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THE ISOLATION AND KINETICS OF PENICILLIN AMIDASE FROM *ESCHERICHIA COLI*

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SUMMARY

1. A penicillin amidase (penicillin amidohydrolase, EC 3.5.1.11) was extracted from *Escherichia coli* NCIB 8743A and purified by precipitation first with $(NH_4)_2SO_4$ and then polyethyleneglycol, followed by chromatography on DEAE-cellulose.

2. The enzyme was shown to be inhibited by excess substrate, benzylpenicillin, and by both of the products of hydrolysis. The inhibition by phenylacetic acid was found to be competitive and by 6-aminopenicillanic acid to be non-competitive.

3. The kinetic and inhibition constants for the enzyme were measured over the range of pH, 7.0–8.5, at 37 °C and at pH 8.0 at 27 °C.

INTRODUCTION

Many microorganisms have been shown to possess penicillin amidase activity[1,2]. Investigations of penicillin amidase (penicillin amidohydrolase, EC 3.5.1.11) of *Escherichia coli* usually have been done with whole bacteria[3–5] and very little has been reported on the kinetics of the isolated enzyme. In a previous paper[6] we described the laboratory-scale isolation of penicillin amidase from *E. coli* ATCC 9637 and reported some kinetic data on the enzyme, including an immobilised form prepared by covalent binding of the enzyme to DEAE-cellulose. Following that feasibility study it was our intention to design an immobilised enzyme reactor for the conversion of benzylpenicillin to 6-aminopenicillanic acid (6-APA). Before this could be done it was necessary to know more about the kinetics of penicillin amidase. Here we describe the large-scale isolation of the penicillin amidase from *E. coli* NCIB 8743A and the kinetics of hydrolysis of benzylpenicillin by this enzyme.

Abbreviation: 6-APA, 6-aminopenicillanic acid.

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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-APA from Sigma Chemicals Ltd, London. Analar grade reagents were used where possible except during the isolation of the enzyme. For this the following were used: $(NH_4)_2SO_4$ (B.P. grade) from Frederick Allen and Co. Ltd, streptomycin sulphate (Medical grade) from Dista Products Ltd, and polyethyleneglycol 6000 from Shell Chemicals Ltd. DEAE-cellulose (Grade DE52) was supplied by W. R. Balston (Modified Cellulose) Ltd.

Enzyme isolation

E. coli (10 kg wet wt), provided by Beecham Research Laboratories, was suspended in 0.01 M phosphate buffer, pH 7.0, to give a final volume of 50 l. The bacteria were disrupted by passing the suspension four times through a Manton–Gaulin homogeniser[7] (Model 15M/8BA) at 500 kg_f/cm^2 operating pressure. The cell debris was removed by passing the suspension through a tubular bowl centrifuge (Model 6P, Sharples Centrifuges Ltd, Camberley, Surrey, Great Britain). Streptomycin sulphate (278 g) was added to the supernatant to precipitate nucleic acids which were removed using the 6P centrifuge. $(NH_4)_2SO_4$ was added slowly to give a 60% saturated solution. After being left overnight, the precipitate (2.5 kg) was collected in the 6P centrifuge and the supernatant discarded. The enzyme-containing precipitate was stored at 2 °C and processed further when required.

The precipitate was redissolved in 0.01 M phosphate buffer, pH 7.0, and fractionally precipitated with polyethyleneglycol. The fraction collected between 10 and 20% (w/v) polyethyleneglycol was redissolved in 0.01 M phosphate buffer, pH 8.0, dialysed against more of this buffer and then passed down a DEAE-cellulose column (45 cm × 7.5 cm diameter). The purified penicillin amidase appeared in the first protein-containing fractions which were pooled, dialysed against 5 mM phosphate buffer, pH 7.5, and freeze–dried. The powder was stored at −20 °C. All of the isolation steps were done at about 5 °C.

