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Penicillin acylase-catalyzed synthesis of ampicillin in ''aqueous solution–precipitate'' systems. High substrate concentration and supersaturation effect

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Abstract

Penicillin acylase-catalyzed ampicillin synthesis via acyl group transfer in aqueous solution is highly dependent on the initial substrate concentration. The solubility of one substrate, 6-aminopenicillanic acid (6-APA), can be advantageously enhanced by the presence of acyl donor, the second substrate. Furthermore, a comparison of enzymatic synthesis in homogeneous solution with synthesis in a heterogeneous system having partially undissolved reactants, reveals major advantages for the latter approach. In this ''aqueous solution–precipitate'' system, accumulation of both products, ampicillin and D-(−)-phenylglycine, proceeds through the formation of their supersaturated solutions. Subsequent precipitation of the product ampicillin positively influences the efficiency of the biocatalytic process. As a result, ampicillin synthesis proceeds in 93% conversion on 6-APA and in 60% conversion on D-(−)-phenylglycine methyl ester.

Keywords: Ampicillin synthesis; Penicillin acylase; Aqueous solution–precipitate; Supersaturation

1. Introduction

Starting from the first report on the reversibility of penicillin acylase-catalyzed hydrolysis of benzylpenicillin [1], the enzymatic synthesis of semi-synthetic antibiotics has been studied extensively. It has been shown that the specificity of penicillin acylase for the acyl donor is not limited to phenylacetic acid alone but encompasses a range of acyl donors, including derivatives of D-(−)-phenylglycine (PG). In principle, this latter feature makes the enzyme capable of synthesizing a wide range of semi-synthetic antibiotics [2–7], all of which contain substituted glycines as side chain moiety. Unfortunately, thermodynamic studies have demonstrated that the equilibrium towards synthesis is highly unfavorable for β-lactam antibiotics bearing an α-amino acid as side chain [8]. Consequently, the condensation of, e.g. PG and

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a β-lactam nucleus cannot be performed efficiently [4,9,10]. Therefore, the efficient synthesis of antibiotics with an α-amino acid side chain can only be accomplished via enzymatic acyl group transfer, i.e. from an activated side chain donor. But in contrast to the direct condensation of the free acid and nucleus, where product gradually accumulates until equilibrium is reached, acyl transfer from donor to acceptor is governed by both kinetics and thermodynamics, and the product accumulation curve shows a clear-cut maximum [11]. In acyl transfer reactions, water acts as a competing nucleophile (Fig. 1), and the acyl donor is partially hydrolyzed in the course of the biocatalytic process. Moreover, hydrolysis of the acyl group transfer product is thermodynamically favorable, and finally, the overall hydrolysis exceeds acyl transfer to 6-aminopenicillanic acid (6-APA). The resulting loss of acyl donor is a major drawback in the industrial application of enzymatic antibiotic synthesis by acyl transfer [12].

Detailed kinetic analyses have revealed quantitative regularities of the enzymatic acyl transfer reactions [13,14], but the impact on the penicillin acylase-mediated synthesis of PG-derived penicillins and cephalosporins has been quite limited. A multitude of strategies directed at improvement of the efficiency of the synthetic reaction have been undertaken:

- Optimization of pH, ionic strength and temperature [15–17]
- Use of an excess of acyl donor with respect to the nucleophile [3]
- Use of cosolvents [18,19]
- Regulation of the thermodynamic water activity [17]
- Regeneration of the acyl donor using methanol as an additional nucleophile [20]
- Application of aqueous two-phase systems for in situ extraction of the reaction product [21]
- Synthesis in frozen solution [22]
- Microenvironmental design of the enzyme active site [23]
- Changing the catalytic properties of penicillin acylase by chemical modification and immobilization [24,25]
- Decreasing the solubility of the product by formation of inclusion complexes [12]
- High reactant concentrations [26,27]

Here, we present our results on the penicillin acylase-catalyzed synthesis of ampicillin in aqueous medium at increasing substrate concen-

