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Purification of isopenicillin N synthetase

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1. Isopenicillin N synthetase was extracted from *Cephalosporium acremonium* and purified about 200-fold. The product showed one major protein band, coinciding with synthetase activity, when subjected to electrophoresis in polyacrylamide gel. 2. An isopenicillin N synthetase from *Penicillium chrysogenum* was purified about 70-fold by similar procedures. 3. The two enzymes resemble each other closely in their M_r, in their mobility on electrophoresis in polyacrylamide gel and in their requirement for Fe^{2+} and ascorbate for maximum activity. 4. Preliminary experiments have shown that a similar isopenicillin N synthetase can be extracted from *Streptomyces clavuligerus*.

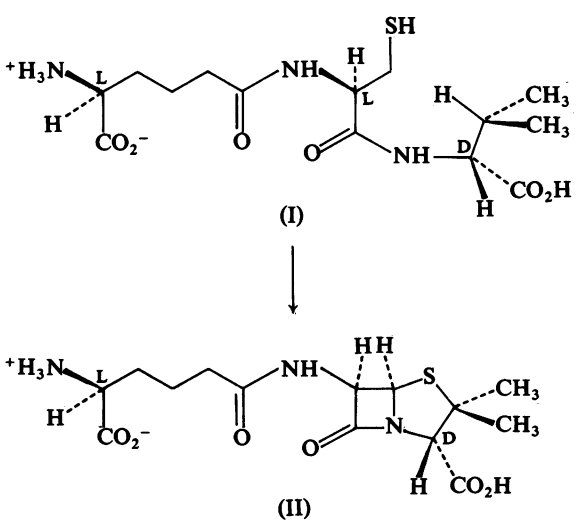
The tripeptide (L-α-amino-δ-adipyl)-L-cysteinyl-D-valine (I) (Loder & Abraham, 1971) is cyclized to isopenicillin N (II) (Scheme 1) in the presence of O_2 (White *et al.*, 1982), Fe^{2+} and a reducing agent by a soluble enzyme (isopenicillin N synthetase) from *Cephalosporium acremonium* (*Acremonium chrysogenum*). The partial purification and some of the properties of this enzyme from *C. acremonium* were reported briefly by Abraham *et al.* (1981) and later by Kupka *et al.* (1983). The further work described in the present paper was done with a higher-yielding strain of the *Cephalosporium* (C0728) than that used previously (C91), and also with strains of *Penicillium chrysogenum* and *Streptomyces clavuligerus*.

Experimental

Materials

Cephalosporin C (potassium salt) was provided by Glaxo Group Research, Greenford, Middx., U.K. (L-α-Amino-δ-adipyl)-L-cysteinyl-D-valine (Fawcett *et al.*, 1976; Baldwin *et al.*, 1981) was used as the free thiol or as the disulphide dimer, which was reduced to the monomer *in situ* with dithiothreitol. Phenyl-Sepharose CL-4B, DEAE-Sepharose CL-6B and Sephacryl S-200 (superfine grade) were from Pharmacia Fine Chemicals, Uppsala, Sweden. Dithiothreitol, bovine liver catalase, protamine sulphate, Brilliant Blue G, Tris and Mops (4-morpholinepropanesulphonic acid) were from Sigma Chemical Co., Poole, Dorset, U.K. Bovine serum albumin was from Armour Pharmaceutical Co., Eastbourne, U.K. Other chemicals were from BDH Chemicals, Poole, Dorset, U.K., and were of AnalaR grade except for $(NH_4)_2SO_4$, which was of AristaR grade.

C. acremonium C0728 was a gift from Glaxo, Ulverston, Cumbria, U.K., and *P. chrysogenum* SC6140 was kindly provided by the Squibb Institute for Medical Research, Princeton, NJ,



Scheme 1. *Cyclization of* (L-α-*amino-δ-adipyl*)-L-*cysteinyl*-D-*valine* (*I*) *to isopenicillin N* (*II*)

U.S.A. *Strep. clavuligerus* A.T.C.C. 27064 was from the type collection.

