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Biochemical Engineering Journal 39 (2008) 450–456

Biochemical Engineering Journal

www.elsevier.com/locate/bej

Production of ricinoleic acid estolide with free and immobilized lipase from *Candida rugosa*

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Received 5 July 2007; received in revised form 4 September 2007; accepted 23 October 2007

Abstract

Ricinoleic acid estolide was produced by using free and immobilized *Candida rugosa* lipase at moderate temperature in a bioreactor. This work describes the immobilization of *C. rugosa* lipase on 10 different supports by covalent binding and physical adsorption, and how of the most suitable immobilized derivative was selected. The comparison was mainly based on the enzyme content and on the activity results. An anion exchange resin was judged to be the most appropriate support and the corresponding immobilization process was investigated and optimized. Although repeated batch reactions using the same derivative are not entirely advisable, the reaction proceeds at a noticeably slower rate and the degree of condensation reached is lower when the same amount of protein as in the derivative is added to the bioreactor in native form.

Keywords: Estolide; Ricinoleic acid; Lipase; Immobilized enzymes; Heterogeneous biocatalysis

1. Introduction

Estolide is a generic name for linear oligomeric polyesters of hydroxyl fatty acids, in which the carboxyl group and hydroxyl group of hydroxyl fatty acids are dehydrated to form oligomers. The estolide made from ricinoleic acid ($18{:}1^{9}$–OH^{12}) is used as a viscosity controller for chocolate and an emulsifier in margarine. It is produced by polymerizing castor bean oil fatty acids at 205–210 °C under a vacuum of 700 mm Hg in a carbon dioxide atmosphere for 8 h [1]. Due to the high reaction temperature, some undesirable side-reactions may occur and the appearance of several by-products may give to the final product an unwanted color and odor, making it unacceptable for the food industry. As an alternative, some researchers have investigated the enzymatic synthesis of ricinoleic acid estolide by the catalytic action of lipase (E.C. 3.1.1.3), which acts in mild reaction conditions, for example, low temperatures and pressures and neutral pH [2,3].

The natural action of lipase in an aqueous medium is to hydrolyze organic esters. If the enzyme is placed in a medium with a low water concentration, the thermodynamic equilibrium will be shifted in the synthetic direction. In principle, complete removal of water from the reaction medium would drastically distort the enzyme conformation and inactivate it. However, it has been found that, in the case of lipase, only a few layers around the enzyme surface are needed. On the other hand, the water formed by the reaction must be removed from the reaction mixture if ricinoleic acid estolide with a high degree of condensation is to be obtained [3].

The present authors have studied the production of ricinoleic acid estolide with free *Candida rugosa* lipase in a batch reactor, obtaining a product with an acid value (AV) of 65 in 48 h [4]. The acid value of the chemically prepared ricinoleic acid estolide is 40, which is equivalent to a mean of five fatty acid residues per molecule of polyricinoleic acid [5], an AV only attainable with immobilized lipase and never with free enzyme [6]. A number of articles have been found on the immobilization of *C. rugosa* lipase on carriers of different physic-chemical characteristics, for many applications [7–12]. The support can affect the partitioning of substrates, products and water in the reaction mixture, and thereby influence the catalytic properties of the enzyme. Effort was therefore devoted to obtaining an immobilized derivative with a high-immobilized protein percentage and enzymatic activity.

After a conscientious bibliographical search, only two papers have been found describing the production of ricinoleic acid estolide with immobilized lipase [3]. The authors of the first paper are also the owners of at least two patents about the process described in the paper [6,13]. However, only one

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doi:10.1016/j.bej.2007.10.013

immobilized derivative was used in these studies and no details about the optimization of the immobilization procedure were given. The best results were obtained when immobilized lipase (60 mg Enzyme/g carrier) was used in repeated batch operations, thus obtaining 425 g estolide/g free enzyme [3].

In the second recently published paper [14] Novozym 435 is used as biocatalyst for ricinoleic acid estolide production. The enzyme used was a lipase (lipase B) from *Candida antarctica* adsorbed on a macroporous resin and commercially produced by Novozymes, North America Inc. (Franklinton, NC), although details concerning the immobilization method are not available.

