Enzymatic glycerolysis–interesterification of palm stearin–olein blend for synthesis structured lipid containing high mono- and diacylglycerol

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Abstract The objective of this research was to evaluate enzymatic glycerolysis–interesterification to synthesize structured lipids (SLs) containing high monoacylglycerol (MAG) and diacylglycerol (DAG) from a palm stearin–olein blend (PS–PO blend). The results showed that the optimum conditions for the solvent to fat ratio, glycerol to fat ratio, and enzyme concentration were 2:1 (v/w), 1.5:1, and 15% (w/w), respectively. The conversion rate of MAG and DAG decreased at a high glycerol to fat ratio, low solvent to fat ratio, and high enzyme concentration due to an increase in viscosity and low agitation effectiveness. The emulsion capacity and stability of the SLs were 60.19% and 96.80%, respectively. The hardness of the SLs increased about 3.1-fold. The MAG, DAG, and triacylglycerol conversion rates were 0.45, 0.48, and 1.02%/h, respectively. Thus, glycerolysis–interesterification of a PS–PO blend increased DAG and MAG concentrations and further improved the hardness, emulsion capacity, and emulsion stability of the SLs.

Keywords Glycerolysis–interesterification · Diacylglycerol · Monoacylglycerol · Structured lipid · Palm stearin–olein blend

Introduction

Structured lipids (SLs) are lipids in which the physical, chemical, or nutritional properties have been modified for specific food and nutraceutical applications (Osborn and Akoh, 2002). SLs provide an effective alternative to produce tailor-made lipids with desired characteristics. They can be synthesized by blending or interesterification of two or more types of fats/oils with certain melting characteristics (Oliveira et al., 2017; Ornela et al., 2016). SLs produced from blending vegetable fats/oils have a disadvantage, such as a lower melting profile and a soft texture (Biswas et al., 2017; Jahurul et al., 2013) because of low stearic acid content. Lauric fats/oils (Biswas et al., 2016; Sonwai et al., 2012) and hydrogenated fat (Abigor et al., 2003; Furlán et al., 2017) are used to increase hardness. However, a disadvantage of hydrogenated oils is that they may contain trans-oil/fat.

Interesterification of blends, based on low and high melting points such as a palm stearin and palm olein (PS–PO) blend, enhances the physicochemical properties of SLs (Soares et al., 2009). Their fatty acid and triacylglycerol composition are very versatile for producing a variety of SL products (Aini and Miskandar, 2007). However, SLs contain very low amounts of monoacylglycerol (MAG) and diacylglycerol (DAG), which prevents any emulsifier properties.

However, MAG and DAG have been used as emulsifiers in various emulsion-based foods, such as chocolate products and shortening (Bornscheuer, 1995; Feltes et al., 2013). They are synthesized by the triacylglycerol (TAG) hydrolysis method (Cheong et al., 2007) or esterification between glycerol and free fatty acids (Byun et al., 2007). A disadvantage of the TAG hydrolysis method is the occurrence of free fatty acids in the product, which are difficult

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to separate. Esterification between glycerol and free fatty acids produces water, which inhibits the esterification reaction (Feltès et al., 2013). MAG and DAG can also be synthesized through the glycerolysis reaction between TAG and glycerol (Krüger et al., 2010). The glycerolysis method may be more effective than other methods, as the reaction between TAG and glycerol produces MAG and DAG and results in the highest yield because each mole of TAG theoretically produces 3 mol of MAG and the reaction leads to the formation of DAG when there is no excess glycerol (Bornscheuer, 1995). In general, glycerolysis is performed on one type of fat/oil (Naik et al., 2014; Saberi et al., 2011) but it is not performed in a fat/oil blend.

The conversion rate of substrate to product is an important factor determining the effectiveness of the reaction. It depends on the solvent to fat ratio, glycerol to fat ratio, and enzyme concentration (Krüger et al., 2010; Naik et al., 2014; Wang et al., 2011).

In this research, SLs containing high MAG and DAG contents were synthesized by enzymatic glycerolysis–interesterification, which combines the interesterification method and glycerolysis method in one reaction system. It is expected that interesterification of the fat/oil blend will enhance the physicochemical properties of the products, while glycerolysis will provide MAG and DAG as emulsifiers. The reaction was initiated by adding *Candida antarctica* lipase, which was immobilized on a hydrophobic macroporous matrix. Factors, such as the solvent, the glycerol to fat ratio, and the enzyme concentration, were evaluated based on fatty acid composition, the conversion rate of MAG, DAG, and TAG; SL hardness; and emulsion capacity and stability.

