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The use of stabilised penicillin acylase derivatives improves the design of kinetically controlled synthesis

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Abstract

The advantages of the use of stabilised derivatives in the kinetically controlled synthesis catalysed by penicillin G acylase has been discussed. Stabilised derivatives were found to be less inhibited by the nucleophile than non-stabilised ones, improving in that way the reaction rate. Also, more resistance under unfavourable conditions (e.g. presence of methanol) was found using stabilised derivatives. Multipoint covalently attached derivatives prepared in the presence of penicillin sulfoxide showed the same hydrolytic/synthetic ratio as the non-distorted enzyme suggesting that the stabilisation strategy is able to increase the enzyme rigidity without promoting significant changes in the enzyme properties.

In addition, the effect of the enzyme source was found to be significant: enzyme derivatives from *Escherichia coli* appeared to have better properties as catalysts of these reactions than enzyme derivatives of penicillin G acylase from *Kluivera citrophila*.

Keywords: Enzymes; Immobilised enzyme derivatives; Organic synthesis; Penicillin G acylase; Stabilised enzyme derivatives

1. Introduction

The employment of enzymes as catalysts of organic synthesis using kinetically controlled strategies is very popular in the literature [1–3]. This strategy of synthesis can be performed in fully aqueous reaction media and, therefore, the use of non-stabilised enzyme derivatives, even soluble enzymes, is frequently possible. The other strategy of synthesis that can be followed using enzymes as catalysts, the thermodynamically controlled one, requires moderately high concentrations of organic solvents and, therefore, very stable derivatives [1–6].

Although the conditions of the reaction medium may be very mild, the overall design of the kinetically controlled synthesis may be much more complex than the thermodynamically controlled one [7–15]. Firstly, it requires the use of an activated acyl donor (as amide or ester) that allows the formation of the acyl–enzyme complex. Secondly, three different reactions are possible: synthesis of the antibiotic (s), hydrolysis of the ester (h_1) and hydrolysis of the antibiotic (h_2). All these reactions determine the maximum yield. Thirdly, h_1 and h_2 can be different if the nucleophile is absorbed or not to the active centre of the enzyme. Fourthly, the yields of synthesis are not stable from a thermodynamic point of view and the synthetic products are potential substrates of the enzyme. Therefore, transient 'maximum yields' are usually obtained using this strategy.

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The maximum yield is controlled by three different parameters:

1.–Degree of saturation of the active centre of the enzyme by the nucleophile. Only when the nucleophile is absorbed onto the active centre of the enzyme is the transferase reaction possible [12].

2.–Maximum ratio (rate of synthesis/rate of hydrolysis of the ester) (maximum $R_{s/h1}$). Proportion of the molecules of enzyme with the nucleophile absorbed in their active centre that yield the synthetic reaction. The experimental value obtained depends on the degree of saturation, the maximum $R_{s/h1}$ is only obtained when the enzyme is fully saturated with nucleophile.

3.–The ratio ('Rate of synthesis'/'rate of hydrolysis of product') ($R_{s/h2}$). The experimental value for this ratio may be dependant on the relative concentration of the different substrates and products (the nucleophile can affect the hydrolysis of the antibiotic, the ester may be an inhibitor of this reaction, ..)

The design of these reactions is very complex because synthetic yields depend on the catalyst properties, on the reaction media and on the catalyst–reaction media interaction. Partial studies performed using yields at fixed times could not reach an adequate understanding of the reaction.

At a first glance, the use of stable derivatives does not seem a critical point in the design of this type of reaction. However, the maintenance of these properties for prolonged periods would be critical to perform an adequate design of the reactor because the yields are determined by the enzyme properties. Slight conformational changes in the enzyme structure, without clear effects in the enzymic activity in simpler reactions (e.g. hydrolysis of simple substrates) could produce important changes in the enzyme behaviour as catalyst of this type of processes, changing not only the rate of reaction, but also the maximum yields (this problem does not exist in thermodynamically controlled synthesis or hydrolytic reactions). Also, the resistance towards distorting agents have allowed in some cases the use the positive effects of some changes in the reaction media to improve the yields by avoiding negative distortions of the enzyme structure [16].

In this paper, using the kinetically controlled synthesis of semi-synthetic antibiotics by penicillin G acylase (PGA) as a model, different enzyme derivatives (different source, different immobilisation conditions) have been assayed as catalysts of the kinetically controlled synthesis of antibiotics.