Assays of penicillin N, isopenicillin N and cephalosporin C

Bioassays were carried out by the hole–plate method (Smith *et al.*, 1967). For the determination of the combined activities of penicillin N and cephalosporin C in fermentation fluids, *Salmonella typhi* strain 'Mrs S' (Felix & Pitt, 1935) was used as the test organism. The concentration of cephalosporin C was determined with *Alcaligenes faecalis* A.T.C.C. 8750 in agar containing β-lactamase I from *Bacillus cereus* (Davies *et al.*, 1974) to inactivate the penicillin N. For assay of the isopenicillin N produced in the cell-free system the test organism was *Staphylococcus aureus* N.C.T.C. 6571 and potassium cephalosporin C (recrystallized, 10 units/mg) was used as the standard. The specific activity of pure isopenicillin N was taken to be 80 units/mg against *Staph. aureus* N.C.T.C. 6571 (Abraham *et al.*, 1981).

Assay of preparations of isopenicillin N synthetase

The reaction mixture (200μl), containing enzyme, (L-α-amino-δ-adipyl)-L-cysteinyl-D-valine (1mM), dithiothreitol (2mM), L-ascorbic acid (1mM), $FeSO_4$ (0.1mM) and catalase (3000 Sigma units/ml) in 0.1M-Mops/NaOH buffer, pH8.0, was incubated at 30°C in 10ml tubes (10cm × 1.4cm) with reciprocal shaking at 150 cycles/min for 10min. The reaction was quenched by the addition of acetone (465μl), which precipitated the protein. The sample was centrifuged, the acetone removed from the supernatant by a stream of air, and the solution assayed for isopenicillin N. The protein content of solutions of the enzyme was determined by the method of Bradford (1976), with bovine serum albumin as the standard.

One unit of synthetase activity was defined as the amount of synthetase required for the formation of 1μmol of isopenicillin N/min under the conditions described.

Polyacrylamide-gel electrophoresis

Analytical electrophoresis was carried out in a slab-gel apparatus from Bio-Rad Laboratories, Richmond, CA, U.S.A., with a discontinuous buffer system (Laemmli, 1970). Stock acrylamide solutions containing 29.2% (w/v) acrylamide and 0.8% (w/v) *NN'*-methylenebisacrylamide were used to cast the stacking gel (3%, w/v; pH6.5) and the resolving gel (7.5%, w/v; pH8.8). Polymerization was catalysed by ammonium persulphate (0.05%) and *NNN'N'*-tetramethylethylenediamine (0.025% for the resolving gel and 0.04% for the stacking gel). The running buffer (pH8.1) contained 1.44% (w/v) glycine and 0.3% (w/v) Tris. The protein samples (10–50μg) contained 10% (w/v) sucrose. Electrophoresis was carried out at a constant current of 30mA and at 4°C for about 3.5h. The protein bands were fixed with 3.5% (w/v) $HClO_4$ and stained with Coomassie Blue R in methanol/acetic acid/water (5:5:1, by vol.). The same methanol/acetic acid/water solution without the dye was used for destaining. Colour densities of the protein bands were measured with a microdensitometer (Joyce, Loebl and Co., Gateshead, U.K.).

For the elution of proteins after electrophoresis the gel was first hardened by storage at −20°C for several hours. Slices (about 3mm × 6mm) were then cut from positions corresponding to those on which protein bands were located on a stained gel run in parallel. Tris buffer, pH8.0, containing 0.5M-NaCl (200–400μl), was added to each slice, and the gel was broken up with a Pasteur pipette while the mixtures were kept at 4°C for 1h. The liquid was then removed for assay of synthetase activity.

Preparative electrophoresis was carried out with an apparatus in which the resolving gel was packed to form an annular vertical tube (about 12cm long), with internal and external diameters of 2.4cm and 3cm respectively, so that cooling could be applied to both surfaces. The buffer system and the compositions of the stacking and resolving gels were as those for the analytical procedure. Electrophoresis was carried out at constant current (30mA) and at 4°C for 16h. During downward migration of the protein (up to 60mg in 10% sucrose) Tris/glycine buffer was pumped laterally at 0.5ml/min through the lowest portion of the gel, which was seated on a Visking membrane, to remove proteins reaching that position.

