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A Pure Enzyme Catalyzing Penicillin Biosynthesis

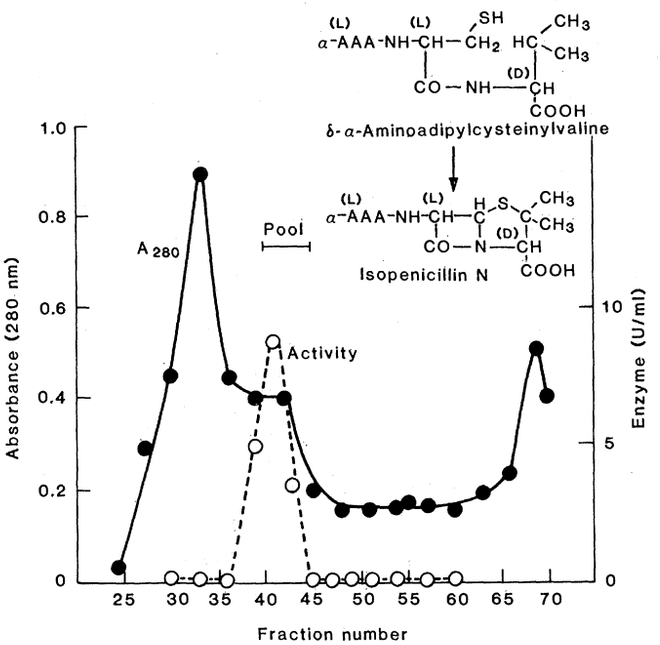
Abstract. *Isopenicillin N synthetase (cyclase) has been purified to homogeneity from* Cephalosporium acremonium *strain C-10. The enzyme has a molecular weight of 40,000 to 42,000 and yields a single band on sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The enzyme was purified in 10 percent yield by a combination of protamine sulfate and ammonium sulfate precipitations, gel filtration, and ion-exchange high-performance liquid chromatography. The purified enzyme can be stabilized with sucrose and stored at −20°C for several weeks without any loss in activity.*

None of the enzymes in the pathway from penicillin N to cephalosporin C (*1*) has been completely purified from any organism producing β-lactam antibiotics. We now describe our purification of a penicillin-forming enzyme isopenicillin N synthetase (cyclase) that converts the tripeptide δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine to isopenicillin N in the presence of O_2, Fe^{2+}, and ascorbate. We have been able to purify the enzyme to a homogeneous protein as determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) (*2*).

Slant cultures of *Cephalosporium acremonium* strain C-10 (*3*) were prepared on CM medium (percent: sucrose, 2; yeast extract, 0.4; peptone, 0.4; $NaNO_3$, 0.3; K_2HPO_4, 0.05; KH_2PO_4, 0.05; KCl, 0.05; $MgSO_4 \cdot 7H_2O$, 0.05; $FeSO_4 \cdot 7H_2O$, 0.001; and agar, 2) and were incubated at 25°C with 60 ± 10 percent humidity for 7 days. Slant growth, suspended in water, was added to a 500-ml Erlenmeyer flask containing 50 ml of C′ seed medium [percent: cornstarch, 4; corn steep liquor, 3; soybean meal, 1; $(NH_4)_2SO_4$, 0.1; $CaCO_3$, 0.3; and methyl oleate, 4.8 by volume]; the *p*H of the medium was adjusted to 7.0, and the mixture was autoclaved 40 minutes. The seed flask was incubated on a rotary shaker (50-mm throw, 25°C, 60 ± 10 percent humidity) for 54 hours. The seed culture (1.2 ml) was then added to 500-ml flasks containing 30 ml of fermentation medium (percent: sucrose, 2; cornstarch, 3; beet molasses, 5; soybean meal, 6; ammonium acetate, 0.8; $CaSO_4$, 1.25; $CaCO_3$, 0.5; and methyl oleate, 3 by volume); the *p*H was adjusted to 6.4 before autoclaving for 40 minutes, and the flasks were incubated as above for 60 hours.

