Biochemical Engineering Journal 71 (2013) 89–96

Contents lists available at SciVerse ScienceDirect

Biochemical Engineering Journal

journal homepage: www.elsevier.com/locate/bej

Regular article

Kinetic model of lipase-catalyzed conversion of ascorbic acid and oleic acid to liposoluble vitamin C ester

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ARTICLE INFO

Article history:
Received 6 August 2012
Received in revised form 18 October 2012
Accepted 2 December 2012
Available online 10 December 2012

Keywords:
Ascorbyl oleate
Lipase
Candida antarctica
Kinetic parameters
Substrate inhibition
Production kinetics

ABSTRACT

The kinetics of L-ascorbyl oleate synthesis catalyzed by immobilized lipase from *Candida antarctica* in acetone was investigated. Significant inhibition of synthesis with an excess of ascorbic acid was observed. Experimental data were successfully fitted with a ping–pong bi–bi kinetic model with substrate inhibition, and related kinetic constants were determined. The kinetic study was performed at optimum experimental factors (temperature, initial water content, and enzyme concentration), which were determined using response surface methodology. Then, a model for predicting product–time progress curves was developed by expanding the obtained ping–pong model with terms describing ester hydrolysis. Kinetic constants of the reverse reaction were determined, and good congruence between the model and experimental data was achieved. Calculated kinetic constants revealed that lipase has the highest affinity for ascorbyl oleate, slightly lower activity with ascorbic acid, and the lowest activity with oleic acid. The obtained results are valuable for elucidating the reaction mechanism and represent an important contribution for reaction optimization and creating strategies to increase the productivity of vitamin C ester synthesis.

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1. Introduction

Vitamin C is well known for its antioxidative properties. Nevertheless, the use of L-ascorbic acid in stabilizing fats and oils is very scarce due to its hydrophilic nature [1]. On the contrary, fatty acid ascorbyl esters are oil-soluble with the same or even enhanced antioxidative properties compared to vitamin C. Mineral acids or lipases can catalyze the esterification process between vitamin C and fatty acid (or its methyl or vinyl ester). At the moment, despite the numerous shortcomings, ascorbyl palmitate is being produced industrially by chemical means [2]. On the other hand, there are many advantages of the biosynthetic process, such as mild reaction conditions, regioselectivity, and the possibility of using immobilized enzymes, resulting in simpler downstream processing [3]. Additionally, obtaining a product in such a way allows it to be labeled as natural and have a higher market value [4]. Although lipase-catalyzed synthesis of ascorbyl esters has already been described by many authors, long reaction times, bio-incompatible solvents, and high price of the enzyme are still some of the main obstacles in the commercialization of the process [5]. Ascorbyl esters derived from unsaturated fatty acids are superior compared to those with saturated hydrocarbon chains in terms of solubility, free radical scavenging capacity and beneficial effects on human nutrition [6]. Therefore, their biosynthesis, especially in GRAS (Generally Recognized as Safe) solvents, is of particular interest [5].

In regard to scale-up and process automation, it is necessary to optimize operating parameters and establish adequate kinetic models for the reactions. Additionally, the type of kinetic model gives valuable information about the reaction mechanism and substrate inhibition which helps in organizing enzymatic processes in such a way as to avoid intrinsic limitations at the molecular level. There are just a few reports of ascorbyl oleate synthesis catalyzed by immobilized lipase from *Candida antarctica* [7–11]. On the other hand, reports on the kinetics of aliphatic ester synthesis are more frequent, but different kinetic models have been proposed. In general, esterification reactions catalyzed by immobilized *C. antarctica* lipase preparation occurs *via* acyl–enzyme intermediates and are most commonly being described by models based on a ping–pong bi–bi or ordered bi–bi mechanism [12]. Additionally, inhibition by one or both substrates was reported on several occasions [13–17].

The main goals of this study were to obtain an adequate kinetic model for the enzymatic synthesis of ascorbyl oleate (Scheme 1) in acetone, determine key kinetic constants, and compare affinity of substrates towards lipase. To investigate inhibition by excess of substrates, concentration was varied in a wide

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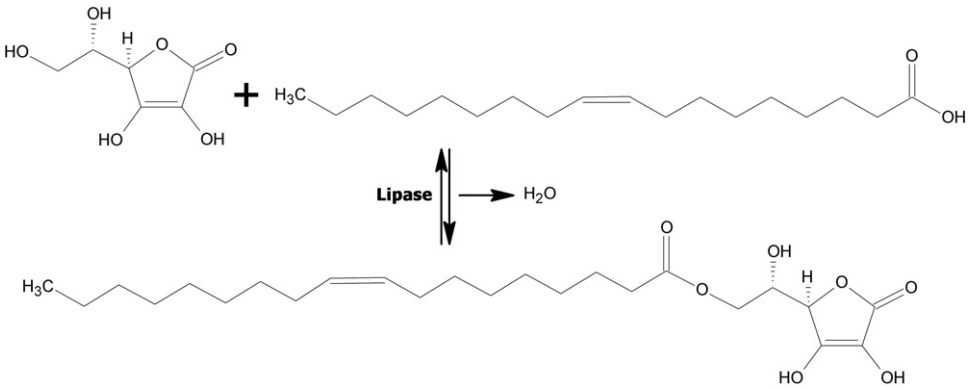
http://dx.doi.org/10.1016/j.bej.2012.12.001



