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RESEARCH ARTICLE

Lipase-catalyzed transesterification of medium-long-medium structured lipid (MLM-SL) using palm olein and tricaprylin in packed-bed Reactor (PBR)

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ABSTRACT

Lipase-catalyzed transesterification between refined bleached deodorized palm olein (RBDO) and tricaprylin to produce medium-long-medium structured lipid (MLM-SL) in a packed bed reactor (PBR) has been investigated. A specific *sn*-1,3 commercial Lipozyme TL IM was used as biocatalyst. Within this study, the progress of transesterification was monitored especially for triacylglycerol (TAG) formation with equivalent carbon number (ECN) of 32, presumably 1,3-dicapryoyl-2-oleoyl-*sn*-glycerol (COC). Transesterification conditions investigated were residence times (*i.e.*, 15, 30, and 60 min) and enzyme loadings (2.0 and 4.5 g). The highest yield of ECN 32 (13%) and transesterification degree (71%) were obtained at residence time of 15 mins for both enzyme loadings. Longer residence time seemed to facilitate lipid hydrolysis over transesterification. This was indicated by the number of peaks appearing in the high-performance liquid chromatography (HPLC) chromatograms and the reduction of fat slip melting point (SMP). Additionally, the highest productivity was obtained at 2.0 g enzyme loading. Conclusively, this study has demonstrated the potential use of packed-bed reactor with immobilized Lipozyme TL IM for continuous synthesis of MLM-SLs especially TAG with ECN32.

Keywords: 1,3-dicapryoyl-2-oleoyl-*sn*-glycerol; Lipase; Palm olein; Packed bed reactor; Structured lipids

INTRODUCTION

Medium-long-medium structured lipid (MLM-SL) is a typical structured lipid that contains medium chain fatty acids (MCFAs, C6-C12) at *sn*-1,3 positions and long chain fatty acid (LCFA, C14-C24) at *sn*-2 position. The presences of MCFA and LCFA on a triacylglycerol (TAG) molecule poses benefits especially for clinical nutrition purposes such as to improve fat malabsorption and managing obesity. MCFAs at *sn*-1,3 positions are directly transported to liver as instant energy sources. In addition to this, structured lipid where LCFA is at *sn*-2 position is also directly absorbed. MLM-SLs are not commonly found from natural resources. Both chemical and enzymatic synthesis are used in an attempt to produce MLM-SLs. The enzymatic interesterification was preferably to synthesize MLM-SL due to its selectivity, less by-products produced, mild reaction conditions, and ease of biocatalysts recovery. Herein, enzyme-based MLM-SL synthesis has gained popularity in recent years (Utama et al., 2019).

The continuous synthesis of MLM-SL was of importance especially at industrial scale. Continuous synthesis leads to the reduction of unproductive times (due to start-, and end-procedures in repetitive batch cycles), and also minimization of batch-to-batch oscillation in product quality (Sitanggang et al., 2016, 2015, 2014a). Generally, MLM-SL synthesis in continuous system was conducted using packed bed reactor (PBR), micro-channels (MC), enzymatic membrane reactor (EMR). PBR has several advantages such as ease of operation, better product control, and high reaction rate and mass transfer (Itabaiana et al., 2013; Silva et al., 2011). In PBR system, flow rate or residence time plays important role for reaction kinetics and thus, volumetric productivity. The operation of PBR requires the enzyme to be immobilized. Herein, another

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consideration for successful and efficient MLM-SL synthesis in PBR is the cost of biocatalyst. Lipozyme TL IM (Novozymes A/S) is an *sn*-1,3 specific lipase originating from *Thermomyces lanuginosus,* and immobilized in a non-compressible silica gel carrier (Yang et al., 2014). It has been reported for its economically low price and larger active side pockets possible for rapid catalysis of lipid transesterification (Basri et al., 2013; Wang et al., 2008). For MLM-SL synthesis, Lipozyme TL IM showed higher operational stability in transesterification reaction as compared to that of acidolysis reaction (Utama et al., 2020).

