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Ultrasonic pretreatment in lipase-catalyzed synthesis of structured lipids with high 1,3-dioleoyl-2-palmitoylglycerol content

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ABSTRACT

Production of structured lipid 1,3-dioleoyl-2-palmitoylglycerol (OPO), from tripalmitin (PPP) and oleic acid (OA) using lipases and ultrasonic pretreatment was conducted. Factors influencing both the ultrasonic conditions and enzymatic reaction were investigated. Optimum conditions could be attained with 6 min pretreatment time, 50% ultrasonic power, 3 s/9 s (work/pause) cycle of ultrasonic pulse, 1:8 PPP/OA molar ratio, 12% enzyme dosage and 50 °C temperature of. At the optimum conditions, the OPO yield of 51.8% could be achieved in 4 h. Studies showed that the OPO content increased to 35.9% in 1 h with ultrasonic pretreatment, in comparison to 4 h without ultrasonic pretreatment. Reuse of Lipozyme RM IM for 10 cycles under ultrasonic irradiation did not cause essential damage to its lipase activity. Reaction kinetic model fitted well with the proposed Ping-Pong mechanism. The apparent kinetic constant (V_m'/K_2) of ultrasound pretreatment reaction was 2.52 times higher than the conventional mechanical stirring, indicating that ultrasound pretreatment enhanced the substrates affinity to the enzyme. This study confirmed that ultrasonic pretreatment was more efficient in OPO production than conventional mechanical agitation.

1. Introduction

Fats and oils are major food constituents. They are also high-energy substances in the human body, and are composed of >95% triglycerides (TAGs). The nutritional value and physicochemical properties of TAGs depend not only on the fatty acid composition, but also on the positional distribution of acyl groups bonded to glycerol [1]. Hence, improvement of the physicochemical properties and nutritive values of TAGs by modification of their fatty acid composition and positional distribution holds promise in nutrition, food and pharmaceutical applications. Structured lipids (SLs), a form of TAGs, have been studied extensively by using chemically catalyzed reactions, enzymatically catalyzed reactions, genetic engineering, or a combination thereof to modify the fatty acid composition, positional distribution in the glycerol backbone, or both [2]. Various SLs have been used to limit the caloric intake of fats and oils, to improve the physicochemical properties of fats, to reduce the absorption of long-chain saturated fatty acids, and to increase the amount of essential fatty acids for specific purposes, such as docosahexaenoic acid and arachidonic acid [3]. 1,3-Dioleoyl-2-palmitoylglycerol (OPO), an important ABA-type SL, is one of the main components of TAGs in human milk [4]. Human milk fat is the richest energy source for infants, supplying essential structural components for the cell membranes of newborns [5]; therefore, numerous investigations have focused on the production of OPO [6].

Enzyme-catalyzed synthesis of SLs has major advantages over chemical synthesis in terms of practical applications such as those involving mild reaction conditions and environmentally friendly processes [7]. Another advantage of enzyme technology is its amenability for use with foodstuffs. Acidolysis is an enzymatic method that is more commonly employed than interesterification for the production of OPO SLs, as its products are easily separable from the final reaction mixtures, consequently decreasing the cost of separation [8]. Acidolysis consists of two steps: the starting TAGs are first hydrolyzed into diacylglycerols and monoacylglycerols, and then the new fatty acids are esterified into the glycerol skeleton to form new TAGs [9]. Acyl migration is a side reaction of esterification that forms byproducts. Acyl migration is affected by many factors such as type of lipase, solubility of the substrate, reaction

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temperature, and reaction time [10]. Attempts have been made to reduce acyl migration during enzymatic acidolysis. These include reduction of reaction time, activation of lipase activity, and diminution of mass-transfer limitations [11].

Ultrasound irradiation, an environmentally friendly method, has gained popularity and potential for use in applications in organic chemistry and in biotechnology [12,13]. This method has been used to accelerate the rates of numerous chemical reactions [14]. However, its effects on enzymatic reactions have been studied less extensively [15]. Studies on enzymatic reactions subjected to ultrasound irradiation used it mainly for pretreatment and for catalytic reactions. Ultrasound pretreatment for enzymatic reactions aids in reducing the particle size and in increasing the substrate–enzyme interface area through its high cavitation energy. This effect is much more obvious in reactions in organic media using enzyme powders and reduces in mass-transfer limitations [16]. The use of ultrasound throughout the reaction allows substrates to access active sites more easily [17]. Conformations of enzymes change upon exposure to ultrasound. Immersion in an ultrasonic cleaning bath is the most common method used in ultrasound-assisted reactions. However, ultrasound energy cannot be completely transferred to a reaction because of its indirect mode of transmission. Furthermore, it is difficult to realize in large-scale production and is uneconomical in industrial application. Thus, ultrasound pretreatment using microtip probe may be an appropriate method for enzymatic reactions [18]. Many investigators utilized ultrasound to conduct enzymatic interesterification and thus obtained promising results [19,20]. Compared with conventional methods, ultrasound pretreatment could improve the biocatalyst performance [21], shorten the reaction time [22], and increase the product yields [23,24].

