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THE PREPARATION AND KINETICS OF IMMOBILISED PENICILLIN AMIDASE FROM *ESCHERICHIA COLI*

D. WARBURTON*, K. BALASINGHAM**, P. DUNNILL AND M. D. LILLY

Biochemical engineering Section, Department of Chemical Engineering, University College London, Torrington Place, London, W.C.1. (Great Britain)

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SUMMARY

1. Penicillin amidase (penicillin amidohydrolase, EC 3.5.1.11) has been immobilised by covalent binding to DEAE-cellulose using 2-amino-4,6-dichloro-*s*-triazine.

2. The immobilised penicillin amidase preparations contained up to 160 mg protein/g. DEAE-cellulose and the bound enzyme retained 45–81% of its activity before attachment.

3. The kinetics of immobilised penicillin amidase were determined at 37 °C and pH 8.0 and the inhibition by 6-aminopenicillanic acid was less than for the free enzyme.

4. There was no evidence of diffusional limitation of the reaction rate.

INTRODUCTION

Benzylpenicillin and other penicillins may be hydrolysed by penicillin amidase (penicillin amidohydrolase, EC 3.5.1.11) from *Escherichia coli* and other bacteria[1] to give 6-aminopenicillanic acid, a key intermediate in the production of new penicillins such as 6-(α-aminophenylacetamido)penicillanic acid. In 1969 we described[2] the laboratory-scale isolation of penicillin amidase from *E. coli* ATCC 9637 and gave some kinetic data on the enzyme, including an immobilised form prepared by covalent binding to DEAE-cellulose. Following that feasibility study we started to investigate the design of an immobilised enzyme reactor for the conversion of benzylpenicillin to 6-aminopenicillanic acid. In a previous paper[3] we described the isolation of the penicillin amidase from *E. coli* NCIB 8743A and the kinetics of hydrolysis of benzylpenicillin by this enzyme. The reaction was found to be inhibited by high concentrations of the substrate and by both products. Here we report the immobilisation of penicillin amidase from *E. coli* NCIB 8743A by covalent binding to DEAE-cellulose and the kinetics of hydrolysis of benzylpenicillin by this immobilised enzyme.

* Present address: Beecham Research Laboratories, Worthing, Sussex, Great Britain.

** Present address: Department of Biochemistry, Faculty of Medicine, University of Ceylon, Peraderiya, Ceylon.

MATERIALS AND METHODS

Materials

The sodium salt of benzylpenicillin was obtained from Dista Products Ltd, Speke, Liverpool. Phenylacetic acid was obtained from Hopkin and Williams Ltd, London, and 6-aminopenicillanic acid from Sigma Chemicals Ltd, London. Analar grade reagents were used where possible.

Penicillin amidase was either prepared in our pilot-plant as described previously[3] or supplied by Beecham Research Laboratories as a wet solid obtained by precipitation with $(NH_4)_2SO_4$. 2-Amino-4,6-dichloro-*s*-triazine was prepared by the method of Kay and Lilly[4] and characterised by measurement of the melting point (235 °C) and infrared spectrum. DEAE-cellulose (Grades DE23 and DE52) were supplied by W.R. Balston (Modified Cellulose) Ltd.

Preparation of aminochloro-s-triazinyl DEAE-cellulose

The method described by Kay and Lilly[4] was used except that the amount of 2-amino-4,6-dichloro-*s*-triazine was increased to 0.4 g/g dry weight of DEAE-cellulose. To ensure complete removal of unreacted triazine the filtered product was washed with an equivolume mixture of acetone and water, followed by water and then stirred in 1 M NaCl for 30 min. After recovering the product by filtration, the NaCl wash was repeated. The product was washed finally with phosphate buffer, pH 7.0, and stored at 2 °C.

Preparation of immobilised penicillin amidase

A solution of penicillin amidase in 0.05 M phosphate buffer, pH 8.0, with a protein concentration of 0.5–7.0 mg/ml depending on the experiment, was added to the aminochloro-*s*-triazinyl DEAE-cellulose and the suspension stirred gently for 18 h at room temperature. The product was recovered by filtration and washed thoroughly with 0.05 M phosphate buffer, pH 8.0, and 5 M NaCl to remove any adsorbed penicillin amidase. The amount of protein attached was determined by measuring by the biuret method the total protein, in the enzyme solution before attachment and in the filtrate and washings after attachment.

