EFFICACY OF SOME COMMERCIAL LIPASES IN HYDROLYSIS OF PALM OLEIN FOR THE PRODUCTION OF FREE FATTY ACIDS AND DIACYLGLYCEROL OIL

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ABSTRACT

Palm olein, which is an abundant cheap vegetable oil, can be used for production of value-added products such as free fatty acids (FFA) and diacylglycerol (DAG) oil. In this study, the efficacy of four commercial lipases (Lipase A, Lipase AY, Lipozyme RM IM and Lipozyme TL IM) and one phospholipase (Lecitase Ultra) in hydrolysis of palm olein was investigated. Lecitase Ultra and Lipozyme RM IM were the most selective enzymes towards hydrolysis of saturated fatty acids. Lipozyme TL IM was found to be selective towards unsaturated fatty acids. Lipase A and Lipase AY were almost non-selective. At optimum hydrolysis condition, FFA and DAG oil yields of 55.0%-94.5% and 5.5%-45.0% were obtained, respectively. The highest FFA or DAG oil yields were obtained using Lecitase Ultra (94.5%) and Lipase AY (45.0%), respectively. The DAG oils contained 39.0%-42.2% DAG and had higher slip melting point, solid fat content and iodine value, and lower content of saturated fatty acids than palm olein. Totally, Lecitase Ultra may be of importance due to its high FFA yield and saturated fatty acid selectivity. However, when a high yield of DAG oil is of interest, Lipase AY will be the best choice.

Keywords: palm olein, hydrolysis, lipase, free fatty acid, DAG oil.

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INTRODUCTION

Lipase or glycerol ester hydrolase, EC (3.1.1.3.), is a renewable biocatalyst broadly employed in production of structured lipids and fatty acids. It catalyses the hydrolysis of ester linkage in triacylglycerols (TAG) leading to the formation of diacylglycerols (DAG), monoacylglycerols (MAG), free fatty acids (FFA) and glycerol. In addition to hydrolysis, lipases can also catalyse esterification and interesterification (transesterification, acidolysis, glycerolysis) reactions (Gandhi, 1997).

Hydrolysis of vegetable oils is especially important for production of FFA that can be used in production of several non-food and food products such as soaps, detergents, health care products, emulsifiers and nutraceuticals (Avelar *et al*., 2013). Compared to conventional non-enzymatic hydrolysis of vegetable oils that requires high reaction temperatures (250°C) and pressures (50 bar), the lipase-catalysed hydrolysis of vegetable oils is more advantageous as it requires mild reaction condition; moreover, it can be performed in a selective manner leading to the enrichment of certain fatty acids (Murty *et al*., 2002; Rupani *et al*.,

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2012). Partial hydrolysis of vegetable oils can also be used for production of DAG oils to which several beneficial health effects have been attributed. There are claims that DAG are similar to TAG in terms of digestibility and energy value. However, DAG are metabolised in a manner that makes them beneficial for human nutrition. The DAG-enriched oil can overcome the problems of obesity and heart diseases owing to its ability to decrease postprandial lipid levels. In addition, consumption of DAG may reduce body weight and accumulation of visceral abdominal fat (Lo *et al*., 2008).

Palm olein, which is a product of the palm oil fractionation, is an abundant cheap vegetable oil. The fatty acids composition of palm olein is similar to palm oil; however, due to its different TAG structure, unlike palm oil, it is liquid at room temperature. The main fatty acids of palm olein are palmitic and oleic acids (about 40% of each acid) (Siew and Faridah, 2000). Due to high amount of saturated fatty acids in palm olein, enhancement of the nutritional quality of palm olein may be of interest. Enzymatic hydrolysis may provide the opportunity to convert palm olein to FFA and DAG oil, *i.e*., production of an important lipid raw material (FFA) in one hand and improving the nutritional quality of palm olein by producing DAG-enriched oil on the other hand (Lo *et al*., 2008; Murty *et al*., 2002).

In recent decade, several commercial lipases have been used in hydrolysis of vegetable oils. The use of commercial phospholipases like Lecitase Ultra (from Novozymes) has also gained some attention for production of DAG-enriched oil (Wang *et al*., 2008; 2009; 2010). Cheong *et al*. (2007) studied the production of DAG-enriched palm olein via partial hydrolysis using Lipozyme RM IM and Lecitase Ultra. Saberi *et al*. (2011) studied the physico-chemical properties of palm olein-based DAG produced by Novozyme 435-catalysed glycerolysis. Though several researches have been published on lipase/phospholipase-catalysed hydrolysis of oils, there is no comprehensive comparison of available commercial lipases in hydrolysis of oils. Accordingly, in this research, performance of several commercially available lipases and one phospholipase in simultaneous production of FFA and DAG oil was compared in terms of fatty acid selectivity, optimum reaction condition, and physico-chemical properties of the product.

