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Effect of temperature on the fatty acid distribution of structured lipids by enzymatic interesterification of fish oil with milk fat fatty acids

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Enzymatic interesterification for the synthesis of structured lipids (SLs) is affected by several factors, one of them is temperature. The aim of this research was to evaluate the effect of temperature on the positional distribution of fatty acids and acylglycerol profiles of SLs from enzymatic interesterification of fish oil with milk fat fatty acids (MFFAs). The method used in this research was explanatory research and continued by descriptive analysis with five different treatments, consist of temperature (30°C, 40°C, 50°C, 60°C, and 70°C). Positional distribution of the fatty acid residues at sn-2 and sn-1,3; and acylglycerol profiles were analyzed in each treatment. The results showed that the increasing temperature increased the incorporation of saturated fatty acids (SFAs) at the sn-1,3 up to a certain temperature and decreased the triacylglycerol (TAG) of SLs. The optimum temperature for the enzymatic interesterification was 50°C. SL was occupied by SFAs about 44.27% at the sn-1,3 while PUFA (EPA and DHA) remained at the position of sn-2 about 29.81%. The acylglycerol profile showed that structured lipid contained triacylglycerols (TAG), diacylglycerols (DAG), and monoacylglycerol (MAG) about 70.25%, 19.83%, and 9.92%, respectively. The higher temperature up to 50 °C increased the SFA content at the sn-1,3 of SLs, but the use of the temperatures above 50 °C reduced the TAG content of SLs that synthesized by enzymatic interesterification of fish oil with MFFAs.

Keywords: Enzymatic interesterification, positional distribution of fatty acid, acylglycerol profile, fish oil, structured lipids

INTRODUCTION

Fish oil is a source of long-chain PUFAs namely docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) which is good for health (Ramezani et al., 2008). However, PUFAs from fish oil had a good nutrition value only they were at the sn-2 position of the glycerol backbone. PUFAs were absorbed in a less efficient metabolism when incorporated at the sn-1,3 position. This was due to the long-chain PUFAs that were metabolized slower in the digestive tract to produced energy. The position of fatty acids at glycerol backbone can be arranged through the synthesis of structured lipids (SLs), where PUFAs incorporated at the sn-2 position and shorter chain fatty acids were occupied at the sn-1,3 position. SLs were synthesized to obtain the modified lipid with a good functional properties, high nutrition values, and desired characteristics (Kim and Akoh, 2015; Subroto et al., 2019b). SLs could be synthesized by interesterification or acidolysis reaction (reaction between triacylglycerols and free fatty acids) (Willis and Marangoni, 2002).

Enzymatic interesterification had been growing rapidly by modifying fat or oil to improve the nutritional value or functional properties (Kavadia et al., 2018). Enzymatic interesterification (acidolysis) carried out by the

addition of saturated fatty acids (SFAs) to the fish oil had a potential to produce the SLs which had a good properties. SFAs were more difficult to oxidize than a fish oil that contained PUFAs. The SFAs can be obtained from milk fat fatty acids (MFFAs) which were rich in myristic, palmitic, and stearic acids. MFFAs contained of SFAs expected to be incorporated at the sn-1,3 while PUFAs from fish oil remained at the position of sn-2. PUFAs were good for health, while SFAs at the position of sn-1 and sn-3 were more resistant to oxidation (Park et al., 2011). PUFAs at the Sn-2 position were absorbed by the intestine in the form of 2-monoacylglycerol. They were more ready to be absorbed than other PUFA derivatives (Shahidi and Ambigaipalan, 2018).

Enzymatic interesterification was affected by several factors. In the previous research, the factor of fish oil to MFFAs ratios and enzyme concentrations for the synthesis of SLs had been studied (Subroto et al., 2019a). One of the other factors that affected enzymatic interesterification was temperature. Temperature could affect the composition and positional distribution of fatty acid on the SLs produced. The reaction rate increased until a certain temperature and the reaction to be stationary but the fatty acid composition remained after the reaction reached the equilibrium. According to Willis and Marangoni (2002), interesterification reaction increased with the increase of temperature. However, higher temperature caused the irreversible denaturation of the enzyme, decreased the enzyme activity, and reduced the reaction rate. In addition, the high temperatures affected the acyl migration (Pacheco et al., 2015).

