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ORIGINAL PAPER

Enrichment of Arachidonic Acid for the Enzymatic Synthesis of Arachidonoyl Ethanolamide

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Abstract Arachidonoyl ethanolamide is an endogenous cannabinoid neurotransmitter that potentially has therapeutic properties. In this study, we report an enzymatic method to synthesize this bioactive ethanolamide. First, free fatty acids were obtained from arachidonic acid-rich oil and then arachidonic acid was enriched by urea inclusion and $AgNO_3$ solution fractionation. Arachidonic acid content was increased from 40.2 to 78.4 % with 27.6 ± 1.8 % total fatty acid mass yield (w/w, relative to total free fatty acid) after urea inclusion. Purification of arachidonic acid by $AgNO_3$ solution fractionation was optimized, and under the optimal conditions, a product with 90.7 % arachidonic acid was obtained with 80.4 % mass yield (w/w, relative to total free fatty acid). Finally, arachidonoyl ethanolamide was synthesized by reacting the purified arachidonic acid with ethanolamine in hexane using Novozym 435 lipase of Novozymes America (Blair, NE), which resulted in the formation of 88.4 ± 0.6 % arachidonoyl ethanolamide with 72.7 ± 2.2 % mass yield. The main novelties of this study are the enrichment of polyunsaturated fatty acids by $AgNO_3$ solution fractionation which has received little attention in recent years, and this is the first time the synthesis of arachidonoyl ethanolamide is reported.

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Introduction

Arachidonoyl ethanolamide, also known as anandamide, belongs to a family of fatty alkanolamides which are of considerable interest due to their wide ranging applications in lubricants, surfactants, cosmetics, and other industries [1, 2]. Moreover, arachidonoyl ethanolamide has unique bioactivities. It is a lipid mediator naturally found in animal and plant tissues [3], and it has been reported to exhibit a variety of cannabimimetic activities in vitro and in vivo [4, 5]. Some recent studies showed that the cannabinoid receptors, CB_1 and CB_2, and the vanilloid-receptor ion channels are cellular binding sites for arachidonoyl ethanolamide, 2-arachidonoylglycerol, and other (yet to be unidentified) endogenous cannabinoids [6]. Arachidonoyl ethanolamide binds to the central CB_1 and peripheral CB_2 cannabinoid receptors through which it is thought to exhibit its analgesic and anti-inflammatory effects [7, 8]. In addition, arachidonoyl ethanolamide is reported to be an endogenous ligand for the vanilloid receptor (TRPV1) that is involved in the transduction of acute and inflammatory pain signals, activating the receptor in a PKC-dependent manner and leading to the perception of pain [9, 10]. Arachidonoyl ethanolamide was also reported to induce hypothermia, analgesia, motor defects [11] and inhibits breast cancer cell proliferation [12].

Fatty acid ethanolamides are usually synthesized by reacting an acyl donor such as a free fatty acid [13–15], a fatty acid methyl ester [2], a triacylglycerol [16], a fatty

acid chloride [17, 18], or a fatty acid vinyl ester [19] with ethanolamine in a solvent or solvent-free system and with sodium methoxide or lipase as the catalyst. In this study, arachidonoyl ethanolamide was synthesized by reacting the purified arachidonic acid from arachidonic acid-oil with ethanolamine in hexane with Novozym 435 as the catalyst because fatty acid chloride, vinyl ester and triarachidonin are much less available and much more expensive than the free fatty acid. Arachidonic acid vinyl ester is commercially unavailable even though the reactions using vinyl ester as acyl donors are more efficient than those using a free fatty acid as the acyl donor. Fatty acid chloride is a corrosive and relatively toxic chemical which may dramatically affect the bioactivity of arachidonoyl ethanolamide if it is not fully removed after purification.

To the best of our knowledge, no report is in the literature that demonstrates the synthesis of arachidonoyl ethanolamide probably because a pure arachidonic acid or its other forms are very expensive even though the method for the synthesis of fatty acid ethanolamides and method improvements have been established [19]. Thus, in order to reduce the cost for the synthesis of arachidonoyl ethanolamide, we enriched the arachidonic acid from an arachidonic acid-rich oil. There are several methods for the enrichment of polyunsaturated fatty acids (PUFA), including enzymatic methods, low temperature crystallization, supercritical fluid extraction and urea inclusion [20], but not many are suitable for scale-up production [21].

