Intermediate production of mono- and diolein by an immobilized lipase from *Candida antarctica*

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Abstract

Lipase from *Candida antarctica*, fixed on macroporous acrylic resin, has been used for the intermediate production of mono- and diolein by hydrolysis of triolein. The effect of altering concentrations of triolein and glycerol and the function of the molecular sieve on the hydrolysis reaction of triolein were investigated. The highest hydrolysis yield was observed for the utmost concentration of triolein, which gave a hydrolysis optimum at the lower reaction time. Raising the concentration of triolein resulted in a 13.7 times increase in mono- and diolein during reaction period. Addition of glycerol to the reaction mixture had a considerably higher positive effect on the production of monolein than that of diolein. The use of a molecular sieve in the mixture was found to be the most effective environment tested, which demonstrated high activity and excellent selectivity toward the formation of intermediate monolein.

Keywords: Diolein; glycerol; hydrolysis; immobilized lipase; molecular sieve; monolein; triolein

INTRODUCTION

Lipases (E.C. 3.1.1.3) are defined as a group of enzymes whose biological function is to catalyze the two ways reactions; the hydrolysis and the synthesis of triacylglycerols (Martinelle and Hult, 1994). The acquired specificity of lipase-catalyzed reactions and the regioselectivity of these enzymes have resulted in their application to many processes. Hydrolyses, synthesis and biotransformation of fats and oils catalyzed by lipases can be carried out at ambient temperatures and pressures, as compared with the high energy costs of conventional chemical processes (Holmberg and Osterberg, 1988; Bjorkling *et al*., 1991; Plou *et al*., 1996).

After unveiling the *Candida antarctica's* lipase structure it was found that this enzyme has potential application in a number of industries (Uppenberg *et al*., 1994). The production of adsorbed *Candida antarctica* Lipase B (CALB) on different supports has also revealed to be very regioselective in the esterification reaction and very enantioselective in the resolution of secondary alcohols via hydrolysis (Hansen *et al*., 1995; Pastor *et al*., 1995) or esterification in organic solvents (Frykman *et al*., 1993). Moreover, lipases are enzymes exhibiting high hydrophobicity; hence, hydrophobic supports enhance their adsorption (Ruckenstein and Wang, 1993).

One of the key applications of immobilized lipases is the production of phenolic lipids, as fat-soluble antioxidants (Karboune *et al*., 2008; Sabally *et al*., 2006; Karboune *et al*., 2005), in compression to the stability of fats and oils, the hydrophilic nature of these compounds reduces their performances (Schuler, 1990). Therefore, the modification of phenols via esterification with aliphatic alcohols, using enzymatic acylation by lipases can be used as a tool to alter solubility in oil based formulae and emulsions (Stamatis *et al*., 2001). However, where lipases are used to catalyze the reactions involved in phenolic acid esterification with triacylglycerols, problems arise from the need to

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maximize the concentration of mono- and diacylglycerols, which act as intermediate substrates for further lipase activity. Most studies concerning lipase-catalyzed hydrolysis of fats have focused on the production of glycerol and fatty acids (Yang and Rhee, 1992; Wang *et al*., 1988), but not on intermediate production of mono- and diacylglycerols as secondary substrates for esterification reactions.

In this work, the possibilities of production of mono- and diolein in hydrolysis of triolein are described. These compounds, as a representative of phenolic derivatives, can be used for further activity of lipase in esterification of cinnamic acid. The solvents used for the enzymatic hydrolysis, hexane and 2-butanone, are non-toxic and can solubilize relatively large amounts of substrates. Various reaction parameters affecting the enzyme's catalytic behavior such as triolein and glycerol concentrations as well as the nature of the secondary substrates produced throughout the enzymatic reactions (Fig. 1) are also examined.

MATERIALS AND METHODS

An immobilized lipase from *Candida antarctica* (Novozym 435), was purchased from Novo Nordisk A/S (Bagsværd, Denmark). The enzyme was fixed on a macroporous acrylic resin with an activity of 10,000 propyl laurate units per solid g (PLU/g). Triolein (99%), *trans*-cinnamic acid (> 99% purity), glycerol as well as oleic acid, monolein and diolein standards, were purchased from Sigma Chemical Co. (St.Louis, MO). Chromatography grade methyl ethyl ketone (2-butanone) was obtained from ACP (Montreal, Qc). Hexane and other HPLC (High Performance Liquid Chromatography) grade solvents as well as molecular sieve of 4-Å (8-12 mesh) were purchased from Fisher Scientific (Fair Lawn, NJ).

