The Malaysian Journal of ANALYTICAL SCIENCES ISSN 1394-2506

SYNTHESIS OF PALM OIL HIGH IN DIACYLGLYCEROL THROUGH DIRECT ESTERIFICATION

(Penghasilan Minyak Sawit yang Kaya dengan Diasilgliserol Melalui Kaedah Pengesteran Terus)

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Abstract

Palm oil (PO) mainly constitutes of 90-98% of triacylglycerol, 2-6% of diacylglycerol (DAG) and 2-5% of monoacylglycerol. This study was carried out to produce PO that is high in DAG through direct esterification using 1,3 positional specific lipase from *Rhizomucor miehei* (RM IM) and *Thermomyces lanuginose* (TL IM) as catalysts. Palm olein oil has been hydrolysed by water and 4% enzyme in a controlled water bath at 300 rpm for 48 hours at a temperature of 60±2^{o}C while the esterification process was carried out in a same condition except it was run for 24 hours only. Samples DAG A1 and DAG B1 were synthesized products of the first replication using 10% enzyme TL IM and 10% enzyme RM IM respectively while samples DAG A2 and DAG B2 were synthesized products of second replication. DAG spots found on the thin layer chromatography (TLC) plates of samples DAG A1, A2, B1 and B2 bigger than the spot of the control sample. Based on high performance liquid chromatography chromatogram peak area, the total DAG accumulation showed significant differences ($p<0.05$) between the usage of enzymes TL IM and RM IM which were 34.28% and 45.67% respectively. The esterification method has clearly increased the DAG content of the control sample which was only 3.17%. Significant differences ($p<0.05$) also existed in the iodine value (IV), melting and crystallization temperature of all the samples. IV of control sample, DAG A and B were respectively 56.00, 35.00 and 30.50. Differential scanning calorimetry curves showed the melting and crystallization temperature were respectively -3.73^{o}C and -5.72^{o}C for samples using TL IM while -4.92 and -6.56 respectively for RM IM. The results concluded that the usage of enzyme RM IM is more effective in the production of PO high in DAG and efficiency of direct esterification process has been proved.

Keywords: palm oil, diacylglycerol, triacylglycerol, *Rhizomucor miehei, Thermomyces lanuginose*

Abstrak

Minyak kelapa sawit secara amnya terdiri daripada 90-98% triasilgliserol, 2-6% diasilgliserol (DAG) dan 2-5% monoasilgliserol. Kajian ini telah dijalankan untuk menghasilkan minyak sawit yang kaya DAG melalui tindak balas pengesteran terus bermangkin lipase spesifik 1,3 daripada *Rhizomucor miehei* (RM IM) dan *Thermomyces lanuginose* (TL IM). Minyak sawit dihidrolisis menggunakan 4% enzim dalam kukus air pada kelajuan 300 rpm pada suhu 60±2^{o}C selama 48 jam. Pengesteran terus pula dilakukan dalam kukus air selama 24 jam pada kelajuan 300 rpm pada suhu 60±2^{o}C. Sampel DAG A1 dan DAG B1 adalah hasil sintesis daripada tindakan 10% enzim TL IM dan 10% enzim RM 1M masing-masing pada replikasi pertama manakala sampel DAG A2 dan DAG B2 pula ialah hasil sintesis dari replikasi kedua. Analisis kromatografi lapisan nipis menunjukkan tompokan DAG yang lebih besar dalam keempat-empat sampel DAG A1, A2, B1 dan B2 berbanding sampel asal. Anggaran purata keseluruhan DAG yang terhasil dalam sampel dengan tindakan enzim TL IM dan enzim RM IM berdasarkan luas puncak daripada kromatogram kromatografi cecair berprestasi tinggi (HPLC) adalah masing-masing sebanyak 34.28% dan 45.67%. Keputusannya juga menunjukkan wujudnya perbezaan bererti ($P< 0.05$) antara kedua-dua sampel DAG A dan B. Nilai iodine dalam minyak sawit, sampel DAG A dan B masing-masing adalah 56.00, 35.00 dan 30.50. Daripada lengkuk kalorimetri pengimbas pembezaan (DSC), suhu purata puncak peleburan dan puncak pengkristalan minyak yang terhasil daripada tindak balas pengesteran terus masing-masing sebanyak -3.73^{o}Cdan -5.72^{o}C untuk sampel A dengan tindakan enzim TL IM. Manakala untuk sampel B dengan tindakan enzim RM IM purata suhu puncak peleburan dan puncak pengkristalan masing-masing sebanyak -4.92^{o}C dan -6.56^{o}C. Suhu peleburan dan pengkristalan dalam sampel kawalan adalah sebanyak 5.06^{o}C dan -1.75^{o}C

