



Insight into microwave assisted enzyme catalysis in process intensification of reaction and selectivity: Kinetic resolution of (*R,S*)-flurbiprofen with alcohols

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ABSTRACT

Biocatalysis has potential applications in pharmaceuticals, fine chemicals and drug industries for the synthesis and separation of chiral compounds. The current work deals with the kinetic resolution of (*R,S*)-flurbiprofen using immobilized *Candida antarctica* lipase B under the influence of microwave irradiation. Flurbiprofen (*R,S*)-[2-(3-fluoro-4-phenyl) phenyl] propionic acid is one of the most widely used non-steroidal anti-inflammatory drugs (NSAID). It exists in two enantiomeric forms as (*R,S*)-flurbiprofen because it has a single stereogenic center. Pharmacological action of flurbiprofen resides with the *S* form by inhibiting Cox-2 activity and results in anti-inflammatory action and hence (*R*)-flurbiprofen causes gastrointestinal infection. Novozym 435 was found to be most active among others for the stereoselective esterification of the *R*-enantiomer in organic reaction media. Microwave irradiation shows a synergistic effect on enzyme catalysis by enhancing both rate of the reaction and conversion of *R*-enantiomer into an ester. Effects of various reaction parameters such as solvent, speed of agitation, mole ratio, type of alcohol, temperature, catalyst loading, and reusability were studied. Maximum conversion of *R*-flurbiprofen ester under microwave irradiation was found to be ~44.4% with enantioselectivity (*E*) of 478, $ee_P = 98.9\%$ and $ee_S = 79.07\%$ in 6 h, whereas in conventional heating the maximum conversion was 17% with enantioselectivity (*E*) of 126, and $ee_S = 20.12\%$ in 6 h. The ping-pong bi-bi mechanism with inhibition by methanol was proposed for the kinetic resolution of (*R,S*)-flurbiprofen and validated against experimental data.

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1. Introduction

Biocatalysis offers a clean and green way to perform traditional chemical processes, as it is environment-friendly. It has various applications in several industries such as pharmaceuticals, chemicals, agrochemicals, and food and beverages for the synthesis and separation of compounds. In recent years, enzymes are widely exploited in the synthesis of enantiomerically pure and commercially valuable pharmaceutical intermediates and drugs. Kinetic resolution by enzymatic method is the most commonly used approach for the resolution of the particular enantiomer as it has the following advantages 1) it requires mild temperature and pressure, 2) shows high regio- and stereo-selectivity, and 3) broad substrate specificity [1]. Lipases are the most commonly used enzymes for the kinetic resolution of racemates, as they are capable

of identifying the stereogenic center, have broad substrate specificity and do not require any cofactor for their activity [2–7].

Lipases are present in most of the organisms, mammals, bacteria, fungi and plants [8]. In organic solvents, lipase catalyzes many reactions that include esterification, interesterification, thioesterification, hydrolysis, *trans*-thioesterification, epoxidation and amidation [2,8]. *Candida antarctica* lipase B belongs to the α/β hydrolase family, and it has a conserved catalytic triad with Serine, Histidine and Aspartate/Glutamate at the active site [9,10]. It consists of 317 amino acids and has a molecular weight of 33.5 kDa [10]. Like other lipases, in non-polar solvents Novozym 435 lacks interfacial activation [11]. In pharmaceutical industry, the resolution of racemic chiral alcohols and acids can be done through the enantioselective esterification by using lipases [12].

Flurbiprofen is a non-steroidal anti-inflammatory chiral drug (NSAID), with two enantiomeric forms [1,13–15]. (*S*)-flurbiprofen inhibits Cox-2 activity (anti-inflammatory activity) and Cox-1 activity (gastrointestinal and renal side effects) whereas (*R*)-flurbiprofen is inactive on both Cox-1 and Cox-2. Hence, (*R*)-flurbiprofen is the cause of gastrointestinal and renal effects [16].

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Nomenclature

E	Enantioselectivity
ee_p	Enantiomeric excess of product
ee_s	Enantiomeric excess of substrate
C	Conversion, %
$[R]$	Concentration of <i>R</i> enantiomer, mol L ⁻¹
$[S]$	Concentration of <i>S</i> enantiomer, mol L ⁻¹
$[A]$	Initial concentration of (<i>R,S</i>)-flurbiprofen, mol L ⁻¹
$[B]$	Initial concentration of methanol, mol L ⁻¹
v	Initial rate of the reaction, mol L ⁻¹ min ⁻¹
v_{max}	Maximum rate of the reaction, mol L ⁻¹ min ⁻¹
K_{mA}	Michaelis constant of (<i>R,S</i>)-flurbiprofen, mol L ⁻¹
K_{mB}	Michaelis constant of methanol, mol L ⁻¹
K_{iB}	Inhibition constant of methanol, mol L ⁻¹

Currently, flurbiprofen is administered as a racemic mixture, but the anti-inflammatory activity of the drug resides in only one enantiomer; therefore it requires resolution of the two enantiomeric forms [16].

