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Effect of enzymatic transesterification using *Mucor miehei* lipase on physicochemical properties of engkabang (*Shorea macrophylla*) fat - canola oil blends

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Abstract

The effect of transesterification of engkabang (*Shorea macrophylla*) fat - canola oil (EF35/CaO65) blend by *Mucor miehei* lipase (1%, w/w oil) in a solvent-free system was investigated at different time intervals of 6 h, 12 h and 24 h. Compositional changes of the samples withdrawn at specified time intervals while the reaction in progress were analysed by chromatography, whereas the polymorphic forms and thermal properties were analysed by using X-ray diffraction and differential scanning calorimetry, respectively. There were increases in the amounts of monounsaturated and triunsaturated triacylglycerol (TAG) molecular groups with concurrent reductions of the proportions of desaturated TAG molecular groups during different time intervals. This changing TAG composition led to changes in crystallisation behaviour and thermal properties of the samples, reducing some enthalpy values. All samples withdrawn at different time intervals displayed both β' and β type crystal polymorphs even though engkabang fat itself was predominantly β-type. In terms of melting, solidification and polymorphic properties, the sample withdrawn at 6 h time interval was found to display the closest similarity to lard (LD).

Introduction

Engkabang fat (EF) is a lipid material extracted from the seeds of *Shorea macrophylla*, which is distributed in several parts of the Borneo Island. Owing to its pleasant flavour and aroma, natives of the Borneo have used this fat to prepare special type of rice (FRIM, 2012). Since it is solid in nature and yellow in colour, it may be useful as a base material for preparation of margarines, shortenings, etc. According to previous investigators, EF consists of high proportions of triacylglycerols such as SOS (53%) and POS (36%) while the rest of triacylglycerol molecular species exist in lesser amounts (Nur Illiyin *et al*., 2013). The nature of EF is almost similar to cocoa butter as both of them contain mixed triacylglycerols such as SOS and POS. The same is also true for other plant-based hard butters such as illipe butter, shea butter, kokum butter and so on. Owing to its solid nature, chances are less for EF to be converted into products such as soft margarine. Plasticity is an essential characteristic that is required for fat ingredients in several bakery products. This justifies the adoption of fat modification techniques such as physical blending and transesterification to improve the application range of EF in food industry. Previously, Nur Illiyin *et al*. (2013) optimised the feasibility of blending EF with an appropriate proportion of monounsaturated liquid oils such as canola oil to produce a fat blend to simulate the properties of lard. In a continued effort for improvement, there would be more possibilities for novel fats with interesting properties, if this binary fat mixture were subjected to enzymatic modification. Except for one report, there is hardly any further effort undertaken to explore the usefulness of

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transesterification on melting and crystallisation properties, and polymorphic behaviour of EF-CaO binary mixture.

Utilisation of enzymes for the development of novel fats and oils is a fruitful area of study. Engagement of enzymes as biocatalysts for transesterification of fats is preferred since enzymes are friendlier to the environment (Aravindan *et al*., 2007). Not all enzymes display the same catalytic behaviour as some of them act in *Sn*-1,3-specific mode, while others act in non-specific manner during transesterification. *Mucor miehei* (Lipozyme RM 1M), a *Sn*-1,3-specific lipase, has been studied by various research groups for structural modification of fats and oils as well as their accompanying physical property changes. Generally, lipases with positional specificity act on the Sn-1 and/or Sn-3 position of the TAG molecules. During the past several decades, transesterification using *M. miehei* lipase has been carried out with mixtures of palm-coconut stearin (Khatoon *et al*., 2012), mixtures of palm stearin-palm olein (Sellami *et al*., 2012) and mixtures of palm mid fraction, palm kernel stearin together with medium chain triacylglycerols (Borhan *et al*., 2011). In most of these studies, prepared novel fats did not contain *trans* fatty acids, which is identified as a risk factor associated with cardiovascular disease (Mozaffarian *et al*., 2009). Yet, there has been very little effort during the past several years to employ enzymatic approach to utilise EF in novel product development. For instance, enzymatic treatment of a binary mixture of EF35/CaO65 using *M. miehei* lipase to find new uses has not been reported previously. In the present work, we investigated the changing pattern of composition and physical properties of EF35/CaO65 binary mixture before and after transesterification with *M. miehei* lipase. This might help in formulating novel fat ingredients having potential applications as replacers for animal-based shortenings.

Materials and methods

Research materials

Authentic samples of EF were obtained from Department of Forestry, Sarawak, Malaysia. Samples of CaO were supplied by Giant Hypermarket, Selangor, Malaysia. LD was extracted using adipose tissues of swine supplied by local slaughter houses located in Sri Kembangan, Malaysia following the method previously described by Marikkar *et al*. (2001). The FA compositions of oils used are given in Table 1. *Mucor miehei* (Lipozyme RM 1M) lipase in the immobilised form was obtained from Novo Nordisk Pharma (Malaysia) Sdn. Bhd. Chemicals and reagents used were either analytical or HPLC grade.

