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Short communication

Use of high acyl donor concentrations leads to penicillin acylase inactivation in the course of peptide synthesis

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Abstract

Enzyme inactivation has been observed in the course of penicillin acylase-catalyzed hydrolysis and aminolysis of D-phenylglycine amide. Inactivation was very sensitive to the D-phenylglycine amide concentration: at pH 9.5, 25 °C and 400 mM substrate, penicillin acylase lost more than 90% of its initial catalytic activity in half an hour, in the presence of 100 mM substrate, 50% of the initial activity in two hours, whereas in the absence of substrate, no significant enzyme inactivation was observed in three hours. Observed enzyme inactivation limits use of high acyl donor concentrations at penicillin acylase-catalyzed peptide synthesis.

Keywords: Penicillin acylase; Enzyme stability; Inactivation by substrate

1. Introduction

Penicillin acylase (penicillin amidohydrolase EC 3.5.1.11) from *Escherichia coli*, which is widely used in the manufacture of β-lactam antibiotics [1–3], is a very stable enzyme, which can run thousands of reaction cycles. It is, however, sensitive to pH, temperature, and organic solvents [4–8]. Penicillin acylase undergoes conformational changes above 35 °C as well as in the alkaline medium [9], which are followed by irreversible enzyme inactivation. An engineered penicillin acylase with altered surface charge (Trp431Arg) is slightly more stable at alkaline pH [10]. Numerous attempts to stabilize the enzyme by chemical modification have been reported [11–14], but there is a dearth of information regarding penicillin acylase stability under reaction conditions. The presence of 6-APA was shown to stabilize penicillin acylase against thermal inactivation at the pH-optimum of catalytic activity, while the addition of phenylacetic acid led to some destabilization: simultaneous addition of both 6-APA and phenylacetic acid resulted in a slight destabilization [15]. No significant differences in the thermal inactivation of soluble and immobilized penicillin acylase were observed in the presence of benzylpenicillin [16].

Recently we have shown that penicillin acylase can be used for effective synthesis of peptides containing non-conventional D-phenylglycyl residue [17]. In the course of these studies, we have observed that enzyme stability is very sensitive to the high acyl donor concentrations and evaluated different factors, which might be responsible for enzyme inactivation. Here, we report the results of our experimental study on the enzyme stability in the hydrolysis and aminolysis of D-phenylglycine amide.

2. Materials and methods

6-Nitro-3-phenylacetamidobenzoic acid (NIPAB) was obtained from Sigma; D-phenylglycine amide and D-phenylglycine were kindly donated by DSM, Geleen, The

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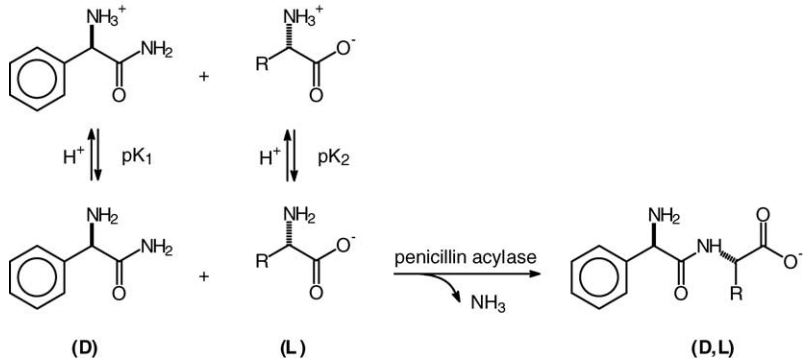

Fig. 1. Scheme of the penicillin acylase-catalyzed synthesis of D-phenylglycyl-peptides from D-phenylglycine amide as an acyl donor and amino acids as nucleophiles.

Netherlands; glycine was obtained from Aldrich. Other chemicals used were of analytical grade. Native (soluble) penicillin acylase from *E. coli* was a gift from DSM Gist, Delft, The Netherlands. The concentration of penicillin acylase active sites was determined by titration with phenylmethylsulfonylfluoride [18] and was equal to 2×10^{-4} M.

