Enzymatic synthesis of palmitoylethanolamide from ketapang kernel oil

E R Gunawan¹, D Suhendra², BA NuansaWindari³, L Kurniawati⁴

Department of Chemistry, Faculty of Mathematics and Science, University of Mataram, Jl. Majapahit No. 62 Mataram, 83125, Indonesia

*Corresponding author: erinryantin@unram.ac.id

Abstract. Palmitoylethanolamide (PEA) has been synthesized from the palmitic acid Ketapang kernel oil (Terminalia catappa L.) through an enzymatic reaction. Usually, PEA was synthesized from pure fatty acids by chemical catalysts. In this study, the raw material used was ketapang kernel oil (renewable and in-edible oil) and the reaction was carried out with lipozyme as a catalyst. The palmitic acid content of ketapang kernel oil is 25-30%. The enzymatic reaction conditions were temperature at 40°C for 24 h, substrate ratio of ketapang kernel oil and ethanolamine of 1 : 3, amount of enzyme (Lipozyme) of 0.15 g and hexane as a solvent. The formation of the PEA was confirmed using Thin layer chromatography (TLC), fourier transform infrared spectroscopy (FTIR) and gas chromatography-mass spectrophotometry (GC-MS). FTIR spectra of the PEA shows the characteristic absorption band at 3340-3100, 1642 and 1270-1216 cm⁻¹ for stretching vibration of O-H, N-H, C=O (carbonyl) and C-N, respectively. The GC-MS chromatogram shows that the peak of PEA appears at retention time of 11.98 minutes.

1. Introduction

Palmitoylethanolamide (PEA) is a natural substance that can be produced by the body, it is very effective and safe to use as a supplement for pain and inflammation [1,2]. PEA or fatty monoethanolamide usually used as non-ionic surfactants in cosmetic/medicine industries [3]. PEA can be synthesized based on fatty acids derived from vegetable oils. In general, vegetable oils that used as basic ingredients is edible oil [4,5]. The focus of this paper is to discuss the synthesis of PEA using non-edible oil. One source of non-edible vegetable oil is ketapang kernel (Terminalia catappa Linn). Ketapang kernel has a high oil content (triglycerides), which is about of 54% [6]. The fatty acid composition of triglycerides palmitic acid (25.05-30.96%), oleic (31.5-38%), stearic (3.1-4.3%) and linoleic (12-21%) [6,7].

So far, many types of fatty acid ethanolamide are produced at high temperatures from the reaction between fatty acids or fatty acid methyl esters with monoethanolamine or diethanolamine by chemical catalysts [8,9]. Synthesis of organic compounds using chemical catalysts requires complex, the presence of by-products and the experiments must also be carried out with caution[10].

An interesting alternative, the catalyst used for PEA synthesis is enzymatically. Enzymatic synthesis has several advantages such as working at lower temperature and pressure [11]. Moreover, the biocatalysts are also easily separated from the product when enzymes are used in the form of solid particles or in an...
immobilized enzyme. The purpose of this study was to investigate the potential of ketapang kernel oil as raw material for enzymatic synthesis of PEA.

2. Methods

All chemicals were of analytical grade; Na\textsubscript{2}SO\textsubscript{4} anhidrous, diethylether, chloroform, ethanolamine (monoetanolamine), Lipozyme RM. IM, Ketapang kernel, n-hexane, ethanol, methanol, acetonitrile, HCl, NaOH 2 M, silica gel, Na\textsubscript{2}S\textsubscript{2}O\textsubscript{3} 0,1 N and aquadest. The equipment used is a glassware in the chemical laboratory, soxhlet extractor, chromatography column, thin layer chromatography(TLC), water bath shaker, rotary evaporator, Spektrofotometer FT-IR Perkin Elmer Model Frontier, HPLC, Waters 1525 (USA), GC-MS Shimadzu Model GCMS-QP2010 Ultra (Japan).

