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An efficient enzymatic modification of cordycepin in ionic liquids under ultrasonic irradiation

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ABSTRACT

A comparative study of the immobilized *Candida antarctica* lipase B (Novozym 435)-catalyzed acylation of cordycepin with vinyl acetate in ionic liquids (ILs) under ultrasonic irradiation and shaking was conducted. The application of ultrasonic irradiation instead of shaking during acylation resulted in an enhanced reaction rate and a higher level of substrate conversion. Among the various ILs examined, 1-butyl-3-methylimidazolium tetrafluorobrate ($[C_4MIm][BF_4]$) was the best medium for the reaction because it produced the highest substrate conversion. In $[C_4MIm][BF_4]$, the optimal ultrasonic power, water activity, and reaction temperature were 120 W, 0.33, and 50 °C, respectively. The acylation of cordycepin in $[C_4MIm][BF_4]$ proved to be regioselective under both conditions: the C5′-OH was acylated. Novozym 435 exhibited a much higher operational stability in $[C_4MIm][BF_4]$, and 58.0% of its original activity was maintained after ten reuse cycles under ultrasonic irradiation. Compared with the cordycepin, the rate of adenosine deaminase-catalyzed deamination was greatly reduced when the 5′-OH was substituted by acetyl group. These results demonstrated that the combined application of ultrasonic irradiation and IL as a medium was an efficient approach for the enzymatic modification of cordycepin.

1. Introduction

Camellia sinensis and *Camellia militaris* have been widely used as a traditional Chinese medicine and a tonic food in East Asia and is also being studied in the West in the past several years, due to its various biological activities [1]. Cordycepin (3′-deoxyadenosine, Scheme 1) is one of the main and most medicinally active components isolated from *C. sinensis* and *C. militaris* [2–4]. Cordycepin is reported to possess many interesting biological and pharmacological activities [1–4]. However, *in vivo* studies have shown that it is hard for cordycepin to exert its pharmacological activities effectively due to its rapid hydrolytic deamination by adenosine deaminase (ADA) [5–7]. Moreover, cordycepin is highly hydrophilic and cannot easily cross cell membranes through passive diffusion. These characteristics necessitate the development of appropriate derivatives of cordycepin.

We recently reported the enzymatic modification of cordycepin by acetylation in organic media [8]. In this report, we established 2-methyltetrahydrofuran (MeTHF) and immobilized *Candida antarctica* lipase B (Novozym 435) as the optimal reaction medium and catalyst, respectively.

MeTHF has many advantages as a solvent [9,10]. In fact, MeTHF is not a safe solvent [11]. MeTHF is a flammable liquid with a mildly irritating odour. Moreover, MeTHF can be easily oxidised to peroxide [11]. The physical and chemical properties of MeTHF hinder its potential use on a large scale as a reaction medium. Furthermore, cordycepin has a very low solubility in most conventional hydrophobic solvents. Therefore, it is necessary to identify an efficient and safe reaction medium for the enzymatic modification of cordycepin.

In recent years, ILs, as green solvents, have been effectively used as media for biocatalytic reactions and are becoming increasingly attractive in such applications [12,13]. Compared with traditional organic solvents, ILs have unique characteristics, such as negligible vapour pressures, excellent solvent properties, and high stabilities [12,13]. More importantly, ILs are capable of dissolving substrates with high polarity, such as carbohydrates, amino acids, and nucleosides [13–15], which are minimally soluble in common organic solvents. This important feature of ILs opens up new opportunities for the biocatalysis of high-polarity substrates in non-aqueous solvents. However, the high viscosity of IL, which is usually two to three orders of magnitude greater than those of conventional organic solvents, results in mass transfer limitations during the reaction [12,13].

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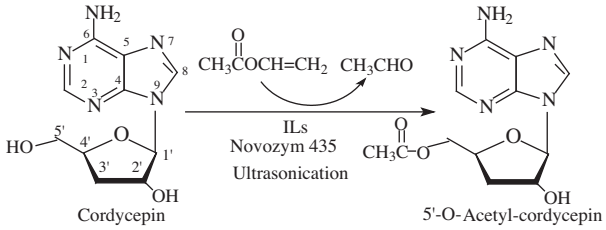

Scheme 1. Novozym 435-catalyzed regioselective acylation of cordycepin with vinyl acetate in ILs under ultrasonic irradiation.

It is well-known that ultrasonic irradiation is a useful tool for enhancing mass transfer in liquid-liquid heterogeneous systems by means of cavitation, mechanical forces, and thermal effects [16]. This means that ultrasonic irradiation can to some extent counteract the effect of high viscosity on reactions in an IL. Moreover, ultrasonication can also be used for the intensification of enzymatic activity [17,18]. Thus, the combination of ultrasonic irradiation and an IL has the potential to improve the performance of Novozym 435 for the modification of cordycepin.

Based on our previous study [8], in the present work, we performed the first evaluation of the influence of ultrasonic irradiation on the Novozym 435-catalyzed acylation of cordycepin with vinyl acetate in ILs (Scheme 1). To evaluate the effect of ultrasonic irradiation on the reaction rationally, we conducted a comparative study between this and the effect of shaking.

