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The Effect of Substrat Ratio Fish Oil and Milk Fat on Synthesis of Structured Lipid by Enzimatic Transesterification

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Abstract*— Structured lipid with saturated fatty acid (SFA) at outer position and polyunsaturated fatty acid (PUFA) at sn-2 position has good dietary and stabilized characteristics. In this research structured lipids was synthesized by enzymatic transesterification between fish oil and milk fat. The reaction was catalyzed by lipase from C*andida antartica* that has randomized specificity to inter esterification. The factor substrat ratio of fish oil and milk fat were studied. Reaction operated at 40 oC for 4 hours, and the enzim concentration was 10 % by substrat. Composition of fatty acid, regiospecificity position of fatty acid, and glyceride profile were determinate. The results showed that the substrat ratio of fish oil: milk fat affect the composition of structured lipid. The more of milk fat added showed that composition saturated fatty acids on structured lipid was increased but the unsaturated fatty acids was decreased. The regiospecificity of structured lipid showed that saturated fatty acid has tendency at outer position and unsaturated fatty acid at sn-2 position and optimum at ratio fish oil: milk fat was 1 : 3 (w/w). In the ratio fish-oil: milk fat 1:3 produced structured lipids in which the sn-2 incorporated by unsaturated fatty acids such as oleic, EPA, and DHA was 22.9; 3.3, and 2.2% respectively. While in position sn-1, 3 incorporated mostly medium chain and saturated fatty acids such as capric, lauric, myristic, palmitic, and stearic acid 5.0; 7.0; 16.6; 31.7, and 9.9 % respectively. The ratio substrate did not affect the profil of glyceride on structured lipid. Triglyceride tend to decreased, and the diglyceride and monogliceride was increased with an increase of milk fat on substrate but not significantly. Structured lipid showed the characteristic like milk fat but high nutrition because rich of PUFA at sn-2 position, so this product may can applied on milk fortification.

***Keywords*— Transeresterification; Fish Oil; Milk Fat; Structured Lipids; Substrat.**

I. INTRODUCTION

Synthesis of structured lipids has grown rapidly in the past decade by modifying lipid primarily to improve the functional properties and nutritional value of a fat or oil. Structured lipids by medium chain fatty acids (MCFA) and saturated fatty acids (SFA) on the external position, against polyunsaturated fatty acids (PUFA) in the midle sn-2 position has nutritional value and excellent absorption (Irimescu et al, 2001b). Medium chain residues easily hydrolyzed in the digestive tract that was absorbed quickly and was used as a source of high energy in the body. PUFA absorbed as 2-MG among the most readily absorbed to synthesis the tissue of hearth, brain, kidney and other vital organs. Structured lipids with saturated residue on the outside and PUFA in sn-2 position is also more resistant to oxidation (Endo et al, 1997).

Fish oil well known as a source of polyunsaturated fatty acids (PUFA), include docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). Various kinds of fish oil has been used since ancient as dietary supplements. Depth studies have been conducted on the nature or the biological function and therapy in DHA and EPA, and various kinds of health products made from or containing fish oil or fish oil derived compounds such as ethyl esters have recently entered the market nutraceutical (Irimescu et al, 2001a). Against, milk fat contains a lot of MCFA and saturated fatty acids (SFA) (National Research Council, 1976), but so far utilized for the synthesis of structured lipids are still very rare. While many distributed PUFA in fish oil. So, fish oil and milk fat has the actual potential for the manufacture of structured lipids with PUFA at the sn-2 position, while SCFA, MCFA and saturated fatty acids in position sn-1, 3.

Various methods of synthesis specific structured lipids by enzymatic has been done. Subroto et al (2008) had synthesized a specific structured lipids which PUFA at sn-2 and MCFA in the sn-1,3 through asidolisis fish oil with lauric acid. Actually structured lipids can also be done through direct transesterification. Torres et al (2002) had synthesized structured lipids by transesterification the corn

oil with tristearin. In this research the synthesis of structured lipids will be done through direct transesterification between milk fat and fish oil with a certain incubation time and temperature using lipase from *Candida Antartica*. PUFA expected would tend to esterified at sn-2 position, while the sn-1 and sn-3 esterified by SCFA, MCFA, and other saturated fatty acids from milk fat. Studies conducted on the ratio of fish oil and milk fat to determine how the composition and position of the fatty acids and the glicerides profiles of structured lipids.

