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Crystalline Bacterial Penicillinase

By M. R. POLLOCK, ANNE-MARIE TORRIANI AND E. J. TRIDGELL

*National Institute for Medical Research, Mill Hill, London, N.W. 7,
and Service de Biochimie Cellulaire, Institut Pasteur, Paris*

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Bacterial penicillinase, which catalyses the hydrolysis of penicillin to the antibiotically inactive penicilloic acid, was first discovered by Abraham & Chain (1940) in a strain of *Escherichia coli* and has since been found in a wide variety of micro-organisms (Abraham, 1951). Several attempts have been made to isolate and purify the enzyme, amongst which the most successful appear to have been those reported by Wainwright (1950), Morgan & Campbell (1947), McQuarrie & Liebmman (1944) and Murao (1950). Although preparations of high activity were obtained by these workers and a crystalline product was claimed by Murao (1950) and by Baudet & Hagemann (1954), no criteria of purity, such as electrophoretic or ultracentrifugal analyses, were applied, and it is difficult to compare the specific activities achieved by different workers because of the varying and often unsatisfactory methods of assay employed. Strains of bacteria vary enormously in the amount of penicillinase they produce and in the extent to which its formation is stimulated by treatment of cells with penicillin. The organism most commonly used so far for isolation attempts has been *Bacillus cereus* strain NRRL 569, which produces and liberates into the medium large quantities of the enzyme after induction with penicillin (see Pollock, 1950).

Pollock & Torriani (1953) succeeded in isolating the induced enzyme from the culture supernatant fluid of this strain, grown in a peptone medium, to a point which subsequent work (Kogut, Pollock & Tridgell, 1956) showed to have been nearly 50 % pure. Experience with peptone showed that the difficulty of eliminating high molecular-weight substances present in the medium outweighed any of its possible advantages, such as increased yield of enzyme, and it was decided in future to use casein hydrolysate.

Late in 1953 a constitutive penicillinase-producing *B. cereus* strain, capable of forming large amounts of the enzyme without penicillin treatment, was isolated by Dr P. H. A. Sneath. This constitutive strain (*B. cereus* 5/B) appeared as a spontaneous mutant (Sneath, 1955) from a penicillin-sensitive, parent strain (*B. cereus* 5), physiologically distinct from *B. cereus* 569.

B. cereus 5/B was found to produce, in the absence of penicillin, penicillinase activities two to three times those of penicillin-induced *B. cereus* 569. Because of the high yield of enzyme, and the fact that penicillin additions to the medium during growth were not necessary, it was decided to use strain 5/B in an attempt to prepare a large batch by growth of cells in casein hydrolysate.

METHODS

Organism. *B. cereus* 5/B (Sneath, 1955), as described, was used.

Medium. This comprised: Casamino Acids (Difco, technical grade), 100 g.; KH_2PO_4 , 32.5 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 2.0 g.; FeSO_4 (0.1%), 5 ml.; KOH to pH 7.3; water to 5 l. Sterilized by autoclaving.

Growth of culture. A portion (2.5 l.) of culture inoculated with spores the previous day and, after incubation, kept at $+2^\circ$ overnight, was used to inoculate 50 l. of medium. The bulk culture was incubated at 35° under conditions of maximum aeration in a large cylindrical culture vessel, designed by Dr Monod for continuous bacterial growth, at the Institut Pasteur, Paris. Growth continued for 6 hr. until a bacterial density of 1.9 mg./ml. was attained. A continuous culture was then started at a medium inflow rate of 20 l./hr., until a total of 100 l. had been collected. The culture was then cooled and the cells were separated in a Sharples centrifuge (temp. $14-15^\circ$).

Penicillinase assay. All samples were diluted in 1% (w/v) aqueous gelatin (Difco: standardized) and penicillinase activity was measured manometrically by the method of Henry & Housewright (1947). A unit of enzyme is taken as that defined by Pollock & Torriani (1953), namely, the quantity capable of hydrolysing 1 μ mole of penicillin in 1 hr. at pH 7.0 and 30° .

N estimations. The Kjeldahl method as modified by Ma & Zuazaga (1942) was used. For specific-activity measurements the enzyme samples were dialysed repeatedly against 0.01 M potassium phosphate pH 7.0 to remove all traces of non-protein N.

