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ARTICLE

Fatty Acid Selectivity of Lipases during Acidolysis Reaction between Oleic Acid and Monoacid Triacylglycerols

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With the aim of determining the fatty acid (FA) selectivity of lipases, a mixture of oleic acid and monoacid triacylglycerols (TAGs) including tricaproin (T6), tricaprylin (T8), tricaprin (T10), trilaurin (T12), trimyristin (T14), tripalmitin (T16) and tristearin (T18) was used as the substrate in acidolysis performed in hexane. Three immobilized lipases, namely, Lipozyme TL IM from *Thermomyces lanoginosus*, Lipozyme RM IM from *Rhizomucor miehei* and Novozym 435 from *Candida antarctica*, were used as biocatalyst. The effects of operating variables including the mole ratio of oleic acid to monoacid TAG, temperature, enzyme dosage and reaction time on incorporation were also investigated. Significantly different incorporation rates were obtained for different TAGs used ($P < 0.05$). Incorporation of oleic acid into TAGs except tricaproin and tricaprylin was higher for all the TAGs with Lipozyme TL IM catalyzed reactions than those of other two enzymes tested. Incorporation of oleic acid decreased as the acyl chain length of FA in the TAG increased with Novozyme 435 catalyzed acidolysis. Compared to the other substrate mixtures, the highest incorporation was observed for oleic acid and tricaproin mixture with three lipases tested. It was shown that the FA selectivity of the lipases is strongly dependent on the acyl chain length of FA in a TAG.

KEYWORDS: Lipase; fatty acid selectivity; acidolysis; triacylglycerol; oleic acid

INTRODUCTION

Lipases [triacylglycerol (TAG) acylhydrolases, E.C. 3.1.1.3] are used as versatile biocatalysts in numerous applications including detergent, food, flavor, pharmaceutical, leather, textile, cosmetic and paper industries. A lot of study has also been conducted on the topic of lipase-mediated reactions in academia. In this respect, production of fats free from trans fatty acids (TFAs), structured lipids (SLs) and kinetic assessments related to lipase-catalyzed reactions has become a very popular research area in the last two decades. Lipase can be used as biocatalyst for hydrolysis, esterification, acidolysis, interesterification and modification of fats and oils. One of the common routes reported in the literature to the synthesis of SLs is based on a simple lipase-catalyzed acidolysis between TAG and FA, leading to exchange of acyl groups (*1*−*9*).

FA selectivity of lipases has been successfully utilized in fat and oil modification. It has been reported that the composition of substrates, nature of solvents, operating parameters and source of lipases could influence the selectivity (*10*). The ability of lipases to discriminate among FAs could be utilized to facilitate new applications. In general, FA selectivity has been assessed by determining the similarity of FAs released by lipase-catalyzed hydrolysis of equimolar mixtures of monoacid TAG, and by use of known synthetic TAG as substrates (*11*). FA selectivity of lipases is related to their ability to distinguish between particular FA or acyl moieties. Knowledge about the substrate selectivity of lipases is therefore essential for their utilization (*12*).

A comprehensive understanding of substrate selectivity patterns of lipases in acidolysis is required to fully enable the development of appropriate strategies to prepare SLs or other products. Recently, we investigated the chain length selectivity of the same lipases during acidolysis between triolein and saturated FAs varying from caproic to behenic (*13*). In most studies to determine the selectivity of the lipases, a mixture of FAs as acyl donor and a certain TAG molecule has been used as the substrate. Here we report an evaluation of the selectivity of lipases in acidolysis between oleic acid and different monoacid TAGs. We aimed to incorporate oleic acid into different monoacid TAGs varying from tricaproin to tristearin using different immobilized lipases, namely, Lipozyme TL IM from *Thermomyces lanoginosus*, Lipozyme RM IM from *Rhizomucor miehei*, and Novozym 435 from *Candida antarctica* lipase B. On the other hand, we tried to find the answer for the question, how will the FA selectivity of lipases be influenced by the molecular weight or the geometry of the substrate combinations of FA and TAG molecules? The incorporation abilities of the enzymes were determined in the presence of hexane. The effect of operating variables such as mole ratio of oleic acid to TAG, temperature, enzyme dosage, and reaction time on the incorporation was also investigated.