II. METHODOLOGY

A. Materials

Fish oil, milk fat, lipase B *Candida antartica* immobilized on immobead 150, recombinant from *Aspergillus oryzae* (54326) was purchased from Novo Nordisk, Porcine pancreatic lipase, 2-mono oleoylglycerol, solven (hexane, petroleum ether, diethyl ether, ethanol, aceton), reagen (KOH, iodium). all solvents and reagents used in these experiments were of analytical grade and were purchased from sigma aldrich, silica gel TLC plates (20 × 20 cm; 60 Å mean pore diameter, 2–25 μm mean particle size, 500 μm thickness, with dichlorofluorescein)

B. Methods

1) Reaction protocol: The reaction mixture consisted of fish oil and milk fat with the ratio 1:1, 1:3, 1:5, 1:10, and 1:15 added with 10% (w/w) immobilized lipase as catalist, then added hexane (1,5 times of the substrat) in a 50-mL sealed erlenmeyer. The erlemeyer was placed in an incubator shaker (300 rpm) maintained at 40°C. The reaction mixture was held at these conditions for 4 hours. After reaction period, the reaction mixture was filtered to separate the immobilized lipase to stop the reaction. A mixture of ethanol and acetone with a ratio of 1:1 (v / v) of 10 ml was added to prevent the emulsion while simultaneously neutralizing the free fatty acids and as the toxic to the enzyme so that if there is residual soluble enzymes could be inactive. To neutralize the free fatty acids, the reaction mixture was titrated with 0.1 M KOH solution with indicator of phenolphathalein (PP) until the color of the solution turn pink. 35 ml of hexane is added to the mixture to extract acilglycerol. The mixture was mixed thoroughly and transferred into a separating funnel. Two layers (fraction of water and hexane fraction) would be separated, and the water layer discarded. Hexane fraction was then added anhydrous sodium sulfate to remove residual water. The next hexane is evaporated using a rotary evaporator at 40 oC 335 mmHg vacuum pressure. Fraction asilgliserol moved into a small bottle to be stored and analyzed.

2) FA composition of products, Park et al (1994) modification

(i) Preparation of FAME: The 200 μl of sample oil or fat methylated by addition of 400 μl BF3-methanol complex in sealed flask. These mixtures were heated on hot plate stirrer at 90oC for 2 hour. The methyl ester of the fatty acids residues were extracted by 500 μl hexane. Hexane fraction was evaporated and than analyzed by gas chromatography.

(ii) Analysis of FAME by GC: The FAME were analyzed using a gas chromatography "Varian 450 – GC" equipped with a wcot fused silica CP-Sil 5 CB column (15 m length, 0.25 mm diameter, 0.25 μm film thickness). The oven temperature was first set at 80°C for 5 min and then raised to 305°C at 10°C/min and held there for 8 min. The injector and the FID temperatures were set at 230°C and 250°C respectively. Ultra-high purity helium was used as a carrier gas at a flow rate of 30 mL/min. The FAME amount of 2 μl were injected to GC. The FAME were identified by comparing their retention times with those of authentic standard and the results were presented as weight percentage of total FAME.

3) Positional distributions of FA in Structured Lipids

A modified version of the methods of Williams *et al.* (1995) and Luddy *et al*. (1964) was employed to release FA from the *sn*-1,3 positions of acylglycerols. This modification involves the use of borax to minimize the possibility of migration of residues from position 2 to positions 1 and 3 of the glycerol backbone. A known weight of TAG and an appropriate (*ca.* 20–50 mg) weight ofporcine pancreatic lipase were added to a 60-mL stoppered flask. Next, 0.65 mL Tris-HCl buffer (1 M, sodium salt, pH 8.0), 0.35 mL sodium borate (0.19 M), 0.1 mL CaCl2 (22%, w/w), and 0.25 mL bile salts (0.1%, w/w) were added. The resulting mixture (pH = 7.91) was maintained at 40°C for 1 min without shaking, then shaken at 300 rpm at 40°C for 7 min. The reaction was stopped by addition of 1 mL acetic acid (0.1 M). The mixture was extracted three times with 1 mL of chloroform.

