Synthesis of Oleoylethanolamide Using Lipase

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ABSTRACT: An effective process for the enzymatic synthesis of oleoylethanolamide is described in this study. The process included purification of a commercial oleic acid product and then optimization of the reaction between the purified oleic acid and ethanolamine in the presence of hexane and a lipase. Under the optimal amidation reaction conditions identified, oleoylethanolamide was obtained with 96.6% purity. The synthesis was also conducted on a large scale (50 mmol of each of the reactants), and oleoylethanolamide purity and yield after crystallization purification were 96.1 and 73.5%, respectively. Compared to the previous studies, the current method of preparing high-purity oleoylethanolamide is more effective and economically feasible. The scalability and ease for such synthesis make it possible to study the biological and nutritional functions of the cannabinoid-like oleoylethanolamide in animal or human subjects.

KEYWORDS: amidation reaction, Novozym 435 lipase, oleic acid purification, oleoylethanolamide, synthesis

■ INTRODUCTION

Fatty acid alkanolamides are an important class of nonionic surfactants suitable for a variety of applications.[1] Their chemical properties vary according to the length of the hydrocarbon chain and the functional groups on the chain. Fatty acid ethanolamides structurally belong to fatty acid alkanolamides, and they are a family of lipids naturally found in both plant and animal tissues.[2]

Fatty acid ethanolamides first attracted attention as lipid mediators in 1957, when it was found that palmitoylethanolamide from soybeans, peanut oil, and egg yolk was an anti-inflammatory factor.[3] Furthermore, palmitoylethanolamide was shown to attenuate pain sensation.[4] Stearoylethanolamide had pro-apoptotic and anorexic effects,[5] and it also exhibited an anti-inflammatory property.[6] Another important fatty acid ethanolamide is oleoylethanolamide, which is a natural analogue of the endogenous cannabinoid anandamide (derived from arachidonic acid) and is synthesized mainly in specific cells.[6] It can be rapidly hydrolyzed by two different endogenous hydrolases,[7] suggesting a function in cellular signaling. Oleoylethanolamide modulated feeding and energy homeostasis, and it was thought to act by binding to peroxisome proliferator-activated receptor-α (PPAR-α).[8] It decreased food intake by inducing a satiety signal, whereas anandamide increased food intake by activating cannabinoid receptor subtype 1 (CB1).[9] Moreover, oleoylethanolamide had lipolytic properties through the inhibition of adipogenesis in adipose tissue[10] and the activation of lipid β-oxidation in muscle.[11] These intriguing biological functions warrant further investigations of oleoylethanolamide in animal and human models. Therefore, synthesis of this compound by an effective method is highly desirable.

Fatty acid ethanolamides can be synthesized from a fatty acyl donor and ethanolamine at temperatures above 100 °C, usually at 180 °C,[12,13] in the absence of sodium methoxide as catalyst or at low temperature with sodium methoxide.[14] The fatty acyl

donors include fatty acid chloride,[8,15,16] free fatty acid,[13] fatty acid methyl ester,[12] and triacylglycerol.[14,17,18] In general, reaction at high temperature will affect the color, odor, and purity of the products. Impure fatty acid ethanolamides are only used as industrial product,[18] not for the study of biological functions in animal systems. It is expensive to use oleoyl chloride to synthesize oleoylethanolamide in gram quantity even though high-purity product could be obtained by this method.[8] In our previous research, we have established a new method for synthesizing palmitoyl- and stearoylethanolamides, in which fatty acid vinyl esters were used as acyl donors. However, vinyl oleate is not readily available commercially, so we had to use oleic acid for the synthesis.

Pure oleic acid is relatively more costly ($54.6 per 5 g quantity, from Sigma-Aldrich Chemical Co.) than a commercial grade oleic acid product ($41.8 per liter quantity, from the same vendor). Because this research is about feasibly producing a large quantity of pure oleoylethanolamide, we decided to start with a relatively cheap starting material. Many methods have been used to obtain pure fatty acids, including supercritical fluid chromatography,[19] low-temperature crystallization,[20] urea inclusion,[21] and molecular distillation.[22] In this study, purification of oleic acid was performed using low-temperature crystallization to remove the 10% of other fatty acids from the commercial oleic acid product. In previous studies, low-temperature crystallization was commonly used to purify the polyunsaturated fatty acids.[20] We expected that a similar purification strategy would also apply to the purification of oleic acid and plan to demonstrate the effectiveness of this procedure.

In this study, the traditional routes for fatty acid ethanolamide synthesis were modified and replaced with lipase-catalyzed reaction, which had been proved effective for the production of hydroxyl fatty amides from hydroxyl fatty acid and ammonia.[23] Hence, the synthesis of oleoylethanolamide included purification of oleic acid from a commercial product and amidation reaction between oleic acid and ethanolamine with a lipase. This method is economical because of the low cost of oleic acid and efficient because of the high selectivity of lipase for the amidation reaction, and the method was improved and proved suitable for large-scale synthesis in a quantity needed for biological function evaluation in animal or human systems.