MATERIALS AND METHODS

Materials

Refined palm olein was generously provided by Partodeneh Khazar (Behshahr, Iran). Commercial lipases Lipozyme TL IM (from *Thermomyces lanuginosa*), Lipozyme RM IM (from *Rhizomucor miehei*) and Lecitase Ultra (the enzyme obtained from the fusion of the genes of *Thermomyces lanuginosa* lipase and *Fusarium oxysporum* phospholipase) were kindly donated by Novo Nordisk (A.S., Denmark). Lipase AY (from *Candida rugosa*) and Lipase A (from *Aspergillus niger*) were purchased from Amano (Nagoya, Japan). High-purity silica gel Grade 923 (100-200 mesh) was purchased from Sigma-Aldrich (St Louis, MO). All other chemicals were of chromatographic or analytical grade and obtained from Merck (Darmstadt, Germany).

Selectivity Assay

Fatty acid selectivity of the enzymes was evaluated at their optimum temperature [45°C for Lipase A, 37°C for Lipase AY, 40°C for Lecitase Ultra, 65°C for Lipozyme RM IM and 70°C for Lipozyme TL IM] as described by the supplier or other researchers (You and Baharin, 2006; Watanabe *et al*., 2003; Cheong *et al*., 2007; Kiatsimkul *et al*., 2006; Wang *et al*., 2008). Briefly, 50 g of palm olein was hydrolysed in the presence of proper amount of enzyme [65 units per gramme oil for Lecitase ultra, Lipase A or Lipase AY, 5% (w/w, oil basis) for Lipozyme TL IM or Lipozyme RM IM] and proper water content [40% (w/w, oil basis) for Lecitase ultra, Lipase A and Lipase AY, 4% (w/w, oil basis) for Lipozyme TL IM and Lipozyme RM IM] at 150 rpm. The reaction was stopped after 5%, 10% and 25% conversion by adding 100 ml of a mixture of methanol and diethyl ether (80:20, v/v). FFA were separated from acylglycerols and analysed for fatty acid composition. Selectivity towards different fatty acids was evaluated by calculation of enrichment number (EN) using the following equation (Kiatsimkul *et al*., 2006):

$$EN = \frac{\%\ \text{normalisation of fatty acid A in FFA fraction}}{\%\ \text{normalisation of fatty acid A in palm olein}}$$

Recovery of FFA and DAG Oil

After the hydrolysis reaction was stopped, 80 ml of 0.5 ml potassium bicarbonate and 15 ml of diethyl ether was added into the reaction mixture. Then the mixture was poured into a separatory funnel and FFA soap (the lower aqueous phase) and DAG oil (the upper organic phase containing acylglycerols) fractions were separated from each other. FFA were recovered from soaps by acidification with sulphuric acid and then solvent extraction with diethyl ether. Finally, the diethyl ether was evaporated from FFA or DAG oil at 45°C using a rotary evaporator (Kiatsimkul *et al*., 2006).

Optimisation of Partial Hydrolysis

Response surface methodology (RSM) was used to optimise the hydrolysis of palm olein for each

enzyme. A central composite design composed of four variables was used to maximise the response (degree of hydrolysis) of enzyme activities. The levels of variables for each enzyme were selected from the literature (You and Baharin, 2006; Watanabe *et al*., 2003; Cheong *et al*., 2007; Kiatsimkul *et al*., 2006; Wang *et al*., 2008). Lipozyme TL IM and RM IM were in the form of immobilised enzymes and had almost similar properties. Lipase A, Lipase AY and Lecitase Ultra were in the form of free enzymes. They were active in almost similar reaction conditions, as well. Accordingly, two sets of RSM experiments were designed: one for the immobilised enzymes and the other one for the free enzymes. Variables included water content [30% to 50% (w/w, oil basis)] for free enzymes, 3% to 7% (w/w, oil basis) for immobilised enzymes, enzyme load [10 to 50 units per gramme oil for free enzymes, 2% to 10% (w/w, oil basis)] for immobilised enzymes, reaction temperature (30°C to 50°C for free enzymes, 40°C to 80°C for immobilised enzymes) and reaction time (2 to 10 hr). Thirty experiments were designed for each enzyme using Minitab 16.2.2 statistical software (LEAD Technologies, Inc.).

Degree of Hydrolysis and DAG Oil Yield

Degree of hydrolysis was expressed as percentage of FFA liberated from oil and determined in accordance with the American Oil Chemists' Society (AOCS) method Ca5a–40 (AOCS, 1996). A blank titration was done as control sample. DAG oil yield was calculated using the following formula:

$$\text{DAG oil yield (\%)} = 100 - \text{FFA\%}$$

Fatty Acid Composition Analysis

Fatty acid methyl esters (FAME) of FFA fraction were prepared as described elsewhere (Christie, 1993). Briefly, 1 ml of methanolic BF3 solution [14% (v/v) BF3 in methanol] was added into 10 mg of sample in a glass vial and shook well for 10 min at room temperature. Then 2 ml of hexane was added into the mixture, and centrifuged (11000 rpm) for 1 min. Finally, the upper layer was washed using 5 ml of distilled water and dried using anhydrous sodium sulphate. The dry hexane solution was then injected into a gas chromatograph. FAME of palm olein or DAG oil were prepared according to the AOCS method Ce2-66 (AOCS, 1996).