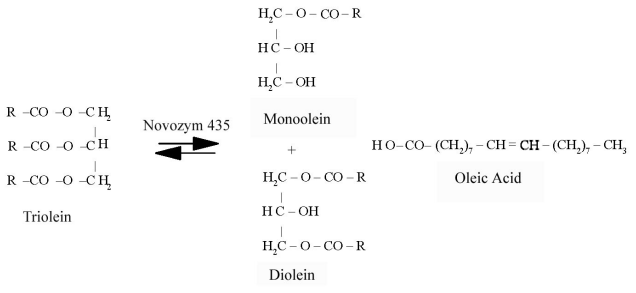

Figure 1. The structural formulae of products developed by hydrolysis of triolein by the lipase catalyzed reaction.

Enzymatic hydrolysis of triolein and transesterification of mono- and diolein with cinnamic acid: The hydrolysis of triolein and transesterification reaction of cinnmamic acid with mono- and diolein was performed in 50 ml Erlenmeyer flasks based on the modified method described by Kermasha *et al*., (1995). A stock solution of triolein (40 mM) was prepared in n-hexane, whereas that of cinnamic acid (26.67 mM) was freshly prepared in 2-butanone to ensure an adequate solubilization. Appropriate proportions of triolein and cinnamic acid stock solutions were mixed with n-hexane to acquire a final concentration of triolein equal to 4, 8, 12, 18 and 24 mM and a fixed concentration of cinnamic acid (4 mM). The total volume was adjusted to 10 ml with a mixture of hexane/2-butanone (85:15, v/v). Following addition of 20 mg of solid immobilized enzyme to the reaction mixture enzymatic reactions were initiated. The incubation of reaction mixtures was carried out under vacuum and continuous shaking (55ºC, 150 rpm), in an orbital shaker (New Brunswick Scientific Co., Inc., Edison, NJ). All reactions were performed in duplicate, and compared with those without enzyme. The total reaction period was 10 days and aliquots were withdrawn at specific time intervals. In order to terminate the enzymatic reaction, the immobilized lipase was removed by decanting off the organic solvent mixtures from the reaction flasks. Glycerol concentration, produced in the reaction mixture, was calculated by molar balance using the measured oleic acid concentration (Karboune *et al*., 2005; Plou *et al*., 1996).

Reverse phase (RP)-HPLC analysis of reaction mixture: The concentration of intermediate mono- and diolein as well as transesterification products were determined by HPLC analysis, based on the method described by Compton and co-workers (2000). The 300 µl aliquots of reaction mixtures were withdrawn and vacuum dried with an Automatic Environmental Speed Vac system (Savant Instruments Inc., Holbrook, NY). The resulting dried mixtures were then re-solubilized in appropriate amounts of iso-propanol (0.3 to 1 ml) before HPLC analysis (Karboune *et al*., 2005).

The Beckman HPLC system (Model 126, Beckman Instruments Inc., San Ramon, CA) consisted of an autosampler (model 507) and was equipped with computerized data handling and integration analysis (System Gold software, version 5.0, Beckman, San Ramon, CA). The HPLC column and detection system were an evaporative light scattering detector (ELSDII, Varex Corporation, Burtonsville, MD and a Zorbax SB-C18 reversed-phase (RP) (250 × 4.6 mm, 5 µm,

Agilent, Wilimington, DE), respectively. Elution was conducted with solvent (A), consisting of a mixture of methanol, iso-propanol, water and acetic acid (190:10:1.5:0.5, v/v/v/v) and solvent (B) composed of iso-propanol, at a flow rate of 1 ml/min. The elution profile was initiated by an isocratic flow of 100% solvent (A) for 10 min, followed by a 10 min linear gradient to 100% solvent (B), which was maintained for a 15 min period before reverting back to the 100% solvent (A). Injected sample volume was 20 µl and the detection of reaction components was performed by the ELSD (drift tube 82ºC, nitrogen flow 40 mm, sensitivity 2, data integration speed 1 MHz). Calibration curves were constructed using different concentrations of cinnamic acid, oleic acid, monolein, diolein and triolein standards (Karboune *et al*., 2005).

