Enzymatic synthesis of structured lipids with behenic acid at the *sn*-1, 3 positions of triacylglycerols

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Abstract A long chain saturated fatty acid (SFA), behenic acid, is incorporated into the *sn*-1, 3 positions of triacylglycerols in palm olein (POo) and high-oleic sunflower oil (HOS) by solvent-free interesterification catalyzed by Lipozyme RM IM. The enzymatic interesterified HOS (EIE-HOS) yielded 76.5% of BOO and BOB as compared to 45.6% in POo (EIE-POo). The *sn*-2 position of EIE-HOS displayed 5.3 mol% of SFA which is significantly lower compared to 13.5 mol% in EIE-POo ($P < 0.001$). The *sn*-1, 3 positions of EIE-POo exhibited greater amount of behenic acid (82.0 mol%) in relation to EIE-HOS (64.0 mol%) ($P < 0.001$). Due to the greater variety of constitutive triacylglycerol, EIE-POo showed greater differences between onset (T_o) and offset temperature (T_f) in the melting endotherms (76.99 °C) as compared to EIE-HOS (68.65 °C), and may offer more intensive cooling sensation and flavor release.

Keywords Enzymatic interesterification reaction · Structured lipids · Behenic acid · *sn*-1, 3 positions · Lipozyme RM IM

Introduction

In a broad sense, structured lipids are triacylglycerols that have been tailor-made by altering the fatty acid composition as well as the positional attachment of fatty acids on

the glycerol moiety using chemicals, enzymatic catalyzed reaction or genetic engineering. Due to environmental and safety issues, biocatalysts are often used to catalyze reactions related to modification of oils and fats. Lipases from the strain of *Rhizomucor miehei* is one of the 1, 3-regiospecific biocatalysts which have been used widely in the synthesis of structured lipids [1–4]. Lipozyme RM IM, was found to perform well in many reaction systems including as 1, 3-specific catalyst [5]. According to Wang et al., Lipozyme RM IM produced highest caprylic acid incorporation ability and the lowest acyl migration among the lipases Novozym 435 from the strain of *Candida Antarctica*, Lipozyme TL IM and Lipase AK [6]. On top of that, milder reaction conditions as well as fewer side reactions were the additional advantages of Lipozyme RM IM [1]. With these modified triacylglycerols produced from biocatalysts, structured lipids are expected to provide enhanced nutritional or functional properties, particularly in food application [7].

According to a report by the World Health Organization in year 2008, there are 500 million obese and 1.4 billion overweight adults around the globe [8]. The most energy-rich component of a diet, dietary fat, which is providing 9 kcal/g compared with the 4 kcal/g of carbohydrate and protein, is often being accountable for adiposity. Consequently, synthesis of low-calorie structured lipids is worthwhile. Specifically in the effort to reduce calorie, structured lipids are commonly designed by taking the approaches of either the relatively low calorie density of medium chain fatty acids or the limited absorption of long chain saturated fatty acids (SFA) in the intestine. Akoh and Yee synthesized low-calorie structured lipids through enzymatic interesterification reaction between tricaprins and tristearin [9]. Meanwhile, Yang et al. synthesized low-calorie structured lipids through incorporation of stearic

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acid mainly into the *sn*-1 and/or *sn*-3 positions of triacetin using immobilized lipase in a solvent-free system [10].

A unique dietary fat, CapreninTM, was introduced into the food market as a potential cocoa butter substitute, as well as one of the low-calorie fats [11]. It was a structured lipid synthesized by random interesterification reaction of behenic acid (~ 45%) and medium chain fatty acids, namely, capric and caprylic acids. Notwithstanding its nutritional benefit in limiting calorie, significant reduction in high-density-lipoprotein cholesterol was observed in hypercholesterolemic men fed with CapreninTM as compared with palm oil/palm kernel oil or butter diet, while the total cholesterol, low-density-lipoprotein cholesterol and serum triacylglycerols remained unaffected [12]. As a consequence, the product is now withdrawn from the market.

However, long chain saturated behenic acid is still perceived as potential substrates in the design of low-calorie structured lipids by virtue of its restricted intestinal absorption after hydrolysis by 1, 3-specific pancreatic lipase. Recently, Kanjilal et al. [13–15] investigated hypcholesterolemic effects of two low calorie structured lipids (5.36 kcal/g), prepared by lipase catalysed interesterification reaction of ethyl behenate with sunflower and soybean oils, respectively, in rats and rabbits. These two structured lipids were designed to have low calorific value and lipid deposition in animal tissues. In [16] found that the diet enriched with 1-behenoyl-2, 3-dioleoylglycerol (BOO) prevented the deposition of visceral fat and hepatic triacylglycerols using Wistar rat model. The observation is consistent with our previous study [17]. We also found that long chain SFA at the *sn*-1, 3 positions of triacylglycerols reduced fat accretion remarkably in C57BL/6 mice model [17]. Thus, in the current investigation, we wish to synthesize low-calorie structured lipids by incorporating behenic acid into the *sn*-1, 3 positions of triacylglycerols in vegetable oils containing high amount of oleic acid, namely, palm olein with iodine value (IV) 56 (POo) and high-oleic sunflower oil (HOS) with the aid of 1, 3-specific Lipozyme RM IM. In contrast to the random stereospecific distribution of behenic acids in CapreninTM, our structured lipids are designed to contain high amount of behenic acid at the *sn*-1, 3 positions of triacylglycerols, while the *sn*-2 position is conserved with high unsaturation for favorable nutritional attributes in terms of blood lipid profile and cholesterol levels in humans.

Materials and methods

Materials

Refined, bleached and deodorized POo and HOS were provided by Intercontinental Specialty Fats Sdn. Bhd. (Selangor, Malaysia). Behenic acid (minimum 80% purity) was obtained from Tokyo Chemical Industry (Tokyo, Japan). Immobilized lipase from the strain of *Rhizomucor miehei* (Lipozyme RM IM, NovozymesTM) (Bagsværd, Denmark). All solvents needed for the preparation as well as analysis including the chromatographic-grade solvents, were purchased from Merck (Darmstadt, Germany). Fatty acid methyl ester and triacylglycerol standard materials were procured from Sigma Chemicals (St. Louis, MO, United States).

