



Monoglyceride biosynthesis from coconut milk with lypase enzyme of sesame seed sprouts as biocatalyst

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Abstract

Monoglycerides are oleochemical compounds that were widely used as emulsifying agents for foods, anti-bacterial and anti-viruses (monolaurin, monocaprin). The objective of this research was to study the optimal reaction conditions in monoglyceride production from coconut milk. To achieve that purpose, the research was carried out with two factors, namely the ratio of coconut milk: glycerol (50:5; 50:10; 50:15 and 50:20) and esterification reaction time (0, 1, 2, 3, 4, 5 and 6 hour). The observation was conducted on free fatty acid levels of hydrolyzed coconut milk, esterification reaction rates, the amount of monoglyceride and fatty acid compositions in monoglyceride. The results showed that the ratio of coconut milk with glycerol of 50: 5 and the reaction time for 4 hour had the optimum esterification reaction rate. The highest of monoglyceride amount was obtained on the reaction time for 4 hours and the ratio of coconut milk with glycerol of 50:5.

Keywords: monoglyceride; coconut milk; lypase; sesame.

Practical Application: Monoglyceride can be used as functional food to increase body immunity. Monoglyceride (monolaurin) also can be used as anti bacterial and anti virus.

1 Introduction

Some oleochemical products from coconut oil are fatty acid esters, amine fatty acids and fatty acids (lauric acid, myristic acid, capric acid) (Indonesia, 2005). Lauric acid, capric acid and myristic acid are very useful as an anti-bacterial (Vetter & Schlievert, 2005) and can inhibit the HIV viruses development (Conrado, 2002). Kabara et al. (1972) added that, lauric acid, capric acid, myristic acid, palmitic acid, linoleic acid, linolenic acid could inhibit the growth of *Pneumococci*, *Streptococcus*, *Micrococci*, *Candida*, *S. aureus*, *S. Epidermis*. Su'i et al. (2015) stated that lauric acid which was isolated from coconut oil could hamper *Salmonella* sp., *E. Coli*, and *Staphylococcus aureus* bacteria at a concentration of 3.13% and *Bacillus stearothermophilus* at 6.25%.

The other chemical oleo products from coconut oil are monoglyceride (monolaurin, monocaprin, monomiristin, monocaprilin, and other monoglyceride) and diglycerides. Monoglyceride and diglycerides are widely used as emulsifier agents (Arbianti et al., 2008). Monoglyceride in the form of monolaurin is as anti-bacterial (Nuraida et al., 2008), anti-bacterial and anti-virus (Conrado, 2002).

Monoglyceride in the form of monolaurin and monocaprin have antibacterial ability higher than in the form of free fatty acids such as lauric acid and capric acid. Whereas in the form of diglycerides (dilaurin, and dicaprine) and triglycerides (trilaurin), they do not have any bacteriostatic activity (Kabara et al., 1972). Monolaurin can also inhibit toxin

productions from *Staphylococcus aureus* and other gram-positive bacteria (Vetter & Schlievert, 2005).

Monoglyceride synthesis has been done by several previous researchers. Luna (2011) had synthesized monoglyceride in the form of monolaurin using raw materials of lauric acid and glycerol with an immobilized lypase enzyme catalyst. Arbianti et al. (2008) made monoglyceride (in the form of monolaurin) through esterification reactions. Esterification used a substrate as lauric acid with glycerol and lypase enzyme from sesame seed powder as catalyst. The making of monoglyceride was using pure lauric acid that was produced from industry and hexane solvents.

Su'i et al. (2014) had isolated lauric acid (in the form of free fatty acids) from coconut milk. Coconut milk was hydrolyzed using lypase enzymes from coconut flesh (endogenous enzymes). The highest lauric acid amount was produced from coconut flesh that was made from coconut milk with the addition of 100% water and was hydrolyzed for 72 hours. The amount of lauric acid was 48.25% from the total oil in coconut milk. That lauric acid had a purity of 53.86% so that it was called a rich lauric acid fraction. Besides lauric acid, it still contained 16.71% myristic acid, 7.86% caprylic acid, 7.76% capric acid and 6.60% palmitic acid.