The hydrolysis yield was calculated on the basis of total concentrations (mM) of monolein, diolein and free fatty acids produced, divided by concentrations of these components, multiplied by 100. The transesterification yield was calculated based on the total peak area of the cinnamoylated lipid products, divided by the area of cinnamic acid in the blank, multiplied by 100 (Karboune *et al*., 2005).

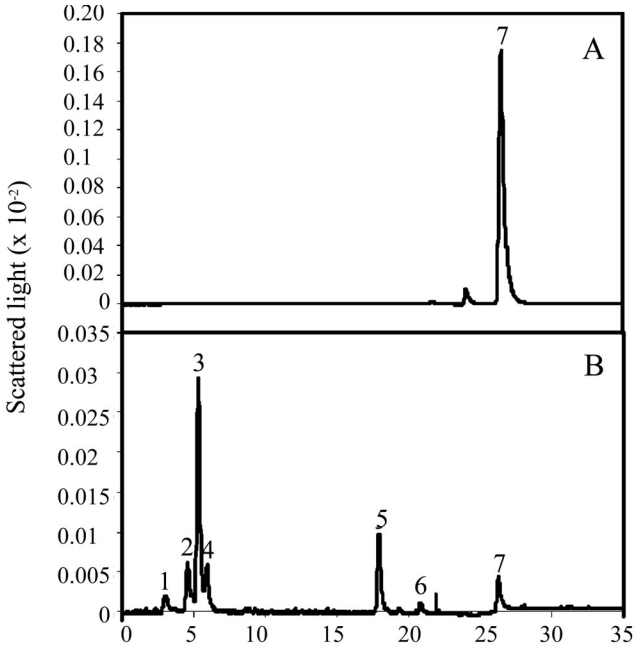

Figure 2. HPLC chromatogram of Novozyme catalysed acidolysis of triolein using triolein (A) and a mixture of hydrolysate products with different retention times; 3.0 cinnamic acid; 4.49 monolein; 5.32 oleic acid; 5.89 products, 17.93 diolein; 20.7 products; 26.22 triolein.

RESULTS

RP-HPLC separation of products developed by enzymatic reaction: The qualitative and quantitative determinations of hydrolysate products including mono- and diolein were performed using a preparative reverse phase column. The hydrolytic activity of the immobilized lipase from *C. antractica* was monitored throughout a total 10 days incubation period. The concentrations of products were measured based on standard curves obtained from different quantities of oleic acid, monolein, diolein and triolein. The individual peak was identified by comparing the retention time of each peak with standard. Figure 2 shows a typical HPLC chromatogram of the initial substrate, triolein (Fig. 2A), as well as total product obtained through the hydrolysis reaction (Fig. 2B). The results (Fig. 2) demonstrate the presence of 7 different fractions some of which resulted from the hydrolysis reaction (peaks 2, 3 and 5) and others represent esterification reactions (peaks 4 and 6). As shown (Fig. 2), the peaks with retention times of 4.58, 5.35 and 18.22 min represent the presence of monolein, oleic acid and diolein, respectively. Figure 2 also demonstrates the presence of two minor peaks (peaks 4 and 6) with retention times of 5.72 and 21.05 min, which are probably related to the development of cinnamoylated lipids in the reaction mixture.

Effect of substrate concentration on the hydrolysis yield: The effect of altering triolein concentrations on the hydrolysis yield, based on the amount of monolein, diolein and oleic acid produced in *n*-hexane was studied. In order to maintain the highest activity of Novozym, *n*-hexane was amended with a slightly polar solvent, 2-butanone, to increase glycerol solubility which is produced through the hydrolytic reaction of the enzyme. The progress of triolein hydrolysis as a function of various concentrations of triolein (4, 8, 12, 18 and 24 mM) is shown in Figure 3. As observed, during the first five days of incubation the percent of hydrolysis yield increased with increasing triolein concentrations. The highest hydrolysis yield (59.0%, 5 days) was obtained for the highest concentration of triolein (24 mM), which indicate the lack of inhibition by triolein. Goto, and co-workers (1992) demonstrated the same results in enzymatic hydrolysis of triolein. However, the results (Fig. 3) also show that only five days of incubation time is enough for the hydrolysis