2.1. Procedure

2.1.1. Extraction of Ketapang Kernel oil

Ketapang kernel was mashed and dried. Then, 60 grams of it are placed in filter paper and put into soxhlet column. The soxhletation process was run for 6 hours using 250 ml of n-hexane and a temperature of 60°C. The extracted oil was evaporated to remove n-hexane solvent by rotary evaporator at 40°C at a rate of 120 rpm. The next treatment, the oil was added anhydrous sodium sulfate to remove the water content and purified by chromatography column. The oil was eluted with n-hexane: diethyl ether (87:13 v / v). After that, the refined oil was weighed with an analytical balance to determine the oil content.

2.2.2. Hydrolysis of Ketapang kernel oil

Amount of 50 g of refined oil was put into a flask round bottom, then it added 100 mL of distilled water and 2 ml of HCl concentrated as a catalyst. The hydrolysis process was carried out at a temperature of 115-130°C for 4 hours. After 4 hours the process was stopped and the solution will be 2 phases, water and organic phase. The product is in the organic phase. The organic phase was separated from the water phase. Then, 50 ml of 2M NaOH was added to the organic phase and stirred at 50°C for 30 minutes. Furthermore, the solution was added with 100 ml 1 M NaCl, separated and neutralized with 2 M HCl to the pH = 1. The next step, the fatty acid in the solution was extracted with n-hexane at 3 times.

2.2.3. Synthesis of PEA

PEA synthesis was carried out by reacting fatty acids (From hydrolysis process) with ethanolamine reagents (primary amines) through enzymatic reaction. The reaction mixture consisted of 1 g of fatty acids, 10 mL n-hexane, 3 g of ethanolamine and 0.15 g commercial enzyme (Lipozyme). The mixture was incubated into horizontal water batch shaker with a rate of 150 rpm at 40°C for 24 hours.

2.2.4. Purification of PEA

The first step, the reaction mixture was separated from enzyme using a vacuum filter. The second step, PEA is in the hexane fraction and it was separated from the water fraction using a separating funnel. N-hexane fraction was cooled in the freezer (<-5 °C) for 5 hours and filtered. The product was dried in a phosphorus pentoxide desiccator for 24 hours.

2.2.5. Characterization of PEA

PEA was characterized with various methods: determination of melting points, TLC for determination of factor Retention, FTIR for and GC-MS.
3. Result and Discussion

3.1. Extraction of Ketapang Kernel oil

The percentage of kernel from ketapang fruit is about of 7.24%. This result is slightly higher when compared to palm oil kernel [12]. For extraction technique; In general, vegetable oil processing in commercial applications is carried out by chemical extraction, one of them by soxhletation method. This method will produce higher yields, faster and less expensive [13]. The solvent used in the soxhletation process is n-hexane (analytical grade). N-hexane and oil have a similar polarity. Purification of ketapang kernel oil was carried out by chromatography column, with eluent of hexane; diethyleter (87:13) [6]. This method is suitable for purifying triglycerides (oil) as has been done by Sitompul et al [14]. The refined oil content of ketapang kernel was 58.07%. This result is almost similar to the palm oil content of 49.3% [15]. Unfortunately, this is less than coconut oil content (82.5%) [16]. However, palm and coconut oil have been widely used as edible oils. The good news, the oil content of ketapang kernel is higher than kapok oil (30.075%) [17] and castor oil (48.320%) [18], which is also non-edible oil.

3.2. Hydrolysis of Ketapang Kernel Oil

The production of fatty acids was carried out through hydrolysis reaction via acid catalyst (concentrated HCl). Hydrolysis of triglycerides to produce fatty acids and glycerol can be seen in the following reactions:

\[
\begin{align*}
\text{Triglyceride} + 3 \text{H}_2\text{O} & \rightarrow \text{Fatty Acids} + \text{Glycerol} \\
\text{Catalyst} & \text{Catalyst}
\end{align*}
\]

At the end of the hydrolysis process, fatty acids and glycerol will separate in different phases. Fatty acids obtained from the ketapang kernel oil are semi-solid form such as butter and white colour. The semi-solid form of fatty acids is caused by the oil content of ketapang kernel, which is dominant by palmitic acid (saturated fatty acid/solid form) and also oleic acid (unsaturated fatty acids/liquid form). The percentage of fatty acids is 88%. The high yield shows that the majority of the fatty acids have been successfully hydrolyzed from triglycerides.). This research was agreement with the work of Weerawatanakorn et al [19].