The aim of this research was to evaluate the effect of reaction temperature on the positional distribution of fatty acids and acylglycerol profiles of SLs by enzymatic interesterification (acidolysis) of fish oil with MFFAs. SFAs from MFFAs were expected to be incorporated at the position of sn-1,3 while the PUFAs from fish oil remained at the position of sn-2.

MATERIALS AND METHODS

Materials

The milk fat rich in SFA and fish oil rich in PUFAs were purchased from the Prince of Peace® Enterprises, Inc. The immobilized lipase from *Mucor miehei* was used as a catalyst. The solvent (hexane, chloroform, petroleum ether, acetone, diethyl ether, ethanol, BF3-methanol complex, and acetic acid), and reagents were analytical grade.

Preparation of milk fat fatty acids

Preparation of milk fat fatty acids (MFFAs) from milk fat was carried out according to the method of Kim and Hill (2006). Milk fat was added with a solution of NaOH (40% w/v) in the mixture of ethanol and distilled water (3:1 v/v). The mixture was then refluxed for 1 h. Aquadest was added to the mixture, then the unsaponified materials were extracted using n-hexane. The saponified materials were then acidified by HCl (6 N) until the pH of 1.0. The aqueous fraction at the lower layer was then removed, while the MFFAs at the upper layer were extracted using n-hexane and then washed with distilled water. The solvent (hexane) was then evaporated and the MFFAs were stored in refrigerator (5-10°C) until they are ready to be used.

Enzymatic interesterification (acidolysis) at various temperature

Enzymatic interesterification (acidolysis) was carried out according to Subroto et al. (2008). Fish oil and MFFAs with the molar ratio of 1:3 were added by immobilized lipase from *Mucor miehei* (10% w/w) as catalyst and n-hexane (1.5 times of the substrates) in a sealed erlenmeyer. The erlenmeyer was placed in an incubator shaker (300 rpm) at various temperature (30, 40, 50, 60 and 70°C) for 4 h. The immobilized lipase was then separated. The remaining free fatty acids were then neutralized by titrated using 0.1 M of KOH. The mixture was added n-hexane to extract acylglycerol. The solvent was then evaporated using a rotary evaporator. Acylglycerol fraction was then stored and analyzed.

Determination of fatty acid composition

The sample (200 µL) was transmethylated by BF3-methanol complex (400 µL) and heated (90°C, 2 h) (Subroto et al., 2018a). The fatty acid methyl ester (FAME) residues were extracted by n-hexane. The FAME was then analyzed by gas chromatography "Varian 450 – GC" using a CP-Sil 5 CB column and an FID detector following to AOCS (2004). The FAMEs were then identified with those authentic standards.

Determination of positional distributions of FA in SLs

The positional distributions of FA were determined according to Torres et al. (2002). SLs (50 mg) was added by porcine pancreatic lipase

into a stoppered flask. The reaction mixture was then added with sodium borate, bile salts, tris-HCl buffer (pH 8.0), and $CaCl_2$. The mixture was then incubated at 40°C for 1 min, then shook using vortex for 7 min. The acetic acid was added to stop the reaction. The reaction mixture was then added with chloroform to extract the 2-monoacylglycerols. The extract was then evaporated and analyzed by thin layer chromatography (TLC). The 2-monoacylglycerols bands were scraped and extracted with hexane. The resulting solutions were analyzed by GC as described above. These provide fatty acid composition at the position of sn-2, while the fatty acid distribution at the sn-1,3 was calculated according to the formula (1).

$$\%\ of\ sn-1{,}3 = \frac{3(\%\ of\ total) - (\%\ of\ sn\text{-}2)}{2}$$

Determination of acylglycerol profile

The acylglycerol profiles of SLs were analyzed using TLC (Fuchs et al., 2011). The sample was spotted to the TLC plate which had activated by heating (105 ^oC, 1 h). The plate was developed by the solution mixture containing petroleum ether, diethyl ether, and acetic acid with a ratio of 60:40:1 (v/v/v). The quantitative analysis measured using "dummy" (S/N 081 124) TLC Scanner Camag 3. The wavelength set at 350 nm, using a D2 lamp, and the speed of 20 mm/sec.