MATERIALS AND METHODS

Materials. Lipases were provided by Novo Nordisk A/S (Bagsvaerd, Denmark). Lipozyme TL IM is *Thermomyces lanuginosa* lipase immobilized on granulated silica particles. Lipozyme RM IM is *Rhizomucor miehei*

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lipase immobilized on anion exchange resin particles. Novozym 435 is *Candida antarctica* lipase type B, immobilized on macroporous acrylic resin beads. Tricaproin (T6), tricaprylin (T8), tricaprin (T10), trilaurin (T12), trimyristin (T14), tripalmitin (T16), tristearin (T18), and oleic acid (C18:1) were purchased Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Fatty acid methyl ester (FAME) mixture (37 component FAME mix) and mono-, di- and triglyceride mixtures were obtained from Supelco (Bellefonte, PA). Sodium sulfate (anhydrous) was supplied by J.T. Baker (Deventer, Holland). All other chemicals and reagents for the analysis were analytic or chromatography grades.

Acidolysis Reaction. Experimental design was similar to those of a previous study (*13*). Binary mixtures of oleic acid (28.2 mg) with each of the TAGs (T6, T8, T10, T12, T14, T16 and T18) were used separately in acidolysis. Reactions were carried out in tightly closed, screw-capped glass vials (20 mL) containing an oleic acid−TAG mixture dissolved in 3 mL of hexane. The vials were incubated in a shaking water bath at 200 rpm. Effects of substrate mole ratios (oleic acid:TAG) ranging from 1:1 to 4:1, temperatures ranging from 40 to 60 °C, reaction times ranging from 3 to 24 h, and enzyme dosages ranging from 5% to 20% (by total weight of substrates) on the incorporation were studied. To determine the effects of different parameters on incorporation of oleic acid into TAG, the default conditions were chosen as the following: substrate mole ratio of 1:1, temperature of 50 °C, reaction time of 6 h, enzyme dosage of 10% and no extra water addition.

At the end of the reaction the suspensions were filtered through a syringe membrane filter (0.45 μm) to remove the enzyme particles, and filtrates (hexane solutions) were used for subsequent analysis.

Analysis of Product. One hundred microliters of the hexane solution was applied to thin-layer chromatography (TLC) plates (20 cm × 20 cm) coated with silica gel 60 F_{254} (Merck) in a thin uniform line by means of an applicator (Linomat 5, Camag, Muttenz, Switzerland). The developing solvent was hexane/diethyl ether/acetic acid (80/20/1, v/v/v). The bands were visualized under UV light after spraying with 0.2% 2,7-dichlorofluorescein in ethanol. The TAG band was scraped off into a screw-capped vial and methylated with 3 mL of 6% HCl in methanol at 75 °C for 2 h (*14*). At the end of the incubation, vials were cooled on ice bags, and 2 mL of hexane was added before centrifugation. The upper phase containing FAMEs was transferred to a vial containing anhydrous sodium sulfate by Pasteur pipet and used for FA composition analysis.

FA Composition Analysis. The FAMEs were analyzed by gas−liquid chromatography. The gas chromatograph (GC) was an Agilent 7890A with a fused capillary column (DB-23, 60 m × 0.25 mm i.d., 0.25 μm film thickness; J&W Scientific, Folsom, CA), an auto injector (Agilent 7683B), and a flame ionization detector (FID) and was operated in split mode with a split ratio of 1:30. The injector and detector temperatures were maintained at 250 °C. The column temperature was held at 140 °C for 5 min and ramped to 240 °C for 10 min at a rate of 4 °C/min. The carrier gas was helium, and the total flow rate was 30 mL/min. The FAMEs were identified with those of standard mixtures (37 FAMEs mixtures, Sigma-Aldrich Inc., St. Louis, MO), and the results are presented as average molar percentage of two determinations.

Statistics. SPSS version 9.0 (SPSS Inc., Chicago, IL) was used to perform statistical calculations. Significant differences in the means of incorporated oleic acid (mol %) into TAGs between three lipases catalyzed acidolysis were determined by using a least significant difference test and analysis of variance procedure ($P < 0.05$).

RESULTS AND DISCUSSION

The common routes reported in the literature to determine the FA selectivity of lipases are based on a simple lipase-catalyzed acidolysis between a single TAG and various FAs (*2*, *5*, *12*, *13*, *15*) or a vegetable oil and FAs (*16*) leading to exchange of acyl groups. Enzymatic synthesis of a new TAG usually involves a two-step reaction: hydrolysis of TAG and then esterification of the FA present in the medium, by a process called acidolysis as shown in **Figure 1**. The side reactions such as re-esterification of FAs hydrolyzed were ignored at the presentations. The acidolysis reaction provides an ester change between the free FA and TAG molecule. The catalytic action of a lipase is governed by

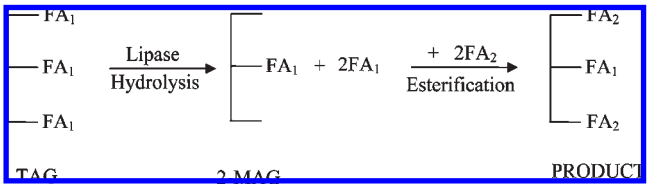

Figure 1. Reaction scheme of acidolysis catalyzed by 1,3-specific lipase.

