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Enhancement of Lipase-catalyzed Synthesis of Caffeic Acid Phenethyl Ester in Ionic Liquid with DMSO Co-solvent ☆

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ABSTRACT

Caffeic acid phenethyl ester (CAPE) is a natural and rare ingredient with several biological activities, but its industrial production using lipase-catalyzed esterification of caffeic acid (CA) and 2-phenylethanol (PE) in ionic liquids (ILs) is hindered by low substrate concentrations and long reaction time. To set up a high-efficiency bioprocess for production of CAPE, a novel dimethyl sulfoxide (DMSO)–IL co-solvent system was established in this study. The 2% (by volume) DMSO–[Bmim][Tf_2N] system was found to be the best medium with higher substrate solubility and conversion of CA. Under the optimum conditions, the substrate concentration of CA was raised 8-fold, the reaction time was reduced by half, and the conversion reached 96.23%. The kinetics follows a ping-pong bi-bi mechanism with inhibition by PE, with kinetic parameters as follows: $V_{max} = 0.89\ mmol \cdot min^{-1} \cdot g^{-1}$, $K_{m,CA} = 42.9\ mmol \cdot L^{-1}$, $K_{m,PE} = 165.7\ mmol \cdot L^{-1}$, and $K_{i,PE} = 146.2\ mmol \cdot L^{-1}$. The results suggest that the DMSO co-solvent effect has great potential to enhance the enzymatic synthesis efficiency of CAPE in ILs.

1. Introduction

Caffeic acid phenethyl ester (CAPE) is one of the most active ingredients of propolis of honeybee hives. It possesses several biological activities, such as antioxidant [1] and anti-inflammatory [2,3], so the application of CAPE in the pharmaceutical industry is increasing due to its potential health benefits. However, CAPE is a rare phenolic compound in nature. Compared with chemical synthesis, enzymatic synthesis of CAPE has several advantages such as high specificity and efficiency, and milder reaction conditions [4]. A process has been proposed [5,6] for CAPE synthesis by lipase-catalyzed esterification between caffeic acid (CA) and 2-phenylethanol (PE) in isooctane. However, the use of organic solvent has some disadvantages, such as toxicity and environmental unfriendliness. Ionic liquids (ILs) as 'green solvents', which are easier to be efficiently reused and can improve the stability, catalytic activity and selectivity of enzymes [7–9], are replacing organic solvents for enzymatic synthesis of CAPE [10,11].

The application of ILs in CAPE enzymatic synthesis was investigated. Wang *et al.* [12] used CA and PE as substrates in the lipase-catalyzed synthesis of CAPE in [Emim][Tf_2N] and obtained 98.76% conversion after 48 h. Ha *et al.* investigated the same reaction and optimized the reaction conditions by response surface methodology [13]. A maximum conversion of 96.6% was obtained after 60 h, but the process presented two major drawbacks: low solubility of CA and long reaction time. The solubility of CA in IL [Emim][Tf_2N] is only 13 $mmol \cdot L^{-1}$, which results in lower synthesis efficiency, higher cost and more energy consumption. In order to improve the solubility of substrate, the lipase transesterification synthesis of CAPE from methyl caffeate (MC) and PE was proposed. Although the solubility of MC is higher (49 $mmol \cdot L^{-1}$ in [Bmim][Tf_2N]) and the conversion is high, this method needs two steps and increases the production cost [3]. Hence, lipase direct-esterification synthesis of CAPE is more attractive for high yield.