Enzymatic interesterification reaction

Esterification between behenic acid and ethanol was employed in the preparation of ethyl behenate. Ethanol and behenic acid (molar ratio = 10:1) were refluxed for 1 h in the presence of sulphuric acid catalyst (0.66 vol% in ethanol). The synthesized ethyl behenate was used as the starting material for the subsequent interesterification reaction without further purification. Ethyl behenate was chosen rather than the usual route of reacting free fatty acids and triacylglycerols, for faster reaction rates and lower reaction temperature. Ethyl behenate has lower melting point than behenic acid, thus, easier removal if unreacted.

POo was mixed with ethyl behenate (90% purity, obtained in previous step) at a molar ratio of 1:3 in a 100-mL Schott bottle with screw cap. The substrates were pre-heated at 65 °C and thereafter the immobilized lipase from Lipozyme RM IM (10% of the total mass of substrates) was loaded. Solvent-free interesterification reaction was performed in an orbital shaking water bath (Model SV1422, Memmert, Germany) at 150 rpm and 65 °C. Individual samples were withdrawn at selected intervals (0.5, 1, 2, 3, 5, 10, 15, 20, 24 h), filtered and subjected to further characterization after removal of catalyst. Interesterification reaction was repeated by using HOS and ethyl behenate.

Purification and isolation of selected structured lipids

The interesterified products were subjected to chromatographic separation using silica gel G column (50 g, Kieselgel 60-200 mesh, Merck Art. No 7731, Darmstadt, Germany). A solvent system of 1% ethyl acetate in hexane

was used to elute unreacted ethyl behenate, while 5% ethyl acetate for the elution of newly synthesized structured triacylglycerols. The remaining free fatty acids and other partial acylglycerols remained undeveloped under the above-mentioned solvent systems.

Triacylglycerol composition

Triacylglycerol composition was determined by reversed-phase high performance liquid chromatography (HPLC) on a Waters chromatographic system (Waters SFO, Milford, USA) equipped with an evaporative light scattering detector (Model 2424, Waters, USA). The separation of the molecular species was effected on a Purospher STAR RP-18 endcapped column (250 mm × 4.6 mm) of 5-μm particle size (Merck, Darmstadt, Germany). Sample injection was executed by a Sample Manager Waters 2757 injector with 20 μL loops (Waters, USA). The detector drift tube temperature was set at 60 °C. Nitrogen gas was used as nebulizing gas at the pressure of 40 psi.

Sample (8 mg) was dissolved in 10 mL of HPLC grade dichloromethane and subsequently vortexed for 1 min. Prior to sample injection, the samples were passed through a filter with pore size of 0.2 μm (Milipore, Bedford, USA). The mobile phase consisted of an isocratic elution of acetonitrile/dichloromethane solvent system (40:60, v/v) for 25 min. The flow rate was set at 1 mL/minute. The data acquisition and processing were executed by MassLynx version 4.1 (Waters, USA). The order of elution of triacylglycerol molecular species was based on the corresponding equivalent carbon number (ECN). Nonetheless, the exact chromatographic peak assignment is not possible merely based on ECN values of triacylglycerols. Peak identification was further affirmed on the plot of the logarithm of the retention volume of triacylglycerols relative to trioleoylglycerol versus the number of double bonds [18]. Relative retention volumes express the retention volume of the other peaks relative to a standard. In the present study, trioleoylglycerol (OOO) was used as the reference molecular species. Henceforth, the detailed chromatographic peak identification is achieved by the approach of logarithms of retention volumes. For example, logarithms of relative retention volume for $PPP = \log_{10} \left(\frac{\text{retention volume of PPP}}{\text{retention volume of OOO}} \right)$.

¹³C NMR regiospecific analysis

Regiospecific analyses of structured lipids were conducted as established in our previous study [19]. ¹³C NMR measurements were performed using a JEOL ECA-400 MHz NMR spectrometer operating at 9.4 T. Manual shimming was employed. A spectral width of 1500 Hz at where the

acyl chain carbonyl carbons resonate, 8192 data points and 90° pulse excitation were applied. Experimental temperature was set at 298.15 K whereas repetition time of 34.5 s was chosen. Total scans per analysis were 128 times. Deconvolution was used as the integration method to separate the overlapped peak and calculate its area.

Total fatty acid composition

Total fatty acid composition was determined by gas chromatography. Fatty acid methyl esters were produced by sodium methoxide-catalyzed transesterification [20]. Methyl ester sample (1 μL) was injected into the gas chromatography (GC-2010A series, Shimadzu) equipped with a flame ionization detector and a BPX70, 30 m × 0.32 mm × 0.25 μm capillary column. An initial temperature of 140 °C was held isothermally for 2 min, and then was increased to 220 °C at a rate of 8 °C per minute. The column was held at the final temperature for 5 min. The oven, injector and the detector ports were set at 140, 240 and 260 °C, respectively. Helium was the carrier gas with a flow rate of 1.10 mL/minute operated at a 50:1 split ratio.

Differential scanning calorimetry

The thermal profiles of structured lipids synthesized were attained by a Mettler Toledo Differential Scanning Calorimetry (DSC) Model DSC823e (Greifensee, Switzerland). Analysis was performed in accordance to the American Oil Chemists' Society official DSC method Cj 1-94 [21]. A 4 to 5 mg of structured lipids was hermetically sealed in an aluminium pan, with an empty pan serving as a reference. The sample was first heated rapidly from 25 to 80 °C and held isothermally for 10 min. Then, the sample was cooled to −40 °C at 10 °C/minute and kept 30 min isothermally. Melting profiles of structured lipids were recorded from −40 to 80 °C at a heating rate of 5 °C/minute. Normal standardization of the instrument was performed with *n*-decane and indium as reference standards.

Slip melting point

Slip melting points (SMP) of the selected structured lipids were analyzed in accordance to AOCS Slip Melting Point Cc 3-25 test method [22]. About 1 g of melted sample was placed in a capillary tube to a height of 1 cm. The tube was cooled overnight at 4 °C and then immersed in a beaker half-full of distilled water. The water level in beaker was 2 cm above the upper edge of sample. The initial bath temperature was adjusted to 10 °C below the expected SMP of the sample. The water bath was agitated with a

magnetic stirrer and heated applying a temperature gradient of 0.5 °C/min. The temperature at which the sample in each tube started to melt and become clear was observed and recorded as its SMP.