RESULTS

Purification of penicillinase

Stage I. The cell-free culture supernatant fluid, containing 2.08×10^8 units of penicillinase/ml., was concentrated to 16 l. by distillation under reduced pressure at 16° . To the concentrate was added very slowly, with constant stirring, 1.63 vol. (26 l.) of acetone at -10° , and the mixture was left 18 hr. at -10° . Most of the supernatant fluid was decanted off and the remainder filtered at $+2^\circ$. The massive precipitate consisted largely of salts (mainly phosphate) from the medium but included nearly all the penicillinase and other exocellular bacterial proteins possibly adsorbed on them. The penicillinase was brought into solution by adding 2 kg. of mixed ice and water to the precipitate, leaving at $+5^\circ$ for 16 hr. and filtering. Most of the salts remained undissolved, but 84% of the enzyme was freed into solution and recovered in 1829 ml. of filtrate.

Stage II. The filtrate was freeze-dried (wt. 86.25 g.) and a proportion (24 g.) taken for further purification, as follows. The powder was mixed with 70 ml. of water and dialysed directly against 0.60 saturated ammonium sulphate at pH 7.0 (two changes of 1 l.) at $+2^\circ$. The matter remaining in-

soluble after equilibration was spun off and discarded, and the enzyme, which remained entirely in solution in the supernatant fluid (90 ml.), precipitated by addition of more ammonium sulphate to produce a saturated solution.

Stage III. The precipitate was dissolved in 30 ml. of 0.1 M potassium phosphate buffer, pH 7.0, and fractionally reprecipitated by dialysis against increasing concentrations of ammonium sulphate at $+2^\circ$. It was found that 62% of the enzyme precipitated between 0.67 and 0.83 saturated ammonium sulphate, so all fractions soluble at 0.67 saturation and insoluble at 0.83 saturation were combined and dialysed against 0.1 M potassium phosphate pH 7.0.

Stage IV. This solution (7.0 ml.) was cooled until ice crystals began to form, and ethanol, previously cooled to -20° , added slowly, drop by drop, with constant stirring, to a final concentration of 40% (w/v). The precipitated enzyme was left overnight at -2° and then spun down, resuspended in water (4.0 ml. final vol.) and just enough M potassium phosphate pH 7.0 added to bring the precipitate into solution at $+2^\circ$. It was found that 77% of the enzyme had been precipitated by this treatment with ethanol and the final aqueous solution had a nitrogen content corresponding to nearly 3% protein. The solution had a pale brown colour and rapidly formed a precipitate on cooling to -2° from $+2^\circ$.

Crystallization. The marked change in solubility with temperature at about 0° raised hopes that crystallization might be achieved by a gradual lowering of temperature from $+2^\circ$. The solution was transferred to a saline bath at -2° to allow slow cooling, but an apparently amorphous precipitate gradually formed. It was left untouched for 2 weeks and was then brought to room temperature. A few crystals were noticed in the solution after the amorphous precipitate had dissolved; the tube was cooled again to -2° , whereupon a crop of columnar crystals of variable size formed during the course of the next few days. The crystals were somewhat difficult to study, since they dissolved fairly rapidly in the mother liquor on warming to room temperature. A sample of crystals was spun down at $+2^\circ$, washed with a small quantity of iced distilled water (in which the crystals were insoluble) and dissolved in 0.1 M potassium phosphate buffer pH 7.0 to form a clear, colourless solution. The specific activity of this solution was 10% higher than that of the original solution before separation of the crystals, and gave an ultraviolet absorption curve typical of a protein, with maximal absorption at 280 m μ . and a ratio of absorption at 280 m μ . to that at 260 m μ . of 1.78. It seemed reasonable to conclude that the crystals were those of penicillinase itself.

A summary of the isolation procedure is given in Table 1. It can be seen that little further purification was achieved by ethanol precipitation.

Recrystallization. The remainder (62.25 g.) of the acetone powder (from stage I) was treated in the same way as described above and a good yield of crystals obtained.

A sample of these crystals (approx. 20 mg.) was suspended in 1.0 ml. of water at $+2^\circ$ and was dissolved by adding the minimum quantity (about

bath and left at -2° (after shaking to dissolve the precipitate). After 4 days crystals of penicillinase began to form and gradually increased over a further period of 6 days (Fig. 1). A sample of crystals was removed, washed with cold distilled water, dissolved in 0.1M potassium phosphate, pH 7.0, and the specific activity found to be 1.48×10^6 units/mg. of N (not significantly different from that of the enzyme after a single crystallization).