Preparation of binary blend

A sample of EF was melted at 60°C in an oven prior to use. EF-3 binary mixture was formulated by blending melted EF with CaO in 35:65 (w/w) ratio.

Enzymatic transesterification

EF-3 (EF35/CaO65) (40 g) samples in triplicate were enzymatically transesterified using *M. miehei* lipase (1%, w/w oil) in a solvent-free system. Reaction conditions were in accordance with the previous optimisation study reported by Nur Illiyin *et al*. (2014). Fat mixtures having the sample and the enzyme were agitated at 200 rpm for 6, 12 and 24 h, respectively in an orbital shaker at 60°C. At the end of each time interval, samples were withdrawn for hot filtration in an oven using Whatman No. 1 filter paper to separate the reacted mixture from the enzyme.

Slip melting point (SMP) determination

AOCS method Cc 3-25 was referred to determine the SMP of the samples (AOCS, 1999).

Analysis of fatty acid profile

A sample portion of oil (0.4 g) was weighed into screw capped glass tubes and 4.0 mL portion of methanol and 0.1 mL portion of methanolic KOH were added. The mixture was heated to 60°C in a water bath for 10 min and allowed to cool. Into this, 2 mL of hexane and 4 mL of distilled water were added, and agitated at 2,500 rpm for 10 min using a vortex. After allowing the content to separate into two layers, the upper layer was injected into a gas chromatograph (Agilent Technologies, Singapore) joined with an FID detector. In the analysis, the polar capillary column DB-wax (with 0.25 mm internal diameter, 30 m length and 0.25 μm film thickness; Agilent Technology, Santa Clara, CA) was used. The oven temperature was programed as follows: initial temperature of 50°C (for 1 min), thereafter increased to 200°C at 8°C/min. Both injector and detector temperatures were maintained at 200°C throughout the analysis. The carrier gas (helium) flow rate was 1.0 mL/min, and the split ratio was 58:1. The identification of the samples' peaks was done with reference to a chromatographic profile containing FAME standards (Supelco, Bellefonte, PA). The percentage of fatty acid was calculated as the ratio of the partial area to the total peaks area (Nur Illiyin *et al*., 2013).

Analysis of TAG composition

Waters Model 2695 liquid chromatograph equipped with a differential refractometer model 2414 as a detector (Waters Associates, Milford, MA) was used to determine the TAG composition of oil samples. The TAG separation was performed on a Merck LiChrospher RP-18 column (5 μm particle size, L × I.D. 25 cm × 3.2 mm). The mobile phase was a mixture of acetone:acetonitrile (63.5:36.5), and the flow rate was 1.5 mL/min. The oven temperature was maintained at 30°C. The injector volume was 10 μL of 5% (w/w) oil in chloroform. Each sample was chromatographed three times, and the data was recorded as area percentages. The identification of TAG peaks of samples was done using a set of TAG standards purchased from Sigma-Aldrich (Deisehofen, Germany) as well as the TAG profiles of lard (Yanty *et al*., 2011), engkabang fat and canola oil (Nur Illiyin *et al*., 2013).

DSC analysis of thermal properties

Analysis of the thermal properties of samples was carried out on a Mettler Toledo Differential Scanning Calorimeter (DSC 823 Model) equipped with a thermal analysis data station (STARe software, Version 9.0x, Schwerzenbach, Switzerland). Prior to analysis, calibration of the instrument was done using indium as the metallic standard, based on the onset temperature of fusion and the heat of indium's fusion. Nitrogen (99.99% purity) was used as the purge gas at a rate of 20 mL/min. Approximately 4-8 mg of molten sample was placed in a standard DSC aluminium pan and then hermetically sealed. An empty hermetically sealed DSC aluminium pan was used as the control. The oil/fat samples were subjected to the following temperature program: 70°C isotherm for 1 min, cooled at 5°C/min to -70°C. The samples were held at -70°C isotherm for 1 min, and heated at 5°C/min to reach 70°C (Nur Illiyin *et al*, 2013).

NMR analysis of solid fat content

Solid fat content (SFC) measurement of samples was carried out using a Bruker Minispec (Model Mq 20) pulse Nuclear Magnetic Resonance (pNMR) spectrometer (Karlsruhe, Germany) based on method AOCS Cd 16b-93 (AOCS, 1999). Before the analysis, the instrument was calibrated using 0%, 31.2% and 72.1% solid SFC calibration standards. The sample in the NMR tube was melted at 70°C for 15 min, followed by chilling at 0°C for 60 min, and then held at each measuring temperature for 30 min prior to measurement (PORIM, 1995). Melting, chilling and holding of the samples were carried out in pre-equilibrated thermostated glycol containing baths, accurate to 0.1°C. SFC measurements were taken in duplicate at 5°C intervals over a range of 0–55°C.