FAME were analysed by a Trace gas chromatograph (Thermo Finnigan, Italy) equipped with a BPX-70 (60 m × 0.25 mm × 0.25 μm) column and flame ionisation detector. Injector and detector were set at 250°C and 270°C, respectively. Injection was performed in a split ratio of 1:80. The oven was set at 175°C for 2 min, increased to 230°C at a rate of 3°C min^{-1} and held at 230°C for 10 min. Nitrogen was used as carrier gas with a flow rate of 0.8 ml min^{-1}.

Determination of DAG Content by Column Chromatography

DAG content of DAG oils was determined according to the AOCS method Cd 11c-93 (AOCS, 1996). The method determines acylglycerol composition by solid-liquid adsorption chromatography using silica gel as the absorbent. The TAG were eluted with 10% diethyl ether in petroleum ether, DAG with 25% diethyl ether in petroleum ether, and MAG with 100% ether. Finally, all eluted portions were de-solvented in a rotary evaporator, weighed and the ratio of each fraction was calculated.

Slip Melting Point Determination

Slip melting point (SMP) was determined in accordance with the AOCS Cc 3–25 open tube melting point method (AOCS, 1996). Capillary tubes filled with samples were stored in a refrigerator (6°C-7°C) overnight before the measurements.

Solid Fat Content Determination

A Minispec mq 20 pulsed nuclear magnetic resonance spectroscope (pNMR, Bruker Corporation, Hamburg, Germany) was employed for solid fat content (SFC) measurement at 10°C, 20°C, 30°C and 40°C according to the AOCS Cd 16b-93 method (AOCS, 1996).

Iodine Value

Iodine value (IV) was calculated from the fatty acid compositions according to the AOCS method Cd 1c-85 (AOCS, 1996).

Statistical Analysis

Experiments were performed in duplicate in a completely randomised design and data were analysed for analysis of variances (one-way) and comparison of means (Duncan›s multiple range test) by SPSS software version 21 (SPSS, Chicago, IL, USA).

RESULTS AND DISCUSSION

Selectivity of Enzymes in Hydrolysis of Palm Olein

Conversion data at 100% hydrolysis will not reveal information on selectivity of lipases, because all of the fatty acids will be released at 100% hydrolysis

(Kiatsimkul *et al*., 2006). Accordingly, reaction times of this investigation were selected to provide about 5%-25% conversion. Fatty acid selectivity of lipases was evaluated by analysis of fatty acid composition of FFA fractions and calculation of EN of different fatty acids. The EN is a measure of the selectivity of the enzyme towards a specific fatty acid. A high EN reflects a high selectivity of the enzyme towards hydrolysing a particular fatty acid (Kiatsimkul *et al*., 2006). As can be seen in *Table 1*, Lecitase Ultra showed the highest amount of EN for palmitic acid and total saturated fatty acids, at 10% hydrolysis. A similar result was also observed by Wang *et al*. (2008) who studied the hydrolysis of soyabean oil using Lecitase Ultra. In addition to Lecitase Ultra, Lipozyme RM IM was also selective towards palmitic, stearic and total saturated fatty acids. Lipozyme TL IM showed the highest selectivity towards oleic, linoleic and total unsaturated fatty acids at 25% degree of hydrolysis. The highest selectivity towards stearic acid was observed for Lipase A (EN=1.37); however considering total saturated and unsaturated fatty acids this enzyme was almost non-selective. Lipase AY did not show any significant selectivity towards total saturated or total unsaturated fatty acids. Based on the data of *Table 1*, EN of all enzymes for stearic acid was higher than palmitic acid. Kiatsimkul *et al*. (2006) reported that Lipase AY and Lipase A had a higher EN for palmitic acid than stearic acid in hydrolysis of soyabean oil, which is in contrast with the results of this study. This may be due to the differences in the fatty acid composition and TAG structures of soyabean oil and palm olein (Basiron, 2005; Hammond *et al*., 2005).

Optimisation of Hydrolysis Process

RSM was used to model and optimise the FFA production, with four reaction parameters: water content, enzyme load, reaction temperature and time. *Tables 2* and *3* show the percentage of FFA produced at each of the 30 experiments designed by RSM. Lecitase Ultra gave the highest FFA yield (78.2%, *Table 2*), followed by Lipozyme RM IM (76.3%, *Table 3*), Lipozyme TL IM (68.7%, *Table 3*), Lipase AY (64%, *Table 2*) and Lipase A (53.0%, *Table 2*).