Scheme 1. Formation of ascorbyl oleate in a reaction of ascorbic acid and oleic acid.

range from 0.05 to 1 M. Prior to kinetic studies, response surface methodology (RSM) and 5-level-5-factor central composite rotatable design (CCRD) were employed to determine the effects of key experimental parameters (initial water content, temperature, ascorbic acid:oleic acid molar ratio, vitamin C concentration, and enzyme content) on the initial rate of the vitamin C and oleic acid esterification reaction, so the kinetic study was performed at optimum conditions. Finally, it was shown that reaction kinetics can be fitted throughout the reaction range with a model that also includes the reverse reaction of ester hydrolysis, and the kinetic constants of the reverse reaction were determined.

2. Materials and methods

2.1. Enzyme and chemicals

Novozym® 435 (lipase from *C. antarctica*, type B immobilized on acrylic resin) was purchased from Novozymes (Bagsvaerd, Denmark). Substrates were L-ascorbic acid (purity 99.7%, Zorka, Šabac, Serbia) and oleic acid (Ph. Eur., NF pure purchased from AppliChem, Darmstadt, Germany). Acetone was used as a reaction medium (99.5%, JT Baker, USA). Substances used for the quantitative HPLC analyses were methanol obtained from JT Baker (USA) and phosphoric acid, purchased from Sigma–Aldrich (Chemie GmbH, Steinheim, Germany); all were HPLC grade.

2.2. Procedure for the enzymatic synthesis

Experiments were carried out in 100 ml capped vials. The reaction mixture consisted of different amounts of ascorbic acid, oleic acid, enzyme, water, and acetone (amounts specified for each experiment separately), so that the total volume was 10 ml. The reactions were conducted in a shaker at 250 rpm and at a temperature in the range from 40 to 60 °C. All experiments were carried out in duplicate, and average values are presented in Figs. All standard deviations were less than 5%. Control samples (without enzyme) were prepared by exposure to the same temperature treatment. The product was not detected in control samples. In experiments with the addition of molecular sieves, 80 mg of zeolite type sieves were added per ml of the reaction medium.

2.3. Kinetic study

Kinetic studies were performed in accordance with experimental plans comprising of 64 experimental points of a matrix (8×8), representing all possible pairs of ascorbic acid and oleic acid concentrations at following set of values: 0.05; 0.1; 0.15; 0.2; 0.3; 0.5; 0.75; 1.

The initial rate was determined as the slope of the reaction curve tangent to the initial stage of the reaction. Because all experiments were performed in duplicate, reaction curves were constructed using average values of the reaction rate for each experimental point. A linear portion of the reaction curve at various substrate concentrations consisted of 4–6 experimental points, where the number of experimental points included was determined by the condition that correlation coefficients of the initial straight line must be above 0.95.

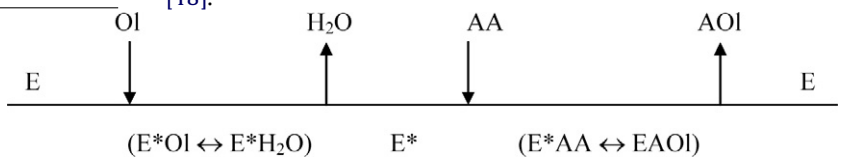
2.4. HPLC analysis

For quantitative analysis of reactants and products, an Akta Purifier HPLC system was used. A reverse phase column (Waters Spherisorb ODS 2-C18, 250 mm × 4.6 mm, 5 μm) was employed. The injection volume of the reaction mixture, diluted fifteen fold, was 10 μl. Methanol/H_3PO_4, 100/0.1 (v/v), was used as eluent with a flow rate of 1 ml/min. The product was detected by a UV detector at 235 nm.

3. Theory and calculations

3.1. Ping–pong bi–bi model

The ping–pong bi–bi mechanism illustrates alternate binding of substrates and release of products in a bi-substrate reaction with two products formed. It is the most frequently postulated reaction mechanism in lipase-catalyzed esterifications [13–19]. The first stage of the reaction is the binding of the acyl-donor (Ol, oleic acid), resulting in the formation of an acyl–enzyme complex. In the next step, the first product (water) is released. Then, the acyl acceptor (AA, ascorbic acid) binds, and in the final step, ester (AOl) is released [18].



Nevertheless, basic ping–pong mechanisms can rarely adequately describe lipase-catalyzed esterifications because inhibition by excess of one of the substrates was frequently reported [14–19].

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