Lauric acid from coconut milk isolation (a rich lauric acid fraction) was able to kill (almost all bacterial cells)

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pathogenic and non-pathogenic bacteria. Lauric acid could destroy pathogenic bacteria of *Salmonella* at a concentration of 3.13%, *Staphylococcus aureus* at a concentration of 6.25% and *E. Coli* at a concentration of 6.25%. Lauric acid could kill non-pathogenic bacteria of *Micrococcus* at a concentration of 30%, *Bacillus stearothermophilus* at a concentration of 50% and *Pseudomonas* at a concentration of 70%. Lauric acid could inhibit (kill some bacterial cells) *Salmonella* sp., *E. Coli*, and *Staphylococcus aureus* at a concentration of 3.13% whereas it may fight against *Micrococcus* at a concentration of 10%, *Bacillus stearothermophilus*, 30% and *Pseudomonas*, 50% (Su'i et al., 2015).

Su'i et al. (2018) had conducted an isolation and esterification activity of sesame seed lipase enzymes (sprouts and not sprouts). The optimal esterification reaction used an enzyme from the germinated sesame seeds. Esterification activity of sesame seed sprouts enzyme was 335.98 u mol FFA/minute/gram sesame seeds. While the seeds that were not germinated were 206.99 u mol FFA/minute/gram sesame seeds.

Esterification reaction was optimum at an enzyme concentration of 75% from oil (substrate) compared with those of 25%, 50% and 100% from oil (substrate). The esterification activity with enzyme concentration of 75% for 1, 2, 3 and 4 hours were 22.50 u mol FFA/mL sample; 43.75 u mol FFA/mL sample; 35.75 mole FFA/mL sample and 38.0 mole FFA/5 mL sample, respectively (Su'i et al., 2018).

Based on the description above, the making of monoglyceride can also be carried out from coconut milk which was hydrolyzed using the lipase enzyme. Furthermore, coconut milk that had been hydrolyzed was added with glycerol and sesame seed lipase enzyme as the catalyst.

The Esterification reaction of monoglyceride formation was greatly influenced by the ratio of glycerol and free fatty acids and esterification reaction time. If the amount of free fatty acids were too much, the esterification reaction would produce a lot of triglycerides. Otherwise, if free fatty acids were too low, the esterification reaction occurred slowly so that the formation of monoglyceride was not optimal. When the reaction time was too long, a lot of triglycerides would be produced.

This research will study the comparison of glycerol with coconut milk and the optimum reaction time for monoglyceride formation.

2 Materials and methods

The materials for this research were coconut variety from Lawang Malang Regency, sesame seeds, distilled water, ion free distilled water. Chemicals were arabic gum, glycerol, hexane, tertiary butanol, ammonium sulfate salt, olive oil, twin 80, petroleum ether, diethyl ether, formic acid, TCA, ethanol, glycerol, NaOH, pp indicator, K₂HPO₄, KH₂PO₄, CuSO₄, KNa-tartrate, HCl.

The tools that used in this study were a set of glassware, micro pipettes, plastic filters, stainless steel knives, stainless steel (Brilliant), mortar, centrifuge, analytical balance (Mettler Toledo AL 204), magnetic stirrers, heaters Janke-Kunkel), oven, pH-meter (Orion 201), space thermometer, UV-Vis spectrophotometer

(Genesys 10 UV series), gas chromatography (HP model 5890 series) with CBPS column.