RESULTS

The fatty acid composition of structured lipids

Fish oil contained PUFAs and SFAs about 30.64% and 38.48%, respectively. The MFFAs contained SFAs about 73.86% and they did not have PUFAs (Table 1). The temperature of the enzymatic interesterification changed the fatty acid composition of SLs. The SFA content of SLs (especially palmitate and stearate) increased at the temperature of 30 ^oC to 50 ^oC then did not change significantly. The reaction reached its optimum temperature at 50 ^oC where the SFA and PUFA content was about 43.48% and 26.21%, respectively. Fish oil contained a higher PUFAs than SFAs in the glycerol backbone. The PUFA content in the SLs was inversely proportional to SFA content. PUFAs content decreased in the temperature range of 30 ^oC to 50 ^oC. SFA content then decreased slightly from 43.48% to 41.10% at the temperature range of 50 ^oC to 60 ^oC, then the SFA content increased again at the temperature range of 60 ^oC to 70 ^oC. This result indicated that the condition had reached equilibrium at temperatures above 50 ^oC.

Positional distribution of fatty acids

The effect of temperature on the fatty acid residues at the sn-2 position can be seen in Table 2. The results showed that the SFA and PUFA content at sn-2 of fish oil was about 31.87% and 37.91%, respectively. The SFA content at sn-2 position increased with an increase of the temperature reaction, and vice versa, the PUFA content decreased. SFA content in the SLs increased in the temperature range of 30 ^oC to 70 ^oC, which was from 39.19% to 44.10%. This indicated that the higher temperature increased the binding of SFAs from the MFFAs to the glycerol backbone of fish oil.

The changes in the distribution of fatty acids at the position of sn-2 further affected the distribution of fatty acids at the position of sn-1,3 (Table 3).

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Table 1. Fatty acid residues in the fish oil, MFFAs, and SLs at various temperature.

Fatty acid	Content (%)						
	Fish oil	MFFAs	30 °C	40 °C	50 °C	60 °C	70 °C
Capric	0.25 ± 0.04	1.97 ± 0.01	0.80 ± 0.14	0.87 ± 0.19	1.06 ± 0.01	1.00 ± 0.01	1.06 ± 0.01
Lauric	0.74 ± 0.12	4.92 ± 0.47	1.98 ± 0.23	2.20 ± 0.52	2.57 ± 0.02	2.34 ± 0.01	2.56 ± 0.04
Myristic	10.47 ± 0.70	14.95 ± 0.68	9.63 ± 0.25	10.31 ± 0.12	9.91 ± 0.13	9.37 ± 0.19	9.82 ± 0.22
Palmitoleic	13.63 ± 1.15	1.80 ± 0.02	11.51 ± 0.57	11.51 ± 0.46	10.01 ± 0.12	9.47 ± 0.49	9.33 ± 0.20
Palmitic	22.48 ± 1.46	39.53 ± 1.96	21.79 ± 0.97	23.58 ± 0.63	23.65 ± 0.77	22.42 ± 0.55	24.39 ± 0.85
Oleic	17.25 ± 0.18	24.35 ± 0.80	19.36 ± 0.93	20.22 ± 1.19	20.30 ± 0.54	19.92 ± 0.16	19.77 ± 0.34
Stearic	5.13 ± 0.49	12.49 ± 1.83	5.21 ± 0.16	5.67 ± 0.02	6.29 ± 0.46	5.96 ± 0.23	6.66 ± 0.38
EPA	18.32 ± 2.5	ND	17.59 ± 1.64	15.53 ± 1.21	13.64 ± 1.63	15.60 ± 0.58	13.19 ± 1.33
DHA	12.32 ± 1.28	ND	12.13 ± 1.62	10.11 ± 0.92	12.57 ± 0.18	13.91 ± 0.67	13.21 ± 0.64
∑ SFA	38.48 ± 1.26	73.86 ± 1.43	39.41 ± 1.76	42.63 ± 0.48	43.48 ± 0.40	41.10 ± 0.59	44.50 ± 1.42
∑ PUFA	30.64 ± 1.75	ND	29.72 ± 1.26	25.64 ± 1.14	26.21 ± 1.81	29.50 ± 1.25	26.40 ± 1.96