2. Materials and methods

2.1. Materials

Cross-linked 6% agarose gels and their glyoxyl derivatives were donated by Hispanagar S.A. (Fax. No. (+34-47)200328, Burgos, Spain). Semi-purified extracts of PGA from *E. coli* (with a 25% degree of purity) were donated by Boehringer Mannheim GmbH (Penzberg, Germany) and semi-purified extracts of PGA from *Kluivera citrophila* (with a 25% degree of purity) were donated by Antibioticos S.A. (Leon, Spain). Glyoxyl agarose CL 6B containing 70 μmol glyoxyl groups/ml support was prepared by Hispanagar S.A. as previously described [17]. Further control of the PGA (amine)–agarose (aldehyde) (PA) preparation was performed as previously reported [18,19]. Properties of this enzyme derivatives are presented in Table 1. 7-Amino cephalosporanic acid (7ACA) and penicillin sulfoxide were kindly donated by CIPAN S.A.R.L. (Lisbon, Portugal). All other reagents and substrates were from Sigma Chemical Co. (St. Louis, MO, USA). Organic cosolvents were analytical grade.

2.2. Synthesis of α-hydroxy antibiotics

The synthesis of these types of antibiotics was chosen as model because of the good solubility of the mandelic acid and the methyl mandelate. 20 ml of different solutions containing 50 mM of 6-amino penicillanic acid (6APA) or 7ACA and 25 mM of D-methyl mandelate in 50 mM phosphate

Table 1
Properties of the different enzyme derivatives used in this work. Penicillin G acylases from *Escherichia coli* (E derivatives) or *Kluivera citrophila* (K1 derivative) were immobilised in glyoxyl agarose beads as previously described [18,19]. E1 and K1 derivatives were immobilised using CL-6-glyoxyl agarose, with 75 μmol glyoxyl groups per ml of support. E0 derivatives were prepared using supports with only 2.5 μmol glyoxyl groups per ml of support. Except when E1 − was prepared, the enzyme was immobilised in presence of 3 mg/ml of penicillin sulfoxide. Residual activity is the percentage of activity preserved during the immobilisation process. Relative activity is the ratio 'stability of enzyme derivative'/'stability of soluble enzyme from *E. coli*'. Esterolytic activity was assayed as described in Methods

	PA derivative			
	E0	E1 +	E1 −	K1
Esterolytic activity	71	1065	630	870
Residual activity	100	100	60	100
Relative stability	1	1000	2000	5000

buffer were cooled to 4°C in a jacket beaker connected to a thermoregulated water-bath. The use of an excess of nucleophile allowed us to keep close levels of saturation of the enzyme by the nucleophile during the first steps of the reaction, increasing the precision in the evaluation of $R_{s/h1}$. When the desired temperature was reached, the pH was adjusted to 7, 1 ml of the solution was removed as a blank and the reaction was started by adding different volumes of the different PA derivatives. pH was monitored throughout. Periodically, samples were removed and substrates and products were identified and analyzed by HPLC using a Konic Instruments (San Cugat, Spain) solvent delivery system with a Spectra Physics SP 8450 detector and a 250 × 4.6 mm RP-18 (5 μm) column (Spherisorb). Samples were eluted isocratically with 30% MeOH (v/v) in 0.067 M sodium phosphate, final pH 3, with a flow rate of 1.2 ml/min. The amounts of reactants were determined from calibration curves using stock solutions. $R_{s/h1}$ values were calculated when less than 15% of the initial concentration of ester has been consumed. Yields are given as the percentage of ester transformed to antibiotic.

One unit of activity was expressed as the amount of enzyme that produces 1 μmol of antibiotic or free acid per hour. In order to compare different enzymes preparations directly, the activity of these derivatives was divided by their activities with *D*-methyl mandelate at pH 7 and 4°C, multiplied by 100. Thus, we have used 'relative activity values'. For the same purpose, reaction courses obtained using different PA derivatives have been standardised, supposing that all the enzyme derivatives have the activity towards D-methyl mandelate that would be obtained using 0.2 ml of the E1 + derivative per experiment.

3. Results

3.1. Synthesis of α-hydroxy penicillin

The standardised reaction-time courses of the kinetically controlled synthesis of α-hydroxy penicillin using the different derivatives described in the introduction are shown in Fig. 1, Fig. 2, Fig. 3, Fig. 4. Comparing these reaction courses, a very different behaviour of the different enzyme derivatives can be easily detected, except between both E1 derivatives.

The direct study of the reaction courses may be too complex to reach a deep enough understanding of what is occurring during the reaction. Therefore, some of the kinetic parameters that determined the maximum yields are included in Table 2.