3.3. Synthesis of PEA

PEA synthesis was carried out at 40°C for 24 hours in a water batch shaker via enzymatic reactions. Lypozime RM IM is used as a biocatalysator. Synthesis of an amide can be done by enzymatic methods or chemical methods [6, 8]. This work was done by enzymatic methods because this method is known as an environmentally friendly, short method and it does not require high temperature and pressure [4, 10, 11].

Separation of products from enzymes is also quite simple by filtering because the enzymes used are immobilized enzyme (solid granules). Immobilized enzymes can be reused after rinsing to reduce synthesis costs. Immobilized enzymes are used in organic synthesis to fully exploit the technical and economical advantages of biocatalysts based on isolated enzymes [20].

Wang et al [8] had compared the two methods, chemical and enzymatic methods to synthesize linoleylethanolamide. Percentage yield of the product are not too much different. However, the advantage of enzymatic method is much simpler.
Synthesis enzymatic reaction of PEA (Lipozyme as a catalyst) can be seen in the following:

\[
\text{CH}_3(\text{CH}_2)_{14}\text{COOH} + \text{H}_2\text{N-CH}_2\text{-CH}_2\text{-OH} \rightarrow \text{CH}_3(\text{CH}_2)_{14}\text{CO-NH-(CH}_2)_2\text{-OH + H}_2\text{O}
\]

Palmitic acid   ethanolamine   PEA

3.4. Purification of PEA

PEA as a product will be solid because it is derived from the palmitic acid, which is a saturated fatty acid that has a solid form. PEA is in the hexane fraction because it has a long fatty acid chain following the properties of 'like dissolve like' (semi/non polar). To purify products of unsaturated fatty acid ethanolamide, such as oleyl and linoleylethanolamide, crystallization method was used. This method was recommended by Japri et al [15] whom had successfully purify saturated fatty acids from palm oil. This method is an efficient method for it is cheap and equipments required are simple.

The purified PEA may still be mixed with Stearoylethanolamide (SEA) because SEA is also solid. However, it can be assumed that most of the products are PEA because the composition of stearate acid in the oil is less than 4%. The products (PEA) have a high percentage yield of 70%. Furthermore, PEA refining was carried out by HPLC.

3.5. Characterization of PEA

Characterization of PEA is done by TLC with eluent used by chloroform: methanol (90:10). The \(R_f\) (Retention factor) value is similar among of the PEA standard and product (0.73). This indicates that PEA has been successfully synthesized from fatty acids of ketapang kernel oil.

The next characterization was determining the melting point of PEA. The purpose of the determination is to identify the purity level of the substance. Based on the measurement, it obtained the average melting point of PEA is 97°C. This value is slightly higher than the melting point of standard PEA (95°C). However, this is consistent with the result from Keppel et al [21] which states that PEA has a high melting point of around 95-98°C.

The successful synthesis of the PEA can also be proven by analysis with FT-IR. Typical peaks in the area at 3340.00 cm\(^{-1}\) which indicate the vibration of OH group. The vibration of secondary N-H group shows one peak at 3100 cm\(^{-1}\). The Amide group has been formed which is proven by the absorption peak of the wave number at 1642.98 cm\(^{-1}\) indicating the vibration of C = O (carbonyl) for amide. The presence of this amide group is supported by the appearance of absorption at wave number of 1555.38 cm\(^{-1}\) (N-H bending) and wave numbers at 1270.6-1216.27 cm\(^{-1}\) for C-N stretching vibration. The similar spectra were also obtained from other researchers who carried out amide synthesis from the oil [3, 6].
Figure 2. FTIR Spectra of PEA

Characterization of PEA was carried out by GC-MS. The retention times \((R_t)\) of PEA standard and PEA products are adjacent at 11.92 and 11.98 minutes. Mass spectrophotometry for PEA gives an estimate of the relative molecular Massa \((M_r)\) of PEA and its fragmentation.