The study was carried out to determine the ratio between hydrolyzed coconut milk and glycerol (50:5; 50:10; 50:15 and 50:20) and the reaction time (0, 1, 2, 3, 4, 5 and 6 hours) which was optimal to produce monoglyceride. The study was undergone in several stages, namely (1) The making of hydrolyzed coconut milk (as a substrate for making monoglyceride); (2) Extraction of sesame seed sprouts enzyme; (3) Esterification reaction of monoglyceride formation; (4) Separation of monoglyceride; and (5) Testing of monoglyceride composition.

2.1 Making of hydrolyzed coconut milk

Old coconuts were shelled, then shredded. The grated coconut were added with distilled water with a ratio of 1:1, then pressed to obtain coconut milk. Next, the sample of the coconut milk was taken for testing its lipase enzyme activity (hydrolysis activity).

Then, the coconut milk was put into a container and closed tightl, then stored in an incubator at 55°C for 72 hours so that hydrolysis was resulted in. After incubation, the amount of free fatty acids in the coconut milk was tested. Free fatty acids were produced from hydrolysis of oil in coconut milk into free fatty acids. Hydrolysis occurred enzymatically because of the endogenous lipase enzymes that were found in coconut milk. This free fatty acid from hydrolysis would act as a substrate in the esterification reaction of monoglyceride formation.

2.2 Enzyme extraction (crude) sesame sprouts (Suhendra et al., 2004)

Sprouts were made by washing sesame seeds using aquades. Then sesame seeds were soaked in a phosphate buffer solution of pH 9.4 for 110 minutes. Furthermore, sesame seeds were drained until no drips of liquid came out. Then drained sesame seeds were germinated in the dark room at a temperature of 20-30 °C and the humidity (RH) of 90-95% for 37 hours.

Sesame sprouts were crushed by adding 0.15 M cold phosphate buffer pH 7.5 (5 mL/g ingredient) . The buffer contained 0.6 M sucrose, 1 mM EDTA, 10 mM KCl, 1 mM MgCl₂. The mixture of sesame sprouts and buffer was crushed until it was homogeneous then it was centrifuged for 30 minutes at 3000 rpm. The precipitate was removed and the filtrate was collected. This filtrate was a crude extract of the sesame sprout lipase enzyme. Before being used, the lipase enzyme was tested in terms of its activity (esterification activity). the enzyme extract was stored in the freezer, before it was used.

2.3 Esterification of monoglyceride formation

Esterification reaction was carried out by preparing a substrate that was a mixture of hydrolyzed coconut milk and glycerol in the ratio of (50:20; 50:15; 50:10 and 50: 5) v/v. Furthermore, the substrate was added with a crude extract of lipase enzyme from sesame seed sprouts with a concentration of 40% (v/v) from the substrate. Before being used, lipase enzymes in coconut milk was tested for its esterification activity.

The mixture of substrate enzyme was incubated at 55 °C with different times, namely 0, 1, 2, 3, 4, 5 and 6 hours. After incubation, each sample was tested for the esterification rate by counting the amount of free fatty acids reacting with glycerol.

2.4 Monoglyceride separation

Esterification product was put into the separating funnel. Petroleum ether was added, then shaken until it was homogeneous and was allowed to stand until it was formed into two fractions. The lower layer was a non oil fraction containing water, lipase enzyme extracts and glycerol. This lower layer was discarded. The top layer was an oil fraction containing petroleum ether, triglycerides, diglycerides and monoglyceride and free fatty acids. This oil fraction was collected to obtain the monoglyceride.

Oil fraction was taken as much as 10 mL and then put into a chromatographic column that contains G 60 silica gel adsorbent. Next, it continue to be eluted gradually where each elution product was collected in different containers. N-hexane elluent was added as much as 200 mL (gradually) to obtain triglyceride and free fatty acid fraction. N-hexane: diethyl ether: formic acid elluent (90: 10: 2) as much as 200 mL were added gradually to obtain diglyceride fraction. N-hexane: diethyl ether: formic acid elluent (80: 20: 2) as much as 200 mL were added gradually to obtain monoglyceride fraction. Each fraction was evaporated to get solvent residue so that the solvent-free fraction was obtained. The volume of the monoglyceride fraction was measured and its composition was identified.