Table 4 shows statistical analysis of RSM designs for various lipases. For Lipozyme TL IM, all the reaction parameters had significant ($P<0.05$) positive effects on FFA production, with time having the greatest effect followed by water, temperature, and enzyme. For Lipozyme RM IM and Lipase AY, all reaction parameters except time, had significant ($P<0.05$) positive effects on FFA production, and temperature had the greatest effect on FFA production. Water, enzyme and time had no significant effects on FFA production by Lecitase Ultra; however, temperature had a significant effect on FFA production. Finally, for Lipase A, temperature

TABLE 1. FATTY ACID COMPOSITION AND ENRICHMENT NUMBER OF RELEASED FREE FATTY ACID (FFA) AFTER PARTIAL HYDROLYSIS OF PALM OLEIN USING DIFFERENT ENZYMES

Treatment		Percentage fatty acid composition (enrichment number) of FFA fractions							
Enzyme	**DH (%)**	**14:0**	**16:0**	**18:0**	**18:1**	**18:2**	**18:3**	**SFA**	**UFA**
No enzyme	0	0.9	42.0	4.1	41.7	11.1	0.2	47.0	53.0
Lipozyme TLIM	5	1.0 (1.11)	41.0 (0.98)	4.6 (1.13)	41.7 (1.00)	11.3 (1.02)	0.3 (1.50)	46.7 (0.99)	53.3 (1.01)
	10	0.9 (1.00)	41.3 (0.98)	4.6 (1.12)	42.3 (1.01)	10.7 (0.97)	0.2 (1.00)	46.8 (0.99)	53.2 (1.00)
	25	1.0 (1.12)	37.5 (0.89)	4.8 (1.18)	44.3 (1.06)	12.0 (1.09)	0.3 (1.51)	43.4 (0.92)	56.6 (1.07)
Lipozyme RMIM	5	1.0 (1.11)	42.6 (1.02)	4.8 (1.17)	40.6 (0.97)	10.6 (0.96)	0.3 (1.50)	48.5 (1.03)	51.5 (0.97)
	10	1.0 (1.12)	40.0 (0.95)	5.4 (1.30)	41.8 (1.00)	11.5 (1.04)	0.3 (1.51)	46.4 (0.99)	53.6 (1.01)
	25	1.0 (1.11)	43.5 (1.04)	4.9 (1.20)	40.3 (0.97)	10.1 (0.91)	0.2 (1.00)	49.4 (1.05)	50.6 (0.95)
Lipase AY	5	1.0 (1.11)	41.8 (1.00)	4.7 (1.15)	41.5 (1.00)	10.6 (0.96)	0.3 (1.50)	47.6 (1.01)	52.4 (0.99)
	10	1.0 (1.12)	40.3 (0.96)	4.9 (1.18)	42.8 (1.03)	10.7 (0.97)	0.3 (1.51)	46.2 (0.98)	53.8 (1.02)
	25	1.0 (1.11)	40.8 (0.97)	5.2 (1.27)	41.7 (1.00)	11.0 (0.99)	0.2 (1.00)	47.1 (1.00)	52.9 (1.00)
Lipase A	5	1.0 (1.12)	41.4 (0.98)	4.9 (1.20)	41.8 (1.00)	10.6 (0.96)	0.3 (1.51)	47.3 (1.01)	52.7 (0.99)
	10	1.0 (1.12)	40.8 (0.97)	4.9 (1.2)	42.2 (1.01)	10.8 (0.98)	0.3 (1.51)	46.7 (0.99)	53.3 (1.01)
	25	1.0 (1.11)	41.0 (0.98)	5.6 (1.37)	41.4 (0.99)	10.7 (0.96)	0.2 (1.00)	47.7 (1.01)	52.3 (0.99)
Lecitase Ultra	5	1.0 (1.12)	43.5 (1.04)	4.8 (1.18)	40.2 (0.96)	10.2 (0.92)	0.3 (1.51)	49.3 (1.05)	50.7 (0.96)
	10	1.0 (1.12)	44.6 (1.06)	4.9 (1.18)	39.0 (0.94)	10.2 (0.93)	0.3 (1.51)	50.5 (1.07)	49.5 (0.94)
	25	1.0 (1.12)	43.2 (1.03)	4.8 (1.18)	40.4 (0.97)	10.3 (0.93)	0.3 (1.51)	49.0 (1.04)	51.0 (0.96)

Note: Enrichment numbers are written within parenthesis.
DH - degree of hydrolysis; SFA - saturated fatty acids (sum of myristic, palmitic and stearic acids); UFA - unsaturated fatty acids (sum of oleic, linoleic and linolenic acids).