Isoelectric focusing was carried out in 5% polyacrylamide gel according to *LKB Instruction Manual* no. 1804.

Growth of C. acremonium

(*a*) Fermentations in 500ml baffled shake flasks containing a chemically defined medium were carried out at 27°C as described by Smith *et al.* (1967) except that an inoculum of *C. acremonium* C0728 was grown for 96–102h and the flasks were shaken at about 160rev./min. The mycelium (about 4g/flask) was harvested at 48h. (*b*) Larger-scale fermentations were carried out in the same medium (15 litres) in a 20-litre fermenter (L.H. Engineering Co., Stoke Poges, Bucks., U.K.) at 27°C with stirring at 400rev./min and an aeration rate of 5 litres/min. After 72h the mycelium was harvested by centrifugation at 4°C (1900*g*) for 30min. The mycelial sediment was washed five times by resuspension in water and filtration through Whatman no. 54 hardened filter paper in a

Buchner funnel. Excess water was removed by pressing the mycelium between sheets of filter paper. The damp-dry mycelium (about 650g) could be stored at −20°C for up to 6 months before use.

Preparation of cell-free extracts

Mycelium was ground in a Dyno-Mill (Glen Creston, Stanmore, Middx., U.K.) with glass beads that had been cleaned with conc. HNO_3 and then with 7X detergent from Flow Laboratories, Rickmansworth, Herts., U.K. One of two procedures was used, depending on the amount of mycelium processed.

(1) *Batch method.* A portion of thawed mycelium (15g) from a shake-flask fermentation was resuspended in 0.1M-Mops buffer, pH8.0, containing KCl (10mM) and $MgSO_4$ (10mM) to a final volume of about 80ml. The suspension was stirred at 0–5°C in a 150ml glass head of a Dyno-Mill with 125ml of 0.10–0.12mm-diam. glass beads at 4500rev./min for 10min. The contents of the batch head were removed and the glass beads allowed to settle at 0°C. The supernatant was decanted and centrifuged (1900*g* for 15min). The beads were stirred with Mops buffer containing KCl and $MgSO_4$ and the supernatant was centrifuged. The two final supernatants were combined.

(2) *Continuous-flow method.* A portion of thawed mycelium (180g) was suspended in 0.1M-Mops buffer (400ml), pH8.0, containing KCl (10mM) and $MgSO_4$ (10mM) at 0°C. The suspension was pumped at a rate of 50ml/min into a 600ml glass continuous-flow head of the Dyno-Mill containing 500ml of glass beads (0.25–0.31mm diam.) and 100ml of 0.1M-Mops buffer, pH8.0, containing KCl (10mM) and $MgSO_4$ (10mM). The agitator discs were rotated at 4500rev./min for a period of 10min while the temperature was maintained at 5–10°C. The crude extract (720ml) was collected.

Growth and extraction of the synthetase from P. chrysogenum and Strep. clavuligerus

P. chrysogenum SC6140 was grown as described by Fawcett *et al.* (1975) and *Strep. clavuligerus* A.T.C.C. 27064 as described by O'Sullivan *et al.* (1979). The former was harvested after 48h and the latter after 24h. Extracts of the mycelia were prepared by the method used for the extraction of *C. acremonium*.

Results

Purification of isopenicillin N synthetase from C. acremonium

Preliminary experiments indicated that *C. acremonium* C0728 was a better source of cell-free isopenicillin N synthetase than were the strains of *P. chrysogenum* and *Strep. clavuligerus* that were available to us. The results of a typical purification of the enzyme from the *Cephalosporium* are described below and in Table 1.

