PRODUCTION OF RICINOLEIC ACID ESTOLIDE WITH FREE AND IMMOBILIZED LIPASE FROM *Candida rugosa*.

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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 *Candida rugosa* lipase on ten 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 205-210ºC under a vacuum of 700 mm Hg in a carbon dioxide atmosphere for 8 hours [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 *Candida 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 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 *Candida 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 X 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 ºC for 60 min and then rinsed with distilled water and dried in an oven for 24 h at 110 ºC.

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 ºC water bath for 2 h. The silanised glass was removed from the bath, washed with distilled water and dried overnight in an oven at 110 ºC. The resulting product may be stored for later use.

Immobilization on glass-glutaraldehyde: 1 g 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 ºC. 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 4ºC 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 two 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 gram of sample. AV corresponds to the carboxyl group concentration in the reaction mixture, which decreases due to the condensation of ricinoleic acid (AV=180).

2.8. Reactor experiments

The enzymatic reaction was carried out in an open jacketed batch reactor (100 ml total volume). Complete mixing was achieved by means of a three-bladed propeller stirrer. The reaction temperature was always kept constant at 40°C [3]. The reaction mixture contained 30 g of

ricinoleic acid and five grams of immobilized lipase. The only water in the reaction system is that soaked in the support. When free enzyme is used as catalyst, a certain amount of water is poured over the lipase before the substrate is added to the reactor. Samples were taken and AV was determined.

3. Results and discussion

3.1. Choice of the immobilized derivative

It has been described that adsorbed lipase on a ceramic carrier SM-10 [3] and the commercial immobilized lipase Novozym are suitable for producing ricinoleic acid estolide. However, the difficulty of acquiring the former support and the small size and low density of the latter, which hinders it separation from the reaction mixture, led the authors to test different immobilization matrices in an attempt to obtain an immobilized derivative which could be successfully used to catalyze the production of ricinoleic acid estolide.

Eight inorganic supports (two types of biolite, Celite R-643, Chromosorb W, non porous glass beads of two particle sizes and porous glass beads of different pore sizes) and two organic carriers (cationic and anionic exchange resins, Dowex 50 X 8 and Lewatit MonoPlus MP 64, respectively) were used.

Twelve different immobilized derivatives were obtained, six of them by physical adsorption and the other six by covalent coupling via the amino groups of the enzyme. Immobilization on glutaraldehyde-activated aminopropyl glass beads was selected because it has been widely used by the authors with different enzymes [15, 18] and has been shown to be very versatile.

The results obtained are shown in Table 1 where the percentages of immobilized protein and the protein contents are summarized. It is important to note that these values are based on the protein content provided by Lowry´s method [16], which showed that the commercial lipase contained only 15% protein. The best results were obtained when porous glass was used as immobilization matrix and covalent binding as coupling method. In these cases enzyme loading increased as the pore size became smaller because of the greater internal surface available for immobilization. The percentage of immobilized lipase obtained by physical adsorption on Lewatit MonoPlus MP 64 was higher than those obtained with porous glass because five times less enzyme was offered for immobilization, so that enzyme loading (mg E/g support) was noticeably lower. Celite R-643 was also shown to be suitable for *Candida rugosa* lipase immobilization.

The five above mentioned immobilized derivatives were used to catalyze the polymerization reaction of ricinoleic acid following the procedure described in Materials and Methods. The results obtained are shown in Figure 1. It can be seen that there was a large difference between the activity of the derivative obtained on the anion exchange resin and the activity of other derivatives. In the case of the anion exchange resin, the acid value dropped from 180 to 50 in 150 hours while the best result of the other derivatives was a fall to 136 in 285 hours (porous glass 75-400). Therefore, the immobilized lipase obtained by physical adsorption onto Lewatit MonoPlus MP 64 was chosen for further studies.

3.2. Choice of support activator

It has been described [19] that the esterifying activity of lipase is high when the enzyme is immobilized on a carrier previously activated with phospholipids, fatty acids or fatty acid esters. In this case, lipase is adsorbed at the water-fat interface on the surface of the support and

the enzyme activity can be efficiently utilized. Table 2 shows the results obtained in the immobilization processes with and without activators, in terms of percentage of immobilized protein and enzyme loading. Similar results were obtained for all the immobilized derivatives, pointing to the null effect of activator on the immobilization process as described in the bibliography [19].