The extracted reaction product were then evaporated and analyzed by thin layer chromatography. The chromatogram was developed with petroleum ether: diethil ether: acetic acid (60 : 40 : 1 v:v:v). The bands were visualized with iodine vapor and heating. The bands coresonding to 2-monoacylglycerols were scraped from the silica plate and extracted with 3 ml hexane, the resulting solutions than methylated by 200 μl addition of 400 μl BF3-methanol complex. These mixtures were heated at 90^{o}C for 2 hour. The methyl ester of the fatty acids residues were extracted by 500 μl hexane and than analyzed by gas chromatography. This protocol provides information concerning the distribution of FA residues at the *sn-2* position. The distribution of FA residues at the *sn*-1,3 positions was then calculated by subtracting the amount of a FA residue at the *sn*-2 position from the total quantity of this FA present in the corresponding unhydrolyzed structured lipid as determined by GC.

4) Glyceride Profiles of Structured Lipids (Martati, 1998 modification)

Structured lipid from interesterification were analyzed of glyceride profiles with Thin Layer Chromatography (TLC) to determine the components of monoglycerides, diglycerides, and triglycerides from the resulting fractions acilglycerol. Silica gel TLC plate before using was activated by heating in 105 oC for 1-2 hours. The sample was applied to the TLC plate and after drying the plate was developed in a tank that has been saturated with developer mixture of petroleum ether: diethyl ether: acetic acid (60:40:1). Development was done along the 1 cm from the top edge of

the plate. TLC plates were then dried and visualized with iodine vapor so visible spots of brown from acilglycerol components. Quantitative analysis performed with TLC Scanner Camag 3 "dummy" (S / N 081 124). The light used is D2 lamp, wavelength set at 350 nm, and a scan speed of 20 mm / sec.

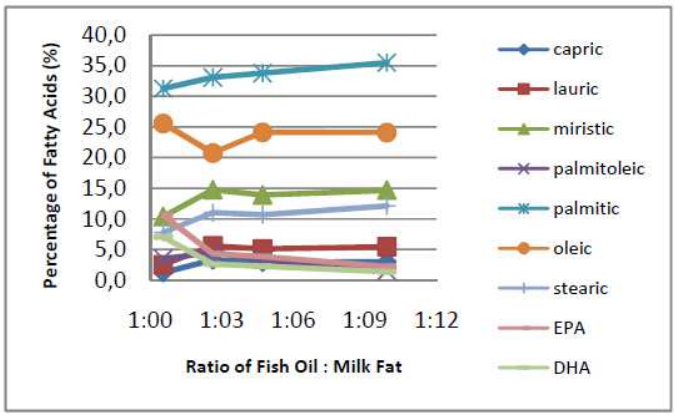
III. Results and Discussion

A. *Effect of Substrat Ratio Fish Oil and Milk Fat on Enzymatic Transesterification to the Composition and Regiospecificity Positions Fatty Acids of the Structured Lipids*