Enzyme assays

The enzymic activities of penicillin amidase solutions obtained at the various stages of the isolation procedure were determined by measuring the amount of 6-APA produced in a reaction mixture containing 20 mg/ml benzylpenicillin in 0.1 M phosphate buffer, pH 7.5, when incubated at 37 °C. The hydroxylamine method of Batchelor *et al.*[8] was used for the estimation of 6-APA.

For kinetic measurements the hydroxylamine method of assay is not very suitable as it is not sufficiently accurate. A second assay was therefore developed, based on that described by Svatek[9]. This depends on the reaction of the free amino group of 6-APA with *p*-dimethylaminobenzaldehyde to form a coloured Schiff's base. The assay may be done in the presence of benzylpenicillin which does not react. 6-Aminopenicilloic acid also produces the chromogen but no penicillinase activity was detected in the purified penicillin amidase so that chromogen formation was entirely due to 6-APA.

Enzyme assays were done in 0.05 M phosphate buffer. Aliquots (0.5 ml) were taken at 15, 60, 120 and 180 s and pipetted into solutions each containing 3.5 ml of reagent solution. This was prepared by adding 0.5 ml of 0.5% (w/v) *p*-dimethylaminobenzaldehyde in methanol to 3.0 ml of a solution made by mixing 2.0 ml of 20% glacial acetic acid with 1.0 ml of 0.05 M NaOH. The absorbance at 415 nm of the reacted solutions was read against a solution containing the reagent solution and 0.5 ml of phosphate buffer. The absorbance was proportional to 6-APA concentration up to about 4 mM (for 1 μmole 6-APA/ml, $A_{415\text{ nm}}^{1\text{ cm}} = 0.10$). With high protein concentrations in the reaction mixture a precipitate may form when pipetted into the acetic acid. This precipitate can be removed by centrifugation.

One unit of enzyme activity was defined as the amount of enzyme required to produce 1 μmole of 6-APA per min.

Protein concentrations were determined by the biuret method of Layne[10] or the Folin method of Lowry *et al.*[11].

All operations involving the use of penicillin, including assays, were done inside a plastic film cabinet fitted with rubber gloves and operating under a negative pressure. This eliminated any risk to workers from inhalation of or contact with penicillin.

TABLE I

THE ISOLATION OF PENICILLIN AMIDASE FROM *E. coli* (10 kg)

Material	*Vol. (l)*	*Protein concn (mg/ml)*	*Total enzyme activity* $\times 10^{-5}$ *(units)*	*Spec. act. (units/mg)*	*Yield (%)*
Disrupted suspension	50.0	28.4	1.32	0.093	100
Extract (after removal of debris)	42.0	31.4	1.16	0.088	88
Supernatant (after streptomycin step)	42.0	19.2	0.87	0.108	66
$(NH_4)_2SO_4$ ppt. (redissolved)	6.0	45.0	0.71	0.26	54
$(NH_4)_2SO_4$ ppt. (redissolved)	1.0	45.0	0.150	0.33	54
Polyethyleneglycol ppt. (redissolved)	0.25	76.0	0.181	0.95	65
Dialysed solution	0.25	62.0	0.114	0.73	41
Pooled fractions from DEAE-cellulose	0.24	4.4	0.067	6.35	25

RESULTS

Enzyme isolation

The results for the isolation of penicillin amidase from a 10-kg batch of *E. coli* are given in Table I. Only part of the material obtained by $(NH_4)_2SO_4$ precipitation was processed further. The remainder was stored for later use. The hydroxylamine assay was used for samples taken during the initial part of the isolation since it is more specific for penicillin amidase than the other assay. The *p*-dimethylaminobenzaldehyde assay was used for all measurements after the $(NH_4)_2SO_4$ precipitation stage. This ac-

counts for the apparent increase in total activity at the start of the polyethyleneglycol precipitation stage. No enzyme activity was lost during the chromatographic stage but only those fractions having a specific activity greater than 5 units/mg were collected and pooled. The highest specific activity measured was 12 units/mg. No penicillinase activity was present in the purified enzyme preparations.