Recently, co-solvent mixtures of organic solvents and ILs have emerged as an efficient approach for lipase-catalyzed reactions [14]. The main advantages of this strategy are to increase substrate solubility and shorten reaction time. In addition, this innovative strategy allows changes in the hydrophobic nature of reaction medium, selectively accumulating the desired product [15]. Dimethyl sulfoxide (DMSO) is the most common solvent used in synthesis chemistry. It is called "universal solvent" and can dissolve numerous substrates. Thus it is usually considered as a co-solvent to improve the solubility of substrates and to promote reaction rates [16,17]. The co-solvent mixture of IL and organic solvent, [Bmim][Br]–DMSO, has been used as a reaction medium for the synthesis of 1-aryl tetrazoles to reduce the reaction time [18]. In our

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preliminary experiment, it is found that the utilization of IL [Bmim][Tf_2N] and DMSO for the synthesis of CAPE is feasible. This strategy not only improves the solubility of CA but also shortens the reaction time. In addition, DMSO is miscible with water while [Bmim][Tf_2N] is immiscible with water. It is easy to remove DMSO by using water to wash the reaction mixture several times. To the best of our knowledge, the lipase-catalyzed esterification of CAPE in the DMSO–[Bmim][Tf_2N] co-solvent system has not been reported.

Moreover, a detailed kinetic analysis for enzymatic synthesis of CAPE has not been carried out, so it is necessary to study the kinetics of lipase-catalyzed synthesis of CAPE and understand the reaction mechanism. Several models have been proposed to explain lipase-catalyzed esterification reactions. A simple model is the Michaelis–Menten mechanism, but it is only valid for the simplest enzymatic reactions. Other two useful models include ping-pong bi-bi and ordered bi-bi mechanisms [19]. The ping-pong bi-bi mechanism, with the first product released between additions of two substrates, seems to agree with experimental results better and is generally accepted [20]. Therefore, the ping-pong bi-bi model is more suitable for investigating the kinetics of lipase-catalyzed CAPE synthesis in the DMSO–[Bmim][Tf_2N] system.

The objective of this study is to synthesize CAPE by lipase-catalyzed esterification via the utilization of [Bmim][Tf_2N] and DMSO. The effects of DMSO content, molar ratio of CA to PE, dosage of lipase, reaction temperature and CA concentration on the CA conversion and CAPE yield are assessed. The synthesis product is identified by liquid chromatography–mass spectrometry (LC–MS) and nuclear magnetic resonance (NMR). A kinetic model is proposed for the esterification of CA with PE catalyzed by Novozym 435 in this co-solvent system.

2. Materials and Methods

2.1. Chemicals and enzyme

CA was purchased from Nanjing Zelang Pharmaceutical Sci. & Tech. Co. Ltd. (Nanjing, China). DMSO was purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). IL [Bmim][Tf_2N] was purchased from Shanghai Cheng-Jie Chemical Co., Ltd. (Shanghai, China), with a residual chloride content less than 50×10^{-6} Novozym 435 (*Candida antarctica* type B lipase immobilized on macro-porous polyacrylate resin, bead size of 0.3–0.9 mm) was purchased from Novozymes (Bagsvaerd, Denmark). All other regents were of analytical purity.

2.2. Enzymatic esterification for CAPE

The enzymatic esterification of CA in co-solvent DMSO–[Bmim][Tf_2N] mixtures was conducted in 5 ml glass vials with screw caps at an agitation speed of 120 r · min^{-1}. CA was fully dissolved in DMSO. PE and 1 ml [Bmim][Tf_2N] were added and stirred until a homogenous solution was obtained. The esterification reaction was initiated after the addition of Novozym 435. Approximately 20 μl of the well-stirred reaction mixture was taken at selected time intervals (0, 12, 24, 48, 60 and 72 h) for HPLC analysis. The effects of DMSO content (0–10%, by volume), mass ratio of CA to Novozym 435 (1:10, 1:15, 1:18, 1:20 and 1:25), molar ratio of CA to PE (1:10, 1:15, 1:20, 1:30, 1:40 and 1:50), reaction temperature (40–85 °C), and substrate concentration of CA (10–65 mmol · L^{-1}) on the CA conversion and CAPE yield were investigated. All experiments were performed in triplicate.