TABLE 2. CENTRAL COMPOSITE RESPONSE SURFACE METHODOLOGY DESIGN AND RESPONSES FOR LECITASE ULTRA, LIPASE A AND LIPASE AY

Run	Factors				Responses (% FFA)		
	Temperature (°C)	Time (hr)	Enzyme (μ g^{-1} of oil)	Water (% w/w of oil)	Lipase AY	Lipase A	Lecitase Ultra
1	50	6	30	40	45.6	54.1	36.0
2	40	6	30	50	44.0	56.2	71.1
3	30	6	30	40	35.4	42.9	26.4
4	40	6	30	40	44.4	53.0	63.3
5	40	6	30	30	37.3	46.3	45.7
6	40	10	30	40	48.5	55.8	78.2
7	40	2	30	40	36.9	46.5	24.7
8	40	6	30	40	44.3	53.2	61.0
9	40	6	10	40	35.2	42.7	45.0
10	40	6	50	40	49.1	60.2	46.5
11	45	8	40	45	53.0	64.0	78.1
12	35	4	40	45	41.0	51.8	45.6
13	45	8	40	35	49.4	58.9	53.1
14	40	6	30	40	44.1	53.5	65.7
15	40	6	30	40	44.5	53.4	63.5
16	45	8	20	45	46.2	55.0	66.3
17	45	4	40	45	47.8	59.0	32.1
18	45	4	20	35	37.3	45.5	23.6
19	35	4	20	35	29.9	38.3	26.9
20	35	8	20	35	35.0	43.2	48.2
21	40	6	30	40	45.0	52.8	59.5
22	45	4	40	35	44.1	54.0	19.8
23	35	4	20	45	34.2	43.3	31.4
24	35	8	40	45	46.6	53.4	56.5
25	40	6	30	40	44.0	52.8	64.6
26	45	4	20	45	41.0	50.0	25.1
27	35	8	20	45	38.7	47.9	53.5
28	35	8	40	35	42.0	48.5	40.1
29	35	4	40	35	37.5	47.1	29.1
30	45	8	20	35	42.5	49.7	40.3

Note: FFA – free fatty acid.

and water content had significant (P<0.05) positive effects on FFA production but time and enzyme load effects were insignificant (*Table 4*).

The quadratic terms of all parameters had negative effects on FFA production (*Table 4*). All quadratic terms, except time and enzyme for Lipozyme RM IM and water for Lecitase Ultra, had significant (P<0.05) effects on FFA production. For Lecitase Ultra, Lipase A and Lipase AY, the quadratic term of time had the greatest effect followed by the quadratic terms of temperature, water and enzyme. Quadratic terms of time, water, temperature and enzyme (in decreasing order) had significant effect on the hydrolysis activity of Lipozyme TL IM (P<0.05). For Lipozyme RM IM, the quadratic term of enzyme had the greatest effect, followed by the quadratic terms of time, temperature and water, respectively (P<0.05). All the interactions between parameters, except interaction between temperature and time for Lecitase Ultra, had insignificant effects on FFA production (P<0.05).

Given the main, quadratic and interaction effects of reaction parameters, the optimal reaction condition of each enzyme in partial hydrolysis of palm olein was determined (*Table 5*). The adequacy of the predictive model was examined by performing suggested experiments at the optimal conditions. Verification results revealed that the predicted values were reasonably close to the observed ones (*Table 5*). Therefore, models were able to predict the degree of hydrolysis of palm olein with high accuracy. Experimentally, at optimal reaction condition, the

TABLE 3. CENTRAL COMPOSITE RESPONSE SURFACE METHODOLOGY DESIGN AND RESPONSES FOR THE LIPASES TL IM AND RM IM

Run	Factors				Responses (% FFA)	
	Temperature (°C)	Time (hr)	Enzyme (% w/w of oil)	Water (% w/w of oil)	Lipozyme TL IM	Lipozyme RM IM
1	70	8	8	40	67.1	67.3
2	60	6	6	50	61.0	66.1
3	50	4	8	60	56.2	62.5
4	70	4	8	60	63.9	66.4
5	50	8	4	40	50.5	53.2
6	70	4	8	40	62.3	63.5
7	70	8	8	60	68.7	70.0
8	70	4	4	60	54.8	60.5
9	50	4	4	60	47.2	52.5
10	70	8	4	40	58.3	57.0
11	50	4	8	40	54.2	59.5
12	50	8	8	60	61.5	66.8
13	70	8	4	60	60.1	60.0
14	50	8	4	60	52.0	56.1
15	60	6	6	50	61.5	66.5
16	60	6	6	50	60.8	66.9
17	70	4	4	40	52.9	53.5
18	60	6	6	50	60.9	66.6
19	50	4	4	40	45.1	49.5
20	50	8	8	40	59.5	63.2
21	60	6	2	50	50.0	56.0
22	80	6	6	50	62.7	63.3
23	40	6	6	50	48.8	56.8
24	60	6	6	50	61.0	66.4
25	60	6	6	30	53.1	56.5
26	60	6	6	70	58.5	62.5
27	60	2	6	50	53.7	63.0
28	60	6	10	50	67.2	76.3
29	60	6	6	50	61.2	66.7
30	60	10	6	50	63.9	70.1

Note: FFA – free fatty acid.

highest FFA yield was obtained by Lecitase Ultra followed by Lipozyme RM IM, Lipozyme TL IM, Lipase A and Lipase AY, respectively.

Using a combination of Lipozyme TL IM and Lipozyme RM IM, Rodrigues and Ayub (2011) reached a 95% yield of soyabean oil hydrolysis under optimal condition, which is comparable with the result of Lecitase Ultra in this study. However, as they reported, the hydrolysis yield of individual enzymes were much lower than that obtained in this study (83% for Lipozyme TL IM compared to 70.4% in this study, and 62% for Lipozyme RM IM compared to 78.2% in this study). The *C. rugosa* lipase has been used for palm oil hydrolysis by Knezevic *et al*. (2002) and a maximum of 74% hydrolysis was obtained, which is much higher than that (Lipase AY, 55%) obtained in this study.