Precipitation with protamine sulphate and $(NH_4)_2SO_4$. A solution of protamine sulphate (80ml; 12%, w/v) in 0.1M-Mops buffer adjusted to pH2.0 was added dropwise with stirring to the crude cell-free extract (720ml) at 0°C so that the final concentration of protoamine sulphate was about 1.2% (w/v). The pH fell from 8.0 to 7.2 during the addition and was raised to 8.0 with 1M-NaOH. The resulting mixture was stirred at 0°C for 45min and then centrifuged at 4°C (20000*g* for 30min).

Table 1. *Purification of isopenicillin N synthetase*

The crude extract was from 180g of damp-dry mycelium of *C. acremonium* C0728. Experimental details of the purification are given in the text. One unit is the amount of enzyme that catalyses the synthesis of 1μmol of isopenicillin N/min (see the Experimental section). The values given are typical of those obtained in several experiments, but in one case the specific activity of the most active product obtained (1200 units/g) was about 1.4× that shown.

Stage	Material	10^3 × Total synthetase activity (units)	Total protein (mg)	10^3 × Specific activity (units/mg)	Purification (fold)
1.	Crude extract	64810	11160	5.8	1
2.	Protamine sulphate precipitate	62460	5858	10.7	1.8
3.	$(NH_4)_2SO_4$ precipitate	50500	1650	30.6	5.3
4.	Phenyl-Sepharose eluate	27960	418	66.9	11.5
5.	DEAE-Sepharose eluate	29950	90	333	57
6.	Sephacryl S-200 eluate	20830	32	657	113
7.	DEAE-Sepharose eluate				
	Fraction 12*	5290	7.5	702	121
	Fraction 13*	9490	10.7	885	152
	Fraction 14*	4070	6.0	673	116

* These fractions are those shown in Fig. 3.

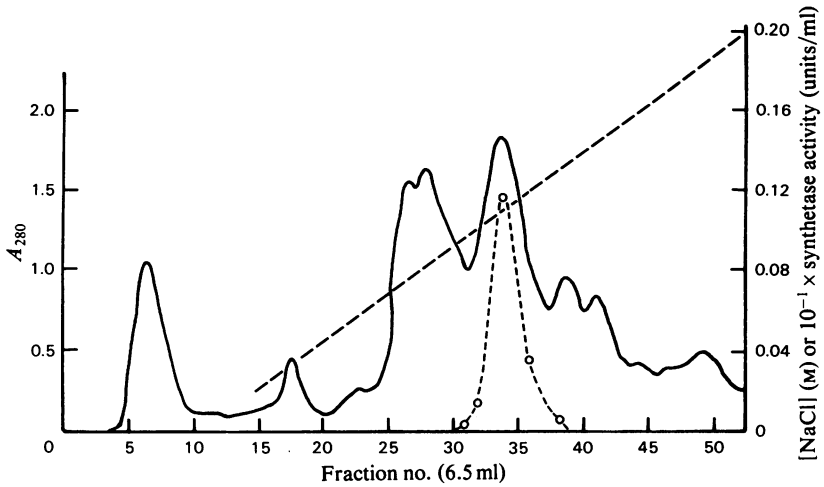


Fig. 1. *Elution of isopenicillin N synthetase from a column of DEAE-Sepharose*
The product chromatographed was from stage 4 of the purification (see Table 1). ——, A_{280}; ——, concn. of NaCl; --O--, synthetase activity.

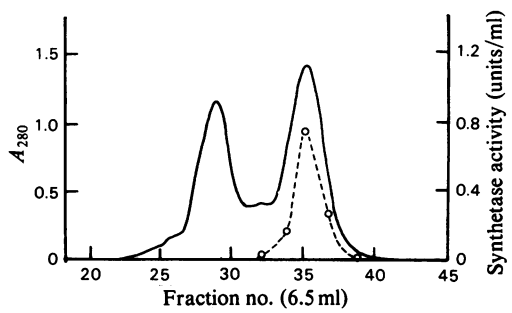


Fig. 2. *Gel filtration of isopenicillin N synthetase through Sephacryl S-200*
The product used was from stage 5 of the purification process (see Table 1). ——, A_{280}; --O--, synthetase activity.