ND = not detected

Table 2; The effect of temperature on the fatty acid residues at the sn-2 position of SLs.

Fatty acid	Content (%)				
	30 °C	40 °C	50 °C	60 °C	70 °C
Capric	ND	ND	ND	ND	ND
Lauric	1.54 ± 0.28	1.48 ± 0.01	2.32 ± 0.55	1.73 ± 0.74	2.04 ± 0.47
Myristic	8.97 ± 0.29	8.50 ± 0.01	9.13 ± 0.57	8.64 ± 0.30	8.63 ± 1.09
Palmitoleic	1.27 ± 0.31	9.52 ± 0.13	9.10 ± 1.07	9.06 ± 1.05	7.75 ± 0.37
Palmitic	22.53 ± 0.98	23.13 ± 0.54	22.73 ±0.18	23.02 ± 0.66	23.62 ± 0.94
Oleic	20.04 ± 0.72	21.01 ± 1.30	19.17 ± 0.75	20.35 ± 1.67	18.93 ± 0.90
Stearic	6.14 ± 0.55	6.90 ± 0.08	7.73 ± 0.29	10.10 ± 0.43	9.80 ± 0.55
EPA	15.83 ± 1.36	15.25 ± 0.28	16.64 ± 0.76	15.45 ± 1.28	15.26 ± 1.74
DHA	14.67 ± 0.29	14.22 ± 2.32	13.17 ± 0.66	11.65 ± 1.72	13.96 ± 1.49
∑ SFA	39.19 ± 0.96	40.01 ± 0.61	41.92 ± 1.24	43.48 ± 1.27	44.10 ± 1.95
∑ PUFA	30.50 ± 1.07	29.46 ± 2.04	29.81 ± 1.42	27.10 ± 1.99	29.21 ± 1.22

ND = not detected

Table 3; The effect of temperature on the fatty acid residues at the sn-1,3 position of SLs.

Fatty acid	Content (%)				
	30 °C	40 °C	50 °C	60 °C	70 °C
Capric	1.20 ± 0.22	1.31 ± 0.28	1.59 ± 0.01	1.50 ± 0.02	1.59 ± 0.01
Lauric	2.19 ± 0.21	2.56 ± 0.78	2.69 ± 0.24	2.65 ± 0.39	2.82 ± 0.18
Myristic	9.96 ± 0.23	11.22 ± 1.18	10.30 ± 0.09	9.74 ± 0.87	10.42 ± 0.88
Palmitoleic	12.13 ± 0.20	12.51 ± 0.76	10.46 ± 0.35	9.67 ± 1.76	10.12 ± 0.49
Palmitic	21.42 ± 1.95	23.81 ± 1.72	24.11 ± 1.25	22.13 ± 1.65	24.78 ± 1.74
Oleic	19.02 ± 1.04	19.82 ± 1.44	20.86 ± 1.18	19.71 ± 1.08	20.19 ± 0.97
Stearic	4.74 ± 0.52	5.05 ± 0.57	5.57 ± 0.46	3.90 ± 0.37	5.08 ± 0.29
EPA	18.47 ± 1.77	15.67 ± 1.68	12.14 ± 1.06	15.67 ± 1.51	12.16 ± 2.28
DHA	10.86 ± 2.58	8.06 ± 1.05	12.27 ± 0.05	15.04 ± 1.86	12.84 ± 1.70
∑ SFA	39.52 ± 1.12	43.94 ± 1.53	44.27 ± 0.47	39.91 ±2.53	44.70 ± 2.11
∑ PUFA	29.33 ± 1.36	23.73 ± 1.73	24.42 ± 2.01	30.70 ± 2.37	25.00 ± 2.56