The accuracy of the models was evaluated by measuring coefficient of determination (R^2 and adjusted R^2 values) and absolute average deviation (AAD). The AAD was calculated using the following equation (Cheong *et al*., 2007):

$$AAD = \left\{\left[\sum_{i=1}^{p}\left(\left|y_{i,exp} - y_{i,cal}\right| / y_{i,exp}\right)\right] / p\right\} \times 100$$

where $y_{i,exp}$ and $y_{i,cal}$ are the experimental and calculated responses, respectively, and p is the number of experimental run. The R^2 must be close to 1.0 and the AAD between the predicted and observed data must be as small as possible. Values of R^2, adjusted R^2 and AAD of the models were 0.96%,

TABLE 4. STATISTICAL ANALYSIS OF RESPONSE SURFACE METHODOLOGY MODELS FOR FREE FATTY ACID PRODUCTION USING DIFFERENT ENZYMES

Terms	Lipozyme TL IM		Lipozyme RM IM		Lipase AY		Lipase A		Lecitase Ultra	
	R	P-value	R	P-value	R	P-value	R	P-value	R	P-value
Constant	-80.88	0.00	-118.16	0.00	-152.7	0.00	-103.17	0.00	-476.8	0.00
Te	1.95	0.00	2.73	0.00	4.15	0.00	3.80	0.00	22.63	0.00
Ti	4.28	0.00	2.79	0.21	2.01	0.05	1.20	0.40	-10.03	0.25
En	1.49	0.00	4.79	0.04	0.75	0.00	0.37	0.19	0.76	0.65
Wa	2.80	0.00	2.37	0.00	3.34	0.00	1.88	0.01	2.64	0.52
Te*Te	-0.01	0.00	-0.02	0.00	-0.04	0.00	-0.04	0.00	-0.34	0.00
Ti*Ti	-0.17	0.00	-0.08	0.34	-0.09	0.00	-0.12	0.01	-0.91	0.00
En*En	-0.01	0.00	-0.11	0.19	-0.00	0.00	-0.00	0.02	-0.05	0.00
Wa*Wa	-0.13	0.00	-0.02	0.00	-0.03	0.00	-0.01	0.01	-0.07	0.07
Te*Ti	-0.00	0.95	-0.01	0.58	0.00	0.62	0.04	0.08	0.45	0.00
Te*En	-0.00	0.78	-0.01	0.58	-0.00	0.41	0.00	0.05	0.02	0.44
Te*Wa	0.00	0.70	0.00	0.58	-0.00	0.56	0.00	0.86	0.05	0.31
Ti*En	0.00	0.95	0.06	0.58	0.00	0.80	-0.01	0.12	0.00	0.99
Ti*Wa	-0.00	0.86	-0.01	0.58	0.00	0.87	0.00	0.82	0.23	0.09
En*Wa	0.00	0.95	-0.01	0.58	0.00	1.00	0.00	0.95	0.04	0.14

Note: R - regression coefficient; Te - temperature (°C), Ti - time (hr); En - enzyme load; Wa - water (%).

TABLE 5. OPTIMAL REACTION CONDITIONS PREDICTED FOR DIFFERENT ENZYMES AND ACYLGLYCEROL COMPOSITION OF THE HYDROLYSED PALM OLEIN AT OPTIMUM CONDITION

Sample	Enzyme	Wa	En	Ti	Te	Observed	Predicted
						FFA (%)	FFA (%)
1	Lipozyme TL IM	5.4	10.0	10.0	73.1	72.6	70.4
2	Lipozyme RM IM	5.0	10.0	10.0	63.0	76.8	78.2
3	Lipase AY	44.9	50.0	10.0	47.3	56.5	55.0
4	Lipase A	50.0	50.0	10.0	50.0	69.9	68.3
5	Lecitase Ultra	50.0	36.0	10.0	43.9	96.0	94.5

Note: Wa - water (%); En - enzyme load; Ti - time (hr); Te - temperature (°C).

0.92% and 1.59% for Lecitase Ultra, 0.96%, 0.91% and 1.79% for Lipozyme RM IM, 0.99%, 0.99% and 3.12% for Lipozyme TL IM, 0.99%, 0.99% and 2.73% for Lipase AY and 0.99%, 0.98% and 2.34% for Lipase A, respectively. This shows that the models were statistically good and had no significant ($P<0.05$) lack of fit.