Solid fat content (SFC)

Solid fat content in selected structured lipids were measured at 5, 10, 20, 30, 35, 40 and 45 °C with a low resolution NMR (Minispec MQ20, Bruker) using IUPAC Solid Fat Content 2.150(a) test method [23]. Both samples were tempered prior to the measurement of SFC to remove all crystallization and melting history. Structured lipids were melted at 80 °C and held at 60 °C for 20 min, then cooled at 0 °C for 90 min. Subsequently, kept overnight at 26.5 °C. The samples were stabilized for 35 min at each measuring temperature before measuring.

Statistical analyses

All statistical analyses on three replicates were carried out using GraphPad Prism 5. One-way ANOVA was used to assess the significance of differences among the groups, whereas Tukey's honestly significant difference test was used for pairwise comparisons. *P* values lower than 0.05 were considered significant.

Results and discussion

Identification of chromatographic peaks

The ECN value is employed to reaffirm the order of HPLC elution of various triacylglycerol molecular species [24]. As exact chromatographic peak assignment is not possible merely based on ECN values of triacylglycerols, the detailed chromatographic peak identification is achieved by the approach of logarithms of retention volumes [18, 25]. In the present study, trioleoylglycerol (OOO) was used as the reference molecular species.

Figure 1 shows a plot of logarithm of the relative retention volumes against the number of double bonds in the triacylglycerol molecule. Trioleoylglycerol (OOO) was used as the reference molecular species. The chromatographic data were derived from the analysis of synthetic mixtures of standard triacylglycerols, POo, HOS and the synthesized structured lipids. By employing the homogeneous standard saturated triacylglycerols, namely, PPP, StStSt and BBB, the chromatographic data were derived from the analysis of POo, HOS and the synthesized structured lipids. In Fig. 1, each plot was joining points representing two different homogenous triacylglycerols on each end, while their mixed triacylglycerols were well-

placed in between on the same plot. For example, BOB and BOO were represented by two points on the line connecting BBB and OOO, and they divided the plot into four equal segments. In addition, BOST was positioned at the mass center of the triangle formed by BBB, OOO and StStSt, as well as remained equidistant from BOB and StOSt (Fig. 1). In other words, the logarithm of the relative retention volume of BOST was equal to half of the sum of those of BOB and StOSt, and also one-third of the sum of BBB, OOO and StStSt.

At constant number of double bond, the logarithm of relative retention volume increased linearly as a function of ECN values. In addition, triacylglycerol molecular species which were eluted prior to OOO displayed negative logarithm values. All plots showed exceptional correlation coefficients ($r = 0.9949\text{--}1.0000$), suggesting the present HPLC experimental condition was robust and well-controlled throughout all analyses. More important, this diagram allows the identification of an unknown chromatographic peak with known relative retention volume. For example, if an unknown peak has its logarithms of relative retention volume of 0.1365 and two number of double bond was plotted on Fig. 1, the point fell on the linear line that connects BBB and OOO. Hence, the peak was identified as BOO.

Triacylglycerol composition

Triacylglycerol composition of structured lipids produced from POo and HOS are given in Tables 1 and 2, respectively. The predominant structured triacylglycerol species that built-up during the reaction were BOO and BOB. There were 13.9% (min) to 27.2% (max) of BOO and 18.4% (min) to 26.7% (max) of BOB in various POo-based structured lipids (Table 1). This was concomitant with 40.4 and 24.0% decrements of native POO and OOO contents under 24-h catalytic action of Lipozyme RM IM, respectively. On the contrary, interesterification reaction of HOS produced relatively higher amounts of BOO and BOB at the expense of OOO, which fell in the range of 33.9 to 46.2 and 23.6 to 33.5%, respectively (Table 2). The OOO contents were reduced from 90.1% in initial HOS to 5.0% in 24-h reaction product.

Among all the undesirable saturated species produced, maximum BBP/BPB of POo-based reaction products (15.8%, Table 1) gained significant higher BBP/BPB when compared with maximum BBP/BPB of HOS-based ($P = 0.001$) reaction products. It may be attributed by the acyl migration of relatively high amount of palmitic acid in POo. Meanwhile, BBP/BPB and BBB were only developed gradually after 5 h of interesterification reaction of HOS with ethyl behenate (Table 2). The latter reached the maximum (7.1%) in 24-h reaction product. Generally,

Fig. 1 Graph of logarithm of the retention volumes of triacylglycerols, relative to trioleoylglycerol (OOO), against the total number of double bonds in the fatty acid acyl chains

minor polyunsaturated species containing linoleic acid, namely LLL and OLL, were diminished in both interesterification reactions.

Triacylglycerol molecular species are grouped into target structured lipids, co-products and reactant triacylglycerols. In the presence of 1, 3-specific enzymes, the target triacylglycerols will be those species with the behenic acid at the *sn*-1 and/or *sn*-3 positions, in which the BOB and BOO make up the chief constituents. Meanwhile, the reactant triacylglycerols represent the species which are natively attainable in POo or HOS starting precursor, whereas the side products are referred to the trisaturated species.

The desired triacylglycerol molecular species generally underwent decrement after reaching maximum at 1 h of reaction (79.3%). These phenomenon could be attributed to the gradual shifting of the reaction from initially 1, 3-specific interesterification reaction to fatty acid acyl chain randomization. The same observations were reported from previous work employing Lipozyme TL IM [26]. Under prolonged reaction, the newly synthesized structured lipids (BOB and BOO) were subjected to successive acyl migration and enzymatic hydrolysis, which were accompanied by the progression of unfavorable saturated species, from 2.3% in 0.5-h to 26.9% in 24-h reaction products. This suggested that more BBP and BBB were formed at the expenses of preferred BOB and BOO. In addition, no significant change was noted in the compositions of BOO and BOB after 5 h of reaction, i.e. 33.6% (5 h) to 37.1% (24 h) ($P = 0.14$). A major shift from enzymatic

interesterification reaction to complete and non-specific hydrolysis was therefore observed.