■ MATERIALS AND METHODS

Materials. All chemicals were purchased from the Sigma-Aldrich Chemical Co. (St. Louis, MO) except the following: Ethanolamine (>99%) was purchased from Fisher Scientific (Fair Lawn, NJ). *Candida antarctica* (Novozym 435) lipase was provided by Novozymes (Blair, NE).

Purification of Oleic Acid. The commercial oleic acid product contained 89.6% oleic acid, 4.2% stearic acid, and 6.2% linoleic acid. Our purification process included two steps: removal of linoleic acid and then stearic acid. Because linoleic acid can be well dissolved in methanol at −23 °C,[24] whereas oleic acid and saturated fatty acids can be crystallized from methanol, optimization experiments were conducted to obtain pure oleic acid by low-temperature crystallization. The commercial oleic acid product of 5 mL was mixed with 5, 10, 15, 20, or 25 mL of methanol at −23 °C for 4 h to investigate the effect of solvent quantity on the purity and yield of oleic acid. Linoleic acid was removed by collecting the crystals by vacuum filtration in a cold room (about 5 °C) to fully remove the liquid phase.

In the second step, stearic acid was removed by placing the mixture (4:1 ratio of methanol to oleic acid) at −18 °C due to the lower solubility of stearic acid in methanol compared to oleic acid.[24] Once the crystal was observed in the solution, an additional 15 min was allowed to fully crystallize the saturated fatty acid. The liquid phase was then collected by vacuum filtration in a cold room, and the crystal phase was discarded. All experiments were conducted in duplicate.

Purification of oleic acid was then conducted on a large scale. Two hundred milliliters of oleic acid was divided into two groups as two replicates. Methanol and oleic acid were mixed at −23 °C for 4 h at a 4:1 volume ratio to remove the linoleic acid, and the crystal phase collected was mixed with methanol in a 1:4 volume ratio at −18 °C to remove saturated fatty acid.

Oleoylethanolamide Synthesis Using the Previously Published Conditions. *Amidation of Ethanolamine with Oleic Acid in the Presence of Excess Ethanolamine as Solvent.* The modified procedure of Kolancilar,[14] in which an excess of ethanolamine was used as solvent, was followed. Oleic acid (1 mmol), ethanolamine (10 mmol), and 50% lipase (relative to total reactants) were placed in a 10 mL round-bottom flask and reacted with agitation at ambient temperature for 6 and 24 h or reacted at 65 °C for 4, 8, and 20 h. The reaction mixture was then washed with 5 mL of distilled water at 6 °C three times to remove the excess amount of ethanolamine, and the lipase was removed by filtration. The oleoylethanolamide product was then derivatized and quantified by GC as described in the following section.

Amidation of Ethanolamine with Oleic Acid in Hexane. This experiment was carried out according to the procedure of using free fatty acid as acyl donor.[25] Ethanolamine (1 mmol) in hexane (1 mL) was mixed with oleic acid (1 mmol) in a 10 mL round-bottom flask in the presence of the lipase (50%, relative to total reactants). Reaction was conducted with agitation at ambient temperature for 4, 8, and 24 h or at 65 °C for 4, 8, and 20 h before hexane was removed under reduced pressure.

These two methods for oleoylethanolamide synthesis were compared so we could select a set of appropriate conditions for optimization of the synthesis.

Optimization of Oleoylethanolamide Synthesis Using the Appropriate Reaction System Identified. The design for the optimization experiments is outlined in Table 1. Effects of the amounts

Table 1. Experimental Design for Optimization of Amidation between Oleic Acid and Ethanolamine[a]

level	X_1 (%)	X_2 (μL)	X_3 (mL)	X_4 (°C)	X_5 (h)
1	10	0	0.5	45	1
2	20	10	1.0	55	2
3	30	20	1.5	65	3
4	40	30	2.0	75	4
5		40	3.0		5

[a] X_1 = lipase, conducted by reacting ethanolamine with oleic acid in 1 mL of hexane without water. All reactions were conducted by reacting 1 mmol of ethanolamine with 1 mmol of oleic acid at 65 °C for 2 h in 1.5 mL of hexane and 10 μL of water with 30% enzyme unless otherwise stated. X_2 = moisture content, conducted by reacting ethanolamine with oleic acid in 1 mL of hexane. X_3 = hexane amount. X_4 = reaction temperature. X_5 = reaction time.

of lipase, hexane, and water, reaction temperature, and time as single factors on the purity of oleoylethanolamide were investigated while other reaction conditions were fixed.