masing-masing. Kesimpulannya, enzim RM IM lebih berupaya menghasilkan minyak kelapa sawit yang kaya DAG dan keberkesanan tindak balas pengesteran terus dalam menghasilkan minyak yang kaya DAG juga telah dibuktikan.

Kata kunci: minyak kelapa sawit, diasilgliserol, triasilgliserol, *Rhizomucor miehei*, *Thermomyces lanuginose*

Introduction

Palm oil is produced from a part of palm fruit (*Elaeis guineensis*) known as mesocarp. The hybrid of *Dura* and *Pisifera* known as *Tenera* is the type of palm fruit cultivated abundantly in Malaysia. Palm oil can be classified either as saturated or unsaturated fat due to the equal amount of saturated and unsaturated fat found in the oil [1]. Saturated fatty acid in the palm oil consist of 44% palmitic acid, and 5% oleic acid. While unsaturated fatty acid are in the form of 39% oleic acid (mono-unsaturated fatty acid) and 10% linoleic acid (polyunsaturated fatty acid) [2]. Triacylglycerol (TAG) is the main component in the palm oil followed by diacylglycerol (DAG) around 6-8% and monoacylglycerol (MAG) with quantity less than 1%.

World Health Organization in the year 2000 has reported that more than one billion adults in the world are in the category of fat while almost 300 million adults are classified as obese. Fat accumulation in the body is always associated with dietary intake that is higher in fat (lipid). The only way to reduce the obese cases in the world is through consumption of low fat food which is very difficult to practice. This is because the foods that taste good is somehow compose of fat. That is why food compose of modified fat through the scientific method is most preferred by consumers [3]. Consumers can eat this modified food products as much as possible without any worries of fat accumulation in the body. Modified fat is mainly compose of short and medium chain fatty acid from MAG and DAG replacing long chain fatty acid from TAG. Fat modification from TAG to DAG and MAG can be done through esterification method. Synthesis of DAG oil is on about the scientific proof that modified fat is good for health.

Some of the advantages of considering DAG oil in the diet over TAG high oil are it controls the triglyceride serum level as well as it suppress fat accumulation in the body [4]. DAG oil compose of unique physicochemical property due to the presence of free hydroxyl group in the structure. DAG oil will increase the usage of fat as energy by reducing the rate of respiration. High fitosterol content in the DAG oil is capable of reducing low density lipoprotein [3]. DAG oil can be produced from free fatty acid and glycerol through direct esterification using 1,3-positional specific lipase from *Rhizomucor miehei* as catalyst [5]. Enzymatic direct esterification is one of the most prominent methods in producing oil that is high in DAG content.

In this study, palm oil that is high in DAG (34-45%) was produced with an enzyme as catalyst in a solvent free condition. Palm oil that is high in DAG content has been synthesized through direct esterification method catalyst by 1,3 positional specific lipase from *Rhizomucor miehei* (RM IM) and *Thermomyces lanuginose* (TL IM).