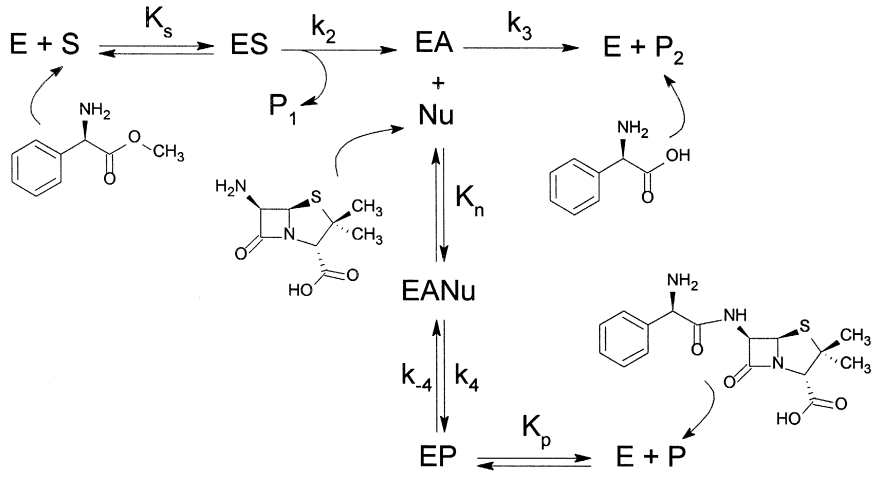

Fig. 1. ''Minimal'' kinetic scheme of penicillin acylase-catalyzed ampicillin synthesis. E: penicillin acylase, S: substrate (D-(−)-phenylglycine amide, PGA), Nu: nucleophile (6-APA), ES and EP: enzyme–substrate and enzyme–ampicillin complexes, EA and EANu: acyl–enzyme and acyl–enzyme–nucleophile complexes, P_1: methanol, P_2: PG, P: ampicillin.

trations. Consideration of the solubilities of the reaction components led us towards a suspension system in which only a fraction of the reaction components is in solution. The merits of this dynamic ''aqueous solution–precipitate'' system in comparison with a homogeneous system are discussed.

2. Materials and methods

2.1. Materials

Penicillin acylase from *Escherichia coli* (ATCC 11105), 6-APA, ampicillin, PG, PGA, and D-(−)-phenylglycine methyl ester (PGM) were from DSM (The Netherlands). The enzyme active site concentration (3×10^4 M) was determined by titration using phenylmethylsulfonyl fluoride as described earlier [28]. Other chemicals and buffer components were from Merck, Darmstadt, Germany. Organic solvents were of analytical grade.

2.2. Analysis

Samples were analyzed by HPLC using a Waters M6000 pump, a 4.6×150 mm^2 Nucleosil C-18 column and a Shimadzu SPD-(−)-6A UV detector at 225 nm. The eluent was prepared by adjusting the pH of a 0.68 g/l solution of KH_2PO_4 in acetonitrile/water (30:70, v/v) containing 0.68 g/l sodium dodecylsulphate to 3.0 with phosphoric acid. The flow rate was 1.0 ml/min.

2.3. Solubility measurements

The solubilities of individual compounds and their mixtures were determined by stirring a corresponding suspension during 1 h in a thermostated 10 ml cell of a pH-stat (Radiometer RTS-622, Copenhagen, Denmark) at 25°C and appropriate pH, kept constant by automated titration using sulfuric acid or sodium hydroxide as titrants unless otherwise stated; the suspensions were centrifuged and the supernatants were diluted with eluent before analysis.

