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Structured Lipids Produced through Lipase-Catalyzed Acidolysis of Canola Oil

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ABSTRACT

Enzymatic acidolysis of canola oil through caprylic acid was investigated to produce certain medium chain Ttriacyliglycerol (TAG) structured lipids (SLs). Lipozyme TL IM, an *sn-1,3* specific *Thermomyces lanuginosa* lipase, and Novozym 435, a non-specific *Candida antarctica* lipase, were utilized as the biocatalysts in a batch reactor. Reaction conditions were designed according to Taguchi's approach, considering three levels of fatty acid to oil ratio (1:1, 2:1, and 3:1), three levels of enzyme load (4, 8 and 12%, w/w), three levels of temperature (45, 55, and 65°C) as well as three levels of reaction time (15, 30 and 45 hours). Results indicated that fatty acid composition of canola oil was modified by the above acidolysis reactions. The highest mole percent of caprylic acid incorporation (37.2 mole%) was obtained after 15 hours of incubation in the presence of Lipozyme TL IM at 55°C, fatty acid to oil ratio of 3:1 and at 12% of enzyme level. However, with Novozym 435 the highest level of incorporation (38.5 mole%) was obtained after 45 hours of reaction at 45°C, fatty acid to oil ratio of 3:1, and at 8% enzyme level. Novozym 435 was able to incorporate more caprylic acid in the oil than did Lipozyme TL IM. SLs prepared using either Lipozyme TL IM or Novozym 435 differed in terms of their TAG compositions. According to the obtained results, SLs produced by use of Lipozyme TL IM lipase contained higher levels of MLM-type (Medium-Long-Medium) triacylglycerols than those produced using Novozym 435 lipase (21.2 and 9.9%, respectively).

Keywords: Acidolysis, Canola oil, Caprylic acid, Lipozyme TL IM, Novozym 435.

INTRODUCTION

Structured Lipids (SLs) are triacylglycerols (TAGs) that have been modified from their natural biosynthetic form by use of chemical or enzymatic methods. SLs are produced through the incorporation of novel fatty acids (FAs) into oil and/or changing the fatty acid location in the glycerol backbone (Iwasaki and Yamane, 2004). Two important sources of fatty acids for the production of nutritionally-enhanced SLs include medium chain fatty acids (MCFAs, 6-12 carbons) and unsaturated long chain fatty acids (LCFAs), which are utilized in the production of MLM-type SLs (TAGs with MCFA in *sn-1* and *sn-3* positions and LCFA in *sn-2* position). MCFAs in *sn-1,3* positions are rapidly hydrolyzed by the pancreatic lipases, reached to and absorbed by the liver *via* the portal vein where they are oxidized for energy. The other products of such action from the pancreatic lipase, 2-monoacylglycerols containing essential LCFAs, are also readily absorbed and

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metabolized. When consumed, MCFAs are not incorporated into the chylomicrons and are therefore not likely to be deposited as adipose tissue (Jenning and Akoh, 2000; Kennedy, 1991). MCFAs are readily oxidized in the liver, constituting a highly concentrated source of energy for premature infants and patients with fat malabsorption diseases like cystic fibrosis (Kennedy, 1991; Bray *et al*., 1980). Unsaturated LCFAs, which have been associated with reduced risk of platelet aggregation and cardiovascular diseases are also needed by the body (Rahimi *et al.*, 2011; Watkins and German, 2008). Therefore, production of SLs containing MCFAs and polyunsaturated fatty acids (PUFAs) might be deemed to improve the nutritional properties of TAGs.

Acidolysis, a type of interesterification, includes the exchange of acyl groups between an acid (FA) and an ester (mainly a TAG) and is an efficient method for the production of SLs (Xu, 2000). Due to the many purification steps required to remove intermediate products, production of SLs through chemical synthesis is rather difficult. Therefore, considering many advantages associated with the enzymatic processes, most research efforts have been directed towards these types of reactions, which are more specific, require less severe reaction conditions and produce fewer/no by-products as compared with non-enzymatic reactions (Farmani *et al.*, 2009). Enzymatic acidolysis is accomplished using lipases which are defined as glycerol ester hydrolases. The reactions catalyzed by *sn-1,3*-specific lipases are more effective for the production of SLs especially MLM-type (medium-long-medium) TAGs. Also, when immobilized, lipases can be reused thereby making them economically more attractive (Weete *et al*., 2008).

Canola oil contains oleic, linoleic and linolenic acids, especially in the *sn-2* position (Przybylski *et al*., 2005). Because of its FA composition which assures both nutritional quality as well as oxidative stability, canola oil has been widely utilized in the production of SLs. Production of MLM-type SLs *via* acidolysis of canola oil through caproic (Zhou *et al*., 2001), caprylic (Xu *et al*., 2000; Akoh and Moussata, 2001; Kim and Akoh, 2005; Xu *et al*., 2005), capric (Xu *et al*., 1998; Xu *et al*., 2002) and lauric (Long *et al*., 1997) acids have been previously described. All previous studies, except for that of Zhou *et al.* (2001), performed while using the lipase from *Aspergillus flavus,* were carried out utilizing the lipase from *Rhizomucor miehei*, Lipozyme RM IM. In this work, lipase-catalyzed acidolysis of canola oil with caprylic acid, using the 1,3-specific lipase from *Thermomyces lanuginosa* (Lipozyme TL IM) was investigated. The reactions were in the meantime, performed using the non-specific lipase from *Candida antarctica* (Novozym 435) and finally a comparison was made in terms of the enzymes' efficacy in the production of SLs.

