CHAPTER IV

Interesterification degree of dual lipase system in acidolysis of palm stearin and oleic acid

4.1 Introduction

Interesterification oftenly involve a single lipase reaction whether in immobilized form or free lipase to produce structure lipid. Immobilized lipozyme TL IM commonly used to act as a biocatalyst to the interesterification reaction. Shekarchizadeh and Kadivar (2012), have interesterified camel hump fat and tristearin using 10% (w/w) TL IM to produce a potential cocoa butter analogue. The reaction which conduct under the presence of supercritical fluid carbon dioxide rather than used organic solvent have produced most similar cocoa butter analogue under its optimized condition paramters.

On the other hand, Lee et al. (2010) studied interesterification by using TL IM to synthesis 1,3-dioleoyl-2-palmitoylglycerol (OPO)-rich human milk fat substitute (HMFS) from tripalmitin-rich fraction and ethyl oleate. The research found to have 31.43% of OPO in HMFS under optimum reaction condition. All this various end-products reactions were employed a single lipase system.

These, have come to an extend whereby researchers seek to understand multiple enzymes activity towards a certain reaction. Wang et al. (2012) catalyzed acidolysis of canola oil with caprylic acid with several enzymes including immobilized lipase and free lipase, lipozyme TL IM, lipozyme RM IM, Novozyme 435, lipase AK, newlase F and lipase AY. All these lipases were screened for their ability to incorporated into the canola oil and act as a single lipase system.

However, back in 2010, Huang et al. used combinations of immobilized lipases, novozyme 435 and lipozyme TL IM to carried out the transesterification of lard for biodiesel production using response surface methodology (RSM). They found that under 5% summed amount of enzyme load, 1.8% of Novozyme 435 and 3.2% lipozyme TL IM generated a markedly positive synergistic action, gives the highest methyl ester yield, 93.5% (Huang et al., 2010).

Thus, this allows more study on multiple enzymes combinations in a single reactions to be done. The effect of combinations of enzymes may varies according to the type of reactions and corresponding parameters to be studied. Based on Ibrahim et al. (2007), a synergistic effect occurred for many lipase combinations but largely depending on the lipase species mixture and their ratios. Ibrahim and his co-researchers investigated a dual lipase system reaction once in interesterification of palm stearin and coconut oil by combining lipozyme TL IM with lipozyme RM IM and lipozyme TL IM with lipase AK. The interaction between lipases was measured according their degree of interesterification and synegystic effect coefficient. The findings showed that, 70% of TL

IM combine with 30% lipase AK shows high interesterification degree where the two lipases plays vital role in catalyzing interesterification of palm stearin and coconut oil. They believe that there was "assisted immobilization" effect of the immobilized lipase towards the co-existed free enzyme.

By seeing the possibility of combination enzymes to promote the reaction rate and obtaining the optimum condition, thus, it has been a favor to other researchers to experiment lipases combination on various type of lipid modification. Application of lipase combination in catalyzing a reaction was extended in acidolysis reaction of palm stearin and oleic acid to see whether the same pattern of synergistic effect from two lipases imply towards this acidolysis reaction of palm stearin and oleic acid. The synergistic effect of both lipases measured as interestification degree of the acidolysis reaction.

4.2 Materials

Lipozyme TL IM was purchased from Novozymes, Germany. Lipase AK Amano 20 purchased from Sigma-aldrich, Japan. Palm Stearin was obtained from Sime Darby Jomalina, Malaysia. Oleic acid, n-hexane of GC grade, chloroform of liqud chromatography (LC) grade, acetonitrile, acetone, and sodium methoxide were purchased from Merck, Germany.