The mycelia in each flask were harvested by centrifugation (8000*g*, 10 minutes), washed twice with 20 ml of ice-cold distilled water, suspended in 10 ml of ice-cold buffer A (50 m*M* tris-HCl, 10 m*M* $MgSO_4$, 10 m*M* KCl; *p*H 7.4 at 25°C) and sonicated (*4*). Cell debris was removed by centrifugation. Phenylmethylsulfonyl fluoride (PMSF) was added to the supernatant (1 m*M*), and the solution was frozen at −70°C until used. Upon thawing, fresh PMSF was added (1 m*M*) and the suspension was centrifuged at 20,000*g* (10 minutes, 2°C) to remove proteins precipitated in the cold.

The crude extract (70 ml from seven flasks) was treated (*5*) with protamine sulfate to remove nucleic acids and with solid $(NH_4)_2SO_4$ (enzyme grade) between 40 and 60 percent of saturation to remove other proteins. The pellet was resuspended in 2 to 4 ml of buffer A, centrifuged (20,000*g*, 10 minutes, 0°C), and placed in an ice bath during collection of the supernatant by pipette (some oily material that did not dissolve remained as a pellet as long as the temperature was kept low). The supernatant was then applied to an LKB Ultrogel AcA 54 column (1.6 by 90 cm) in buffer A at 4°C. Fractions were collected in tubes to which 50 percent sucrose (enzyme



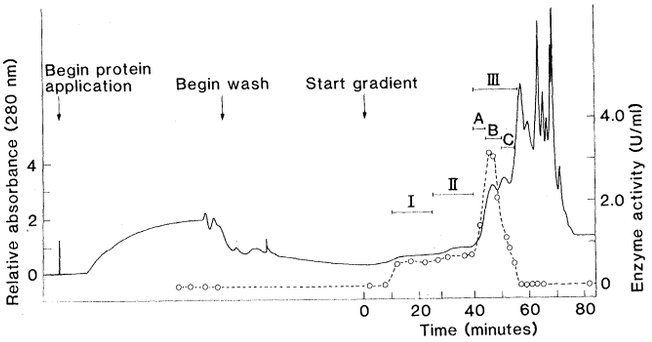


Fig. 1 (left). Gel filtration on LKB Ultrogel AcA 54. Fractions (40 drops = 2.3 ml) were collected at 10 ml/hour. Scheme depicting the reaction catalyzed by the enzyme is shown in the upper part of the figure. Fig. 2 (right). Ion exchange by HPLC (Waters Protein-Pak DEAE-5 PW). Diluted, *p*H-adjusted enzyme (40 ml) was applied at 1.0 ml/min. Detection of absorbency at 280 nm was by Waters model 440 absorbance detector set at 0.2 absorbance units full-scale. Enzyme was eluted by a 1-hour gradient (No. 10 on Waters model 660 solvent programmer).

grade) in buffer A had been added; the final sucrose concentration was 10 percent. The tubes were kept frozen after removal of equal portions for assay. Fractions exhibiting enzymatic activity were pooled, adjusted to *p*H 8.1 at 25°C with potassium hydroxide, and diluted with an equal volume of ice-cold distilled water.

The enzyme preparation was kept on ice, and samples were applied at a rate of 1.0 ml per minute to a high-performance liquid chromatography (HPLC) column equilibrated in buffer I (25 m*M* tris-HCl, 5 m*M* $MgSO_4$, 5 m*M* KCl, 5 percent sucrose; *p*H 8.1). The column was washed with buffer I at a rate of 1.0 ml per minute for 30 to 60 minutes until absorbancy at 280 nm became stabilized. The enzyme was eluted from the column with a 1-hour gradient (curve 10 on Waters model 660 solvent programmer) from 0 to 100 percent buffer II (250 m*M* tris-HCl, 10 m*M* $MgSO_4$, 10 m*M* KCl, 10 percent sucrose; *p*H 8.1). Fractions were collected and kept on ice. The enzyme activity was found in three fractions: two minor (at 15 to 35 minutes, respectively) and one major peak (at 45 minutes).

The active fractions were pooled separately and stored frozen at −20°C with no measurable loss of activity for at least 2 weeks. Enzyme activity was measured (*4*) with *Micrococcus luteus* (ATCC 381) as the test organism for the bioassay. Earlier studies have confirmed that the bioactive product is isopenicillin N by HPLC; ^3H incorporation; ^1H, ^{13}C, and ^{17}O nuclear magnetic resonance spectroscopy; and mass spectrometry (*6*).