From the literature available till date it shows that this reaction is very slow under conventional heating [1,13,14,17]. Siódmiak et al. [36] have reported that Novozym 435 exhibited 35.7% conversion and $E=90.5$ in 96 h at 35 °C [16]. To meet the industrial requirements, there is a need to intensify this slow reaction with high enantioselectivity. To enhance the performance, various strategies have been adopted and microwave irradiation is one such approach [12]. Hence we decided to study the effect of microwave irradiation on the kinetic resolution of (*R,S*)-flurbiprofen using a suitable biocatalyst.

Microwave irradiation has gained importance in biotransformation as a green and clean process by increasing yield and selectivity with a significant reduction in time [12,15,18–23]. The mechanism of heating under microwave irradiation is due to ionic conduction or dipole rotation (resulting in instantaneous localized superheating) whereas, conduction and convection occur in conventional heating [18]. An increase in temperature produces greater movement of molecules that leads to a larger number of energetic collisions and hence enhances the rate of the reaction and conversion [18]. Yadav and co-workers have successfully used microwave irradiation for a number of reactions [8,12,19,24].

In the present work, kinetic resolution of (*R,S*)-flurbiprofen using various commercially available immobilized lipases has been studied under the influence of microwave irradiation. We are first time reporting kinetic resolution of (*R,S*)-flurbiprofen under microwave irradiation in comparison with conventional heating. The effect of various reaction parameters such as solvent, different types of lipases, speed of agitation, mole ratio, type of alcohols, temperature, catalyst loading and catalyst reusability on rate of reaction and conversion under microwave irradiation was studied systematically. The Lineweaver-Burk plot was made by varying concentrations of substrate to demonstrate the reaction mechanism and kinetics.

2. Materials and methods

2.1. Chemicals and enzymes

All enzymes were used as reported by Kamble and Yadav [11]. All chemicals were purchased from the reputed and renowned firms and used without further purification. (*R,S*)-Flurbiprofen was a gift sample from IPCA Laboratories, Mumbai India. All solvents were used of HPLC grade and purchased from Thomas Baker (Chemicals) Pvt. Ltd. Mumbai.

2.2. Experimental set-up and procedure

2.2.1. Conventional heating

The reactions were carried out under mechanically agitated fully baffled glass reactor as reported by Kamble et al. [19]. A typical reaction mixture consisted of 0.2 mmol (*R,S*)-flurbiprofen, 0.6 mmol methanol, diluted up to 20 mL with toluene as solvent. The reaction mixture was agitated at 75 °C for 15 min at a speed of 400 rpm, and then 50 mg of Novozym 435 was added to initiate the reaction. The reaction was carried out up to 6 h. Samples were withdrawn periodically, filtered and analyzed using HPLC. All experimental data are an average of triplicate values within a standard deviation (SD) of $\pm 5\%$.

2.2.2. Microwave reactor

The reactions were carried out in a commercially available system (Discover, CEM Model-1245) and reaction conditions such as temperature and pressure were controlled by a feedback system. A constant power of microwave irradiation 50 W was provided. The reactor used for the reaction was of 120 mL capacity with 4.5 cm i.d. and six-bladed pitched turbine impeller for agitation [12,25]. A constant temperature of ± 1 °C was maintained throughout the reaction time. A typical reaction mixture of lipase catalyzed kinetic resolution of (*R,S*)-flurbiprofen consisted of (0.2–0.6) mmol (*R,S*)-flurbiprofen and (0.2–1.2) mmol methanol, diluted up to 20 mL with different solvents. The reaction was carried out at different temperatures (35–95) °C and speeds of agitation (200–500) rpm. Then the reaction was initiated by adding immobilized lipase of different concentrations (20–60) mg and carried out up to 6 h. All experimental data are an average of triplicate values within a standard deviation (SD) of $\pm 5\%$.

2.3. Enzyme kinetics

The kinetics of lipase catalyzed enantioselective resolution of (*R,S*)-flurbiprofen was evaluated by studying various operational parameters (Scheme 1). Effect of concentrations of (*R,S*)-flurbiprofen and methanol on the rate of the reaction using Novozym 435 was studied systematically. To study the enzyme kinetics only initial rates of reaction were considered. During the initial period only (*R*)-flurbiprofen methyl ester was obtained ($ee_p = 99.9\%$). The initial rate of conversion of (*S*)-flurbiprofen can be considered to be negligible as there was no significant conversion.

2.4. Analytical studies

2.4.1. HPLC analysis and structural identification

The analysis was done by HPLC (Agilent 1260 infinity) with UV detector at 254 nm. The column used for the analysis was Chiralpak IB (4.6 mm ϕ \times 250 mmL, particle size 5 μ m) from Daicel Chemical India, Ltd. The mobile phase composition used was *n*-hexane/tert-methyl butyl ether/2-propanol/trifluoroacetic acid (70:30:0.4:0.1 v/v/v/v) with a flow rate of 1 mL/min. The temperature of the column was operated at 25 °C. The samples withdrawn were diluted with solvent (1:1 v/v). A calibration curve was prepared using different concentrations of (*R,S*)-flurbiprofen. The chromatography was done in isocratic mode (Supplementary information, Fig. A1 and A2). The product of the enzymatic biotransformation was confirmed by GC-MS (Thermo Fischer-TRACE™ 1300) (Supplementary information, Fig. A3). The following parameters were calculated using the equations described in the literature [16]:

- The Enantiomeric ratio (E),

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