4.3 Methods

4.3.1 Production and Purification of Modified Palm Stearin using Short Path Distillation

Acidolysis reaction of palm stearin and oleic acid conducted by following Nagachinta and Akoh (2012), Kadivar et al. (2014) and Kamar et al. (2011) under 250 rpm orbital shaking water bath at 60°C. The up-scale reaction was conducted in 500g bioreactor according to method Koh et al. (2008) using the optimum condition of four parameters obtained using RSM, 3 hours time reaction, 10% enzyme load, 5:5 ratio of lipase AK towards Lypozyme TL IM and 1:3 substrate molar ratio of palm stearin towards oleic acid. The hot reaction mixture was added anhydrous sodium sulphate and filtered using filter paper Whatman No.1 at 50°C.

Excess fatty acid from modified palm stearin was removed using short path distillation as it can cause rancidity to the fat by following Artiin et al. (2009). The melted reaction mixture was transferred to the reservoir and introduced drop wise into the wiped film KD6 30624204 short path distillation apparatus (Germany), to separate solvent at 80°C under atmospheric pressure. Solvent-free product was distilled at 180°C at 700 mTorr at a feed rate of 100 ml/h. The reservoir heated with external hot water at 85°C to keep the sample in a liquid state. The internal cooling trap condenser temperature maintained at 5°C, which then condensed the evaporated free fatty acids. The purified modified palm stearin was then preceded to triacylgliserols (TAG) analysis.

4.3.2 Enzyme Reusability

Enzyme reusability was done by following the method of Oliveira and Rosa (2006) without washing the enzyme with solvent. The process was conducted during the upscale of acidolysis of palm stearin and oleic acid in the stirred tank 1 kg bioreactor. The reaction was performed under optimized parameters as stated in method 4.3.1. The enzyme was collected after the filtration and then reused to catalyze the next batch of acidolysis reaction. This procedure was done repeatedly with the same condition till 5 times reactions of acidolysis. The products for each batch were collected and denoted as batch1 (S1), batch 2 (S2), batch 3 (S3), batch 4 (S4) and batch 5 (S5).

4.3.3 Determination of TAG Composition for Interesterification Degree

Determination of TAG according to Koh et al. (2009) with the use of different detector. The sample melted up at 60°C prior to sample preparation. Sample (10%) prepared by weighing 0.1 ml of the sample into an Eppendorf tube and making up to 1 ml with the solubilization solvent (1:1 acetone-chloroform mixture). After that, samples were filtered before being put into vials and injected. The running time for each sample was 50 minutes. Samples were determined by High Performance Liquid Chromatography (HPLC) using a WATERS series model equipped with evaporating light scattering detector (ELSD) and RP-18e, LiChroCART 250-4.6 column (75mm × 5µm × 4.6mm). The column maintained at 35°C. The mobile phase, a mixture of acetone/acetonitrile (65:45, v/v) at a flow rate of 1.0 ml/min. Samples were purified by passing through a

silica gel column and eluting with acetone. The solvent was evaporated and 5µl was injected into the HPLC. The peaks were identified by comparing the retention times with the standards and literature values. The values reported as relative percentages for individual TAG. The analysis was carried out in triplicate.

4.3.4 Interesterification Degree of Acidolysis of Palm Stearin and Oleic Acid

Interesterification degree (ID) calculated by following the method reported by Ibrahim et al. (2007). The change of relative contents of the two groups was viewed as an index denoting the reaction evolution. According to Ibrahim et al. (2007) the ID obtained were by equivalent carbon number (ECN) group yet for this acidolysis reaction, as it promote changes on sole peak of TAG, thus the calculation were based on area of the sole peak TAG from the HPLC chromatograms that have the highest and the lowest changes. For example, peaks 'a' had the major increment while peak 'b' was the component that shows major decrement. Therefore, the degree of interesterification (ID) can be simplified as;

$$ID = \frac{\text{area of peak a}}{\text{area of peak b}}$$

4.4 Results and Discussion

4.4.1 TAG composition

The triacylglycerols (TAG) compositions monitored based on HPLC chromatograms were oleic-oleic-oleic or triolein (OOO), palmitic-oleic-oleic (POO), palmitic-palmitic-

oleic (PPO) and tripalmitin or palmitic-palmitic-palmitic (PPP). The change in increment of OOO and POO, and decrement of PPO and PPP was expected after the acidolysis reaction. These changes then will promote to lower the melting point of the purified modified palm stearin.

