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Study of Vitamin C ester synthesis by immobilized lipase from *Candida* sp.

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Abstract

Oleoyl ester of L-ascorbic acid was synthesized by using immobilized lipases from *Candida* sp. A series of solvents, such as ethanol, tetrahydrofuran, pyridine, butanol, tertiary amyl alcohol (*t*-amyl alcohol), hexanol, octanol and hexane (log *P* from −0.24 to 3.5) were investigated for the reaction, and *t*-amyl alcohol was found to be the most suitable from the standpoint of the substrate concentration and the enzyme activity. And the equilibrium of the reaction was affected by the addition of the molecular sieves and the temperature. Reaction carried out at 55 °C and with 50 g/l of 4 Å molecular sieves is good for the enzyme to keep its activity and for making the equilibrium go to the product. The kinetic model was studied and the result showed that the reaction can be described by Ping-Pong mechanism. Parameters value of V_m and K'_m were obtained. Last, the pure products of the reaction were attained and determined by IR spectra, mass spectrometry and ^{1}H NMR spectra.

Keywords: Ascorbic acid; Oleic acid; Lipase; Enzymatic synthesis; Kinetic model

1. Introduction

Lipases that widely used as catalysts in organic synthesis were mainly from microbials. Lipases not only catalyze hydrolysis but also esterification, transesterification, and so on. Each lipase demonstrates its distinct substrate specificity, regioselectivity, and steroselectivity. Enzyme-catalyzed reactions are superior to conventional chemical methods owing to mild reaction conditions, high catalytic efficiency and the inherent selectivity of natural catalysts, further, use of immobilized enzyme simplifies the downstream processing [1,2]. The number of reports concerning the use of immobilized lipase as catalysts in organic synthesis is increasing considerably [3].

Vitamin C and oleic acid have been used extensively in food, cosmetic and medicine. Vitamin C work as natural antioxidant, its solubility is good in water, however, the hydrophilic character reduces its effectiveness in stabilizing fats, oils, and has reported as a serious disadvantage if an aqueous phase is present [4]. To alter the solubility of Vitamin C, it was converted into oil-soluble fatty acid ester [5]. Fatty acid esters of ascorbic acid would occupy an important place as potential antioxidants and as surfactants in high-fat-content food and cosmetics. Recently, the synthesis of ascorbyl derivatives catalyzed by immobilized lipase has been reported, in which more attention has been paid to the synthesis of ascorbyl palmitate catalyzed by lipase B from *Candida antarctica* [6–8]. At present there were no reports on the

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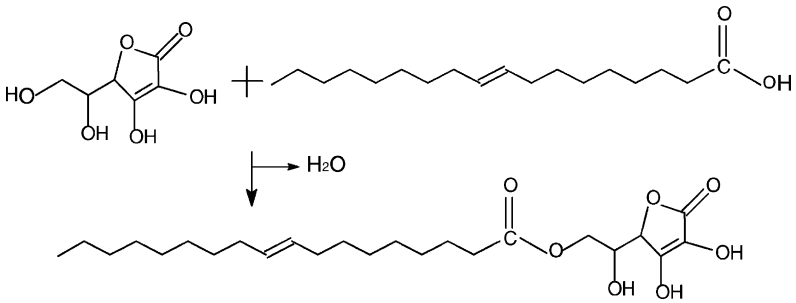

Fig. 1. Synthesis of oleoly L-ascorbic acid ester catalyzed by lipase in organic media.

lipase-catalyzed synthesis of oleoyl ester of ascorbic acid. In this study, oleoyl ester of L-ascorbic acid was synthesized by immobilized lipase from *Candida* sp. Enzymatic reaction condition was optimized, and the kinetic model can be described by Ping-Pong mechanism. Product was purified and determined. The reaction that we have been studied is shown in Fig. 1.

2. Materials and methods

2.1. Biological and chemical materials

Lipase was from *Candida* sp. and immobilized by adsorption on siliceous earth [9]. For immobilization, siliceous earth (5.0 g) were wetted with 20 ml 0.1 M sodium phosphate buffer (pH 7.2), mixed with 4.0 g of crude lipase (20 U/mg). The suspension was on a shaker at 100 rpm for 24 h, the immobilized enzyme was collected by filtration, washed with 0.01 M sodium phosphate buffer (pH 7.2), and air-dried to constant weight. The activity of the immobilized lipase was determined (8 U/mg).

The solvents were all analytical grade. Oleic acid (more than 90% pure), was obtained from Aldrich, USA. The purity of L-ascorbic acid was over 99%.

2.2. General procedure for the enzymatic synthesis

Reactions were conducted in 25 ml screw-caped glass vials. Under standard conditions, 2 mmol of Vitamin C was reacted with 1.5 mmol of oleic acid, in the presence of 300 mg of lipase in 10 ml of solvent. The reactions were carried out in a shaker fitted with a thermostat and protected from light. These conditions were used except when otherwise stated in the text.