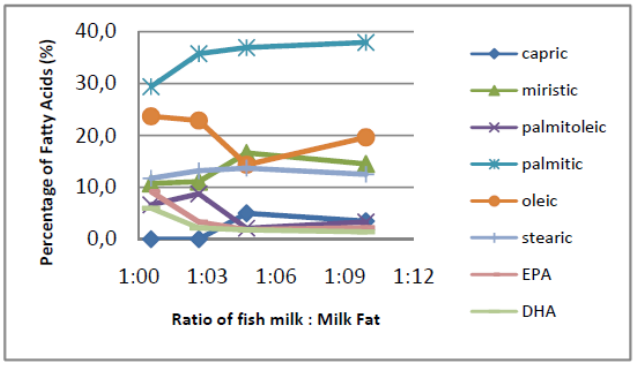
Substrate was the material which would react with the enzyme to resulting the product, so the enzymatic reaction (enzymatic interesterification) course is influenced by the condition of the substrate, in this case was the ratio between the fish oil milk fat. In general, if the milk fat was added at the excess, the reaction would tends to be dominated by fatty acids of milk fat and saturated fat on the incorporation of structured lipids and it would be expected to be mostly incorporated in the sn-1 and sn-3 of glycerol backbone. But keep in mind also that with the addition of more milk fat will also affect the regiospecificity of structured lipids that produced as the possibility of some saturated fatty acids from milk fat would be incorporated at sn-2 of the glycerol backbone.

In the enzymatic reaction, the more substrateswere used would the more the product was produced, until finally the addition of substrate has no effect on the product. The addition of substrate will be increasing the load or side product during the catalytic enzyme is available, but when the load exceeds the amount of the enzyme substrate that was then the excess will not be catalyzed substrate again, so it will not affect the amount of product. Even excess substrate can actually inhibit lipase activity (Willis and Marangoni, 2002).

In this study the ratio of fish oil and milk fat affect the composition and the regiospecificity of the of fatty acids structured lipid as in Fig 1. Fig 1 shown that the greater addition of milk fat in the transesterification reaction resulting of saturated fatty acids such as capric acid, lauric, myristic, palmitic, and stearic increased, while unsaturated fatty acids, especially EPA and DHA decreased. This was due to milk fat contains a lot of saturated fatty acids, so that with the addition of milk fat will be a greater increased in the content of saturated fatty acids and unsaturated fatty acids to be dropped. The chane of composition occurs in both at the total fatty acids at position sn-1, 2, 3 and in the middle at sn-2. At the ratio fish oil and milk fat 1:1, actually produce structured lipids which EPA and DHA were high, but most still incorporated in the sn-1 and sn-3 so that it is did not desirable and structured lipids that produced still has a strong fishy smell. And the ratio fish oil and milk fat: 1:3 content of EPA and DHA mostly at the sn-2, while saturated fatty acids mostly incorporated at the exterior or the sn-1 and sn-3 at the glycerol backbone. While the ratio of milk fat higher make the saturated fatty acids too high and also located at sn-2 so that it is less than desirable.



(a)



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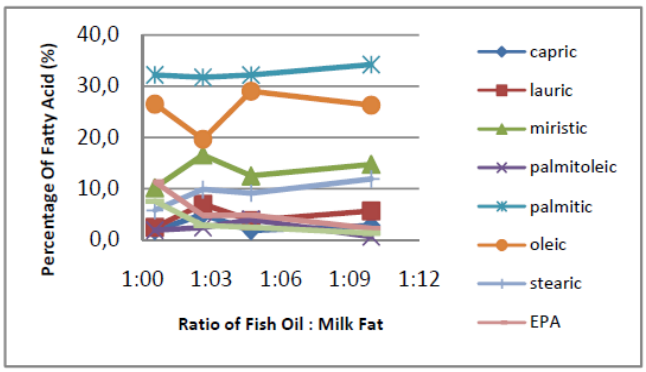

(c)

Fig 1. The effect of ratio fish oil and milk fat to the composition and regiospecificity fatty acids of the structured lipid. a. The composition of total fatty acids, b. The composition of fatty acids at the sn-2, c. The composition of fatty acids at th sn-1 and sn-3.z