Materials and methods

Materials

Palm olein (PO) and palm stearin (PS) were obtained from PT Smart (Tbk, Indonesia). Macroporous *Amberlite IRA-96 free base*, *C. antarctica* lipase B, and molecular sieves were obtained from Sigma-Aldrich (St. Louis, MO, USA). 2-Phenylpropionaldehyde, glycerol, tert-butanol, and hexane were obtained from Merck KGaA (Darmstadt, Germany).

Immobilization of lipase on macroporous hydrophobic matrix

The lipase was immobilized on the macroporous hydrophobic matrix according to our previous study (Hilmanto et al., 2016). Adsorption was performed at 30 °C in a shaker water bath at 150 strokes/min for various times (1,

2, 3, 4, and 5 h). The adsorbed protein was calculated as follows.

$$\text{Adsorbed Protein} = \frac{\text{Initial Protein} - \text{Non adsorbed Protein}}{\text{Adsorbent Matrix}} \quad (1)$$

Effect of solvent to substrate ratio on conversion of MAG and DAG

PS was added to PO at a ratio of 60:40 (w/w) in a batch stirred tank reactor (BSTR). Glycerol was then added to the mixture at a glycerol to fat molar ratio 1.5:1. Tert-butanol was added at various ratios of solvent to substrate (1.5:1, 2:1, and 3:1 v/w). The reaction was initiated by adding the immobilized lipase (10% w/w), and the mixture was incubated at 50 °C (Naik et al., 2014; Wang et al., 2011). A column containing a molecular sieve (12% of the total reactants) was connected to the BSTR. After the reaction, the immobilized lipase was separated and the filtrate was stored for further analysis. The conversion rates of MAG, DAG, and TAG were determined as the slope of the linear part of product concentration versus reaction time curve.

Effect of glycerol to fat ratio on conversion of MAG and DAG

A mixture of palm stearin–palm olein was prepared at a ratio of 60:40 (w/w). Glycerol was added to the fat at a glycerol to fat molar ratios of 1:1, 1.5:1, 2:1, and 2.5:1. Tert-butanol was added to the substrate at a solvent to substrate ratio of 2:1 (v/w). The reaction was initiated by adding the immobilized lipase (10%) to the BSTR at 50 °C for 24 h. A column containing a molecular sieve was connected to the BSTR. After the reaction, the immobilized lipase was separated, and the filtrate was stored for further analysis. The conversion rates of MAG, DAG, and TAG were determined as the slope of the linear part of product concentration versus reaction time curve.

Effect of lipase concentration on conversion of MAG and DAG

A mixture of palm stearin–palm olein was prepared at a ratio of 60:40 (w/w). Glycerol was added to the fat at a glycerol to fat molar ratio 1.5:1. Tert-butanol was added to the substrate at a solvent to substrate ratio of 2:1 (v/w). The reaction was initiated by adding various concentrations of the immobilized lipase (10, 15, and 20%). It was performed in a BSTR at 50 °C for 24 h. A column containing a molecular sieve was connected to the BSTR. After the reaction, the immobilized lipase was separated and the filtrate was stored for further analysis. The conversion rates

of MAG, DAG, and TAG were determined as the slope of the linear part of product concentration versus reaction time curve.

Determination of fatty acid composition

Fatty acid composition was analyzed by gas chromatography (GC) after methylation of the fatty acids to fatty acid methyl esters (FAMES). About 200 μ l of fat was methylated by adding 400 μ l of BF₃-methanol complex in a sealed flask. The mixture was heated for 2 h at 90°C. The FAME residues were extracted with 500 μ l hexane. The FAMES were analyzed by a Shimadzu GC-2010 equipped with a focused silica column of CP Sil 8 CB (30 m length, 0.25 mm diameter, 0.25 μ m film thickness), according to the AOCS Official Method Ce 1-62 (AOCS, 2004). All samples were analyzed in duplicate.

Determination of slip melting point and melting point

The AOCS method Cc. 3.25 (AOCS, 1997) was used to determine the slip melting point (SMP), and the AOCS official method Cc 1-25 (AOCS, 1997) was used to determine the melting point (MP).