The stability of penicillin acylase was studied at different pH-values in a buffer free medium, in the hydrolysis of D-phenylglycine amide at different initial substrate concentrations and in the course of D-phenylglycyl transfer. A typical experiment was carried in a thermostated reaction vessel at 25 °C; the pH was kept constant by automatic titration. Aliquots of the reaction or incubation mixture (10–50 μM) were continuously taken (depending on the rate of enzyme inactivation) and the residual penicillin acylase activity was measured spectrophotometrically (on a Varian Cary 3 Bio spectrophotometer) in an independent experiment using the colorimetric substrate NIPAB (0.2 mM) in a 1 mL cuvette at the standard conditions (25 °C, 0.01 M phosphate buffer pH 7.5). The presence of the substrate (D-phenylglycine amide) as well as the reaction products (D-phenylglycine and ammonia) in the aliquot taken from the incubation mixture for the residual catalytic activity measurement did not interfere with the colorimetric assay due to the dilution and pH control as was demonstrated by an independent control experiment. The concentration of substrates and products in the course of penicillin acylase-catalyzed peptide synthesis was monitored by HPLC using a Waters 6000A pump, a Phenomenex Luna C18(2) column (250 mm × 4.6 mm, 5 μm) and a Waters M481 LC detector at 208 nm with 7 mM phosphate pH 3.0, containing 40% (v/v) acetonitrile and a 0.7 g/L of sodium dodecylsulphate, as the eluent. The flow rate was 0.5 mL/min. Retention times (in min): D-phenylglycine (5.34), D-phenylglycyl-glycine (8.12), D-phenylglycine amide (8.8).

3. Results and discussion

The object of this study was enzyme inactivation in the course of penicillin acylase-catalyzed peptide synthesis by enzymatic acyl transfer from D-phenylglycine amide to amino acids as external nucleophiles (Fig. 1). In order to be sure that this inactivation is not just a simple effect of pH, we have first investigated the stability of the enzyme in the appropriate pH interval 9.0–10.5 using 200 mM glycine as a buffer (Fig. 2). Penicillin acylase was stable for at least four hours at pH ≤9.0 (Fig. 2, curve 1), but lost more than 90% of its activity in one hour at pH 10.5 (Fig. 2, curve 4). As the pK_a values of the amino group of free amino acids are in the range 9.0–9.8, it seemed likely that a pH of 9.5 would be optimal for penicillin acylase-catalyzed peptide synthesis (Fig. 1), and therefore, we studied enzyme stability more carefully at this pH (Fig. 3). Indeed, penicillin acylase was quite stable at its incubation in a buffer free medium at pH 9.5 (Fig. 3, curve 1). We have then studied penicillin acylase stability in the presence of each substrate (glycine and D-phenylglycine amide). In the presence of 200 mM glycine, no significant enzyme inactivation was observed at pH 9.5 (Fig. 3, curve 5). In contrast, the penicillin acylase-catalyzed hydrolysis of D-phenylglycine amide at this pH was accompanied by a rapid inactivation (Fig. 3, curves 2–4). Inactivation was very sensitive to the substrate concentration: with 400 mM D-phenylglycine amide practically total inactivation was observed in half an hour, whereas with 100 mM substrate in two hours penicillin acylase retained more than 50% of its initial catalytic activity. It is noteworthy that the extent of in-

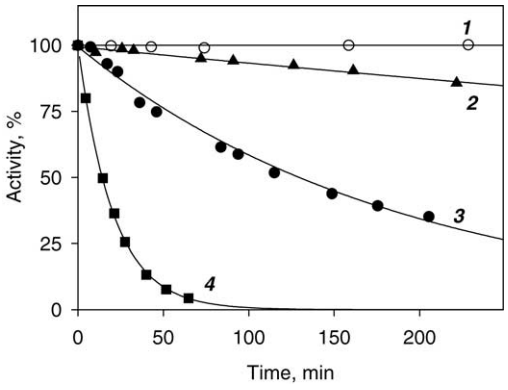

Fig. 2. Penicillin acylase stability in alkaline medium at 25 °C, 0.2 M glycine. (1) pH 9.0; (2) pH 9.5; (3) pH 10.0; and (4) pH 10.5.