2.3. HPLC analysis

Quantitative analyses of reactants and products were conducted using HPLC with an SCL-10AVP system from Shimadzu, Japan. The detector was SPD-10AVP UV-Vis detector. A reversed-phase column (Hewlett Packard XDB-C18, 250 mm × 4 mm, 5 μm) was used. A 10 μl volume of the proper dilution of the reaction mixture was injected, a mixture of methanol/water/H_3PO_4, 85/15/0.1 (v/v/v), was used as eluent at 40 °C with a flow rate of 1 ml/min, products were detected by a UV detector at 280 nm.

Initial reaction rates were determined by plotting the product concentration as a function of reaction time and determining the slope of the line at times close to zero. The rates were expressed as μmol of product formed per minute per gram of immobilized lipase.

2.4. Purification of reaction product

At the end of the reaction, after filtration, the reaction mixture was evaporated under reduced pressure. The remaining mixture was purified by reversed-phase column (300 mm × 15 mm, filled with Lichroprep RP-18, 40–63 μm, Merck, Germany), a mixture of methanol/water/H_3PO_4, 80/20/0.1 (v/v/v), was used as the eluent with a flow rate of 4 ml/min, the products were detected by a UV detector at 280 nm.

2.5. Structural analysis

In order to confirm that the reaction product is exactly the oleoyl L-ascorbic acid, the structure analysis has been done. The isolated product was

finally identified by spectral studies. IR spectra were recorded on Nicolet Magna-IR550. The mass spectrometry data were obtained on Perkin-Elmer SCIEX API100. The ^{1}H NMR spectra were recorded on a Bruker Am 500 spectrometer (Karlsruhe, Germany) at 500 MHz.

3. Results and discussion

3.1. Optimization of organic solvent

Organic solvent influenced the relatively solubility of the substrates, thus it would affect the synthesis of the oleoyl ester of ascorbic acid. The polarity of L-ascorbic acid is very different from those of oleic acid and oleic ester of L-ascorbic acid. Because of this significant difference in polarity, such a solvent need to be found, in which there were relatively high solubility of oleic acid, L-ascorbic acid and product [10]. Moreover, the hydrophobicity of the organic solvent greatly influenced the enzyme activity and the substrate specificity of the lipase [11]. Different organic solvents have different ability to distort the essential water layer around immobilized lipase. Log P (polarity constant, the partition coefficient of the solvent between in octanol and water) is widely used to represent the characteristics of the organic phase, and to predict enzymatic activity [12,13]. It is generally reported that solvents with log $P < 2$ are less suitable for biocatalytic purpose [14]. However *t*-amyl alcohol (log $P = 1.15$) is the optimal organic solvent in this reaction of all the solvents that had been studied. This is mainly due to that, the solvents with high polarity may strip water from enzyme molecules easily, and the enzyme can not get enough water for keeping its active configuration, and also, L-ascorbic acid has very low concentration in solvents with high log P (Table 1). The reaction product was not inspected in other solvents.

Table 1
The effect of solvent on reaction

Solvent	Log P	Product concentration (g/l)
Ethanol	−0.24	0
Tetrahydrofuran	0.49	0
Pyridine	0.71	0
Butanol	0.80	0
t-Amyl alcohol	1.15	16.8
Hexanol	1.80	0
Octanol	2.90	0
Hexane	3.50	0

The reactions were carried out at 50 °C, with 2 mmol oleic acid and 1.5 mmol L-ascorbic acid, with 0.3 g of immobilized enzyme in 10 ml different solvents for 24 h.

3.2. Effect of molecular sieves on the product concentration

In esterification reaction, water content of the medium not only effects the rate of reaction but also the equilibrium position. Suitable water content can keep the enzyme active configuration, but more water will inhibit the equilibrium move to the product. Thus, it was essential to have a method to control the water content or the thermodynamic water activity during the process of the reaction. To continuously remove the water and improve the reaction product concentration, the effect of molecular sieves on product concentration was studied. In the reaction, molecular sieves not only dried the reaction mixture but also shifted the equilibrium to the synthesis by adsorbing the water formed [15,16]. The product concentration reached 18.5 g/l after10 h of reaction with 50 g/l of 4 Å molecular sieves (Fig. 2).