The four immobilized derivatives were tested for activity in a batch reactor, following the procedure described in Materials and Methods. The results obtained are shown in Figure 2, where the beneficial effect on the enzymatic activity provoked by ricinoleic acid and soybean lecithin is pointed out. Estolide with an AV close to 40 was obtained after 100 hours of reaction. Slightly better results were achieved when soybean lecithin was used, and so this phospholipid was chosen as activator for further experiments. It is important to emphasize that soybean lecithin is also the activator used by the Japanese researchers in the only immobilized lipase described in the literature to obtain ricinoleic acid estolide [3]. However, as mentioned before, there are no papers on the use of other activators, so we can only suppose that the authors used their intuition or that they have a lot of unpublished studies.

3.3. Influence of enzyme concentration and pH

The support Lewatit MonoPlus MP 64 is a weakly basic anion exchange resin and its nature suggests that any adsorption of proteins would be governed by electrostatic forces. Therefore, changing the pH value should have a large impact on adsorption. On the other hand, the carrier is based on a styrene-divinylbenzene copolymer which, together with the phospholipids used as activator, could confer a certain hydrophobic character to the support. In this case, the adsorption could be controlled by hydrophobic interactions and the amount of protein

adsorbed would not be significantly influenced by pH changes in the protein solutions used for adsorption.

In order to explain which interaction predominates in the adsorption of lipase on Lewatit MonoPlus MP 64, several immobilized derivatives were obtained using lipase solution concentrations varying between 2 and 30 mgE/ml (which corresponds to 0.03–4.5 mg Lowry's protein/ml) dissolved in different buffers in a pH range from 4 to 7. The results obtained are shown in Figure 3. It can be seen that for each pH value assayed, an increase in protein concentration up to 4 mg/ml enhanced the driving force for the adsorption and increased the amount of protein adsorbed correspondingly. At pH ≥ 6 and when protein concentration was higher than 4 mgE/ml, the increase slowed down gradually. Lipase adsorption was improved at pH ≤ 5 and, in this case, the amount of enzyme adsorbed continuously increased as protein concentration increased (up to 30 mgE/ml), which means that higher enzyme concentrations could be used, resulting in an immobilized derivative of 14 mgE/g support. In the article of Yoshida [3] describing estolide production, two different derivatives with 60 and 120 mgE/g support were obtained, the authors considering that the entire enzyme is attached to the support during the immobilization process. The maximum capacity of the polypropylene carrier used by Gitlesen [20] was about 220 mgE/g support; however, this parameter could not be verified because we found difficulties when trying to dissolve higher enzyme concentrations in the immobilization buffer. Nonetheless, the maximum capacity of the Lewatit MonoPlus MP64 support at pH ≤ 5 was not reached. Other authors obtained immobilized derivatives of lipase with a lower (0.17 mgE/g [12]; 0.071-0.129 mgE/g [8]; 0.25-6.5 mgE/g [9]) or higher (150-1000 mgE/g [11]) enzyme contents.

Lipase immobilization is clearly affected by pH changes in the absorption solution, suggesting that electrostatic interactions are the main driving force in the adsorption of lipase by Lewatit MonoPlus MP

64. The isoelectric point (pI) of *Candida rugosa* lipase is 4.6-4.7 [21], so when the pH is lower than pI, the enzyme takes on a positive charge. Since the absorption process is favored at pH values lower than 5.0, the carrier has a negative charge, revealing that exchange sites of the support have a pK of less than 4.0. For pH values between 4.6 and 5.0, both enzyme and carrier have negative charges and in such a situation the lipase could possess localized areas of high positive charge on its surface (i.e. discrete binding sites) or other forces (hydrophobic forces) could play a role in the binding process.

On the other hand, the shape of the adsorption isotherms show a sigmoidal shape at pH = 7, indicating that enzyme-enzyme forces are more important than the support-enzyme forces, and the typical Langmuir trend at pH # 5 indicates that at the carrier surface the support-protein interactions are stronger than protein-protein ones [10]. No trend is clear at pH = 6 (data not shown).

3.4. Effect of mixing in the immobilization process

It has been described that lipase adsorption to solid surfaces follows several steps. The lipase molecule in the bulk phase must be transported to the surface, either by convection or diffusion. Even in well-stirred systems there exists a stagnant layer close to the surface that must be penetrated by diffusion. The lipase is then adsorbed at the solid surface at a given rate. After adsorption, macroscopic or microscopic rearrangements in protein structure can occur. Desorption of adsorbed protein is not common and the process is apparently irreversible [20].

In a standard experiment of protein adsorption, the activated support is placed in a jacketed column reactor and enzyme solution is added and recycled for 48 hours at 4°C. Although adsorption processes are usually slow, two days seemed to be sufficient to reach equilibrium.