Protein was determined by the method of Bradford (*7*) and by absorbancy at 280 nm. Purity and size were determined by SDS-PAGE procedures according to the method of Laemmli (*8*). The SDS low molecular weight kit (Sigma) was used for standards. The molecular weight of the native enzyme was determined by gel filtration on LKB Ultrogel AcA 54. Bovine serum albumin, ovalbumin, chymotrypsinogen, and ribonuclease A were used as molecular weight standards.

The native cyclase obtained from gel filtration had an apparent molecular weight of 41,000 ± 1,000 (Fig. 1). The enzyme activity coincided with the second major protein peak. The activity in the pooled fractions from gel filtration, when subjected to DEAE-HPLC ion exchange, was resolved into three areas of activity (Fig. 2) that contained 16 percent of the activity of the crude extract. Two-thirds of this activity (fractions I and II and fraction A of peak III) consisted of an electrophoretically homogeneous enzyme as shown by SDS-PAGE (Fig. 3). The molecular weight of the enzyme was determined by this method to be 40.5 ± 0.5K, which agrees well with the molecular weight of the native enzyme. Although the SDS-PAGE shows no apparent differences in electrophoretic mobility of the three pure enzyme fractions, there is a difference in specific activity between the first two fractions and fraction A of peak III (Table 1).

Table 1. Purification of cyclase from crude extract; N.D., not determined.

Treatment	Volume (ml)	Total protein (mg)	Total enzyme (U)	Yield (%)	Specific activity (U/mg)	Purification
Crude extract	68	58	245	100	4.2	1.0
Protamine sulfate precipitation	67	54	270	110	5.0	1.2
Ammonium sulfate precipitation	3.4	51	235	96	4.6	1.1
Gel filtration	20	6.2	100	41	16	3.8
HPLC fraction						
I	15	0.069	8.5	3.5	123	29
II	15	0.081	10	4.1	123	29
IIIA	4	0.032	5.5	2.2	170	40
IIIB	5	0.158	7.3	3.0	47	11
IIIC	6	N.D.	7.0	2.8	N.D.	N.D.
Total HPLC recovery:			38	16		
Fractions I + II + IIIA recovery:			24	9.8		

The difficulty that has arisen in attempts to purify this enzyme has probably been caused by the instability of the enzyme during purification. After gel filtration, the enzyme rapidly lost activity at 4°C (more than 50 percent in 24 hours) although it could be stored at −20°C overnight. Any further purification increased instability, and even freezing such purified fractions caused complete loss of activity. The addition of 5 to 10 percent sucrose slowed the inactivation at 4°C and allowed long-term storage in the frozen state.

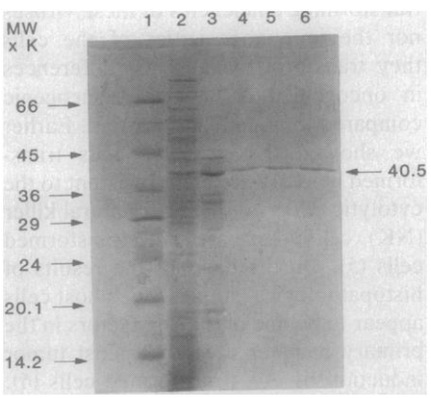


Fig. 3. Eleven percent SDS-PAGE (*9*) of enzyme preparations. (Lane 1) Molecular weight markers (Sigma); (lane 2) crude extract; (lane 3) enzyme pool after gel filtration; (lane 4) fraction I from HPLC; (lane 5) fraction II from HPLC; and (lane 6) fraction A of peak III from HPLC. The protein was stained with Coomassie blue R-250.

This instability led to our use of the newly developed DEAE-HPLC column. Although a similar elution pattern was observed with DEAE-Sepharose CL-6B, the enzyme (or enzymes) was incompletely separated from contaminating proteins. Most damaging was a loss of activity (80 to 100 percent), probably caused by excessive dilution of the enzyme (final concentrations were approximately 1 μg/ml) and the time (more than 10 hours) required for separation. On the other hand, HPLC was conducted in less than 2 hours and resulted in little, if any, dilution.