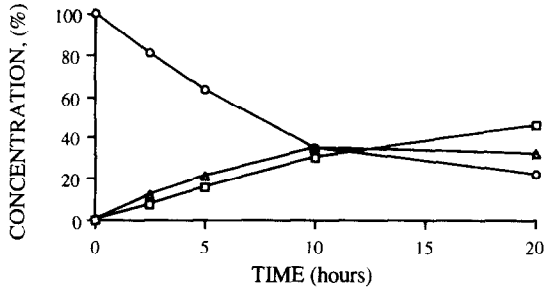

Fig. 1. Synthesis of α-hydroxy penicillin catalysed by E0 derivative. E0 derivative properties are described in Table 1. The reaction course has been standardised supposing that E0 derivatives have the same hydrolytic activity in absence of nucleophile as E1 + derivatives. Experiments were carried out as described in methods. Concentrations of products and substrate are given as percentage with regard to the initial ester concentration. Circles: ester, triangles: antibiotic, squares: acid.

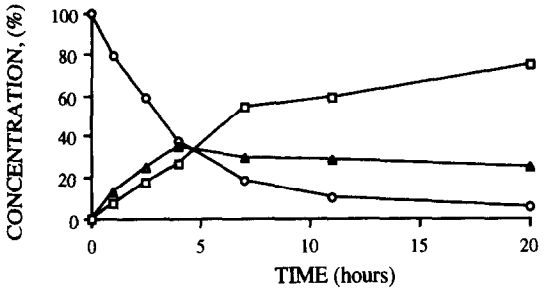

Fig. 2. Synthesis of α-hydroxy penicillin catalysed by E1 + derivative. E1 + derivative properties are described in Table 1. Experiments were carried out as described in Methods. Concentrations of products and substrate are given as percentage with regard to the initial ester concentration. Circles: ester, triangles: antibiotic, squares: acid.

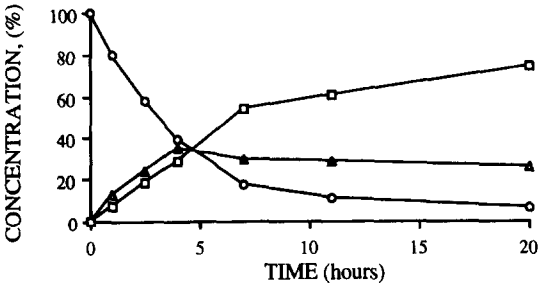

Fig. 3. Synthesis of α-hydroxy penicillin catalysed by E1 − derivative. E1 − derivative properties are described in Table 1. The reaction course has been standardised supposing that E1 − derivative has the same hydrolytic activity in absence of nucleophile as E1 + derivative. Experiments were carried out as described in Methods. Concentrations of products and substrate are given as percentages with regard to the initial ester concentration. Circles: ester, triangles: antibiotic, squares: acid.

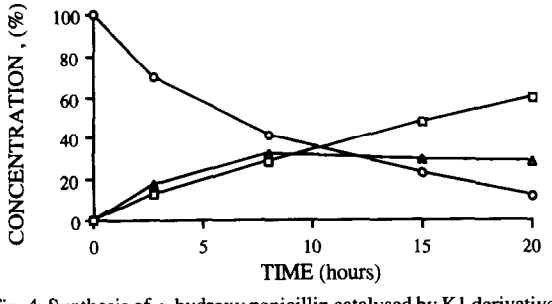

Fig. 4. Synthesis of α-hydroxy penicillin catalysed by K1 derivative. K1 derivative properties are described in Table 1. The reaction course has been standardised supposing that K1 derivative has the same hydrolytic activity in absence of nucleophile as E1 + derivative. Experiments were carried out as described in Methods. Concentrations of products and substrate are given as percentages with regard to the initial ester concentration. Circles: ester, triangles: antibiotic, squares: acid.

All derivatives E have the same $R_{s/h1}$ value (around 1.7). Using the three derivatives, the rate of synthesis and hydrolysis of the antibiotic seems to reach similar values when around 62% of the initial ester and 31% of the initial antibiotic nucleus have been consumed, given a maximum yield of around 35%. The theoretical maximum yield at this moment (considering that the degree of saturation of the enzyme by the nucleophile remained close to the initial level during all the reaction time and that h_2 was 0) was around 39%. Therefore, the previous average value of h_2 seemed to be around 10% of the synthetic activity during the initial steps of the synthetic process. However, the decrease in the overall rate of ester consumption promoted a significant decrease of the synthetic activity, suggesting a strong competence between the ester and the antibiotic by the active centre of the enzyme. The consequence of this was that h_2 became higher than s, and the concentration of antibiotic started decreasing when a high percentage of the ester remained in the reaction mixture.