Refined bleached deodorized palm olein (RBDO) is always considered as a potent substrate to produce MLM-SL. It is due to high content of oleic acid at *sn*-2 position (May and Nesaretnam, 2014). The consumption of oleic acid has shown positive effect for the prevention of cardiovascular diseases (Mele et al., 2018; Ong and Goh, 2002). In addition to this, caprylic acid has been shown to be more effective to increase plasma ketone for rapid energy sources as compared to other MCFAs (Vandenberghe et al., 2017). The incorporation of caprylic acid into RBDO is expected to yield MLM-SL with equivalent carbon number (ECN) of 32, presumably1,3-dicapryoyl-2-oleoyl-*sn*-glycerol (COC). In our previous work (Utama et al., 2020), we reported the transesterification of RBDO and tricaprylin in batch system that could obtain 16.75% MLM-SL with ECN 32. In addition, Lai et al. (2005) reported that acidolysis reaction between RBDO with caprylic acid in PBR system catalyzed by Lipozyme IM 60 produced 35.3% of ECN 32. However, studies about MLM-SL synthesis using RBDO and tricaprylin in PBR system are limited. Based on this rationale, this study was aimed to synthesize MLM-SL using RBDO and tricaprylin under PBR system. The synthesis was catalyzed by Lipozyme TL IM. The effect of residence time and enzyme loading were also investigated within this work.

MATERIALS AND METHODS

Materials

RBDO with iodine value (IV) of 60 was obtained from PT. Salim Ivomas TBK, Indonesia. Tricaprylin, molecular sieve 4 Å and triglyceride standard mixture (tricaprin, tricaprylin, trilaurin, trimyristin, and tripalmitin) were purchased from Sigma-Aldrich, Singapore. Lipozyme TL IM was obtained from Novozyme A/S, Denmark. Hexane, chloroform, ethanol, octanol, sodium hydroxide, acetonitrile, and acetone were analytical grade and purchased from Merck, Germany.

Synthesis of MLM-SL in continuous system (packed bed reactor)

The schematic design of PBR system is shown in Fig. 1. The reactor system was consisted of substrate reservoir, peristaltic pump (BT 100-IF longer Peristaltic Pump, Baoding longer Precision Pump Co., Ltd), column, water bath (Stephen Hacke, Germany), and product reservoir. Packed bed reactor column (ID =11 mm and H = 80 mm) with jacketed wall was made from glass material. The upper and lower ends of column were equipped with filter which was impermeable for the biocatalyst resins. The column was packed with either 2.0 or 4.5 g of biocatalysts. For 2.0 g of enzyme loading, molecular sieve 4 Å (Sigma-Aldrich) was used as "dummy enzyme" to avoid catalysts floating within the column. The mixture of substrates (RBDO and tricaprylin with molar ratio of 1:1) was pumped into the reactor from the upper-end of the column. Three different residence times were realized (i.e., 15, 30, and 60 min) within this study. The residence time was calculated according to Levenspiel (1999) and Sitanggang et al. (2014b) as follows (eq. 1).

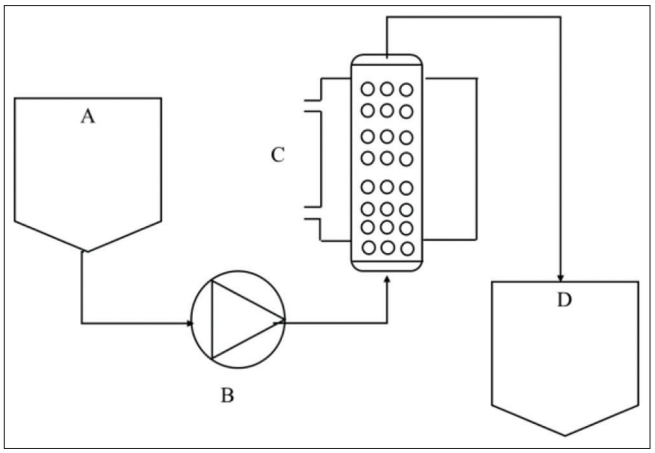

Fig 1. Schematic design of reactor system: (A) sample reservoir, (B) peristaltic pump, (C) packed bed reactor, and (D) product reservoir.

$$\tau = \frac{V}{v_0} \quad (1)$$

where = residence time (s), = working volume of the reactor (m^3) and = volumetric flow rate (m^3/s). The temperature of reaction (50°C) was maintained by circulating water continuously into substrates reservoir and jacketed column of PBR. Samples were taken from product reservoir after 3 h of reaction (without recycle procedure). Productivity of MLM-SL synthesis especially for ECN32 was calculated as follows:

$$P = PA\left\{\frac{\left(\frac{V}{\tau}\right)}{[E_0]}\right\} \quad (2)$$

Where = productivity ($\frac{m^3_{ECN32}}{g_{[E]} \times h}$), PA = percentage area of ECN32 and [E_0] = enzyme weight (*i.e.*, 2.0 or 4.5 g).

