ENZYMATIC BIOSYNTHESIS OF RICINOLEIC ACID ESTOLIDES

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ABSTRACT

Candida rugosa lipase has been shown to have sufficient activity to catalyse the enzymatic synthesis of ricinoleic acid estolides in a batch reactor. The water requirements of the reactor change during the reaction: at the beginning of the process a minimum amount of water is necessary but, later, the reaction mixture must be dried out to obtain an estolide with a high degree of condensation. The influence on the reaction rate of variables, such as water content, enzyme concentration and mixing devices, was established and optimised. Using an initial water content of 144000 ppm and a lipase concentration of 13.33 mgE/g ricin, and maintaining the temperature at 40°C by mean of hot air circulation and using a three-bladed propeller stirrer as mixing device, an estolide of ricinoleic acid with an acid number of 65 was obtained in 48 hours.

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 [1, 2]. Estolides have been reported to occur in nature [3] or to be synthesised at high pressure/temperature from castor oil or its hydroxy acid, ricinoleic acid ($18:1^{9}$ $-OH^{12}$) [4]. The estolide made from ricinoleic acid (Figure 1) is a useful substance with many applications in industry. For example, it is used as a viscosity controller for chocolate and an emulsifier in margarine, as a cutting oil base in metal processing, and as pigment dispersant in paint, ink and cosmetics [2].

However, chemically synthesised estolides present problems of coloration and odour due to the high reaction temperatures needed. Such products are therefore unsuitable for the food industry. As an alternative, some researchers have investigated the enzymatic synthesis of ricinoleic acid estolides by the catalytic action of lipase (EC. 3.1.1.3), which acts in mild reaction conditions, for example, low temperatures and pressures and neutral pH [2, 5].

The natural action of lipase in an aqueous medium is to hydrolyse organic esters. If the enzyme is placed in a medium with a low water concentration, the thermodynamic equilibrium can 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 the amount of water necessary for enzyme activity might be very small and, in the case of lipase, just a few layers around the enzyme surface are needed [6]. On the other hand, the water formed by the reaction must be removed from the reaction mixture if ricinoleic acid estolides with a high degree of condensation are to be obtained [2].

The formation of estolides by lipase-catalysed reactions depends strongly on the position of the hydroxyl moiety and slightly on the chainlength and concentration of the hydroxy acid. Reactions involving estolide formation from ricinoleic acid or hydrolysis of estolides have been catalysed successfully by "random lipases", i.e., those that lack 1,3-positional selectivity (e.g. *Candida rugosa, Chromobacterium viscosum, Pseudomonas* sp. and *Geotrichum candidum*), but have not been catalysed by 1,3-specific lipases (e.g. *Rhizopus* sp., *Rhizomucor miehei,* porcine pancreatic lipases, etc.). This may be related to the inability of several 1,3-selective lipases to attack secondary alcohols [7, 8].

The main objective of this work was to determine the optimum conditions for the enzymatic synthesis of ricinoleic acid estolides. For this the optimal initial water content and enzyme concentration were studied. Moreover, the influence of water removal and way of mixing on the reaction performance were also investigated. Although lipases are quite stable in organic solvents, where the enzymatic reaction can be conducted at a lower temperature [5], the present

experiments were carried out in a low-water aqueous system, without organic solvent to obtain non-toxic products [9].

MATERIALS AND METHODS

Materials

Lipase from *Candida rugosa* (819 Units/mg solid) and ricinoleic acid (99 %) were purchased from Sigma-Aldrich. The ricinoleic acid (~ 65%) was supplied by Fluka. Other chemicals were of analytical grade and used without further purification.

Methods

Measurement of the reaction extension: Acid value (AV) [10] was used as an index to show the degree of reaction. AV corresponds to the carboxyl group concentration in the reaction mixture, which decreases due to the condensation of ricinoleic acid (AV = 180). It was also confirmed by HPLC (see below) that the decrease in AV reflected the condensation of ricinoleic acid.