$(NH_4)_2SO_4$ (256g) was added to the above supernatant (570ml) with stirring at 0°C over a period of 10min to give a 60%-saturated solution. The mixture was stirred for a further 20min and then centrifuged at 4°C (20000*g* for 15min). Additional $(NH_4)_2SO_4$ (134g) was then added to the supernatant with stirring at 0°C during 10min (to give 85% saturation). The mixture was stirred for a further 30min and was then centrifuged at 4°C (20000*g*, 20min) to obtain pellets of proteins which contained isopenicillin N synthetase.

Chromatography and gel filtration. All these procedures were carried out at 4°C. The pH of all buffers was 8.0

1. The precipitated crude enzyme was resuspended in 0.1M-Mops buffer, pH8.0, containing 0.8M-$(NH_4)_2SO_4$. The turbid solution (55ml) was applied to a phenyl-Sepharose column (21.5cm × 2.6cm) that had been equilibrated with the same buffer. The column was then washed with this buffer (200ml) at a flow rate of 0.4ml/min, which removed inactive proteins that were not adsorbed on the matrix. The synthetase was eluted with 0.1M-Mops buffer containing 0.5M-$(NH_4)_2SO_4$. The protein in the fractions containing the synthetase (270ml) was precipitated by $(NH_4)_2SO_4$ (85% saturation). Oily material, possibly lipoprotein, was removed at this stage.

2. The precipitated protein was suspended in 50mM-Tris/HCl buffer, pH8.0, to give a solution (7.8ml) which was dialysed for 5h against 10 litres of the same buffer. The solution was then applied to a DEAE-Sepharose column (35cm × 1.6cm) that had been equilibrated with the Tris buffer. Elution was carried out with a linear NaCl gradient (0–0.2M) and a flow rate of 0.5ml/min, and the eluate was monitored by measurement of absorbance at 280nm and by bioassay (Fig. 1). Fractions (each 6.5ml) containing most of the isopenicillin N synthetase activity (32–37) were combined (46ml) and the synthetase was precipitated with $(NH_4)_2SO_4$.

3. A solution of the precipitated protein in 50mM-Tris/HCl buffer (2.4ml) was applied to a Sephacryl S-200 column (105cm × 2.6cm) previously equilibrated with the same buffer. Elution was carried out with this buffer at a flow rate of 18ml/h. Fractions (each 6.5ml) containing isopenicillin N synthetase activity (34–39) were combined (Fig. 2).

4. The solution obtained after gel filtration was applied to a DEAE-Sepharose column (12cm × 0.9cm) that had been equilibrated with the Tris buffer. An NaCl gradient (0–0.25M) in the same buffer was used for elution at a flow rate of 0.45ml/min (Fig. 3). The fractions (each 6.5ml) containing isopenicillin N synthetase were stored at −20°C. The total enzyme activity obtained at this stage was about 30% of that in the crude extract (Table 1). In a number of other experiments the specific activity of the product varied from 0.7 to 1.2 units/mg of protein.

Samples of the purified synthetase could be kept in Mops or Tris buffer, pH8.0, at −20°C without significant loss of activity over at least 2 months. The enzyme could also be kept in the solid state after dialysis against 5mM-NH_4HCO_3 and subsequent freeze-drying, during which less than 10% of the total activity was lost.

Preparative electrophoresis in polyacrylamide gel. A 1.4-fold purification (from 220 units/g to 310 units/g) was achieved by preparative electrophoresis of partially purified synthetase. At least 60% of the total enzyme activity, however, was lost during the electrophoresis in each of three experiments. Analytical electrophoresis of the resulting product showed that a large increase had occurred in the intensity of a band corresponding to a protein with slightly greater mobility than that of the synthetase. Other minor proteins of lower mobility disappeared.