TAG composition analysis

TAG composition analysis was conducted according to Utama et al. (2020). The TAG peaks were identified using TAG mixture standard peaks and ECN value. ECN can be obtained as CN-2(DB), where CN shows the total amount of carbon in the TAG molecule without glycerol, and DB is number of double bonds on the TAG molecule (Holčapek et al., 2005). The change of tricaprylin concentration before and after interesterification was used to determine transesterification degree (TD) and as follows (eq. 3):

$$TD = \frac{(P_E - P_O)}{P_O} \quad (3)$$

where P_O and P_E were percentage area of tricapylin prior to- and after reaction, respectively.

Determination of acylglycerol fractions

The acylglycerol fractions were determined by AOCS Official Method Cd 11b-91 (AOCS, 2017) with minor modifications. The acylglycerol fractions were analyzed using a Hewlett Packed Series 6890 autoinjector gas chromatography system equipped with a flame ionization detector (FID) and DB-5HT column (L = 15 m, ID = 320 nm, and thickness = 0.1 μm).

Differential scanning calorimetry

Melting and crystallization point of blending and the produced structured lipids were determined using differential scanning calorimetry **(**DSC) (model TA-60, TA instrument, New Castle) according to Saberi et al. (2011). The crystallization was indicated by peaks in cooling curves, whereas melting points were indicated by heating curves.

Slip melting point (SMP)

Slip melting point (SMP) was determined according AOCS Official method Cc 3-25 (AOCS, 2017b). The measurements were run in triplicate and reported as a mean ± standard deviation (SD).

Statistical analysis

Data are presented as mean ± standard deviation. The effects of different treatments (i.e., residence time and enzyme loading) on the observed parameters were analyzed by one-way analysis of variance (ANOVA) and followed by Duncan posthoc test using IBM SPSS 20 software.

RESULTS AND DISCUSSION

TAG compositions of structured lipids

The formation of MLM-SL was determined by comparing peaks (i.e., TAG composition) between TAG mixture standard and transesterification products. In the blended mixture (i.e., RBDO:tricaprylin (1:1)), the dominant TAGs were mainly those with ECN > 42. TAGs of blended mixture were dominated by tricaprylin (CCC), palmitic-oleic-oleic (POO), palmitic-oleic-palmitic (POP), and palmitic-linoleic-oleic (PLO). After transesterification reaction, these TAGs reduced, leading to the emergences of several new TAG species especially with ECN 32, 38, and 40 (see Fig. 2b-d). This change was presumed as the results of caprylic acid incorporation (mono- or di-substitution) within TAG molecules found in RBDO. During batch transesterification using the same substrates and biocatalyst, several new TAG species were also produced including ECN 30, 32, 34, 36, 38, 40, and 42 (Utama et al., 2020). Moreover, ECN 32 also showed highest TAG concentration among other TAG species in batch-wise. Within this work, the incorporation of caprylic acid into RBDO catalyzed by Lipozyme TL IM also showed higher possibility to produce COC which is indicated by higher chromatogram areas of ECN 32 as compared to that of blended mixture's peak area (Fig. 2a-d). Herein, COC was selected as TAG of interest and to represent MLM-SL in this study.

In continuous reaction, residence time plays a key role to determine the rate of disappearance or formation of chemical species of interest. In this study, 15 min of residence time was considered as the optimum residence time for both enzyme loadings due to high concentrations of ECN 32 (Fig. 3). The increase in residence time had no influence on the concentration of TAG dominant. Yang et al. (2014) reported a range of residence time of 30-40 min as optimum condition to produce MLM-SL using soybean oil medium chain triacylglycerol (MCT) catalyzed by Lipozyme TL IM in PBR system. In addition, Xu et al. (2002) also reported that Lipozyme TL IM-catalyzed interesterification between fish oil and MCT in PBR system showed the degree of reaction reached equilibrium at 30-40 min of residence times.