3 Results and discussion

3.1 The making of substrate

The making of monoglyceride was done from hydrolyzed coconut milk substrate. Coconut milk was taken from grated coconut which was added with water (1: 1) gradually then it was squeezed to obtain coconut milk. The amount of coconut milk was 1250 mL per kg grated coconut. Substrate obtained from every 1 kilogram of coconut is presented in Table 1.

Furthermore, coconut milk was tested in terms of its lipase enzyme activity. The results showed that the lipase enzyme activity (hydrolysis) in coconut milk was 0.129 $\mu\text{mol}/\text{minute}/\text{mL}$ coconut milk. According to Su'i (2012), coconut milk had lipase enzyme activity of 1.41 unit/mg protein. Therefore, it could be concluded that there was enzyme activity in coconut milk.

Endogenous lipase enzymes in coconut milk would catalyze hydrolysis reaction to break down the oil in coconut milk into free fatty acids. Coconut milk that contained free fatty acid was called hydrolyzed coconut milk. The higher lipase enzyme activity in coconut milk, the more free fatty acid would be produced.

3.2 Free fatty acid in substrate (hydrolyzed coconut milk)

Free fatty acid in substrate was very important to form the monoglyceride. Monoglyceride formed from free fatty acid with glycerol through esterification reaction. The greater of free fatty acid, the higher the chance to form monoglyceride would be. The level of FFA hydrolyzed coconut milk is presented in Table 2.

Table 2 showed that during incubation (hydrolysis) there had been an increase in FFA levels from 0.06 $\mu\text{mol}/\text{mL}$ to 0.21 $\mu\text{mol}/\text{mL}$. The results from Su'i et al. (2014) revealed coconut milk that was hydrolyzed using an endogenous lipase enzyme for 72 hours produced free fatty acid of 0.15 $\mu\text{mol}/\text{mL}$.

It was consistent with Tambun's (2002) research about hydrolysis of palm oil by endogenous lipase enzyme. The amount of free fatty acid production was higher with longer hydrolysis.

3.3 Lipase enzyme isolation of sesame seed sprouts

Lipase enzyme of sesame sprouts was used in esterification reaction to form monoglyceride. The results of the enzyme lipase extract of sesame seed sprouts could be seen in Table 3.

A crude extract of lipase enzyme of sesame seed sprout was obtained by 3550 mL from 450 grams of dried sesame seeds. This enzyme extract was approximately 7.89 times from the sesame seeds weight. This was caused in the enzyme isolation process, the sprouts weight increased 2 times from dried sesame seeds weight. Next, sesame sprouts were added with 5 mL buffer solution per gram of sesame sprouts.

The results of Su'i et al. (2018) showed that the amount of lipase enzyme extract of sesame sprouts was 380 mL from 50 grams of sesame seeds or 7.6 times sesame seeds weight.

Esterification activity from crude extracts of lipase enzyme of sesame sprouts in this study was 2.89 $\mu\text{mol}/\text{min}$ per mL of enzyme or 22.8 $\mu\text{mol}/\text{min}/\text{gram}$ sesame seeds. The results of Su'i et al. (2018) showed that enzyme lipase activity of sesame seed sprouts was 35.53 $\mu\text{mol}/\text{min}/\text{gram}$ of sesame seeds. According to Suhendra et al. (2004), lipase enzymes activity in sesame sprouts was 7 $\mu\text{mol}/\text{min}$. The result of this study was a little bit lower than than that of Suhendra et al. (2004). It was probably caused by the fact that the sesame seeds used were not similar.

Table 1. The amount of substrate obtained from every one kilogram of coconut.

Material	Amount
Grated coconut	1 kg
Hydrolyzed coconut milk	1,250 mL

Table 2. The Level of FFA coconut milk for incubation.

