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Kinetic and thermodynamic investigation of enzymatic L-ascorbyl acetate synthesis

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ABSTRACT

Kinetics and thermodynamics of lipase-catalyzed esterification of L-ascorbic acid in acetone were investigated by using vinyl acetate as acyl donor. The results showed that L-ascorbic acid could generate inhibition effect on lipase activity. A suitable model, Ping-Pong Bi-Bi mechanism having substrate inhibition, was thus introduced to describe the enzymatic kinetics. Furthermore, the kinetic and thermodynamic parameters were calculated from a series of experimental data according to the kinetic model. The inhibition constant of L-ascorbic acid was also obtained, which seemed to imply that enhancing reaction temperature could depress the substrate inhibition. Besides, the activation energy values of the first-step and the second-step reaction were estimated to be 37.31 and 4.94 kJ/mol, respectively, demonstrating that the first-step reaction was the rate-limiting reaction and could be easily improved by enhancing temperature.

1. Introduction

L-Ascorbic acid has been used extensively in food, pharmaceutical and cosmetic fields. However, its poor liposolubility limited the application (Liu et al., 1996; Watanabe et al., 2010). To cope with this problem, many derivatives of L-ascorbic acid have been synthesized. For example, modification of L-ascorbic acid via esterification was a useful way to alter its solubility (Reyes-Duarte et al., 2011; Watanabe et al., 2012; Karmee, 2009). Recently, there were many reports concerning the synthesis of ascorbyl esters by using lipase as catalyst in organic solvent (Lerin et al., 2012; Chang et al., 2009; Zhang et al., 2011; Treichel et al., 2010). In order to identify the optimal conditions for the lipase-catalyzed esterification, it was essential to investigate the reaction mechanism, kinetics and thermodynamics of this reaction.

Lipase was widely used as a biocatalyst to catalyze multisubstrate-multiproduct reactions (Batistella et al., 2012). Moreover, complex kinetic mechanisms have been proposed to provide descriptions of the lipase-catalyzed reactions. Many studies showed that lipase-catalyzed esterification reaction could be described by a Ping-Pong kinetic model (Awang et al., 2004; Chulalaksananukul et al., 1990, 1992). Especially, several mechanisms have been proposed to explain lipase-catalyzed reactions containing substrate inhibition (Al-Zuhair, 2005; Mestri and Pai, 1995; de Castro et al., 1997). In these studies, a dead-end complex formatted by one of the substrate and lipase was introduced to explain substrate inhibition phenomenon.

So far, many ascorbyl esters such as ascorbyl oleate (Reyes-Duarte et al., 2011), ascorbyl palmitate (Burham et al., 2009), ascorbyl linoleate (Watanabe et al., 2008) and ascorbyl benzoate (Lv et al., 2007) have been synthesized by enzymatic esterification in organic solvent. However, the studies on the enzymatic kinetics and thermodynamics were rarely reported. In this paper, it was found that L-ascorbic acid, one of the substrate, could generate inhibition on the activity of Lipozyme TLIM lipase. Thus the kinetics of lipase-catalyzed esterification of L-ascorbic acid using vinyl acetate as acyl donor was investigated. The kinetic model of the Ping-Pong mechanism having substrate inhibition was built and the kinetic and thermodynamic parameters were evaluated. These results would provide some valuable informations in enzymatic synthesis of L-ascorbyl ester.

2. Materials and methods

2.1. Materials

This enzyme, Lipozyme TLIM (Novo Industri, Bagsvaerd, Denmark), was a preparation of a *Thermomyces lanuginosus* lipase immobilized on silica gel. L-Ascorbic acid (vitamin C, Vc) was

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purchased from Sigma Chemical Co. (St. Louis, MO, USA). Vinyl acetate (VAc, 99%) was obtained from Fuchen Chemical Co. (Tianjin, China). Analytical grade acetone was from Kewei Chemical Co. (Tianjin, China).

2.2. Experimental procedure

For the standard reaction, Lipozyme TLIM (20 mg) was added to a reaction mixture containing L-ascorbic acid and vinyl acetate in 1 mL acetone. The concentrations of L-ascorbic acid and vinyl acetate were, respectively, varied from 3 to 12 mmol/L and 6 to 22 mmol/L. Then the reaction mixture of different substrate molar ratio was incubated in a temperature-controlled shaker at 170 rpm at a certain temperature. After 2 h, 50 μL of the reaction mixture was withdrawn and evaporated, and then the residue was dissolved in methanol/water (5/5, v/v) and analyzed by HPLC. All experiments were conducted in triplicate and the mean values were calculated.

2.3. HPLC analysis

The reaction resultants were analyzed using high-performance liquid chromatography (HPLC, ChuangXinTongHeng Science & Technology Co. Ltd., China) with a C-18 column (ZORBAX 300SB-C18 4.6 mm ID × 250 mm (5 μm), Agilent Technologies, Palo Alto, CA) and a UV detector at 254 nm. A 20-μL diluted sample was injected, and the elution was done with methanol/water/acetic acid (50/50/0.1, v/v/v) at a flow rate of 0.5 mL min^{-1}. Reaction conversion was calculated in terms of the mole percentage of esterification, based on the ratio of consumed L-ascorbic acid to the total amount of L-ascorbic acid before reaction.