In general, the increase in enzyme concentration in reacting medium leads to the increased reaction rate. Zhang et al. (2001) reported that interesterification degree was positively influenced by the enzyme loading and reached equilibrium at 6% of enzyme loading. However, within this work, two levels of enzyme loading (*i.e.*, 2.0 and 4.5 g) had no effect on the product concentration obtained. The results for different enzyme loadings also showed similar patterns

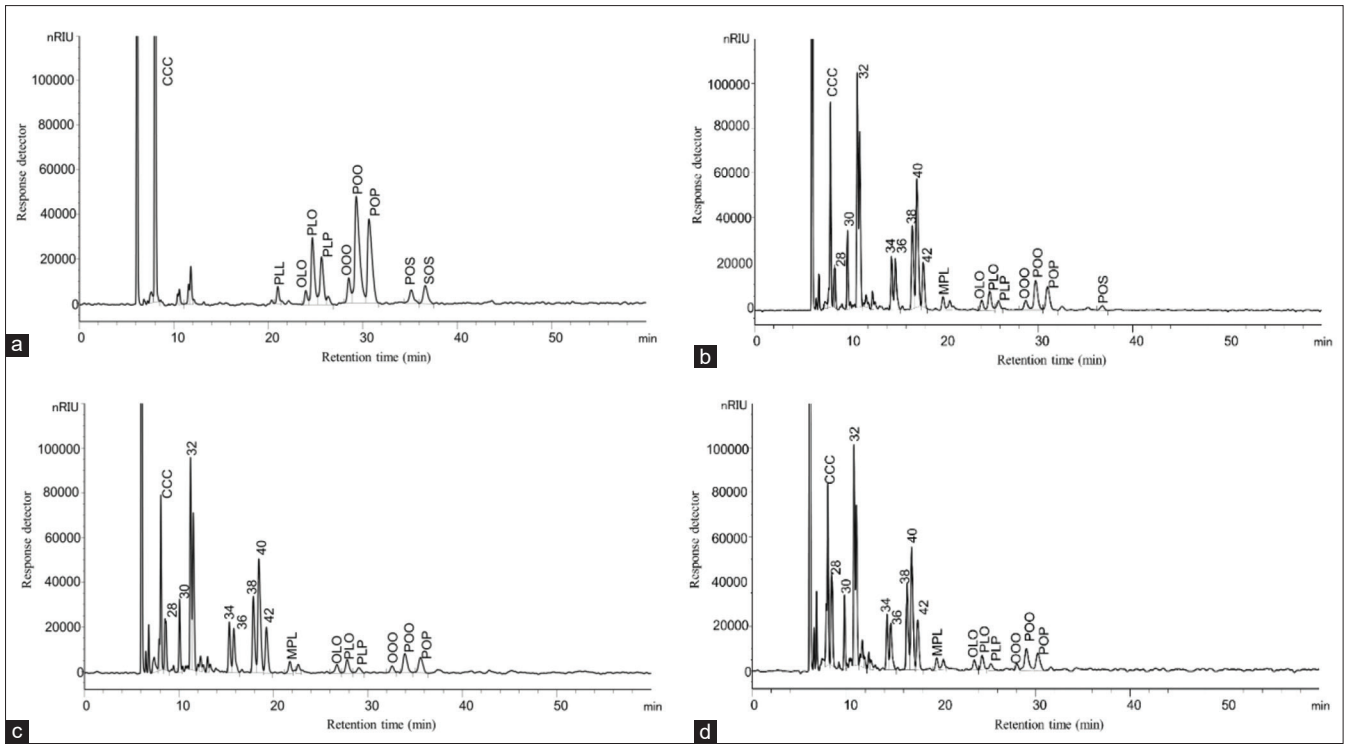

Fig 2. Chromatograms of (a) blending RBDO : tricaprylin (1:1), (b) τ = 15 min, (c) τ = 30 min, and (d) τ = 60 min with enzyme loading of 4.5 g and temperature of 50°C. TC/CCC = tricaprylin; MPL = myristic-palmitic-linoleic; OLO = oleic-linoleic-oleic; PLO = palmitic-linoleic-oleic; PLP = palmitic-linoleic-palmitic; OOO = oleic-oleic-oleic; POO = palmitic-oleic-oleic; POP = palmitic-oleic-palmitic.