Effect of Enzyme Concentration. Optimization of the amount of enzyme was conducted by reacting ethanolamine (1 mmol) with oleic acid (1 mmol) at 65 °C for 2 h in 1 mL of hexane with 10, 20, 30, and 40% lipase and without the use of water.

Effect of Moisture Content. Optimization for moisture content was conducted by reacting ethanolamine (1 mmol) with oleic acid (1 mmol) at 65 °C for 2 h in 1 mL of hexane with 30% lipase. Water was added in the reaction mixture at 0, 10, 20, 30, and 40 μL (0, 2.8, 5.6, 8.4, and 11.2%, relative to total reaction mixture).

Effect of Hexane Quantity. Optimization for the amount of hexane was conducted by reacting ethanolamine (1 mmol) with oleic acid (1 mmol) at 65 °C for 2 h in 0.5, 1, 1.5, 2, or 3 mL of hexane with 30% lipase and 10 μL of water.

Effect of Reaction Temperature. Ethanolamine and oleic acid were mixed at equal molar ratio (1 mmol), and the reaction was carried out with agitation at 45, 55, 65, and 75 °C for 2 h in 1.5 mL of hexane and 10 μL of water with 30% lipase.

Effect of Reaction Time. Equal molar ratio (1 mmol) ethanolamine and oleic acid was mixed at 65 °C in 1.5 mL of hexane and 10 μL of water with 30% lipase to optimize the reaction time in the 1−5 h range.

Synthesis of Oleoylethanolamide on a Large Scale. The reaction conditions used were exactly the same as the optimal conditions established on 1 mmol scale. Ethanolamine (50 mmol) and oleic acid (50 mmol) were mixed at 65 °C for 6 h in 75 mL of hexane with 30% lipase and 0.5 mL of water. Hexane of 150 mL was then added to the system after lipase was removed by filtration. The mixture was placed at 6 °C for 1 h to crystallize oleoylethanolamide from hexane.

All optimization experiments were conducted in duplicate, and the results were expressed as means ± standard deviation.

Preparation of Methyl Esters of Fatty Acids and Oleoylethanolamide Derivative for GC Quantification. One drop of oleic acid of commercial or purified product was mixed with a 14% boron trifluoride−methanol solution in a 5 mL glass vial at 70 °C for 5 min, and then 2 mL of hexane was added to the mixture to extract the fatty acid methyl esters. The anhydrous reaction product of the oleoylethanolamide (about 5 mg) was placed into a 2 mL glass vial for producing its ether derivative for GC quantification. Pyridine (0.5 mL) was added followed by hexamethyldisilazane (0.15 mL) and trimethylchlorosilane (0.05 mL). The mixture was shaken for 15−30 s and allowed to stand for 1 h or stored in a freezer (0 °C) overnight to allow the upper layer phase turn clear.[26] GC was used to quantify

oleoylethanolamide. The purity of oleoylethanolamide was calculated according to the peak area ratio.

Derivatives of fatty acids and oleoylethanolamide were quantified by using an HP 5890 series II capillary GC (Hewlett-Packard) equipped with a flame ionization detector (FID) and using a 30 m × 0.25 mm ×0.25 μm (length × i.d. × film thickness) fused silica bonded phase capillary column SP-1 (Supelco, Bellefonte, PA). The carrier gas (helium) flow rate was 32.3 mL/min, and the split ratio was 7. The oven temperature for oleic acid quantification was programmed from 140 to 230 °C at a rate of 5 °C/min, then programmed from 140 to 300 °C at a rate of 10 °C/min, and then held at 300 °C for 5 min for oleoylethanolamide quantification. The injector and detector temperatures were set to 250 °C for fatty acid analysis and to 300 °C for oleoylethanolamide quantification.

NMR Analysis for Structure Confirmation. ^{1}H NMR qualitative analysis of the oleoylethanolamide product was done by using a Varian MR-400 spectrometer (Foster City, CA) with CDCl$_3$ as solvent and TMS as the internal standard (chemical shift of 0 ppm).

Statistical Analysis. All data were analyzed by one-way ANOVA. Differences among the means were compared at $P = 0.05$ using Tukey's test. Different letters labeled in the figures indicate significant differences for the specific quality parameter.

■ RESULTS AND DISCUSSION

Purification of Oleic Acid. The solubility of oleic acid at −20 °C is 4.02 g oleic acid/100 g methanol, compared to 233 g linoleic acid/100 g methanol. The solubility of stearic acid at −20 °C is 0.011 g/100 g methanol.[24] Thus, if methanol and the commercial oleic acid were mixed at −23 °C, oleic acid and stearic acid should crystallize from methanol, whereas linoleic acid is still dissolved in methanol. If methanol and the partially purified oleic acid were mixed at −18 °C, stearic acid should be crystallized from methanol very quickly, whereas oleic acid should remain relatively soluble in the methanol during a short crystallization time. Thus, stearic acid can be removed by collecting the liquid phase.