Determination of hardness

Hardness was determined according to Biswas et al. (2017) using a TA.XT Plus texture analyzer. The fats were stored at 5°C for 24 h and the hardness measurements were performed at 25°C. Hardness was determined at a distance of 4 mm and speed of 1 mm/s for 4 s. The samples were measured in triplicate.

Determination of emulsion capacity and stability

Emulsion capacity and emulsion stability were determined according to Cano-Medina et al. (2011). Heating and centrifugation were used to determine emulsion stability.

Determination of hydrolytic and esterification activities of immobilized lipase

Esterification and hydrolytic activities of the immobilized lipase were determined according to Hilmanto et al. (2016).

Analysis of acylglycerols

Thin layer chromatography (TLC) was used to analyze the composition of acylglycerols (MAG, DAG, and TAG) (Fuchs et al., 2011). The samples were applied to an activated TLC plate and developed using a mixture of hexane:

ethyl ether: acetic acid (80:20:2 v/v/v) as the mobile phase. The TLC plate was subsequently dried. Coomassie Blue R-350 (0.02%, w/v) in a mixture containing water: methanol: acetic acid (6:3:1; v/v/v) was used to stain the samples. A Camag TLC Scanner III, which was equipped with winCATS Planar Chromatography software was used to quantify the results.

Protein assay

Protein analysis was determined according to the Lowry method (Lowry et al., 1951). Bovine serum albumin was used as the standard.

Statistical analysis

The data were analyzed by one-way analysis of variance. Tukey's test was applied to detect the differences. *P* values < 0.05 were considered significant.

Results and discussion

Effect of adsorption time on immobilized *C. antarctica* lipase activity

The adsorbed protein did not increase significantly as adsorption time increased from 1 to 5 h (Fig. 1). However, hydrolytic and esterification activities increased 2.84 times and 1.45 times as adsorption time increased from 1 to 4 h, respectively. Further increases in adsorption time resulted in a decrease in hydrolytic and esterification activities of 1.21 times and 1.28 times, respectively. These results suggest that the adsorption of lipase on the matrix surface was very quick (< 1 h). Increasing the adsorption time

Fig. 1 Effect of adsorption time on the adsorbed protein (black diamond symbols), esterification activity (white square symbols), and hydrolytic activity (black square symbols) of the immobilized *Candida antarctica* lipase on the macroporous hydrophobic matrix. Adsorption was performed at 30 °C, and the initial enzyme concentration was 12 mg/mL

provided more chances for hydrophobic interactions between the active sites of lipase and the surface of the hydrophobic matrix. As a result, the structure of the enzyme changed from closed to open. The hydrophobic matrix also improved the microenvironment for the enzymatic reactions (Chen et al., 2012), which increased immobilized enzyme activity (Fig. 1). Further increasing the adsorption time decreased lipase activity, which may have been due to a decrease in enzyme stability. Thus the optimum conditions for immobilized lipase were reached at an adsorption time of 3 h.

Effect of solvent to substrate ratio on conversion of MAG and DAG

Adding a solvent to the substrate affected the MAG and TAG conversion rates (Fig. 2). The conversion rates increased with an increase in the solvent to substrate ratio from 1.5:1 to 2:1 (Fig. 2A, C). The increased conversion rates of MAG and TAG were about 1.78 times and 1.25 times, respectively. The DAG conversion rate was not significantly different. The reactant system was more viscous at the low solvent ratio. Therefore, molecular diffusion and mass transfer were low. Adding more solvent increased the solubility of the substrates, mixture homogeneity and stability, and substrate diffusivity due to the change in the polarities of the fat and glycerol mixture. Therefore, mass transfer increased due to lowering of the

viscosity (Naik et al., 2014). However, a further increase in the solvent to substrate ratio (3:1) increased the DAG and TAG conversion rates, but it decreased the MAG conversion rate (Fig. 2). Thus, a 2:1 the solvent to substrate ratio was chosen for further experiments.