Transesterification of fish oil with milk fat which saturated fatty acids were incorporated in the sn-1 and sn-3, while the unsaturated fatty acids in the sn-2 optimum at the ratio fish oil and milk fat was 1:3. Basically transesterification of fish oil with milk fat using Candida antartica lipase while more and more milk fat was added, the supply of saturated fatty acids which are also the higher and saturated fatty acids was expected incorporated in the position at sn-1 and sn-3 at the glycerol backbone fish oil, but the reaction was run competitively since the release of fatty acids from fish oil and milk fat will also competetion for re-esterified into glycerol backbone. Therefore, in order to incorporation of saturated fatty acids in fish oil, required

excessive amounts of milk fat. But keep in mind while the saturated fatty acid that was too high would also increasing the potential for saturated fatty acids incorporated at the sn-2 and that it did not expected. Therefore the condition of excessive amount of milk fat was certainly a need to be set at a certain ratio so that should not be too much saturated fatty acids were at sn-2. Therefore, the ratio of substrate that gives optimal results while the ratio of fish oil and milk fat was 1:3. At the ratio of the fish-oil and milk fat: 1:3 produced structured lipids in which the sn-2 occupied by unsaturated fatty acids such as oleic, EPA, and DHA were 22.9%; 3.3%, and 2.2% respectively. While at the position sn-1 and sn-3 incorporated by most of medium chain saturated fatty acids such as capric, lauric, myristic, palmitic, and stearic 5.0%, 7.0%; 16.6%; 31.7%, and 9.9 % respectively.

B. The Effect of Substrat Ratio Fish Oil and Milk Fat on enzymatic Transesterification to Glyceride Profile of Stuctured Lipids

Substrate ratios of fish oil and milk fat can affect the condition of the substrate or in the reaction system. With the increasing number of milk fat containing fatty acids with shorter carbon chain will increase the polarity of the system so that the condition can affect the balance of the reaction or the reaction would produces side products and intermediate products such as diglycerides and monoglycerides. Glyceride profiles of the structured lipid were affected by the ratio of the substrate was shown in Fig 2.

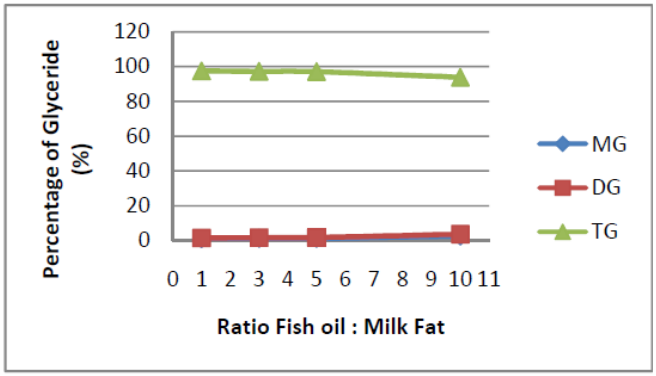

Fig 2. The effect ratio of the fish oil and milk fat to glycerides profile of the structured lipids. The amount of fish oil remains while the amount of milk fat was arranged (eg 3 means the ratio of Fish oil and milk fat was 1:3). MG: monoglycerides, DG: diglycerides, TG triglycerides

Based on Fig 2 shows that the greater addition of milk fat tended to decreasing of triglyceride composition, while diglycerides and monoglycerides tend to rise. This was demonstrated during reactions occur partial hydrolysis on structured lipid. Increased milk fatty acids will reduce the amount of triglycerides, this may be caused by the activity of the lipase enzyme from *Candida antartica* have specifications hydrolyze short-chain fatty acids relative to the more easily hydrolyzed than long-chain fatty acids, so there are some short and medium-chain fatty acids were hydrolyzed and non-esterified again. Milk fat contains a lot of shorter-chain fatty acids can also increase the polarity of the system which also can increase the activity of lipase to hydrolyze fatty acids from the glycerol backbone, which may contribute to the occurrence of partial hydrolysis of triglycerides will be decreased while the mono-and diglycerides will be increased. In this study, the use of milk fat also has a free fatty acid were higher than fish oil, free fatty acids will also affect the balance of the reaction and can stimulate the occurrence of partial hydrolysis. So with greater milk fat was added, then TG was decreased while the MG and DG were increased.