MATERIALS AND METHODS

Materials

Refined, bleached and deodorized (RBD) canola oil was purchased from Behshahr Industrial Co. (Tehran, Iran). Lipozyme TL IM, a commercial silica-granulated *sn-1,3* specific lipase from *Thermomyces lanuginosa*, and Novozym 435, a non-specific *Candida antarctica* lipase immobilized on a macroporous acrylic resin, were kindly donated by Novo Nordisk A/S (Denmark). Caprylic acid (purity at > 98%) and Fatty Acid Methyl Ester (FAME) standards ($C_{8:0}$, $C_{13:0}$, $C_{14:0}$, $C_{16:0}$, $C_{18:0}$, $C_{18:1}$cis-9, $C_{18:2}$cis-9, cis-12 and $C_{18:3}$cis-9, cis-12, cis-15) were purchased from Merck Chemical Co. (Darmstadt, Germany). Porcine pancreatic lipase (EC 3.1.1.3) was procured from Sigma Chemical Company (St. Louis, MO). All other chemicals, of analytical grade, were also obtained from Merck Chemical Co.

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Drying of the Oil

To avoid/minimize the competing hydrolysis reaction, water content of the oil was reduced prior to the acidolysis reactions being carried out. For the purpose, the oil was mixed with anhydrous sodium sulfate at a 5:1 ratio in a conical flask and allowed to stay overnight, after which it was filtered out.

Acidolysis Rreaction

Variables affecting the reaction and their ranges were selected from literature, with the experimental conditions being designed according to Taguchi's statistical approach (Roy, 1990). Different reaction conditions applied during the study are presented in Table 1. In general, the acidolysis reactions were carried out on an orbital shaker at 200 rpm shaking frequency. The flasks contained 5.0 g dried canola oil already dissolved in 10.0 mL of hexane. At the end of each experiment, with the shaking stopped, the enzyme was allowed to fully settle at the bottom of the flask. The reaction mixture was then immediately filtered through a Wattman No. 4 filter paper to have the enzyme particles recovered.

Removal of Free Fatty Acids and Partial Acylglycerols

Removal of extra free FAs from the filtrate of the reaction mixture was performed according to Hamam and Shahidi (2005) by titrating the oil (soluble in hexane) with 0.2 M NaOH in the presence of phenolphthalein. Acylglycerols (including mono-, di- and tri-acylglycerols) were then extracted by having the mixture shaken with hexane (50 mL) in a separatory funnel. The top layer (hexane) was collected and passed through a bed of anhydrous sodium sulfate to have any residual water removed. The acylglycerol fraction was then recovered by evaporating the solvent (hexane) using a vacuum oven. To remove the remaining soaps and partial acylglycerols (mono- and di-acylglycerols), the method reported by Farmani *et al.* (2008) was employed. To perform that, acylglycerol fraction was mixed with an equal volume of 96% (v/v) ethanol (at 40–50°C) in a test tube. The ethanol phase was then removed by the use of a syringe and the procedure repeated for five more times. Following this, the traces of ethanol were removed by passing a stream of nitrogen gas through the oil.

Fatty Acid Composition

To determine the FA compositions of the original and of the produced oils, they were derivatized according to Huang *et al.* (2006) using a solution of sodium methoxide (0.5 M) in methanol and analyzed through gas chromatography. For the purpose, 20 mg of purified sample was dissolved in 500 µL hexane in a small test tube equipped with a tight sealing cap and then mixed with 250 µL solution of sodium methoxide. The tube was then tightly capped, and vortexed for 1 minute, after which 500µL of saturated sodium

Table 1. Reaction conditions (designed according to the Taguchi's experimental approach) considered for the enzyme-catalyzed acidolysis of canola oil with caprylic acid.

Run no.	Enzyme load (%, w/w)	Acid to oil ratio	Temperature(˚C)	Time (h)
1	4	1:1	45	15
2	8	1:1	55	30
3	12	1:1	65	45
4	4	2:1	55	45
5	8	2:1	65	15
6	12	2:1	45	30
7	4	3:1	65	30
8	8	3:1	45	45
9	12	3: 1	55	15

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chloride solution was added to the mixture and shaken again for 15 seconds. After 10 minutes past, a small quantity of anhydrous sodium sulphate was added and 50 μL of hexane as well as a predetermined concentration of internal standard (10 μg mL^{-1} methyl tridecanoate in hexane) incorporated into each sample. The mixture was then allowed to stand for 30 minutes after which the hexane layer was passed through a 0.45 μm filter and kept in a 0.5-mL vial for GC analysis. Identification and quantification of FAMEs were carried out through a Shimadzu gas chromatograph (model 14-A, Kyoto, Japan) equipped with a flame ionization detector. An RT-2560 capillary chromatographic column (100 m×0.25 mm×0.2 μm) from Restec (Bellefonte, PA) was employed to separate the FAMEs. Nitrogen was employed as the carrier gas. The oven temperature was first set at 100°C where it was held for 4 minutes and then raised to 240°C at 3°C min^{-1} and held steady for 5 minutes at 240°C. The injector and detector temperatures were set at 240 and 225°C, respectively. FAMEs were identified by comparing their retention times with those of their standard mixture and the results presented as mole percentages. Iodine Values (IV) of the SLs were determined from the FA composition data according to method Cd 1c-85 from AOCS (1996).

Design of the Study

Treatments were designed as based on the Taguchi's statistical approach and the effect of four different parameters (each in 3 levels), namely: reaction temperature, reaction time, enzyme load and FA:oil ratio on the acidolysis reaction was evaluated. Table 1 shows the reaction conditions applied.

Determination of Positional Distribution of Fatty Acids in Triacylglycerols

Positional analysis of FAs was performed according to a pancreatic lipase hydrolysis procedure described through method Ch3-91 by AOCS (1996). TAG composition of samples was determined based on the 1,3-random, 2-random hypothesis approach described by Fomuso and Akoh (2002).

