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Stabilization of a tetrameric enzyme (α-amino acid ester hydrolase from *Acetobacter turbidans*) enables a very improved performance of ampicillin synthesis

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Abstract

The stabilized derivative of the enzyme α-amino acid ester hydrolase from *Acetobacter turbidans* has been found to be very adequate as biocatalyst of the synthesis of the very relevant antibiotic ampicillin. This enzyme resulted much more adequate than the Penicillin G Acylase (PGA) from *Escherichia coli* (the most used enzyme). The stabilization of the enzyme was required because under optimal conditions (absence of phosphate and 40% of MeOH), no-stabilized derivatives or soluble enzyme from *A. turbidans* become very rapidly inactivated. Under these conditions, this new stabilized derivative exhibited a very high selectivity for the transferase activity compared to the esterase one, as well as a very low hydrolytic activity towards the antibiotic. Moreover, this new biocatalyst did not recognize L-phenylglycine as substrate in the synthetic process. By using the racemic mixture of D/L phenylglycine methyl ester, 85% of the D-ester could be transformed to ampicillin. In contrast, the enzyme from *E. coli* exhibited a high hydrolytic activity for the ampicillin yielding low synthetic yields. This enzyme also resulted much less enantioselective producing both isomers of the antibiotic.

Keywords: Stabilization of multimeric enzymes; Enzymatic synthesis of ampicillin; Stereospecific synthesis; Enzyme specificity

1. Introduction

The range of conditions for studying enzymes in biotransformations is many times restricted by the low stability of these catalysts under conditions very different than physiological ones. This problem reaches a special relevance when using multimeric enzymes, composed by several subunits that must be associated to exhibit catalytic activity. These enzymes are mainly inactivated by subunit dissociation and this phenomenon become more important under determinate experimental conditions (extreme pH,

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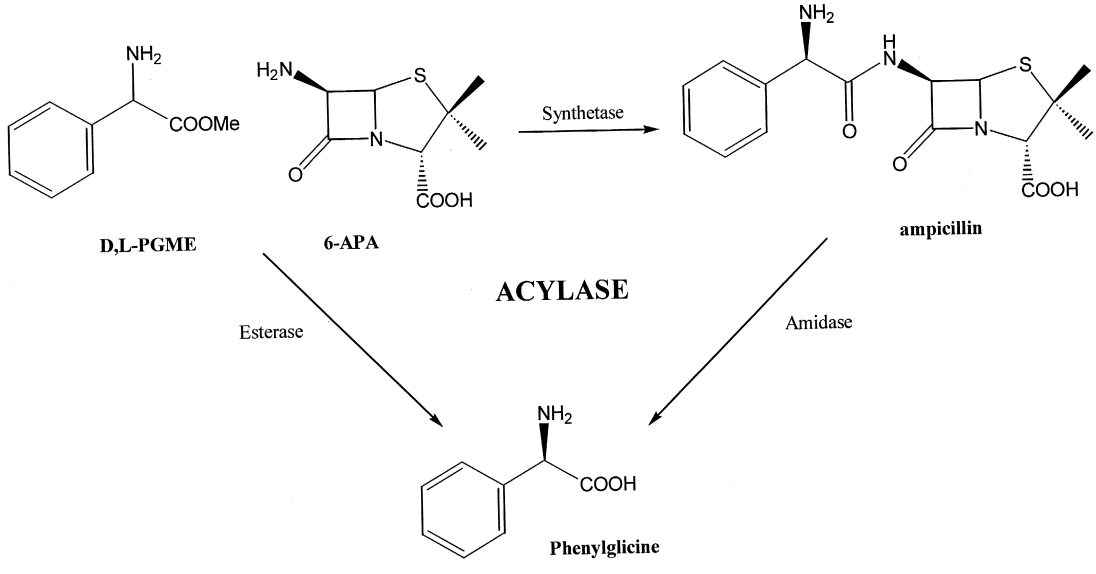
presence or absence of some compounds, presence of organic cosolvents, high temperature, etc.) [1]. Thus, it may be possible that the particular condition where the enzyme might perform its catalytic function in the most effective way fully coincide with conditions where that multimeric enzyme tends to dissociate. In this way, the enzyme would be inactivated and those conditions have to be excluded when studying the performance of the biocatalyst even at laboratory scale. From this point of view, the preparation of derivatives of multimeric enzymes with a fully stabilized structure may be a critical point in the design of biotransformations catalyzed by these enzymes.