Materials and Methods

Materials

Palm olein cooking oil (*Seri Murni*) purchased from the market has been used for this study. The two catalysts that were used for this study 1,3 positional specific lipase from *Rhizomucor miehei* (RM IM) and *Thermomyces lanuginose* (TL IM) were supplied by Novozymes Malaysia Sdn Bhd. All other solvents and reagents were analytical or chromatographic grades.

Enzymatic Hydrolysis

A total of 150 g of palm oil was transferred into a 250 ml conical cone and followed by the addition of 60% water and 4% enzyme TL (based on the quantity of oil). The mixture has been placed on a vortex for uniform distribution of the enzyme. The mixed sample, then was transferred into the test tubes and was left it in the water bath with temperature 60±2ºC for 48 hours at the speed of 300 rpm. The samples were strained out to separate the enzyme and the glycerol. The resultant from this process was known as hydrolysate. Finally glycerol was added to the hydrolysate. The steps were repeated with enzyme RM [6].

Direct Esterification Of Glycerol With 1,3-Specific Lipase TL IM and RM IM

Direct esterification was a solvent free process. In this study, 2 replicates of direct esterification of hydrolysate with glycerol were conducted. The results obtained through this process were labelled as DAG A1 and DAG A2 respectively. A total of 100 ml of hydrolysate compose of 1.33 mol free fatty acid and 0.22 mol glycerol in the stoichiometri ratio 2:1 (free fatty acid : glycerol) were added in 250 ml conical cone followed by the addition of 10% enzyme *Thermomyces Lanuginosa* (weight/ substrate weight). The mixture was placed in the water bath with temperature of 60±2ºC at the speed of 300 rpm. After 24 hours, palm oil that is high in DAG content was obtained. The steps were repeated with enzyme *Rhizomucor miehei* that were labelled as DAG B1 and DAG B2. Finally, Thin Layer Chromatography (TLC) and High Performance Liquid Chromatography (HPLC) analysis were conducted on this oil to determine the extraction efficiency of the two lipases [7].

Thin Layer Chromatography

Thin Layer Chromatography (TLC) separations of the palm olein cooking oil were done on silica gel coated with aluminum layer supplied by Merck. After developing the plate with a mixture of n-hexane/diethyl ether/ glacial acetic acid (70/30/1.5, v/v/v), the migration of MAG, DAG, TAG and FFA was observed by lightly spraying the plate with potassium permanganate 0.5% (w/v) that was visualized under UV light [8].

High Performance Liquid Chromatography

The synthesized samples were analyzed in HPLC to compare the percentage of MAG, DAG and TAG before and after the direct esterification process. The column used was reversed-phase C18 (4.6 x 250 mm, 5 µm), and the mobile phase was acetonitrile:aceton (55:45 v/v). The flow rate was controlled at 1ml/hour.

Differential Scanning Calorimetry (DSC)

The melting and crystallization profile of the samples were determined using a Mettler Toledo Differential Scanning Calorimeter model DSC 822e. Calibration was undertaken with an indium standard. About 5 mg of precisely weighed (± 0.005mg) oil sample was placed in the sealed DSC pan and was melted at 70^{o}C for 30 minutes before cooling to 0^{o}C, after which it was held for 90 minutes before being transferred to the DSC head. The pan was held at -40^{o}C for 5 minutes prior to measurement. DSC melting curves were recorded at a heating rate of 5^{o}C/min from -50^{o}C to a maximum temperature of 40^{o}C.

Iodine Value

Iodine value showed the degree of unsaturation of the constituent fatty acids in an oil or fat and is thus a relative measure of the unsaturated bonds present in the oil or fat [9]. Iodine value is expressed in grams of iodine absorbed by100 grams of oil or fat. 0.5 gm of oil was weighed and transferred into Iodine flask followed by addition of 20 ml of siklohexane and 25 ml of Wij's solution. The flask was covered by aluminum foil and left in a dark place. After half an hour 20 ml of pottasium iodide solution (15%) was added in the flask and titrated against 0.1 N sodium thiosulphate solution. Upon the appearance of yellow color, 1 ml of starch indicator was added in the flask and again titrated against the sodium thiosulphte solution from the burette until the disappearance of blue color. The above procedures were repeated without any sample and the reading was noted for blank titration. Iodine value determined by the following equation (1):

$$\text{Iodine value} = (V1 - V2) \times N \times 12.69 / Wt \quad (1)$$

where,

V1 - Volume of thiosulphate required by blank (ml)
V2 - Volume of thiosulphate required by sample (ml)
N - Normality of thiosulphate
Wt - Weight of the sample

