Enzymatic Glycerolysis of Palm Kernel Olein and Palm Kernel Stearin in different Ratios for Monolaurin Synthesis

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Abstract: Monolaurin is known as a monoglyceride of lauric acid, which act as an emulsifier and antimicrobial. One potential of monolaurin raw materials is palm kernel oil (PKO), which can be fractionated into palm kernel olein (PKOo) and palm kernel stearin (PKS). This study aims to determine the enzymatic glycerolysis influence of PKOo and PKS mixture in different ratios and time, on monoacylglycerol (MAG), diacylglycerol (DAG), and triacylglycerol (TAG) profiles. The Glycerolysis process of PKOo-PKS fat blends were carried out in different ratios of (60:40, 40:60, and 20:80 w/w) using commercial lipases (Lypozime RM IM) at 50°C, with an oil:glycerol molar ratio of 1:4, in a tert-butanol solvent system, between 3 to 24 hours. The results showed that the glycerolysis of PKOo and PKS in different ratios yielded varying product reactions with similar compositions (1.8–3.9% MAG, 73.2–76.4% DAG, and 20.7–24.2% TAG). A higher and not significant MAG was observed during the period of 24 hours. Also, the monolaurin was obtained at the ratio of 40:60 PKOo:PKS, within the glycerolysis time of 3 to 24 hours, at approximately 2.18±0.59% and 3.47±0.62%, respectively. The FTIR analysis also showed that the monolaurin sample was identical to the standard type, with the formation of OH group at wave number and cluster C=O ester of 3368.64 cm\textsuperscript{-1} and 1734.03 cm\textsuperscript{-1}, respectively.

1. Introduction

Monolaurin is a monoglyceride of lauric acid, which is naturally found in breast milk, at 5.8% of fat. It serves as an essential fat and immune system boosters for infants \cite{1} has two hydroxylic and one lauryl groups. Based on these classification, monolaurin is categorized as non-ionic emulsifier containing hydrophilic and hydrophobic groups, in its molecular structure \cite{2}. While as an emulsifier, it is used as food industry-based emulsion and in the pharmaceutical industry. This compound is also capable of acting as an antimicrobial agent \cite{3}, and food preservative.

The production of commercial monolaurin uses lauric acid esterification with chemical catalysts. Meanwhile, the use of these catalysts also possess several drawbacks, such as occurring at high temperatures (220-260°C). The energy consumption is also observed to be higher and not environmentally friendly, as the product is further found to be darker in colour \cite{4}. Therefore, the development of monolaurin synthesis started through the use of enzymatic catalysts. Based on the enzymatic use of lipase in this process, several advantages are being detected. These includes the occurrence of reaction at a lower temperature as well as the production of higher quality and purity.

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The sources of monolaurin are known as lauric acid, methyl lauric, as well as coconut and palm kernel oils (PKO). Meanwhile, the use of lauric acid and methyl lauric are known to have some disadvantages, due to their unavailability. This causes the relatively high price of the sources, due to only being obtained from hydrolysis or transesterification of coconut or PKO. Therefore, the most potential monolaurin samples are derived from both coconut and PKO. Meanwhile, the palm kernel oil has higher productivity advantages than other sources in Indonesia.

Monolaurin synthesis with PKO and coconut oil also uses ethanolysis [5,6], hydrolysis-continued esterification [7], or Glycerolysis [8–10] reactions. Meanwhile, the use of ethanol is feared to still be contained in the product, during the ethanolysis method. The use of oil in the hydrolysis-continued esterification reaction is also observed to take longer, leading to the production of responsive inhibitors. Also, the triglyceride glycerolysis reaction is the most economical method of monolaurin synthesis. Based on the use of specific lipase at sn 1 and 3, this method is known to produce 2-monoglyceride products.