Table 1. *Isolation and purification of Bacillus cereus 5/B penicillinase*

Stages (as in text)	Successive fractions	Activity (units/ml.)	Vol. (ml.) or wt. (g.)	Total activity (units)	Specific activity (units/mg. of non-dialysable N)	Yield (%)
	Culture supernatant	2.08×10^3	100 000 ml.	2.08×10^8	—	100
I	Concentrated supernatant	1.25×10^4	16 000 ml.	2.01×10^8	—	97
	Acetone powder (28% of total)	—	24 g.	5.0×10^7	2.83×10^5	86
II	Total soluble in 0.60 saturated (NH_4) ₂ SO ₄ at $+2^\circ$	1.67×10^6	30 ml.	5.0×10^7	8.45×10^5	86
III	Combined fractions precipitated between 0.67 and 0.83 satur- ated (NH_4) ₂ SO ₄ at $+2^\circ$	4.8×10^6	7 ml.	3.37×10^7	1.34×10^6	58
IV	Fraction precipitated with 40% ethanol in 0.1M phosphate	5.95×10^6	4 ml.	2.37×10^7	1.43×10^6	41
	Crystals	—	—	—	1.59×10^6	—
	After recrystallization	—	—	—	1.48×10^6	—

(a)

(b)

Fig. 1. Twice-crystallized penicillinase from *B. cereus* 5/B. a, $\times 67$; b, $\times 120$.

0.01 ml.) of M potassium phosphate, pH 7.0 and then left in a test tube with open end, suspended in a small sealed bath (12 ml.) of 30% (v/v) ethanol at -2° . This technique (Low & Richards, 1954), designed to assist crystallization, allows very gradual equilibration of a volatile precipitant between bath and solution. After 8 days a slight, amorphous precipitate formed in the enzyme solution, which was removed from the ethanol

Properties of the enzyme

Ultracentrifuging. A sample of the solution before separation of crystals was investigated by Hall & Ogston (1956; see Addendum to the next paper) and was found to behave in strictly homogenous fashion, giving a probable molecular weight of 35 200.

Electrophoresis. A solution of the crystals was investigated in the Perkin-Elmer Tiselius apparatus

at pH 8.4 and 5.0 (Fig. 2), and the results are summarized in Table 2. Boundary analysis showed a single, apparently symmetrical peak under all conditions. The relatively slow mobility towards the cathode at pH 5.0 showed that the isoelectric point must be only slightly above pH 5.0.

Fig. 2. Tracings of boundary patterns obtained on electrophoresis of crystalline *B. cereus* 5/B penicillinase at pH 8.4 (glycine-NaCl, $\mu=0.2$).

at pH 7.0, the enzyme is completely stable (for at least several weeks at 0°) at a concentration of 0.1 m μ g./ml. (measurable by a micro-assay method to be described later).

Its molecular activity (turnover no., 1.48×10^5 molecules of substrate hydrolysed/min. by 1 molecule of enzyme at 30°) is one of the highest amongst known enzymes, 1 mg. destroying 77 g. of penicillin in 1 hr. at 30°.

Although its pH/activity curve and Michaelis affinity constant were found not to be significantly different from those of the *B. cereus* 569 induced penicillinase (Pollock & Tridgell, unpublished), its apparently greater solubility in high concentrations of ammonium sulphate suggested that it might be distinct in its physicochemical properties. Indeed, it proved to be quite easily distinguishable from the 569 enzyme both by physicochemical and

Table 2. Summary of physicochemical properties of purified *Bacillus cereus* 5/B penicillinase

Electrophoretic mobility (cm. ² /v/sec.):	
At pH 8.4 (glycine-NaCl; $\mu=0.2$)	-3.48×10^{-5}
At pH 5.0 (KH ₂ PO ₄ -NaOH; $\mu=0.1$)	$+3.6 \times 10^{-6}$
Sedimentation constant $S_{20,w}$ (see Hall & Ogston, 1956)	2.82×10^{-13}
Diffusion constant (see Hall & Ogston, 1956)	7.80×10^{-7}
Molecular weight (see Hall & Ogston, 1956)	35 200
Specific activity (units/mg. of N) (mean value for crystals)	1.53×10^6
Molecular activity (moles of penicillin hydrolysed/mole of enzyme/min. at 30°)	1.48×10^6
$E_{280m\mu}^{1\%}$ at a concn. of 1.0 mg. of N/ml.	7.35

Amino acid content. A semi-quantitative analysis of the crystals by paper chromatography, after hydrolysis in 6N-HCl at 105° for 36 hr., using a technique based on that described by Roland & Gross (1954), showed an unusually high proportion of glutamic and aspartic acids (together accounting for nearly 15 % of the total weight). Neither of the S-containing amino acids—cystine (assayed as cysteic acid) and methionine (assayed as the sulphoxide)—was present in detectable quantity.