Results and Discussion

Enzymatic hydrolysis was the main step involve in the production of palm oil high in DAG whereby it break down the complete triglyceride structure into FFA and glycerol. In this study, the efficiency of breaking the triglyceride

bond was observed by the usage of 1,3 positional specific lipase from *Rhizomucor miehei* (RM IM) and *Thermomyces lanuginose* (TL IM). Figure 1 obviously showed that enzyme TLIM produce more FFA which was about 35%/24 hours compare to less than 5%/24 hours by enzyme RMIM.

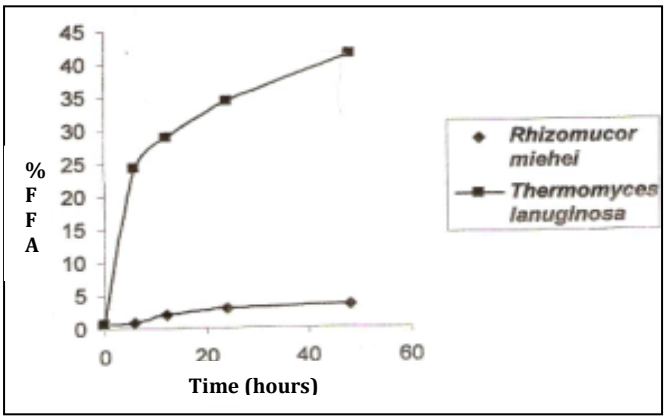


Figure 1. Rate of hydrolysis by 1,3 positional specific lipase from *Rhizomucor miehei* (RM IM) and *Thermomyces lanuginose* (TL IM).

Two samples of palm oil high in DAG have been synthesized through the direct esterification process. DAG A1 and DAG B1 were obtained through direct esterification respectively from TLIM and RMIM while the second replicate products were DAG A2 and DAG B2. The results showed that RMIM produce palm oil with 45.6% DAG (average of DAG B1 and DAG B2) while TLIM with 34.28 % DAG (average of DAG A1 and DAG A2). The direct esterification method that was used in this study has been proved effective since the original palm olein cooking oil (*Seri Murni*) contain only 3.17% DAG (Figure 2). Scheme 1 and 2 showed the hydrolysis reaction and direct esterification process involve in the production of palm oil high in DAG.

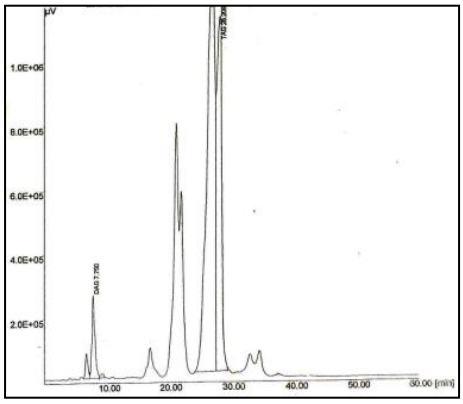
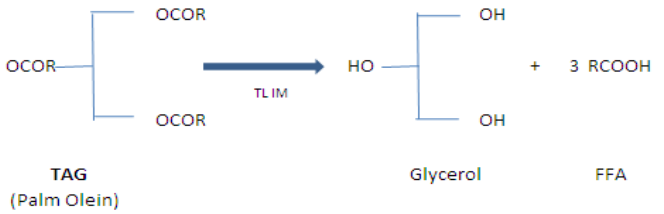
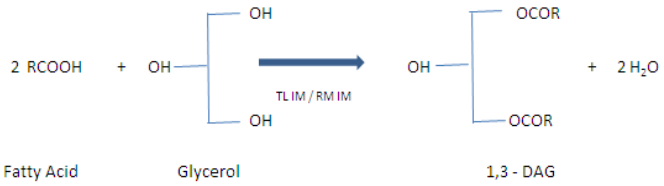