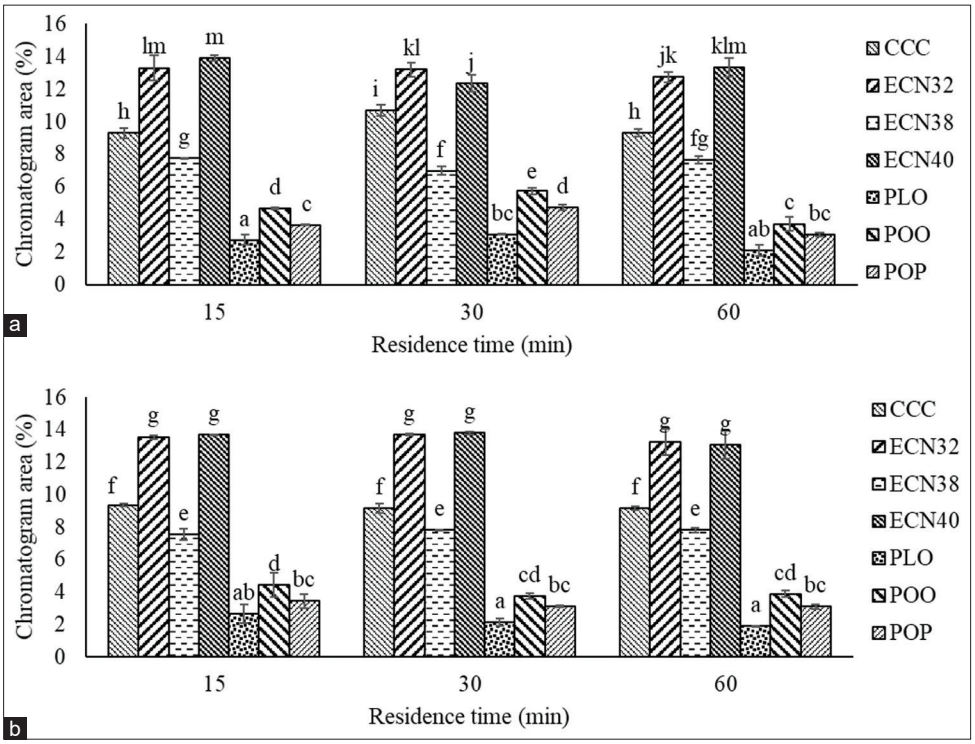

Fig 3. Effect of residence time (τ) on the TAG profiles of the structured lipids. Enzyme loading of 2.0 g (a), and 4.5 g (b). Different letters indicate a significant difference ($p<0.05$).

especially for the reduction trend of initial dominant TAGs and the increase trend of new TAGs (Fig. 3). Two enzyme loadings employed within this study might be presumably excessive. This could be indicated by relatively short reaction times to reach concentration plateau for both enzyme loadings (*i.e.*, approximately 15 min, see Fig. 4).

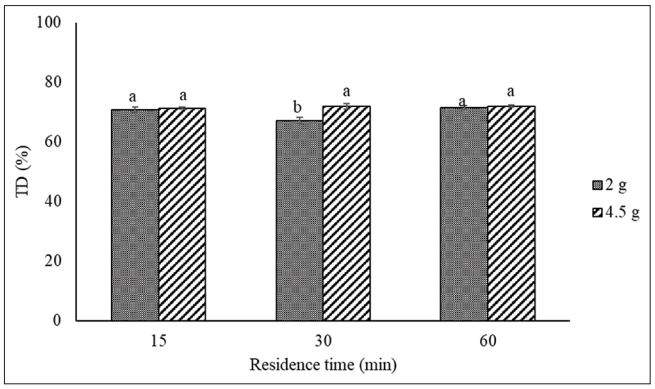

Fig 4. Effect of residence time (τ) on transesterification degree (TD). Different letters indicate a significant difference (p<0.05).

Productivity of structured lipid (ECN 32) synthesis

For optimum residence time (*i.e.*, τ = 15 min), enzyme loading (E_0) of 2.0 g had volumetric flow rate (v_0) of 4.33 x 10^{-9} m^3/s. Assuming the density of the reactor did not change throughout the reaction time and the percentage area of ECN 32 was 13.84% (see Fig. 3), the calculated productivity was about 3.33 x 10^{-10} $m^3/g_{[E]}.s$. For enzyme loading (E_0) of 4.5 g, the optimum residence time was also the same with (E_0) of 2 g. However, the volumetric flow rate was raised to 6.5 x 10^{-9} m^3/s. Hence, with percentage area of ECN 32 of 13.55%, the calculated productivity based on eq. (2) was 1.67 x 10^{-10} $m^3/g_{[E]}.s$. Based on the productivity value, enzyme loading 2.0 g was considered as the optimum condition.