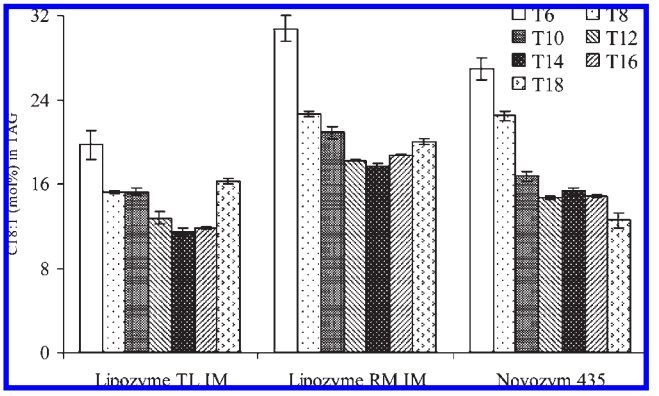

Figure 2. Effect of lipases on incorporation (mol %) of oleic acid into different monoacid TAGs. The reaction conditions are as follows: substrate mole ratio, 1:1; temperature, 50 °C; reaction time, 6 h; and enzyme dosage, 10%.

some conformational changes that occur at the active site of the enzyme during acyl−enzyme complex formation as explained in the report by Pleiss et al. (*17*). The geometry of these binding sites of the lipases has a strong effect on the selectivity. For this reason, the three-dimensional structure of a substrate has to be fit to this region of the enzyme. Therefore, the binary mixtures of oleic acid and different TAGs which construct different geometrical structures may result in different amounts of acyl−enzyme complex formation. Probably due to the above reasons, we detected different amounts of incorporations in such reactions.

The results obtained from acidolysis reactions between oleic acid and different monoacid TAGs catalyzed by Lipozyme TL IM, Lipozyme RM IM and Novozym 435 are shown in **Figure 2**. The acidolysis was performed at the default conditions as follows: substrate mole ratio, 1:1; temperature, 50 °C; reaction time, 6 h; and enzyme dosage, 10%. The highest incorporation ratios (19.74, 30.72, and 26.93% for Lipozyme TL IM, Lipozyme RM IM, and Novozym 435-catalyzed acidolysis, respectively) were obtained when the binary mixtures of oleic acid and tricaproin were used as substrate. It was observed that the incorporation of oleic acid into TAG except T16 and T18 decreased as the chain length of the FA in the TAG increased with Lipozyme TL IM and Lipozyme RM IM-catalyzed acidolysis, while decreasing trends were observed with Novozym 435-catalyzed acidolysis. The highest to lowest incorporation rates were obtained for Lipozyme RM IM, Novozyme 435and Lipozyme TL IM-catalyzed acidolysis in general when binary mixtures of oleic acid and different TAGs. The incorporation ability of Lipozyme TL IM was significantly lower than those of other two enzymes ($P < 0.05$) when a mixture of oleic acid and T6 or T8 was used.

Several factors that have influence on lipase activity have been discussed in our previous report (*13*). The factors include free energy changes between the substrates, the intermediate states and products, variation in pH values that may affect three-dimensional structure of the enzyme responsible for the catalytic activity, effect of the chain length of a FA on its solubility in water

which determines the direction of the reaction (hydrolysis or esterification), water concentration, and finally the physical state of the substrate may affect the reactivity of a FA. In contrast to our findings from the current study, it has been found that the lipases showed a bell-shaped distribution for acidolysis reactions with a maximum at around C12−C16 in the previous report (*13*). It was assumed that the above factors might cause some differences between the FA selectivity of lipases determined in two studies.