The ideal triacylglycerol species constituted more than 80% of total triacylglycerols in all HOS-based intermediates, and the principal constituents (BOB and BOO) accounted for 66.6% (24 h) to 76.5% (2 h) of total triacylglycerols. These reflected a promising yield achieved in the present synthesis of structured triacylglycerols, with the highest yield found in the 2-h reaction product. In addition, another encouraging observation would be the negligible amounts of undesirable fully saturated species within the first 5 h of enzymatic interesterification reaction. These remained an additional benefit as those fully saturated species might jeopardize the nutritional attributes of the structured lipids and exert detrimental effects on blood cholesterol and lipid profiles [27]. Besides, the major reactant triacylglycerol, OOO, which constituted 90.1% in HOS precursor (Table 2), depreciated greatly within 0.5 h of interesterification reaction. In contrast to those produced from POo, all triacylglycerol categories based on HOS did not experience remarkable changes throughout the duration of interesterification reaction. The interesterification reaction of HOS reached equilibrium at a faster rate than that of POo.

Positional distribution of fatty acids

Positional fatty acid compositions of lipase-catalyzed interesterification reaction intermediates produced from POo and HOS are presented in Tables 3 and 4, respectively. As a consequence of the incorporation of behenic

Table 1 Triacylglycerol compositions of palm olein IV 56 (POo) and its interesterification intermediates catalyzed by Lipozyme RM IM

ECN	Molecular species	Composition of total triacylglycerols (mol%)	Intesterification intermediate products									
			POo	0.5 h	1 h	2 h	3 h	5 h	10 h	15 h	20 h	24 h
41.4	LIL	2.2 ± 0.1	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
43.4	OLL	0.9 ± 0.0	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
43.6	PLL	0.7 ± 0.0	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
45.4	OOL	7.5 ± 0.1	3.3 ± 0.2	1.1 ± 0.1	1.8 ± 0.1	2.3 ± 0.0	1.2 ± 0.1	2.7 ± 0.1	2.0 ± 0.1	4.7 ± 0.2	1.6 ± 0.0	1.6 ± 0.0
45.6	POL	12.1 ± 0.1	2.8 ± 0.1	nd	nd	nd	nd	nd	nd	nd	nd	nd
47.4	OOO	31.7 ± 0.2	10.3 ± 0.3	7.1 ± 0.1	7.4 ± 0.1	8.8 ± 0.2	8.6 ± 0.2	7.7 ± 0.1	6.3 ± 0.1	6.0 ± 0.1	7.7 ± 0.2	7.7 ± 0.2
47.6	POO	42.8 ± 0.2	9.8 ± 0.2	3.7 ± 0.2	3.1 ± 0.1	3.1 ± 0.1	3.2 ± 0.1	3.4 ± 0.1	2.2 ± 0.1	2.5 ± 0.1	2.4 ± 0.1	2.4 ± 0.1
47.8	POP	2.2 ± 0.1	3.8 ± 0.2	4.5 ± 0.2	4.3 ± 0.0	4.6 ± 0.1	9.7 ± 0.2	4.7 ± 0.2	4.1 ± 0.1	3.3 ± 0.1	3.3 ± 0.2	3.3 ± 0.2
51.6	BLO	nd	4.3 ± 0.1	5.1 ± 0.2	6.1 ± 0.1	3.5 ± 0.1	3.4 ± 0.1	3.4 ± 0.1	2.4 ± 0.1	3.2 ± 0.1	2.4 ± 0.1	2.4 ± 0.1
51.8	BLP	nd	8.5 ± 0.2	10.9 ± 0.4	10.8 ± 0.1	10.3 ± 0.2	10.5 ± 0.2	8.4 ± 0.1	7.0 ± 0.1	6.7 ± 0.1	7.0 ± 0.2	7.0 ± 0.2
53.6	BOO	nd	27.2 ± 0.3	25.9 ± 0.3	20.2 ± 0.1	20.2 ± 0.2	15.8 ± 0.2	15.8 ± 0.2	14.8 ± 0.2	13.9 ± 0.4	15.7 ± 0.2	15.7 ± 0.2
53.8	BOP	nd	2.6 ± 0.1	3.3 ± 0.1	3.1 ± 0.1	4.3 ± 0.2	5.5 ± 0.2	5.9 ± 0.1	6.6 ± 0.1	8.4 ± 0.1	6.7 ± 0.1	6.7 ± 0.1
55.8	BOSi	nd	3.9 ± 0.2	4.5 ± 0.2	4.4 ± 0.1	4.5 ± 0.1	4.6 ± 0.2	3.7 ± 0.2	4.6 ± 0.1	5.2 ± 0.2	3.1 ± 0.1	3.1 ± 0.1
57.8	BLB	nd	2.7 ± 0.1	2.8 ± 0.1	2.5 ± 0.1	3.1 ± 0.2	5.3 ± 0.1	2.7 ± 0.2	3.9 ± 0.2	2.7 ± 0.1	3.2 ± 0.2	3.2 ± 0.2
59.8	BOB	nd	18.4 ± 0.2	26.7 ± 0.2	26.3 ± 0.2	25.9 ± 0.3	19.4 ± 0.2	21.3 ± 0.3	21.8 ± 0.3	19.9 ± 0.3	20.0 ± 0.3	20.0 ± 0.3
60.0	BBP/BPB	nd	2.3 ± 0.1	4.3 ± 0.2	6.9 ± 0.2	6.5 ± 0.2	8.5 ± 0.2	12.1 ± 0.3	13.4 ± 0.2	13.1 ± 0.2	15.8 ± 0.2	15.8 ± 0.2
62.0	BBSi/BSiB	nd	nd	nd	nd	nd	nd	2.1 ± 0.1	2.9 ± 0.1	2.1 ± 0.1	2.6 ± 0.1	2.6 ± 0.1
66.0	BBB	nd	nd	nd	3.2 ± 0.1	2.8 ± 0.2	4.1 ± 0.1	6.1 ± 0.2	8.1 ± 0.1	8.4 ± 0.2	8.5 ± 0.2	8.5 ± 0.2

IV iodine value, POo palm olein IV 56, ECN equivalent carbon number, nd not detected, P palmitic, St stearic, O oleic, L linoleic, B behenic

^aEquivalent carbon number (ECN) = CN - 2 × DB - 0.2 × NUFA, where CN is the total number of carbons in acyl chains, DB is the total number of double bonds and NUFA is the number of unsaturated fatty acids in the triacylglycerol molecule

Table 2 Triacylglycerol compositions of high-oleic sunflower oil (HOS) and its interesterification intermediates catalyzed by Lipozyme RM IM