Acylglycerol fraction after transesterification

Despite of its small amount is required (*i.e.*, microaqueous system), water still has important role during lipase-catalyzed interesterification. In lipase-catalyzed interesterification reaction, water was included in the enzyme materials or substrates. High content of water in system will shift the progress of reaction towards hydrolysis. Herein, the formations of new TAGs in transesterification are accompanied by the formations of by-products such as diacylglycerol (DAG), monoacylglycerol (MAG) and the fatty acid (FA) in reaction system. Kadhum and Shamma (2017) determined the formation acyl glycerol complexes as results of lipase-catalyzed interesterification. Moreover, Hermansyah et al. (2010) reported that hydrolysis of triacylglycerol by the enzyme was a stepwise process in order to obtain DAG, MAG, and glycerol in which enzyme-substrate complexes and FA are formed at each reaction step.

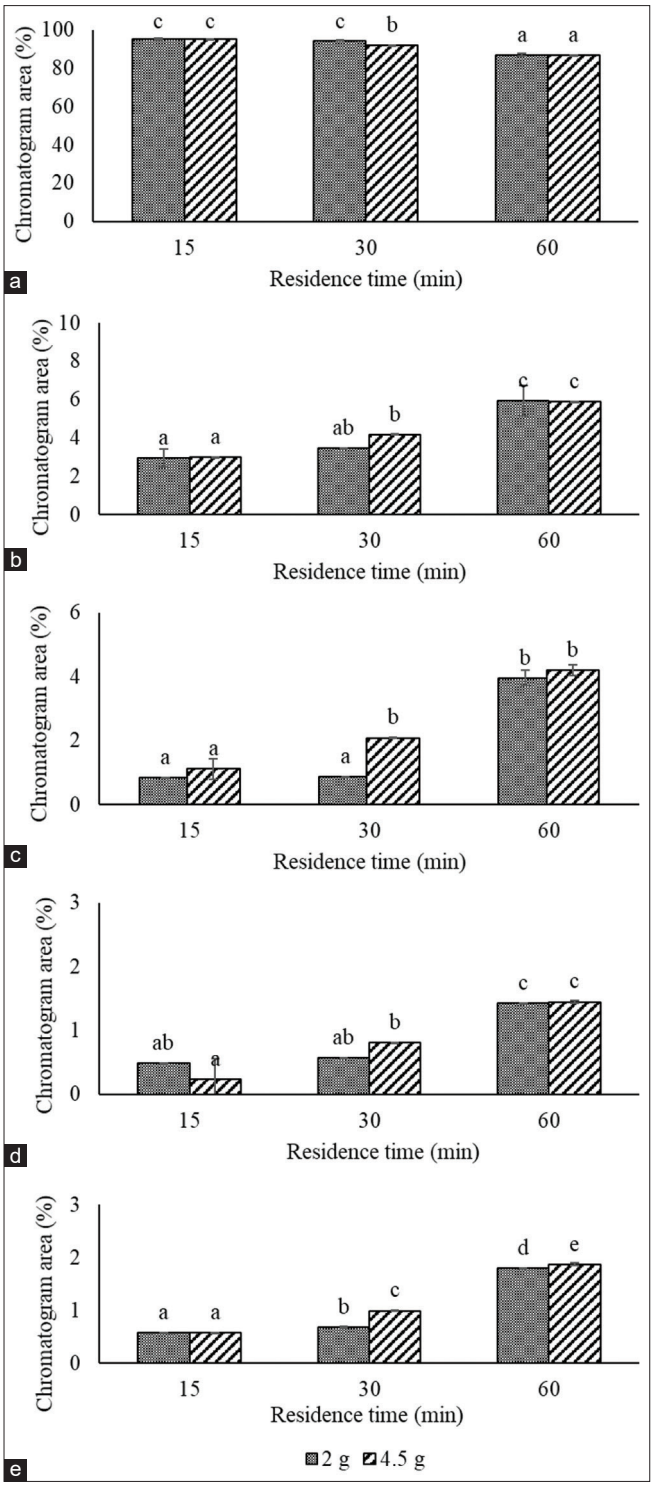

Fig 5. Effect of residence time and enzyme loading on (a) TAG, (b) DAG, (c) MAG, (d) fatty acid, and (e) glycerol concentration of structured lipid. Different letters indicate a significant difference (p<0.05).