In contrast to our findings, it was reported that Lipozyme TL IM is not selective toward different monoacid TAGs in interesterification reactions (*18*). However, experimental design in this study was unlike that we used, and moreover, the selectivity of the Lipozyme TL IM was investigated in ester change reactions (interesterification) between two monoacid TAGs. Alhir et al. (*19*) reported that hydrolysis preference of lipase from *Penicillium caseicolum* from higher to lower was in the order tributyrin, tricaproin, tricaprylin, trilaurin, trimyristin and tripalmitin. Vaysse et al. (*20*) expressed the lipase selectivity with specific constants, and reactivity of the FAs ranged as follows: C8 > C10 > C12 > C14 > C18 > C16. Likewise, it has been reported that the lipase activity decreased with the increasing chain length of a FA in TAG (*21*). Our findings are in agreement with these reports.

Among the lipases examined, Lipozyme RM IM from *Rhizomucor miehei* showed the highest acidolysis activity and it was preferred as a catalyst for the subsequent experiments. In order to determine the effect of reaction parameters on incorporation of oleic acid into different monoacid TAGs, different substrate mole ratios, temperatures, reaction time and enzyme dosages were tested. However, optimization of these parameters was not aimed to achieve the highest incorporation rate. From this point of view, we only determined the border of incorporation attitude of the lipases at different reaction conditions.

Substrate Mole Ratio (Oleic Acid to TAG). The mole ratio of oleic acid to TAG was varied from 1:1 to 4:1. A three-dimensional plot summarizes the incorporation rates (mol %) of oleic acid at different substrate mole ratios for varying monoacid TAGs (**Figure 3**). There were clear decreases in incorporation of oleic acid as the chain length of FA in TAG increased with the exception of substrate mole ratios of 2:1, 3:1 and 4:1 for T16, T18, and T18, respectively. As the substrate mole ratio increased, oleic acid incorporation increased for T12. However, the reaction reached a steady state at a substrate mole ratio of 3:1 for the TAG molecules greater than T12. Among the experimental parameters, the highest oleic acid incorporation was achieved at the substrate mole ratio of 4:1 when the binary mixture of oleic acid and T6 was used. Similarly, a previous study has shown that higher substrate mole ratios would certainly shift the reaction equilibrium to the products and improve the acyl incorporation (*22*).

As mentioned earlier, acidolysis generates unbounded FAs released from TAG structure by hydrolysis. Higher oleic acid incorporation was observed when the mixture of oleic acid and TAG containing short chain FAs was used. It was reported that the acidolysis activity of the lipase from *Rhizomucor miehei* decreased when the substrate (peanut oil to caprylic acid) mole ratio was greater than 1:2. In contrast to our findings, this phenomenon has been attributed to substrate inhibition caused by acidity (*23*). In light of these findings it could be inferred that the importance of geometrical structure of the substrate (FA + TAG) has a priority on the FA selectivity of a lipase in acidolysis.

Reaction Temperature. Variation in the reaction temperature could affect the enzyme stability, the affinity of an enzyme for its substrate, and the preponderance of competing reactions. To minimize the thermal degradation risk, thermostability of

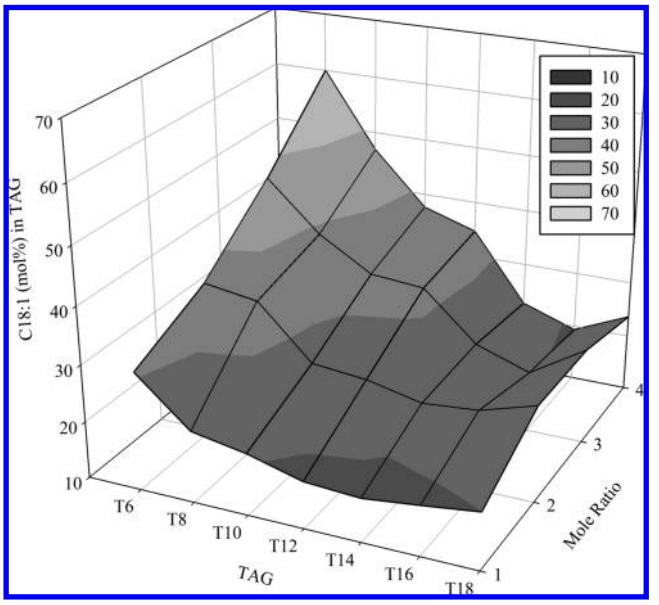

Figure 3. Interaction of different monoacid TAGs with substrate mole ratio on incorporation (mol %) of oleic acid. The reaction conditions are as follows: reaction temperature, 50 °C; reaction time, 6 h; and enzyme dosage, 10%.