In this study, we chose urea inclusion and then liquid–liquid $AgNO_3$ solution fractionation to enrich arachidonic acid from a commercial arachidonic acid-rich oil. In the urea inclusion step, saturated and monounsaturated fatty acids easily complex with urea, while the non-urea complexed fraction is enriched with PUFA. Thus, all saturated and most monounsaturated fatty acids are expected to be removed. Urea inclusion fractionation is a robust pre-fractionation step because of the use of inexpensive renewable materials (urea and ethanol as solvent) [22]. In $AgNO_3$ solution fractionation step, Ag^+ can complex with double bond. The complexing ability with Ag^+ is higher with the increasing number of double bonds in fatty acid structure. Thus, this method can be used to separate fatty acids with different degrees of unsaturation. Purification of arachidonic acid by $AgNO_3$ solution fractionation should further remove all monounsaturated fatty acids and partially remove linoleic and linolenic acids. The theory of using an $AgNO_3$ solution to separate fatty acid esters with different unsaturation degree has been established [23], but liquid–liquid $AgNO_3$ solution fractionation has received little attention for separating free fatty acids except for a study reporting the method in Japanese [24]. This method is different from using an $AgNO_3$-loaded column chromatograph [25] or using hollow-fiber membranes [26] to enrich PUFA. For this research, we intended to develop economical and effective methods for the purification of polyunsaturated fatty acid and for the synthesis of a bioactive polyunsaturated fatty acid ethanolamide.

Materials and Methods

Materials

All chemicals were purchased from the Sigma-Aldrich Chemical Co. (St. Louis, MO) except the following: Arachidonic acid-rich oil (≥40 %) was from Cargill (Minneapolis, MN) and it is a fungal oil extracted from *Mortierella alpina* ALK-1. Ethanolamine (>99 %) was purchased from Fisher Scientific (Fair Lawn, NJ). *Candida antarctica* (Novozym 435) lipase B was provided by Novozymes (Blair, NE). This is a lipase immobilized on a macroscopic acrylate and has a declared activity of 10,000 PLU (propyl laurate unit)/g.

Enrichment of Arachidonic Acid

Preparation of Free Fatty Acid from Arachidonic Acid-Rich Oil

Free fatty acid (FFA) was prepared from arachidonic acid-rich oil according to the method of Wanasundara and Shahidi [27]. In brief, 100 g arachidonic acid-rich oil (treated with butylated hydroxytoluene, i.e. BHT at 300 ppm concentration) was hydrolyzed by using 300 mL of a KOH solution containing 25 g KOH, 60 mL water and 220 mL ethanol at 60 °C for 1 h under nitrogen. After hydrolysis, 100 mL distilled water was added and the unsaponifiable matter was extracted in hexane and discarded. The aqueous layer containing saponifiable matter was subsequently acidified to pH 1.0 by using 6 M HCl. The resulting solution was extracted with hexane. The hexane layers were pooled and anhydrous Na_2SO_4 was added to remove water. Free fatty acid was obtained after the solvent was evaporated. This saponification procedure was used to process two batches of oil.

Preparation of Arachidonic Acid Concentrate by Urea Inclusion

According to a published procedure [27] and our own preliminary optimization trial results, free fatty acid (2 g) was mixed with urea (2 g) in 95 % aqueous ethanol (10 mL). The mixture was heated at 65 °C with stirring until the mixture turned into a clear homogeneous solution. Initially, the urea-fatty acid complex was allowed to crystallize at

room temperature and then the system was kept at 0 °C for 12 h for further crystallization. The crystals formed were separated from the liquid with a Büchner funnel. The filtrate was diluted with water (5 mL) and acidified to pH 4.0 with 6 M HCl followed by extraction with hexane. The residual urea or any contaminants in the liquid was thus removed by discarding the aqueous phase and collecting the hexane phase. The extracted organic layer was dried with anhydrous Na_2SO_4, concentrated on a rotary evaporator and analyzed by GC. This purification was repeated to obtain a large quantity of concentrated free fatty acid.