X-ray diffraction analysis

The polymorphic forms of fat crystals were determined using a wide angle X-ray diffraction (WAXD) machine (D8 Advance Bruker AXS, Karlsruhe, Germany). Before analysis, samples were melted at 70°C for 10 min, followed by chilling at 4°C for 24 h, and then held at 25°C for 30 min. The power used was 40 kv, 40 mA with the source of beam from Cu Kα1 X-ray beam (λ = 0.15406 Å). Samples were scanned from 15°2θ to 25°2θ, increasing with a step size of 0.025°/0.1 sec (Ribeiro *et al*., 2009). Short spacing on the X-ray film was measured with an Evaluation Diffract plus software. The short spacings of the β′ form were at 4.2 and 3.8 Å while that of the β form was at 4.6 Å (D'Souza *et al*., 1991).

Statistical analysis

All results from analyses were expressed as the mean value ± standard deviation. Data were statistically analysed by one-way analysis of variance (ANOVA) using Tukey's test of MINITAB (version 14) statistical package at 0.05 probability level.

Results and discussion

Melting point characteristics

Melting point is the temperature at which a fat transforms from solid to liquid. The data given in Table 1 shows the melting points of EF, LD and EF-3 (EF35/CaO65) blend before and after transesterification. All substrates were found to remain in the liquid state since the transesterification process was conducted in a solvent-free system at 60°C. EF had a melting point of 34.5°C while the non-transesterified EF-3 blend displayed melting point of 30.5°C. Non-transesterified EF-3 displaying melting point at lower temperature was mainly due to compositional changes caused by CaO. A declining trend was observed in the SMP of transesterified blends as the transesterification time was varied from 6 to 24 h. Previously, Sellami *et al*. (2012) also observed a decline in SMP values among palm stearin/palm kernel olein mixtures when subjected to transesterification by lipases. The slight decrease in SMP could be due to the TAG compositional changes caused by transesterification. Previously, Aravindan *et al*. (2007) discussed that *M. miehei* lipase would cause fatty acid exchange within and in between TAG molecules during transesterification which in turn affects their melting characteristics. According

Table 1. Slip melting points (SMP) and fatty acid compositions of CaO, EF, LD and EF-3 (EF:CaO = 35:65) blend before and after transesterification using Mucor miehei lipase.

	SMP (°C)	C14:0	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3	C20:0	C20:1	Others
CaO	-	-	4.6 ± 0.0^{d}	0.2 ± 0.0^{b}	1.7 ± 0.0^{d}	61.5 ± 0.1^{a}	21.5 ± 0.0^{a}	8.0 ± 0.0^{a}	0.5 ± 0.1^{d}	1.0 ± 0.0^{a}	0.9 ± 0.0^{cd}
EF-3	30.5 ± 0.0^{b}	-	8.6 ± 0.0^{c}	0.2 ± 0.0^{b}	18.3 ± 0.0^{b}	51.0 ± 0.1^{b}	14.1 ± 0.0^{d}	4.8 ± 0.3^{b}	1.1 ± 0.0^{b}	0.8 ± 0.0^{a}	1.6 ± 0.2^{b}
EF-3 (6 h)	29.8 ± 0.3^{b}	-	8.9 ± 0.0^{c}	0.2 ± 0.0^{b}	18.5 ± 0.2^{b}	50.4 ± 0.2^{c}	14.5 ± 0.0^{cd}	4.5 ± 0.0^{b}	0.9 ± 0.0^{bc}	0.7 ± 0.0^{a}	1.0 ± 0.0^{bcd}
EF-3 (12 h)	28.8 ± 0.3^{b}	-	8.9 ± 0.0^{c}	0.2 ± 0.0^{b}	18.0 ± 0.2^{b}	51.0 ± 0.2^{b}	14.6 ± 0.0^{cd}	4.6 ± 0.3^{b}	0.9 ± 0.0^{bc}	0.7 ± 0.0^{a}	0.9 ± 0.3^{cd}
EF-3 (24 h)	28.8 ± 0.3^{b}	-	8.9 ± 0.0^{c}	0.2 ± 0.0^{b}	18.3 ± 0.1^{b}	50.6 ± 0.1^{bc}	14.9 ± 0.0^{c}	4.6 ± 0.2^{b}	0.9 ± 0.0^{bc}	0.7 ± 0.0^{a}	0.9 ± 0.3^{cd}
LD	29.5 ± 0.0^{b}	1.4 ± 0.0^{a}	23.8 ± 0.0^{a}	2.0 ± 0.0^{a}	13.0 ± 0.0^{c}	40.2 ± 0.0^{d}	17.3 ± 0.0^{b}	-	0.7 ± 0.0^{cd}	-	2.5 ± 0.0^{a}
EF	34.5 ± 0.0^{a}	-	16.1 ± 0.0^{b}	-	48.0 ± 0.0^{a}	33.0 ± 0.0^{e}	0.9 ± 0.0^{e}	-	2.1 ± 0.1^{a}	-	-

Data are means of two replicates (n = 2) ± standard deviation. Means within each column with different superscript letters are significantly ($p < 0.05$) different. CaO: canola oil; LD: lard; EF: engkabang fat; EF-3 (6 h): 35% of interesterified blend for 6 h; EF-3 (12 h): 35% of interesterified blend for 12 h; EF-3 (24 h): 35% of interesterified blend for 24 h.

to the results of the present work, EF-3 (6 h) mixture showed a SMP value closely similar to LD (29.8°C). However, no significant difference ($p > 0.05$) was found between the melting point values of EF-3 before and after transesterification.