2. Materials and methods

2.1. Chemical and biological materials

Cordycepin (purity > 99%) was purchased from Phytochemical Laboratory, College of Life Science, South China Normal University, Guangzhou, China. Vinyl acetate (>99%) was purchased from Sigma-Aldrich (St. Louis, MO, USA). The ILs 1-alkyl-3-methylimidazolium tetrafluorobrate ($[C_n MIm][BF_4]$, n = 2, 4, or 8) and 1-alkyl-3-methylimidazolium hexafluorophosphate ($[C_n MIm][PF_6]$, n = 2, 4, or 6) were purchased from Sigma-Aldrich (Shanghai)Trading Co. Ltd. (Shanghai, China). Novozym 435 (from *Candida antarctica* lipase B, immobilized on a macroporous acrylic resin) was kindly donated by Novozymes China (Beijing, China). The catalytic activity of Novozym 435 was 10,000 PLU (Propyl Laurate Units) g^{-1} containing 1–2% (w/w) water. Adenosine deaminase (5.0 units/mL, from calf intestinal mucosa) was purchased from Beijing Qisong Biotechnology Co., Ltd. (Beijing, China). One unit will deaminate 1.0 μmole of adenosine to inosine per min at pH 7.5 at 25 °C. All of the other reagents were obtained from commercial sources and were of analytical grade.

2.2. Ultrasonic experimental set-up

The experiments were conducted in a round-bottomed flask that was sealed and clamped inside an ultrasonic bath (20 kHz, 0–400 W; Type NP-B-400-15; Newpower Co. Ltd., Kunshan, China). The internal dimensions of the ultrasonic bath were 30 × 25 × 25 cm. The ultrasonic pulse sequence was 10 s on and 5 s off. The bath temperature can be maintained within ±1 °C. In our study, the height of the water bath was 22 cm from the bottom, and the 50 mL reaction flask with sample was located in the centre of the bath and fixed at 16 cm from the bottom.

In the shaking case, an air-bath shaker was used, and the shaking rate was maintained constant for all of the experimental conditions at 250 rpm. The shaker temperature can be maintained within ±1 °C.

2.3. General procedures for the enzymatic acylation of cordycepin

A typical enzymatic acylation reaction was conducted in 20 mL of ILs with a specified a_w value containing 4.0 mmol (1.0 g) of cordycepin, 40.0 mmol of vinyl esters, and 3000 U of Novozym 435 at 50 ± 1 °C under ultrasonic irradiation or shaking. Aliquots (20 μL) were withdrawn at specified time intervals from the reaction mixture, filtered through a 0.45-μm syringe filter to remove the biocatalyst, and then diluted 100 times with methanol prior to HPLC analysis. The HPLC analysis was conducted according to a method reported previously [8]. The retention times for cordycepin and 5′-acetyl cordycepin were 2.81 and 5.69 min, respectively (ESI Figs. S1 and S2).

In the control reaction, which was performed using the above procedure without the enzyme, no chemical acylation reaction was detectable. All of the experiments were performed at least in triplicate, and the results are reported as the mean ± standard deviation.

2.4. Water activity (a_w) control

The water activity of each reaction system was controlled according to a previously reported method [19]. The initial water activities of the reaction medium, the substrate and the enzyme were controlled by gaseous equilibrium with different saturated salt solutions in separate closed containers at 25 °C. The following salts were used: LiBr (a_w = 0.07), LiCl (a_w = 0.11), $MgCl_2$ (a_w = 0.33), $Mg(NO_3)_2$ (a_w = 0.54), NaCl (a_w = 0.75), and KCl (a_w = 0.84).

2.5. Operational stability (reusability) of Novozym 435

The stability of Novozym 435 during the batch acylation reactions was investigated. Reaction conditions: 4.0 mmol of cordycepin, 40.0 of mmol vinyl acetate, 3000 U of Novozym 435, 20 mL of $[C_4MIm][BF_4]$, a_w = 0.33, ultrasonic power = 120 W, 4 h. After each batch reaction, the immobilized lipase was recovered by filtration, washed three times with acetone, and used in the next batch (4 h per batch) with new substrates. The conversion achieved in the first batch was set to 100. The relative activity was expressed as the relative conversion.

2.6. Adenosine deaminase-catalyzed deamination of cordycepin and acetyl cordycepin

The adenosine deaminase (ADA) catalyzed-deamination of cordycepin and acetyl cordycepin was investigated. The reaction conditions were the following: 1.0 mmol of cordycepin or acetyl cordycepin, 10 U of ADA, 100 mL phosphate buffered saline (PBS, 0.05 M, pH 7.0), 37 °C.

2.7. Product recovery and structure determination

After reaction, vial was removed and 20 mL of methanol was added to the viscous reaction mixture. The mixture was immediately filtered through a 0.45 μm PTFE filter membrane to remove the lipase beads. The filtrate was evaporated under vacuum. Cold ethanol (ethanol to reaction mixture ratio of 4:1 by vol) was then added to precipitate the product and the solids were filtered. The product was purified by semi-preparative HPLC using the mixture of methanol–water (3:7, v/v) as an eluant.

The position of the acylation in an enzymatically prepared ester was determined by NMR. The ^1H and ^{13}C NMR spectra (ESI Figs. S3 and S4) were recorded on a Bruker AVANCE AV-500 instrument. DMSO-d_6 was used as the solvent. Elemental analysis was measured using CHN-O-Rapid elemental analysis apparatus (Heraeus, Germany). Accurate is within 0.3% (ESI Table S1).