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Lipase-catalyzed synthesis of palmitanilide: Kinetic model and antimicrobial activity study

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ABSTRACT

Enzymatic syntheses of fatty acid anilides are important owing to their wide range of industrial applications in detergents, shampoo, cosmetics, and surfactant formulations. The amidation reaction of *Mucor miehei* lipase Lipozyme IM20 was investigated for direct amidation of triacylglycerol in organic solvents. The process parameters (reaction temperature, substrate molar ratio, enzyme amount) were optimized to achieve the highest yield of anilide. The maximum yield of palmitanilide (88.9%) was achieved after 24 h of reaction at 40 °C at an enzyme concentration of 1.4% (70 mg). Kinetics of lipase-catalyzed amidation of aniline with tripalmitin has been investigated. The reaction rate could be described in terms of the Michaelis–Menten equation with a Ping–Pong Bi–Bi mechanism and competitive inhibition by both the substrates. The kinetic constants were estimated by using non-linear regression method using enzyme kinetic modules. The enzyme operational stability study showed that Lipozyme IM20 retained 38.1% of the initial activity for the synthesis of palmitanilide (even after repeated use for 48 h). Palmitanilide, a fatty acid amide, exhibited potent antimicrobial activity toward *Bacillus cereus*.

1. Introduction

Fatty acid anilides are used as detergents, shampoos, cosmetics, lubricants, foam-control agents, fungicides, antioxidant agents, bioactive agents, corrosion inhibitors, and water repellents [1,2]. Owing to their possessed strong inhibitory activities against human macrophage, hepatic and intestinal acyl-CoA: cholesterol acyltransferase (ACAT), it is expected as antiarteriosclerotic and hypocholesterolemic agent for use in humans [3]. Palmitanilide is used in the preparation of antislip and antiblock additives in polyethylene films and as flow improvers for oils owing to their low reactivity and good thermal properties [4]. Fatty acid anilides can be prepared by amidation of natural oil. Feairheller et al. [5] synthesized palmitanilide by reacting aniline with tallow. The reaction was carried out at 50–60 °C for 72 h using a molar ratio of oil and amine of 1:10. However, only a 7% yield of palmitanilide was obtained, the method is not cost-effective.

Anilides are produced industrially from the fatty acids and by heating them at 150–170 °C during 2–3 h in an agitated vessel with a means of removing excess of amine or water [6]. The current production processes of palmitanilide is based on the reactions of fatty acids with anhydrous ammonia under high-temperature (about 200 °C) and high-pressure (340–700 kPa) conditions [7]. An additional purification step is also required to obtain pure fatty acid amides, i.e., without any side products. In view of these disadvantages of these processes, an attempt has been made to develop an enzymatic reaction as an alternative low-cost and low-energy-consuming industrial process. Lipases have been successfully employed as the catalysts in the synthesis of fatty acid amides [8,9]; e.g., oleamide from oleic acid [10]. However, the synthesis of palmitanilide from tripalmitin using lipase Lipozyme IM20 is one of the very few cases of enzymatic synthesis of fatty amides.

Palmitanilide can inhibit bacterial growth (an antibacterial agent) [11,12]. In this study, the amidation of aniline with tripalmitin was investigated in organic solvents using Lipozyme IM20, as the biocatalyst. To optimize the production of palmitanilide, various parameters influencing enzyme activity, such as reaction temperature, time, and aniline/tripalmitin molar ratio were investigated. The kinetic parameters of amidation were assayed to evaluate the practicality of the developed method. Moreover, the antibacterial activity of palmitanilide was investigated using *Bacillus cereus*, *Escherichia coli*, and *Salmonella enterica* subsp. *arizonae* as the index organisms. The inhibition zones of palmitanilide were compared to the commercial antibiotic of reference disc. Finally, the

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residual enzyme activity after repeated operations was assayed to evaluate the practicality of the developed method.

2. Materials and methods

2.1. Materials

Five commercially available lipases were used. *Pseudomonas cepacia* lipase (Amano PS) and *Penicillium camemberti* lipase (Amano G) were purchased from Amano International Enzyme Co. (Nagoya, Japan). *Mucor miehei* (Lipozyme IM20) and *Candida antarctica* (Novozym 435) lipases were obtained from Novo Nordisk Inc. (Danbury, CT, USA). *Candida cylindracea* type VII lipase was obtained from Sigma Chemical Co. (St. Louis, MO, USA). Lipozyme IM20 and Novozym 435 were used as the immobilized enzymes. Aniline, palmitic acid, palmitic anhydride, palmitic acid ethyl ester, and tripalmitin were obtained from Sigma. All other chemicals used were of reagent grade.