Fatty acid composition

Nature's fatty acids are of different kinds; their distribution might change depending on the source of origin. In Table 1, fatty acid composition of EF-3 (EF35/CO65) blend before and after transesterification using *M. miehei* lipase are compared against CO, EF, and LD. Fatty acid composition of EF-3 consisted of oleic acid (51.0%) followed by stearic (18.3%) and linoleic (14.1%) acids. Higher proportion of oleic acid in EF-3 was mainly due to CO, which consisted of predominantly oleic acid (61.5%) followed by linoleic acid. In this blend, stearic became the second most dominant fatty acids due to the presence of EF, which consisted of about 48.0% stearic acid. Transesterification of EF-3 using *M. miehei* lipase for three different time intervals (6 h, 12 h and 24 h) did not seem to cause remarkable changes in fatty acid distribution. This was mainly due to the fact that overall fatty acid compositions of fat are not altered by transesterification; but rather it brings changes in positional distribution of fatty acids within a TAG molecule (Siew *et al*., 2007).

Compositional changes in TAG

There is a tendency for TAG compositional changes in fats subjected to transesterification by *M. miehei* lipase since its action is positional specific. In Table 2, TAG distribution of EF-3 (EF35/CO65) blend, before and after transesterification is compared against CO, EF and LD. Before transesterification, EF-3 had OOO (19.3%) as the most dominant TAG molecular species, followed by SOS (18.4%), OOL (15.0%), and SPO (12.9%). Higher proportion of OOO in EF-3 was mainly due to CO, which consisted of predominantly OOO (30.1%) followed by OOL (23.1%). In this blend, SOS became the second most dominant TAG molecular species due to the presence of EF, which consisted of about 53.1% SOS. Intermolecular and intramolecular rearrangements of fatty acids are common phenomena in enzymatic transesterification and hence TAG composition of the substrates is always subject to change (Aravindan *et al*., 2007). In the present work, after transesterification, proportions of SOS and SPO were remarkably reduced along with some other disaturated TAG molecules. This has led to a reduction in the total proportion of disaturated TAG molecular group. Interestingly, total proportion of disaturated TAG molecular group of EF-3 (24 h) and LD were similar. Meanwhile, several TAG molecular species belonging to the monosaturated TAG molecular group such as POL, POO and SOO showed slight increases in their proportions. As a result, there was a significant increase in the proportion of total monosaturated TAG molecular group.

Profile of solid fat content

Profiling of solid fat content of oils, fats and their transesterified blends would greatly help in determining their suitability for food processing and development (Siew *et al*., 2007). Physical properties such as firmness, hardness and consistency of lipid-

Table 2. TAG composition of canola oil, lard, EF and EF-3 (EF:CaO = 35:65) blend before and after transesterification using Mucor miehei lipase.