Figure 15 shows comparisons amount of TAG between palm stearin (PS) and purified modified palm stearin (PMPS). Based on Figure 15, POO and OOO showed an increment for PMPS compared to PS sample while PPP and PPO had decreased. The increment in OOO and POO and decrement in PPO and PPP gave a general idea that both lipases effectively functions as a catalyst to incorporated the oleic acid and rearrange the fatty acids of the TAG in palm stearin during acidolysis reaction.

FIGURE 15: Amount of OOO, POO, PPO and PPP between PS and PMPS

*PS = palm stearin. PMPS = Purified modified palm stearin
*OOO = triolein, POO = palmitic-oleic-oleic, PPO = palmitic-palmitic-oleic, PPP = tripalmitin
*One-way ANOVA denoted that there was a significance difference among all samples for all four TAG, $p<0.005$.

Lipase enzymes cleave the fatty acid at the glycerol bond at *sn*-1,3 position according to their regiospecificity, thus promoting the rearrangement of fatty acids to occur and allows the incorporation of oleic acid to the glycerol bond to form new triacylglycerol. The increase in OOO and POO amount of 7.33% and 32.27% respectively for PMPS sample. Besides, after modification reaction, the percentage of PPP from PS has decreased from 35.14% to 14.78%. The amount of PPO did not have much difference (44.63% and 44.14%) for PS and PMPS. This may due cutting down of PPP TAG happen as much as PPO TAG. The difference between samples in TAG composition was less distinct as a result of random rearrangement of oleic acid during the reaction as supported by De Clercq et al. (2012).

Based on increase and decrease of TAG in the Figure 15, this shows that there was cleave of fatty acids at the *sn*-1,3 position and incorporation of oleic acid towards the glycerols backbone. The PPP mainly rearranged to PPO or POO or OOO, followed by PPO and POO to produce more POO and OOO, thus promoted a high increment mainly in OOO. This supported by Karabulut et al. (2009) where the study stated that incorporation of oleic acid into monoacid TAG except tricaprin and tricaprylin was high for all TAG with lipozyme TL IM catalyzed reactions than other two enzymes tested, lipozyme RM IM and Novozymes 435. However, because of the reaction parameters and dual lipase system used in this acidolysis reaction differ from Karabulut et al. (2009) which used single lipase system with different parameters and substrate, thus the result may different in the amount and percentage of incorporation.

Although the acidolysis reaction focused on incorporation oleic acid in position *sn*-1,3 of TAG, based on increment of OOO and POO yet the large increase of OOO shows that there was possibility of migration of oleic acid towards *sn*-2 position during the reaction. Besides, Wang et al. (2012) found out the same pattern in acidolysis of canola oil with caprylic acid. They discovered that although the lipases used in the study have been shown to be 1,3 specific lipases, the present of caprylic acid at the sn-2 position in the end product implied that movement of caprylic acid into the *sn*-2 position may occur during lipase-catalyzed acidolysis (Wang et al., 2012).

