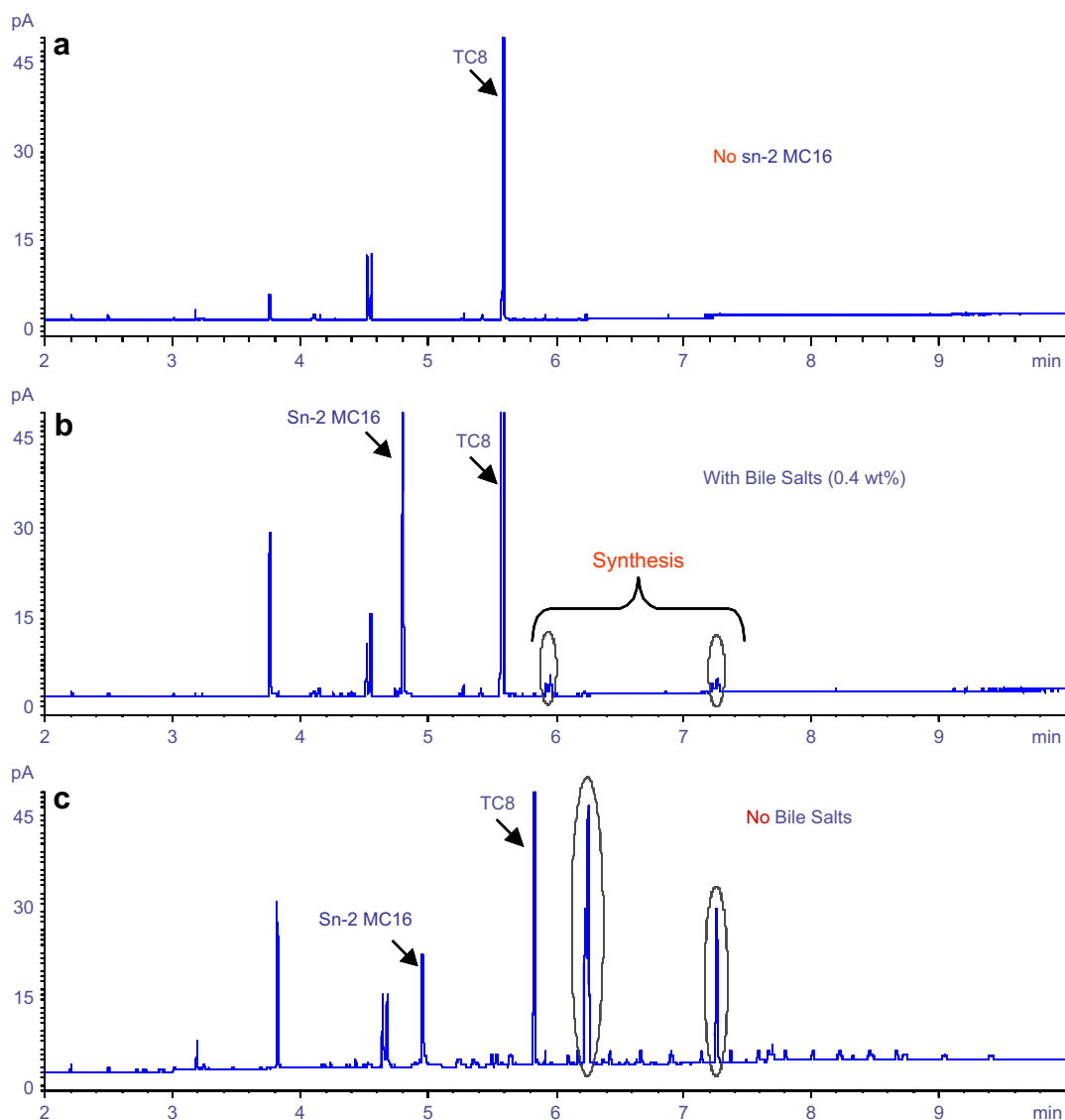
Fig. 4. Chromatograms obtained by GC-FID after 3 days of biocatalysis at room temperature performed by lipase from *Rhizomucor miehei* in a phosphate buffer/decane system in the presence of surface active agents.

generally Sn-1,3 regiospecific enzymes, hydrolytic interface reactions during digestion and fat metabolism could be self-limiting, controlled by the amount of interfacially accumulated Sn-2 monoacylglyceride. Consequently, complete fat digestion and metabolic conversion depend on the successful removal of the highly surface active components to assure the access of the substrate to the interface. This is achieved by bile salt micelles in the intestinal tract and by specific transport proteins in the metabolic processes. Failure of removal (by inhibition or lack of transporters) will lead to ceasing of the lipolytic reaction, self-limiting the amount of surface active material liberated in the body.

