

Ketoprofen Resolution by Enzymatic Esterification and Hydrolysis of the Ester Product

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Abstract Immobilized *Candida antarctica* lipase was used to catalyze the separation of ketoprofen into its components by means of esterification followed by the enzymatic hydrolysis of the ester product. In this study, ketoprofen underwent esterification to ethanol in the presence of isooctane. When the reaction was complete, 58.3% of the ketoprofen had been transformed into an ester. The ketoprofen remaining in solution after the reaction was complete consisted primarily of its *S*-enantiomer (83.0%), while the 59.4% of the ketoprofen component of the ester consisted of its *R*-enantiomer. We then subjected the ester product to enzymatic hydrolysis in the presence of the same enzyme and produced a ketoprofen product rich in the *R*-enantiomer; 77% of this product consisted of the *R*-enantiomer when 50% of the ester had been hydrolyzed, and 90% of it consisted of the *R*-enantiomer when 30% of the ester had been hydrolyzed. By contrast, the *R*-enantiomer levels only reached approximately 42 and 65%, respectively, when 50 and 30% of the racemic ester was hydrolyzed under the same conditions.

Keywords: ketoprofen, resolution, esterification, hydrolysis, lipase, *Candida antarctica*

INTRODUCTION

The possibility of using enzymes to separate a racemic compound into its components to obtain optically pure enantiomers of that compound has been studied extensively over the past decade [1-5]. Generally, only one enantiomer is targeted; the other enantiomer usually remains in solution or becomes a component of the product of the reaction. The outcome varies with the enantiomer selectivity of the enzyme used, because the product is often racemized chemically or enzymatically for repeated use as the starting material. However, when both enantiomers are desired, it might be beneficial to subject the remaining substrate or the product containing the other enantiomer to further enzymatic activity to obtain a product that is rich in the other enantiomer. This is especially effective for achieving a high concentration of the other enantiomer when the selectivity of the enzyme for that enantiomer is low and the substrate has already been partially degraded in the last step of the enzymatic process.

Ketoprofen is a nonsteroidal antiinflammatory drug whose biological activity is associated solely with its *S*-enantiomer [6]. Its *R*-enantiomer is used as a toothpaste additive to prevent periodontal disease [7,8]. We investigated the degradation of ketoprofen into its components by enzymatic esterification followed by enzymatic hydrolysis of the ester product using commercially available

immobilized *Candida antarctica* lipase.

MATERIALS AND METHODS

Materials

Immobilized *C. antarctica* lipase B (EC 3.1.1.3), *Candida rugosa* lipase, and ketoprofen were purchased from Sigma (St. Louis, MO, USA). Ketoprofen ethyl ester was prepared following the method described by Kim *et al.* [9]. High-performance liquid chromatography (HPLC) grade organic solvents were purchased from Merck (Whitehouse Station, NJ, USA). All other chemicals used in this study were of a reagent grade and obtained commercially.

Enzymatic Esterification of Ketoprofen

Ketoprofen 800 mg was dissolved in a solution containing isooctane:acetone (95:5, v/v) 40 mL, ethanol 920 μ L, and immobilized *C. antarctica* lipase 800 mg. The reaction mixture was maintained in a shaking water bath (180 rpm) at 45°C for 8 h. At this point, 58.3% of the substrate had been converted to ketoprofen ethyl ester. The lipase was then removed by filtration, and the filtrate was mixed with an equal volume of 1 M NaHCO₃ for approximately 20 min. This mixture was then separated into 2 phases through a separating funnel: the organic phase was collected and evaporated in a rotary evaporator under reduced pressure to obtain the ketoprofen ethyl

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ester, in which the majority (59.4%) of the ketoprofen component consisted of its *R*-enantiomer; the aqueous phase was acidified to a pH of 2 by adding 1 M H₂SO₄ to cause the unreacted ketoprofen – which consisted primarily of its *S*-enantiomer (83.0%) – to precipitate out of solution so that it could be collected by filtration, washed with deionized water, and dried.

Enzymatic Hydrolysis of the Ketoprofen Ester

Ketoprofen ethyl ester 200 mg was dissolved in 0.1 M phosphate buffer (pH 7.0) 10 mL, and immobilized *C. antarctica* lipase 100 mg was added. The mixture was kept in a shaking water bath (180 rpm) at 45°C for 2 h, then underwent filtration to remove the lipase. The filtrate was acidified to a pH of 2 by adding 1 M H₂SO₄. The reaction product (ketoprofen) along with the remaining ester, was extracted using ethyl acetate (2 × 10 mL) and subjected to HPLC analysis.

Analytical Methods

Ketoprofen was analyzed by HPLC using a Chiracel OJ-H column (4.6 × 250 mm). Samples (5-μL) were eluted by a mixture of *n*-hexane:2-propanol:acetic acid (90:10:0.5, v/v/v) at a rate of 1.0 mL/min, and detected at a wavelength of 254 nm. The enantiomer selectivity (*E*) of the enzyme was calculated using the following formula:

$$E = \frac{\ln[(1-C)(1-ee_s)]}{\ln[(1-C)(1+ee_s)]}$$

where *C* is the substrate conversion rate and *ee_s* is the proportion of the enantiomer, acted on by the enzyme that remains in the substrate [10]. All data are presented as the mean of at least triplicate experiments and were reproducible within ± 10%.