The synthesis of antibiotics, e.g., ampicillin, may be a very interesting process currently carried out by chemical routes and where the use of enzymes may have a great relevance because of the reduction of protection/deprotection steps and elimination of toxic and contaminant reagents and solvents currently used in chemical synthesis [2–5]. The advantages of the use of enzymes in this kind of process may be further reinforced taking into account advantages arising from the substrate specificity of enzymes. In the case of ampicillin, for example, an enzyme exhibiting a very high enantioselectivity towards D-phenylglycine methyl ester (D-PGME), should make feasible the development of a kinetically controlled stereoselective process starting from the racemic mixture of the acylating ester. This enzymatic process may be very competitive with the current chemical one.

We have evaluated the possibility of performing this stereospecific enzymatic synthesis (Scheme 1) by using D,L-PGME and 6-amino penicillanic acid (6-APA) as substrates. In this process, the enzyme may catalyze three different reactions [2–5] and the yields are determined by two parameters: (i) the ratio between the rates of synthesis of the antibiotic and hydrolysis of the ester, and (ii) the ratio between the rates of synthesis and hydrolysis of the antibiotic.

Therefore, the yields obtained by using this synthetic approach strictly depend on the enzyme catalytic properties, which may be further modulated by the experimental conditions. As resulting from previous reported studies, the tetrameric enzyme α-amino acid ester hydrolase from *Acetobacter turbidans* has been found very adequate for the synthesis of ampicillin [6]. To perform these studies, a new derivative of this enzyme with the quaternary structure fully stabilized has been utilized [7] and compared to the



Scheme 1. Kinetically controlled stereoselective synthesis of ampicillin.

most commonly used enzyme, the penicillin G acylase (PGA) from *Escherichia coli*.

2. Materials and methods

Purified α-amino acid ester hydrolase from *A. turbidans* (ATCC 9325) was prepared as previously described [6]. 6-APA, PGA from *E. coli* as well as D, L and D/L-PGME were kindly donated by Antibioticos (Leon, Spain). 4% cross-linked amino agarose beads (MANAE) activated with 5 or 40 μmol/ml was a gift from Hispanagar (Burgos, Spain) and prepared as previously described [8]. 25% glutaraldehyde solution was from Merck. All other reagents were from Sigma (St. Louis, MO, USA). Aldehyde dextran (MW 18,000) was prepared by its full oxidation with sodium periodate as previously described [9]. PGA from *E. coli* was immobilized as previously described [10].

2.1. Preparation of glutaraldehyde agarose

A total of 10 ml of 6% cross-linked-MANE-agarose activated with 10 or 75 μmol amino groups/ml were suspended in 90 ml of 11% glutaraldehyde v/v, 100 mM sodium phosphate pH 7. The gel was gently stirred for 14 h and then washed with a great excess of distilled water. This activated support was used immediately after preparation.

2.2. Preparation of α-amino acid ester hydrolase derivatives from A. turbidans

A strategy designed in our laboratory was utilized to stabilize the quaternary structure of the enzyme [11]. Ten milliliters of glutaraldehyde-agarose actived with 40 μmol/ml were added to 90 ml of a solution containing 20 international units of enzyme in 100 mM sodium phosphate pH 7 (activity was determined by the hydrolysis of 10 mM D-PGME in 25 mM sodium phosphate pH 6.5 at 4°C). After 12 h under continuous stirring at 4°C, 500 ml of 200 mM sodium bicarbonate pH 8.5 and 800 mg of solid sodium borohydride were added to the immobilization solution. This suspension was stirred for 1 h and the derivative was washed with an excess of distilled water.

2.3. Modification of the of α-amino acid ester hydrolase derivatives from A. turbidans with aldehyde dextrans

Ten milliliters of derivative prepared as above described were added to 90 ml of 100 mM phosphate pH 7. Twenty milliliters of the aldehyde–dextran solution (1.8 mM) were then added. After 24 h of stirring, 500 ml of 200 mM sodium bicarbonate pH 8.5 and 800 mg of solid sodium borohydride were added to the immobilization solution. This suspension was stirred for 1 h, and the derivative was washed with an excess of distilled water. The immobilization process was controlled by evaluating that the derivative prepared does not release any subunit of enzyme when boiled in SDS [11].