Optimization of Purification of Arachidonic Acid Concentrate by $AgNO_3$ Solution Fractionation

Arachidonic acid concentrate obtained by the urea inclusion treatment was purified further by $AgNO_3$ solution fractionation. The ranges of parameters were selected based on the results from a previous study [23]. The effects of fatty acid concentration in hexane, fatty acid addition amount, methanol volume fraction, temperature and time on fatty acid distribution ratio (D) were investigated individually by changing only one factor for each experiment while the other factors were kept constant.

The D value is defined by the equation:

$$D = [\text{PUFA}]_w/[\text{PUFA}]_o \quad (1)$$

where $[\text{PUFA}]_o$ is the mass of a PUFA in hexane (organic) phase that was determined by GC with methyl heptadecanoate as the internal standard. The $[\text{PUFA}]_w$ term is the mass of the PUFA in the aqueous (water) phase that was calculated according to the material balance from the initial PUFA mass and the mass of the PUFA in hexane [23].

All treatments were performed in duplicate unless otherwise specified and the results were expressed as means ± standard deviations.

The effect of free fatty acid concentration in hexane on D urea fractionation concentrated free fatty acid (0.6 g) was diluted to different concentrations (0.05–0.4 g/mL) with hexane. The diluted free fatty acid solutions (containing 0.6 g) were mixed with 5 mL of 2 M $AgNO_3$ aqueous solution at 20 °C for 2 h under constant stirring to investigate the effect of free fatty acid concentration in hexane on the D value.

The effect of free fatty acid amount on D The same $AgNO_3$ aqueous solution (5 mL of 2 M) was mixed with different volumes (1–6 mL) of 0.1 g/mL free fatty acid hexane solution at 20 °C for 2 h under constant stirring.

The effect of methanol volume fraction in $AgNO_3$ aqueous solution on D $AgNO_3$ was dissolved in different concentrations of methanolic aqueous solutions (0–40 %) to make 2 M solution. Subsequently, 5 mL of 2 M $AgNO_3$ methanol aqueous solution was mixed with 1 mL of 0.1 g/mL free fatty acid solution at 20 °C for 2 h under constant stirring.

The effect of treatment temperature on D 5 mL of 2 M $AgNO_3$ in 30 % methanolic aqueous solution was mixed with 1 mL of 0.1 g/mL free fatty acid hexane solution at different temperatures (−5 to 20 °C) for 2 h under constant stirring.

The effect of incubation time on D 5 mL of 2 M $AgNO_3$ in 30 % methanolic aqueous solution was mixed with 1 mL of 0.1 g/mL free fatty acid hexane solution at −5 °C for different time periods (0.5–2 h).

After complex formation between $AgNO_3$ and PUFA, hexane was separated and another 1 mL precooled hexane (−10 °C) was added to wash the aqueous phase. The aqueous extracts were separated from hexane and then extracted twice with 5 mL ethyl ether at 50 °C. The ether phase was pooled and fatty acid was determined by GC after evaporating the solvent. Mixed fatty acid standards were used as external standard to identify the GC peaks. Methyl heptadecanoate was used as the internal standard to quantify fatty acids and calculate the respective D values.

Synthesis of Arachidonoyl Ethanolamide

Arachidonoyl ethanolamide was synthesized based on our previously optimized procedure and conditions with appropriate modification [15]. In brief, 1 mmol arachidonic acid and 1 mmol ethanolamine were mixed with agitation under nitrogen in 1 mL hexane with 30 % (w/w, relative to total reactants) Novozym 435 as catalyst. The reaction was conducted at 50 °C for 6 h. Water (5 mL) and hexane (5 mL) were then added to the system after lipase had been removed with a Büchner funnel by vacuum filtration. The hexane phase was separated from the aqueous phase. Arachidonoyl ethanolamide was then quantified by GC as described below.

Preparation of Fatty Acid Methyl Esters and Arachidonoyl Ethanolamide Derivative for GC Quantification

One drop of free fatty acids 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 containing amide (about 5 mg) was placed in a 2-mL glass vial and treated with pyridine (0.5 mL), hexamethyldisilazane (0.15 mL) and trimethylchlorosilane (0.05 mL). The mixture was shaken for 15–30 s and then allowed to stand for 30 min. GC was used for quantitative analysis. The purity of the arachidonoyl ethanolamide was calculated based on its peak area

relative to the total peak area, because all products from the main reaction or side reactions would elute and be identified under the GC conditions used [15, 19].