HPLC analysis of the ricinoleic acid: Ricinoleic acid was dissolved in absolute ethanol. A 10 μl aliquot was injected into a Nova-Pack C_{18} (4 μm particle size) reverse-phase column. Samples were eluted with acetonitrile / 30 Mm phosphoric acid (80:20 v/v) at 30°C, at a flow rate of 1.5 ml/min and detected by their absorbance at 205 nm. Area values were converted to concentrations using a calibration curve (ricinoleic acid (mg/ml) = $1.79 \times \text{area} \times 10^{-6}$).

Determination of water content: The water content in the reaction solution was determined by the Karl-Fisher method with a water content meter.

Reactor experiments

The enzymatic reaction was carried out in a batch reactor (100 ml total volume). Complete mixing was achieved either by orbital shaking or by means of a three-bladed propeller stirrer. The reaction temperature was always kept constant at 40° C [2], which has been found to be the optimal reaction temperature. The heating system was an incubator provided with hot air circulation. The reaction mixture contained 30 g of ricinoleic acid and the appropriate amounts of enzyme and water. Samples were taken and AV and ricinoleic acid concentrations were determined

RESULTS AND DISCUSSION

Because the synthesis of ricinoleic acid estolide is a dehydration-condensation reaction, the reaction reaches equilibrium and stops when the water content in the reaction mixture increases as a result of the water formed during the reaction. It has been reported [2] that the polarity of the bulk reaction mixture becomes lower as condensation progresses and most water molecules associated to the polar groups of ricinoleic acid are now free to maintain the configuration of lipase. Therefore, the optimal water content decreases during the reaction course and water must be removed from the reaction mixture.

By comparing experiments conducted at similar initial conditions of enzyme concentration, water content and stirring speed, but using different dehydration methods (spontaneous evaporation, a vacuum aspirator and hot air current), hot air was found to be the best way of removing excess water and obtaining estolides with a high degree of condensation (data not shown).

First, the influence of a hot air current on the water content of ricinoleic acid was studied. For this, 30 g of ricinoleic acid were mixed in open flasks with different volumes of water to give initial water concentrations between 10000 and 510000 ppm. Then, they were placed in an incubator with an orbital shaking apparatus. The incubator was thermostated to 40°C using hot air circulation. The total water content of the mixture was followed by taking samples and titrating using the Karl-Fisher method. The results show that, in all cases, most of the water was eliminated during the first two to ten hours, depending on the initial water concentration. After 24 hours, the water content had stabilised at about 2500 ppm in all the experiments.

The presence of a small amount of water is necessary for the expression of the enzyme activity because water is essential for maintaining the configuration of the enzyme in the proper form [7]. On the other hand, too high a water content might displace the equilibrium towards the hydrolysis reaction. Therefore, the influence of the initial water content on the condensation reaction should be studied. A set of experiments was carried out varying the initial concentration of water in the reaction mixture between 4000 ppm and 510000 ppm. The two lowest water contents were achieved by drying commercial ricinoleic acid (water content 10000 ppm). All the reactions were carried out with 30 g of

ricinoleic acid and 200 mg of lipase from *Candida rugosa.* The appropriate amounts of water were added when initial water contents higher than 10000 ppm were assayed.

It can be observed from Figure 2 that the ricinoleic acid condensed even when the minimum initial water content (4000 ppm) was used. This result is in accordance with the only previous report found in the literature, which mentions that the minimum water content required for expressing lipase activity lies between 1800 and 4200 ppm for ricinoleic acid estolide biosynthesis [2].

As the water content was increased from 4000 ppm to 77000 ppm, so the decrease in AV became more pronounced. This was because the initial water concentration in the reaction mixture was not sufficient to maintain the enzyme in a catalytically active conformation and the enzymatic activity increased with increasing concentrations of water. On the other hand, when the water concentrations were varied between 77000 ppm and 310000 ppm, no significant changes in the AV/time slopes were observed. In these four experiments, the water in the reaction mixture enabled the enzyme to catalyse the synthetic reaction at a very high reaction rate. However, when 15 ml of water were added to the reactor (to provide a water concentration of 510000 ppm) the reaction rate decreased. In this case, the high water concentrations favoured the reverse reaction (law of mass action). From these experiments it was not possible to establish the optimum water content because high reaction rates were observed for a wide range. However, it can be assumed that the addition of water to the commercial ricinoleic acid benefits the condensation reaction as long as the initial amount of added water is lower than 0.5 ml H_2O/g ricinoleic acid (initial water concentration lower than 510000 ppm). These results agree with previously published findings [2] only from a qualitative point of view because these authors reported optimum water contents noticeably lower than ours (between 3000 ppm and 17000 ppm). This difference could be explained by differences in the ricinoleic acid used as substrate of the enzymatic reaction. Another reason could be the fact that the above mentioned authors use of an immobilised lipase in a ceramic support and, in this microenvironment, the amount of water required by the enzyme to express its catalytic activity might be lower. In view of those results, a water