2.3. Analysis of products by LC–MS and NMR

Mass spectrometry of product CAPE was performed according to a previously described protocol on an LC–PAD–MS (Thermo Fisher system) [21,22]. The CAPE product (5 mg) was dissolved in DMSO-d_6 (0.5 ml). ^{1}H NMR spectra were recorded on a Bruker AVANCE 400 instrument operated at ^{1}H resonance frequency of 400 MHz (see Fig. A1). The chemical shifts were reported relative to tetramethylsilane as the internal standard. The NMR data of CAPE were as follows: ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.62 (s, 1H), 9.15 (s, 1H), 7.44 (d, J = 15.9 Hz, 1H), 7.34–7.25 (m, 4H), 7.22 (ddd, J = 5.7, 4.2, 1.9 Hz, 1H), 7.04 (d, J = 2.0 Hz, 1H), 6.99 (dd, J = 8.2, 2.0 Hz, 1H), 6.76 (d, J = 8.1 Hz, 1H), 6.22 (d, J = 15.9 Hz, 1H), 4.32 (t, J = 6.9 Hz, 2H), 2.95 (t, J = 6.9 Hz, 2H). The LC–MS and NMR data for CAPE were in accordance with those in literature [3,12].

2.4. HPLC analysis of CA and CAPE

The concentrations of CA and CAPE were determined by an HPLC system with a constant flow pump (2PB0540, Beijing Satellite Factory, Beijing, China), a UV–vis detector (L-7420, Techcomp Co. Ltd., Shanghai, China) and an N-2000 workstation (Hangzhou Mingtong S&T Ltd., Hangzhou, China). The chromatographic conditions were as follows: HC-C_{18} column (250 mm × 4.6 mm, i.d.; 5 μm, W. R. Grace & Co., Deerfield, IL, USA); detection wavelength of 325 nm; flow rate of 1.0 ml · min^{-1}; and mobile phase, acetonitrile/water (50:50, v/v). 20 μl of the sample was taken from the reaction mixture and diluted using 980 μl methanol for HPLC analysis. Before injection, all samples were filtered through a 0.45 μm filter. According to the literature [12], the CA conversion and CAPE yield of the lipase-catalyzed reaction are calculated as follows.

$$\text{CAPE yield} = \frac{\text{moles of CAPE}}{\text{initial moles of CA}} \times 100\% \quad (1)$$

$$\text{CA conversion} = \frac{\text{consumptive moles of CA}}{\text{initial moles of CA}} \times 100\%. \quad (2)$$

2.5. Kinetic study of lipase-catalyzed synthesis of CAPE in the co-solvent system

The kinetics of esterification was investigated by examining the effect of concentrations of CA and PE on the initial reaction rate. CA concentration was varied at different fixed concentrations of PE and *vice versa*. The concentrations of CA and PE varied in the range of 10–65 mmol · L^{-1} and 0.04–0.8 mol · L^{-1}, respectively [3].

2.6. Statistical analysis

Triplicate experiments were conducted for each parameter. The standard deviations of the measures were calculated to check the reliability of results. The statistical analyses were performed using the ANOVA method. Significant differences ($p < 0.05$) between the means were determined.

3. Results and Discussion

3.1. Lipase-catalyzed synthesis of CAPE in different media and selection of reaction system

The solubility of CA is important in the lipase-catalyzed synthesis of CAPE in IL. Table 1 shows the results of lipase-catalyzed synthesis of CAPE in different reaction media. Benzene, chloroform and *n*-hexane have poor solubility for CA, so the lipase–catalyst synthesis of CAPE in these systems is negligible. Although *tert*-butanol presents higher solubility for CA, pure organic solvents have some disadvantages such as toxicity, environmental unfriendliness and lower yield.

In our preliminary study, the CAPE yield in IL [Bmim][PF_6] is only 4.73%, while [Bmim][Tf_2N] and [Emim][Tf_2N] are more effective than other ILs in the lipase-catalyzed synthesis of CAPE. Table 1 gives the CAPE yields in [Bmim][Tf_2N] and [Emim][Tf_2N], 65.22% and 64.55%, respectively. [Bmim][Tf_2N] is relatively effective due to its hydrophobicity,