Therefore, the kinetically controlled synthesis of α-hydroxy penicillin catalysed by penicillin G acylase is very complex, and it is going to depend on a delicate balance of competence by the active centre of the enzyme among the different substrates and products. However, all the E derivatives gave the same maximum yields. This fact suggests that the properties of the enzyme have been fairly preserved during the multipoint interactions between the enzyme and the support. The only difference among these derivatives was that the activity of E1 derivatives were less decreased by the presence of the 6APA (E1 derivatives have

Table 2
Behaviour of the different PA derivatives as catalysts of kinetically controlled synthesis of α-hydroxy penicillin. Experimental conditions: 50 mM phosphate pH 7, 4°C, 50 mM 6APA and 25 mM D-methyl mandelate. Other specifications as described in Methods

Derivative	Initial relative rates			$R_{s/h1}$
	Ester consumption	Hydrolysis	Synthesis	
E0	14	5	9	1.7
E1+	32	12	20	1.7
E1−	30	11	19	1.7
K1	23	10	13	1.3

around 30% of the activity in absence of nucleophile, E0 derivatives only 14%). This antibiotic nucleus has been described as a mixed inhibitor of penicillin G acylase in the hydrolysis of esters [12]. Apparently, the inhibition promoted by 6APA may be partially founded upon the promotion of a conformational change in the PA, and the increase of enzyme rigidity promoted by the multipoint covalent attachment allowed to preserve higher levels of activity.

Using PA from *K. citrophila*, the $R_{s/h1}$ was lower than using E derivatives (around 1.3). The maximum yield reached was also a little lower than using E derivatives (31–32%). Again, h_2 and s seemed to reach similar values when around 40–41% of the ester left. This means that the maximum theoretical yield at that moment was around 34–35%, suggesting that the value of h_2 compared with the value of s given by this derivative was close to that of E derivatives. However, this derivative was more strongly inhibited by 6APA than by E1 derivatives: the synthetic activity was 65% of that of the E1 derivatives.

3.2. *Effect of the presence of methanol*

Kasche has described a positive effect of methanol on the yields of synthesis of semi-synthetic antibiotics following a kinetically controlled strategy [20]. For this reason, the effect of this alcohol has been studied when different enzyme derivatives were employed.

Table 3 shows some of the results obtained. With all derivatives, the $R_{s/h1}$ increased in the presence of 20% methanol. However, differences were observed for each enzyme derivative.

The effect of the rigidity of the enzyme derivative can be checked by comparing the results obtained using E1 and E0 derivatives. Methanol decreased the synthetic activity of the E0 derivative (now, it is 66% of that obtained in absence of methanol) while the multipoint covalently attached derivative had a lower decrease in activity (only 10% of the synthetic activity was lost using E1 derivative). This suggested that the decrease in the enzyme activity caused by methanol can be, at least partially, promoted by deleterious conformational changes in the PGA structure, and stabilised derivatives were more resistant to the changes in the reaction media. However, $R_{s/h1}$ values and the synthetic yields obtained with E1 + and E0 derivatives were very close to each other (around 50%).

Table 3
Effect of 20% methanol in the behaviour of the different PA derivatives as catalysts of kinetically controlled synthesis of α-hydroxy penicillin. Experimental conditions: 20% methanol in 50 mM phosphate pH 7, 4°C, 50 mM 6APA and 25 mM D-methyl mandelate. Other specifications as described in Methods

Derivative	Relative rates			$R_{s/h1}$
	Ester consumption	Hydrolysis	Synthesis	
E0	8.5	2.5	6	2.4
E1 +	25	7	18	2.4
E1 −	26	9	17	1.9
K1	19	6	13	2.3

When E1 − derivatives were studied, it was found that the overall rate of the reaction was more preserved but the $R_{s/h1}$ obtained was lower than when using E0 or E1 + derivatives. This enzyme derivative was partially distorted during the multipoint covalent attachment (activity against penicillin G decreased by a 40%) and it is more stable than E1 derivative (therefore, we can assume that it is more rigid) [18,21]. The results seem to suggest that this more 'rigid' derivative was more resistant to the deleterious effect of methanol on the enzyme structure. To explain the lower effect of methanol on the E1 − derivative, the exact mechanism of this cosolvent should be determined. If the main effect of methanol is to induce a conformational change in the enzyme structure, the higher rigidity of this derivative may avoid this theoretical positive conformational change as well as the negative. If the main mechanism was the substrate recycling, as proposed by V. Kasche, a lower affinity for the methanol produced by the distortions during the enzyme immobilisation might explain the results. The conformational change induced by methanol is a more likely theory because this can also explain the higher resistance to methanol of E1 derivatives. Anyway,