2.4. Kinetically controlled synthesis of antibiotics

After dissolving the desired amounts of 6-APA and 2.5 mM D-PGME in the reaction medium utilized, the pH was adjusted at the desired value by adding concentrated ammonia. The reaction mixture was cooled at 4°C and 1 ml of enzyme derivative were then added to 20 ml of solution under magnetic stirring. When methanol was used, pH values were given without corrections as pH_{app}. Reactions were followed by HPLC analyses recording the absorbance at 254 nm. Mobile phase was 10% MeOH/90% 100 mM ammonium phosphate pH 5. The column was a kromasil C8 (150 × 4.6 mm) produced by Analisis Vinicos (Spain). At a flow of 2 ml/min, retention times were 1.25 min for phenylglycine, 3 min for 6-APA, 11 min for D-PGME and 22 min for ampicillin. Identification and evaluation of the concentration of substrates and products were performed by comparison with standard solutions of authentic samples. For L-ampicillin, a standard solution of ampicillin was considered for quantification, while the identification of this acylation product was performed by HPLC analysis by comparing with a

solution of L-ampicillin prepared by acylation of 6-APA with L-PGME catalyzed by the PGA from *E. coli*.

2.5. Enantioselectivity of the acylases from E. coli and A. turbidans

The enantioselectivity of the derivative prepared with the acylases from *A. turbidans* and *E. coli* was evaluated by measuring, separately, the initial rate of hydrolysis of the different enantiomers of PGME. Reactions were performed at pH 6.5 and room temperature in presence of 2.5 mM of ester. After dissolving the ester in distilled water, the pH was adjusted at 6.5 by adding concentrated ammonia. The reaction mixture was cooled at 4°C under magnetic stirring and 1 ml of enzyme derivative was added to 20 ml of solution. The hydrolysis rates were evaluated monitoring the acid formation at the beginning of the reaction by HPLC analysis performed as above reported for the synthetic reactions.

3. Results

3.1. Synthesis of ampicillin catalyzed by stabilized derivatives of α-amino acid ester hydrolase from A. turbidans

The immobilized and stabilized α-amino acid ester hydrolase was used as catalyst in the kinetically controlled synthesis of ampicillin. This derivative was compared with the free enzyme and with the most commonly used PGA from *E. coli* [2,5]. In this study, the enzymatic reactions were performed operating in presence of a large excess of 6-APA in order to compare the different catalysts ensuring an almost constant concentration of this nucleophile as well as a good saturation level of the enzymatic active centers.

Both soluble acylase and stabilized derivative could be used under standard conditions (presence of phosphate ions, pH 6.5 and 4°C) yielding very similar results (Fig. 1). However, the yields obtained were too low for being interesting.

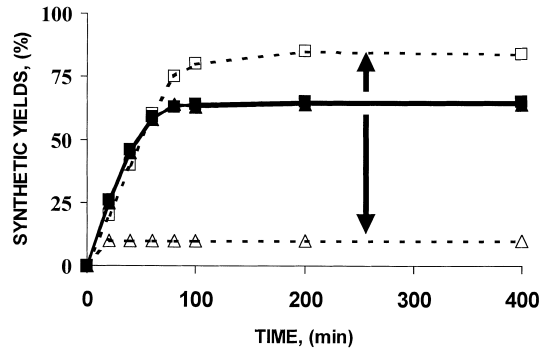


Fig. 1. Kinetically controlled synthesis of ampicillin catalyzed by stabilized (squares) or free enzyme from *A. turbidans* (triangle). Experiments were carried out at pH 6.5, 4°C in 0.1 M phosphate (solid line) or in the absence of phosphate (dashed line) 50 mM 6-APA and 2.5 mM were used as substrates.

By eliminating the phosphate ions from the reaction medium, it was observed that the stabilized derivative gave much higher yields (Fig. 1) while the soluble enzyme stopped the reaction long before consuming all the ester. This seems to be related with some role played by the phosphate ions in the stabilization of the quaternary structure of the enzyme. Consequently, the use of a stabilized derivative in the conditions where yields become interesting, resulted necessary even for laboratory studies.

Fig. 2 shows the comparison between the performance in the ampicillin synthesis of PGA from *E. coli* and the stabilized derivative of the enzyme from *A. turbidans*. In these reactions, the use of methanol (40%) was considered for improving yields, in agreement with previous reported results [12]. Under these conditions, the enzyme from *A. turbidans* gave much higher yield (85%) than the enzyme from *E. coli* (36%). Using the first acylase, the rate of antibiotic synthesis was much higher than the rates of ester and antibiotic hydrolyses. In contrast, utilizing PGA from *E. coli*, a rapid hydrolysis of the already synthesized ampicillin was observed (Fig. 2).

3.2. Stereospecificity of the α-amino acid ester hydrolase from A. turbidans

In a preliminary study, the enantioselectivity of the enzymes from *E. coli* and *A. turbidans* were

compared, considering the hydrolysis of the D and L-PGME. The enzyme from *E. coli* exhibited a very low stereospecificity under optimal conditions (around 2) while the stereospecificity of the enzyme from *A. turbidans* resulted much higher (around 20). Thus, the use of this new enzyme derivative for the synthesis of ampicillin starting from racemic D,L-PGME was considered.