Physico-chemical Properties of DAG Oil

Table 6 shows the DAG oil yield and the level of MAG, DAG and TAG of the product of each enzyme at optimum reaction condition. The highest and lowest yields of DAG oil were obtained by Lipase AY and Lecitase Ultra, respectively. The highest and lowest MAG contents were observed in the product of Lipozyme RM IM and Lecitase Ultra, respectively. The DAG oils of Lecitase Ultra and Lipase A or Lipase AY contained the highest and lowest amount of DAG, respectively. Hydrolysis of palm olein using Lipozyme RM IM or Lecitase Ultra gave the lowest and highest amount of TAG, respectively. Although the highest content of TAG was observed in the product of the Lecitase Ultra, the best result was obtained with this enzyme as it contained the highest amount of DAG and the lowest amount of MAG. Lipozyme RM IM was better than Lipozyme TL IM, Lipase A or Lipase AY were, in terms of DAG content. Considering the DAG yield together with the DAG oil yield, if the production of DAG is of interest, the higher DAG yield of Lecitase Ultra may not be of importance as the yield of DAG oil was

TABLE 6. PHYSICO-CHEMICAL PROPERTIES OF ACYLGLYCEROL FRACTIONS OBTAINED FROM PARTIAL HYDROLYSIS OF PALM OLEIN USING DIFFERENT ENZYMES

Property	Palm olein	Sample prepared using				
		Lipozyme TLIM	Lipozyme RMIM	Lipase AY	Lipase A	Lecitase Ultra
Acylglycerol yield (%)	-	29.6	21.8	45.0	31.7	5.5
Acylglycerol composition (%)						
MAG	-	9.1c	11.2a	9.7b	9.3bc	3.5d
DAG	-	40.5b	40.8b	39.0c	39.1c	42.2a
TAG	-	49.9c	47.7d	51.2b	49.9c	53.6a
Fatty acid composition (%)						
14:0	1.0a	0.7a	0.8a	0.8a	0.7a	0.6a
16:0	41.8a	40.0b	37.5d	38.3c	38.1c	37.3d
18:0	4.1a	4.2a	3.9a	4.0a	4.1a	3.9a
18:1	41.5d	42.4c	44.6a	43.9b	44.0b	44.9a
18:2	11.0a	10.9a	11.4a	11.3a	11.3a	11.4a
18:3	0.2a	0.2a	0.2a	0.2a	0.2a	0.2a
SFA	47.1a	45.1b	42.4d	43.2c	43.1c	42.1d
UFA	52.7d	53.9c	56.7a	55.8b	55.9b	57.0a
IV	53.6d	56.4c	59.3a	58.4b	58.5b	59.5a
SMP (°C)	17.8d	41.0a	39.2b	40.5a	39.6b	37.2c

Note: Values with different superscripts in each row were significantly different at $p<0.05$.
SFA - saturated fatty acids (sum of myristic, palmitic and stearic acids); UFA - unsaturated fatty acids (sum of oleic, linoleic and linolenic acids); IV - iodine value; SMP - slip melting point.

only 5.5% (*Table 6*). In this regard, Lipase AY with a DAG oil yield of 45.0% and DAG yield of 39.0% may be more interesting. However, when a high yield of FFA is needed, Lecitase Ultra will be the best choice. Wang *et al*. (2008) produced DAG-enriched oil with 42.64% DAG via partial hydrolysis of soyabean oil using Lecitase Ultra. In another work, Wang *et al*. (2010) reported accumulation of 26.51% DAG in the product of Lecitase Ultra-hydrolysed soyabean oil. The acylglycerol fraction produced by Lipozyme RM IM had a DAG content of 40.8%, which was higher than that (34.17%) obtained by Awadallak *et al*. (2013) who studied palm oil hydrolysis under ultrasound irradiation. Cheong *et al*. (2007) reported a DAG yield of 32.0% in hydrolysis of palm oil using Lipozyme RM IM, which was also lower than that obtained in this study.

Fatty acids composition of the DAG oils obtained from hydrolysis of palm olein is shown in *Table 6*. The DAG oils had higher unsaturated fatty acid and lower saturated fatty acid content than palm olein had. This was in agreement with the results published in the literature (Cheong *et al*., 2007; Wang *et al*., 2008). In this context, Lecitase Ultra, which showed the highest saturated fatty acid-selectivity might be of great importance especially in production of DAG oils with reduced saturated fatty acid content.

IV of palm olein and DAG oils obtained from different enzymes are given in *Table 6*. As can be seen in *Table 6*, IV increased after partial hydrolysis of palm olein using all enzymes. This is due to the increase of unsaturated fatty acids in DAG oils. The DAG oils obtained using Lecitase Ultra or Lipozyme RM IM, which had the highest unsaturated fatty acid content, had also the highest IV.

SMP of DAG oils are shown in *Table 6*. The SMP of palm olein before partial hydrolysis was about 22.5°C. As can be seen in *Table 6*, partial hydrolysis of palm olein resulted in an increase in SMP of the product. The highest and lowest SMP was obtained for the product of Lipozyme TL IM and Lecitase Ultra, respectively. As the amount of saturated fatty acids in DAG oil increased, the corresponding SMP also increased, similar to the results of Saberi *et al*. (2011). The higher SMP of DAG oils compared to palm olein could be attributed to the different structure of MAG, DAG and TAG. In fact, MAG and DAG, due to their higher melting points, may play a

role in hardening of vegetable oils. The DAG oil produced by Lecitase Ultra had the lowest SMP among the product of all enzymes (may be due to the lower amount of MAG) (Soares *et al*., 2009).