Statistical Analysis

All the experiments for the preparation of SLs were designed according to Taguchi's experimental approach and analyzed by use of Minitab software (model 14.1). The experiments were all performed in duplicate. Data were processed for the Analysis of Variances (ANOVA) with the means compared, using MSTAT-C software and at a significance level of $P< 0.05$. The Signal to Noise ratio (S/N), a measure of robustness that can be used to identify the control factor settings for minimum impact of noise on the response, was determined using the formula $10\times\log(\bar{Y}^2/S^2)$, where $\bar{Y}$ and S^2 stand for the slope and mean square of the error, respectively (Roy, 1990).

RESULTS AND DISCUSSION

Effect of Reaction Conditions

The ANOVA Tables prepared for the effects of various reaction conditions of the study on acidolysis of canola oil (Table 2) indicated that for both enzymes, acid to oil ratio plus reaction time were the most effective parameters for an incorporation of caprylic acid into canola oil. The other two parameters (enzyme load and reaction temperature) were exert to have lower impact (still considerably high) when compared with the two parameters mentioned above. For Lipozyme TL IM, enzyme load and reaction temperature were, respectively, the third and the fourth most effective parameters. With Novozym 435, reaction temperature was observed as more effective than enzyme load in the acidolysis reaction.

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Table 2. Analysis Of Variance (ANOVA) of caprylic acid incorporation into canola oil in the acidolysis reaction with Lipozyme TL IM (the top Table) and Novozym 435 (the bottom Table).

Lipozyme TL IM					
Source	DF[a]	ANOVA SS[b]	Mean square	*F* value	*Pr*> *F*
Mole ratio	2	1076.821	538.410	190.44	0.0001
Enzyme load	2	52.967	26.483	9.37	0.0063
Reaction temperature	2	16.214	8.107	2.87	0.1088
Reaction time	2	63.887	31.943	11.30	0.0035
Error	9	25.445	2.827		
Total	17	1235.336			
Novozym 435					
Source	DF	ANOVA SS	Mean square	F value	*Pr*> *F*
Mole ratio	2	476.351	238.175	46.42	0.0001
Enzyme load	2	26.684	13.342	2.60	0.1284
Reaction temperature	2	120.671	60.335	11.76	0.0031
Reaction time	2	149.471	74.735	14.57	0.0015
Error	9	46.180	5.131		
Total	17	819.357			

[a] Degree of Freedom, [b] Sum of Square.

The effect of molar ratio of substrates on the incorporation of caprylic acid into canola oil is shown in Figure 1. When the molar ratio of caprylic acid to canola oil was changed from 1:1 to 3:1, the incorporation of caprylic acid into canola oil was increased for both the enzymes. Higher acid to oil ratio directs the reaction equilibrium towards a synthesis of the product (Ozturk *et al*., 2010). Comparing the acidolysis reactions conducted by use of the two enzymes indicated that caprylic acid incorporation by

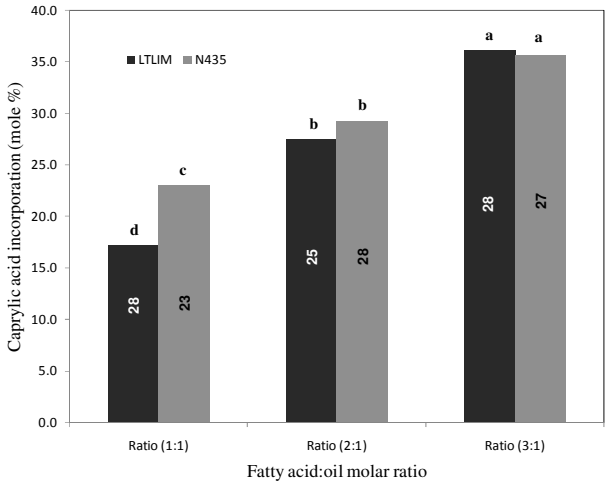

Figure 1. Main effect of the ratio of fatty acid to oil on incorporation of caprylic acid into canola oil during acidolysis reaction. LTLIM, reactions catalyzed through Lipozyme TL IM; N435, reactions catalyzed through Novozyme 435; bars are shown as Mean±SD. Signal to noise ratios are presented as data labels. Means denoted with the same letter are not significantly different (P> 0.05).

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Novozym 435 (22.4 mole%) at an acid to oil ratio of 1:1 was significantly higher than that incorporated by Lipozyme TL IM at the same acid to oil ratio (17.2 mole%). However, no significant differences were observed between the two enzymes when acid to oil ratio was either at 2:1 or 3:1 levels.

The effect of enzyme load on the incorporation of caprylic acid into canola oil is shown in Figure 2. With 12% enzyme load of Lipozyme TL IM, incorporation of caprylic acid reached its maximum level of 30.0%. The increase in the incorporation of acyl through increase in the enzyme load has already been documented (Zhao *et al*., 2007; Kim *et al*., 2010). Zhao *et al.* (2007) reported that higher enzyme load can improve the incorporation of acyl in the acidolysis. They found out the highest incorporation of capric acid into lard in the presence of 12% Lipozyme TL IM, however the analysis of variance indicated that the incorporation of capric acid achieved at 5% enzyme load, was not significantly different from other enzyme loads tested. Using Novozym 435 in the current study, the highest level of caprylic acid incorporation was obtained at 8% enzyme level, beyond which no significant increase in the acyl incorporation was observed (in comparison with that at 8% enzyme load). This is, possibly, due to a deficiency in the available water for the hydration of the enzyme (Hamam and Shahidi, 2005). At 4 and 8% enzyme loads in the current study, Novozym 435 resulted in greater levels of caprylic acid incorporation into canola oil than did Lipozyme TL IM at similar enzyme loads. No significant differences were observed between the performances of the two enzymes when used at enzyme loads of 12%. Overall, optimum enzyme loads were estimated as 8% for Novozyme 435 and 12% for Lipozyme TL IM.