For blending product, acyl glycerol fraction only consisted of TAGs and DAGs. After transesterification reaction, changes on acylglycerol fractions were observed (Fig. 5). Within 15 min of residence time, TAG concentration slightly increased while DAG concentration decreased. In addition to this, MAG, glycerol, and FA were also detected during the reaction. Increased residence time (*i.e.*, 30 and 60 min) enhanced DAG, MAG, glycerol, and FA formation. This might be due to facilitation of a longer contact time between the initial and produced TAGs with enzyme

molecules that favored hydrolysis reaction. Higher amounts of side products from the transesterification might be detrimental especially for the separation of the produced structured lipids. In addition to this, formation of FA could lead to pH shift that levels off the stability of the enzyme.

Different enzyme loadings relatively showed similar concentrations of acylglycerol fractions. In contrast, Zhang et al. (2001) reported that the increase of enzyme loading had positive impact on the increased formation of FAs and DAGs. This was due to a higher amount of water from the enzyme materials involved during the reaction. Moreover, in higher enzyme loading, such higher active pockets were also available to perform hydrolysis on the TAGs.

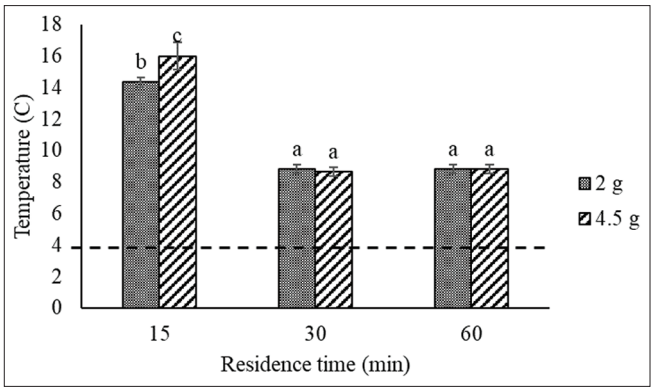

Fig 6. Slip melting point of blending product (dashed line) and structured lipid products. Different letters indicate a significant difference (p<0.05).

Thermal profile of structured lipid product

The concentrations of MAGs and DAGs may influence melting point, crystal formation and/or temperature, and the hardness of lipids (Basso et al., 2010; Saberi et al., 2011). In this work, SMP of the transesterification product shifted in parallel to the changes in the compositions of acylglycerol fractions. Generally, the increasing of DAGs concentration will reduce slip melting point of lipid (Fig. 6). SMP of blending product was 4.33°C. At 15 min of residence time, SMP was higher than at 30 and 60 min. At 15 min of residence time, the formations of TAGs (also MLM TAG) were favored whereas for longer residence times the hydrolysis was pronounced. Thus, the concentrations of DAGs were higher than TAGs for these longer residence times (*i.e.*, 30 and 60 min). The effects of DAGs and MAGs concentration on SMP also depend on types of fatty acid (*i.e.*, length of carbon chain, saturated or unsaturated) and isomeric positions of fatty acids. Siew (2002) reported that 1, 2 isomers of DAG was found to be more effective to reduce melting point as compared to that of 1,3 isomers of DAG. Moreover, Subroto et al. (2019) mentioned that higher concentrations of saturated fatty acids in DAG and MAG structures could increase melting point of lipids.

The information about melting and crystallization temperature of fats is important for designing their applications in food products. Within this study, transesterification was also found to reduce melting and crystallization point of blended product (Fig. 7 and Table 1). Furthermore, the obtained structured lipid showed a smaller range in melting temperatures and a wider range in crystallization temperatures as compared to that of