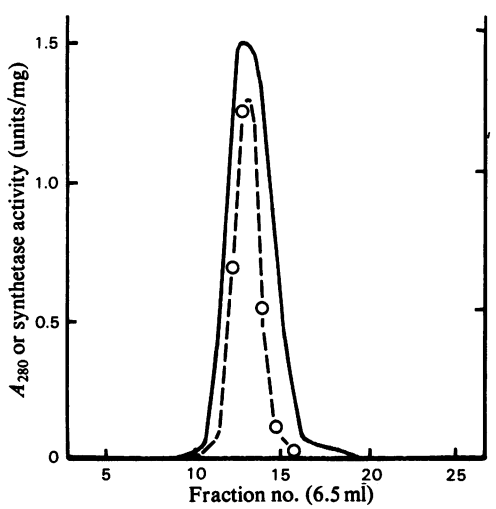


Fig. 3. *Chromatography of isopenicillin N synthetase on DEAE-Sepharose*

The product chromatographed was from stage 6 of the purification process. ——, A_{280}; --○--, synthetase activity.

Partial purification of the synthetase from P. chrysogenum and from Strep. clavuligerus

The specific activity of the isopenicillin N synthetase in crude extracts of *P. chrysogenum* was about one-half that in corresponding extracts of *C. acremonium*. Purification by procedures similar to those listed up to stage 6 in Table 1, but with omission of chromatography on phenyl-Sepharose, yielded a product with a specific activity of about 150 units/g in a yield of 16%. By preparative electrophoresis in polyacrylamide gel this specific activity was raised to about 240 units/g.

The specific activity of the synthetase in crude extracts of *Strep. clavuligerus* was only about 7% of that in extracts of *C. acremonium*. In view of this low activity, purification was not carried beyond precipitation with $(NH_4)_2SO_4$. The specific activity at this stage was 3.1 units/g of protein.

Discussion

Crude isopenicillin N synthetase from *C. acremonium* was relatively unstable at a pH below 7.0, and this imposed a limitation on the procedures that could be used for its purification. It was much less stable than the purified enzyme between pH7 and pH8, presumably because of the presence of proteinases in mycelial extracts. When a crude extract was dialysed against 50mM-Mops buffer, pH7.2, the assay values for protein decreased by about 70% in 43h and the formation of amino acids was shown by electrophoresis of the diffusate on paper at pH1.8.

The presence of an aminopeptidase in crude extracts was revealed when the tetrapeptide glycyl-(L-α-amino-δ-adipyl)-L-cysteinyl-D-valine was tested as a substrate with crude preparations of the synthetase. When incubated with these preparations for 60min the tetrapeptide yielded a product with activity against *Staph. aureus*. The ammonia-desorption chemical ionization mass spectrum of the *N*-ethoxycarbonyl and carboxymethyl derivative of this product (Loder & Abraham, 1971) was identical with that of the corresponding derivative of authentic isopenicillin N (Adlington *et al.*, 1983), showing a protonated molecular ion (MH^+) with *m/z* 460. Hence the *N*-terminal glycine residue of the tetrapeptide had been removed by hydrolysis during the incubation.

Kupka *et al.* (1983) reported that a partially purified preparation of isopenicillin N synthetase converted glycyl-(L-α-amino-δ-adipyl)-L-cysteinyl-D-valine into a product that behaved like isopenicillin N on high-pressure liquid chromatography, and that a purified preparation also did so, although less readily. However, when the tetrapeptide was incubated with the purified enzyme

obtained in the present work, no generation of activity against *Staph. aureus* was detected, although a synthetic preparation of *N*-glycylisopenicillin N showed activity similar to that of isopenicillin N. We conclude that the aminopeptidase activity is not a property of the synthetase, but is due to an enzyme that can be separated from the latter and that converts the tetrapeptide into (L-α-amino-δ-adipyl)-L-cysteinyl-D-valine, the precursor of isopenicillin N (Scheme 1).

The purified isopenicillin N synthetase (Table 1, stage 7) gave one major band and three very minor bands on analytical electrophoreis in polyacrylamide gel at pH 8. The relative intensity of the major band increased with the specific activity of the enzyme sample (Fig. 4). The major band of the sample with the highest specific activity accounted for about 83% of the total colour density. Only the material in the major band showed synthetase activity, and this band was absent after electrophoresis of enzyme samples that had become inactive on storage at 4°C. Isoelectric focusing revealed one major band corresponding to a protein with pI 5.0.

