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Lipozyme 435-catalyzed synthesis of eicosapentaenoyl ethanolamide in a solvent-free system

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ABSTRACT

Eicosapentaenoyl ethanolamide (EPEA) is a lipid signaling molecule. In this study, an effective process is described to synthesize EPEA by enzymatic amidation using eicosapentaenoic acid ethyl ester (EPA-EE) as acyl donor with lipase as catalyst. The reaction conditions were optimized. When the amidation reaction was conducted at 70 °C for 1 h in a solvent-free system with agitation by reacting 2 mmol fatty acid ethyl ester with 3 mmol ethanolamine in presence of 10% Lipozyme 435 as a catalyst, fatty acid ethanolamides was formed at 62.5% molar yield. This was the first time reporting that Lipozyme 435 lipase was used as catalyst for enzymatic amidation. In addition, compared to previous methods using free fatty acid as acyl donor for fatty acid ethanolamide synthesis, study using fatty acid ester as acyl donor is limited and the use of fatty acid ester avoids ion pair formation between free fatty acid and ethanolamine. Finally, we found that Lipozyme 435 lipase had a higher tolerance toward polar ethanolamine as compared to Novozym 435 and Lipozyme RM IM.

1. Introduction

Fatty acid ethanolamides are an important class of nonionic surfactants, which are widely used in lubricants, surfactants and detergents, cosmetics, and the related industries [1,2]. Fatty acid ethanolamides as biosurfactants are a particularly attractive class of compounds that are potential substitutes for emulsifiers derived from petroleum due to highly environmental acceptance, skin tolerance, good biologic degradability and low toxicity [3]. Typically, they are used as anti-slip and anti-block additives for polyethylene films, coatings for paper, lubricant additives, printing ink additives, water repellants for textiles, mold release agents, defoaming agents and flow improvers [2].

In addition to the use as nonionic surfactants, studies conducted over the last twenty years have given numerous compelling evidences that fatty acid ethanolamides serve as new and additional class of endogenous signaling molecules and they are lipid mediators naturally found in animal and plant tissues [4–6]. Fatty acid ethanolamides were first discovered as lipid mediators in 1957. Palmitoyl ethanolamide was found in soybeans, peanut oil, and egg yolk and was shown to exhibit an anti-inflammatory activity [7]. Saturated and monounsaturated fatty acid ethanolamides are major components in animal and plant tissues and has been reported to show a variety of biological activities such as anti-inflammatory and anorexic effects, modulation of feeding and energy homeostasis, and attenuating pain sensation [8,9]. Similarly, polyunsaturated fatty acid ethanolamides also exhibit various biological functions. For example, eicosapentaenoic acid (EPA) can be converted to eicosapentaenoyl ethanolamide (EPEA), which has been shown to have anti-cancer activity [10]. In addition, EPEA also plays a new role in organismal aging and acts as a metabolic signal that couples nutrient availability with growth and lifespan [11]. Other activities of EPEA include anti-proliferative [12] and anti-inflammatory properties [13]. For these reasons, there is now a great interest in the synthesis of polyunsaturated fatty acid ethanolamides such as EPEA and docosahexaenoyl ethanolamide (DHEA).

In general, fatty acid ethanolamides are prepared by the reactions of free fatty acid [14–17], fatty acid chloride [18–20] with ethanolamine. The most common method is to use free fatty acid as acyl donor to synthesize pure fatty acid ethanolamides since fatty acid chloride is a corrosive, relatively toxic and expensive chemical. When the free fatty acid is selected as acyl donor, fatty acid ethanolamides are usually prepared by enzymatic amidation in a solvent. Even though the enzymatic synthesis using free fatty acid

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as acyl donor is a relatively effective process, the amidation reaction conducted in a solvent usually needs 30% (w/w, relative to total reactants) lipase or more [14,15,17]. The high lipase load may be attributed to the formation of ion pair from equivalent moles of ethanolamine and free fatty acid. The ion pair is a salt, has a high melting point and cannot be dissolved in organic solvents. The lipase has a low catalytic activity to ion pair to form fatty acid ethanolamides. Fatty acid ethanolamides can also be synthesized by using fatty acid vinyl ester as acyl donor [21]. However, unsaturated fatty acid vinyl ester is not readily available commercially.

The study on biological activities of EPEA has received little attention compared to other fatty ethanolamides probably, because little commercial EPEA is available and expensive. Therefore, an effective method for EPEA synthesis needs to be studied. To establish an efficient method for fatty ethanolamide synthesis and avoid the formation of ion pair, we chose fatty acid ethyl ester rich in eicosapentaenoic acid ethyl ester (EPA-EE) rather than free fatty acid as acyl donor to prepare EPEA. Moreover, we investigated the feasibility of using solvent-free system due to low melting point of EPEA. The effects of the removal of ethanol, type of lipase, ethanolamine amount, reaction temperature and reaction time on fatty acid ethanolamide content in the crude mixture were examined.