RESULTS AND DISCUSSION

Effect of Acetone Content on Enzymatic Esterification of Ketoprofen

It has been well recognized that enzymatic activity is generally enhanced when carried out in a hydrophobic solvent, while hydrophilic solvents enhance enzyme enantiomer selectivity. The use of mixed solvents is thus a good way to improve enzyme enantiomer selectivity without causing a significant loss in enzyme activity. We screened several polar organic solvents (including acetone, acetonitrile, diethyl ether, and ethylene chloride) by mixing each one with isooctane, but observed no significant difference in the enantiomer selectivity of the enzyme (immobilized *C. antarctica* lipase; data not shown). We chose acetone for this experiment because of its low toxicity. Ethanol was used as the acyl acceptor; a 5:1 molar ratio of ethanol:ketoprofen proved to be opti-

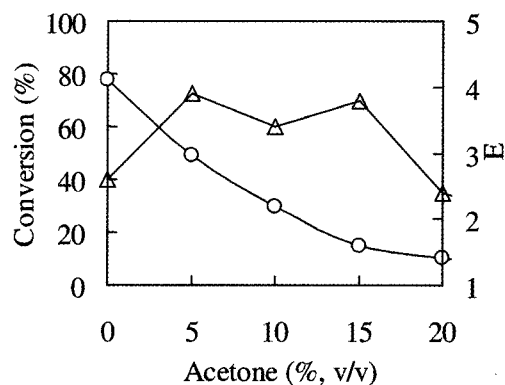


Fig. 1. Effect of acetone concentration on the rate of substrate conversion and enzyme enantiomer selectivity (*E*) during ketoprofen esterification. 100 mg ketoprofen; 115 μL ethanol (5 times the molarity of ketoprofen); 100 mg *C. antarctica* lipase; 20 mL solvent (isooctane: acetone); temperature: 37°C; shaking speed: 180 rpm; reaction time: 3 h. (○): ketoprofen conversion; (△): enantiomer selectivity (*E*).

mal for this reaction [11].

The rate of substrate conversion decreased rapidly as the acetone content increased, and enzyme enantiomer selectivity peaked when the acetone content reached 5% (v/v) (Fig. 1). When the acetone content was increased to 20% (v/v), the rate of substrate conversion decreased to 10%, which is much lower than the rate of 80% that is seen in the absence of acetone. This suggests that enzyme activity is dramatically reduced in the presence of a high concentration of acetone. The presence of acetone improved the solubility of ketoprofen in isooctane, thereby facilitating substrate conversion. The hydrophilic acetone, however, might remove water from the enzyme surface, thereby deactivating the enzyme. In the subsequent study, the acetone content was fixed at 5% (v/v).

Effect of Reaction Temperature on Esterification

It is well known that temperature affects both the speed of enzymatic activity and the enantiomer selectivity of the enzyme. In this experiment, the rate of substrate conversion increased continuously with a rise in temperature, while enzyme enantiomer selectivity reached a maximum (*E* = 4) at 45°C (Fig. 2). As the temperatures exceeded 50°C, the enantiomer selectivity of the enzyme decreased drastically with the increasing temperature. This might have been caused by the evaporation of acetone, which has a boiling point of 56°C. The markedly increased rate of substrate conversion at 60°C might be due, in part, to a change in the composition of the reaction medium that accompanied the evaporation of acetone. The reaction temperature was kept at 45°C in the subsequent study.

Time Course for the Enzymatic Esterification of Ketoprofen

Both the rate of substrate conversion and the majority

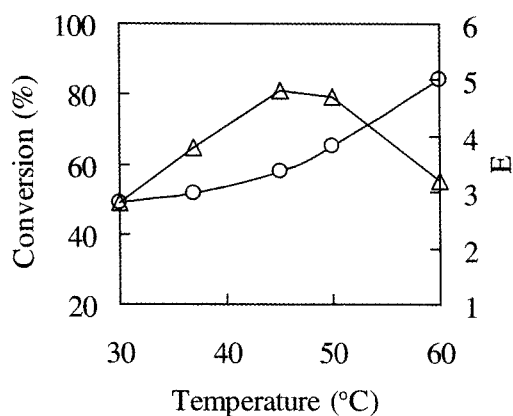


Fig. 2. Effect of the reaction temperature on the rate of substrate conversion and enzyme enantiomer selectivity (E) during ketoprofen esterification. *C. antarctica* lipase: 100 mg; ketoprofen: 100 mg; ethanol: 115 μ L (5 times the molarity of ketoprofen); solvent (isooctane/acetone, 95:5, v/v): 20 mL; shaking speed: 180 rpm; reaction time: 3 h. (○): ketoprofen conversion; (△): enantiomer selectivity (E).