Triacylglycerol (TAG) is found to be the main composition in PKO, with its value approximately at 95% fat. The main TAG composition of PKO is also known to be 20.46%, 17.77%, and 16.88% of lauric-lauric-lauric (LaLaLa/trilaurin), lauric-lauric-myristic (LaLaM), and capric-lauric-lauric (CaLaLa), respectively [11]. This oil also contains 50% lauric acid [12]. Based on being a monolaurin raw material, the use of PKO has several disadvantages, i.e., the inconsistency of quality, which is affected by the processing and storage condition of the kernel. The PKO is further fractionated to 60–70% Palm Kernel Olein (PKOo) and 30–40% Palm Kernel Stearin (PKS), with the amount of lauric acid at 39.7–48% and 56–59.7%, respectively [12]. Based on the amount of the acid composition, PKOo and PKS potentially serve as the raw material of monolaurin synthesis. Although the availability of PKOo is higher, its lauric acid content is found to be lower. Therefore it is necessary to mix both PKOo and PKS, in order to increase the value of lauric acid and consistent quality of fat mixture. The mixture also produces a significant change in trilaurin and lauric acid, with the acidic compound at the sn-2 position. Also, the PKOo:PKS ratios of 60:40, 40:60, and 20:80 is found to produce the trilaurin and lauric acid values of 23.72 ± 0.6% and 55.33 ± 0.41%, 24.58 ± 0.56% and 57.01 ± 0.15%, as well as 26.93 ± 0.19% and 59.92 ± 0.09%, respectively. Based on the position of the acid at sn-2, the values of PKOo and PKS at 60:40, 40:60, and 20:80, are 42.29%, 50.71%, and 51.84%, respectively [13]. Furthermore, differences in the values of trilaurin and lauric acid present in the mixture are found to affect the amount of monolaurin produced. Based on the use of specific lipase at sn 1 and 3 during the process of glycerolysis, the trilaurin present in the fat mixture is converted into monolaurin (a mixture of 2 and 1 monolaurin) and dilaurin, as intermediate products. Meanwhile, the influence of PKOo and PKS mixture on the composition of monoacylglycerol (MAG), diacylglycerol (DAG), and triacylglycerol (TAG), is still unknown.

The amount of monolaurin produced is also affected by the time of glycerolysis, which is an essential factor used in producing MAG and DAG [14]. The increased reaction time also improves the amount of monolaurin. Also, glycerolysis is found to reach dynamic equilibrium at 8 hours, as the product tends to be constant with higher reaction time [15]. Based on different ratios and time, this study aims to determine the influence of PKOo and PKS mixture on MAG, DAG, and TAG profiles, during enzymatic glycerolysis.

2. Materials and Methods

2.1. Materials
The PKOo, PKS, and refined glycerin were obtained from PT Wilmar, Indonesia. Also, Lipozyme RM IM and Molecular sieves were purchased from Sigma-Aldrich (St. Louis, MO, USA). The tert-butanol was also obtained from Merck KGaA (Darmstadt, Germany).

2.2. Preparation of fat blends
The materials were heated at 70°C for 30 mins, in order to homogenize all oil fractions before mixing. Based on the results, the liquefied products were mixed at PKOo:PKS ratios of 60:40, 40:60, and 20:80 (w/w), homogenized at 70°C for 15 mins, and further stored in a refrigerator.
2.3. Monolaurin synthesis

A 10 g mixture of PKOo and PKS in different ratios (60:40; 40:60; and 20:80) was mixed with glyceral, in the proportion of 1:4. The tert-butanol was further added to the mixture, with the ratio of 1.5 for oil and molecular sieve of 12% glycerol. Furthermore, the mixture was placed in the batch stirred tank reactor at 50°C, and mix at a speed of 200 rpm. Lipozyme RM IM with 5% w/w of oil was further added to the mixture, and reacted for 3 and 24 hours. The separation of the product from the enzyme and glyceral was carried out through centrifugation at 3000g for 5 mins, and then extracted from the solvent using a rotary evaporator. This product also analyzed the composition of MAG, DAG, and TAG, by using gas chromatography (GC). According to Nitbani et al. [5] the products of glycerolysis were obtained through the isolation or purification process of monolaurin. These products dissolved in hydroalcoholic (ethanol:water=8:2), with a ratio of 1:9 v/v. The mixture was further extracted, by using n-hexane with a balance of 1:3 v/v. This mixture dissolved in the hydroalcoholic phase, and then evaporated to obtain monolaurin, which was weighed and analyzed using FTIR. Additionally, the experiment was conducted with duplicates.

2.4. Statistical analysis

The completely randomized design was used as an experimental design. The experiment was conducted with duplicates. The resulting data is then performed to an analysis of variance and if there is a significant difference between the treatment followed by a Duncan Multiple Range Test (DMRT) level 5%.

3. Result and Discussion

3.1. Compositions of MAG, DAG, and TAG

Based on different ratios, the composition of MAG, DAG, and TAG from the enzymatic glycerolysis of PKO-PKS mixture are shown in Figure 1.

![Figure 1](image-url)  
**Figure 1.** The composition of MAG, DAG, and TAG results of PKOo: PKS glycerolysis with different ratios (60:40; 40:60; and 20:80) at 50 °C, oil: glyceral molar ratio of 1:4, solvent amount 1.5x oil, enzyme concentration 5% of oil weight, stirring speed 200 rpm with a time of 3 hours (A) and 24 hours (B).