SFC (the quantity of fat crystals in a fat) curve is one the most important physical properties of fat determining its plasticity and melting behaviour (Dian *et al*., 2007). The SFC of fats at 10°C-40°C before and after partial hydrolysis are shown in *Figure 1*. Hydrolysis of palm olein caused a decrease in SFC at 10°C; however, DAG oils had higher SFC at 20°C-40°C as compared to palm olein. Similar to our results, Saberi *et al*. (2011) reported lower SFC at 10°C and higher SFC at 20°C-40°C for DAG prepared from palm oil, olein or stearin. The increase in SFC of the hydrolysed oil may be due to the increased amount of MAG and DAG that have higher melting points than TAG. Cheong *et al*. (2009) reported that lard-based DAG had higher melting temperature than lard. In other words, with similar fatty acid composition, DAG oil has higher melting point than TAG oil. Therefore, it can be concluded that the higher melting temperature of hydrolysed palm olein, compared to the initial palm olein, is more related to the acylglycerol structures rather than fatty acid composition (Cheong *et al*., 2009; Miklos *et al*., 2013). In general, DAG oils were found to have flatter SFC profiles indicating more uniform consistency over a wide range of temperature. This property makes them suitable for production of plastic fats (Nor Aini *et al*., 1995).

The DAG oils obtained by different enzymes had almost similar SFC curves, which was in agreement with Soares *et al*. (2009). Lipozyme TL IM, Lipase A and Lipase AY produced DAG oils with higher amount of saturated fatty acids than other commercial enzymes. Accordingly, these products had almost higher SFC. In contrast, the product of Lecitase Ultra or Lipozyme RM IM that had lower saturated fatty acids content showed a lower SFC.

CONCLUSION

The ability of four commercial lipases and one phospholipase in hydrolysis of palm olein was evaluated. Lecitase Ultra and Lipozyme RM IM preferentially released more saturated fatty acids in hydrolysis reaction. The hydrolysis reaction conditions were optimised using RSM and the highest degree of hydrolysis was obtained by Lecitase Ultra (94.5%) followed by Lipozyme RM IM, Lipozyme TL IM, Lipase A and Lipase AY, respectively. FFA produced by hydrolysis of palm olein may find applications as raw material in food or chemical industry. Partial hydrolysis caused an increase in DAG content of the acylglycerol fraction (DAG oil) of the hydrolysed oil, which may improve the nutritional quality of the product. The DAG oil contained lower content of saturated fatty acids and showed increased SMP, SFC and IV. This can widen the application of palm olein in food industry especially in formulation of shortenings and margarines.

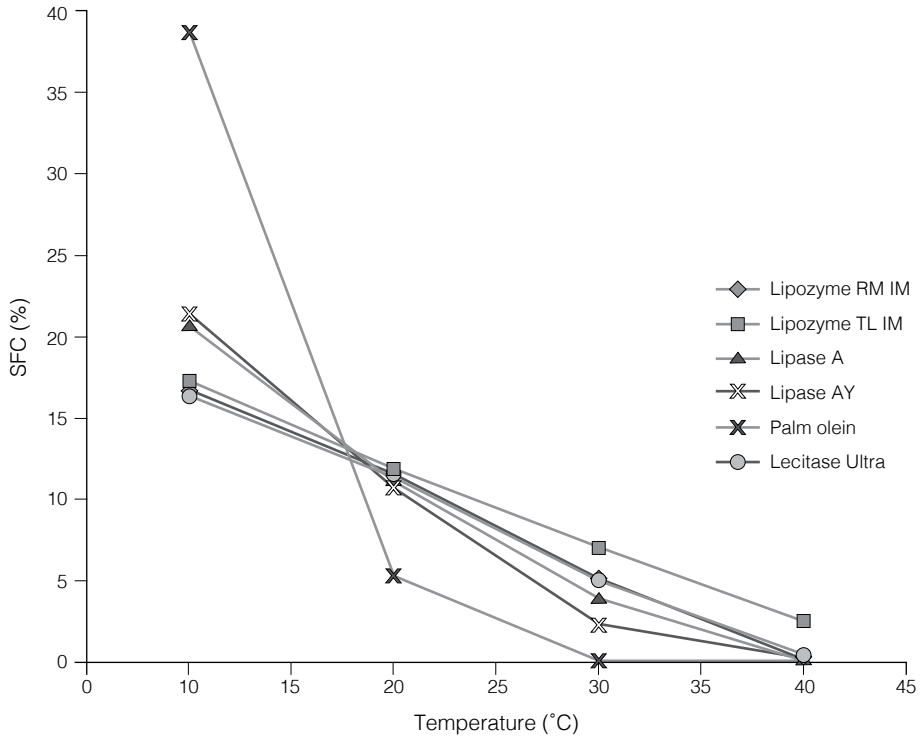

Figure 1. Solid fat content (SFC) curve of palm olein and acylglycerol fractions of palm olein hydrolysis.

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