Enzyme assays

Immobilised penicillin amidase was assayed in a baffled 1-l perspex tank fitted with a turbine impeller and automatic pH control by alkali addition. The reaction rate was found to be independent of the impeller speed over the range 300–900 rev./min and a speed of 500 rev./min was used in all other experiments. The temperature of the reactor contents was controlled by placing the tank in a thermostatically controlled water bath. After equilibration of the immobilised enzyme suspended in phosphate buffer (total volume 450 ml), 50 ml of equilibrated benzylpenicillin solution was added. Samples (0.5 ml) were taken by means of a pipette, fitted with a sintered glass disc on the inlet, at 0, 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 min after addition of the benzylpenicillin. The samples were assayed for 6-aminopenicillanic acid using *p*-dimethylamino-benzaldehyde as described previously[3]. The initial velocities of reaction were defined as the quantity of 6-aminopenicillanic acid produced in μmoles/ml per min and were found to be linear up to 3 min.

After reaction the immobilised enzyme was recovered by filtration, washed thoroughly with 0.05 M phosphate buffer, pH 8.0, to remove any benzylpenicillin or products remaining and was stored at 2 °C if not required for immediate further use.

RESULTS

Immobilisation of penicillin amidase

The results for five different preparations of immobilised penicillin amidase are given in Table I. The activity of each preparation was the maximum reaction rate calculated from Lineweaver–Burk plots. Prepn 1 was made with enzyme that had been purified by ion-exchange chromatography but the remaining preparations were done with a dialysed ammonium sulphate precipitated material.

TABLE I

THE IMMOBILISATION OF PENICILLIN AMIDASE BY REACTION WITH AMINO-DICHLORO-*S*-TRIAZINYL DEAE-CELLULOSE

The spec. act. (μmoles/min per mg) of the initial enzyme solutions were 2.5 for Prepn 1 and 1.75 for Prepns 2–5.

Prepn No.	*Support material (grade and weight)*	*Protein (g)*		*Protein content (mg/g support)*	*Activity retention (%)*	*Activity/g support (μmoles/min)*
		initial	*attached*			
1	DE 52, 1.5 g (dry)	0.39	0.24	160	45	117
2	DE 23, 4.75 g	1.27	0.76	159	81	80
3	DE 23, 4.75 g	0.127	0.127	27	71	32
4	DE 23, 4.75 g	1.32	0.63	132	47	71
5	DE 23, 36.0 g	10.3	3.27	91	72	85

Allowing for the difference in specific activities of the initial enzyme solutions that were used, there does not seem to be any advantage in using DE52 cellulose. DE23 cellulose therefore was used for all other experiments since it has better mechanical properties. Prepns 2 and 3 were made to investigate the kinetic parameters and properties of preparations with different protein contents. As penicillin amidase is not adsorbed by DEAE-cellulose at pH 8.0, non-enzymic protein was preferentially attached in Prepn 2. This effect was not seen in Expt 3 where all the available protein was attached. In Expt 4 the initial enzyme solution was pretreated with DEAE-cellulose at pH 8.0 to remove some non-enzymic protein prior to the immobilisation step. Prepn 5 was made under identical conditions to Prepn 2 but the amount handled was about eight times greater.

Effect of pH on the activity of immobilised penicillin amidase

The pH–activity profile was determined by measuring the reaction rate with 0.01 M benzylpenicillin at pH values up to 8.5 in 0.05 M phosphate. Tris buffer (0.05 M) was used for another measurement at pH 8.5 and also one at pH 9.0. The rate at pH 8.5 was identical in phosphate and tris buffer. The activity profile is shown in Fig. 1.

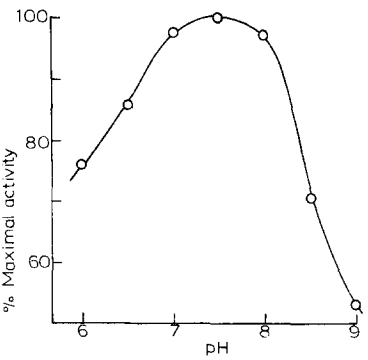
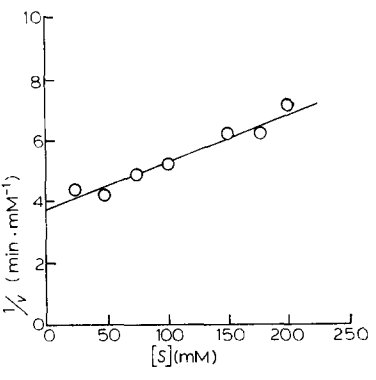


Fig. 1. pH–activity profile of immobilised penicillin amidase when assayed in the presence of 0.01 M benzylpenicillin and 0.05 M phosphate or Tris buffer.