2.4. Ampicillin synthesis

Reactions were carried out in a thermostated cell of a pH-stat (Radiometer RTS-622, Copenhagen, Denmark or Titrino 718, Metrohm, Switzerland) at 25°C under permanent stirring. After adding the appropriate amounts of PGM and 6-APA to 10 ml of water and incubation in a thermostated cell during 20 min at pH 6.3, the reaction was started by adding 100 μl of penicillin acylase solution to the reaction mixture; the pH was kept constant by automatic titration. Samples of 20–100 μl were taken from the reaction mixture, added to the corresponding amount of eluent (980–900 μl) in order to dilute the sample and to stop enzymatic reaction, and subjected to HPLC analysis. In case of heterogeneous systems, two samples were analyzed: one was removed by Chromafil 0.45 μm filter (Bester, Amstelveen, The Netherlands) in order to separate solids from the solution, and the other aliquot of the heterogeneous system was taken for characterization of the whole reaction mixture.

3. Results and discussion

In order to provide a basis for understanding the results of subsequent coupling experiments, we first measured the solubility of the individual reaction components over the relevant pH range 4–8 (Figs. 2 and 3). Ampicillin appeared to be the poorest soluble compound at pH values lower than 7.3, whereas at higher pH, its solubility surpasses the solubility of the second lowest soluble component, PG. The solubility of PGA was in contrast to the ester dependent on the titrant used: its solubility was nearly pH-independent when using hydrochloric acid, but in case of sulfuric acid, the solubility of PGA was similar to the ester and decreased from pH 5–8 (Fig. 2). It should be taken into account that this

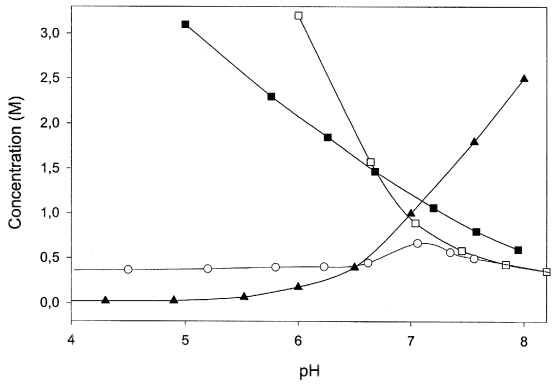

Fig. 2. pH dependence of the solubility of 6-APA (▲), PGM (■) and PGA using sulfuric (□) and hydrochloric (○) acid as titrant at 25°C.

pH effect is mainly due to the diminishing fraction of the protonated form of the molecules. The effect on the concentration of the important reactive species, the neutral molecule [4], is believed to be only minor. From these observations, several conclusions may be drawn:

- Precipitation of PG is nearly inevitable in the course of enzymatic ampicillin synthesis starting from high initial substrate concentrations at any pH
- Ampicillin precipitation can be effective only below pH 7.5
- The high solubility of both investigated acyl donors, PGM and PGA, compared to the low solubility of 6-APA, provides a good possibility to have an excess of the acylating agent over the nucleophile at pH values below 7.0

Since the solubility of 6-APA increases substantially above pH 6.5, the efficiency of the acyl group transfer to 6-APA will increase with 6-APA concentration. At lower pH, practical systems can be envisaged with partially undissolved 6-APA.

On the other hand, basic conditions will retard the precipitation of ampicillin and, in this way, contribute to its hydrolysis as well as to the degradation of the β-lactam system. Hence, the pH for the biocatalytic process should be chosen at maximum nucleophile reactivity of 6-APA but below the value at which the solubility as well as degradation of ampicillin start to quench effective synthesis. From an inspection of Figs. 2 and 3, it was concluded that optimum conditions could be in the pH range 6–7.

As the solubility of the reactants can be different in complex mixtures, we also measured the influence of considerable (up to 0.8 M) concentrations of the side chain donors on the solubility of 6-APA as well as ampicillin. The solubility of ampicillin nearly doubled in the presence of 0.8 M PGM but the effect of the amide was less (Fig. 4). The solubility of 6-APA also increased in the presence of the acyl donors (Fig. 5), which should positively affect the effi-