Figure 2. Chromatogram of palm olein oil



Scheme 1. Enzymatic hydrolysis reaction



Scheme 2. Direct esterification reaction

The spots on the TLC plates were directly observed after the development but since the compounds were colorless it was visualized under UV light [10]. The spots interfered with the fluorescence and appeared as a dark spot on a glowing background which was outlined with a pencil to mark their locations as shown in Figure 3. In both the TLC plates developed with palm olein cooking oil and synthesized DAG oil, the TAG migrates the most quickly followed by FFA, 1,3-DAG, 1,2-DAG and MAG. TLC separation of the palm olein cooking oil showed that majority of the lipid molecular species was TAG which was observed by the biggest spot on the TLC plate. TLC separation of the synthesized DAG A1, DAG A2, DAG B1 and DAG B2 showed that majority of the lipid molecular species was DAG.

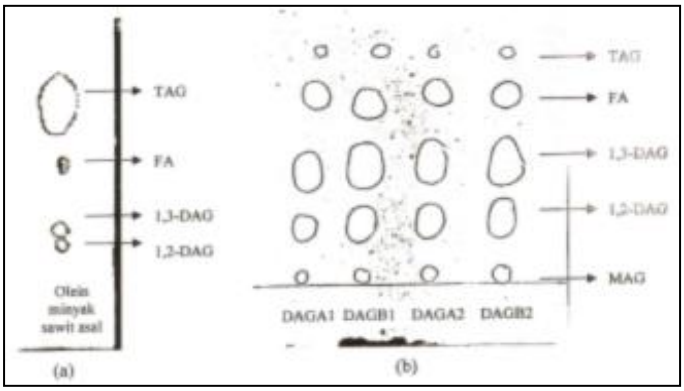


Figure 3. TLC plate showed lipid separation of palm olein cooking oil (a). TLC plate showed lipid separation of DAG A1, DAG A2, DAG B1 and DAG B2 (b).

On the contrary, the TAG spot was much smaller on the TLC plate that was developed with DAG A and DAG B. The qualitative result of the TLC method showed that the TAG content in the palm olein oil was much higher with low DAG content. While after the esterification process the DAG content was higher with biggest spot that was formed on the TLC plate that developed with DAG A and DAG B. 1,3 positional specific lipase from *Rhizomucor*

miehei is very much effective in producing DAG oil high in 1,3-DAG [11]. The total time that was taken for this lipid separation was 45 minutes.

Table 1 below showed the percentage of TAG, DAG and MAG found in palm olein oil, DAG oil A and DAG oil B through HPLC analysis. Palm olein oil showed the highest percentage of TAG which was about 44.40 followed by 3.17 DAG content. MAG was absent in this control. DAG B that was synthesized by RM IM has the highest percentage of DAG content with 45.67 compared to DAG A with only 34.28 DAG content. The difference between the DAG content in DAG A and B was due to the efficiency of the enzymes that were used during the direct esterification process [11].

Table 1. Comparison of TAG, DAG and MAG percentage found in palm olein oil, DAG A and DAG B

Samples	**%TAG**	**%DAG**	**%MAG**
Palm olein oil	44.40 [a]	3.17 [c]	0 [c]
DAG A (TL IM)	17.42 [b]	34.28 [b]	24.52 [a]
DAG B (RM IM)	15.25 [b]	45.67 [a]	17.34 [b]

*Values of the same column, followed by the same letter (a,b,c) are not statistically different ($p < 0.05$)

Statistical analysis showed that TAG content differed significantly ($p < 0.05$) between the palm olein oil, DAG A and DAG B, while no significant difference in the TAG content between DAG A and DAG B. Significance difference ($p < 0.05$) were observed in the percentage of DAG and MAG in between all the three samples of palm olein oil, DAG A and DAG B.