Based on Figure 1, the composition of MAG, DAG, and TAG at different ratios (60:40, 40:60, and 20:80), did not show a significant difference. The reaction time of 3 and 24 hours also produced MAG, DAG, and TAG compositions that did not have significant differences. Also, the TAG conversion results in several DAG (73-74%) and MAG approximately produced 3-3.19%. This was due to the use of the sn-1,3 enzyme, which specifically decomposed fatty acids, and produced 2,3-DAG and 1,2-DAG at sn 1 and 3 positions, respectively. Also, the values of DAG produced was found to be high, as it was assumed not to be totally hydrolyzed into MAG by the lipozyme RM IM enzyme. Furthermore, glycerolysis was a complicated process, which included hydrolysis, esterification, and isomerization of MAG and DAG. Based on the initial 5 h, hydrolysis dominated the process, accompanied by
esterification and isomerization [16]. The higher molar fraction of TAG compared to glycerol was found to also enhance the synthesis of dilaurin. Based on the higher production of DAG, the free fatty acids did not totally react to the glycerol. This was because most glycerol were found to react with fatty acids obtained from TAG, in order to produce monoacylglycerol during glycerolysis reactions [17]. Theoretically, 1 mole of TAG and 2 moles of glycerol provided 3 moles of MAG during this process [18]. However, the yield of monoacylglycerol depends on equilibrium conditions, due to several occurrences [19]. Glycerolysis was also influenced by the concentration of enzymes, molar ratio of oil:glycerol, amount of solvent, and the temperature of the response [20]. Also, the molar ratio of fat:glycerol determined the glycerolysis products that were produced. Based on the excess and limited use of glycerol in this reaction, MAG and DAG were found to be high, respectively [21]. According to the equilibrium law, an increase in the glycerol content shifted the balance towards the production of MAG [18]. Based on the study of Solasea et al. [18] a glycerol:oil mole ratio of 3:1 was selected as optimum value for MAG production. The low yield of MAG was also caused by low concentrations of enzymes. This study was also found to use an enzyme concentration of 5%, compared to the 5-10% of total reactants (oil and glycerol) used in previous studies. The MAG yield also increased with higher lipase concentration, which was due to the greater number of active sites [22]. Solasea et al. [22], also found that the glycerolysis of sardine oil was carried out using Lipozyme 435, at a load of 5 and 10 wt%. This was further conducted with a glycerol:oil mole ratio of 3:1, in order to produce MAG at 90 and 80%, for the lipase load of 10 and 5%, respectively.

The conversion of TAG to MAG and DAG at 3 and 24 h showed no significant difference on all PKOo and PKS ratios (Figure 2). This was due to the decreasing activities of the lipase enzymes. Excess glycerol also inhibited the enzymatic activity due to its polar nature, which often led to inactivation [23]. Also, the glycerol content directly affected the lipase activity, due to its influence on the polarity of the reaction medium [18]. Based on the studies of Mustafa et al. [24], there was no further increase in the percentage of conversion after 3 hours of reaction. This was observed in the esterification of lauric acid and glycerine into glycerine laurate, using the lipolyme RM IM enzymes. Furthermore, the conversion percentage dramatically increased in the initial 30 mins of the reaction, as approximately 70% of the original fatty acids were converted into esters [24]. Based on the use of Novozymes 435 (10% oil weight) at 65°C for 7 hours, the enzymatic glycerolysis of PKO and glycerol at a molar ratio of 1:1 produced 4.00 ± 0.09% MAG, 88.41 ± 0.19% DAG, and 7.60 ± 0.10% TAG, after purification [8]. The reaction reached dynamic equilibrium at the eighth hour, as well as essentially maintained the type and content of products formed in the reversible response constant [15]. Based on the reaction times above 36 hours, the amount of monolaurin observed was not growing [10]. The glycerolytic reaction of VCO and glycerol reported no significant increase in the amount of MAG and DAG, due to using the Lipase Candida antartica (Novozymes 435) that was continuous for approximately 48 h [25].

3.2. The amount of free fatty acids
The amount of free fatty acids produced during the enzymatic glycerolysis of PKOo and PKS mixtures are shown in Table 1.

| Time of glycerolysis (hour) | PKOo: PKS Ratios* |
|----------------------------|-------------------|
|                            | 60:40             | 40:60             | 20:80             |
| 3                          | 0.85±0.14a        | 0.76±0.01a        | 0.69±0.09a        |
| 24                         | 0.97±0.13a        | 0.89±0.11a        | 0.87±0.10a        |

*value is average ± SD (n=2). Average on row or column, followed by the same letter, indicates a not significant difference in the Duncan Multiple Range Test (DMRT).

Based on Table 1, the free fatty acids produced during the glycerolysis process were insignificantly different. This showed that the hydrolysis of oil was very tiny, leading to the minimal
production of these compounds. Free fatty acids were also the result of oil hydrolysis, which was carried out by the water derived from glycerol, in the reaction system. Although several studies added water to glycerol in order to increase the amount of MAG, it was also found to enhance free fatty acids [18].