content of 77000 ppm is considered adequate for an enzyme concentration of 6.67 mgE/g ricin.

Next, another set of experiments was conducted to study the influence of the enzyme concentration on the reaction rate. Because the main role of water is to maintain the enzyme configuration, it may be thought that an increasing amount of enzyme should accompany an increasing amount of water. In all cases the water concentration was maintained in the range where the reaction rate was maximal (see Figure 2). Therefore, another series of experiments was planned, in which both enzyme and water concentration were simultaneously increased. The results of this experimental series are shown in Figure 3A. As can be seen, a substantial improvement in the reaction rate was achieved when added water and enzyme concentrations were doubled but no additional enhancement was observed when the concentration values were increased threefold. As a consequence, it was concluded that no faster processes can be expected by increasing the biocatalyst concentration.

Even at the 40°C used, the viscosity of the reaction medium was quite high, and it may be thought that the reaction process was controlled by mass transport phenomena. Another experimental series was carried out with the same water and lipase concentrations as in the previous series but using a three-bladed propeller stirrer instead of orbital shaking for mixing. The results are shown in Figure 3B, where it can be seen that the reaction rate improves only in the case of the lowest enzyme and water concentrations. Comparing both figures, it can be concluded that when the lower enzyme concentration was used the condensation reaction progressed under diffusional control because the reaction rate was improved by more efficient mixing. However, when larger enzyme concentrations (double and triple) were assayed no improvement in reaction rate was observed by changing the mixing device due to kinetic control. Therefore, the optimum enzyme concentration lies between 6.67 and 13.33 mgE/g ricin. These results could not be compared with others because no previous studies have been found in the literature.

In the experiments described in this paper, samples were analysed by HPLC to measure the ricinoleic acid and to check whether the decrease in AV reflects the condensation of this fatty acid. As an example, Figure 4 shows the

variation with time of both ricinoleic acid percentages in the commercial fatty acid and AV. After 30 hours of reaction, the monomer had almost completely disappeared from the reaction mixture and the further AV decreases measured could be attributed to oligomers formation.

It has been reported that the acid value (AV) of ricinoleic acid estolide prepared by conventional chemical methods is 40 [4] and that this AV is only attainable by using immobilised lipase and never with free enzyme [11]. The acid value is related with the degree of polymerisation and each particular application of the estolide will require a different AV value. Using the reactor configuration described in this paper and the best reaction conditions determined (water and enzyme concentrations), an AV value of 65 was reached after 48 hours. This reaction time is shorter than that reported previously [11] where ricinoleic acid estolides with AV of 60 were produced with free lipase after 150 hours of reaction. In spite of this improvement, the results presented in the present paper can probably be improved, especially the reaction time, which needs to be shortened for the process to be interesting from an industrial point of view.