Melting and crystallization, two commonly used physical events to characterize thermal behavior of oil samples, require the intake or release of thermal enthalpy [12]. DSC is the most suitable to determine these physical properties. Statistical analysis showed significance difference in the melting temperature of all the three samples. The results from table 2 indicated that palm olein oil has highest melting point compare to DAG A and DAG B. This is due to the highest TAG content in palm olein oil that delay the breaking down process of molecules that formed with strong bond.

Melting point is interrelated with iodine value of a particular sample. Table 2 showed that palm olein oil has high iodine value of 56.0 with high degree of unsaturation due to higher TAG content. The results also showed that DAG B has lower iodine value (30.50) compare to DAG A (35.0). The DAG B that is high in DAG content has lower unsaturated fatty acids that decrease the iodine value [13]. This has been proved by a study conducted by [14], stated that melting point of oil is highly influenced by number of carbons in a hydrocarbon chain and the degree of unsaturation. That explains why palm olein oil with higher degree of unsaturation has higher melting point compare to DAG A and DAG B.

Melting point of the samples has decreased after the direct esterification process due to the presence of DAG and MAG. The bonds between molecules in oil sample will be weak with the increase in DAG and MAG content subsequently will reduce the melting point of the sample [15]. This also explains that DAG B with higher DAG content (45.67%) has lower melting point -4.92^{o}C compare to DAG A with lesser DAG content (34.28%).

As for crystallization point, statistical analysis result showed that the crystallization temperature of palm olein oil differed significantly ($p<0.05$) with DAG A and DAG B while no significance difference in between DAG A and DAG B. Table 2 showed that the crystallization point of palm olein oil was higher (-1.75 ^{o}C) compare to DAG A (-5.72 ^{o}C) and DAG B (-6.56 ^{o}C) after the esterification process.

Table 2. Iodine value, melting and crystallization point of oil samples before and after esterification process.

Samples	Melting Point (^oC)	Crystallization point (^oC)	Iodine value
Palm olein oil	5.06 [a]	- 1.75 [a]	56.0 [a]
DAG A (TL IM)	- 3.73 [b]	- 5.72 [b]	35.0 [b]
DAG B (RM IM)	- 4.92 [c]	- 6.56 [b]	30.5 [c]

*Values of the same column, followed by the same letter (a,b,c) are not statistically different ($p < 0.05$)

Oil with higher DAG content capable to form crystals faster compare to oil with higher TAG content [16]. So DAG B with higher DAG content capable in forming crystals at a faster rate at a temperature of -6.56^{o}C followed by crystallization of DAG A and palm olein oil. This also explains the higher crystallization temperature of palm olein oil that is high in TAG content [17].

Conclusion

Enzyme *Rhizomucor miehei* (RM IM) is more effective in the production of PO high in DAG with 45.67% compare to *Thermomyces lanuginose* (TL IM) that produced oil with only 34.28% of DAG content. The increased DAG content in the synthesized oil has lower unsaturated fatty acids that decreased the iodine value (30.5 for RM IM and 35.0 for TL IM). Subsequently it produced palm oil high in DAG with low melting point (- 3.73 ^{o}C for TL IM and - 4.92 ^{o}C for RM IM) and crystallization point (- 5.72 ^{o}C for TL IM and – 6.56 ^{o}C for RM IM). Efficiency of direct esterification process by the usage of catalysts *Rhizomucor miehei* (RM IM) and *Thermomyces lanuginose* (TL IM) also has been proved.

Acknowledgement

Authors would like to express deep gratitude and appreciation to the professors, associate professors, lecturers and staffs of the Food Science Programme from UKM.

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