To test the hypothesis of self-limiting enzyme reaction through the accumulation of mono-acylglycerides, we undertook a ''digestion'' experiment in the TNO gastro-intestinal model [28]. This model is frequently used in nutritional studies and mimics digestive processes occurring in the intestine. Studying the processes with added enzymes and bile salt micelles at biological concentrations and pH allows monitoring the digestive processes in stepwise fashion at various positions along the gastro-intestinal tract. Fig. 5 assembles the results from digestion trials of tricaprylin (TC8) with and without addition of Sn-2 monopalmitin (Sn-2 MC16). The results reveal a large
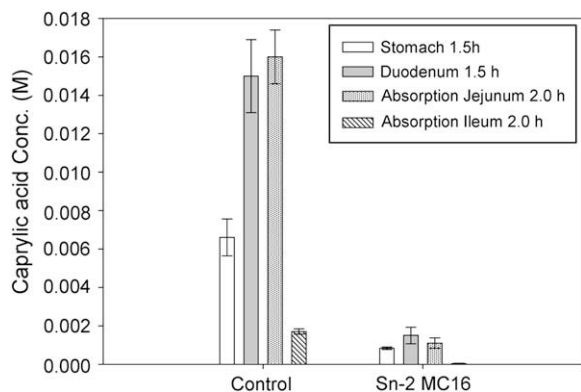
Fig. 5. Caprylic acid concentration generated upon digestion of tricaprylin with/without Sn-2 monopalmitin (Sn-2 MC16) in a gastrointestinal model system (TIM from TNO). The experiments were conducted at 37 °C.

and significant difference in the liberation of fatty acids and extent of the lipolytic reactions.

The control experiment without addition of Sn-2 monoacylglycerides results in a normal ''digestion'' of the oil with large amounts of caprylic acid measured at the various positions from stomach to ileum within a time frame of 1.5–2 h. In contrast, the experiment with added Sn-2 monoacylglycerides (Sn-2 MC16) liberated significantly less caprylic acid. The impact of enzymatic reactions of gastric and pancreatic lipases is strongly reduced at all measuring positions, indicating that the oil was only slightly digested. These results were obtained in the presence of bile salt micelles which were not able to remove completely the added and produced Sn-2 monoacylglyceride from the oil interface. Repetition of the experiments with olive oil and Sn-2 monopalmitin gave similar results showing that possible differences in solubility of monoacylglycerides in the oil phase (TC8 versus olive oil) do not change the experimental outcome.

In conclusion, lipolytic interface reactions exhibit a self-limiting effect due to simultaneous changes in interface composition and accumulation of highly interfacial active components at the interface. The *in situ* production of highly surface active intermediates and end products reduces the access of the substrate to the activated enzyme and inhibits further enzymatic conversion. Depending on the reactivity of the components accumulating at the interface, the enzyme can switch its reaction mode from hydrolysis to synthesis and vice versa. It is worthwhile mentioning that synthesis, i.e. esterification proceeds despite the aqueous environment due to specific physical and chemical conditions in the interface. Furthermore, the

self-limiting process can be controlled by removal agents like bile salt micelles and transport proteins or by the use of non-regiospecific lipases. It is important to keep in mind that even in the case of non-regiospecificity, the production or presence of highly interfacially active additives will stop the lipolytic conversion. The results presented in our study point towards the possibility of controlling lipolytic reactions through compositional control of oil interfaces which can be realized through specific modification of food emulsions or of oil interfaces in food matrices. We are, however, fully aware of the fact that the *in vivo* situation is complicated and that one should be careful in extrapolating results from model reactions to the biological situation.

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