Derivatives of fatty acids and arachidonoyl ethanolamide 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 fatty acid methyl esters determination was programmed from 140 to 250 °C at a rate of 5 °C/min. The oven temperature was programmed from 140 to 300 °C at a rate of 12 °C/min, and then a hold at 300 °C for 5 min for arachidonoyl ethanolamide quantification. The injector and detector temperatures were set to 250 °C for fatty acid analysis and to 300 °C for arachidonoyl ethanolamide quantification.

NMR Analysis for Structure Confirmation

^{1}H-NMR qualitative analysis of the arachidonoyl ethanolamide 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 using Origin 8.0 software. Differences among the means were compared at $P = 0.05$ using Tukey's test.

Results

Urea Inclusion Enrichment of Polyunsaturated Fatty Acids

The initial arachidonic acid-rich oil contained 40.2 % arachidonic acid, 2.2 % linolenic acid, 13.4 % linoleic acid, 11.5 % oleic acid, 5.6 % palmitic acid, 7.7 % stearic acid, 3.5 % docosanoic acid, 10.0 % tetracosanoic acid and 5.9 % unidentified peaks (Table 1). Urea inclusion resulted in a marked increase in arachidonic acid content from 40.2 to 78.4 % (Table 1) but only with 27.6 ± 1.8 % yield (w/w, relative to total free fatty acid). All saturated fatty acids and most oleic acid were removed during this process. Linolenic acid and linoleic acid were 4.2 and 13.7 %, respectively, after urea inclusion purification. These results are similar to those from another study [27].

Arachidonic Acid Enrichment by AgNO3 Solution Fractionation

After the removal of saturated and monounsaturated fatty acids by urea fractionation, arachidonic acid concentrate was purified further by $AgNO_3$ solution fractionation. The main impurities to remove in this purification step are linoleic, linolenic acids and an unknown PUFA (Table 1). *D* values of arachidonic and linolenic acids were selected as indicators for the efficiency of fractionation since it is more difficult to remove linolenic acid compared to linoleic acid. The treatment conditions were optimized by investigating the effects of fatty acid concentration in hexane, free fatty acid addition amount, methanol volume fraction in aqueous $AgNO_3$ solution, incubation temperature and time on *D* value of linolenic and arachidonic acids. The results are presented in Figs. 1, 2, 3, 4 and 5. In general, the best conditions for separation are those that lead to high *D* value for arachidonic acid and low *D* values for linolenic acid.

The effect of the free fatty acid concentration in hexane on *D* value of linolenic and arachidonic acids were not significant ($P > 0.05$) (Fig. 1). The apparent maximum mean value for arachidonic acid was observed at 0.1 g/mL free fatty acid concentration, while *D* value of linolenic at this concentration was relatively low. Thus, 0.1 g/mL was selected as optimal concentration for the next optimization experiments.

The high free fatty acid addition amount may result in a decrease in the *D* value since the complexing of Ag^+ to the

Table 1 Fatty acid composition before and after fractionation and recovery of arachidonic acid

Fatty acid composition (%)[a]	16:0	18:0	18:1	18:2	18:3	20:4	22:0	24:0	Others	20:4 recovery[b] (%)
Initial material	5.6	7.7	11.5	13.4	2.2	40.2	3.5	10.0	5.9	100
After urea inclusion	0	0	0.3 ± 0.1	13.7 ± 0.6	4.2 ± 0.3	78.4 ± 1.4	0	0	3.4 ± 0.7	53.8 ± 2.6
After $AgNO_3$ solution fractionation	0	0	0	0	2.2 ± 0.4	90.7 ± 0.2	0	0	7.2 ± 0.1	93.0 ± 3.1

[a] 16:0: palmitic acid, 18:0: stearic acid, 18:1: oleic acid, 18:2: linoleic acid, 18:3: linolenic acid, 20:4: arachidonic acid, 22:0: docosanoic acid, 24:0: tetracosanoic acid