Reaction temperature is an important parameter in the incorporation of novel FAs into oils during their lipase-catalyzed acidolysis (Karabulut *et al*., 2010). In general, increasing the temperature increases the rate of interesterification, but high temperatures can also reduce the reaction rate due to an irreversible denaturation of the enzyme (Karabulut *et al*., 2009). The effect of reaction temperature on the acidolysis of canola oil with caprylic acid is shown in Figure 3a. Incorporation level through Novozym 435 increased from 26.6 to 32.8 mole% when temperature increased from 45 to 55°C. A similar trend was observed for Lipozyme TL IM, although the extent of caprylic acid incorporation was lower than

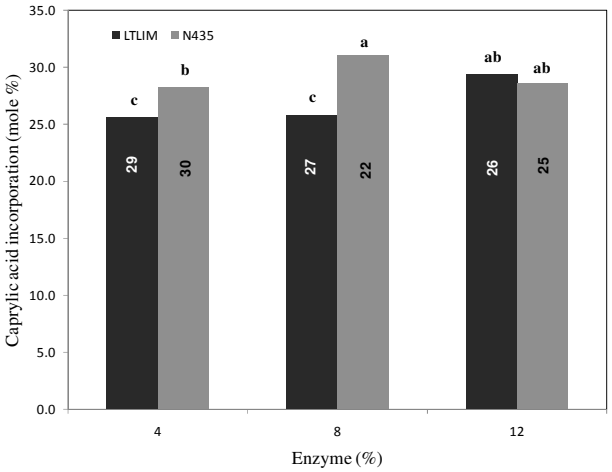

Figure 2. Main effect of enzyme load on incorporation of caprylic acid into canola oil during acidolysis reaction. LTLIM, reactions catalyzed through Lipozyme TL IM; N435, reactions catalyzed through Novozyme 435; bars are shown as Mean±SD. Signal to noise ratios are presented as data labels. Means denoted with the same letter are not significantly different (P> 0.05).

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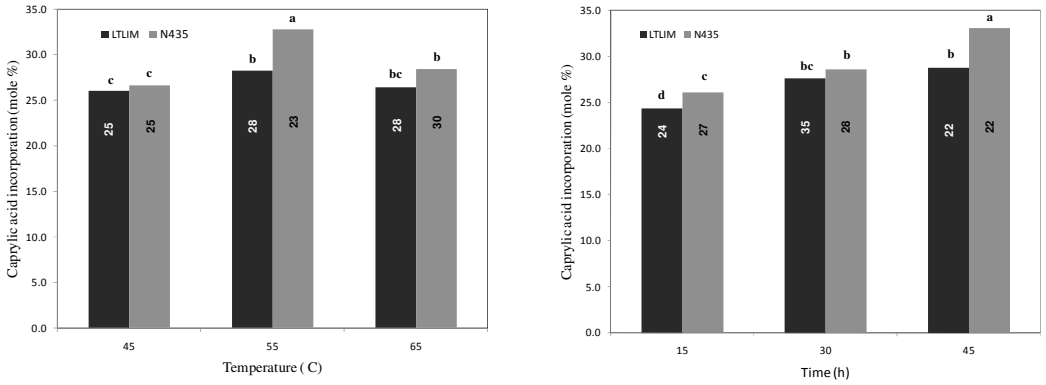
that for Novozym 435. However, further increase in the reaction temperature (to 65°C) resulted in a decrease in the caprylic acid incorporation for Novozym 435, which may be attributed to the partial deactivation of the non-thermophile enzyme (Novozym 435) at the higher temperature (Zhao *et al*., 2007). Such effect was not observed with Lipozyme TL IM, which was a thermophile enzyme. Therefore, it may be concluded that in the acidolysis of canola oil with caprylic acid, Novozym 435 was more sensitive to temperature than Lipozyme TL IM. Zhao *et al.* (2007) reported a higher level of activity for Lipozyme TL IM at 55°C for incorporating capric acid into lard. In a different study, Kim *et al.* (2001) used Novozym 435 as a biocatalyst for the interesterification of Conjugated Linoleic Acid (CLA) ethyl ester and tricaprylin, reporting the highest activity of Novozym 435 at 55°C. These results confirm the findings in the current study.

Studying the reaction time is very important to determine the optimal rate of reactions to reduce the process cost. Effect of reaction time on the incorporation level of caprylic acid into canola oil is shown in Figure 3b. With Lipozyme TL IM, the highest level of incorporation was obtained after 30 hours of reaction (27.7 mole%), after which no further improvement was observed in the incorporation level. This finding suggests that the equilibrium was reached at/before 30 hours of reaction. In agreement with the results of this study, Zhao *et al.* (2007) reported that capric acid incorporation was time-dependent where a maximum of 40.7 mole% was obtained after 72 hours of incubation, not significantly higher than that obtained for a 24 hours duration. Similar results have also been reported by Yankah and Akoh (2000), where a maximum level of caprylic acid incorporation was obtained after 12 hours past of the reaction. In the current study, Novozym 435 resulted the highest caprylic acid incorporation after 45 hours of incubation (33.1 mole%) than did Lipozyme TL IM (28.8). Kim *et al*. (2001) reported the highest activity of Novozym 435 after 10 hours of incubation in the interesterification of CLA ethyl ester and tricaprylin.

Chemical Properties of Structured Lipids

FA compositions of the SLs produced at the various conditions of this study are shown in Table 3. For Lipozyme TL IM, the highest level of caprylic acid incorporation (37.2 mole%) was obtained when the



(a) (b)

Figure 3. Main effect of reaction temperature(a) and time (b) on incorporation of caprylic acid into canola oil during acidolysis reaction. LTLIM, reactions catalyzed through Lipozyme TL IM; N435, reactions catalyzed through Novozyme 435; bars are shown as Mean±SD. Signal to noise ratios are presented as data labels. Means denoted with the same letter are not significantly different (P> 0.05).