2.2. Optimization of palmitanilide synthesis in organic solvents

Palmitanilide was synthesized using a 15-mL reaction vessel via the amidation of aniline (50 mM) with tripalmitin (25 mM) in isopropyl ether. The reaction mixture (5 mL) contained 1.3% (70 mg) biocatalyst. The reactions were performed at 40 °C with orbital shaking (250 rpm). Several experiments were conducted to optimize the reaction conditions such as enzyme source, reaction time (3–48 h), reaction temperature (30–60 °C), molar ratio (0.2–5.0) of aniline to tripalmitin, and reaction solvent to obtain the best yield of palmitanilide. The effects of several parameters on the palmitanilide yield were studied while keeping all the other conditions constant. The residual enzyme activity was assayed using the optimized experimental conditions after 1–3 cycles of operations to evaluate the reaction conditions developed. Yield of palmitanilide prepared under each set of conditions was calculated from the following equation:

$$\text{Yield of palmitanilide}(\%) = \frac{\text{Mole of palmitanilide}}{\text{Mole of aniline}} \times 100$$

2.3. Purification of palmitanilide

The purification protocol for the amide was as follows. The immobilized enzyme was removed from the reaction mixture via filtration through a 0.1 mm sieve. The solvent was then removed by evaporation in a rotary evaporator. The residual solid was dissolved in 1 mL of chloroform to give a final concentration of solids of 20 mg/ml. Further purification was accomplished with a chromatography column on silica gel (240–400 mesh size). The raw product was loaded onto the silica gel column. The palmitanilide was eluted with methanol–dichloromethane (1:32, v/v; 6 mL), followed by monopalmitin and dipalmitin eluted with pentane–dichloromethane (2:1, v/v; 12 mL). The flow-rate was kept at 10–12 mL/min. Fractions were collected and solvent removed by rotary evaporator (R200A, Büchi, Flawil, Switzerland) at 30 °C. The residue was chromatographed by gas chromatography and pure product was obtained.

2.4. Kinetic constants determination

For determining the kinetics of synthesis of palmitanilide, reaction mixtures were prepared with the range of substrate concentration 25–125 mM. Reaction was initiated by addition of 70 mg of Lipozyme IM20 in each of the reaction mixtures. The reaction temperature was maintained at 40 °C. The initial reaction rates obtained at various aniline and tripalmitin concentrations were fitted to Michaelis–Menten kinetics with Ping–Pong Bi–Bi mechanism by non-linear regression using Microsoft Excel Software (version 5.0; Microsoft Corporation, Redmond, WA, USA). The rate equation for Ping–Pong Bi–Bi mechanism with inhibition by aniline and tripalmitin is as follows:

$$\nu = \frac{V \max [A][\mathrm{TP}]}{[A][\mathrm{TP}] + K_{\mathrm{mA}}[\mathrm{TP}]\left[1 + \frac{[\mathrm{TP}]}{K_{\mathrm{iTP}}}\right] + K_{\mathrm{mTP}}[A]\left[1 + \frac{[A]}{K_{\mathrm{iA}}}\right]} \quad (1)$$

where, ν is the rate of reaction, $V_{\max}$, maximum rate of reaction, [A], initial concentration of aniline, [TP], initial concentration of tripalmitin, K_{mA}, Michaelis constant for aniline, K_{mTP}, Michaelis constant for tripalmitin, K_{iA}, inhibition constant for aniline, and K_{iTP} is the inhibition constant for tripalmitin.

2.5. Bacterial cultures

B. cereus ATCC 11778, *E. coli* CCRC 10675, and *S. enterica* subsp. *arizonae* ATCC 13314 were obtained from the Food Industry Research and Development Institute, Department of Bioresources Collection and Research Center, Hsinchu, Taiwan, and employed as the test organisms. *B. cereus* was grown aerobically in 15 mL conical glass flasks containing 2 mL of nutrient broth (NB, Difco, Detroit, MI, USA) at 37 °C for 12 h. Aliquots (40 μL) of this culture were aseptically transferred to 4 mL of fresh medium and incubated at 37 °C to the mid-logarithmic phase (absorbance ~0.6 at 660 nm). After two successive transfers of the test organism in NB at 37 °C for 12 h, the activated culture was again inoculated into 100 mL NB at 37 °C for 12 h. Cells were then harvested by centrifugation (8000 × *g* for 10 min), suspended in 10 mL of 0.85% NaCl to ca. 10^{10} cfu/mL, and used as the inoculum.

2.6. Determination of antibacterial activity of palmitanilide

The antibacterial activity of palmitanilide was tested on the Gram-positive (*B. cereus* ATCC 11778) and Gram-negative strains (*E. coli* CCRC 10675, and *S. enterica* subsp. *arizonae* ATCC 13314) by the disc diffusion method [13]; The suspension (10 mL) containing 10^{10} cfu/mL of bacteria was poured onto the Luria broth (LB) agar plate. The broth cultures of bacteria were freshly prepared for each assay overnight (24 h, 37 °C). The LB agar plates (20 mL) were prepared, allowed to set, and then their surface was dried (37 °C, 30 min). The broth cultures were vortexed for 30 s, and 500 μL of the culture was immediately removed spread over the agar surface, and dried (37 °C, 15 min). Palmitanilide (10 μL, 60 mM, 19.9 mg/mL) was pipetted onto a 6-mm sterile disc (Oxoid), and the disc was placed onto the surface of the prepared agar plate. Following 24 h of incubation at 37 °C, the diameter of the inhibition zone for each dilution was then recorded in mm (including the disc). For the control plates, 10 μL of nanopure water was pipetted onto the disc. The assays were completed in triplicate and repeated independently three times. The assays were also repeated using commercially available antibiotic (ampicillin, 60.1 mM, 21 mg/mL) for the positive controls.

2.7. Enzyme leakage study

Immobilized lipase was subjected to the leakage study as described by Ozyilmaz and Gezer [14] spectrophotometrically at 280 nm in triplicate. 100 mg of immobilized lipase was added to 4 mL of isopropyl ether and was agitated on shaker at 150 rpm for 12–72 h, where pure isopropyl ether was used as blank. The