ECN	Molecular species	Composition of total triacylglycerols (mol%)										
		HOS										
		Interesterification intermediate products										
		0.5 h	1 h	2 h	3 h	5 h	10 h	15 h	20 h	24 h		
41.4	LLL	0.5 ± 0.1	nd	nd	nd	nd	nd	nd	nd	nd	nd	
43.4	OLL	0.6 ± 0.1	nd	nd	nd	nd	nd	nd	nd	nd	nd	
45.4	OOL	1.9 ± 0.1	2.3 ± 0.1	2.0 ± 0.1	2.6 ± 0.1	3.1 ± 0.1	1.9 ± 0.1	2.7 ± 0.1	2.1 ± 0.1	2.4 ± 0.3		
47.4	OOO	90.1 ± 0.3	13.0 ± 0.2	9.7 ± 0.1	8.3 ± 0.1	7.0 ± 0.2	6.2 ± 0.2	6.7 ± 0.2	6.8 ± 0.3	5.0 ± 0.1		
47.6	POO	4.6 ± 0.2	3.6 ± 0.1	2.7 ± 0.1	2.4 ± 0.2	3.3 ± 0.1	3.4 ± 0.0	3.2 ± 0.1	3.0 ± 0.2	3.2 ± 0.3		
47.8	POP	2.0 ± 0.0	nd	nd	nd	nd	nd	nd	nd	nd		
49.6	StOO	0.3 ± 0.0	nd	nd	nd	nd	nd	nd	nd	nd		
51.6	BLO	nd	3.4 ± 0.1	3.7 ± 0.2	4.2 ± 0.3	3.5 ± 0.1	3.4 ± 0.1	4.7 ± 0.1	4.0 ± 0.2	3.8 ± 0.2		
51.8	BLP	nd	1.5 ± 0.1	1.2 ± 0.1	1.2 ± 0.2	0.7 ± 0.0	1.2 ± 0.0	1.7 ± 0.1	1.5 ± 0.2	2.1 ± 0.2		
53.6	BOO	nd	45.8 ± 0.3	45.8 ± 0.3	46.2 ± 0.2	43.0 ± 0.2	40.1 ± 0.2	34.1 ± 0.3	34.4 ± 0.3	33.9 ± 0.4		
53.8	BOP	nd	2.6 ± 0.1	1.6 ± 0.1	1.6 ± 0.0	1.6 ± 0.1	1.4 ± 0.1	1.7 ± 0.1	1.9 ± 0.1	2.3 ± 0.3		
55.8	BOS _t	nd	1.5 ± 0.1	1.3 ± 0.1	1.2 ± 0.1	1.9 ± 0.1	1.5 ± 0.1	2.0 ± 0.1	1.6 ± 0.0	2.3 ± 0.0		
57.8	BLB	nd	2.7 ± 0.1	2.8 ± 0.1	2.5 ± 0.0	2.8 ± 0.2	2.6 ± 0.1	3.3 ± 0.2	3.1 ± 0.1	3.3 ± 0.3		
59.8	BOB	nd	23.6 ± 0.3	29.0 ± 0.2	30.3 ± 0.1	31.3 ± 0.2	32.2 ± 0.3	33.3 ± 0.4	33.5 ± 0.3	32.7 ± 0.4		
60.0	BBP/BBP _B	nd	nd	nd	nd	nd	2.2 ± 0.2	1.4 ± 0.1	1.2 ± 0.1	1.8 ± 0.1		
66.0	BBB	nd	nd	nd	nd	1.5 ± 0.1	3.9 ± 0.1	5.2 ± 0.1	6.7 ± 0.2	7.1 ± 0.1		

Equivalent carbon number (ECN) = $CN - 2 \times DB - 0.2 \times NUFA$, where CN is the total number of carbons in acyl chains, DB is the total number of double bonds and NUFA is the number of unsaturated fatty acids in the triacylglycerol molecule

HOS high-oleic sunflower oil, ECN equivalent carbon number, nd not detected, P palmitic, St stearic, O oleic, L linoleic, B behenic

acid into glycerol backbone, total SFA contents of all reaction intermediates were generally higher than that of the starting precursors, while the total monounsaturated fatty acid compositions depreciated.

In Lipzyme RM IM-catalyzed interesterification reaction of POo with ethyl behenate, content of SFA in various reaction products varies from 78.0 mol% (24 h) to 87.7 mol% (3 h) at the *sn*-1, 3 positions of triacylglycerols (Table 3). It reached the maximum (87.7 mol%) after 3 h of reaction. This was also associated with a minimum level of monounsaturated fatty acids at the similar positions. On the other hand, SFA compositions at the *sn*-1, 3 positions of triacylglycerols in HOS-based reaction intermediates

fell in the range of 55.1–64.0 mol% (Table 4). The maximum content (64.0%) of SFA at the *sn*-1, 3 positions of HOS-based reaction intermediates remained significantly lower than that produced from POo ($P < 0.001$). In addition, it reached the maximum composition (64.0 mol%) only after 2 h of interesterification reaction under the catalytic action of similar enzyme (Table 4).

In an ideal 1, 3-specific lipase catalyzed interesterification reaction, the fatty acid composition at the *sn*-2 position should be preserved or altered at a minimum extent. Yet, changes over time were seen in the present study. SFA content gradually increased with time, whereas the monounsaturated counterparts at the similar position

Table 3 Positional fatty acid compositions of palm olein IV 56 (POo) and its interesterification intermediates catalyzed by Lipzyme RM IM