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reaction was conducted at 55°C with an acid to oil ratio of 3:1, using 12% enzyme load for a 15 hours duration of incubation (run 9). This level of caprylic acid incorporation was not significantly different from that of run 8, which was conducted at 45°C with an acid to oil ratio of 3:1, using 8% enzyme load and 45 hours of incubation. According to Table 3, acidolysis of canola oil with both enzymes resulted in changes in the concentrations of all FAs for the various conditions of the study. However, with Lipozyme TL IM, changes in the levels of oleic, stearic and palmitic acids were larger than those of linoleic and linolenic acids, which may be due to *sn-1,3* specific action of the enzyme resulting in exchanges occurring only at those positions. Large levels of oleic, stearic and palmitic acids in canola oil are present at *sn-1,3* positions (Przybylski *et al*., 2005). For lipids produced using Novozym 435 lipase, the highest caprylic acid incorporation (38.5 mole%) was obtained for run 8, which was conducted at 45°C with an acid to oil ratio of 3:1 using 8% enzyme load for 45 hours of reaction. Higher activity of Novozym 435 in comparison with Lipozyme TL IM is evident within all conditions throughout the study. According to the results reported by Kim *et al.* (2001), who found that Novozym 435 exerted a higher activity level than did Lipozyme RM IM (a 1,3-specific lipase) when performing the enzyme-catalyzed acidolysis of tricaprylin with CLA, *vs.* findings from the current study, the higher activity of Novozym 435 could be explained by its non-specific action on TAGs. In another study, Kim *et al*. (2002) reported that in the lipase-catalyzed acidolysis of perilla oil with caprylic acid, Lipozyme TL IM was more effective in the incorporation of acid than Lipozyme RM IM. Therefore, based upon the above reasoning, an order of activity as Novozym 435> Lipozyme TL IM> Lipozyme RM IM can be suggested for the acidolysis reactions.

The iodine values for different products obtained by way of the acidolysis reactions in this study are also given in Table 3. The iodine levels obtained for the different runs during the study were significantly lower than those for canola oil (118.5), used as the starting material for SL production. Due to the higher caprylic acid incorporation through Novozym 435, the products carry lower iodine values as compared with those through Lipozyme TL IM.

Table 4 shows the positional distribution of FAs among TAGs of canola oil and the products of Lipozyme TL IM as well as Novozym 435. Palmitic, stearic and oleic acids were mainly accumulated in *sn-1,3* positions while linoleic and linolenic acids were abundant in *sn-2* positions of canola oil TAGs. Composition-wise (i.e., regardless of the distribution ratios), *sn-2* position of canola oil TAGs were totally (~98%, w/w) composed of unsaturated FAs (oleic, linoleic and linolenic acids) where oleic and linoleic acids constituted over 80% (w/w) of FA in *sn-2* positions. Current results agree well with those reported by Xu *et al.* (1998) and Xu *et al.* (2000). This makes canola oil a good starting material for the production of MLM-type SLs. TAGs produced using Lipozyme TL IM and Novozym 435 were similar in terms of their FA compositions. However, they were significantly different in terms of positional distributions of FAs (Table 3). Caprylic acid was mainly incorporated at *sn-1,3* position (with *sn-1,3*:*sn-2* ratio of 4:1) when the reaction was catalyzed by Lipozyme TL IM. On the other hand, the *sn-2* position (with this enzyme) was mainly composed of unsaturated FAs (86.5%). These results confirm the *sn-1,3* specificity of Lipozyme TL IM lipase and also the successful production of MLM-type SLs. However, despite the use of a *1,3* specific lipase, 12.5% of caprylic acid was found at *sn-2* position. This finding demonstrates that some acyl migration has taken place during the process, which is common in most reactions of this type. Temperature, water content, type of organic solvent, enzyme load, type of lipase, type of support for enzyme immobilization, type of the acyl donor and the process time as well influence acyl migration (Senanayake and

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Table 3. Fatty acid compositions and Iodine Values (IV) of the structured lipids produced at the various conditions of this study (Table 1) through the enzyme-catalyzed acidolysis of canola oil with the aid of caprylic acid.