For the removal of linoleic acid, the effect of the volume ratio of methanol to oleic acid on the purity and yield of oleic acid is shown in Figure 1. Crystallization conducted with a 4:1 volume
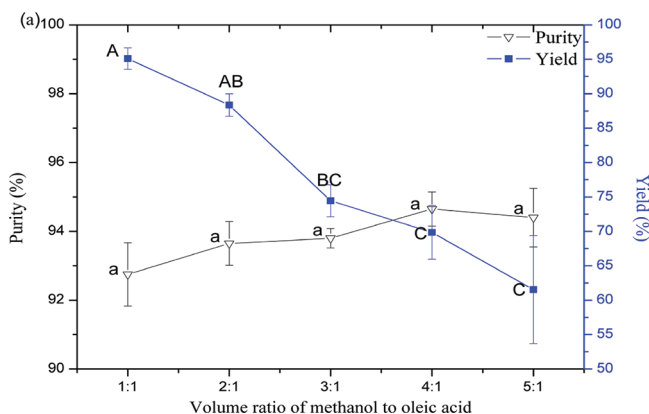
Figure 1. Effects of volume ratio of methanol to oleic acid on the purity and yield of oleic acid after the removal of linoleic acid. Different letters on each individual curve indicate significant difference at $P = 0.05$.

ratio for 4 h resulted in removal of most of the linoleic acid. The purity was not significantly affected by the amount of solvent used. However, the larger the volume of solvent used, the more the lipid was lost in the solvent. By this first purification step, the purity of oleic acid was increased from 89.6 to 94.7%, and linoleic acid and stearic acid were reduced

from 6.2 to 1.9% and from 4.2 to 3.4%, respectively. For the second purification step, a low temperature of −18 °C was effective to quickly separate the stearic acid crystals from methanol, so we obtained 96.7 ± 0.8% oleic acid with 62.2 ± 5.4% overall yield. Linoleic and stearic acids were at 2.1 and 1.2% in the final product.

For the purification of oleic acid on a large scale (100 mL), the final purity and yield were 97.0 ± 0.4 and 59.7 ± 1.8%, respectively. This result shows an excellent feasibility and effectiveness of the crystallization purification process.

Selecting Reaction Conditions for Oleoylethanolamide Synthesis. The previous reactions conducted for the synthesis of fatty acid ethanolamide typically result in the formation of undesirable color and odor even though the addition of deodorizers and antioxidants has been suggested to improve the product quality.[14,27,28] Thus, in the present study, we compared the feasibility of using excess ethanolamine and hexane as solvents to synthesize oleoylethanolamide by using a lipase. Using excess ethanolamine as a solvent was shown to be effective for the synthesis of fatty acid ethanolamide when triacylglycerol was used as the acyl donor.[14,27]

The comparisons of using different solvents for the amidation reaction are shown in Tables 2 and 3. In the present
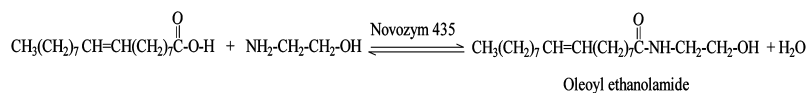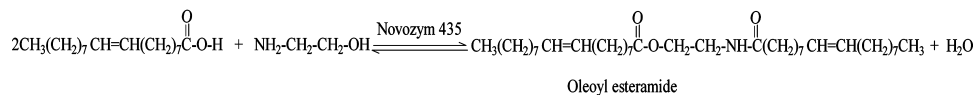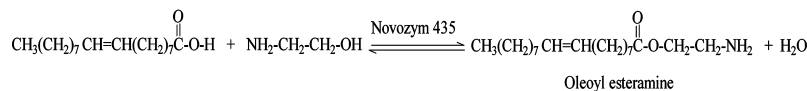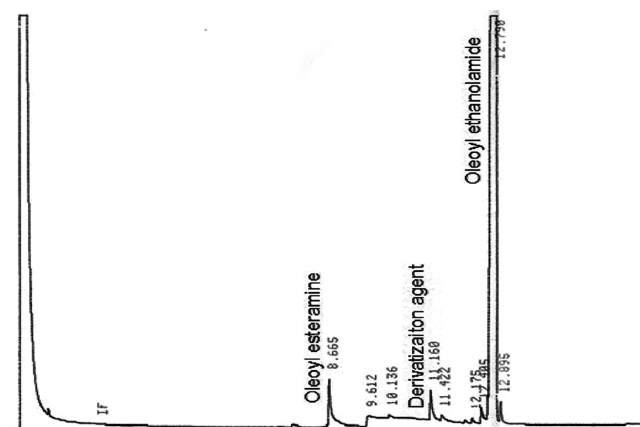
Table 2. Synthesis of Oleoylethanolamide in Excess (10×) Ethanolamine as Solvent

	temperature				
	ambient		60 °C		
	6 h	24 h	4 h	8 h	20 h
oleic acid (%)	67.2	65.1	57.2	61.5	58.8
oleoylethanolamide (%)	23.1	29.6	39.4	37.4	40.1