IV. Conclusions

Transesterification reaction between fish oil and milk fat that cause displacement acyl wherein the medium chain fatty acids and saturated fats tend to be on the outside position (sn-1 and sn-3), while unsaturated fatty acids are at the center position (sn-2) was optimum at the ratio of fishoil and milk fat was 1 : 3. In the ratio fish-oil: milk fat 1:3 produced structured lipids in which the sn-2 incorporated by unsaturated fatty acids such as oleic, EPA, and DHA was 22.9; 3.3, and 2.2% respectively. While in position sn-1, 3 incorporated mostly medium chain and saturated fatty acids such as capric, lauric, myristic, palmitic, and stearic acid 5.0; 7.0; 16.6; 31.7, and 9.9 % respectively. The ratio substrat did not affect the profile of glyceride on structured lipid. Triglyceride tend to decreased, and the diglyceride and monogliceride was increased with an increase of milk fat on substrate but not significantly.

References

[1] Endo, Y., S. Hoshizaki, dan K. Fujimoto., 1997. Oxidation and Synthetic Triacylglicerols Containing Eicosapentaenoid and Docosahexaenoic Acid: Effect of Oxidation system and Triacylglicerol Structure. J. Am. Oil. Chem.

[2] Irimescu, R., K. Furihata, K. Hata, Y. Iwasaki, dan T. Yamane. 2001a. *Utilization Of Reaction Medium-Dependent Regiospecifity Of Candida Antartica Lipase (Novozyme 435) For The Synthesis Of 1,3-Dicapryloyl-2-Docosahexaenoyl (Or Eicosapentaenoyl) Glycerol. J. Am. Oil. Chem. Soc. 78 (3) :285 – 289*

[3] Irimescu, R., K. Furihata, K. Hata, Y. Iwasaki, dan T. Yamane. 2001b. *Two-Step Enzymatic Synthesis of Docosahexaenoic Acid-Rich Symmetrically Structured Triacilglycerols Via 2-Monoacylglycerols. J. Am. Oil. Chem. Soc. 78 (7) :743 – 748* M. Wegmuller, J. P. von der Weid, P. Oberson, and N. Gisin, “High resolution fiber distributed measurements with coherent OFDR,” in *Proc. ECOC’00*, 2000, paper 11.3.4, p. 109.

[4] Luddy, F.E., R.A. Barford, S.F. Herb, P. Magidman, and R.W. 1964. Riemenschneider, Pancreatic Lipase Hydrolysis of Triglycerides by a Semimicro Technique, *J. Am. Oil Chem. Soc. 41*:693–(2002) The IEEE website. [Online]. Available: http://www.ieee.org/

[5] Martati, E. 1998. *Hidrolisis Minyak Hati Ikan Cod dengan Lipase Teramobil dari Mucor miehei*. Thesis. Program Pascasarjana Universitas Gadjah Mada. Yogyakarta.M. Shell. (2002) IEEEtran homepage on CTAN. [Online]. Available: http://www.ctan.org/tex-archive/macros/latex/contrib/supported/IEEEtran/

[6] National Research Council, 1976, Fat Content and Composition of Animal Products, Printing and Publishing Office, National Academy of Science, Washington, D.C., ISBN 0-309-02440-4; p. 203, online edition.

[7] Park, P. W. dan R. E. Goins. 1994. In Situ Preparation of Fatty Acids Metil Ester for Analysis of Fatty Acids Composition in Food. J. of Food Science. 59 (6): 1262 –1266

[8] Subroto, E, C. Hidayat, dan Supriyadi. 2008. Interesterifikasi enzimatis minyak ikan dengan asam laurat untuk sintesis lipid terstruktur. Jurnal Teknologi dan Industri Pangan, vol 19: 151 - 156A.

[9] Torres, C. F., H. S. Garcia, J. J. Ries, dan C. G. Hill, 2001. Esterification of Glicerol With Conjugated Linoleic Acid and Long-

Chain Fatty Acid from Fish Oil. J. Am. Oil. Chem. Soc. 78 (11) : 1093 – 1098

[10] Williams, J.P., M.U. Khan, and D. Wong. 1995. A Simple Technique for the Analysis of Positional Distribution of Fatty Acids on Diand Triacylglycerols Using Lipase and Phospholipase A2, J. Lipid Res. 36:1407–1412 (1995).