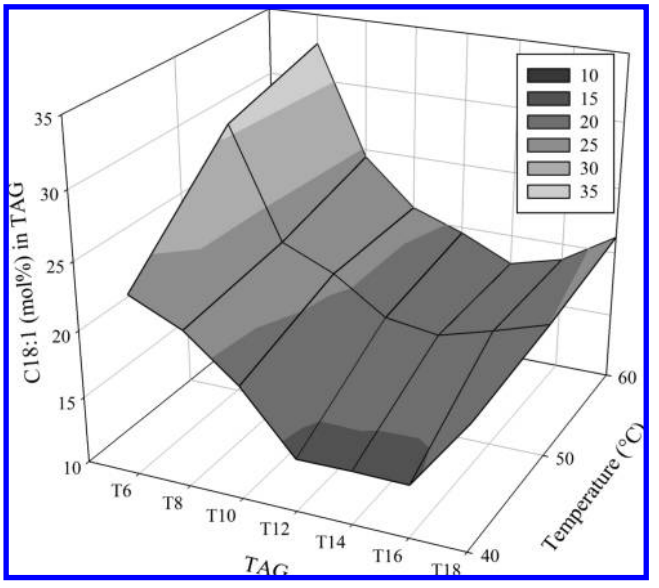

Figure 4. Interaction of different monoacid TAGs with reaction temperature on incorporation (mol %) of oleic acid. The reaction conditions are as follows: substrate mole ratio, 1:1; reaction time, 6 h; and enzyme dosage, 10%.

enzymes is considered as one of the most important factors in industrial use (*24*). To determine the effect of reaction temperature on FA selectivity of Lipozyme RM IM, temperatures of 40, 50, and 60 °C were tested. It was reported that higher temperatures favor higher yields for endothermic reactions due to the shift of thermodynamic equilibrium toward products. An elevated temperature can also make the operation easy, since a higher temperature increases the solubility of the substrate and decreases the viscosity of the solutions (*25*). Three-dimensional plots for the interaction of different monoacid TAGs with reaction temperature on incorporation (mol %) of oleic acid are shown in **Figure 4**. Usage of three different reaction temperatures resulted in significant differences in oleic acid incorporation ($P < 0.05$) when

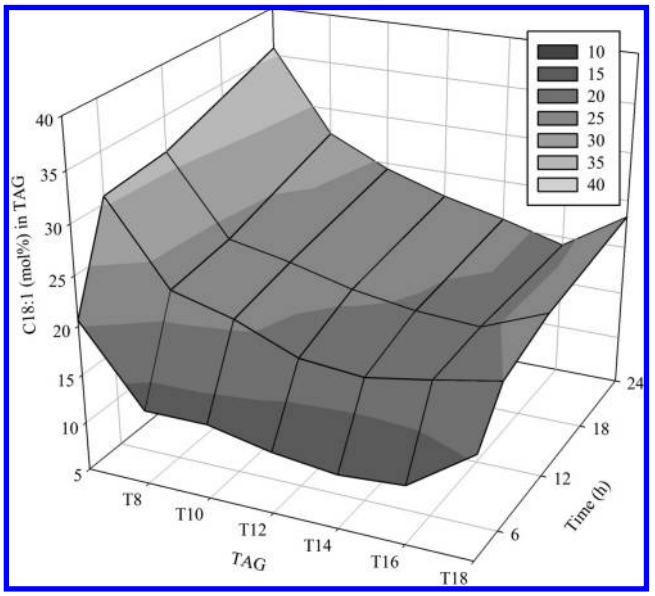

Figure 5. Interaction of different monoacid TAGs with reaction time on incorporation (mol %) of oleic acid. The reaction conditions are as follows: substrate mole ratio, 1:1; reaction temperature, 50 °C; and enzyme dosage, 10%.

the binary mixture of oleic acid and T12 was used. The incorporation rate change was higher when the reaction temperature was increased from 40 to 50 °C compared to from 50 to 60 °C. Oleic acid incorporation into all TAGs was increased with the increasing temperatures. It was reported that higher temperatures reduce the viscosity of the lipid and consequently increase the substrate and product transfer on the surface or inside the enzyme particles (*7*). Besides, a higher amount of incorporation was obtained with the mixtures of oleic acid and TAGs including short chain FAs. These findings indicate that the incorporation of oleic acid into a TAG molecule depends on the chain length of FA in the TAG structure at all reaction temperatures.