Under the conditions of assay and with the most active preparation of the synthetase obtained in the present work, the synthesis of isopenicillin N from (L-α-amino-δ-adipyl)-L-cysteinyl-D-valine in 10 min corresponded to a rate of approx. 38 μmol/min of protein. The rate of production of isopenicillin N by the synthetase extracted from a given weight of mycelium was at least an order higher than the rate of production of penicillin N and cephalosporin C together by the same weight of mycelium at the time of harvesting. This suggests that the intracellular enzyme was not functioning under optimal conditions in the strain of *C. acremonium* used, possibly because the synthesis of (L-α-amino-δ-adipyl)-L-cysteinyl-D-valine from amino acids was rate-limiting, or cofactors were deficient, or both.

The M_r (32000) reported for the synthetase from *C. acremonium* (Abraham *et al.*, 1981) is similar to that found by gel filtration in the present work for the enzyme from *P. chrysogenum*. [Dr. D. Perry has recently obtained a value for M_r of 40000 ± 1000 by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. Kupka *et al.* (1983) and Hollander *et al.* (1984) reported M_r values of about 31000 and 40000 for enzyme preparations from *C. acremonium* strains CW 19 and C-10 respectively. They did not comment on this apparent discrepancy, but comparable differences in M_r values for other proteins have been obtained by gel filtration and electrophoresis (Berks *et al.*, 1982).] A partially purified preparation of the latter enzyme contained a protein with the same mobility on electrophoresis as the enzyme from *C. acremonium*.

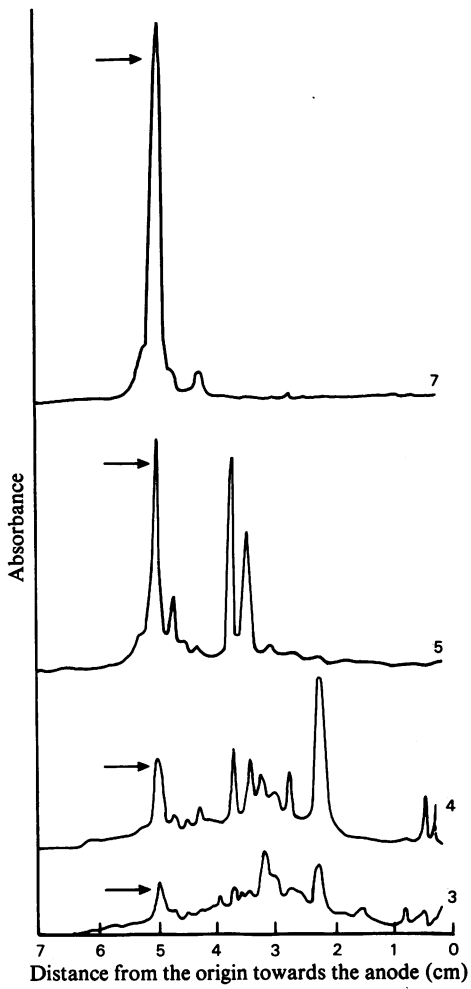


Fig. 4. *Gel electrophoresis of preparations of isopenicillin N synthetase*

Tracings 3, 4, 5 and 7 show the colour densities of protein bands (stained with Coomassie Blue) from samples at the stages of purification corresponding to those listed in Table 1. The enzyme coincided with the band (→) 5 cm from the origin.

The synthetases from both of these fungi and *Strep. clavuligerus* are stimulated by the same cofactors. In requiring Fe^{2+} and ascorbate for maximum activity they resemble the enzymes that catalyse the ring expansion of penicillin N to deacetoxycephalosporin C (Kupka *et al.*, 1983), the oxidation of the latter to deacetylcephalosporin C (Brewer *et al.*, 1977), and the oxidation of the cephalosporin ring system in *Streptomyces* spp. to yield 7α-methoxycephalosporins (O'Sullivan & Abraham, 1980). But they differ from the latter oxygenases in having no requirement for 2-oxoglutarate.

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