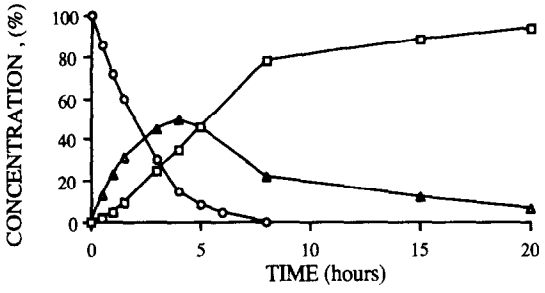

Fig. 5. Behaviour of E1+ derivative using 7ACA as nucleophile. E1+ derivative properties are described in Table 1. Experiments were carried out as described in methods. Concentrations of products and substrate are given as percentages with regard to the initial ester concentration. Circles: ester, triangles: antibiotic, squares: acid.

lower synthetic yields were obtained using this derivative (47–48%).

K1 synthetic activity remained unalterable in presence of methanol, while the hydrolytic activity decreased significantly. Thus, the $R_{s/h1}$ and synthetic yields were now very similar to those obtained with E1 derivatives, although the synthetic activity was 70% of that of E1 derivatives.

3.3. Use of 7ACA as nucleophile

7ACA has been assayed as substrate of this type of synthesis for the different derivatives. Fig. 5 shows the reaction course using E1+ derivative. A very high reaction rate was observed (the initial consumption of ester was around 3 times higher than using 6APA). The maximum yield was reached when 27% of the initial ester was left and its value was around 50% (showing that the s value is greater than the h_2 value when lower ester concentration is presented when compared to the use of 6APA). Very rapid hydrolysis of the antibiotic was observed after the maximum yield was reached. Table 4 shows some of the kinetic values reached with the different parameters. Very high $R_{s/h1}$ values were found with all the derivatives. E derivatives had $R_{s/h1}$ values over 7, very similar in all of them. This means that the maximum theoretical yield after 73% ester consumption ranged between 63 and 66%, and suggests that the average of h_2 had been around 15% of the average of s. Therefore, different balance of enzyme inhibitions seemed to be obtained when 7ACA is used.

Table 4
Effect of the nucleophile in the behaviour of the different PA derivatives as catalyst of kinetically controlled synthesis. Experimental conditions: 50 mM phosphate pH 7, 4°C, 50 mM 7ACA and 25 mM D-methyl mandelate. Other specifications as described in Methods

Derivative	Relative rates			$R_{s/h1}$
	Ester consumption	Hydrolysis	Synthesis	
E0	18	2	16	8
E1+	100	12	88	7.3
E1−	103	12.5	90.5	7.2
K1	33.5	5.5	28	5.5

Under these conditions, 7ACA did not have any effect on the rate of consumption of ester remaining when E1 derivatives were employed, and the values of initial synthetic activity reached were very high (next to the hydrolytic one in absence of nucleophile). However, E0 derivatives presented activities (synthetic and hydrolytic ones) of around 20% of those of the stabilised derivatives. Again, the simplest explanation is that the increase in the enzyme rigidity is able to prevent undesired distortions of the enzyme structure.

K1 derivatives gave better $R_{s/h1}$ value with 7ACA than with 6APA, but it was lower than using E derivatives (the $R_{s/h1}$ obtained was 5). The effect of 7ACA on K1 derivative activity compared to that of the E1 derivatives was significantly higher than that promoted by 6APA. In this case, its synthetic activity was 30% of that of E1 derivatives.

4. Conclusions

The very significant importance of the enzyme derivative in the kinetically controlled synthesis has been clearly demonstrated in this paper. Thus, the extrapolation of results obtained with one enzyme preparation to other enzyme preparations does not seem possible.

The use of rigid enzyme derivatives can greatly improve the design of this process. For example, the use of E1 derivatives allowed to keep higher levels of activity in the presence of high antibiotic nucleus concentrations. Also, these derivatives

lost less activity in the presence of methanol.

The multipoint covalent attachment of the PGA to glyoxyl agarose gels seems to preserve almost unaltered the properties of the active centre of the enzyme, but the increase in the enzyme rigidity allows it to prevent undesired changes that can affect the enzyme activity.

Opposite to the results obtained in the thermodynamically controlled synthesis [22], enzyme derivatives of PGA from E. coli seem to have better properties than PGA from K. citrophila as catalysts of synthesis of semi-synthetic antibiotics following a kinetically controlled strategy.

Therefore, E1+ derivatives appeared to be very suitable derivatives for catalysing these reactions: they gave the highest activity, the best $R_{s/hl}$ and a resistance to the effect of methanol on the enzyme activity.

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