Table 3. Synthesis of Oleoylethanolamide in Hexane as Solvent

	temperature				
	ambient temperature			60 °C	
	4 h	8 h	24 h	4 h	8 h	20 h
oleic acid (%)	51.8	43.3	28.5	0	0	0
oleoylethanolamide (%)	45.5	54.1	69.9	96.8	94.5	93.2

study, <41% oleoylethanolamide was produced when the reactions were conducted at ambient temperature or 60 °C for 24 and 20 h with 50% lipase in the presence of a large excess of ethanolamine as solvent. Increasing the reaction temperature was more effective than time in improving the oleoylethanolamide content in the mixture. The low purity of oleoylethanolamide was due to low conversion rate of oleic acid (Table 2). In comparison, when hexane was used as solvent to synthesize oleoylethanolamide at ambient temperature for 24 h, 69.9% oleoylethanolamide was obtained. When the reaction temperature was increased to 60 °C, there was 96.8% oleoylethanolamide produced in the system in only 4 h, and almost 100% of the oleic acid was converted (Table 3). The impurities in the final reaction mixture could be from side reactions as shown in Figure 2, and the formation of an esteramine is shown in a GC chromatogram (Figure 3). The presence of esteramine in such a reaction mixture was also suggested by others.[28] Thus, the hexane system was identified and chosen to optimize the synthesis of oleoylethanolamide in the next experiments.

Main reaction:

$$CH_3(CH_2)_7CH=CH(CH_2)_7\overset{O}{\underset{\|}{C}}\text{-O-H} + NH_2\text{-}CH_2\text{-}CH_2\text{-OH} \underset{\text{Novozym 435}}{\overrightarrow{}} CH_3(CH_2)_7CH=CH(CH_2)_7\overset{O}{\underset{\|}{C}}\text{-NH-}CH_2\text{-}CH_2\text{-OH} + H_2O$$

Oleoyl ethanolamide

Side reactions:

$$CH_3(CH_2)_7CH=CH(CH_2)_7\overset{O}{\underset{\|}{C}}\text{-O-H} + NH_2\text{-}CH_2\text{-}CH_2\text{-OH} \underset{\text{Novozym 435}}{\overrightarrow{}} CH_3(CH_2)_7CH=CH(CH_2)_7\overset{O}{\underset{\|}{C}}\text{-O-}CH_2\text{-}CH_2\text{-}NH_2 + H_2O$$

Oleoyl esteramine

$$2CH_3(CH_2)_7CH=CH(CH_2)_7\overset{O}{\underset{\|}{C}}\text{-O-H} + NH_2\text{-}CH_2\text{-}CH_2\text{-OH} \underset{\text{Novozym 435}}{\overrightarrow{}} CH_3(CH_2)_7CH=CH(CH_2)_7\overset{O}{\underset{\|}{C}}\text{-O-}CH_2\text{-}CH_2\text{-NH-}\overset{O}{\underset{\|}{C}}(CH_2)_7CH=CH(CH_2)_7CH_3 + H_2O$$

Oleoyl esteramide

Figure 2. Possible reactions between ethanolamine and oleic acid.

Figure 3. GC chromatogram of a synthesis product of oleoylethanolamide.

GC was employed to measure oleoylethanolamide derivatives. The peak at 11.1−11.2 min (Figure 3) was attributed by the silylation reagent rather than sample because this peak also can be found when only silylation reagent was injected into GC. This observation has also been reported by other researchers.[29]

^{1}H NMR spectroscopy of oleoylethanolamide shows the following: δ 0.88 (t, 3H, CH_3), 1.26 (t, 20H, 10 × CH_2), 1.63 (t, 2H, CH_2CH$_2$CONH), 2.01 (m, 4H, CH_2CH=CHCH_2), 2.20 (t, 2H, CH$_2$CH_2CONH), 3.43 (p, 2H, HOCH$_2$CH_2NH), 3.72 (t, 2H, HOCH_2CH$_2$NH), 5.34 (t, 2H, CH$_2$CH= CHCH$_2$), 5.9 (s, 1H, CH$_2$CH$_2$CONH). No ^{1}H NMR peak of $-\text{COOC}H_2\text{C}H_2\text{N}H_2$ (δ 4.2−4.4, 2H) or $-\text{COOCH}_2\text{CH}_2\text{N}H_2$ (δ 1.1−1.5, 2H) can be observed. Thus, we confirmed the structure and purity of oleoylethanolamide.