Determination of kinetic parameters

The kinetic parameters of immobilised penicillin amidase at pH 8.0 and 37 °C were determined in the same way as described previously[3] for the soluble enzyme. The substrate inhibition constant, K_s, was obtained from a plot of $1/v$ against [S] (Fig. 2). The values of V and K_m were obtained from a Lineweaver–Burk plot (Fig. 3). For the inhibition constant, K_i, of phenylacetic acid, $1/v$ was plotted against phenylacetic acid concentration for two different substrate concentrations, 5 and 10 mM (Fig. 4).



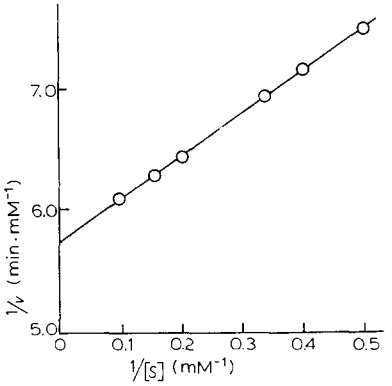
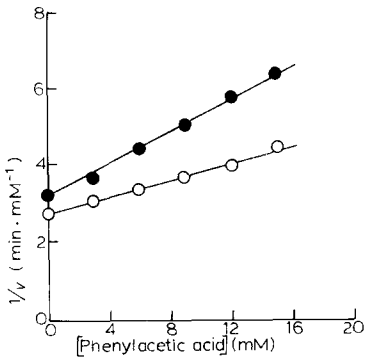


Fig. 2. The inhibition of initial reaction rate (v) of Prepn 1 by high concentrations of benzylpenicillin [S].

Fig. 3. The effect of benzylpenicillin concentration, [S] on the initial reaction rate (v) of Prepn 1.

Finally for the inhibition constant, K_i, for 6-aminopenicillanic acid, v_0/v_i was plotted against 6-aminopenicillanic acid concentration, v_0 and v_i being the initial reaction rates in the absence and presence of the 6-aminopenicillanic acid (Fig. 5). The results for two substrate concentrations, 5 and 10 mM, are given. The good agreement between the two sets of results confirms that the inhibition is strictly non-competitive. K_i is calculated from the slope which is equal to $1/K_i$. The values of the kinetic parameters of both the immobilised and soluble penicillin amidase are given in Table II.



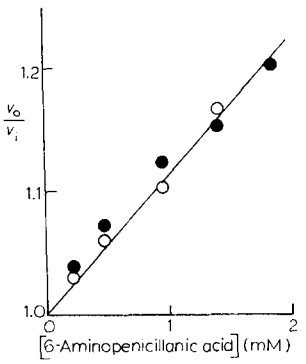


Fig. 4. The effect of phenylacetic acid concentration on the initial reaction rate, v, of Prepn 1 for two different benzylpenicillin concentrations: ●, 5 mM; ○, 10 mM.

Fig. 5. The variation of v_0/v_i (the ratio of initial reaction rates in the absence and presence of 6-aminopenicillanic acid) with 6-aminopenicillanic acid concentration for Prepn 1 and two different benzylpenicillin concentrations: ●, 5 mM; ○, 10 mM.

All of the above results were for Prepn 1. In view of the large change in K_i for 6-aminopenicillanic acid that occurred on immobilisation of the enzyme, values of K_m and K_i for 6-aminopenicillanic acid were also determined for Prepns 2 and 3. Within experimental error, the values of K_m and K_i (6-aminopenicillanic acid) for the two preparations were identical, *i.e.* 0.63 mM and 9.0 mM, respectively.