[b] Recovery is calculated at each step

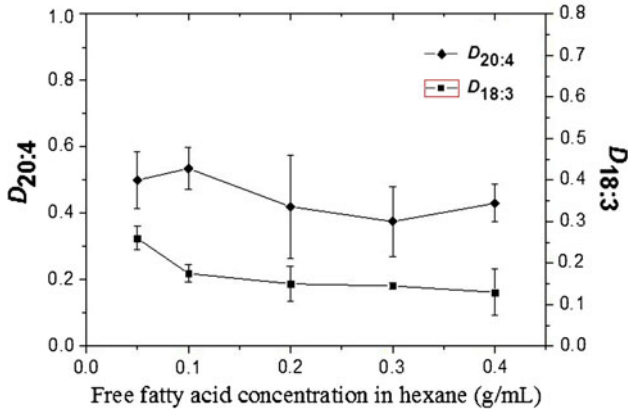

Fig. 1 The effect of free fatty acid concentration in hexane on the *D* value. The *D* value represents the mass ratio of a PUFA in the aqueous phase to the hexane phase. Conditions: different concentration fatty acid solutions (containing 0.6 g) were mixed with 5 mL of 2 M $AgNO_3$ aqueous solution at 20 °C for 2 h; 20:4, arachidonic acid; 18:3, linolenic acid

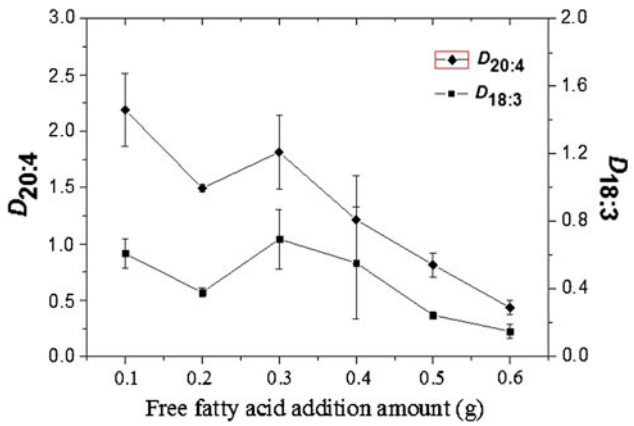

Fig. 2 The effect of free fatty acid addition amount on the *D* value. Conditions: 5 mL of 2 M $AgNO_3$ aqueous solution was mixed with 0.1 g/mL free fatty acid hexane solution at 20 °C for 2 h; 20:4, arachidonic acid; 18:3, linolenic acid

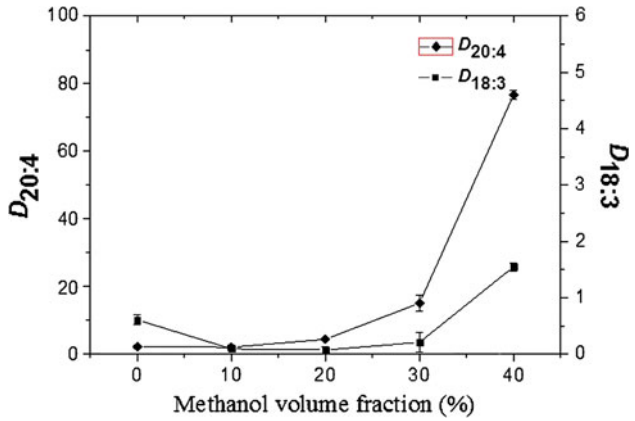

Fig. 3 The effect of the methanol volume fraction in water on the *D* value. Conditions: 5 mL of 2 M $AgNO_3$ methanol aqueous solution was mixed with 1 mL of 0.1 g/mL free fatty acid solution at 20 °C for 2 h; 20:4, arachidonic acid; 18:3, linolenic acid

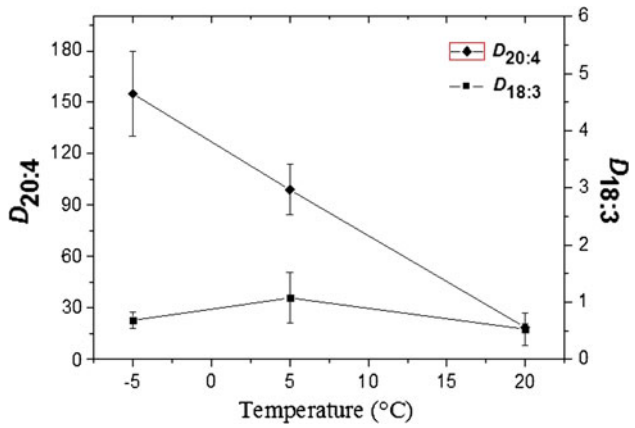

Fig. 4 The effect of the fractionation temperature on the *D* value. Conditions: 5 mL of 2 M $AgNO_3$ in 30 % methanol aqueous solution was mixed with 1 mL of 0.1 g/mL free fatty acid hexane solution for 2 h; 20:4, arachidonic acid; 18:3, linolenic acid