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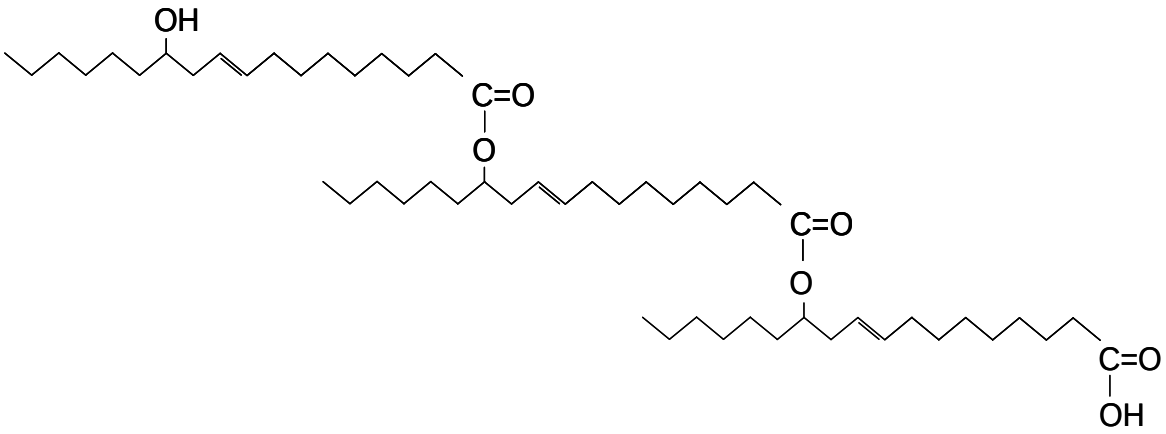

Figure 1.- Estolide of ricinoleic acid.

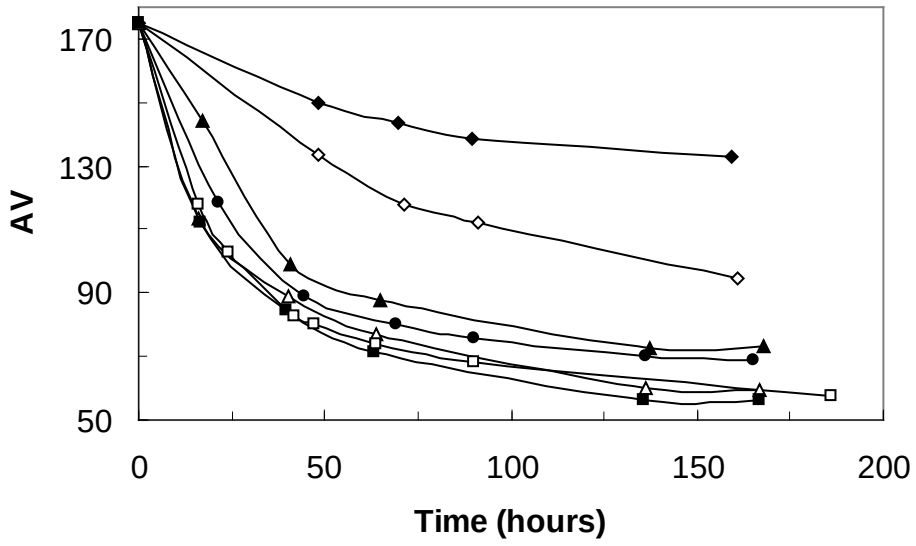

Figure 2.- Kinetic of the condensation reaction at different initial water concentrations. Reaction conditions:

Enzyme concentration = 6.67 mgE/g ricinoleic acid.

Initial water content = 4000 ppm (◆), 5800 ppm (◇), 10000 ppm (▲), 77000 ppm (Δ), 110000 ppm (■), 310000 ppm (□) and 510000 ppm (●).