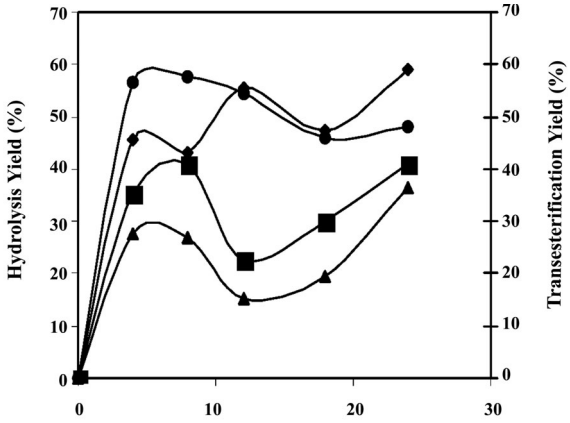

Figure 3. Effect of altering the concentration of triolein on hydrolysis and transesterification yields; (◆) hydrolysis yield, 5 days; (●) hydrolysis yield, 10 days; (■) transesterification yield, 10 days; (▲) transesterification yield, 5 days.

yield to reach maximum, which otherwise decreases with increasing time of the reaction.

Effect of triolein concentration on the development of different molecular species: The molecular species produced during 5 and 10 days incubation of triolein with Novozym 435 are presented in Table 1. The hydrolytic activity of tested lipase on triolein hydrolysis shows (Table 1) the main variation in the concentration of hydrolysates developed at more than 5 days of incubation. At low concentrations of triolein (4 and 8 mM), increasing the reaction time resulted in the concomitant increase in the concentration of monolein, diolein and oleic acid. When the substrate concentrations were increased, the profiles changed. Altering the triolein concentrations to more than 8 mM demonstrated a decrease in the concentrations of hydrolysate products upon elongation of reaction time. Maximum hydrolysis which was the most obvious effect occurred at elevated triolein concentrations (24 mM, 5 days). In the highest concentration of triolein (24 mM), oleic acid was the most hydrolysate produced (22.79 mM, 5 days). Moreover, the results (Table 1) show that only five days of incubation time is enough to reach maximum concentration of 2.10mM for monolein and 9.71 mM for diolein. These concentrations decrease with an increase in reaction time up to 10 days. In the the highest triolein concentration and the lowest reaction time, optimum hydrolysis occurred (Table 1). It makes this hypothesis that the higher quantities of triolein are necessary to activate the enzyme. Indeed, 6 times increase in the triolein concentration resulted in approximately a 10 times increase in the average hydrolysate products. To solve the problem with substrate limitations, it is necessary to understand the actual phenomenon involved.

Effect of the addition of glycerol on hydrolytic activity of the Novozym: It is clear from the results (Table 2) that in the reaction mixture having high glycerol produced, the high triolein concentration gives hydrolysis optimum at lower time. As first sight, glycerol, which is an end product of hydrolysis, seems responsible by itself for the increase in the enzyme activity (Plou *et al*., 1996). In order to confirm this phenomenon, one series of enzymatic reactions was conducted with two different amounts of glycerol. Activities of the catalytic preparations were tested in a

Table 1. Concentration of monolein, diolein and oleic acid produced through enzymatic hydrolysis of triolein during 5 and 10 days of incubation time at 55ºC, using Novozym 435. The values obtained by a Lazer scattered-light detector at sensitivity 1 and 82ºC.

		Concentration of Triolein (mM)[a]				
Product (mM)	Time (Day)	4	8	12	18	24
Monolein	5	0.29	0.26	0.70	0.56	2.10
	10	0.74	0.66	0.49	0.41	1.10
Diolein	5	0.57	1.91	4.10	5.79	9.71
	10	0.98	2.64	3.65	4.97	6.26
Oleic acid	5	2.49	3.95	10.12	9.96	22.79
	10	3.45	7.53	10.29	9.85	14.92
Triolein	5	0.09	1.43	3.58	7.20	5.12
	10	0.12	1.85	2.30	4.55	5.64

[a]Initial concentration of triolein in the reaction mixture containing hexane/2-butanon (80:20 v/v) and Novozym 435.