Effect of glycerol to fat ratio on conversion of MAG and DAG

Synthesis of SLs by the glycerolysis–interesterification reaction requires glycerol to convert TAG to MAG and DAG. The MAG and TAG conversion rates increased as the glycerol to fat ratio was increased from 1:1 to 1.5:1 (Fig. 3A, C). The MAG and TAG conversion rates increased about 1.33 times and 1.25 times, respectively. However, the DAG conversion rate was not significantly different. An increase in the glycerol fraction from 1:1 to 1.5:1 lead to an increase in the reactant equilibrium and the MAG and TAG conversion. However, a further increase in the glycerol to fat ratio (2:1) did not have a significant effect on the MAG, DAG, or TAG conversion rates (Fig. 3). The conversion rates decreased at a higher glycerol to fat ratio (2.5:1). This was due to an increase in the reactant mixture viscosity at a higher glycerol fraction. The viscosity of the reaction mixture increased about 1.6 times as the glycerol to fat molar ratio was increased from 1:1 to 2.5:1 (Fig. 3). An increase in viscosity affected the mass transfer of the substrates to the enzymes. Glycerol is

Fig. 2 Effect of the solvent to substrates ratio, 1.5:1 (black diamond symbols), 2:1 (black square symbols), and 3:1 (cross symbols) on monoacylglycerol (MAG) content (A); diacylglycerol (DAG) content (B); and triacylglycerol (TAG) content (C) in the product mixture. Glycerolysis–interesterification was performed at palm stearin–olein (PS–PO) ratio of 60:40 (w/w), a glycerol to fat molar ratio of 1.5:1, and 10% immobilized lipase at 50°C for 24 h

Fig. 3 Effect of the glycerol to fat molar ratio, 1:1 (black diamond symbols), 1.5:1 (black square symbols), 2:1 (black triangle symbols), and 2.5:1 (cross symbols) on monoacylglycerol (MAG) content (A); diacylglycerol (DAG) content (B); and triacylglycerol (TAG) content (C) in the product mixture. Reaction was performed at palm stearin–olein (PS–PO) ratio of 60:40 (w/w), a solvent to substrate ratio of 2:1 (v/w), and 10% immobilized lipase at 50°C for 24 h

hydrophilic; hence, it can interfere with the reaction system because the fat and immobilized lipase are favor in hydrophobic. Thus, a high amount of glycerol formed a coating around the enzyme, which inhibited enzyme activity, and lowered the conversion rates of MAG, DAG, and TAG (Cheong et al., 2007; Naik et al., 2014). Thus, the glycerol to fat molar ratio of 1.5:1 was chosen for further experiments.

Effect of lipase concentration on conversion of MAG and DAG

The conversion rates of TAG, MAG, and DAG increased as enzyme concentration was increased from 10 to 15% (Fig. 4). The increases in the MAG, DAG, and TAG conversion rates were about 1.40 times, 1.29 times, and 1.29 times, respectively. However, a further increase in the enzyme concentration (20%) resulted in a decrease in the MAG, DAG, and TAG conversion rates. In general, the MAG, DAG, and TAG conversion rates increased as enzyme loading increased. Nonetheless, the product conversion decreased at a 20% enzyme concentration (Fig. 4), suggesting that agitation was less effective due to high solid particle concentrations in the reaction system; therefore the conversion rate decreased. This result is in agreement with Krüger et al. (2010), who showed that yield does not increase with an increase in enzyme concentration

above a certain amount. This was due to poorer mixing of the reaction mixture, which affected the mass-transfer limitations. Thus, a 15% enzyme concentration was chosen for enzymatic glycerolysis of the PS–PO blend in the BSTR at a solvent to substrate ratio of 2:1 (v/w) and a glycerol to fat molar ratio of 1.5:1 at 50°C for 24 h. These conditions resulted in MAG, DAG, and TAG conversion rates of 0.45, 0.48, and 1.02%/h, respectively (Fig. 4). The MAG, DAG, and TAG concentrations were 11.39%, and 27.24%, and 59.60%, respectively.

Fatty acid compositions of MAG, DAG, and TAG

The fatty acid composition affected the physical properties of fat. MAG and DAG in the SLs had different fatty acid compositions compared with those of TAG and the SLs (Table 1). MAG and DAG contained higher total saturated fatty acid contents, particularly palmitic and stearic acids, than TAG and SLs. Total unsaturated fatty acids, especially oleic acid on MAG and DAG, was lower than TAG and the SLs. This was because *C. antarctica* lipase has higher specificity toward saturated fatty acids than unsaturated fatty acids (Kirk et al., 1992). Accordingly, these fatty acids reacted with glycerol to produce MAG and DAG; therefore, the saturated fatty acids in MAG and DAG were higher in content than TAG and the SLs.