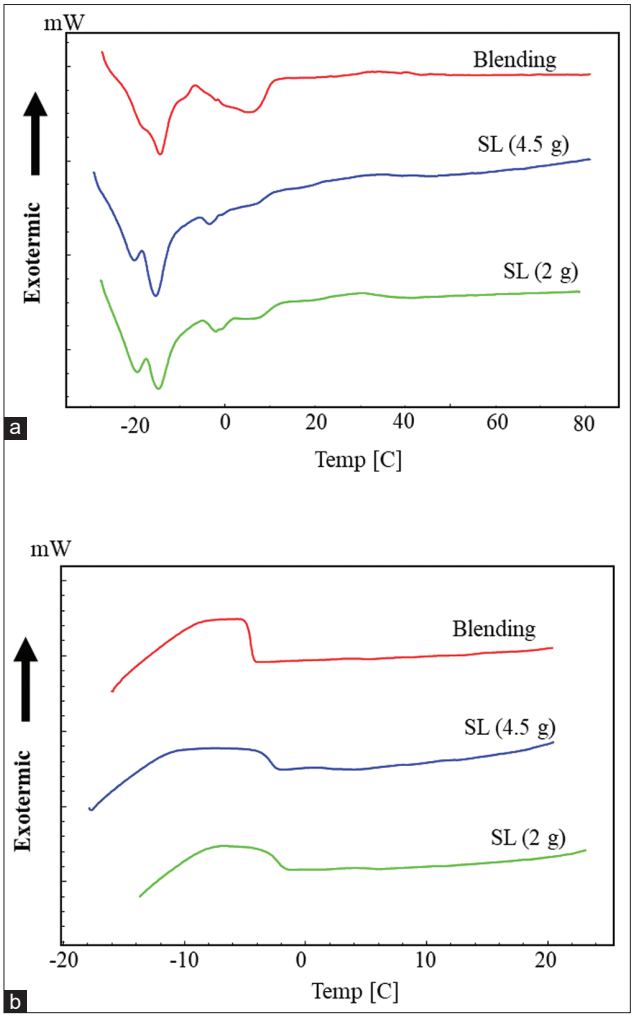

Fig 7. Differential scanning calorimetry (DSC) of melting (a) and crystallization (b) curve of blending and structured lipid products (SL).

Table 1: Thermal profiles of blending and structured lipid products

Sample	Melting				Crystallization			
	Onset (°C)	Peak (°C)	Endset (°C)	Δh (J/g)	Onset (°C)	Peak (°C)	Endset (°C)	Δh (J/g)
Blending	-5.04	5.31	10.26	-10.50	-5.65	-4.18	-12.46	7.79
SL 2.0 g	-4.23	-1.99	1.59	-1.25	-1.70	-6.68	-10.89	4.14
SL 4.5 g	-4.97	-3.41	-1.37	-0.50	-2.35	-7.68	-13.66	4.76

blending products. Moreover, for 4.5 g of enzyme loading showed a lower melting and a crystallization temperature as compared to that of 2.0 g enzyme loading.

CONCLUSIONS

Lipase-catalyzed transesterification reactions can be used to synthesize a potential lipid of MLM type-structured lipid, 1,3-dicapryoyl-2-oleoyl-*sn*-glycerol (COC, ECN 32). Within this study, the enzyme loadings utilized might be excessive as indicated by little or no effect on the formation of TAGs. Higher residence times (*i.e.*, 30 and 60 min) showed to decrease the concentrations of TAGs. This consequently influenced the slip melting point of the transesterified products. Conclusively, in continuous transesterification, residence time τ of 15 min, and enzyme loading (E_0) of 2.0 g were selected as the optimum conditions to obtain the highest productivity for COC formation.

Abbreviations

COC	: 1,3-dicapryoyl-2-oleoyl-*sn*-glycerol / caprylic-oleic-caprylic
DAG	: Diacylglycerol
DSC	: Differential scanning calorimetry
ECN	: Equivalent carbon number
FFA	: Fatty acid
HPLC	: High-performance liquid chromatography
IM	: Immobilized
LCFA	: Long chain fatty acid
MAG	: Monoacylglycerol
MCFA	: Medium chain fatty acid
MLM-SL	: Medium-long-medium structured lipid
MPL	: Myristic-palmitic-linoleic
OLO	: Oleic-linoleic-oleic
OOO	: Oleic-oleic-oleic;
PBR	: Packed-bed reactor
PLO	: Palmitic-linoleic-oleic
PLP	: Palmitic-linoleic-palmitic
POO	: Palmitic-oleic-oleic
POP	: Palmitic-oleic-palmitic
RBDO	: Refined bleached deodorized palm olein
SL	: Structured lipid
SMP	: Slip melting point
sn-	: Stereospecific number
TAG	: Triacylglycerol
TC/CCC	: Tricaprylin
TD	: Transesterification degree
TL	: *Thermomyces lanuginosus*

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Authors' contributions

QDU conducted the research, analyzed the data, and drafted the manuscript. ABS, DRA, and PHA supervised the research, reviewed the manuscript, and provided comments to enhance the quality of manuscript. All authors read and approved the final manuscript.

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