Runs	Enzyme used	Fatty acid composition (Mole%)								IV
		CA	PA	SA	OA	LA	LnA	SFA	UFA	
1	LTLIM	12.4±0.5 ^{k}	3.9±0.1 ^{b}	1.9±0.2 ^{b}	51.2±1.4 ^{b}	22.0±0.4 ^{b}	8.5±0.7 ^{b}	18.2 ^{h}	81.8 ^{b}	104.3±2.3 ^{b}
	N435	16.4±0.5 ^{j}	3.7±0.1 ^{b}	1.9±0.1 ^{b}	48.8±0.8 ^{c}	21.6±0.3 ^{b}	7.8±0.1 ^{c}	21.8 ^{g}	78.2 ^{c}	99.7±0.7 ^{c}
2	LTLIM	18.1±0.2 ^{i}	3.5±0.1 ^{bc}	1.8±0.1 ^{b}	48.1±0.4 ^{c}	20.8±0.5 ^{cd}	7.6±0.3 ^{c}	23.5 ^{g}	76.5 ^{c}	97.2±0.6 ^{c}
	N435	25.6±0.4 ^{f}	2.5±0.2 ^{de}	1.2±0.1 ^{cd}	43.5±0.4 ^{e}	19.7±0.3 ^{ef}	7.6±0.1 ^{c}	29.3 ^{e}	70.7 ^{e}	91.3±0.2 ^{de}
3	LTLIM	21.0±1.1 ^{h}	3.8±0.1 ^{b}	1.9±0.2 ^{b}	45.9±0.4 ^{d}	20.0±0.5 ^{ef}	7.4±0.5 ^{cd}	26.7 ^{f}	73.3 ^{d}	93.4±0.6 ^{d}
	N435	25.3±1.4 ^{f}	2.6±0.1 ^{de}	1.3±0.1 ^{c}	43.7±0.8 ^{e}	19.2±0.2 ^{f}	7.8±0.3 ^{c}	29.2 ^{e}	70.8 ^{e}	91.2±0.8 ^{de}
4	LTLIM	29.4±1.4 ^{e}	2.9±0.1 ^{de}	1.2±0.1 ^{cd}	40.4±0.2 ^{f}	19.0±0.4 ^{f}	7.0±0.6 ^{de}	33.6 ^{d}	66.4 ^{f}	85.9±0.3 ^{f}
	N435	35.5±1.3 ^{c}	2.8±0.1 ^{de}	1.2±0.1 ^{cd}	37.1±0.5 ^{ghi}	17.1±0.8 ^{gh}	6.4±0.2 ^{f}	39.4 ^{bc}	60.6 ^{h}	78.2±1.0 ^{gh}
5	LTLIM	23.4±1.2 ^{g}	3.9±0.6 ^{b}	1.9±0.1 ^{b}	42.5±0.3 ^{e}	21.4±0.2 ^{bc}	6.8±0.1 ^{ef}	29.3 ^{e}	70.7 ^{e}	91.3±0.4 ^{de}
	N435	26.9±0.7 ^{f}	2.6±0.3 ^{de}	1.3±0.1 ^{c}	42.3±0.3 ^{e}	19.4±0.6 ^{ef}	7.6±0.1 ^{c}	30.8 ^{e}	69.2 ^{e}	89.8±0.7 ^{ef}
6	LTLIM	29.8±0.7 ^{e}	2.8±0.1 ^{de}	1.3±0.2 ^{c}	39.1±0.1 ^{fg}	20.1±1.1 ^{de}	6.8±0.1 ^{ef}	33.9 ^{d}	66.1 ^{f}	86.2±1.6 ^{f}
	N435	25.2±0.6 ^{f}	2.4±0.1 ^{e}	1.2±0.1 ^{cd}	43.8±0.5 ^{e}	19.6±0.1 ^{ef}	7.7±0.1 ^{c}	28.9 ^{e}	71.1 ^{e}	91.7±0.3 ^{de}
7	LTLIM	35.0±0.3 ^{c}	3.1±0.2 ^{cd}	1.3±0.1 ^{c}	36.9±0.8 ^{hi}	17.2±0.2 ^{gh}	6.5±0.2 ^{ef}	39.4 ^{bc}	60.6 ^{h}	78.4±0.4 ^{gh}
	N435	33.1±0.3 ^{d}	2.7±0.1 ^{de}	1.2±0.1 ^{cd}	38.7±0.2 ^{g}	17.7±0.2 ^{g}	6.6±0.1 ^{ef}	36.9 ^{c}	63.1 ^{g}	81.1±0.1 ^{g}
8	LTLIM	36.0±2.2 ^{bc}	2.6±0.4 ^{de}	1.0±0.1 ^{d}	36.2±2.2 ^{hik}	17.6±0.6 ^{g}	6.7±0.2 ^{ef}	39.6 ^{bc}	60.4 ^{h}	79.1±1.7 ^{g}
	N435	38.5±3.2 ^{a}	2.3±0.4 ^{e}	1.1±0.2 ^{d}	35.6±1.2 ^{ik}	16.2±0.9 ^{h}	6.3±0.4 ^{f}	41.9 ^{a}	58.1 ^{i}	75.1±2.9 ^{h}
9	LTLIM	37.2±1.5 ^{ab}	2.3±0.1 ^{e}	1.2±0.1 ^{cd}	34.8±0.9 ^{k}	17.7±0.4 ^{g}	6.7±0.1 ^{ef}	40.7 ^{ab}	59.3 ^{hi}	78.1±1.4 ^{gh}
	N435	35.3±1.5 ^{c}	2.2±0.1 ^{e}	1.2±0.1 ^{cd}	37.7±0.4 ^{gh}	17.1±0.8 ^{gh}	6.6±0.2 ^{ef}	38.6 ^{bc}	61.4 ^{gh}	79.3±0.8 ^{g}
Blank	-	0.0	4.7±0.3 ^{a}	2.1±0.1 ^{a}	58.9±0.3 ^{a}	24.5±0.1 ^{a}	9.7±0.1 ^{a}	6.9 ^{i}	93.1 ^{a}	118.5±0.4 ^{a}

The values indicate mean±standard deviation of two replications.

^{a,b,c,d,e,f,g,h,i,k}: In each column, means with the same letters are not significantly different (P> 0.05). CA= Caprylic acid; PA= Palmitic acid; SA= Stearic acid; OA= Oleic acid; LA= Linoleic acid; LnA= Linolenic acid; SFA= Saturated fatty acids (sum of caprylic, palmitic and stearic acids); UFA= Unsaturated fatty acids (sum of oleic, linoleic and linolenic acids);. LTLIM= Lipozyme TL IM, N435= Novozym 435.