Effect of temperature on the activity of immobilised penicillin amidase

Benzylpenicillin is very unstable at high temperatures even at pH 7–8 so that the useful range over which penicillin amidase can be used is limited to below about

TABLE II

KINETIC PARAMETERS OF FREE AND IMMOBILISED PENICILLIN AMIDASE AT 37 °C AND pH 8.0

Penicillin amidase	K_s *(M)*	$K_m \times 10^4$ *(M)*	$K_i \times 10^3$ *(M) for*	
			6-Aminopenicillanic acid (M)	*Phenylacetic acid (M)*
Free	0.27	6.7	7.1	4.8
Immobilised	0.25	6.3	9.0	4.6

40 °C. Nevertheless it was of interest to examine the effect of temperature on the activity of immobilised penicillin amidase and to compare the results with those for the soluble enzyme. The maximum reaction rate, V at each temperature was obtained from initial rate measurements using 12 mM benzylpenicillin in 0.05 M phosphate buffer, pH 8.0. The results for both immobilised enzyme Prepn 4 and the soluble enzyme are shown in Fig. 6 where $\log_{10} V$ is plotted against $1/T$. The slopes are the same indicating that there has not been a change in the activation energy for the reaction when the enzyme is immobilised. The activation energy was calculated to be 8.55 kcal/mole.

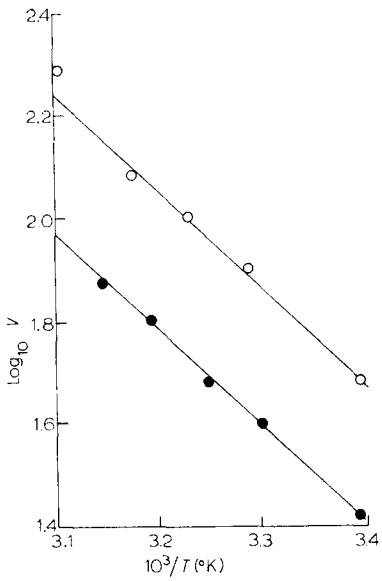


Fig. 6. The effect of temperature on the initial reaction rate for the free enzyme (○) and Prepn 4 (●).

DISCUSSION

Using aminodichloro-*s*-triazine as a linking agent we have immobilised penicillin amidase by attachment to DEAE-cellulose to give preparations with protein contents up to 160 mg/g support and with retentions of activity on immobilisation of 45–81%. These results compare favourably with those for many other immobilised enzymes[5].

With some immobilised enzymes, the reaction rate has been limited by the rate of diffusion of the substrate to the enzyme[4,6,7]. The insignificant effect of diffusional limitation on the reaction rate of the present immobilised penicillin amidase preparations was confirmed by (1) no effect of impeller speed on the reaction rate, (2) no sign of curvature in Lineweaver–Burk plots, (3) no change in the activation energy of reaction on immobilisation and finally (4) no change in the apparent K_m of the immobilised enzyme with a 2.5-fold difference in activity of the preparation. These observations were not surprising since even with the high protein contents and retentions of activity achieved the activities of the preparations were lower than those for which serious diffusional limitation has been observed.

The binding of an enzyme to a charged support may alter the pH–activity profile especially at low ionic strengths[8]. With the present immobilised penicillin amidase a shift in the pH optimum from about 8.2 to 7.65 was observed when the enzyme was assayed in 0.05 M phosphate buffer. Previously with immobilised penicillin amidase from *E. coli* ATCC 9637 we observed a shift to pH 7.4 but a lower substrate concentration was used for the measurements. At high substrate concentrations the shift in pH–activity profile will be less. When the substrate has a net charge, binding of an enzyme to a charged support may also influence the K_m. Immobilisation of the

penicillin amidase from Strain 9637 led to a decrease in the apparent K_m and an apparent increase in the substrate inhibition constant, K_s. With the enzyme from Strain 8743A very little change in K_m or K_s occurred on immobilisation but there was a decrease in the non-competitive inhibition by 6-aminopenicillanic acid. One possibility, that the enzyme preparation contained a component having a higher K_i for 6-aminopenicillanic acid which was preferentially bound, is unlikely. Although only a part of the available enzyme was bound in Prepn 2, it had a similar K_i for 6-aminopenicillanic acid as Prepn 3 where all the enzyme was bound. The change in K_i for 6-aminopenicillanic acid is particularly interesting as it is inhibition by this product that mainly influences the rate of progress of a batch reaction[9].

The use and stability of these immobilised penicillin amidase preparations in batch and continuous-flow stirred tank reactors for the conversion of benzylpenicillin to 6-aminopenicillanic acid is described elsewhere[9].

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