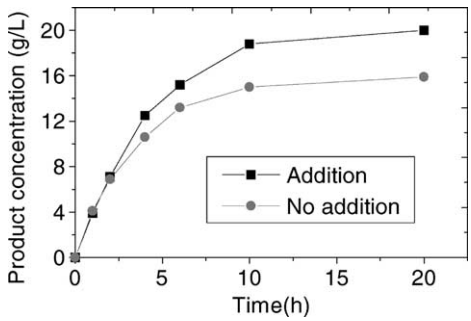

Fig. 2. Effect of molecular sieves on the product concentration. This is the time course of lipase-catalyzed synthesis of oleic ester of L-ascorbic acid with molecular (■) sieves and without molecular sieves (●). The reaction was carried out with 1.5 mmol L-ascorbic acid and 2 mmol oleic acid in 10 ml *t*-amyl alcohol at 50 °C, with or without 50 g/l of 4 Å molecular sieves, and with 0.3 g of immobilized lipase for 24 h.

3.3. Effect of temperature on the initial rate and the product concentration of the reaction

Temperature has a significant effect on the equilibrium of the reaction, and on the activity and stability of immobilized lipase. As shown in Fig. 3, for the initial rate, the most suitable temperature was 60 °C, but for the product concentration the optimal temperature was 55 °C. The product concentration increased with increasing temperature until 55 °C and then decreased slowly. Fig. 3 illustrates that the optimal temperature was 55 °C, and that the immobilized lipase has good thermostability.

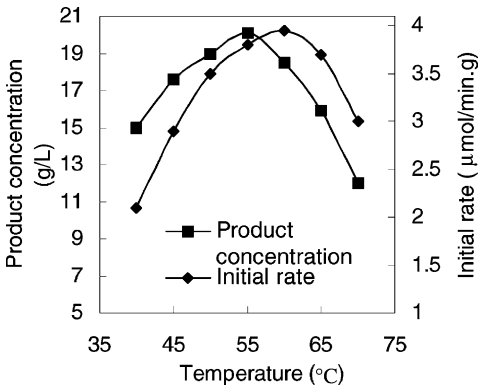

Fig. 3. Effect of temperature on the initial rate (◆) and the product concentration of the reaction (■). The reaction was carried out with 1.5 mmol L-ascorbic acid and 2 mmol oleic acid in 10 ml *t*-amyl alcohol, with 50 g/l of 4 Å molecular sieves, and with 0.3 g of immobilized lipase at different temperature for 24 h.

3.4. Effect of shaking speed on the initial rate

The effect of shaking speed on the initial rate of the reaction is illustrated in Fig. 4. The initial rate followed the increase of the shaking speed when it was less than 200 rpm, and the initial rate reached a maximum at 200 rpm. Above this speed the initial rate remained almost constant. This can be regarded as that the initial rates of reaction were no longer limited by the mass-transfer limitation of immobilized enzyme at shaking speeds above 200 rpm.

3.5. Effect of the concentration of oleic acid and enzyme on the initial rate and the product concentration of reaction

Fig. 5 shows the effect of the concentration of substrate. When the oleic acid concentration was in the range of 0 and 250 mM, the initial rate increased with the increase of oleic acid. Above 250 mM concentration of oleic acid, the initial rates were almost constant. This probably can be due to that (i) the support of the immobilized enzyme has begun to limited the more substrates transfer; (ii) the low constant concentration of L-ascorbic acid limited the initial rates; (iii) all the active sites of the enzyme that can be utilized were saturated with substrates. From Fig. 6, we knew that the main reason was second, it is mainly due to that the concentration of L-ascorbic acid is constant, so it limited the initial rates when the enzyme concentration was over 30 g/l. The initial rate and the product concentration of the reaction reached the maximum when the oleic acid concentration was 250 mM.

3.6. Kinetic mechanism

Previous studies have shown that the reaction of esterification immobilized lipase-catalyzed could be described by the Ping-Pong kinetic models [17–19]. In this reaction, initially, if the product concentrations are zero, and the little water formed in the first step of the lipase-catalyzed esterification was removed by the molecular sieves, the expression for initial reaction rate is

$$v = \frac{V_m}{1 + K_A/[A](1 + [B]/K_{IB}) + K_B/[B]} \quad (1)$$

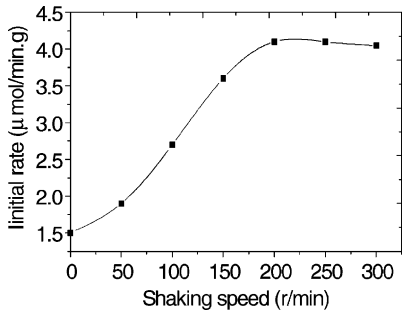

Fig. 4. Effect of shaking speed on the initial rate (■). The reactions were carried out at 55 °C and with 1.5 mmol L-ascorbic acid and 2 mmol oleic acid with 50 g/l of 4 Å molecular sieves, and with 0.3 g of immobilized lipase at different shaking speed in 10 ml *t*-amyl alcohol.