Optimization of Oleoylethanolamide Synthesis. The optimization of oleoylethanolamide synthesis was to find a set of reaction conditions to decrease the esterification and promote the amidation reaction. The possible reaction routes for amidation of ethanolamine with oleic acid are presented in Figure 2. Three reactions may occur in the system. The amidation reaction is predominant, and it results in the formation of oleoylethanolamide and water. The purity of oleoylethanolamide may be improved by removal of water by vacuum; however, the high volatility of ethanolamine is problematic (boiling point of 170 °C).[28] The main side reaction product is esteramine, resulting from an equal molar esterification reaction between ethanolamine and oleic acid. In general, spontaneous acyl migration will happen when a high concentration of esteramine is produced.[28,30] Because the acyl

migration step is very fast, the reaction appears to proceed via direct amidation. The formation of esteramide is not favored due to the limited amount of free fatty acid in the reaction system. Therefore, the impurities in the final product may include ethanolamine, oleic acid, and esteramine. Under the optimal reaction conditions, almost all oleic acid and ethanolamine were converted. Thus, the main impurity is fatty acid esteramine as shown in the GC chromatogram (Figure 3).

The results for optimization of oleoylethanolamide synthesis are presented in Figures 4−8. Figure 4 shows the effect of lipase
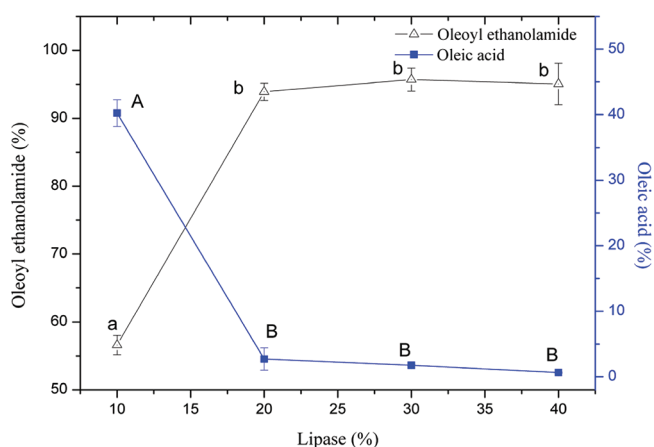
Figure 4. Effect of Novozym 435 lipase concentration on the content of oleoylethanolamide and oleic acid in the final amidation product. Different letters on individual curves indicate significant difference at P = 0.05.

concentration on the conversion of oleic acid. The content of oleoylethanolamide was increased and oleic acid was decreased dramatically when lipase was increased from 10 to 20% (relative to total reactants). Even though 30% lipase did not give significantly higher conversion than that of 20%, we chose 30% lipase as optimal enzyme condition to ensure complete reaction.

Moisture content was then optimized because it affects enzyme activity. Oleoylethanolamide content decreased significantly when the moisture content was increased from 20 to 40 μL (Figure 5). The low oleoylethanolamide content was a result of low oleic acid conversion due to reaction equilibrium. No significant differences in oleoylethanolamide and oleic acid contents were observed between 0 and 20 μL water addition. In general, enzyme needs 0−5% moisture content for catalytic
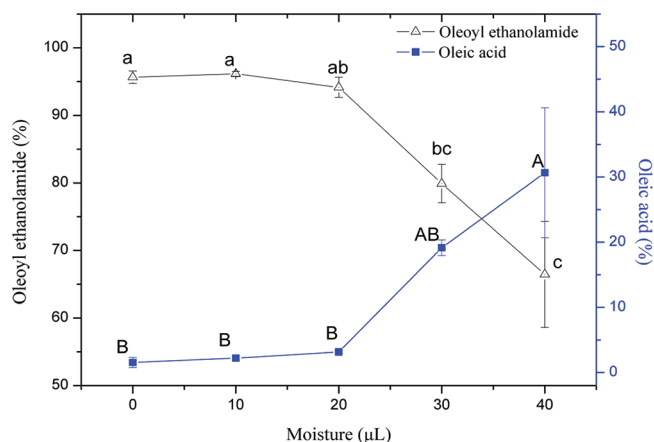
Figure 5. Effect of moisture on the content of oleoylethanolamide and oleic acid in the final amidation product. Different letters on individual curves indicate significant difference at $P = 0.05$.

activity.[31] In our study, we determined that 10 μL of water (2.8%) was the most appropriate condition. In this reaction, there was 18 μL of water produced if oleic acid and ethanolamine were fully reacted; therefore, this water may also contribute to the moisture that is needed for enzyme activity. The moisture content of the enzyme itself was measured to be 1.56%. On the basis of how much enzyme was used (30%) in the 1 mmol reaction system, a total of 1.6 mg of water was contained in the enzyme. This is relatively a small contribution, considering the 10 mg of water added (as an optimum) to the system and the 18 mg of water produced from the amidation reaction.