Sample	Composition (mol%) ^a				
	<i>Sn</i> -position	Saturated	Monounsaturated	Polyunsaturated	
Precursor	POo	1, 3	71.0 ± 0.4	26.1 ± 0.3	2.9 ± 0.3
		2	7.4 ± 0.9	74.3 ± 0.9	18.3 ± 0.2
		1, 2, 3	50.3 ± 0.4	41.8 ± 0.5	7.9 ± 0.2
	0.5 h	1, 3	82.0 ± 0.3	14.8 ± 1.1	3.2 ± 0.7
		2	13.5 ± 1.2	66.3 ± 0.7	20.2 ± 1.8
		1, 2, 3	58.8 ± 0.3	32.3 ± 0.9	8.9 ± 1.3
	1 h	1, 3	84.5 ± 0.8	12.5 ± 1.0	3.0 ± 1.8
		2	17.3 ± 0.6	61.1 ± 1.2	21.6 ± 0.9
		1, 2, 3	63.1 ± 0.4	28.0 ± 1.5	9.0 ± 1.2
	2 h	1, 3	83.7 ± 1.1	12.8 ± 1.0	3.5 ± 0.9
		2	22.5 ± 0.5	60.9 ± 1.9	16.6 ± 2.4
		1, 2, 3	63.3 ± 1.5	28.8 ± 0.5	7.9 ± 1.2
	3 h	1, 3	87.7 ± 0.9	9.3 ± 1.0	3.0 ± 0.1
		2	27.8 ± 0.0	58.5 ± 0.8	13.7 ± 0.8
		1, 2, 3	69.5 ± 0.5	24.3 ± 0.7	6.2 ± 0.3
Interesterification intermediate products	5 h	1, 3	83.0 ± 0.3	13.7 ± 1.4	3.3 ± 1.1
		2	35.0 ± 0.1	50.7 ± 0.9	14.3 ± 1.0
		1, 2, 3	66.9 ± 0.3	26.1 ± 0.8	7.0 ± 0.7
	10 h	1, 3	79.8 ± 1.5	16.3 ± 0.5	3.9 ± 2.1
		2	48.4 ± 0.4	44.7 ± 1.5	6.9 ± 1.8
		1, 2, 3	69.4 ± 0.9	25.8 ± 1.1	4.9 ± 1.9
	15 h	1, 3	78.4 ± 0.6	16.9 ± 0.5	4.7 ± 0.2
		2	56.8 ± 0.7	35.6 ± 0.6	7.6 ± 1.3
		1, 2, 3	71.2 ± 0.1	23.1 ± 0.3	5.7 ± 0.4
	20 h	1, 3	78.4 ± 0.8	17.1 ± 0.5	4.5 ± 0.4
		2	59.5 ± 1.6	30.8 ± 0.5	9.7 ± 1.1
		1, 2, 3	72.3 ± 1.2	21.5 ± 0.5	6.2 ± 0.6
	24 h	1, 3	78.0 ± 0.2	17.1 ± 0.1	5.0 ± 0.1
		2	66.8 ± 0.7	26.1 ± 0.1	7.1 ± 0.6
		1, 2, 3	74.3 ± 0.2	20.0 ± 0.0	5.7 ± 0.2

IV iodine value, POo palm olein IV 56, nd not detected

^a¹³C NMR results are mean of three replicates ± standard deviation

Table 4 Positional fatty acid compositions of high-oleic sunflower oil (HOS) and its interesterification intermediates catalyzed by Lipozyme RM IM

Sample	Composition (mol%) ^a				
	<i>Sn</i> -position	Saturated	Monounsaturated	Polyunsaturated	
Precursor	HOS	1, 3	11.6 ± 0.3	76.5 ± 0.2	11.9 ± 0.5
		2	nd	86.5 ± 0.4	13.5 ± 0.7
		1, 2, 3	7.8 ± 0.3	79.8 ± 0.3	12.4 ± 0.7
	0.5 h	1, 3	58.5 ± 2.0	35.9 ± 1.6	5.5 ± 0.6
		2	nd	85.0 ± 0.9	15.0 ± 0.9
		1, 2, 3	38.6 ± 1.3	52.6 ± 1.2	8.8 ± 0.4
	1 h	1, 3	60.4 ± 0.8	34.1 ± 0.6	5.5 ± 0.1
		2	nd	81.5 ± 0.2	18.5 ± 0.2
		1, 2, 3	40.1 ± 0.3	50.0 ± 0.2	9.9 ± 0.1
	2 h	1, 3	64.0 ± 1.1	31.7 ± 0.8	4.3 ± 0.3
		2	5.3 ± 0.4	81.8 ± 1.3	12.9 ± 0.1
		1, 2, 3	44.3 ± 0.4	48.6 ± 0.1	7.2 ± 0.3
	3 h	1, 3	60.7 ± 0.3	34.0 ± 0.5	5.4 ± 0.4
		2	9.1 ± 1.3	74.4 ± 2.7	16.5 ± 1.7
		1, 2, 3	41.9 ± 0.3	48.7 ± 0.8	9.4 ± 1.0
Intesterification intermediate products	5 h	1, 3	64.0 ± 0.4	30.7 ± 0.2	5.4 ± 0.6
		2	11.4 ± 1.2	72.0 ± 0.4	16.6 ± 0.8
		1, 2, 3	45.4 ± 0.5	45.3 ± 0.1	9.3 ± 0.6
	10 h	1, 3	62.4 ± 0.6	32.4 ± 0.5	5.1 ± 0.2
		2	26.2 ± 0.3	59.4 ± 0.7	14.4 ± 1.1
		1, 2, 3	50.3 ± 0.5	41.4 ± 0.7	8.2 ± 0.4
	15 h	1, 3	60.8 ± 0.3	32.5 ± 0.2	6.8 ± 0.4
		2	32.5 ± 1.2	57.4 ± 0.9	10.1 ± 0.3
		1, 2, 3	50.7 ± 0.4	41.3 ± 0.1	7.9 ± 0.4
	20 h	1, 3	55.9 ± 0.3	39.4 ± 1.1	4.7 ± 1.4
		2	37.1 ± 1.3	52.4 ± 0.9	10.5 ± 2.0
		1, 2, 3	49.6 ± 0.6	43.8 ± 0.9	6.6 ± 1.6
	24 h	1, 3	55.1 ± 0.5	39.0 ± 0.4	5.9 ± 0.9
		2	45.8 ± 0.4	44.1 ± 1.2	10.2 ± 0.8
		1, 2, 3	52.0 ± 0.4	40.7 ± 0.1	7.3 ± 0.3

HOS high-oleic sunflower oil, nd not detected

^a¹³C NMR results are mean of three replicates ± standard deviation

(mainly consisted of oleic acid) decreased considerably for both POo- and HOS-based structured lipids. For instance, SFA content at the *sn*-2 position of POo-based reaction product increased from 7.4 mol% in POo to 13.5 mol% within 0.5 h of reaction (Table 3). In the interesterification reaction of HOS with ethyl behenate, it was promising to note that the saturation level at the *sn*-2 position of structured lipids was still remained undetectable after 1 h of reaction (Table 4). The SFA content at this position was ranging from undetected to 46.0 mol% in the 24-h product (Table 4). Polyunsaturated fatty acids at the *sn*-1, 3 positions of POo-based reaction product did not increase

significantly ($P = 0.115$) during the 24 h reaction (Table 3). The polyunsaturated fatty acids at the *sn*-1, 3 positions of 24-h POo-based reaction product was 5.0 mol%, which increased only 2.1 mol% from its starting precursor POo (2.9 mol%). In contrast, polyunsaturated fatty acids at the *sn*-1, 3 position of HOS-based reaction product dropped from 11.9 mol% in precursor to 4.3 mol% after 2 h of reaction ($P < 0.001$, Table 4).