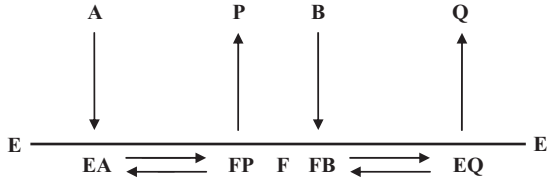
3. Kinetic model

Two kinetic mechanisms (Mukesh et al., 1997; Maugard et al., 2000) were supposed in the present study. The first was called Ping-Pong Bi-Bi mechanism. The second model was assumed Ping-Pong Bi-Bi mechanism having substrate inhibition. They are described as follows.

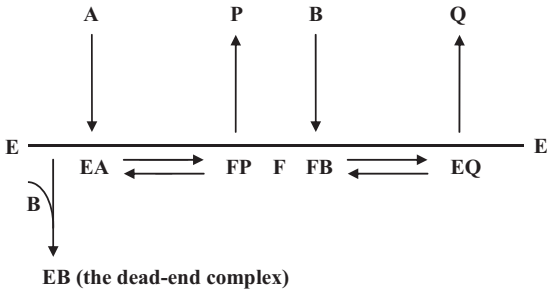
3.1. Ping-Pong Bi-Bi mechanism

Previous studies have shown that the reaction of lipase-catalyzed esterification could be described by the Ping-Pong kinetic model (Lima et al., 1996). To understand the Ping-Pong Bi-Bi mechanism better, a schematic representation of this reaction mechanism using the Cleland notation (Lima et al., 1996) was given in Scheme 1. As shown, acyl donor (A) is bound to the enzyme at first to form the acyl-enzyme complex. As soon as one product (P) is formed and then released, acyl acceptor (B) binds to the modified enzyme (substituted form of the free enzyme) to form the second product (Q). The reaction rate equation is given by Eq. (1) (Lima et al., 1996):

$$v = \frac{V_{max}[A][B]}{K_m^A[B] + K_m^B[A] + [A][B]} \quad (1)$$



Scheme 1. Schematic representation of the Ping-Pong Bi-Bi mechanism.



Scheme 2. Schematic representation of the Ping-Pong Bi-Bi mechanism having substrate inhibition.

where v is the enzymatic reaction rate of the esterification; V_{max} is the maximum rate of the esterification; $[A]$ is the concentration of A; $[B]$ is the concentration of B; K_m^A and K_m^B are the Michaelis constants of A and B, respectively.

3.2. Ping-Pong Bi-Bi mechanism having substrate inhibition

Several recent studies (Al-Zuhair, 2005; Mestri and Pai, 1995; de Castro et al., 1997) on enzymatic esterification containing substrate inhibition attempted to describe the reaction mechanism by a modified Ping-Pong kinetic model (Scheme 2). Compared with Scheme 1, the step of a dead-end complex formation between substrate and free enzyme has been added in Scheme 2. Based on this proposed mechanism, the expression for the reaction rate is given by Eq. (2) (Yadav and Borkar, 2008):

$$v = \frac{V_{max}[A][B]}{K_m^A[B] + K_m^B[A] + [A][B] + (K_m^A[B]^2/K_i^B)} \quad (2)$$

where K_i^B is the inhibition constant of B; other notations are the same as that in Eq. (1).

4. Results and discussion

4.1. Kinetics of enzymatic esterification of L-ascorbic acid

Lipase-catalyzed esterification of L-ascorbic acid was carried out at 40 °C for 2 h by changing substrate concentration and the results were listed in Supplementary Information (Table S1). After that, the reaction rates were calculated and shown in Fig. 1. As can be seen, for a given vinyl acetate concentration, the reaction rate decreased strangely with the increase in L-ascorbic acid concentration, which indicated that, to some extent, L-ascorbic acid had an observable inhibition effect on the enzymatic reaction. For example, lipase

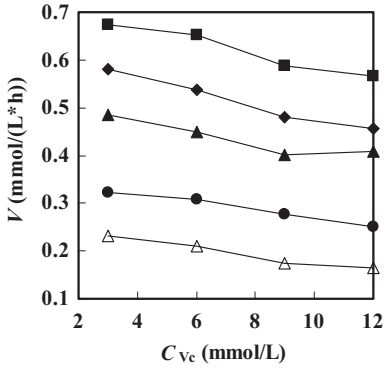


Fig. 1. Effect of L-ascorbic acid (Vc) concentration on the reaction rate at different vinyl acetate (VAc) concentration at 40 °C. (Vinyl acetate concentrations: (△) 6 mmol/L, (●) 10 mmol/L, (▲) 14 mmol/L, (♦) 18 mmol/L, (■) 22 mmol/L.)