Determination of kinetic parameters

Since the kinetic parameters were required for later optimisation studies, measurements were made at various values of pH and temperature. After consideration of the stability of the subtrate and products we chose pH values of 7.0, 7.5, 8.0 and 8.5 and a temperature of 37 °C. A further set of results for pH 8.0 at 27 °C was obtained.

The penicillin amidase from *E. coli* 9637 was inhibited by high concentrations of benzyl penicillin. A similar effect was observed with the present penicillin amidase from *E. coli* 8743A. The substrate inhibition constant, K_s, was determined from plots of $1/v$ against $[S]$. The results for pH 7.0 are given in Fig. 1. The values of V and K_m at each pH were determined from Lineweaver–Burk plots. The plot for pH 7.5 is shown in Fig. 2.

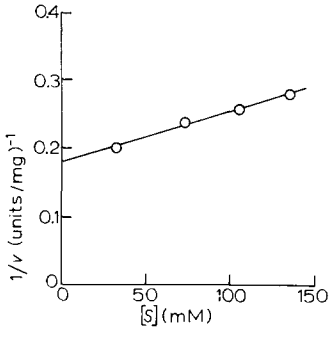


Fig. 1. The inhibition of initial reaction rate (v) by high concentrations of benzylpenicillin, $[S]$, at pH 7.0 and 37 °C.

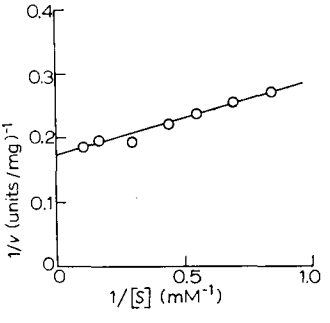
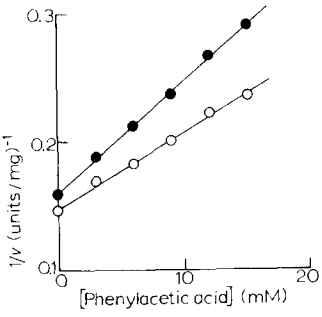


Fig. 2. The effect of benzylpenicillin concentration, $[S]$, on the initial reaction rate, v, at pH 7.5 and 37 °C.

Preliminary experiments had shown that the enzyme was inhibited by its products. To investigate this further, the reaction velocities were measured at two different substrate concentrations in the presence of varying amounts of phenylacetic acid or 6-APA. For the experiments with phenylacetic acid, $1/v$ was plotted against the phenylacetic acid concentration[12]. The results for pH 8.5 are given in Fig. 3. The alternative plot of $1/v$ against $1/[S]$ for two concentrations of product was not used as measurements of initial reaction rates at low substrate concentrations were less accurate. The inhibition constant, K_i, at each pH was determined from Fig. 3 and similar plots by extrapolation of the lines to the point of intersection, when the phenylacetic acid concentration equals $-K_i$. At this point $1/v$ was found to be equal to $1/V$ for that preparation of enzyme confirming that the inhibition was strictly competitive.



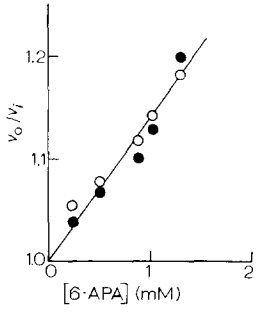


Fig. 3. The effect of phenylacetic acid concentration on the initial reaction rate, v, at pH 8.5 and 37 °C for two different benzylpenicillin concentrations. 7.5 mM, ●; 15 mM, ○.

Fig. 4. The variation of v_0/v_i (the ratio of initial reaction rates in the absence and presence of 6-APA) with 6-APA concentration at pH 8.0 and 37 °C for two different benzylpenicillin concentrations: 7.5 mM (●); 15 mM (○).