The build-up of saturation level at the *sn*-2 position of triacylglycerol in HOS-based structured lipids was relatively slower than those produced from POo. Fatty acid compositions at the different *sn*-positions seemed to

converge as a function of time, indicating the evolution of reaction from the ideal 1, 3-specific interesterification reaction to undesirable randomization. This might result from the acyl migration from the *sn*-2 position to *sn*-1 and *sn*-3, or possibly by a change in the specificity of the enzyme [26]. In the course of reaction, the acyl chain at the *sn*-2 position of triacylglycerol was postulated to migrate to the *sn*-1, 3 positions by virtue of the relatively higher stability of secondary hydroxyl group compared with the primary counterpart. Consequently, this was followed by the incorporation of behenic acid into *sn*-2 position. Thus, both the undesirable acyl migration and enzyme specificity was the major cause for variation in fatty acid composition at the *sn*-2 position.

Under current experimental conditions, 1, 3-specific interesterification reaction was preceded in the first three to 5 h, yet it was thereafter surpassed by randomization under prolonged reaction. Fatty acid randomization of fatty acids was found to be kinetically slower than the 1, 3-specific interesterification reaction. Therefore, a variety of structured lipids could be tailor-made by controlling the reaction duration at the optimized condition.

Selection of ideal structured lipid

Current justification on the ideal structured lipids among all intermediates is centered on the composition of targeted structured triacylglycerol species, as well as the saturation level at the *sn*-2 position. It is predominantly important to keep the degree of saturation at the *sn*-2 position of triacylglycerol at minimum, as the dietary fats with high saturation at the above-mentioned position has been reported to be cholesterolemic and atherogenic [27–29].

In the interesterification reaction of POo, the amount of the targeted triacylglycerol species reached the maximum after 1 h of reaction (79.3%). However, its saturation level at the *sn*-2 position was 17.3 mol% (Table 3). Thus, a compromise between the saturation level at the *sn*-2 position and the amount of target structured lipids has reached. Consequently, the reaction product after 0.5 h of interesterification reaction which gained significantly lower saturation than 1 h of interesterification ($P = 0.008$) (13.5 mol% saturation at the *sn*-2 position) is selected as the ideal product due to low saturation level at *sn*-2 position. On the other hand, the highest amount of desired structured triacylglycerols (87.3%) in concomitant with the negligible side products was found in the 2-h reaction intermediate produced from HOS. The amount of desired structured triacylglycerols by 2-h reaction was significantly higher than 1-h ($P = 0.001$), 3-h ($P = 0.005$) and 5-h ($P = 0.001$) reaction intermediates. It was noteworthy that its saturation level at the *sn*-2 position of triacylglycerol was only 5.3 mol% (Table 4). Therefore, this is the

preferred structured lipid produced from the enzymatic interesterification reaction of HOS with ethyl behenate.

For the ease of discussion, EIE-POo is thereafter denoted as the selected structured lipid produced from the enzymatic interesterification reaction of POo with ethyl behenate, whereas EIE-HOS represents those produced from HOS. In general, EIE-HOS (87.3%) displayed significantly greater amounts of target triacylglycerol species compared with EIE-POo (67.6%). Besides, the absence of co-products signified an additional advantage for the former.

In the context of positional fatty acids, EIE-HOS exhibited saturation levels of 5.3 mol% (Table 4) decreased to 4.3 mol% at the *sn*-2 position after purification. These were significantly lower compared with the decrement of EIE-POo which dropped from 13.5 mol% to 3.3 mol% ($P < 0.001$, Table 3). After purification, saturation levels EIE-POo drastically dropped to 3.3 mol% at the *sn*-2 position. On the contrary, due to the nature and characteristic of POo, EIE-POo (82.0 mol%, Table 3, 0.5 h) showed significantly greater amounts of SFA at the *sn*-1, 3 positions of triacylglycerol compared with EIE-HOS (64.0 mol%, Table 4, 2 h) ($P < 0.001$). Nevertheless, EIE-HOS (71.0 mol%, 2 h) obtained greater amount of SFA at the *sn*-1, 3 positions compared with EIE-POo (63.8 mol%, 0.5 h) ($P = 0.0023$). This remains one of the essential criteria for prospective low-calorie structured lipids.

Total fatty acid composition

The total fatty acid compositions of starting precursors and their selected ideal structured lipids were also analysed. Owing to the high efficiency of Lipozyme RM IM, the most abundant fatty acid in EIE-POo was behenic acid (36.7%, $P < 0.001$). On the contrary, oleic acid still remained the most abundant fatty acid in EIE-HOS (50.6%) by virtue of its extremely high content in HOS starting precursor (83.2%). The behenic acid content in EIE-HOS was found to be 35.5, which has no significant difference when compared with EIE-POo ($P = 0.07$). However, the incorporation of behenic acid was also reflected in the concomitant increase of total saturation from 45.7% in POo to 63.9% in EIE-POo as well as from 8.3% in HOS to 44.0% in EIE-HOS. As a whole, the EIE-POo was primarily constituted of three major fatty acids, namely palmitic (20.0%), oleic (30.6%) and behenic acids (36.7%), whereas only oleic and behenic acids were found predominantly in EIE-HOS (50.6 and 35.5%, respectively).