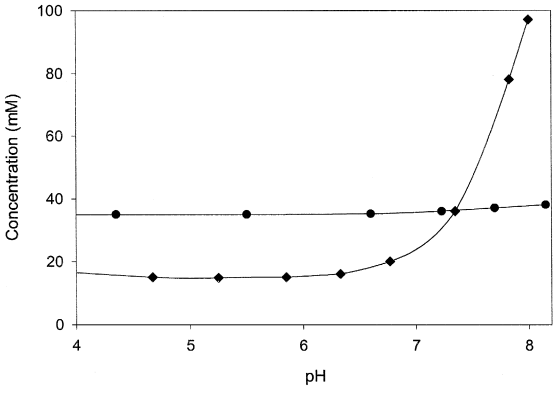

Fig. 3. Solubility of ampicillin (♦) and PG (●) as function of pH at 25°C.

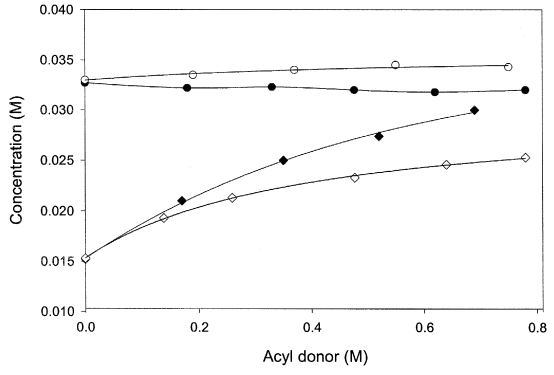

Fig. 4. Solubility of ampicillin (diamonds) and PG (circles) as function of the concentration PGM (filled symbols) and PGA (open symbols) at pH 6.3 and 25°C.

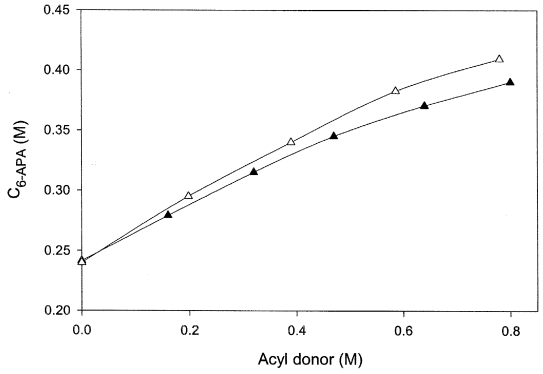

Fig. 5. Solubility of 6-APA as function of the concentration PGM (▲) and PGA (△) at pH 6.3 and 25°C.

ciency of acyl transfer to the β-lactam nucleus. This solubility effect increases with pH (Fig. 6). PGA and PGM affected the solubility of 6-APA to approximately the same extent. The solubility of PG was hardly affected by the pH or the presence of its ester or amide. Moreover, due to its weak binding at the active site of penicillin acylase [29], no effect on the course of the reaction would be expected.

In order to understand the biocatalytic ampicillin synthesis in heterogeneous systems, we carried out enzymatic conversions at increasing initial substrate concentrations. First, we studied the influence of product precipitation. To this end, we compared enzymatic ampicillin synthesis in a system with only one precipitating product, PG (Fig. 7), with a system where both reaction products precipitated (Fig. 8). In both

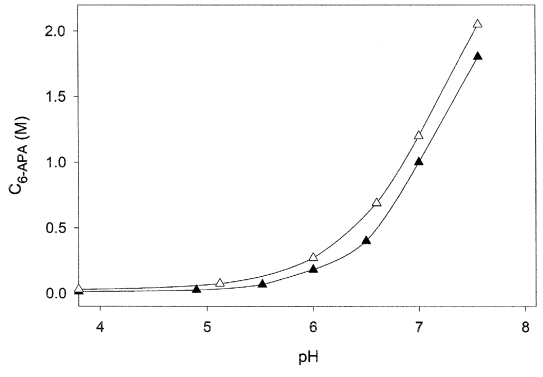

Fig. 6. Solubility of 6-APA at 25°C (▲) and in the presence (△) of 500 mM PGM as function of the pH.