Therefore, the main objectives of the present work were:

- To immobilize of *C. rugosa* lipase using different supports and immobilization methods, the results obtained allowing us to choose the "best" immobilized lipase.
- To optimize the immobilization process by studying several variables: influence of support activators, effect of changing the immobilization pH, influence of the enzyme concentration and effect of mixing on the immobilization process.
- To compare of the behavior of immobilized and free lipase in terms of ricinoleic acid estolide production, including the reuse of the immobilized derivative.

2. Materials and methods

2.1. Enzyme and substrate

Lipase form *C. rugosa* (819 units/mg solid) was purchased from Sigma–Aldrich. Ricinoleic acid (∼80%) was supplied by Fluka.

2.2. Immobilization reagents and activators

γ-APTES ((3-aminopropyl) triethoxysilane) and glutaraldehyde (25%) were purchased from Sigma–Aldrich. Oleic acid (>58%) was acquired from Riedel-de Haën. Soybean lecithin was of commercial grade from Santiveri S.A., Spain.

2.3. Immobilization supports

Uncoated porous glass beads (PG 75-40, PG 700-400 and PG 1000-400) and acid-washed non-porous glass beads (≤106 μm and 425–600 μm) were acquired from Sigma. Biolita L2,7 and P3,5 (biolite) were a kind gift from Ondeo Degrémont, Bilbao. Chromosorb W (30–60 mesh) and Celite R-643 were from Johns Manville Products. Cationic and anionic exchange resins (Dowex 50 × 8 and Lewatit MonoPlus MP 64, respectively) were supplied by Fluka.

Other chemicals were of analytical grade and were used without further purification.

2.4. Immobilization by covalent binding

The immobilization process was carried out according to the following steps [15]:

Preparation of the carrier: Glass beads were washed in 5% HNO_3 at 80–90 °C for 60 min and then rinsed with distilled water and dried in an oven for 24 h at 110 °C.

Support activation: To 1 g of clean glass beads, 18 ml of water was added along with 2 ml of γ-APTES (10% v/v) and the pH was adjusted to between pH 3 and 4 with 6N HCl. After adjustment, the mixture was placed in a 75 °C water bath for 2 h. The silanized glass was removed from the bath, washed with distilled water and dried overnight in an oven at 110 °C. The resulting product may be stored for later use.

Immobilization on glass-glutaraldehyde: One gram of silanized glass was made to react in a jacketed column reactor (2.5 i.d. and 30 cm length) with 25 ml of glutaraldehyde 2.5% in 0.05 M phosphate buffer, pH = 7. The reactor was equipped with a sinterized glass plate placed 5 cm from the bottom. The solution was recycled for 60 min with a peristaltic pump and the glass-glutaraldehyde washed with 25 ml of the same buffer. Enzyme solution (50 ml, 10 mg/ml) was then added to the reactor and the enzyme solution recycled overnight at 4 °C. The derivative was then washed three times with 0.1 M phosphate buffer, pH 7. The immobilized derivative was suspended in the same buffer and stored at 4 °C until use.

2.5. Immobilization by physical adsorption

When used, 1 g of support was mixed with 10 ml of an activator suspension (20 mg/ml) in an Erlenmeyer flask and placed in an orbital shaker overnight at room temperature. Three activators were tested: soybean lecithin, ricinoleic acid and oleic acid. One gram of support (as purchased or activated) was washed with 10 ml of distilled water and then transferred to the above mentioned jacketed column reactor. The enzyme solution (10 ml, 10 mg/ml in acetate buffer 0.01 M, pH 5) was then added to the reactor and recycled for 2 days at 4 °C. The immobilized derivative was washed twice with the same buffer and stored at 4 °C. When the influence of pH was studied, acetate buffer 0.01 M was used to adjust the pH values to 4, 4.5 and 5 and phosphate buffer 0.01 M was used for pH values of 6 and 7.

2.6. Protein determination

The amount of protein initially offered and in the wash-liquid after immobilization was determined by Lowry's procedure modified by Hartree [16], using bovine serum albumin as standard. The amount of coupled lipase was the difference between the amount of the initial enzyme added and the amount of enzyme in the wash-liquid.

2.7. Measurement of the reaction extension

Acid value (AV) [17] was used as an index to show the degree of reaction. The acid value is the number of milligrams of potassium hydroxide necessary to neutralize the free acids in 1 g of sample. AV corresponds to the carboxyl group concentration in the reaction mixture, which decreases due to the condensation of ricinoleic acid (AV = 180).