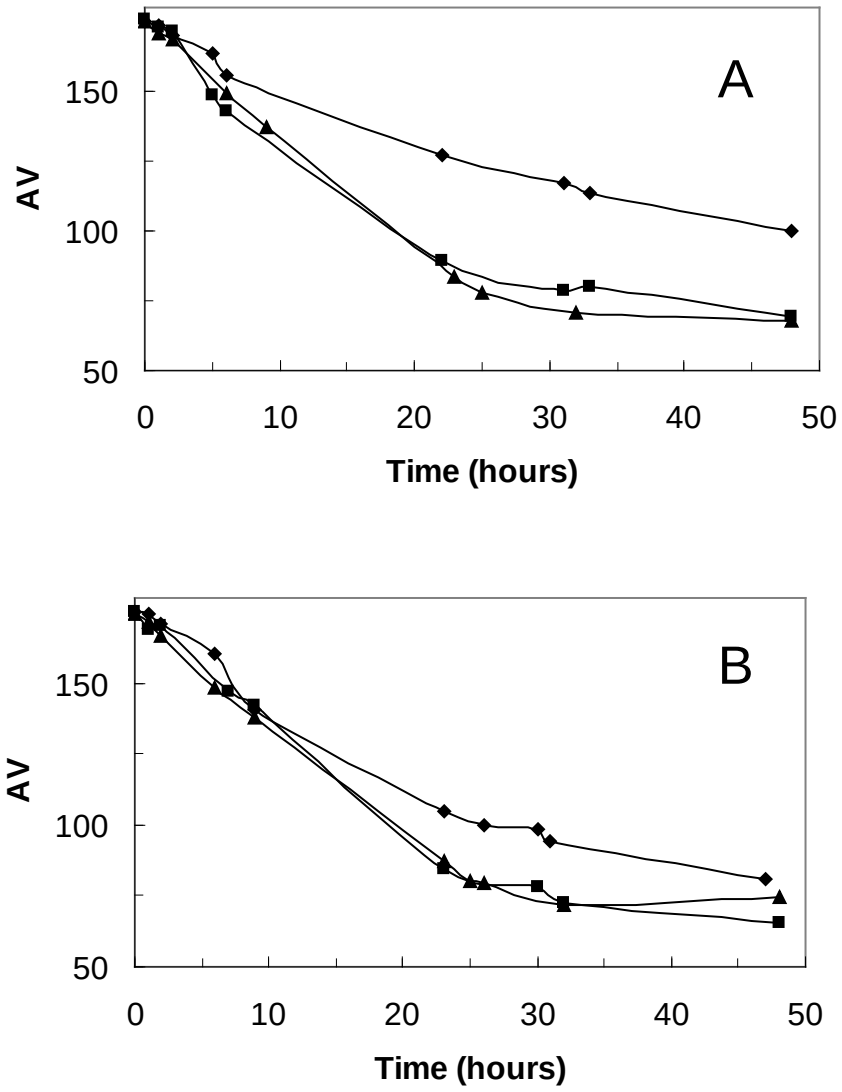

Figure 3.- Time course of the condensation reaction. Conditions:

(◆) [Enzyme] = 6.67 mgE/g ricin. [H_2O] = 77000 ppm.

(■) [Enzyme] = 13.33 mgE/g ricin. [H_2O] = 144000 ppm.

(▲) [Enzyme] = 20.00 mgE/g ricin. [H_2O] = 211000 ppm.

A Orbital shaker.

B Three-bladed propeller stirrer.

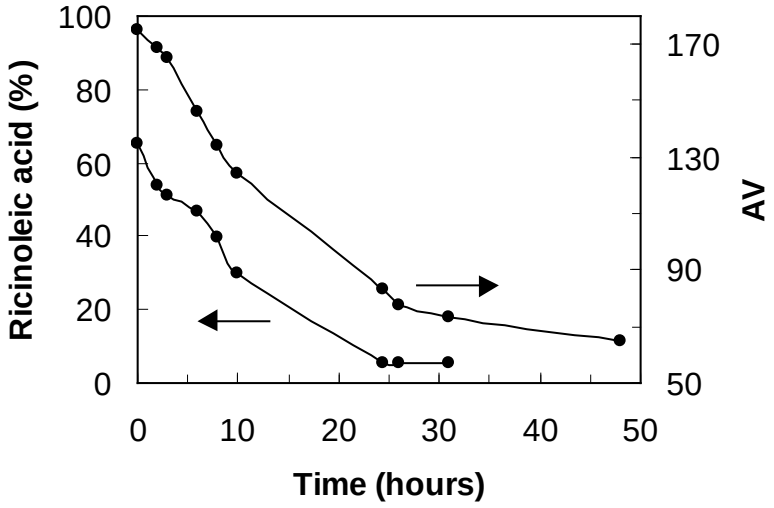

Figure 4.- Time course of the condensation reaction of ricinoleic acid. Reaction conditions: [Enzyme]=13.33 mgE/g ricin. [H_2O]=144000 ppm. Three-bladed propeller stirrer.