	CaO	EF-3	EF-3 (6 h)	EF-3 (12 h)	EF-3 (24 h)	LD	EF
Triunsaturated							
LnLnL	1.3 ± 0.0^{a}	0.5 ± 0.1^{b}	0.4 ± 0.1^{b}	0.4 ± 0.0^{b}	0.4 ± 0.0^{b}	-	-
LLLn	3.2 ± 0.5^{a}	1.9 ± 0.3^{b}	1.8 ± 0.1^{b}	1.8 ± 0.4^{b}	1.9 ± 0.1^{b}	0.9 ± 0.0^{c}	-
LLL	9.0 ± 0.2^{a}	5.4 ± 0.0^{b}	5.9 ± 0.0^{b}	6.1 ± 0.1^{b}	5.9 ± 0.0^{b}	0.3 ± 0.0^{c}	-
OLLn	7.8 ± 0.2^{a}	5.6 ± 0.0^{b}	5.7 ± 0.1^{b}	5.6 ± 0.0^{b}	5.4 ± 0.0^{b}	-	-
OOLn	10.8 ± 0.2^{a}	7.3 ± 0.0^{b}	7.5 ± 0.3^{b}	6.6 ± 0.1^{c}	6.4 ± 0.2^{c}	-	-
OOL	23.1 ± 0.2^{a}	15.0 ± 0.1^{b}	14.2 ± 0.2^{c}	14.6 ± 0.1^{d}	14.4 ± 0.0^{c}	4.4 ± 0.0^{e}	-
OOO	30.1 ± 0.0^{a}	19.3 ± 0.4^{b}	18.6 ± 0.2^{c}	19.2 ± 0.2^{b}	18.7 ± 0.1^{c}	4.1 ± 0.0^{d}	-
OOGa	1.1 ± 0.0^{a}	0.7 ± 0.1^{b}	1.0 ± 0.2^{a}	0.7 ± 0.1^{b}	0.8 ± 0.1^{b}	-	-
OLL	-	-	-	-	-	4.4 ± 0.0^{a}	-
Sub Total	86.4 ± 1.1^{a}	55.7 ± 0.4^{b}	55.1 ± 1.0^{c}	55.0 ± 0.2^{d}	53.9 ± 0.2^{e}	15.9 ± 0.0^{f}	-
Monosaturated							
PLLn	0.9 ± 0.0^{a}	0.1 ± 0.0^{c}	0.8 ± 0.1^{b}	0.6 ± 0.0^{b}	0.7 ± 0.0^{b}	-	-
POL/SLL	5.3 ± 0.6^{b}	3.1 ± 0.0^{c}	3.8 ± 0.4^{b}	4.1 ± 0.1^{b}	4.5 ± 0.0^{b}	22.6 ± 0.1^{a}	-
POO/SOL	5.9 ± 0.1^{b}	4.0 ± 0.2^{d}	5.3 ± 0.2^{c}	5.5 ± 0.1^{c}	6.1 ± 0.2^{b}	22.1 ± 0.1^{a}	0.2 ± 0.1^{e}
SOO	1.7 ± 0.2^{c}	1.1 ± 0.1^{d}	3.3 ± 0.5^{b}	2.8 ± 0.2^{b}	4.0 ± 0.0^{a}	3.3 ± 0.1^{b}	1.0 ± 0.0^{d}
PLL	-	-	-	-	-	7.0 ± 0.0^{a}	-
Sub Total	13.8 ± 0.5^{c}	8.3 ± 0.3^{d}	13.2 ± 0.2^{c}	13.0 ± 0.1^{c}	15.3 ± 0.1^{b}	55.0 ± 0.5^{a}	1.2 ± 0.9^{e}
Disaturated							
PSLn	0.2 ± 0.1^{a}	0.1 ± 0.0^{a}	0.4 ± 0.2^{a}	0.3 ± 0.1^{a}	0.5 ± 0.1^{a}	-	-
SPO	-	12.9 ± 0.1^{c}	11.7 ± 0.1^{d}	11.8 ± 0.1^{d}	11.3 ± 0.1^{d}	14.3 ± 0.2^{b}	36.0 ± 0.2^{a}
SOS	-	18.4 ± 0.1^{b}	15.7 ± 0.7^{d}	16.0 ± 0.3^{c}	15.2 ± 0.1^{d}	1.0 ± 0.1^{e}	53.1 ± 0.8^{a}
PPL	-	-	-	-	-	3.1 ± 0.1^{a}	-
PPO	0.2 ± 0.0^{e}	2.5 ± 0.0^{d}	3.0 ± 0.2^{cd}	2.9 ± 0.1^{c}	2.8 ± 0.1^{c}	8.5 ± 0.4^{a}	6.6 ± 0.1^{b}
SOA	-	1.4 ± 0.0^{a}	-	-	-	-	-
Sub Total	0.4 ± 0.1^{e}	35.4 ± 0.2^{b}	28.9 ± 0.0^{c}	28.2 ± 0.1^{c}	26.9 ± 0.1^{d}	26.9 ± 0.1^{d}	95.7 ± 0.6^{a}
Trisaturated							
PPP	-	-	-	-	-	0.5 ± 0.1^{a}	-
PPS	-	0.2 ± 0.0^{b}	0.3 ± 0.1^{b}	0.3 ± 0.0^{b}	0.2 ± 0.0^{b}	0.8 ± 0.1^{a}	-
SSS	-	0.2 ± 0.0^{c}	0.8 ± 0.1^{a}	0.7 ± 0.3^{b}	0.9 ± 0.1^{a}	1.0 ± 0.1^{a}	-
Sub Total	-	0.4 ± 0.0^{c}	1.1 ± 0.0^{b}	1.0 ± 0.3^{b}	1.1 ± 0.1^{b}	2.2 ± 0.0^{a}	-
UK	-	0.3 ± 0.0^{b}	-	-	0.3 ± 0.1^{b}	-	3.1 ± 0.3^{a}

Data are means of three replicates (n = 3) ± standard deviation. Means within each row with different superscript letters are significantly ($p < 0.05$) different. O: oleic; P: palmitic; L: linoleic; Ln: linolenic; S: stearic; Ga: gadoliec; A: arachidic; UK: unknown; CaO: canola oil; LD: lard; EF: engkabang fat; EF-3 (6 h): 35% of interesterified blend for 6 h; EF-3 (12 h): 35% of interesterified blend for 12 h; EF-3 (24 h): 35% of interesterified blend for 24 h.

based products are dependent on their SFC profiles (Shin *et al*. 2010). SFC profiles of EF-3 (EF35/CaO65) blend before and after transesterification using *M. miehei* lipase are compared against CO, EF and LD in Figure 1. The initial SFC of CaO was 0.2% at 0°C and after being mixed with EF, the formulated EF-3 had SFC value of 36.0% at 0°C. The dramatic increase of SFC from 0.2 to 36.0% caused by significant increases in desaturated TAG molecules (from 0.36% to 35.39%) with concurrent decreases in the proportions of triunsaturated TAG molecules (from 86.25% to 55.73%) as seen in Table 2. This kind of co-relationship between solidification characteristics of fat with their constituent TAG molecules was mainly due to differing melting points based on degree of unsaturation. This is in accordance with the observations made in other studies reported by different research groups (Reshma *et al*., 2008; Shin *et al*., 2010; da Silva *et al*., 2013).