Fig. 4 Effect of enzyme concentration, 10% (black diamond symbols), 15% (black square symbols), 20% (black triangle symbols) on monoacylglycerol (MAG) content (A); diacylglycerol (DAG) content (B); and triacylglycerol (TAG) content (C) in the product mixture. Reaction was performed at palm stearin–olein (PS–PO) ratio of 60:40 (w/w), a solvent to substrate ratio of 2:1 (v/w), and a glycerol to fat molar ratio of 1.5:1, at 50 °C for 24 h

Table 1 Fatty acid composition in the structured lipids (SLs), MAG, DAG, and TAG

Fatty acid	Structured lipids (%)	MAG ⁽¹⁾ (%)	DAG (%)	TAG (%)
C14:0	1.02 ± 0.07	Not detected	Not detected	Not detected
C16:0	49.05 ± 0.15 ^{b(2)}	56.46 ± 0.12 ^d	47.64 ± 1.84 ^a	51.65 ± 0.36 ^c
C18:0	5.12 ± 0.11 ^a	11.32 ± 0.60 ^c	15.75 ± 4.25 ^d	7.49 ± 2.17 ^b
C18:1	44.81 ± 0.11 ^d	32.22 ± 0.72 ^a	36.61 ± 2.41 ^b	40.86 ± 1.80 ^c

⁽¹⁾MAG monoacylglycerol, DAG diacylglycerol, TAG triacylglycerol

⁽²⁾Different letters indicated significantly different values ($p < 0.05$)

Physical properties of structured lipids in palm stearin–olein blend

SLs had a higher SMP and MP compared to the PS–PO blend due to high MAG and DAG content (Table 2). MAG and DAG have a higher MP than TAG (Lo et al., 2008; Zhang et al., 2014). Therefore, fats containing high MAG and DAG contents have a higher MP than those with lower concentrations of MAG and DAG. MAG and DAG contained higher saturated fatty acid content than TAG (Table 1). The MAG and DAG saturated fatty acids contributed to SMP and MP. The increase in SMP and MP resulted in a 3.1-fold higher hardness of the SLs than the PS–PO blend (Table 2).

Glycerolysis–interesterification of the PS–PO blend resulted in an increase in emulsion capacity of the product (Table 2), which was about 1.56 times higher than that of the PS–PO blend. The emulsion stability value was 96.80%. The increase in emulsion capacity was due to an

Table 2 Physical properties of the structured lipids

Sample	PS–PO blend	Structured lipids
Monoacylglycerol (%)	Not detected	8.59 ± 2.31
Diacylglycerol (%)	15.03 ± 3.75 ^{a(1)}	26.90 ± 1.92 ^b
Triacylglycerol (%)	84.97 ± 3.75 ^b	64.51 ± 0.47 ^a
Slip melting point (°C)	38.17 ± 0.29 ^a	38.67 ± 0.29 ^b
Melting point (°C)	44.00 ± 1.00 ^a	44.67 ± 0.58 ^a
Hardness (N)	1.82 ± 0.31 ^a	5.63 ± 0.59 ^b
Emulsion capacity (%)	38.48 ± 1.70 ^a	60.19 ± 4.17 ^b
Emulsion stability (%)	Not detected	96.80 ± 0.23
Hydrophilic–lipophilic balance	4.67 ± 0.25 ^a	8.05 ± 0.44 ^b

⁽¹⁾Different letters indicated significantly different values ($p < 0.05$)

increase in MAG and DAG concentrations (Table 2). MAG and DAG have hydroxyl groups, so they can act as emulsifiers. The HLB value of the SLs was 8.05. Emulsifiers, which have a low HLB are usually used for water-in-oil

emulsions, whereas high HLB emulsifiers are used for oil-in-water emulsions (Losada-Barreiro et al., 2013). The SLs formed an emulsion, which was relatively stable to heat and centrifugation.

In summary, the optimum conditions for the solvent to fat ratio, glycerol to fat ratio, and enzyme concentration were 2:1 (v/w), 1.5:1, and 15% (w/w), respectively. The conversion rate of MAG and DAG decreased at a high glycerol to fat ratio, low solvent to fat ratio, and high enzyme concentration. Finally, glycerolysis–interesterification of a PS–PO blend increased DAG and MAG concentrations and further improved the hardness, emulsion capacity, and stability of the SLs.

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