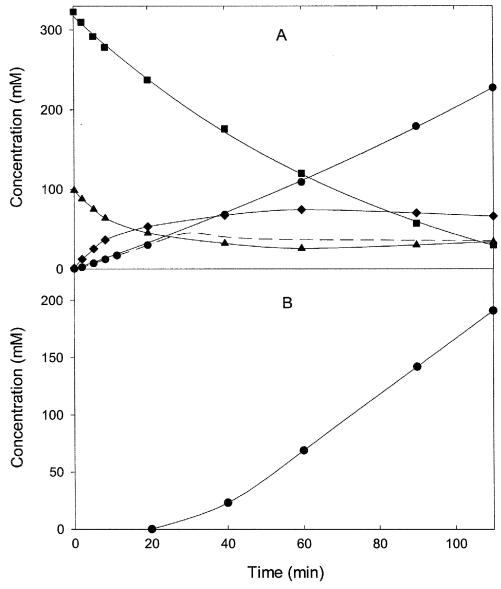

Fig. 7. Penicillin acylase-catalyzed ampicillin synthesis at 25°C and pH 6.3 from a homogeneous solution of 100 mM 6-APA and 500 mM PGM with precipitation of only PG during the process. (A) Total concentration in reaction mixture; concentrations of the components in the solution are presented by dotted lines. (B) Concentration in the precipitate. PGM (■), 6-APA (▲), ampicillin (♦), PG (●).

cases, reactions were started under homogeneous conditions at pH 6.3. As can be seen from Fig. 7, the maximum accumulated ampicillin concentration does not reach saturation at low nucleophile concentration (100 mM). Under these conditions, acyl transfer to 6-APA was far from optimum, and in spite of a 3.2-fold excess of side chain donor over the antibiotic nucleus, only 75% conversion of 6-APA to ampicillin was reached and only 25% of the side chain donor was converted to ampicillin. When the reaction was carried out under the same conditions with a 6-fold molar excess of side chain donor, the conversion of the nucleophile did reach 90%, but only 15% of the donor was converted into ampicillin; the surplus was hydrolyzed to PG (data not shown).

When the initial 6-APA concentration was increased to saturation (280 mM), the efficiency of the enzymatic acyl transfer improved significantly (Fig. 8) and both reaction products, ampi-

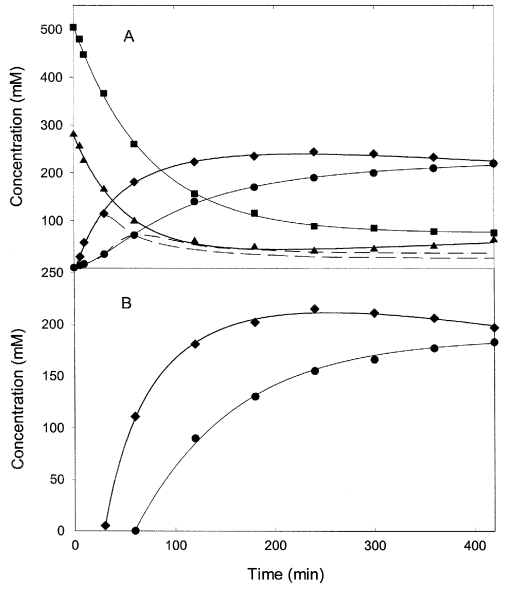

Fig. 8. Penicillin acylase-catalyzed ampicillin synthesis at 25°C and pH 6.3 from a homogeneous solution of 300 mM 6-APA and 500 mM PGM with precipitation of ampicillin and PG during the process. (A) Total concentration in reaction mixture; concentrations of the components in the solution are presented by dotted lines. (B) Concentration in the precipitate. PGM (■), 6-APA (▲), ampicillin (♦), PG (●).