Thermal properties

The melting curve of POo consisted of a broad endothermic peak made up of a variety of triacylglycerols, ranging from relatively saturated POP to highly unsaturated LLL in the melting temperature range of -35.14 – 25.78 °C. The melting point of BOO is 31 °C and BOB is 53 °C [16]. The melting point of BOB is higher than that of BOO because BOB contains two behenic fatty acids chain attached to glycerol backbone. Due to the incorporation of behenic acid, the melting endotherm was shifted to the higher temperature region in EIE-POo and depicted four distinct endothermic peaks which corresponded to the transition temperatures of 18.83 , 33.39 , 41.17 , and 49.19 °C (Fig. 2). The two endothermic peaks at a lower temperature were the major features of the structured lipid, namely BOO and BOB which remained the principal constituents in the target triacylglycerol species (27.2 and 18.3% , respectively, Table 1). In contrast, HOS showed a single distinct tall endothermic peak at -7.73 °C, owing to the high content of OOO (90.1% , Table 2). A wider melting range (-26.64 – 42.01 °C) was noted in the endotherm of EIE-HOS compared with HOS (-22.47 to -0.44 °C) illustrated the production of great variety of new triacylglycerols at the expense of OOO in the HOS.

In POo, a major exothermic peak at 1.71 °C with a shoulder peak at -5.76 °C was noted in its crystallisation thermogram (Fig. 3). A significant shift to the higher temperature region was exhibited in the exotherms of EIE-POo compared with that of POo. A major exothermic plateau consisting of three merging peaks was observed for EIE-POo. Owing to the high degree of unsaturation, the exotherm of HOS displayed a crystallization profile lied

beyond the cooling temperature programme exercised in the present instrumentation. Consequently, the T_f of the crystallization curve of HOS was not attainable. However, the crystallization exotherm of EIE-HOS displayed two major exothermic regions; the higher-temperature region defined crystallization of the behenin fraction, while the lower-temperature region pertained to the crystallization of the olein fraction. Besides, the behenin fraction showed a plateau consisting of three merging peaks.

EIE-POo exhibited greater differences between T_o and T_f in their melting endotherms (76.99 °C) compared with EIE-HOS (68.65 °C) by virtue of the greater variety of triacylglycerol species in the former. Moreover, in the endotherms of EIE-POo, the higher melting T_f as well as the broader endothermic peaks in the high-temperature region (above 37 °C) compared with that of EIE-HOS, reflected the saturated species present in EIE-POo were relatively greater than those contained in EIE-HOS. These minor species might be caused by the undesirable acyl migration evolved during interesterification reaction. On the contrary, both T_o and T_f of EIE-POo were higher than EIE-HOS during crystallization. The latter contained more unsaturated species, for instance OOO, which crystallized at a lower temperature range.

Generally, structured lipids melted at a broader range than their crystallization. Meanwhile, the T_f of melting endotherms appeared to be 1.3 to 3.4 times greater than the T_o of crystallization exotherms of all oil samples. The above-mentioned observations could be elucidated in terms of the complex phenomenon in polymorphism of oils, which was dependent on their thermal history. This triggered a longer duration on the melting process and reached completion at a higher temperature than that of

Fig. 2 Differential scanning calorimetry melting thermograms of the starting precursors (POo and HOS) and their structured lipids (EIE-POo and EIE-HOS)

Fig. 3 Differential scanning calorimetry crystallization thermograms of the starting precursors (POo and HOS) and their structured lipids (EIE-POo and EIE-HOS)

crystallization. Conversely, crystallization was presumed to be influenced only by the chemical composition of the oil was much simpler than the melting profile.

Slip melting point

The slip melting point of purified EIE-POo is recorded at 39.80 °C while that of purified EIE-HOS is 41.40 °C. HOS based structured lipids consists higher content of total BOO and BOB (76.4%) while POo based structured lipids only contained 45.5%. Thus, slip melting point purified EIE-HOS slightly higher than purified EIE-POo (1.60 °C) due to its composition.

Solid fat content

Figure 4 showed the SFC curves of selected EIE-POo and EIE-HOS solid fat content determined by IUPAC 2.150(a) method. The density of solid lipids is higher than the density of liquid lipid. Thus, there was increased in density when structured lipids crystallized and decreased when it melted. At 10 °C, both structured lipids exhibited low spreadability at refrigerator temperature with a value greater than 32% of solid fat content [30, 31]. The SFC of EIE-POo was higher than the solid fat content of EIE-HOS except between 27.5 and 39.0 °C. EIE-POo exhibited greater hardness compared with EIE-HOS due to the greater solid fat content between 5 and 27.5 °C. Moreover, EIE-POo displayed a large difference in SFC between 20 and 33 °C. Hence, EIE-POo may offer more intensive cooling sensation and flavor release in our mouth. At the range of 33–38 °C, both samples have high solid fat content (more than 3.5%) which is related to waxy sensations

[32–34]. Generally, good performance is expected when SFC is between 15 and 25% at the usage temperature as to avoid an excess of liquid and oiliness sensation in baking shortening. In confectionery purpose, EIE-POo is a good structured lipid due to high containing of BOO and BOB but large difference in SFC between 20 and 33 °C. EIE-POo may provides better mouthfeel but less absorb by intestinal mucosa.

Structured lipids containing high amounts of BOO and BOB have been successfully synthesized through lipase catalyzed interesterification reaction. Owing to their physical properties at room temperature, they confers desired rheological properties to bakery, confectionery or as a base for *trans*-fat-free products. Moreover, they may serve as the hard stock for physical blending with other softer cooking oils with the aim of reducing obesogenic effects of the latter. While in standpoints of the high content of symmetrical triacylglycerols, namely, BOB, the structured lipids can also be treated as the cocoa butter equivalent for chocolate and confectionery coatings. Their differential effects in the prevention of obesity are mainly depending on their melting behavior, triacylglycerol composition as well as the composition of long chain SFA at the *sn*-1, 3 positions of triacylglycerols. As a whole, the structured lipids still display the fat properties, but associate with lower risk towards the excessive fat deposition. In the present study, the reaction products from EIE-HOS may have a lower risk towards fat deposition because of the higher amount of BOO and BOB (76.5%) and lower *sn*-2 position of saturation (5.3 mol%) as compared to that from EIE-POo ($P < 0.001$). EIE-HOS obtained higher content of BOB (30.3 mol%) than EIE-POo ($P < 0.001$). However, in terms of sensory, EIE-POo may offer less waxy

Fig. 4 Solid fat content curve of purified selected structured lipids (EIE-POo and EIE-HOS) depending on temperature. Purified selected structured lipids (Times symbol): EIE-HOS; (Triangle symbol): EIE-POo

sensations due to its slip melting point that is nearer to body temperature (37 °C) and more intensive cooling sensation and flavor release in mouth owing to its large difference in SFC between 20 and 33 °C.

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