The amount of hexane was optimized because solvent affects the solubility or dispersibility of the final product and enzyme and reactant concentrations. A low hexane amount results in low product solubility, whereas a high hexane amount dilutes the enzyme and the reactants and may affect the reaction negatively. Figure 6 shows the oleoylethanolamide content had
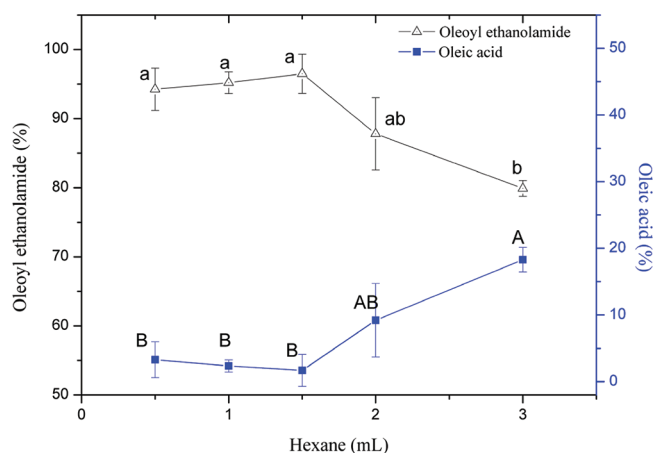
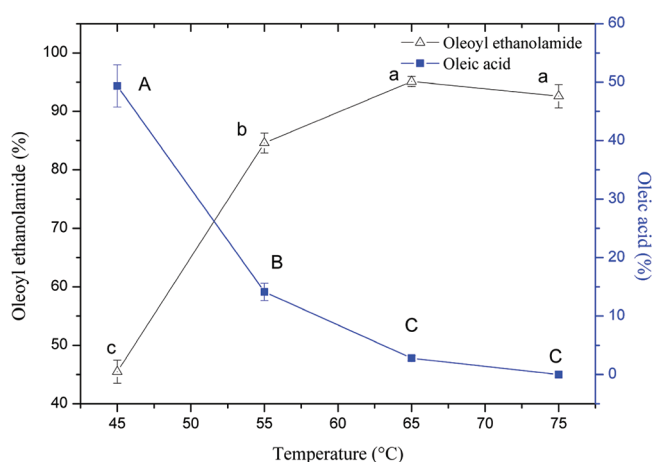

Figure 6. Effect of hexane on the content of oleoylethanolamide and oleic acid in the final amidation product. Different letters on individual curves indicate significant difference at $P = 0.05$.

a slight increasing trend in the presence of 0.5−1.5 mL of hexane but decreased dramatically when more hexane was used. The reaction conducted in the presence of 1.5 mL of hexane as solvent resulted in a high purity of oleoylethanolamide. Therefore, 1.5 mL of hexane was chosen as the optimal

quantity of solvent when 1 mmol of each of the reactants was used.

Reaction temperature affects reaction rate, enzyme activity, and solubility of oleoylethanolamide product. The maximal oleoylethanolamide content was observed at 65 °C with 30% lipase in 1.5 mL of hexane and 10 μL of water (Figure 7).

Figure 7. Effect of reaction temperature on the content of oleoylethanolamide and oleic acid in the final amidation product. Different letters on individual curves indicate significant difference at $P = 0.05$.

Therefore, 65 °C was chosen as the optimal reaction temperature.

The reaction time from 2 to 5 h did not affect the content of oleoylethanolamide and oleic acid significantly (Figure 8). The
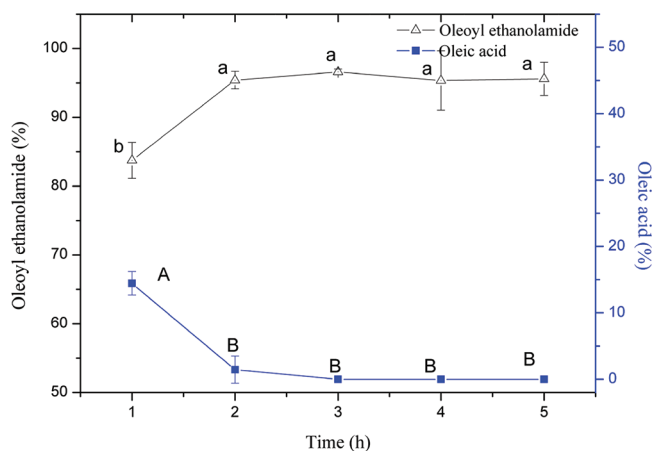
Figure 8. Effect of reaction time on the content of oleoylethanolamide and oleic acid in the final amidation product. Different letters on individual curves indicate significant difference at $P = 0.05$.

maximum content of oleoylethanolamide was observed at 2 or 3 h, and almost 100% oleic acid was converted. Therefore, when 1 mmol of oleic acid and 1 mmol of ethanolamine were mixed at 65 °C for 3 h with 30% lipase in 1.5 mL of hexane and 10 μL of water, the purity of oleoylethanolamide was 96.6 ± 0.4% and all oleic acid was consumed. This is an exceptional conversion reaction catalyzed by an enzyme.