cillin and PG, precipitated from the reaction mixture. Final conversions of 87% on 6-APA and 47% on side chain donor at the maximum of the ampicillin accumulation curve were reached in this way. When we monitored the composition of the heterogeneous reaction mixture, as well as the composition of reactants in solution, we noted that precipitation of the products proceeded via supersaturation (Fig. 8): ampicillin precipitated first and was followed by PG. We observed that the ampicillin supersaturation was reduced substantially when that of PG reached an optimum. Furthermore, we observed that the precipitation of ampicillin influenced the progress curve of PG accumulation, which shows a typical S-shaped form (Fig. 8A) in contrast to the dependence observed in a system without precipitation of ampicillin (Fig. 7). The supersaturation of PG and its consequent precipitation did not influence the formation of ampicillin, as could be expected. Based on the data presented above, we can conclude that a continuous maximum 6-APA concentration in the solution and low solubility of the product ampicillin are extremely important for an effective acyl transfer process.

Thus, a further improvement of the enzymatic ampicillin synthesis could be achieved by starting from a suspension of 6-APA (Fig. 9). This regime allows for a maximum 6-APA concentration at saturation value and consequently maintains optimal acyl transfer efficiency for a longer period. As can be judged from Fig. 9B, the composition of the solid phase shows a noteworthy dynamic character, which results in formation of pure, crystalline ampicillin during the period between exhaustion of solid 6-APA ($t = 70$ min) and precipitation of PG ($t = 110$ min). As the catalyst is present in a soluble form, this material can be recovered by simple

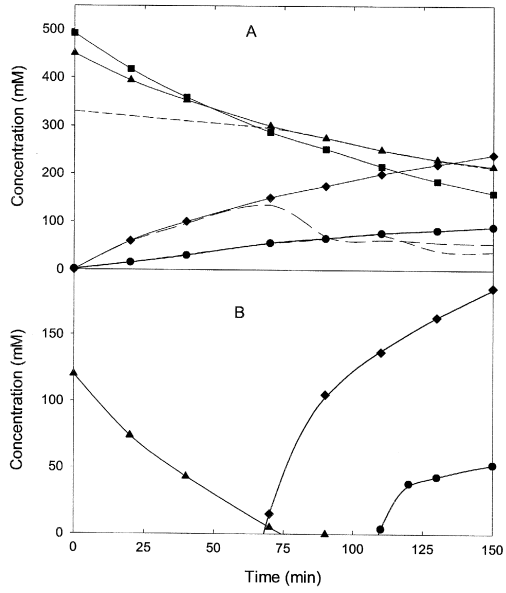

Fig. 9. Penicillin acylase-catalyzed ampicillin synthesis at 25°C and pH 6.3 from a heterogeneous solution of 450 mM 6-APA and 500 mM PGM with precipitation of ampicillin and PG during the process. (A) Total concentration in reaction mixture; concentrations of the components in the solution are presented by dotted lines. (B) Concentration in the precipitate. PGM (■), 6-APA (▲), ampicillin (♦), PG (●).

filtration.[1] After exhaustion of solid 6-APA, a situation like the one depicted in Fig. 8 develops, in which the nucleophile concentration decreases.

The above results led us to combine both regimes by adding additional PGM (200 mM) to the reaction mixture. At maximum accumulation of ampicillin, the heterogeneous reaction mixture consisted of 420 mM ampicillin, 200 mM PG, 30 mM 6-APA and 70 mM PGM. For ampicillin, this is equivalent to a conversion of 93% on 6-APA (starting concentration 450 mM) and 60% on PGM (total amount added 690 mM).

We conclude that the dynamic ''aqueous solution–precipitate'' system presented here is a practical concept for efficient enzymatic ampicillin synthesis. Enhanced solubility of 6-APA in the starting solution due to the presence of acyl donor and supersaturation phenomena in the course of ampicillin and PG formation are the most important factors for optimization of this heterogeneous biocatalytic process. These parameters will be accommodated in a kinetic model for enzymatic ampicillin synthesis and will be reported in subsequent publications.

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[1] When immobilized enzymes are used, clean separation between crystalline reaction products and biocatalyst can be achieved using sieving techniques [30].

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