Shahidi, 2002). Novozym 435 is a non-specific lipase and during a reaction involving this enzyme one expects a random distribution of caprylic acid in the produced TAGs. Positional analysis of TAGs demonstrated such expectation as confirmed, since 31.3% of caprylic acid (about one third) was located at *sn-2* position and 68.7% (about two thirds) located at *sn-1,3* position (Table 4). Cases of *1,3*-specific actions of Lipozyme TL IM (Ozturk *et al*., 2010) and non-specific action of Novozym 435 (Jimenez *et al.*, 2010) have been reported. TAG compositions of MCTs produced by lipase-catalyzed acidolysis of canola oil with caprylic acid are given in Table 5. As observed for FA composition, similar contents of MCTs were produced following acidolysis, using either Lipozyme TL IM or Novozym 435. However, application of Lipozyme TL IM led to the production of higher levels of MLM-type TAGs. This reveals the higher potential of *sn-1,3*-specific lipases in the production of MLM-type SLs, as compared with such non-specific lipases as Novozym 435. The main MCTs in SLs, produced using Lipozyme TL IM were OOC, COC, CLeO and CLeC, where O, C and Le represent, respectively, oleic, caprylic and linoleic acids. When using Novozym 435, CCO and OOC were indicated as extending the greatest contribution to the MCT structure of the produced SLs. SLs prepared using Lipozyme TL IM contained higher levels of MLM and MLL (medium-long-long) and lower quantities of MMM (medium-medium-medium), MML (medium-medium-long) and LML (long-medium-long) TAGs than those prepared using Novozym 435 lipase. The differences in TAG compositions of SLs are due to the different positional specificities of Lipozyme TL IM and Novozym 435 lipases. In fact, as mentioned earlier in the current study, Lipozyme TL IM is an *sn-1,3*-specific lipase (Ozturk *et al*., 2010) while Novozym 435 a non-specific lipase (Jimenez *et al.*, 2010). Zhou *et al*. (2001) studied the acidolysis of canola oil with caproic acid application using the lipase from *Rhizomucor miehei* (Lipozyme RM IM). Under optimized conditions (reaction time: 17 hours, temperature: 65°C, substrate ratio (acid to oil): 5 to 1, enzyme load: 14%, w/w, and water content: 10%, w/w), the resultant SLs contained 55 mole% mono-incorporated caproic acid and 55 mole% di-incorporated TAGs. In a different study (Xu *et al*., 2000), acidolysis of canola oil with caprylic acid, using the lipase from *Rhizomucor miehei* (Lipozyme RM IM) in a packed-bed reactor resulted in 40.1% incorporation of caprylic acid into canola oil, slightly higher than those obtained in the course of the current study (38.5% for Novozym 435 and 37.2% for Lipozyme TL IM). According to Xu *et al.* (2000), under an optimized condition (flow rate: 1 mL min^{-1}, temperature: 60°C, substrate molar ratio: 5 to 1, water content: 0.20% added), only 3.4% caprylic acid was found in *sn-2* positions of the produced SLs. The acidolysis reaction catalyzed by Lipozyme TL IM, as obtained in the current study (Table 2), resulted in accumulation of more caprylic acid in the *sn-2* positions of the SLs (13.4%). A comparison of the data reported by Xu *et al.* (2000) and those obtained in this study may indicate a higher *sn-1,3* specificity of the lipase from *Rhizomucor miehei* (Lipozyme RM IM) than lipase from *Thermomyces lanuginosa* (Lipozyme TL IM).

CONCLUSIONS

The acidolysis reaction between canola oil and caprylic acid was investigated, using the *sn-1,3* specific lipase Lipozyme TL IM *vs.* the non-specific lipase Novozym 435. Novozym 435 indicated a higher capability to incorporate more caprylic acid in the oil than did Lipozyme TL IM. SLs produced by Lipozyme TL IM and by Novozyme 435 were different in terms of their TAG

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Table 4. Positional distributions of fatty acids in canola oil and structured lipids obtained through enzyme-catalyzed acidolysis of canola oil with the aid of caprylic acid.

Sample	Fatty acid position	Fatty acid (%, w/w)							
		CA	PA	SA	OA	LA	LnA	SFA	UFA
CO	TAG	---	4.7±0.3 ^{b}	2.2±0.1 ^{b}	58.9±0.3 ^{b}	24.5±0.1 ^{c}	9.7±0.1 ^{d}	6.9 ^{g}	93.1 ^{b}
	sn-2	---	2.1±0.1 ^{d}(14.5*)	0.4±0.1 ^{f}(6.4)	49.5±0.2 ^{c}(28.0)	32.1±0.2 ^{a}(43.7)	16.1±0.2 ^{a}(55.5)	2.5 ^{h}	97.7 ^{a}
	sn-1,3	---	6.2±0.1 ^{a}(85.5**)	3.1±0.1 ^{a}(93.6)	63.5±0.3 ^{a}(72.0)	20.4±0.4 ^{d}(56.3)	6.6±0.2 ^{e}(44.5)	9.3 ^{f}	90.5 ^{c}
SL LTLIM	TAG	37.1±1.5 ^{cd}	2.3±0.1 ^{d}	1.2±0.1 ^{de}	34.8±0.9 ^{fg}	17.7±0.4 ^{e}	6.7±0.1 ^{e}	40.6 ^{c}	59.2 ^{f}
	sn-2	12.5±0.5 ^{e}(11.2)	0.8±0.1 ^{e}(11.8)	0.1±0.0 ^{f}(2.4)	41.9±1.2 ^{d}(40.1)	29.8±0.4 ^{b}(56.1)	14.8±0.4 ^{b}(73.6)	13.4 ^{e}	86.5 ^{d}
	sn-1,3	49.5±1.9 ^{a} (88.8)	3.1±0.1 ^{c}(88.2)	1.8±0.1 ^{bc}(97.6)	31.3±0.8 ^{h}(59.9)	11.6±0.8 ^{h}(43.9)	2.6±0.2 ^{g}(26.4)	54.4 ^{a}	45.5 ^{h}
SL N435	TAG	38.5±3.2 ^{bc}	2.3±0.4 ^{d}	1.1±0.2 ^{e}	35.6±1.2 ^{ef}	16.2±0.9 ^{f}	6.3±0.4 ^{e}	41.9 ^{c}	58.1 ^{f}
	sn-2	36.1±2.1 ^{d}(31.3)	0.5±0.1 ^{e}(6.4)	0.1±0.0 ^{f}(1.6)	33.1±1.3 ^{gh}(30.1)	19.8±0.5 ^{d}(40.7)	10.4±0.7 ^{c}(55.0)	36.7 ^{d}	63.3 ^{e}
	sn-1,3	39.7±3.7 ^{b}(68.7)	3.3±0.7 ^{c}(93.6)	1.6±0.4 ^{cd}(98.4)	36.9±1.2 ^{e}(69.9)	14.3±1.1 ^{g}(59.3)	4.2±0.3 ^{f}(45.0)	44.6 ^{b}	55.4 ^{g}

* (% fatty acid at the *sn-2* position/% fatty acid in triacylglycerols×3)×100.