double bond may be saturated, which may cause the remaining free fatty acid stay in the hexane phase. Our results in Fig. 2 show that significant differences were observed for both acids from a 1 to 6-mL addition of the 0.1 g/mL free fatty acid ($P < 0.05$). The *D* values were unexpected at a 2-mL addition amount even though there was no significant difference compared to its subsequent value due to the large standard deviations ($P > 0.05$). The difference in *D* values between linolenic and arachidonic acids was the greatest at 1 mL. Thus, 1 mL of 0.1 mg/mL was selected as the optimal fatty acid addition amount.

Subsequently, the effect of methanol volume fraction in the polar phase was investigated. The addition of methanol may dramatically affect the *D* value of linolenic and arachidonic acids since it is difficult for the nonpolar free fatty acid to get into polar $AgNO_3$ phase when only water is used to dissolve $AgNO_3$. Our results showed that the *D* value of arachidonic acid was not changed from 0 to 20 % but markedly increased from 20 to 40 % methanol addition (Fig. 3). *D* value of linolenic acid decreased slightly from 0 to 30 % but sharply increased subsequently. The results agree with the previous study [23]. In order to keep the complexing of linolenic acid with Ag^+ at a low level, 30 % methanol was selected for the further optimization experiments.

Temperature may also affect the complexing of Ag^+ with double bond. The increase in temperature is expected to weaken the binding between double bond and Ag^+. Fig. 4 shows the effect of temperature on the *D* value. *D* of arachidonic acid dramatically increased with a decrease in

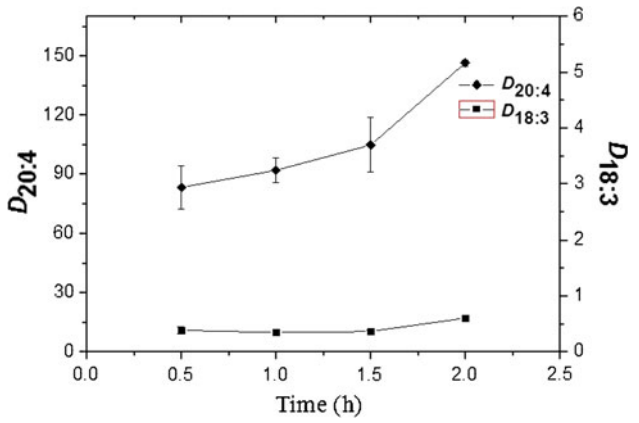

Fig. 5 The effect of the fractionation time on the *D* value. Conditions: 5 mL of 2 M $AgNO_3$ in 30 % methanol aqueous solution was mixed with 1 mL of 0.1 g/mL free fatty acid hexane solution at −5 °C; 20:4, arachidonic acid; 18:3, linolenic acid

temperature, suggesting a stable complex formation at low temperatures ($P < 0.01$). In contrast, the *D* value of linolenic acid did not change significantly from 20 to −5 °C ($P > 0.05$) probably because of competitive complexing of arachidonic and linolenic acids with Ag^+. This result is beneficial to the separation of arachidonic and linolenic acids since the reduction of temperature will increase the complexing of arachidonic acid with Ag^+ greatly, but would not increase the formation of Ag^+-linolenic acid complex. As shown in Fig. 4, the *D* value increased from 20 to 160 when the temperature was changed from 20 to −5 °C for arachidonic acid. Thus, −5 °C was selected as the optimal temperature for the last experiment.

Finally, the effect of time of complexing was optimized (Fig. 5). The incubation time did not greatly affect the *D* value of linolenic acid. The maximum was observed at 2 h for arachidonic acid. There was an increase in the *D* value between 1.5 and 2 h for arachidonic acid, although it was not statistically significant ($P > 0.05$), there was also a slight increase in linolenic acid complexing. In order to keep a low complexing of linolenic acid with Ag^+, 1.5 h was selected as the optimal value.