To check whether the adsorption process had been completed and to optimize the immobilization time, an experiment was carried out in which samples were taken from the supernatant and analyzed for enzyme. In parallel, the same amounts of activated support and enzyme solution were poured together into an Erlenmeyer flask and the mixture was placed in the cool room at 4°C. This supernatant was also sampled and analyzed for protein. The results of both experiments are shown in Figure 4, in which enzyme concentration in the supernatant is represented against time (note that time scales are different). Although the final enzyme concentration was exactly the same in the column reactor and Erlenmeyer flask, the equilibrium concentration in the former device was reached after less than 10 hours, while two days were necessary to complete the immobilization step in the cool room.

3.5. Comparison of free and immobilized lipase

To recapitulate, the immobilization procedure of *Candida rugosa* lipase was optimized and the best results, taking into consideration enzyme consumption and the activity of the resulting derivative, were obtained when 50 ml of enzyme solution (10 mg/ml in acetate buffer 0.1 M, pH=5.0) was mixed with 5 g of the anion exchange resin Lewatit MonoPlus MP 64 previously activated with soybean lecithin, and recycled in a column reactor for 10 hours at 4°C or left to stand in the cool room for two days. Under these conditions an immobilized derivative with almost 8 mgE/g support was obtained. Furthermore 5 g of resin soaks up 3 ml of water.

In order to compare the specific activity of the immobilized lipase and the free enzyme, two experiments were carried out using the same amount of protein as catalyst; that is, one experiment was performed with the immobilized derivative and the other was prepared with the same amount of protein adsorbed on the support. In Figure 5 the evolution of the acid values measured in both reactors are represented.

After 24 hours, the AV of the reaction with native lipase fell to half of its initial value, while for the reaction catalyzed with the immobilized enzyme the AV fell to one third of its initial value. In addition, the final AV attained (close to 40) was noticeably lower when immobilized lipase was used as catalyst. In the case of Yoshida [3], the estolide produced per unit weight of lipase was 425 g estolide/g free enzyme, when immobilized derivative (60 mgE/g) was used in five consecutive batches. In our case, 750 g of estolide are obtained per gram of free enzyme in an only run, and this amount may be increased if the immobilized derivative is evaluated and proved for reuse. A similar comparison could not be established with the results of Kelly and Hayes [14] because they use a commercial immobilized lipase and the derivative enzyme content is not revealed.

3.6. Reuse of the immobilized derivative

Immobilization provides an attractive opportunity for the multiple use of the same enzyme. In order to establish the reusability of our immobilized derivative, successive polymerization reactions were planned. The purpose of this experiment was to assess the multiple use of the immobilized lipase by examining the evolution of AV with time in several consecutive experiments. The results are shown in Figure 6. During the first 24 hours, AV decreases almost 120 units in the first use, 70 units in the second run, and only 48 in the third run. However, after 7 days the reached AV in the second use of the derivative is almost the same than that in the first utilization.

In order to ascertain whether this activity loss is due to enzyme desorption or inactivation an additional experiment was carried out. After 24 hours in a normal polymerization process with immobilized derivative, the reactor content was divided in two halves so that the support remains in the original reactor. Then 15 g of ricinoleic acid was added to both reactors and the evolution of AV was registered. In the

original reactor a decrease of AV with time was observed and in the second reactor no variation of AV with time was detected (data not shown). In consequence it can be affirmed that activity loss in reuse experiments was caused by enzyme deactivation.

4. Conclusions

Among the wide variety of procedures assayed, adsorption on Lewatit Monoplus MP 64 was demonstrated to be the best method for immobilizing *Candida rugosa* lipase for the production of ricinoleic acid estolide. Previous treatment of the support with soybean lecithin has no effect on enzyme loading but improves derivative activity. The immobilization procedure was optimized, pH of the coupling solution being the most critical immobilization parameter.

The specific activity of the immobilized lipase is much higher than that of the free enzyme and leads to an estolide with a superior degree of polymerization (low acid value). In addition the immobilized derivative could be re-used although some operational deactivation was detected.

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FIGURE CAPTIONS

Figure 1.- Change in acid-value as a function of time for estolide synthesis catalyzed by five different immobilized derivatives.

Figure 2.- Influence on the evolution of acid value with time of the addiction of three different activators during the lipase immobilization process.

Figure 3.- Influence of buffer pH and the offered protein concentration on the enzyme loading for the immobilization of lipase on Lewatit-Monoplus MP 64.

Figure 4.- Time course of protein absorption in the cool room (bottom time scale) and in the column reactor (top time scale).

Figure 5.- Change in acid-value as a function of time for estolide synthesis catalyzed by free and immobilized lipase.

Figure 6.- Synthesis of estolide by repeated batch operation.