Substrate	FFA (m mol/mL)
Before being hydrolyzed	0.06
Hydrolyzed coconut milk	0.21

Note: Hydrolysis time was 72 hour and temperature was 55 °C.

Table 3. Extraction of sesame seed sprouts enzyme.

Material	Amount
Sesame seed	450 g
Sesame sprout	900 g
Enzyme extract	3,550 mL
Enzyme activity	2.89 $\mu\text{mol}/\text{minute}$

3.4 Optimal esterification reaction

The results showed that esterification reaction would increase with longer reaction time. Then, it decreased after a certain reaction time. Esterification reaction rates at several coconut milk with glycerol comparison are shown in Table 4.

Table 4 showed esterification reaction rates at all coconut milk: glycerol comparison increased until 4th hours. After that, the reaction rate decreased. It was the influence of feedback that enzymatic reaction decreased.

According to Galliard (1971), the activity of potato lipase enzyme would decrease in activity due to the production of enzyme activity inhibitor compounds, or by-products from reaction product or inactivation of enzyme with longer incubation.

These result was in accordance with Pahoja et al. (2001) which produced similar phenomenon where the incubation time up to 180 minutes would increase lipase enzyme activity of *Caesalpinia bonducella* L. But if incubation was continued, lipase enzyme activity would decrease.

Su'i et al. (2013) reported that hydrolysis lipase enzyme activity of coconut kentos increased with the longer reaction time. The reaction time of 90 minutes produced the most fatty acid which was 40.20% of total first fatty acid. If the incubation was continued for 120 minutes, the amount of fatty acid produced decreased to 29.54%.

While the ratio of coconut milk with glycerol showed that the ratio of coconut milk with glycerol 5:50 showed the highest reaction rate, then it was followed by that of 10:50 and the lowest one was that of 15:50. The more the amount of coconut milk, the greater the reaction rate would be.

Arbianti et al. (2008) research showed that dilaurin formation was increasing to the ratio of glycerol: lauric acid 3: 3. If glycerol was increased again, no significant increase of dilaurin happened.

According to Granner et al. (2003), the increase of substrate concentration influenced complex enzymes substrate formation to form products. Thus, if the substrate concentration was higher, the formation of product was faster.

In the study of Granner et al. (2003), several different substrate concentrations (2, 6, 10.14 and 18%) against the free fatty acid

produced were tested. The results showed that the increase of substrate concentration up to 10% could increase free fatty acids (FFA) production. But if the substrate concentration was more highly increased, the FFA production would decrease.

The results of this study were supported by Pahoja et al. (2001) reporting that the increase of substrate concentration up to 10% would increase lipase activity of *Caesalpinia bonducella* L. If substrate concentration was more increased above 10%, there would be a decrease in the lipase enzyme activity. Decreasing enzyme activity would reduce the production also (in this case was FFA).

3.5 Monoglyceride separation from hydrolyzed coconut milk esterification product

This study used the best ratio of hydrolyzed coconut milk: glycerol (50: 5) with esterification reaction time of 1 to 6 hours. The results of monoglyceride separation may be seen in Table 5.

Separation results showed that the highest monoglyceride was obtained in the 4 hours esterification reaction time, which was 17% of the amount of oil in hydrolyzed coconut milk.

According to Arbianti et al. (2008), esterification reaction of dilaurin formation from lauric acid and glycerol using lipase enzyme of sesame seed was optimal at 18 hours. If the reaction time was lengthened, there was no increase in the amount of dilaurin.

If the esterification reaction was continued, monoglyceride would decrease. This was consistent with testing result of esterification reaction rate (Table 4) that the esterification reaction rate was highest at 4 hours. Esterification reaction was a reaction between free fatty acid in coconut milk with glycerol to form monoglyceride. The higher the esterification reaction rate, more monoglyceride would be produced.

If the reaction was continued, monoglyceride formed would react with free fatty acid to form diglycerides and triglycerides.