Furthermore, the optimized processing conditions for the enrichment of arachidonic acid concentrate were used to conduct a purification experiment at −5 °C for 1.5 h by mixing 1 mL of 0.1 g/mL free fatty acid in hexane with 5 mL of 2 M $AgNO_3$ in 30 % methanolic aqueous solution. Under these optimal conditions, 90.7 ± 0.2 % arachidonic acid was obtained with an 80.4 ± 2.8 % yield (w/w, relative to total free fatty acid).

Synthesis of Arachidonoyl Ethanolamide

The amidation reaction between arachidonic acid and ethanolamine after 6 h resulted in the formation of 88.4 ± 0.6 % arachidonoyl ethanolamide with a 72.7 ± 2.2 % yield after purification. The main impurities were α-linolenoyl ethanolamide and a minor esteramide as observed in another study [15].

Qualitative analysis of arachidonoyl ethanolamide was done by using ^1H NMR. NMR analysis gave the expected amide peak (δ 5.9, 1H, $–CH_2CH_2$ CONH–) and we did not see any NMR peak of $–COOCH_2CH_2NH_2$ (δ 4.2–4.4, 2H) and $–COOCH_2CH_2NH_2$ (δ 1.1–1.5, 2H). Therefore, we confirmed the structure of arachidonoyl ethanolamide.

Discussion

The simplest and most efficient method for PUFA enrichment is reported as urea inclusion. The saturated and monounsaturated fatty acids readily complex with urea and crystallize out of solution on cooling, while PUFA are left in the liquid. Thus, PUFA can be separated from saturated and monounsaturated fatty acids by filtration to remove the crystalline fatty acid-urea.

The starting material's fatty acid composition can significantly affect the purity of the final products. Higher purity arachidonic acid can be obtained from the starting material with saturated fatty acids as the main impurities compared to those with PUFA as the main impurities since arachidonic acid and saturated fatty acids have larger differences in solubility in solvents, and complexing ability with urea and $AgNO_3$. In this study, urea fractionation removed all saturated and most monounsaturated fatty acids.

In the subsequent step, $AgNO_3$ solution fractionation was used to further purify urea fractionation concentrated free fatty acid. The separation of free fatty acid by $AgNO_3$ solution fractionation is based on the complexing of Ag^+ with fatty acid's double bond. The complexing ability of Ag^+ with fatty acid increases with the increasing degree of unsaturation. The effects of fatty acid concentration in hexane, free fatty acid addition amount, methanol volume fraction in aqueous $AgNO_3$ solution, temperature and time on *D* (distribution) values of linolenic and arachidonic acids were systematically investigated in order to identify the most effective purification strategy. Our results showed that the methanol volume fraction and the incubation temperature were the most significant variables among five factors.

The addition of methanol affects the polarity of $AgNO_3$ solution. Polar aqueous phase can not dissolve nonpolar free fatty acid, which may result in a low opportunity for the interaction between Ag^+ and fatty acid's double bond. The addition of methanol in the aqueous phase decreases the polarity and therefore encourages the formation of more lipid–$AgNO_3$ complex. In addition, the polarity of

free fatty acid increases with the increase in the number of double bond of free fatty acid. Therefore, PUFA with high unsaturated degree have better opportunity to partition into the aqueous phase to interact with Ag^+ due to its higher polarity compared to saturated, monounsaturated fatty acids and PUFA with lower unsaturation degree.

The temperature affects the stability of the complex. On one hand, complexing of Ag^+ with a double bond will weaken when the temperature is elevated. On the other hand, hexane had a higher ability to extract PUFA from the methanolic aqueous phase at elevated temperatures. Therefore, the increase in temperature is unfavorable for the formation of a complex. The results of our single factor optimization experiments provide a set of appropriate central points for a further multiple factor optimization study. Interactions among these factors may exist, and the scale of purification can also contribute to the identification of the optimal conditions. Therefore, larger scale and multi-factor optimization experiments should be carried out to more accurately identify the optimal conditions before commercial scale production.

Enrichment of PUFA usually needs a combination of multiple methods to fully remove impurities [28]. The most common combination was low temperature crystallization (or winterization) and urea inclusion [28, 29]. Low temperature crystallization can be first used to remove the most of saturated and partial monounsaturated fatty acids at −10 to −20 °C and then urea inclusion can be used to remove remaining saturated and most monounsaturated fatty acids. In this study, we also need to remove the linoleic and linolenic acids. It is difficult to remove these acids by crystallization and urea treatment since urea does not form complexes well with PUFA (Table 2), while the low temperature crystallization only partially removes linoleic acid when the fractionation was conducted at −80 or lower [30]. Enzymatic methods are a green way to enrich PUFA, but it is almost impossible to enrich PUFA to 90 % purity [31, 32]. Preparative HPLC can also be an effective method to enrich PUFA content to 90 %, but it is not economical [33].