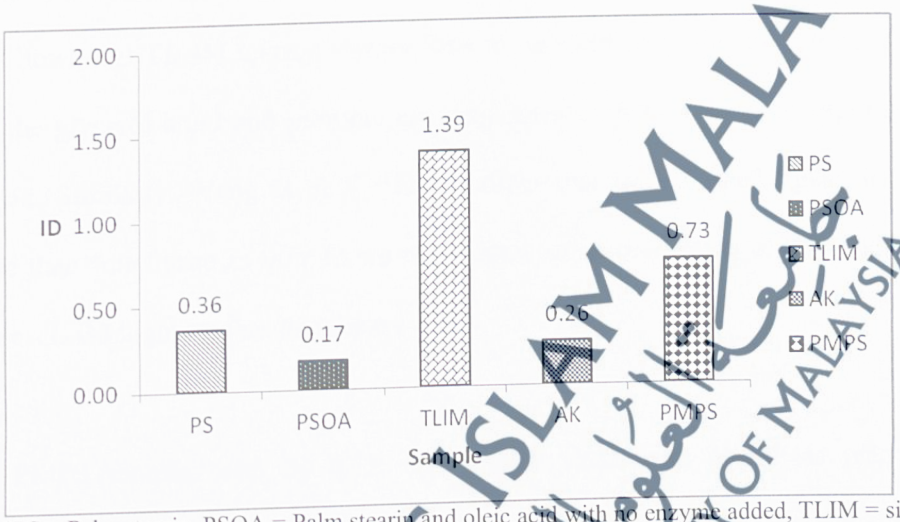
In addition, Zhang et al. (2004) and Xu et al. (1998) reported that the fatty acid at *sn*-2 position remain unchanged except that acyl migration took place with a higher content of byproducts in the system. Whereby, in this case, the supply of oleic acid was plenty and excess. The molar ratio of palm stearin and oleic acid in the reaction can create a condition excess of oleic acid in the system as well as together with other cleaved fatty acid, the free palmitic acid, thus promoted the acyl migration to the *sn*-2 position.

4.4.2 Interesterification degree of lipase AK Amano and Immobilized lipase TL IM in Acidolysis of Palm Stearin and Oleic Acid

Interesterification degree shows the enzymes activity and thus effectiveness of dual lipase enzyme system in acidolysis reaction. This resulted by the highest increment in TAG, which in this case POO, divide by PPO (the least decrease after the modification). The

comparisons of interesterification degree of PS, PSOA, TL IM, AK and PMPS shown in Figure 16.

FIGURE 16: Comparisons of Interesterification degree (ID) of certain sample.



*PS = Palm stearin, PSOA = Palm stearin and oleic acid with no enzyme added, TLIM = single lipase TLIM reaction, AK = single lipase AK reaction, PMPS = purified modified palm stearin with dual lipase reaction

Among those samples, single lipase reaction, TL IM, have the highest degree of interesterification (1.39). Modified samples, PMPS was in the middle range of the ID while PS, PSOA and single run lipase, AK grouped in lower ID. From the data obtained, it shows that palm stearin and substrate mixture without the enzyme that undergo identical condition reactions have lower ID because the rate of acidolysis reaction was very low. Oppositely, there was a contradiction in ID values between single lipase system reaction by using TL IM and AK. Sample TL IM shows high ID (1.39) while AK have very low ID (0.26). The PMPS sample have average ID (0.73) whereby in between the two sample of single lipase reaction TL IM and lipase AK ID.

Based on the graph, it shows that TL IM alone effectively functions in acidolysis reaction of palm stearin and oleic acid. The cleave of glycerol bond and incorporation of oleic acid into the bond have occurred in high degree within 3 hours of reaction. Compared to single lipase AK, it happened to react slowly as a biocatalyst within 3 hours reaction. This pattern shows that TL IM takes a shorter time to be effectively function to cut the fatty acid at the glycerol bond and promote rearrangement of fatty acid to incorporate more oleic acid. Similarly, Wang et al. (2012) reported that immobilized lipase was more effective than free lipase as they found that degree of incorporation of caprylic acid by lipozyme TL IM higher rather than lipase AK.

As for PMPS sample, with ID 0.73, this sample underneath acidolysis reaction by following the optimize condition of all parameters. Thus, for 3 hours of reaction time with 50% of TL IM and 50% lipase AK in total enzyme, the reaction degree was still within higher range of interesterification degree, however still lower as compared to TL IM alone. This may due to the enzyme ratio where the lower amount of TL IM slows down the enzyme activity in acidolysis reaction.