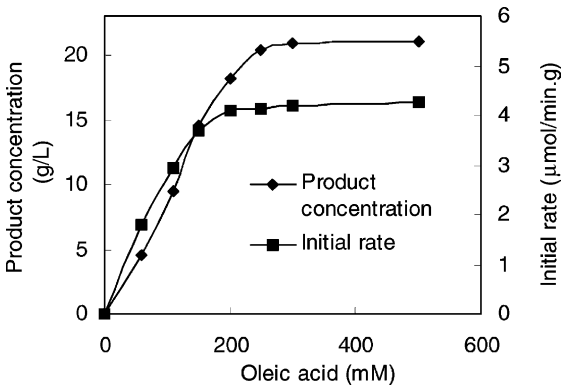

Fig. 5. Effect of the concentration of oleic acid on the initial rate (■) and the product concentration (◆) of the reaction. The reaction was carried out at 55 °C with 1.5 mmol L-ascorbic acid and different concentration of oleic acid in 10 ml *t*-amyl alcohol, with 50 g/l of 4 Å molecular sieves, and with 0.3 g of immobilized lipase for 24 h.

where υ is the initial reaction rate, V_m the maximum reaction rate, [A] and [B] are the concentrations of the oleic acid and the L-ascorbic acid, K_A and K_B are the Ping-Pong constants for the oleic acid and the L-ascorbic acid, K_{IB} is the inhibition constant for the L-ascorbic acid [20]. In the reaction, the concentration of L-ascorbic acid can be regarded as a constant. So the initial reaction rate equation can be expressed simply as

$$\upsilon = \frac{V_m[A]}{[A] + K'_m} \quad (2)$$

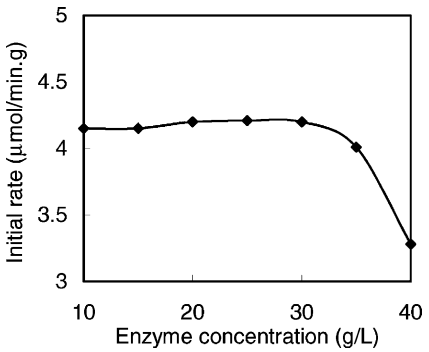

Fig. 6. Effect of the concentration of immobilized enzyme on the initial rate (◆). The reaction was carried out at 55 °C with 1.5 mmol L-ascorbic acid and 2.5 mmol oleic acid in 10 ml *t*-amyl alcohol, with 50 g/l of 4 Å molecular sieves, and with different concentration of immobilized lipase.

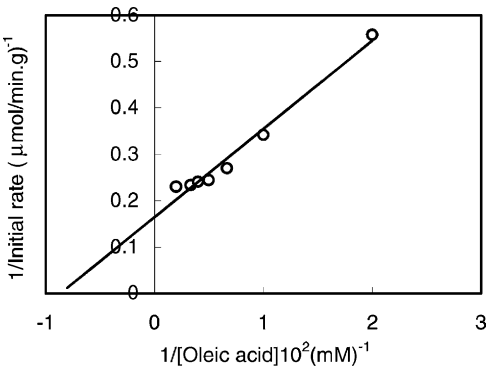

Fig. 7. Reciprocal initial velocity of the reaction vs. reciprocal oleic acid concentration. The conditions of reaction were same to Fig. 5.

where υ is the initial reaction rate, V_m the maximum initial reaction rate, K'_m the apparent Michaelis constant. The Lineweaver–Burk plot of the reciprocal initial rate versus the reciprocal concentration of oleic acid was linear (Fig. 7). The parameters values for V_m and K'_m can be obtained by non-linear regression, $V_m = 6.1$ μmol/min g, $K'_m = 1.16 \times 10^2$ mmol/l.

3.7. Structural analysis

The purity of isolated product was over 98% oleoly ascorbic acid. IRυ (CO–O ester) 1740 cm^{-1}. Mass spectrometry data also gave a molecular ion at m/z = 463.2 $[M_1 + Na]^+$, M_1 (440.2) corresponded exactly to molecular mass of oleoyl L-ascorbic acid. ^1H NMR (500 MHz, $CDCl_3$) δ 4.78 (H-4, 1H), 4.25 (H-6, 2H), 4.37 (H-5, 1H), 5.30 (H-9′and H-10′, 2H), 2.35 (H-2′, 2H), 2.0 (H-8′and H-11′, 4H), 1.65 (H-17′, 2H), 1.28 (H-3′–H-7′ and H-12′–H-16′, 20H), 0.88 (H-18′, 3H).

4. Conclusion

Ascorbyl oleoyl ester was obtained by immobilized lipase catalytic synthesis from *Candida* sp. To the best of our knowledge, the condition and kinetic mechanism of the reaction were studied for the first time, the optimal reaction conditions were found and the kinetic was agreed with Ping-Pong mechanism, the constants value of the kinetic model were obtained. And the product were purified and identified, but its properties and application need further studies.

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