The optimization of oleoylethanolamide synthesis was conducted on the basis of a single-factor experimental design.

We recognize that interactions among these factors may exist. The optimal reaction condition may be different if a multifactor response surface experimental design is used to generate a prediction model. However, there are certain concerns and limitations of such an approach.[32] An advanced optimization experiment, particularly if conducted on a larger scale, may utilize a sophisticated model to accurately determine the best parameters under the conditions that may be different from laboratory experimental settings.

The application of the optimal reaction conditions identified on a small scale (1 mmol reactants) did not produce the expected result for large-scale synthesis. When 50 mmol of ethanolamine was reacted with 50 mmol of oleic acid at 65 °C for 6 h with 30% lipase in the presence of 75 mL of hexane and 0.5 mL of water, the final purity of oleoylethanolamide was about 79%, which was much lower than the purity expected. The enzyme was removed by filtration, and the product was purified by crystallization at 6 °C for 1 h in 150 mL of hexane. The purity and yield of oleoylethanolamide in the crystallized product were 96.1 ± 0.6 and 73.5 ± 3.7%, respectively. The residual oleic acid was completely removed after crystallization. This is an example of the need for a further optimization experiment on a larger reaction scale.

Most of the earlier papers are focused on the synthesis of saturated fatty acid alkanolamides, and the synthesis of unsaturated fatty acid alkanolamides with enzymes has received little attention. The purity of commercial alkanolamides for surfactant purpose that were synthesized by reacting ethanolamine with free fatty acid at high temperature (100−180 °C) [13] was low (about 80%),[18] and the product was of undesirable color and odor quality. In addition, oleic acid and oleoylethanolamide may be oxidized at such high temperatures. To our knowledge, there are two publications in the literature that are closely related to our work. Tufvesson et al.[28] established a method for the synthesis of lauroylethanolamide (melting point of 89 °C) by using the Novozym 435 lipase to catalyze the amidation reaction at milder temperature (90 °C) in a solvent-free system. The final purity of lauroylethanolamide in their reaction mixture was 95% with 97% conversion. In contrast, we used a solvent system at a lower temperature for oleoylethanolamide synthesis. We had to use a solvent to make the final product disperse (melting point of 95 °C) at a temperature that will not significantly denature the enzyme, so in practice the enzyme can be reused. In another study, Plastina et al.[25] also used hexane as solvent to synthesize oleoylethanolamide, but the reaction at ambient temperature resulted in a longer reaction time and low oleic acid conversion. In addition, they used preparative HPLC for the purification of oleoylethanolamide, which is unsuitable for large-scale synthesis, and using pure oleic acid for oleoylethanolamide synthesis is rather expensive. Compared to their method, our procedure also showed that purer ethanolamide can be obtained with almost 100% oleic acid conversion and >95% oleoylethanolamide yield compared to their 90% 18:1 conversion and 88% yield on a small scale. We have shown that our method can be scaled to 50 mmol for each of reactants; we used much less lipase, 30% compared to 65% in theirs, and we have demonstrated that recrystallization is a much simpler and scalable method for oleoylethanolamide purification at large scale compared to preparative HPLC. This recrystallization step is unique and has not been reported by others. Therefore, we have significantly improved the

procedure and efficiency of the synthesis and product purification over the existing work.

The amidation reaction could also be conducted in the presence of a condensation agent, such as 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride.[33] However, this agent is toxic and, therefore, undesirable. In our previous research, stearoyl- and palmitoylethanolamides were successfully synthesized by reacting fatty acid vinyl esters with ethanolamine in excess ethanolamine,[34] so in the future vinyl oleate can be synthesized and used in such a synthesis route.

The synthesis method we report in this work is effective and scalable. It is economical because oleic acid can be purified by crystallization from methanol instead of using a commercially pure oleic acid. It is effective and efficient because enzyme was used as catalyst and the reaction can be made almost complete. The commercially available pure oleoylethanolamide is very expensive ($144 per 10 mg, from Sigma-Aldrich Chemical Co.), which may limit the study of biological and nutritional properties of oleoylethanolamide. The method we report herein allows the production of this compound in a large quantity at low cost.

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