We compared the results from our combined purification methods with that from a typical method as previously reported (Table 2). It shows that the combination of low temperature crystallization and urea inclusion could not fully remove the saturated and monounsaturated fatty acids. Thus, the removal of linoleic and linolenic acids to provide high purity arachidonic acid will not be possible using conventional methods, even though low temperature crystallization also has a high recovery of PUFA as a mixture and it is a scalable method. $AgNO_3$ solution fractionation has a high degree of separation and recovery of arachidonic acid from a mixture of PUFA. Thus, $AgNO_3$ solution fractionation is more effective than other common methods.

We have shown that urea inclusion and $AgNO_3$ solution fractionation can highly enrich the arachidonic acid from arachidonic acid-rich oil. The theory and mechanism of liquid–liquid $AgNO_3$ solution fractionation was established by Teramoto et al. [23], who used pure PUFA esters rather than free fatty acids to investigate the distribution of esters between water and organic phases. To date, there has been no application of this method except the commonly used Ag^+-loaded column chromatography and a publication in Japanese in 1998 [24, 25, 34]. We have demonstrated that liquid–liquid $AgNO_3$ solution fractionation is an effective method to remove linoleic and linolenic acids from arachidonic acid. This method is much simpler compared to Ag^+-loaded column chromatography.

For synthesis of arachidonoyl ethanolamide, about 60–90 % fatty acid alkanolamides are usually obtained using the traditional chemical methods, which present many problems in terms of yield, color, and odor [14]. Recently, we improved the chemical synthesis by using vinyl fatty ester as the acyl donor with sodium methoxide as the catalyst to synthesize bioactive fatty acid ethanolamides [19]. However, arachidonic acid vinyl ester is commercially unavailable. Thus, the enzymatic method is an alternative method for the synthesis of arachidonoyl ethanolamide. Novozym 435 has shown itself to be an effective biocatalyst for the amidation reaction between

Table 2 Fatty acid composition of fish oil before and after fractionation reported by Patil and Nag [28]

Fatty acid composition (%)	Saturated	Monounsaturated	EPA and DHA	Recovery of EPA and DHA (%)
Initial material	48.8 ± 3.61	38.9 ± 1.96	10.9 ± 0.84	100
After acetone treatment[a]	4.2 ± 0.39	73.0 ± 3.3	18.3 ± 1.47	82.2
After urea inclusion[b]	1.8 ± 2.17	6.9 ± 4.27	78.2 ± 4.89	47.0

EPA eicosapentaenoic acid, *DHA* docosahexaenoic acid

[a] The separation was carried out by dissolving the free fatty acid in 12 % (w/v) acetone followed by refrigeration at −10 °C for 12 h

[b] Acetone treated free fatty acid (2 g) was added to 8 g urea in 20 mL of 95 % ethanol. The urea-free fatty acid complex was kept in a refrigerator at 5–7 °C for 18 h

arachidonic acid and ethanolamine and the method is scalable and simple for the synthesis of large quantity of arachidonoyl ethanolamide.

Conclusions

The synthesis of arachidonoyl ethanolamide, a lipid mediator in animal and plant tissues that exhibits multiple biological functions, was considered difficult (the pure arachidonoyl ethanolamide sold by Sigma-Aldrich is $67.3 per 5 mg), because the base materials such as pure arachidonic acid and triarachidonin are expensive or unavailable. In this study, we combined two purification methods to enrich arachidonic acid from an arachidonic acid-rich oil and demonstrated the effectiveness of the methods. The liquid–liquid $AgNO_3$ solution fractionation is shown to be an effective method to remove other polyunsaturated contaminating fatty acids, which are difficult to remove by traditional methods. The enzymatic synthesis optimized from our previous study has proven itself to be suitable for making the arachidonoyl ethanolamide. Thus, we demonstrated a feasible method to obtain a bioactive lipid that may have great potential in studies and applications for animal and human health enhancement.

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