From the result obtained for ID based on Figure 16 it can be concluded that TL IM was effectively function to cleave fatty acid in glycerol bond and incorporate oleic acid into it even within short reaction time given. But then, the combination of lipase AK and TL IM within 3 hours gives drawbacks to the TL IM effectiveness. This research however have a 5:5 ratio between enzyme compared to Ibrahim et al. (2007) which have 7:3 TL IM towards lipase AK respectively. Thus, the lower ID may also due to low amount of TL

IM ratio to catalyze the reaction and act as carrier to free lipase to enhance the lipase AK activity.

The results contradict to what defines synergistic effect of dual lipase system in Ibrahim et al. (2007) worked whereby they found that the combination of both TL IM and lipase AK promoted higher ID value and automatically enhance fatty acid rearrangement. As based on the table, in this acidolysis reaction, the ID showed synergystic effect between the two lipases however, the TL IM alone is the most effective for this acidolysis reaction and the combination between the two not assisting the enzyme activity for the reaction, yet enhance the capability of lipase AK to be more effective within short time.

Dual lipase system pull back ability of TL IM to cleave and incorporate more oleic acid into TAG bond, however the TL IM may focus on helping lipase AK to enhance it capability as catalyst within that short time. Thus, in this case, the dual lipase system may not effectively increase the interesterification degree within short time yet, the immbobilize TL IM enhance the capability of non-immobilied lipase to perform more. A study from Ibrahim et al. (2007) supported this finding whereby there was "assisted immobilization" effect of the immobilized lipase towards the co-existed free enzyme. Besides, the PMPS sample was taken from the combinations of all 5 batches of enzyme reusability reaction whereby there might be high degree of interesterification in the first batch and it keeps reducing by time as the reuse of enzyme may affect the enzyme activity. Interesterification degree of all samples including 30 experiment from RSM samples were shown in Table 11.

TABLE 11: The interesterification degree (ID) of all samples and its parameters

RUN	ID (POO/PPO)	enzyme ratio*	enzyme load (%)	substrate molar ratio*	Reaction time (h)
1	1.65	9	12	3	7
2	1.26	11	10	5	5
3	2.09	5	12	3	7
4	2.39	7	14	5	5
5	1.09	7	10	1	5
6	0.75	9	8	7	3
7	1.46	7	10	9	5
8	1.70	7	10	5	5
9	2.45	7	10	5	9
10	2.28	5	12	7	3
11	1.83	9	12	7	7
12	0.78	7	10	5	1
13	1.24	9	12	7	3
14	1.89	5	8	3	7
15	0.94	5	12	7	7
16	1.64	7	10	5	5
17	1.79	7	10	5	5
18	1.22	9	12	3	3
19	1.26	5	8	3	3
20	1.52	7	10	5	5
21	1.83	5	8	7	7
22	2.43	3	10	5	5
23	1.82	5	12	3	3
24	1.10	9	8	7	7
25	1.40	7	10	5	5
26	1.12	5	8	7	3
27	1.23	9	8	3	7
28	0.80	7	6	5	5
29	0.85	9	8	3	3
30	1.37	7	10	5	5
PS	0.36	0	0	0	3
PMPS	0.73	5	10	3	3
PSOA	0.17	0	0	3	3
TLIM	1.39	0	10	3	3
AK	0.26	0	10	3	3

*enzyme ratio: denote as ratio of lipase AK. Example, 9 = 9 lipase AK:1 TL IM. Substrate molar ratio: denote as ratio of oleic acid. Value of palm stearin stick at ratio 1. Example, 3=1 palm stearin:3 oleic acid.

According to the result in this research, sole lipase AK gives ID of 0.26 within 3 hours reaction. Ibrahim et.al., (2007) reported that sole lipase AK catalyzed reaction was very slow, of which, in their findings, the interesterification degree was less than 0.5 even after 8 hours. As seen in run 2, however, the ID obtained was 1.26 for ratio 11:0 (exceeded 100% of lipase AK, 0% of TL IM) with longer reaction time given (5 h), high supply of oleic acid as acyl donor, (1:5, for palm stearin to oleic acid respectively).