ND = not detected

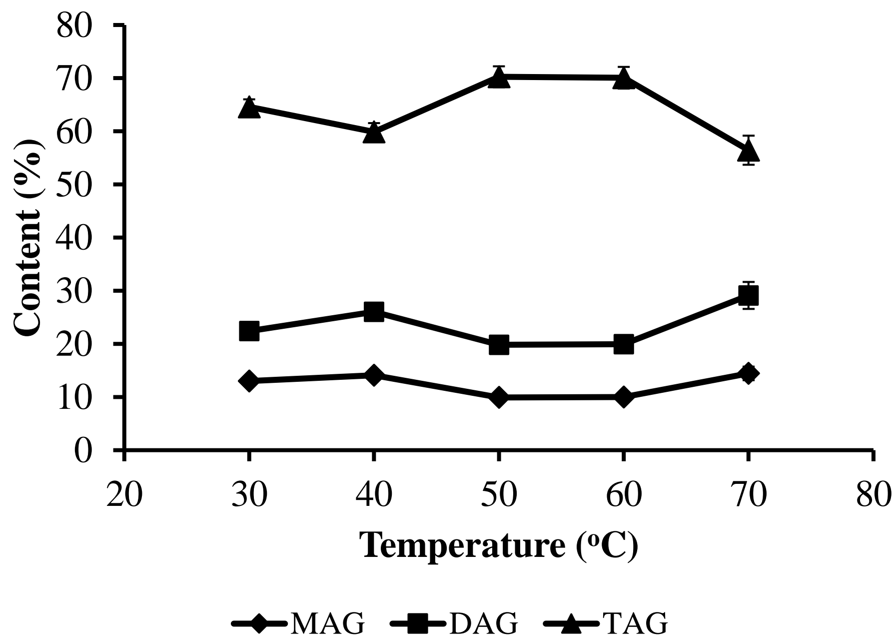

Figure 1; The effect of temperature on the MAG (♦), DAG (■), and TAG content (▲) of SLs.

The SFA content at the sn-1,3 position increased while the PUFA content decreased until at the temperature of 50 °C. The higher temperature caused the PUFA content at sn-1,3 decreased until the temperature of 50 °C, but it increased again at the temperature of 60 °C. This was due to re-esterified of PUFA that had been released to the glycerol backbone of SLs

Acylglycerol profile

The effect of temperature on the acylglycerol profile showed that the different temperatures resulted in a changing of acylglycerol profile, as shown in Fig 1. Acylglycerol profile of fish oil consisting of MAG, DAG, and TAG was about 5.56%, 8.30%, and 86.14%, respectively. The higher reaction temperature resulted in the TAG content to decrease in the temperature range of 30 °C to 40 °C. The TAG content increased again at the temperature range of 40 °C to 60 °C, then decreased at the temperature of 70 °C. This indicated that the higher temperatures caused an incomplete interesterification and encouraged partial hydrolysis of TAGs in fish oil.

DISCUSSION

The SFA content of the SLs increased with the increase of temperature. This was due to the reaction rate of enzymatic interesterification increased until achieved the equilibrium, but the reaction decreased if the enzyme was inactivated. The increasing of reaction temperature increased the rate of interesterification (Willis and Marangoni, 2002). This result was in line with Subroto et al. (2008) who succeeded in interesterification of fish oil with lauric acid achieved optimum conditions at a temperature of 50 °C. This result was the same as that of Kim and Hill (2006), the acidolysis increased with the increase of temperature until 50 °C. The higher temperature increased the lipase activity so that the interesterification reaction run fast and resulted in an increasing of SFA content in SLs as long as the substrate was available. Reactions ran fast over at a certain temperature until the maximum rate was reached, then to equilibrium. Some enzyme proteins could undergo denaturation at a higher temperature (>60 °C) which resulted in a decreased of lipase activity and inhibited the interesterification reaction. The enzymatic interesterification reaction at various temperatures succeeded in increasing the SFA content to the SLs.