Figure 3. Structure of PEA

Fragmentation observed for palmitoylethanolamide is the following \(m/z\) 300 \([M]^+\) is the base peak that shows \(M_r\) of PEA, \(m/z\) 284 \([M-16]^+\) release of OH group \((17-1H)\), \(m/z\) 272 \([M-28]^+\) release of carbonyl,
m/z 256 [M-44] release of CH$_2$-CH$_2$-OH, (45-1H), m/z 239 [M-60]$^+$ release NH-CH$_2$-CH$_2$-OH, m/z 213 [M-87]$^+$ release of CO-NH-CH$_2$-CH$_2$-OH (88-1H). This fragmentation is in accordance with studies from Angelini et al. [22] regarding PEA contaminant and Yun et al. [23] which discuss GC-MS determination of palmitic acid.

4. Conclusion
PEA has been successfully synthesized from palmitic acid of ketapang kernel oil and characterized through observation of melting point value, functional group and ion fragmentation.

Acknowledgements
This project was financed by a grant from Directorate General of Research and Development Strengthening, The Ministry of Research Technology and Higher Education Republic of Indonesia, Scheme “Unggulan Perguruan Tinggi 2018”.

References
[1] Gabrielson L, Mattsson S and Fowler C J 2016 Br J Clin Pharmacol. 82 932
[2] Skaper S D, Facci L, Fusco M, Valle M F D, Zusso M, Cost B and Giusti P 2014 Inflammopharmacol. 22 79
[3] Adewuyi A, Oderinde R, Rao B V S K and Prasad R B N 2012 J. Surfact. Deterg. 15 89
[4] Suhendra D, Gunawan E R and Kusumawati L 2019 Rasayan J.Chem. 12 (2) 765
[5] Nisya F N, Prjono D and Nurkania A 2017 IOP Conf. Series: Earth and Environmental Science 65
[6] Gunawan E R, Suhendra D, Nurita A D and Komalasari D 2017 Asian J. of Chem. 29 (10) 2107
[7] Lasekan O Ng S, Muhammad K S, Hussain N and Sulaiman R J. Food Sci. Technol. 52 (10): 6623
[8] Wang X, Cen Y, Jin Q and Wang X 2013 J. Oleo. Sci. 62 (6) 427
[9] Manurung R., Sinaga R. And Simatupang R T 2013 Int. J. Res. Sci. Eng. Tech. 2(9), 4205
[10] Gunawan E R, Suhendra D, Trisnaris and Kurniawati L 2018 J. of Physics: Conf. Series 1095 012014
[11] Suhendra D, Gunawan E R, Nurita A D, Komalasari D and Ardianto T 2017 J. Oleo. Sci. 66(3), 209
[12] Setiawati W B, Atmaji P and Anggoro D D 2012 Proceeding Insinas 182
[13] Tambun R, Purba R R H and Ginting H K 2017 Matter. Sci. Eng 237 012032
[14] Sitompul J P, Gusdinar T, Anggadiredja K, Rahman H and Tursino 2018 J. Eng. Technol. Sci. 50 (1) 87
[15] Japir A A, Salimon J, Derawi D, Yahaya B H, Bahadi M, Al-Shuja’a S and Yusop M R 2018 OCL 25(2): A203
[16] Lan T T B and Hoa P N 2015 Bio. Chem. Res. 2015 258
[17] Gunawan E R, Suhendra D and Hasanah B R M 2017 Am. J. App. Sci. 14 (12) 1146
[18] Nangbes J G, Nvau J B, Buba W M. And Zukdimma A N 2013 Int. J. Eng. Sci. 2 (9) 105
[19] Weerawatanakorn M, Janporn S, Ho C T, Chavasit V and Terminalia Linn 2015 J. Sci. Technol. 37 (5) 507
[20] Mohamed E H, Tamer T M and Ahmed O M 2016 Int. J. of Current Pharmaceutical Review and Research 7(6) 385
[21] Keppel Hesselink J M, Tineke de Boer and Witkamp R F 2013 Int. J. Inflammation. 2013 151208: 1
[22] Angelini R, Argueta D A, Piomelli D and DiPatrizio N V 2017 Cannabis Cannabinoid Res. 2(1) 123
[23] ALY, Li Z, Zhuo Y, Wang Y, Li R, Zhang F and Yang X 2016 Chin. J. Pharm. Anal. 36 (10) 1875