Table 2. Concentration of glycerol calculated by molar balance using the measured oleic acid concentration during 5 and 10 days of incubation time at 55ºC using Novozym.

	Concentration of Triolein (mM)[a]				
Time (Day)	4	8	12	18	24
5	0.83±0.12[b]	1.32±0.31	3.37±0.13	3.32±0.01	7.60±0.12
10	1.15±0.11	2.51±0.22	3.43±0.18	3.28±0.09	4.97±0.09

[a]The reaction mixture of different concentrations of triolein incubated for 5 and 10 days at 55ºC using Novozym 435. [b]Standard deviation calculated from 3 replications.

Table 3. Effect of molecular sieve (200 mg) on the extent of hydrolysis and production of Mono- and Diolein and Oleic acid produced through enzymatic hydrolysis of triolein during 5 and 10 days of incubation time at 55ºC using Novozym. The values obtained by a Lazer scattered-light detector at sensitivity 1 and 82ºC.

Triolein[a] (mM)	Time (Day)	Oleic acid (mM)		Monolein (mM)		Diolein (mM)		Triolein (mM)	
		C[b]	S[a]	C[b]	S[a]	C[b]	S[a]	C[b]	S[a]
4	5	2.49	4.62	0.29	0.49	0.57	0.37	0.09	0.10
	10	3.45	5.64	0.74	0.52	0.98	0.40	0.12	0.15
8	5	3.95	8.61	0.26	0.91	1.91	0.95	1.43	0.45
	10	7.53	10.44	0.66	0.86	2.64	1.18	1.85	1.06

[a]The initial concentration of triolein in the reaction mixture. [b]The reaction mixture containing triolein and cinnamic acid as well as Novozym, but without molecular sieve. [c]The reaction mixture containing initial substrates, triolein and cinnamic acid, which incubated in the presence of Novozym 435 and molecular sieve.

batch hydrolysis reaction with addition of 2 and 4 mM glycerol to 10 ml of *n*-hexane/2-butanone mixture (85/15 v/v) in the presence of the enzymatic preparation. The concentration of mono- and diolein as well as oleic acid was measured and was compared to a control preparation, carried out without addition of commercial Novozym 435.

Effect of molecular sieve on the hydrolysis of triolein: Previous studies, conducted by Dossat and co-workers, (1999), have demonstrated that the adsorbed glycerol, which is a product of triolein hydrolysis, on supporting material of immobilized lipases decreases the availability of the water adsorbed onto the support leading to a decrease in the thermodynamic water activity from its optimum value and consequently to a decrease in catalytic activity. In order to assess this hypothesis in relation with hydrolytic activity of the Novozym, the hydrolysis reaction of triolein was carried out at two different concentrations of triolein (4 and 8 mM) in the presence of a constant amount of molecular sieve (200 mg), which is expected to adsorb water produced in the reaction mixture. The results showed an overall increase in the production of monolein with a concomitant decrease in the concentration of diolein (Table 3). During 5 days, in the presence of 8 mM triolein, the levels of mono- and diolein were monitored. The results indicated that the concentration of monolein increased sharply (2.5 times) in comparison with the mixture without the molecular sieve. At all tested concentrations of triolein, the amount of diolein was decreased from 35 to 59%. At the higher concentration of triolein (8 mM), a reduction of approximately 50% in diolein concentration was observed without any need to leave the system for a longer period. Table 3 data analysis show that there is a selectivity towards formation of monolein as opposed to diolein, especially at the higher triolein concentration (8 mM) and lower reaction time (5 days). Plou and co-workers (1996) observed the same trend in hydrolysis of triolein by an immobilized lipase on a support of very low aquaphilicity, such as celite.