In this paper, lipase-catalyzed synthesis of SLs is reported. This approach effectively enriches the OPO content via ultrasound pretreatment in organic media using microtip probe. This work mainly focuses on the reaction parameters that affect lipase-catalyzed synthesis of OPO subjected to ultrasound. A comparison of the OPO content and its effect on lipase reusability was made between ultrasound irradiation and conventional stirring methods under optimal conditions. Kinetic study was also investigated at the optimal experimental conditions to determine the apparent kinetic parameters for reactions under either ultrasound pretreatment or conventional mechanical stirring. This work is a comprehensive study on OPO production and on monitoring of acyl migration during pretreatment ultrasonic irradiation.

2. Materials and methods

2.1. Materials

Tripalmitin (>85% purity) and oleic acid (>99% purity) were purchased from Tokyo Chemical Industry Co., Ltd. The immobilized lipases Lipozyme RM IM (immobilized on anion exchange resin) from *Rhizomucor miehei*, Lipozyme TL IM (immobilized on silica gel) from *Thermomyces lanuginosus*, and Novozym 435 (immobilized on polyacrylic resin) from *Candida antarctica* were purchased from Novozymes (Bagsvaerd, Denmark). HPLC-grade hexane and acetonitrile were obtained from CNW (Düsseldorf, Germany). All TAG standards (PPP, OPO, 1,2-dipalmitoyl-3-oleoylglycerol (PPO), 1,3-dipalmitoyl-2-oleoylglycerol (POP), 1-palmitoyl-2, 3-dioleoylglycerol (POO) and triolein (OOO); Larodan Fine Chemicals, Malmö, Sweden) were dissolved in hexane to a concentration of 5 mg/mL and then stored at −20 °C. Analytical grade *n*-hexane and other solvents were purchased from Sinopharm Chemical Reagent (Shanghai, China).

2.2. Equipment

Ultrasound pretreatment reactions were carried out in a long-necked round-bottom flask (10 mL) in a thermostated water bath (Pharmacia, Sweden) with a microtip probe (3 mm diameter), and a sonotrode (Sonics Vibracell, VCX 130, 130 W, 20 kHz, USA). The power for the reaction could be adjusted from 20 to 60%. Lipase-catalyzed acidolysis reactions were performed in a thermostated water bath with magnetic stirrer (Aohua Instrument Co., Ltd., Changzhou, China). An Agilent 1200 series HPLC system was used for the separation of TAGs. It was equipped with a G1312A bin pump, a G1379B degasser, a G1329 Aautosampler, and a G1316 Athermostated column compartment (Agilent Corporation, Palo Alto, CA). MS used for analysis was performed on an API 4000 Q-Trap hybrid triple quadrupole/linear ion trap mass spectrometer with an APCI interface (AB SCIEX, Foster City, CA, USA).

2.3. Lipase-catalyzed acidolysis with ultrasonic pretreatment

A typical reaction was carried out by combining 0.1 g of PPP, 0.21 g of commercial OA (1:6 PPP/OA mole ratio), 0.0248 g of lipase (8% by total weight of substrates), and 3 mL of hexane with 4A molecular sieves (50 mg/mL). Hexane was previously dried with molecular sieves (100 g/L) for at least 48 h. The mixtures were placed into a 10 mL round-bottom flask. The flask was firstly placed in a thermostated circulating water bath with microtip probe for ultrasound pretreatment. It was then transferred to a water bath with magnetic agitation at 200 rpm for further reaction. During the reactions, samples were withdrawn from the mixture every hour to analyze for reaction progress. All analyzes were performed in triplicate and the results were reported as the mean standard deviation.

2.4. Reuse of the enzyme

At the end of each round of acidolysis, the immobilized lipase was washed thrice with *n*-hexane and then separated from the substrate by vacuum filtration. It was then dried for 10 h at 40 °C (in an oven) and reused in a subsequent new reaction.

2.5. Analysis and determination of TAG products by silver ion HPLC atmospheric pressure chemical ionization tandem mass spectrometry (HPLC–APCI–MS/MS)

During synthesis, OPO was obtained. However, TAGs containing oleic acid at the sn-2 position (i.e. POP, POO, and OOO) were also produced because of acyl migration. In this study, Lipozyme RM IM that we used is wildly known as a sn-1,3 specific lipase, which hydrolyzes the ester bonds at sn-1 and sn-3 positions of TAG, but not sn-2 position. Acyl migration from sn-1 and sn-3 to sn-2 position or remigration from sn-2 to sn-1,3 can take place [25]. Fig. 1 shows a proposal for the scheme and mechanism of the current lipase-catalyzed acidolysis reaction. Silver ion HPLC has proven to be an effective means to monitor acyl migration and to control the product quality of the reaction. Thus, HPLC using a Varian ChromSpher 5 Lipids silver ion chromatography column (250 mm × 4.6 mm i.d., 5 μm particle size; Agilent Technologies, Milan, Italy) was performed to separate TAGs. Isocratic elution with hexane/acetonitrile mobile phase (99.4:0.6 v/v) was done at 1.5 mL/min flow rate of, 10 μL injection volume, and 30 °C oven temperature, in accordance with our previous report [26]. MS was carried out in positive APCI mode under the following conditions: curtain gas, 137.9 kPa; nebulizer current, 27.58 kPa; temperature, 450 °C; scan mode, multiple reaction monitoring-enhanced product ion (MRM-EPI); scan rate, 4000 μ/s; pressure of ion source gas 1, 344.75 kPa; declustering potential, 85 V; collision energies,

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