The experiments on inhibition by 6-APA were complicated by the need to assay 6-APA formation in the presence of high concentrations of 6-APA. For this reason the maximum 6-APA concentration used was kept below 2 mM to ensure reasonably accurate rate measurements. This limited the usefulness of $1/v$ *vs* $[I]$ plots and an alternative was adopted where v_0/v_i was plotted against $[I]$, v_0 and v_i being the initial reaction rates in the absence and presence of inhibitor[12]. An example for pH 8.0 is shown in Fig. 4. Since the results for the two different substrate concentrations fit the same straight line, the inhibition is strictly non-competitive and the slope equals $[I]/K_i$.

The values of V, K_m, K_s and K_is for 6-APA and phenylacetic acid obtained at each pH are given in Table II. The values of V are all for one particular enzyme preparation. In a separate set of experiments V was measured at pH 8.0 over the temperature range 20–50 °C. When log V was plotted against $1/T$ (T = absolute temperature) a straight line was obtained from which the activation energy for the reaction was calculated to be 8.55 kcal/mole.

TABLE II

THE KINETIC PARAMETERS OF PENICILLIN AMIDASE

Temp. (°C)	*pH*	*V* (units/mg)	$10^4 \times K_m$ (*M*)	$10 \times K_s$ (*M*)	$10^3 \times K_i$	
					6-APA (*M*)	*Phenylacetic acid* (*M*)
37	7.0	6.3	7.7	2.5	5.9	4.7
	7.5	6.6	7.4	2.7	6.5	4.8
	8.0	7.1	6.7	2.7	7.1	4.8
	8.5	7.1	8.0	2.8	5.3	5.1
27	8.0	4.6	7.7	2.5	5.7	5.8

DISCUSSION

The laboratory scale isolation procedure[6] previously used for *E. coli* ATCC 9637 has been successfully scaled-up fortyfold for the enzyme from *E. coli* NCIB 8743A. On the larger scale the yield after the $(NH_4)_2SO_4$ precipitation stage was higher than that

previously reported for the small scale (43%). Also the purification factor (2.8×) was greater. These improvements combined with a more active extract led to a final purified enzyme preparation with a specific activity several times greater than previously obtained with strain ATCC 9637[6].

The kinetics of the penicillin amidase of strain NCIB 8743A while still cell-bound have been examined by Cole[5]. The K_m values for benzylpenicillin reported here for the isolated enzyme are much lower than that for the enzyme when present in the intact cell (30 mM at pH 8.2 and 50 °C). Values for the K_m of penicillin amidase isolated from *E. coli* have been reported by Brandl[13] (1.35–1.59 mM at pH 8.0 and 30 °C) and Self *et al.*[6] (7.7 mM at pH 7.0 and 37 °C). The enzyme from strain NCIB 8743A seems to differ in two other ways from the enzyme described by Brandl. The optimum pH for our enzyme was between pH 8.0 and 8.5 which agrees with that for whole cells[5] but is higher than that reported by Brandl (pH 7.0). Also the activation energy for the hydrolytic reaction (8.55 kcal/mole) reported here is lower.

There have been several previous reports of inhibition of bacterial penicillin amidase by substrate or products. The present enzyme seems to be less sensitive to substrate inhibition than the enzyme from strain ATCC 9637 where K_s was 0.13–0.17 M at pH 7.0 and 37 °C. Chiang and Bennett[14] have shown that phenylacetic acid is a competitive inhibitor and 6-APA a non-competitive inhibitor of the penicillin amidase of *Bacillus megaterium*. Phenylacetic acid has been shown to inhibit the penicillin amidase from *E. coli* by Kaufmann[15] and Szentirmai[16]. We have now demonstrated that the inhibition is competitive and that 6-APA also inhibits non-competitively. This inhibition pattern is therefore similar to that for the enzyme from *B. megaterium* but the inhibition constants are very different, in both cases being lower for the *E. coli* enzyme. Whereas K_s and K_i for phenylacetic acid are relatively unaffected by pH, the K_i for 6-APA varies markedly with pH. This is particularly significant since the inhibition by 6-APA has the greatest effect on the reaction rate during the production of 6-APA. This aspect and the effect of immobilisation on the kinetic constants of penicillin amidase from strain NCIB 8743A are described in detail elsewhere[17,18].

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