2. Materials and methods

Fatty acid ethyl ester containing 74.7% EPA-EE and 21.8% docosahexaenoic acid (DHA) ethyl ester (336 of average molecular weight) was purchased from Wuxi Xunda Marine Biology Co., Ltd. (Wuxi, China). Stearoyl ethanolamide standard was purchased from Sigma–Aldrich Chemical Co., Ltd. (St. Louis, MO). Ethanolamine was obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Novozym 435 (lipase B from *Candida antarctica*, immobilized on a macroporous acrylic resin), Lipozyme 435 (a recombinant lipase from *C. antarctica*, expressed on *Aspergillus niger*, and immobilized on Lewatit VP OC 1600), and Lipozyme RM IM (lipase from *Rhizomucor miehei*, immobilized on an anionic exchange resin) were obtained from Novozymes (Beijing, China). All other reagents including solvents were analytical grade and purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

2.1. Optimization of fatty acid ethanolamide synthesis

There are five parameters optimized to obtain maximum fatty acid ethanolamide yield. The five parameters include the effects of ethanol removal from reaction system, the type of lipase, addition amount of ethanolamine, reaction temperature and reaction time. When the reaction conditions were optimized, one factor was changed at different levels, whereas the other factors were kept constant. After one of the factors was optimized, the optimal value of this factor was employed for the next factor optimizations. All reactions were run in duplicate unless otherwise specified. The results were expressed as the mean ± standard deviation (SD).

2.1.1. The effect of the removal of ethanol

The reaction for the effect of the removal of ethanol from the reaction system was conducted at 50 °C for 3 h by reacting 2 mmol fatty acid ethyl ester with 2 mmol ethanolamine with 10% Novozym 435 lipase (relative to the weight of the total reactants) as catalyst. The amidation reaction was performed in a solvent-free system with or without the removal of ethanol. When the strategy of ethanol removal was employed, the reaction was carried out at 1.33×10^3 Pa in 15 mL round-bottom flasks rotating at 150 rpm on a vacuum-rotary evaporator. At the end of the reaction, the lipase was removed by vacuum filtration. The crude reaction product was diluted to 1 mg/mL with hexane and subsequently quantified by HPLC as described in the following section.

2.1.2. The effect of the type of lipase

Based on the former optimization results, strategy of ethanol removal was used for the selection of the type of lipase. The amidation was performed at 1.33×10^3 Pa in 15 mL round-bottom flasks rotating at 150 rpm on a vacuum-rotary evaporator using the following conditions: 2 mmol fatty acid ethyl ester, 2 mmol ethanolamine, 10% lipase, 50 °C for 3 h. The selected commercial lipases for the reaction optimization were Novozym 435, Lipozyme 435 and Lipozyme RM IM.

2.1.3. The effect of ethanolamine amount

Based on the former optimization results, Lipozyme 435 was used for the further reactions. Fatty acid ethyl ester (2 mmol) was reacted with ethanolamine in a solvent-free system at 50 °C for 3 h. Ethanolamine addition amount was changed from 2 to 6 mmol. Lipase (10%, relative to the weight of the total reactants) was used as catalyst to start the amidation reaction. Subsequently, the mixture was stirred by a vacuum-rotary evaporator rotating at 150 rpm and the pressure of reaction system was set at 1.33×10^3 Pa. In addition to investigate the effect of ethanolamine amount on fatty acid ethanolamide content, the effect of ethanolamine amount on lipase tolerance toward was also compared. The selected lipases included Lipozyme 435 and Novozym 435.

2.1.4. The effect of reaction temperature

Based on the former optimization results, 3 mmol of ethanolamine was selected for the next optimization reactions. The amidation reaction was carried out for 3 h by mixing 2 mmol fatty acid ethyl ester with 3 mmol ethanolamine in a solvent-free system using 10% Lipozyme 435 lipase as catalyst. The reaction was conducted at 25–80 °C to investigate the effect of reaction temperature. Subsequently, the reaction mixture was stirred at 1.33×10^3 Pa by a vacuum-rotary evaporator rotating at 150 rpm.

2.1.5. The effect of reaction time

Based on the former optimization results, 70 °C was selected as optimal temperature for the study on the effect of reaction time. The amidation reaction was performed at 70 °C by reacting 2 mmol fatty acid ethyl ester with 3 mmol ethanolamine in a solvent-free system with 10% Lipozyme 435 as catalyst. Subsequently, the reaction mixture was stirred at 1.33×10^3 Pa by a vacuum-rotary evaporator rotating at 150 rpm. The sample was taken hourly from the reaction system once the reaction was started.

2.2. HPLC analysis of fatty acid ethanolamide

Amidation product before and after purification was analyzed by HPLC-ELSD, using a Waters 1525 liquid chromatographic system (Waters Corp., Milford, MA, USA) equipped with a LiChrospher Si column (25 cm × 0.46 cm, 5 μm particle size, Sigma–Aldrich Corp., K.K., Tokyo, Japan) at 35 °C, and eluted with a binary gradient of solvent A (1:99 of isopropanol/hexane, v/v) and solvent B (1:1:0.01 of isopropanol/hexane/acetic acid, v/v/v) at 0.8 mL/min. Samples were diluted to 1 mg/mL and analyzed based on the following gradient profiles: B was increased from 0 to 10% over 10 min and increased further to 20% from 10 to 20 min. Finally, B was held at 20% for 10 min. Total run time was 30 min.

Since, HPLC cannot separate fatty acid ethanolamides with different fatty acid chain lengths, all fatty acid ethanolamides came out from HPLC as a peak. Stearoyl ethanolamide was used as external standard to qualify fatty acid ethanolamides. The peak was identified according to the HPLC retention time. Since, ethanolamine