The fatty acid distribution was affected by the temperature of enzymatic interesterification. This was due to the increased of lipase activity so that increased the amount of SFA binding at sn-2 of SLs. The higher reaction temperature caused the increasing of SFA content at the position of sn-2 and SFA content became higher than PUFA content. The use of sn-1,3 specific lipase ideally resulted in binding of SFA only at the position of sn-1,3 and SFA content at the position of sn-2 did not increase with the increasing of temperature. Increasing the SFA content at the position of sn-2 can be caused by the acyl migration. Acyl migration was the transfer of acyl groups to the glycerol backbone from sn-1,3 to sn-2 (Silva et al., 2012). The use of high temperatures caused partial denaturation of lipase proteins so that the enzyme specificity decreased. Acyl migration caused the highest SFA content at sn-2 when the temperature was 70 °C. Factors that caused acyl migration were high reaction temperature, water content, and substrate ratio (Pacheco et al., 2015).

The SLs were expected to have a high content of PUFA at Sn-2 while SFA at sn-1.3 position. The temperature of 50 °C was the most suitable for the synthesis of SLs by enzymatic interesterification. At this condition, it had also reached its optimum temperature by producing a high amount of PUFA (EPA and DHA) content at the sn-2 position, where SFA content at the sn-1,3 position. The SLs that contained SFAs at the sn-1,3 position were more resistant to oxidation (Endo et al., 1997).

High temperature decreased the TAG content in SLs. The decreased in TAG content indicated that there was a partial hydrolysis of TAG resulted the DAG and MAG as the intermediate products. This can also be triggered by acyl migration. Acyl migration caused the acyl transfer in TAG from sn-2 to sn-1 or sn-3. Acyl migration occurred imperfectly caused several sn-1, sn-2, or sn-3 positions did not bind fatty acids, then produced MAG or DAG (Turon et al., 2003). The increased in TAG at 50 °C caused a decreased in DAG and MAG. This showed that the possibility of the reaction was not entirely acidolysis, but also esterification due to the reaction between the hydroxyl group in MAG and DAG with MFFAs. Based on the acylglycerol profile, the equilibrium occurred at a temperature of 50 °C which produced the highest TAG of 70.3%. The portion of the acyl groups in the TAG was hydrolyzed again while MAG and DAG increased. Factors that affected the reaction to partial hydrolyzed were temperature, product and by-products produced, pH, and water content (Rajendran et

al., 2009). In addition, TAG was hydrolyzed easier while MAG was easier to be esterified, so the reaction led to the formation of DAG through either partial hydrolysis of TAG or partial esterification of MAG. SLs produced by the enzymatic interesterification reaction at a temperature of 50 °C contained TAG, DAG, and MAG contents about 70.25%, 19.83%, and 9.92%, respectively. These acylglycerols (MAG and DAG) were good as an emulsifier for the various types of emulsion-based foods (Subroto et al., 2018b), and had the beneficial effects on health (Lo et al., 2008).

CONCLUSION

Temperature up to 50 °C increased the SFA content at the sn-1,3 and temperatures above 60 °C reduced TAG content of SLs. Hence, the optimum temperature for enzymatic interesterification (acidolysis) of fish oils with MFFAs was reached at 50 °C. The SLs occupied by SFAs at the position of Sn-1,3 about 44.27% and PUFAs remained at the position of Sn-2 about 29.81%. High temperature decreased the TAG content and increased the DAG and MAG content in SLs. The reaction temperature of 50 °C resulted the SLs with the TAG, DAG and MAG content about 70.25%, 19.83%, and 9.92%, respectively.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

ES and AR designed the research and wrote the manuscript. RI, TE, and MA performed data analysis and reviewed the manuscript.

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