Fig. 3 shows the reaction courses in the synthesis of ampicillin catalyzed from 5 mM D/L-PGME and 50 mM 6-APA. As expected, the enzyme from *A. turbidans* only utilized D-PGME in the synthetic reaction under the experimental conditions used. Thus, when using the D,L racemic mixture, the yields were similar to those obtained with the pure enantiomer: more than 85% of D-PGME was transformed into antibiotic (Fig. 3a). L-ampicillin was not detected after consumption of 50% of the initial ester (detection limit around 0.25% under analysis conditions). Thus, in this acylation reaction, the enantioselectivity of the enzyme towards the D-isomer was found to be higher than 200.

On the contrary, PGA from *E. coli* gave both isomers of ampicillin, as expected from the low steospecifcity showed by this enzyme. A final ampicillin yield (regarding the ester transformed into antibiotic) of only 17% (maximum yield should be 50%) contaminated with a 3–4% of L-ampicillin was, in fact, observed. In both reactions, Pheglycine-PGME dimmer was not detected, perhaps as consequence of the high excess of 6-APA used.

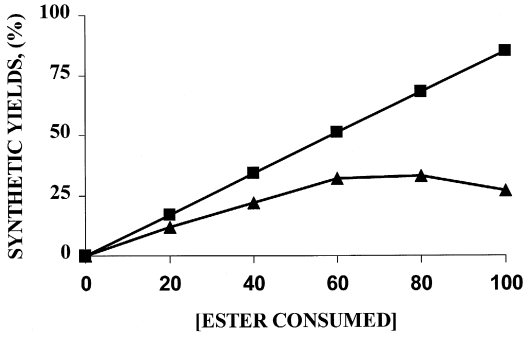


Fig. 2. Comparison between the synthesis of ampicillin reaction catalyzed by derivatives of different enzymes. Experiments were performed in 40% MeOH at 4°C and pH 6.8 using 50 mM 6-APA and 2.5 mM D-PGME. Triangles: stabilized derivative of PGA from *E .coli* (here MeOH was omitted). Squares: stabilized derivative of acylase from *A. turbidans*.

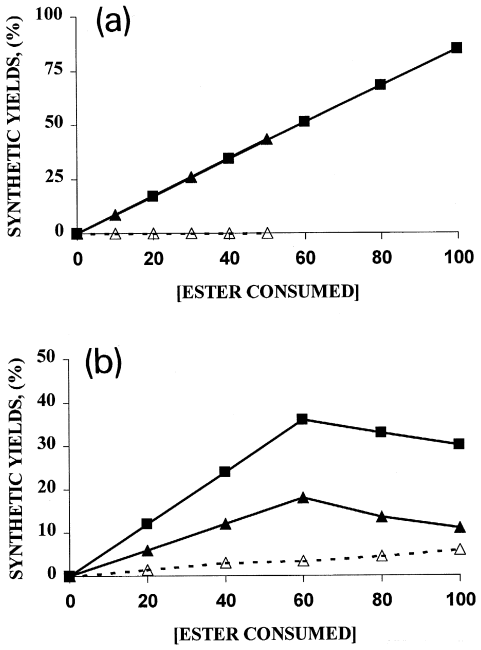


Fig. 3. Synthesis of ampicillin from racemic mixture of D/L PheglyOMe catalyzed by the enzyme from *A. turbidans* (a) or *E. coli* (b). Experiments were performed in 40% MeOH at 4°C and pH 6.8 using 50 mM 6-APA and 2.5 mM of D (squares) or D/L (triangles) PheglyOMe (for *E. coli* derivative, MeOH was omitted). Solid lines/solid symbol: yields of ampicillin; dashed lines/empty symbol: yields of L-ampicillin.

4. Conclusions

Stabilization of multimeric enzymes enable the use of these catalysts under dissociation conditions and that, in many cases, may be a critical point in the design biotransformations.

The stabilized derivative of the enzyme α-amino acid ester hydrolase from *A. turbidans* has been found to be very adequate as biocatalyst for the synthesis of the very relevant antibiotic ampicillin. This derivative, under optimal conditions, exhibit a very high selectivity for the transferase activity compared to the esterase one, as well as very low hydrolytic activity towards the antibiotic alredy syn-

thetized. Furthermore, this enzyme did not recognize L-phenylglycine as substrate in the synthetic process, opening to the possibility of developing an enantioselective synthesis of ampicillin. Further studies are in progress in order to develop a suitable process to obtain the complete transformation of high concentrations of 6-APA into ampicillin. The results obtained will be a matter on forthcoming papers [13].

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