DISCUSSION

Pure *B. cereus* 5/B penicillinase forms a colourless solution in phosphate buffer, pH 7.0, and shows an ultraviolet absorption spectrum typical of a protein, with maximum absorption at 280 m μ . and no evidence of any characteristic prosthetic group. It is insoluble in distilled water and low concentration of salts (<0.01M phosphate). It is quite stable on dialysis and gives no indication of needing any metal activator. On dilution below a concentration of 0.02 mg./ml. in water or aqueous solution of phosphate pH 7.0 considerable (though variable) irreversible inactivation occurs. This can be completely prevented by incorporation of 1 % (w/v) gelatin in the dilution fluid. In 1 % gelatin,

immunological criteria, as is shown in the next paper (Kogut *et al.* 1956).

Total protein-N estimations on the culture supernatant fluid from a small batch grown at 35° in conical flasks, aerated by shaking and with the same order of final enzyme yield as in the 'bactogen' culture, showed (Kogut *et al.* 1956) that *B. cereus* 5/B penicillinase constituted 26 % of the exocellular protein N liberated by the cells into the medium.

SUMMARY

1. Penicillinase has been isolated from the culture supernatant fluid of a *B. cereus* mutant forming large amounts of the enzyme constitutively, and crystallized after fractionation with ammonium sulphate and precipitation with 40 % ethanol.

2. On ultracentrifugal and electrophoretic analysis the purified enzyme appeared to consist of a single species of protein with an isoelectric point a little above pH 5.0, and a molecular weight of 35 200.

3. The enzyme is stable on dialysis, and there was no indication of the presence of any characteristic prosthetic group or need for a co-enzyme or other specific activator.

We wish to express our thanks to Dr Melvin Cohn for much valuable advice during the preliminary work on methods of purification, and one of us (M.R.P.) is greatly indebted to Dr Jacques Monod of the Institut Pasteur, Paris, for the hospitality of his laboratory in which much of the earlier work on isolation of penicillinase was carried out. We are also grateful to Dr T. S. Work for help in the chromatographic estimation of amino acids. The photographs were taken by Mr M. R. Young.

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Purification of Penicillin-Induced Penicillinase of *Bacillus cereus* NRRL 569: A Comparison of its Properties with those of a similarly Purified Penicillinase produced Spontaneously by a Constitutive Mutant Strain

BY MARGOT KOGUT,* M. R. POLLOCK AND E. J. TRIDGELL
National Institute for Medical Research, Mill Hill, London, N.W. 7

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Ever since the phenomenon of microbial enzyme adaptation began to be studied seriously it has been of obvious interest and importance to determine the nature of the substance or substances responsible for the increase in enzyme activity that occurs after treatment of cells with the specific inducer. Although it has often been assumed—in many cases not unreasonably—that the development of enzyme activity corresponded to the production of an equivalent amount of some distinct, enzymically active protein, direct proof of this assumption (for *Escherichia coli* β -galactosidase induction) was not provided until quite recently (Cohn & Monod, 1951). A further query, which could not be answered until the induced enzyme had been isolated and purified, was whether it showed any peculiarities or distinctive features that might place it in a class apart from 'ordinary' (constitutive) enzymes produced by cells spontaneously in the absence of inducer. More specifically this question may take the form of the problem as to whether a spontaneously produced (i.e. basal or

constitutive) enzyme belongs to the same molecular species as the catalytically similar enzyme evoked after addition of a specific inducer. If the 'spontaneous' and the induced enzymes consisted of different types of protein molecule, the mechanisms for their production would probably be quite distinct; and the inducer would have to be considered as initiating the formation of a new kind of protein instead of merely increasing the rate of production of a molecular species already formed to some extent in its absence. The 'unitary hypothesis', discussed by Cohn & Monod (1953), which postulates basically similar mechanisms for the formation of constitutive and induced enzymes, would be thus greatly weakened.

This question, which we have attempted to answer in this paper, can be best approached by the comparative study of a purified induced enzyme and the catalytically similar enzyme formed by the same, or closely related, cells in the absence of inducer. Ideally, such a comparison should be made between purified induced enzyme and the constitutive moiety (usually referred to as 'basal enzyme') produced by the same cells without

* Present address: Agricultural Research Council, Unit for Microbiology, University of Sheffield.