Based on the use of Lypozime RM IM (10% oil) at 50°C for 20 h, the glycerolysis sardine oil and glycerol at a mole ratio of 1:3 increased FFA by 5.3 and 32.5%, for WGC (water glycerol content) at 7 and 24%, respectively. Furthermore, the highest MAG yield was obtained, due to the addition of 12% water in glycerol [18].

Based on the use of Lipozyme TL IM (7% w/w oil and 5 ml alcohol) at 60°C and 200 rpm for 24 h, the MDG (mono-diglyceride) synthesis using RBDPO (refined bleached deodorized palm oil) and glycerol with a molar ratio of 1:5, produced 40.16% MAG, 49.92% DAG, 10% TAG, and 0.16% FFA [14].

3.3. Yield of monolaurin
Glycerolysis products are separated by using hydroalcoholic solvents, in order to obtain monolaurin. The result of monolaurin yield produced is shown in Table 2.

| Time of glycerolysis (hour) | PKOo: PKS ratios |
|-----------------------------|------------------|
|                             | 60:40            | 40:60            | 20:80            |
| 3                           | 1.96±0.81a       | 2.18±0.59a       | 1.24±0.02a       |
| 24                          | 2.91±1.49a       | 3.47±0.62a       | 2.05±1.26a       |

*a-value is average ± SD (n=2). Average on row or column, followed by the same letter, indicates a not significant difference in the Duncan Multiple Range Test (DMRT) 5%.

Based on Table 2, the yield of monolaurin was insignificantly different. Meanwhile, a PKOo:PKS ratio of 40:60 obtained the highest yield at 3.47%, during a period of 24 h. The low output of monolaurin produced in this study was mainly caused by the glycerolytic reaction of the DAG product (Figure 1). Also, the yield obtained was lower, compared to previous studies. Based on the use of Lypoizme TL IM (5% oil) at 35°C for 12 h, the synthesis of 2-monolaurin from coconut oil produced a yield of 35% through ethanolysis, at oil:ethanol ratio of 1:4 [5]. The synthesis of 1-monolaurin at lauric acid and glycerol at 1:1 also produced a yield of 27.89%, based on the use of a 5% pTSA catalyst at 130°C for 6 h [26]. Furthermore, the synthesis of lauric acid and glycerol with mole ratio of 1:1 produced a yield of 17.25%, based on using the Rhizopus spp. lipase at 50°C for 72 h [27]. Based on using a 20% Carica papaya lipase at 45°C for 36 h, the monolaurin synthesis from coconut oil with crude glycerol at 1:8 in ethanol also produced a yield of 58.35% [10].

3.4. Analysis of FTIR (Fourier Transform Infra Red) monolaurin
Analysis of FTIR monolaurin after purification is shown in Figure 2. According to Figure 2, the monolaurin sample was analyzed by FTIR, after purification. It also indicated that the OH group occurred at a wavenumber, C=O ester, C-O-C, CH Stretch vibration, as well as Methyl and methylene group of 3368.64 cm⁻¹, 1734.03 cm⁻¹, 1174.05-993.03 cm⁻¹, 2853.21-2922.01 cm⁻¹, and 1461.04-1380.46 cm⁻¹, respectively. The wavenumber of 719.77 cm⁻¹ also indicated the rocking vibration from (CH2)_n. Furthermore, the FTIR analysis showed that the sample monolaurin was identical to the standard monolaurin, which was found to be in line with previous studies. Widiyarti et al., also found a new group at wavenumbers of 3224.98 cm⁻¹ and 3290.56 cm⁻¹. This indicated that the asymmetric stretching vibration of the hydroxyl and carbonyl groups were similar at wavenumber of 1730.15 cm⁻¹ [28]. Also, Sangadah et al. obtained the C=O ester and OH clusters at wavelengths of 1748 cm⁻1 and 3650-3200 cm⁻³, respectively [29].
Figure 2. Analysis of FTIR monolaurin from glycerolysis with a PKOo-PKS fat blend with ratio of 40:60, glycerolysis time of 24 hours, at 50 °C, the molar ratio of oil: glycerol 1:4, amount of solvent 1.5x oil, enzyme concentration 5% w/w oil, stirring speed 200 rpm.

4. Conclusion

It is concluded that the glycerolysis of PKOo-PKS mixtures in different ratios produced similar compositions (1.8-3.9% MAG, 73.2-76.4% DAG, and 20.7-24.2% TAG). The reaction at a period of 24 h produced higher MAG, which were insignificant. Also, the PKOo:PKS ratio of 40:60 at a period of 3 and 24 h obtained monolaurin yields of 2.18±0.59% and 3.47±0.62%, respectively. The FTIR analysis also showed that the sample of monolaurin was identical to the standard type, with the formation of OH group and C=O ester having wavenumbers of 3368.64 cm⁻¹ and 1734.03 cm⁻¹, respectively.

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