DISCUSSION

The activity profile of Novozym in relation to triolein hydrolysis shows that the main variation in hydrolysis

products occurred during 10 days of incubation (Fig. 3). At the low concentration of triolein (4 mM), the maximum hydrolysis yield of triolein is 56.4%. This decline was steady at higher concentrations of triolein with curve slopes of 0.52% and 1.46% for 12 and 18 mM triolein, respectively. However, with a triolein concentration of 24 mM, a slight increase in hydrolysis yield appears, after 10 days of reaction. The most obvious effect is that the decrease in hydrolysis yield occurs with a concomitant increase in the transesterification yield of triolein with cinnamic acid (Fig. 3). At 24 mM of triolein, the highest transesterification yield is obtained at 10 days of incubation. Figure 3 results clearly shows that increasing the concentration of triolein shifts the maximum transesterification time from 5 to 10 days. These results demonstrate that the enzyme activity is influenced by changes in triolein concentration. The results can create impressions that Novozym 435 has maximal activity at a certain triolein concentration. One must bear in mind that using high substrate concentrations will affect the reaction medium. Moreover, the reaction is not conducted in ''pure'' solvent, since the portion of substrate, triolein, is substantial. If this only affects the polarity of the medium or has a direct impact on the enzyme action is not clear. Increased substrate concentrations, for example, can act as inhibitor or influence the pH (Wehtje and Adlercreutz, 1997). On the other hand, the initial rate of hydrolysis of triolein (Fig. 3) shows that the initial reaction rate could be correlated with the hydrophobicity of the solvent (Naoe *et al*., 2004). More polar organic solvents can strip more water off an enzyme and thereby dehydrate the catalyst so that activity is destroyed. In contrast, solvents having a high log P (lower polarity) value do not detach water from the enzyme. These solvents distort the water-enzyme interactions merely by penetration of the organic solvent into the essential water layer or by pushing aside the essential water. This is the base of solvent dependency in enzymatic reactions (Lanne, 1987).

The profile of mono- and diolein production as a function of various concentrations of triolein (4, 8, 12, 18 and 24 mM) is shown in Figure 4. The results clearly demonstrate a substantial increase in the concentration of mono- and diolein from 0.86 mM to 11.8 mM (13.7 times) in only five days of the reaction period. However, Plou, and co-workers (1996), using a non-specific lipase from *Candida rugosa*, have reported on the complete hydrolysis of triolein to glycerol and fatty acid. This disagreement might be due to the different solvent and enzyme used. By increasing the incubation time from 5 to 10 days, the extent of hydrolysis based on concentrations of mono- and diolein declined from 11.8 to 7.43 mM. This represents a shift in enzymatic reaction equilibrium from hydrolysis to transesterification (Fig. 3).

Figure 5 results clearly shows the independency of oleic acid production in relation to the amount of added glycerol. Increasing the levels of glycerol have similar effects on oleic acid concentration in the reac-

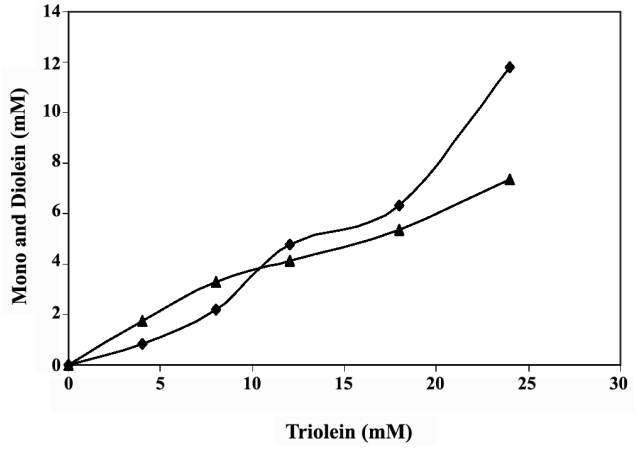

Figure 4. Effect of raising the concentration of triolein on the production of monolein and diolein at 5 days (◆) and 10 days (▲) in the presence of Novozym 435.