3.6 Composition of monoglyceride

The types of fatty acid in monoglyceride were tested using Gas Chromatography (GC). The composition of fatty acid in monoglyceride could be seen in Table 6.

The types of fatty acid in monoglyceride had different tendencies. Short and medium chain fatty acids (monocaproin to monomiristin) tend to increase during esterification. Whereas long chain fatty acids (monopalmitin to monostearin) tend to decrease during esterification.

Monocaprilin and monocaprin increased until the 5th hour of esterification, after that there was a decrease. While the increase in monolaurin occurred until the 6th hour esterification. Monomiristin, the increase only happened until the 2nd hour, after that there was a decrease.

Short-chain and medium-chain fatty acid increased the amount of monoglyceride during esterification. It was caused by the fact that esterification reaction was easier to occur in fatty acid with shorter C atom chains than the long ones.

Table 4. Esterification reaction rates of hydrolyzed coconut milk with glycerol comparison at different reaction times.

Esterification Time (hour)	Reaction Rate at Coconut Milk: Glycerol Comparison (v/v) (u mol FFA/mL coconut milk)		
	50:5	50:10	50:15
1	27.59	16.94	10.95
2	35.96	39.34	27.78
3	46.71	45.92	44.92
4	57.24	39.62	46.44
5	40.68	32.76	44.01
6	29.39	25.20	36.13

Note: v : volume.

Table 5. Monoglyceride Separation of Esterification With Hydrolyzed Coconut Milk Substrate.

Reaction Time (hour)	Oil Fraction of Esterification Product (mL)	Monoglyceride			
		mL	g	% v	% w
0	10	1.4	1.47	14.29	16.65
1	10	0.75	0.79	8.11	9.52
2	10	1.1	1.18	12.29	14.27
3	10	1.4	1.41	14.00	15.34
4	10	1.7	1.71	17.00	18.67
5	10	1	1.06	10.00	12.30
6	10	1.35	1.47	13.85	16.33

Note : v: volume ; w: weight.

Table 6. Fatty Acid Composition in Monoglyceride.

Monoglyceride	The amount of monoglyceride (%) at Esterification time (hour)						
	0	1	2	3	4	5	6
Monocaproin	-	-	-	-	-	1.73	-
Monocaprilin	1.64	2.82	11.82	6.02	16.27	19.19	13.59
Monocaprin	1.76	3.04	8.37	5.41	9.79	10.92	9.19
Monolaurin	16.82	26.52	45.53	44.64	51.14	52.30	54.81
Monomiristin	6.92	10.17	16.68	16.25	13.93	12.52	13.66
Monopalmitin	11.73	11.72	7.51	8.80	2.38	1.38	4.36
Monolinolein	24.63	16.80	4.62	18.89	2.00	0.66	3.77
Monoolein	36.50	28.93	2.09	-	2.07	0.69	0.20
Monostearin	-	-	3.38	-	2.42	0.60	0.27

The highest type of monoglyceride was monolaurin, lauric acid. The type of fatty acid in monoglyceride was strongly influenced by the type of free fatty acid produced from hydrolyzed coconut milk. According to Su'i et al. (2014), coconut milk which was hydrolyzed for 12 hours using endogenous lipase enzyme of coconut milk contains the highest lauric acid, then myristic acids contained were 52.33% and 16.65%.

4 Conclusion

Conclusion of this research was that the highest esterification reaction rate obtained in the ratio of glycerol with hydrolyzed coconut milk of 5:50 and the esterification reaction time for 4 hours with reaction rate was 57.24 u mol FFA/mL coconut milk. The highest amount of monoglyceride obtained at 4 hours esterification time was 18.67% (w/w). Monolaurin was the highest monoglyceride type in monoglyceride product that was obtained at 6 hours esterification reaction time and the ratio of glycerol with hydrolyzed coconut milk of 5:50 was 54.81%.

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For further research, it is recommended to study the antibacterial test and monoglyceride immunity.

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