According to data presented in Figure 1, SFC values of EF-3 tended to decline gradually upon increasing the temperature from 0 to 35°C. Although the SFC profile of EF-3 was comparably similar to LD within the range of 0-35°C, their values were

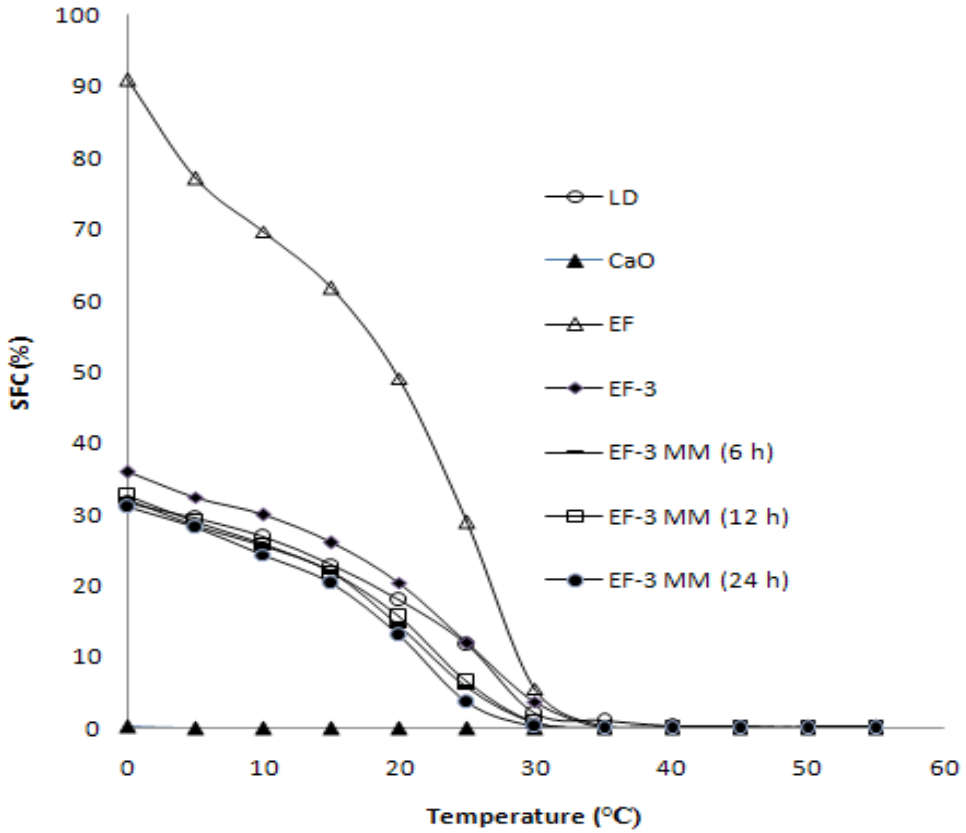

Figure 1. Solid fat content profiles of lard (LD), canola oil (CaO), engkabang fat (EF) and interesterified blends of engkabang-canola oil (EF:CaO = 35:65) with *Mucor miehei* lipase. CaO: canola oil; LD: lard; EF: engkabang fat; EF-3 (6 h): 35% of interesterified blend for 6 h; EF-3 (12 h): 35% of interesterified blend for 12 h; EF-3 (24 h): 35% of interesterified blend for 24 h.

always higher than those of LD. As previously discussed, this was mainly due to differences in melting points of TAG molecules present in both LD and EF-3. However, SFC values of EF-3 and LD were equal at 25°C. A number of previous studies indicated that the solidification behaviour of fat blends might be changed after being subjected to enzymatic transesterification. For instance, da Silva *et al*. (2013) observed the changing behaviour of SFC profile in continuous enzymatic transesterification of blends containing different proportions of LD and soybean oil. Re-distributions of fatty acid within TAG molecules during transesterification could be attributed to the changing behaviour of SFC profile of transesterified blends. As shown in Figure 1, all three transesterified blends displayed slightly lower SFC values than original EF-3 within the range of 0-35°C. After the transesterification, the increase in amounts of monosaturated TAGs with the concurrent drop in amounts of disaturated TAGs (Table 2) could be attributed to this observation. Among the three transesterified blends, EF-3 (6 h) displayed almost similar SFC values to those of LD within the temperature ranges of 0-15°C and 30-40°C.