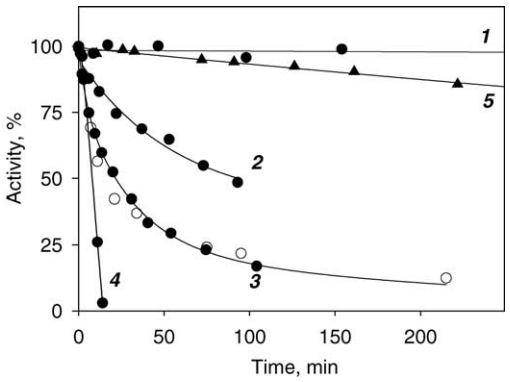

Fig. 3. Penicillin acylase stability (pH 9.5, 25 °C) in a buffer free medium (1), at the enzymatic hydrolysis of D-phenylglycine amide (2, 3, and 4: hydrolysis at 100, 200, and 400 mM D-phenylglycine amide concentration, respectively), in the course of enzymatic synthesis of D-phenylglycyl-glycine from 200 mM D-phenylglycine amide and 200 mM glycine (◯) close to curve 3) and in the presence of 200 mM glycine (5).

activation was roughly the same in D-phenylglycine amide hydrolysis and in the synthesis of D-phenylglycyl-glycine (Fig. 3, curve 3, open circles). The products of both enzymatic reactions (synthesis and hydrolysis) do not seem to be responsible for enzyme inactivation, since in the synthesis of D-phenylglycyl-glycine (Fig. 4) there was an accumulation of the new product up to 160 mM concentration, but no additional enzyme inactivation was observed (Fig. 3, curve 3). The influence of D-phenylglycine on the stability of the enzyme was studied in a separate experiment (data not shown). Penicillin acylase was just slightly inactivated at saturated D-phenylglycine concentration analogous to that observed in the presence of glycine (Fig. 3, curve 5), but this effect cannot explain inactivation of the enzyme in the course of D-phenylglycine amide hydrolysis. So, we can conclude that D-phenylglycine amide is a quite strong penicillin acylase inactivation agent in the alkaline medium, especially at its high

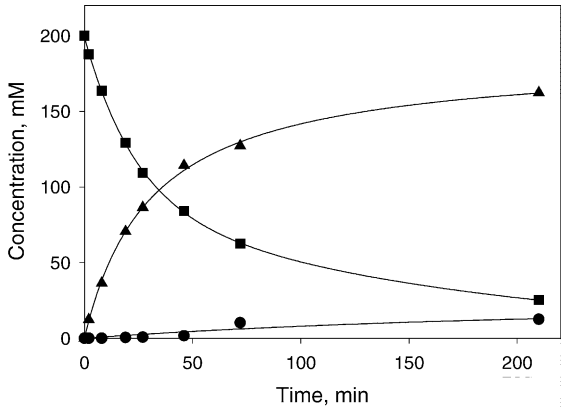

Fig. 4. Penicillin acylase-catalyzed synthesis of D-phenylglycyl-glycine from D-phenylglycine amide and glycine at equimolar (200 mM) concentrations of reagents (pH 9.5, 25 °C). (■) D-Phenylglycine amide; (▲) D-phenylglycyl-glycine; and (●) D-phenylglycine.

concentrations. This fact limits the use of high acyl donor concentrations in penicillin acylase-catalyzed synthesis of D-phenylglycyl-peptides. It should be mentioned that in the penicillin acylase-catalyzed synthesis of β-lactam antibiotics in neutral and slightly acidic reaction medium no remarkable enzyme inactivation was observed even in much more concentrated D-phenylglycine amide solutions (up to 1 M) [19]. It shows that while using penicillin acylase in alkaline medium, we should take into account an additional factor related to the enzyme inactivation at high substrate concentrations. Further studies are aimed at revealing the mechanistic details of this inactivation.

Acknowledgements

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