** 100 – S^2 fraction

The values indicate Mean±Standard deviation of two replicates.

^{a,b,c,d,e,f,g,h} : In each column, values with the same letters are not significantly different (P> 0.05). CO= Canola oil; CA= Caprylic acid; PA= Palmitic acid; SA= Stearic acid; OA= Oleic acid; LA= Linoleic acid; LnA= Linolenic acid; SFA= Saturated fatty acids (sum of caprylic, palmitic and stearic acids); UFA= Unsaturated fatty acids (sum of oleic, linoleic and linolenic acids); SL LTLIM= Structured lipid obtained using Lipozyme TL IM; SLN435= Structured lipid obtained using Novozym 435, TAG= Triacylglycerol.

Table 5. Triacylglycerol species found in the structured lipids obtained through the enzyme-catalysed acidolysis of canola oil with the aid of caprylic acid.

Sample	TAG (%, w/w)														MLM/MCT
	CCC	OOC	CCO	OCO	CCLe	COLe	OCLe	CLeO	CLnO	COC	CLeC	CLnC	MCT	MLM	
SL LTLIM	3.1 ^{b}	13.0 ^{a}	3.9 ^{b}	1.2 ^{b}	1.4 ^{b}	4.8 ^{a}	0.9 ^{b}	9.2 ^{a}	4.6 ^{a}	10.3 ^{a}	7.3 ^{a}	3.6 ^{a}	63.3 ^{a}	21.2 ^{a}	33.5 ^{a}
SL N435	5.7 ^{a}	9.7 ^{b}	10.6 ^{a}	4.9 ^{a}	4.1 ^{a}	3.7 ^{a}	3.8 ^{a}	5.8 ^{b}	3.0 ^{b}	5.2 ^{b}	3.1 ^{b}	1.6 ^{b}	61.2 ^{a}	9.9 ^{b}	16.2 ^{b}

^{a,b}: In each column, values with the same letters are not significantly different (P> 0.05). C= Caprylic acid; P= palmitic acid; S= Stearic acid; O= Oleic acid; Le= Linoleic acid; Ln= Linolenic acid; MCT= Medium chain triacylglycerol; MLM= Medium, long; Medium triacylglycerol; SL LTLIM,= Structured lipid obtained using Lipozyme TL IM, SL N435= Structured lipid obtained using Novozym 435.

compositions. Application of the *sn-1,3* specific Lipozyme TL IM lipase led to the production of more MLM-type TAGs and was more efficient in producing SLs of MLM structure than the non-specific Novozym 435 lipase. Although optimum enzyme loads for the acidolysis of canola oil as found in this study were 8% for Novozym 435 *vs.* 12% for Lipozyme TL IM, considering the costs of the enzymes, an enzyme load at 4% can be suggested for such purposes without any substantially considerable compromise sustained in the incorporation level.

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تهیه لیپیدهای بازسازی شده به کمک اسیدولیز آنزیمی روغن کلزا

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چکیده

اسیدولیز آنزیمی روغن کلزا با اسید کاپریلیک به منظور تولید لیپیدهای بازسازی شده با تری آسیل گلیسرول های زنجیر متوسط مورد بررسی قرار گرفت. الیپوزیم تی ال آی ام، یک لیپاز۱،۳ ویژه از ترومومایسس لانوگینوزا و نووزیم ۴۳۵ یک لیپاز ناویژه کاندیدا آنتارکتیکا به عنوان بیوکاتالیست در یک واکنشگر بچ مورد استفاده قرار گرفتند. شرایط واکنش بر اساس طرح تاگوچی طرح ریزی شد و نسبت مولی اسید چرب به روغن در سه سطح (۱ به ۱، ۲ به ۱ و ۳ به۱)، محتوای آنزیم در سه سطح (۴، ۸ و ۱۲٪)، دما در سه سطح (۴۵، ۵۵ و ۶۵ درجه سیلیوس) و زمان در سه سطح (۱۵ ، ۳۰ و ۴۵ ساعت) در نظر گرفته شد .نتایج نشان داد که ترکیب اسید چرب روغن کلزا بوسیله واکنش اسیدولیز مورد بحث در بالا اصلاح گردید. بالاترین درصد مولی اسید کاپریلیک (۳۷/۲٪) بعد از ۱۵ ساعت واکنش در حضور لیپوزیم تی ال آی ام در دمای ۵۵ درجه، نسبت مولی اسید چرب به روغن ۳ به ۱ و سطح آنزیم ۱۲٪ به دست آمد. در حالیکه، با نووزیم ۴۳۵ بالاترین درصد جابجایی (۳۸/۵٪) بعد از ۴۵ ساعت از واکنش، نسبت اسید به روغن ۳ به ۱ و سطح آنزیم ۸٪ به دست آمد. در کل، نووزیم ۴۳۵ قادر بود که در مقایسه با لیپوزیم تی ال آی ام مقادیر بیشتری از اسید کاپریلیک را در روغن وارد کند. لیپیدهای بازسازی شده بدست آمده بوسیله لیپوزیم تی ال آی ام و نووزیم ۴۳۵ در زمینه ساختار تری آسیل گلیسرول هایشان متفاوت بودند. براساس نتایج این مطالعه، لیپید بازسازی شده بوسیله لیپوزیم تی ال آی ام مقادیر بیشتری از تری آسیل گلیسرول های با وضعیت های اسیدهای چرب از نوع متوسط-بلند-متوسط در مقایسه با نووزیم ۴۳۵ دارا بودند (به ترتیب ۲۱/۲ %و ۹/۹%).

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