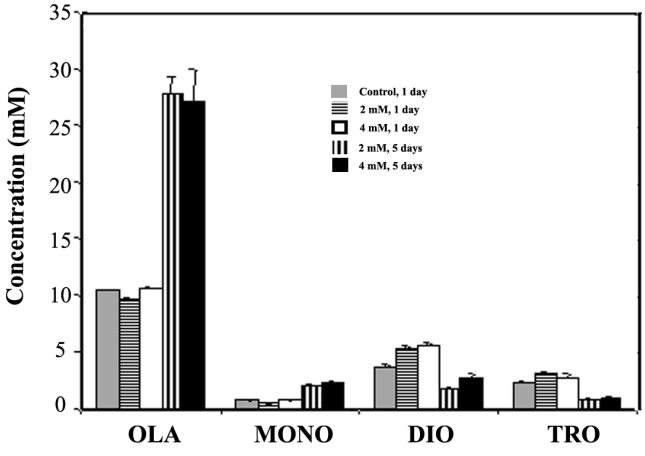

Figure 5. The effect of addition of glycerol on hydrolysis of triolein at different concentrations and time intervals on oleic acid (OLA), monolein (MONO), diolein (DIO) and triolein (TRO).

tion mixture. Addition of glycerol has a considerably higher positive effect on monolein concentration. Monolein increases from 0.49 mM in the control to around 2 mM in the tested reaction mixture. As can be seen in Figure 5, the less hydrophobic the substrates, the less negative is the effect of glycerol adsorbed onto enzymatic support. Altering the concentration of monolein may be due to the presence of the slightly polar solvent, 2-butanone, in the reaction mixture; since, a slightly polar solvent can increase semi-polar substrates in the reaction medium and hence remove them continuously from the enzymatic support (Colombie *et al*., 1998). However, there was a substantial reduction in the production of diolein during incubation (5 days). The results show that the production of diolein is time dependent, which demonstrates an initial increase in diolein concentration during the first day of incubation, following a decline with increasing time (Fig. 5). The formation of a hydrophilic layer of adsorbed glycerol seems to be the more valid explanation for alteration of the hydrolytic activity of lipase. The reduction of diolein at an elevated time (5 days) might be due to the over production of glycerol which is absorbed onto the enzymatic support. It could be hypothesized that the thicker the hydrophilic layer in the supported environment, the lower the activity of the reaction involving highly hydrophobic substrates. The accumulation of adsorbed glycerol molecules generates a hydrophilic barrier around the immobilized catalyst leading to diffusion limitations of the hydrophobic substrate from the medium to the enzyme. This hypothesis has already been proposed by Balcao (1996) in the case of reactions producing water to explain the decrease in activity during continuous esterification reactions. The results (Fig. 5) show that the hydrolysis of triolein by lipase is inhibited by oleic acid but not by glycerol, as also previously reported by Goto and co-workers (1992). A part of the oleic acid produced forms a complex with the lipase at the interface and thus inhibits the hydrolysis reaction (Goto *et al*. 1992).

A study of the mode of hydrolytic action of the Novozym 435 using different concentrations of triolein and glycerol was performed in this research. The remarkable differences observed during the time course of triolein hydrolysis can be partly ascribed to the well-known substrate specificity of lipases. The hydrophobicity of the matrix used for immobilization of the Novozym, acrylic resin, seems to be essential in both the hydrolysis yield and selectivity in these processes. Some authors have suggested that hydrophobic materials are suitable supports for lipase immobilization during the hydrolysis of fats (Plou *et al*., 1996). Moreover, because lipases are enzymes exhibiting high hydrophobicity, hydrophobic supports enhance their adsorption. It is clear from the results that the best concentration of triolein for obtaining a mixture of monolein and diolein is 24 mM. This concentration of triolein in the reaction mixture fulfills two conditions: (a) increasing the hydrophobicity of the reaction mixture and (b) depressing water removal from the enzymatic support; hence, enhancing the hydrolytic activity of lipase. Concerning the study on the effect of glycerol added to the reaction mixture, the profiles of triolein hydrolysis show that glycerol is the main product of the reaction for all triolein concentrations tested. It is also evident from the results that the application of the molecular sieve to the reaction mixture enhances the formation of monolein and minimizes the complete hydrolysis of intermediate glycerides. It could be concluded that the controlled hydrolysis of triacylglycerols using a suitable lipase immobilized on a convenient support, preferably hydrophobic, can become an industrial alternative to the synthetic or glycerolytic routes. An immobilized lipase has potential application in the production of mono- and diacylglycerols mixtures, which can be used as secondary substrates for esterification reaction.

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