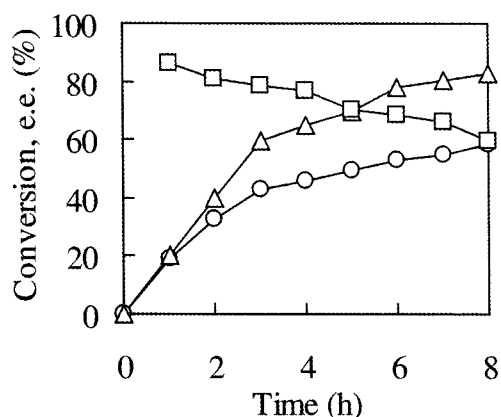


Fig. 3. Time course for the enzymatic esterification of ketoprofen. Ketoprofen: 800 mg; ethanol: 920 μ L (5 times the molarity of ketoprofen), *C. antarctica* lipase: 800 mg; solvent (isooctane/acetone, 95:5, v/v): 40 mL; temperature: 45°C; shaking speed: 180 rpm. (○): ketoprofen conversion; (△): S-enantiomer concentration in the ketoprofen remaining in solution; (□): R-enantiomer concentration of the ester product.

enantiomer concentration in the ketoprofen remaining in solution increased over a 6-h period, then remained relatively stable; by contrast, the majority enantiomer concentration in the ester product decreased gradually over the same period of time (Fig. 3). Within 8 h, 58.3% of the substrate had been transformed into the ester product; correspondingly, the concentration of the majority enantiomer in the ketoprofen remaining in solution and in the ester product reached 83.0 and 59.4%, respectively. The reduced rate of substrate conversion might have been due to the reaction (which is reversible) reaching equilibrium; this, in turn, may have occurred as a result of water formation deactivating enzyme activity. We tried to improve the concentration of the majority enantiomer in the keto-

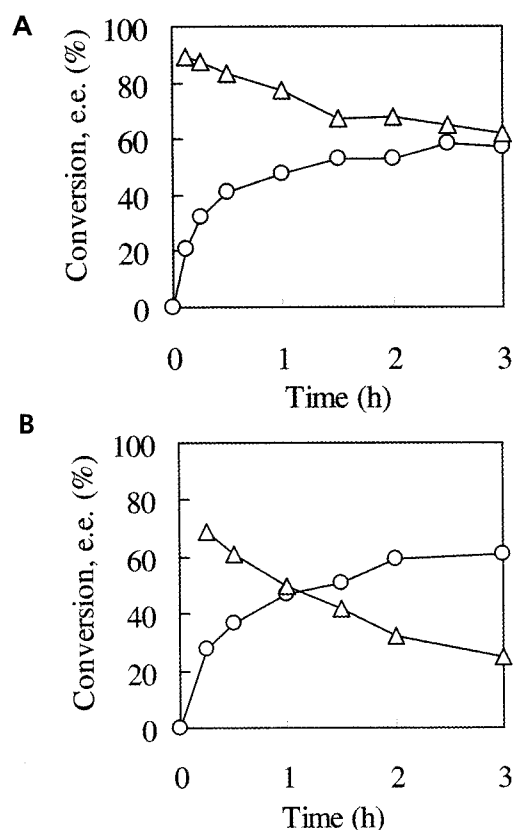


Fig. 4. Time course for the enzymatic hydrolysis of ketoprofen ethyl ester. Ketoprofen ethyl ester: 200 mg; *C. antarctica* lipase: 100 mg; buffer (pH 7.0): 10 mL; temperature: 45°C; shaking speed: 180 rpm. (○): ester conversion; (△): product concentration; (A) R-ketoprofen ethyl ester concentration = 59.4%, (B) racemic ester.

profen remaining in solution by inducing additional esterification in the presence of *C. rugosa* lipase, which has shown a preference for the S-enantiomer [9,12]. We had to discontinue this effort, however, because the enzyme activity was too low. Our subsequent work focused on increasing the concentration of R-ketoprofen by inducing the hydrolysis of the ester product, which was rich in R-enantiomers (59.4%), in the presence of lipase.

Time Course for Enzymatic Hydrolysis of the Ester

When the amount of time required for the hydrolysis of the ketoprofen ethyl ester (Fig. 4A) is compared with that for the hydrolysis of the racemic ester (Fig. 4B), it becomes clear that the substrate conversion rate increased over time and the R-ketoprofen concentration in the product decreased within the first 1.5 h of the reaction, stabilizing thereafter. An evaluation of enantiomer concentrations that were seen as a result of the lipase hydrolysis of the ester product revealed that the R-ketoprofen concentration in the product reached 77% when 50% of the ester had been transformed and 90% when 30% of the ester had been transformed (Fig. 4A). By contrast, when the enantiomer concentrations result-

ing from the hydrolysis of the racemic ester were evaluated, it was found that the *R*-ketoprofen concentration in the product only reached 42% when 50% of the ester was transformed and 65% when 30% of the ester was transformed (Fig. 4B). Therefore, enzymatic hydrolysis of the ester product resulted in a high concentration of *R*-ketoprofen.

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