Polymorphic behaviour

In the diffractograms presented in Figure 2, polymorphic behaviour of EF-3 (EF35/CaO65) blend before and after transesterification using *M. miehei* lipase are compared against CaO, EF and LD. In modifying fats and oils, the polymorphic phases are important to fulfil the requirements of finished product specifications. Particularly, in the development of margarine, β′ polymorph is preferred over the β form as it provides smaller crystals that give smoothness to end products. According to the diffractogram presented in Figure 2, EF was found to display crystals in β form where the XRD pattern represents a strong peak at around 4.60. This may be due to the TAG molecular species of EF which were dominated by SOS molecular species (Table 2). On the other hand, LD displayed both β- and β'-forms of polymorphs, out of which the β‘ polymorph was dominant. Based on Table 2, this polymorphic behaviour of LD was attributed to the presence of TAG molecular species, namely SPO and PPO in higher amounts (22%). As shown in Figure 2, β form, which is represented by a strong peak at around 4.60, was exhibited by EF-3 before transesterification. According to Ribeiro *et al*.

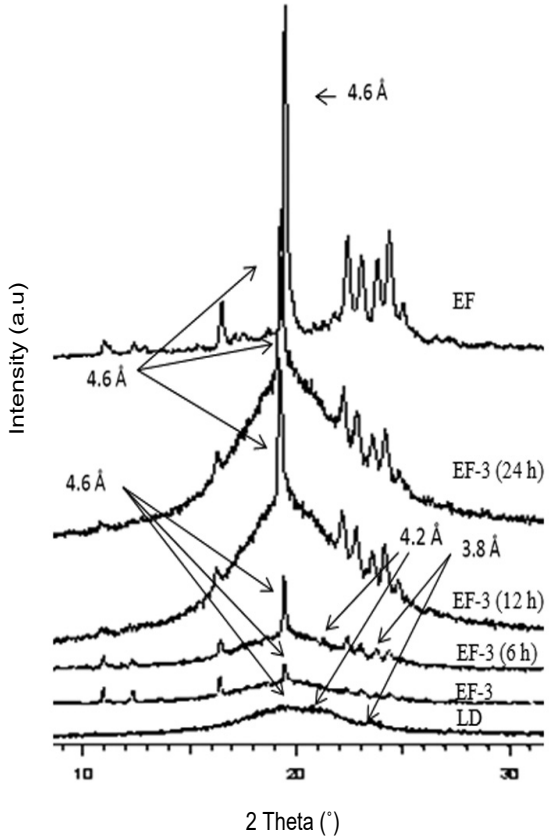

Figure 2. WAXD-patterns of lard (LD), engkabang fat (EF) and interesterified blends of engkabang-canola oil (EF:CaO = 35:65) with *Mucor miehei* lipase. CaO: canola oil; LD: lard; EF: engkabang fat; EF-3 (6 h): 35% of interesterified blend for 6 h; EF-3 (12 h): 35% of interesterified blend for 12 h; EF-3 (24 h): 35% of interesterified blend for 24 h.

(2009), the polymorphic behaviour of fat blends could be modified through transesterification to exhibit predominance of β' polymorph. In the present work, owing to transesterification by *M. miehei* lipase, the blends of EF-3 (6 h) tended to exhibit β' and β forms which were the same polymorphs as LD (Figure 2). In this case, significant reductions were observed in the proportions of TAG molecules such as SPO, SOS, SOA, OOL, and OOO while significant increases were noticed in the amounts of TAG molecules such as POL, POO, SOL and SOO. A number of previous studies indicated that the polymorphic behaviour of fat blends might be changed after being subjected to enzymatic transesterification. For instance, da Silva *et al*. (2013) observed that fat blends having LD and soybean oil tended to show β' polymorph after being subjected to enzymatic transesterification. According to Reshma *et al*. (2008), these could be due to factors such as duration of crystallisation, fatty acid combination of the TAG molecules, final temperature and time duration. These might influence the occurrence of polymorphic forms of specific nature in a mixed TAG system. As previously discussed by Sato and Ueno (2005), crystalline habit and polymorphic forms of fats might be affected by slight changes in the distribution of TAG composition caused by transesterification process.

Characterization of thermal properties by DSC

In the DSC curves presented in Figure 3, thermal behaviour of EF-3 (EF35/CaO65) blend before and after transesterification using *M. miehei* lipase are compared against CaO, EF and LD. Assessment of melting characteristics using DSC has been a vital part of research on development of novel fats for specific needs. The onset of crystallisation, number and positions of thermal transitions, and end of melting are some of the thermodynamic parameters studied when developing a plant-based fat substitute for LD. Particularly, evidence related to the occurrence of polymorphic transitions can be easily derived from DSC melting curves (Nassu and Gonçalves, 1999). The melting curve of EF was characterised by a major transition at 26.6°C and minor transitions at -7.2°C, 11.8°C, and 31.2°C. This could be due to EF possessing SOS (53.1%) as the most dominant TAG, followed by SPO (36.0%) and PPO (6.6%). As shown in Table 2, mixing of EF with CaO caused a decline in the amounts of SOP and SOS and increases in the amounts of OOO, OOL and