This shown that lipase AK took longer time of reaction to act effectively in this acidolysis reaction to do the cleave and promotes incorporation of oleic acid to glycerol bond. Longer time reaction given (5 h) also can be seen in run 22 but with higher portion of TL IM percentage. This range ofreaction time alco can increased the interesterification degree of combination enzyme, which comprised 30% lipase AK and 70% TL IM (ID 2.43) compared to PMPS (ID 0.73). This gives an idea that by increasing the reaction time, it may increase the lipase AK and TL IM activity for the acidolysis of palm stearin and oleic acid.

Besides, PMPS and sole reaction enzyme samples have a substrate molar ratio of 1:3 (palm stearin towards oleic acid respectively). But in run 22, the ratio was 1:5 (palm stearin towards oleic acid respectively). Thus, the excess supply of oleic acid also may contribute to more incorporation of oleic acid to the glycerol bond resulting on formation of more OOO and POO TAG and reduce lots more PPP and PPO TAG which then produce higher ID for run 22. This is in line with the findings from the study on acidolysis of sunflower oil with a palmitic-stearic mixture from Carrín and Crapiste

(2008), as they stated that the incorporation of palmitic and stearic acid into sunflower oil increased as the mole ratio increased until reached its maximum level.

Reusability enzymes can be monitored as well based on the ID. Table 12 shows the ID of 5 batch samples from acidolysis reaction by using the reused enzyme. The ID shows a decreased by batch. The highest ID obtained from the first time used the enzyme, 0.98, while lowest ID obtained in the fifth batch of the reaction by using the same enzyme collected from the previous reaction, 0.28. Thus, the effectiveness of the enzyme have steep changes in 2nd usage as a catalyst from 0.98 to 0.57. This indicates that by reusing the enzyme, the enzyme activity to leave fatty acids and incorporates more oleic acid into the glycerol bond reduced. These also can be seen from the lower amount of POO and the higher amount of PPP in 2nd batch until 5th batch.

TABLE 12: Reused enzyme batch samples' ID

Sample	OOO	POO	PPO	PPP	ID (POO/PPO)
S1	6.28	43.30	44.38	6.04	0.98
S2	4.53	29.12	51.05	11.13	0.57
S3	3.67	27.40	54.77	16.27	0.50
S4	2.54	23.25	56.20	17.23	0.41
S5	1.11	16.52	59.95	18.87	0.28

*all batch are using optimum condition reaction parameters, 3h reaction time, 5:5 TL IM ratio to lipase AK, 1:3 substrate molar ratio of palm stearin to oleic acid, and 10% enzyme load.

Based on Huang et al. (2010), they found that combination of lipase, Novozyme 435 and TL IM, gives little loss of enzymatic activity after was continuously used for 20 batch cycles (about 400h in total). Compared with acidolysis of palm stearin and oleic acid by the batch reaction, the used of combination lipase still cannot overcome the decrease in

enzyme activity. This may due to the enzyme used consist of an immobilized enzyme and the other was the free lipase compared to Huang et al. (2010), whereby both used immobilized lipase. These result explained that by using the enzymes repeatedly, it lowered the enzyme activity.

4.5 Conclusion

From both TAG and ID value, it shows that all those four parameters play a vital role in the enzyme activity to cleave the glycerol bond and incorporates the oleic acid in the TAG. Reaction time plays an uppermost influence on ID in dual lipase system in this acidolysis reaction. Dual lipase system in this acidolysis reaction may lower the TL IM effectiveness as biocatalyst in acidolysis however, it enhance the capability of lipase AK activity in the reaction within shorter reaction time. In short, dual lipase system of TL IM and lipase AK will give higher ID by having longer time reaction and higher substrate molar ratio (or high oleic acid ratio). Besides, repeated used of the lipases may reduce the enzyme activity. The enzymes ID decrease from one batch to the next batch, thus, effect the incorporation of oleic acid. The combination of enzymes cannot overcome the reduce in the effectiveness of enzyme when reused it, however, the result maybe different by prolong the time reaction and increase in oleic acid supply.