As explained in our previous report (*13*), the physical state of the substrates may affect their reactivity during acidolysis reactions. While the T6 and T8 are in liquid form at room temperature, TAGs containing longer chain length FAs have higher melting points which cause increase in viscosity. Thus, the solid state could cause obstacles to the access of substrate to the active site of the lipase during acidolysis, even if it is solved in an organic solvent. Hence, higher oleic acid incorporation was observed when the binary mixtures of oleic acid and T6 or T8 were used.

Reaction Time. Effects of various reaction times ranging from 3 to 24 h on the amount of oleic acid incorporated into different TAG structures with Lipozyme RM IM were investigated. Three-dimensional plots for the interaction of different monoacid TAGs with reaction time on incorporation (mol %) of oleic acid are shown in **Figure 5**. As the chain length of FA in TAG increased, except tristearin, the amount of oleic acid incorporated in TAGs varied from tricaproin to tristearin decreased at the longer reaction times. The amount of oleic acid incorporated into the same TAG was found to be statistically significant ($P < 0.05$) when the reaction time was increased. The highest increases were observed when the reaction time increased from 3 to 6 h for all TAG species. The reaction reached a steady state at 12 h of reaction for the binary mixtures of oleic acid and T16. There was no significant difference ($P < 0.05$) between the reaction times of 12 and 24 h at the same substrate set. The highest incorporation was obtained (35.75%) when the mixture of oleic acid and T6 was used.

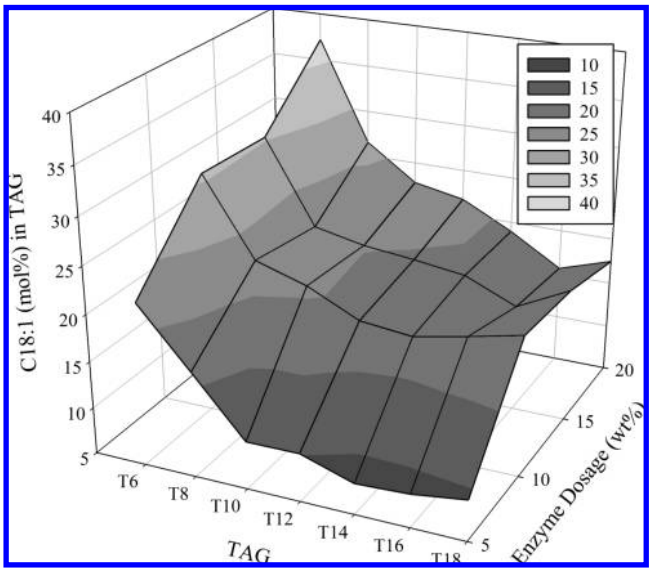

Figure 6. Interaction of different monoacid TAGs with enzyme dosage on incorporation (mol %) of oleic acid. The reaction conditions are as follows: substrate mole ratio, 1:1; reaction temperature, 50 °C; and reaction time, 6 h.

Enzyme Dosage. To show the differences in the amount of oleic acid incorporated into different TAGs with Lipozyme RM IM, different enzyme dosages based on the weight of total reactants ranging from 5 to 20%, with 5 increments, were tested. Three-dimensional plots for the interaction of different monoacid TAGs with reaction time on incorporation (mol %) of oleic acid are shown in **Figure 6**. As the chain length of FA in TAG increased, the amount of oleic acid incorporated into TAG decreased with the exception of T18 for all enzyme dosages tested. The highest differences were obtained with the changing of enzyme dosage from 5 to 10% for all TAG species. Incorporation of the oleic acid into T6 and T18 decreased after enzyme dosages higher than 10%. Similar observations have been reported in acidolysis with different substrates (*2*, *5*, *13*, *25*).

In conclusion, FA selectivity of the lipases depends on many factors. In a previous study, we have pointed out that the acyl chain length of FA had an impact on selectivity of lipases tested. Lipases, namely, Lipozyme TL IM from *Thermomyces lanoginosus*, Lipozyme RM IM from *Rhizomucor miehei*, and Novozym 435 from *Candida antarctica* lipase B had preference for the FAs varied from C12 to C16 when they were used as acyl donor (*13*). In the present study, it was shown that acyl chain length of FA in TAG structure has also a strong impact on lipases' FA selectivity. Among different TAGs (varied from tricaproin to tristearin) the short chain FA containing ones were preferred by the lipases in acidolysis with oleic acid. This conclusion could be used to modify the FA composition of oils and fats with desirable TAG structure in designing new products. The results of both studies may provide useful information for future lipase applications and will be conducted in academia and industry.

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