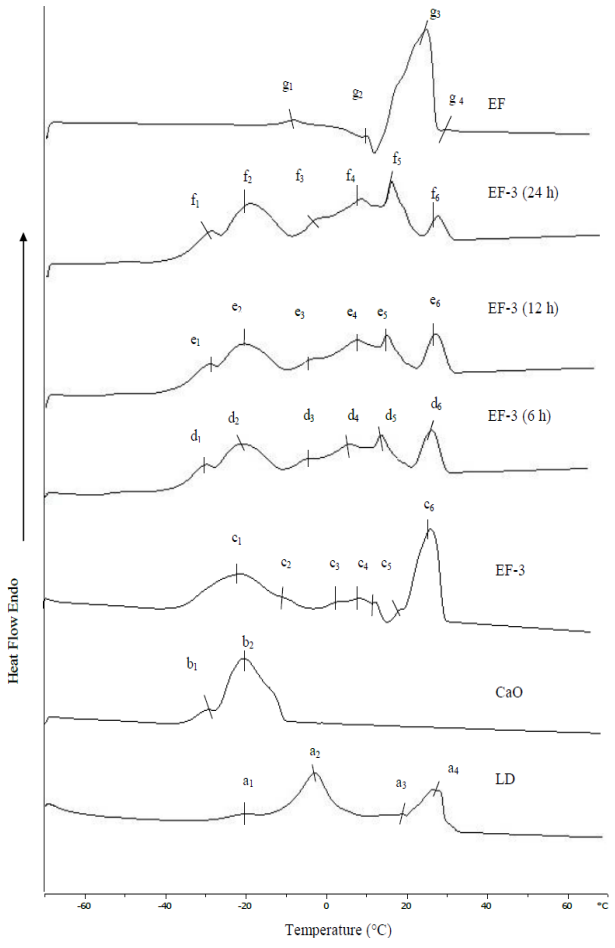

Figure 3. DSC heating curves of lard (LD), engkabang fat (EF) and interesterified blends of engkabang-canola oil (EF:CaO = 35:65) with *Mucor miehei* lipase. CaO: canola oil; LD: lard; EF: engkabang fat; EF-3 (6 h): 35% of interesterified blend for 6 h; EF-3 (12 h): 35% of interesterified blend for 12 h; EF-3 (24 h): 35% of interesterified blend for 24 h.

OOLn. These changes in TAG composition could be attributed to the changing melting profile of EF-3 blend. Before transesterification, EF-3 displayed a high-melting transition at 29.0°C (c_6), and a low-melting transitions at -22.0°C (c1); in between these two extremes a set of multiple transitions (c_5, c_4, c_3, c_2, etc.) were also found. The melting curves of the transesterified blends namely, EF-3 (6 h), EF-3 (12 h) and EF-3 (24 h) showed significant deviations from that of EF-3 with respect to thermal transitions as well as other associated features. For instance, with the increasing length of transesterification, the major thermal transition of EF-3 at 29.0°C (c_6) was found to decrease in size while the minor thermal transition of EF-3 at 10.5°C (c_5) was found to increase. After transesterification of 6 h (d_5, 11.2°C), 12 h (e_5, 11.7°C) and 24 h (f_5, 12.02°C), the positions of these thermal transitions also slightly shifted. In addition, the transition c_1 of EF-3 turned out to become a doublet peak comprising d_1 and d_2 in the range of -40 to -30°C after transesterification, which is in conformity with the changes taking place in polymorphic forms of EF-3, as indicated in the diffractogram presented in Figure 2. According to Ribeiro *et al*. (2009), the changing crystalline behaviour of EF-3 could be attributed to the TAG compositional changes caused by enzymatic transesterification. It could be assumed that the increasing of particular type of TAG molecular species through transesterification might contribute to these changes. The transition from β to β' polymorph could also result from smaller crystal formation. For example, the proportion of monosaturated TAG molecular groups (POL/SLL, POO/SOL, etc.) significantly increased ($p < 0.05$) with concurrent decreases in the proportions of disaturated TAG molecular group (SPO, SOS, etc.) (Table 2).

Conclusion

In the present work, the effect of transesterification by *Mucor miehei* on the rearrangement of the TAG composition of EF-3 (EF35/CO65) has been demonstrated. Results showed that the physical properties of the transesterified blends were strongly influenced by rearrangement of fatty acids within TAG molecules. Between time intervals from 6 to 24 h of transesterification, slight decline in SMP and SFC values were noticed. EF-3 (6 h) displayed almost similar to LD with regard to SFC values within the temperature ranges of 0-15°C as well as 30-40°C. Likewise, EF-3 (6 h) showed β' and β forms, which were similar to the polymorphic forms displayed by LD. Although the transesterified fat blends showed only a few similarities to LD in terms of the DSC thermal profile, it was clear that the peak maxima of EF-3 and EF-3 (6 h) showed the closest value to LD, which had the peak maxima at 29.3°C (a_4).

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