The cyclase was also destroyed by proteolytic enzyme (or enzymes) in the crude extract. In the absence of PMSF, we observed degradation of the enzyme to 39K, 25K, and lower molecular weight forms with concomitant decreases in activity.

There are at least two possible explanations for the multiple fraction pattern shown by HPLC-DEAE. It is unlikely that the multiple fractions are an aberration of the HPLC procedure since we have seen similar elution patterns on DEAE-Sepharose CL-6B. The three species may represent limited proteolytic (active) products of the original gene product resulting in slightly different affinities for a DEAE matrix. Alternatively, they may be cyclase isozymes, especially since strain C-10 is an industrial high-producing strain that has been subjected to multiple rounds of mutagenesis. Deriving the primary sequence of the three forms should resolve this question.

The cyclase reaction is intriguing to chemists who have been trying to elucidate the mechanism of this oxidative cyclization (*9*). Intermediates have been proposed, but most have failed to stand

the test of further experimentation. The availability of pure enzyme may facilitate the discovery of the reaction mechanism, some 55 years after the discovery of penicillin (*10*).

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Differential NK Cell and Macrophage Killing of Hamster Cells Infected with Nononcogenic or Oncogenic Adenovirus

Abstract. *Hamster cells infected with highly oncogenic human adenovirus type 12 (Ad12) were resistant to lysis by natural killer cells and macrophages, compared to cells infected with nononcogenic adenovirus type 2 (Ad2). The data suggest that early adenovirus gene expression in hamster cells results in preferential survival of Ad12, compared to Ad2, infected cells in vivo, thus providing an explanation for the differences in the oncogenicities of these two transforming viruses.*

Adenoviruses (Ad's) are common human pathogens and were the first human viruses observed to be oncogenic in rodents (*1*). On the basis of their oncogenicities for newborn hamsters, human Ad's were classified as highly oncogenic, weakly oncogenic, and nononcogenic (*2*). In spite of the marked differences in the tumor-inducing capacities of the viruses, Ad serotypes from both highly oncogenic and nononcogenic subgroups transform hamster and rat cells to a neoplastic state in vitro with approximately equal efficiency (*3*). Cell lines transformed in vitro by highly oncogenic Ad's (for example, Ad12) efficiently induce tumors in syngeneic adult hamsters, whereas cell lines transformed by nononcogenic Ad's (for example, Ad2) are either nontumorigenic or induce tumors only in immunodeficient hosts (*4*). All such Ad-transformed cells are highly immunogenic (*3*). Therefore, neither the transforming efficiencies of these viruses nor the immunogenicities of the cells they transform explains the differences in oncogenicities of highly oncogenic compared to nononcogenic Ad's. Earlier we showed that hamster cells transformed by Ad12 are more resistent to the cytolytic effects of hamster natural killer (NK) cells than are Ad2-transformed cells (*5*). On the basis of the results of histopathological studies, such host cells appear to be one of the key factors in the primary hamster defense against tumor induction by Ad2-transformed cells (*6*). Lytic infection by adenoviruses has not been shown to induce increased cellular susceptibility to lysis by NK cells or other host effector cells. We now report an increased cytolytic susceptibility in hamster cells infected with Ad2 compared to Ad12-infected cells, a phenomenon that is linked to the cellular accumulation of Ad2-encoded early polypeptides (T antigens). This suggestion of functional differences in the early gene products of Ad2 and Ad12 in the context of the host cellular immune response provides a new explanation for the differences in the tumor-inducing capacities of these two Ad serotypes.

To determine the cytolytic susceptibilities of cells infected with nononcogenic Ad2 and highly oncogenic Ad12, we used quantitative assays of target cell lysis by normal (nonimmune) hamster spleen cells (the functional hamster analog of the NK activity observed in other species) and bacillus Calmette-Guerin (BCG)–activated hamster macrophages in which the cytolytic susceptible cell line Ad2HE1 was used as a sensitive control (*5, 7*). The cytolytic activity of these preparations of spleen cells from unsensitized hamsters exhibits several of the properties of NK cells from other species, such as the lack of a requirement for specific sensitization for expression of lytic activity, the lack of genetic restriction, and the persistence of activity after depletion of adherent cells (*5*). Secondary LSH hamster embryo cells (HEC's) prepared from the inbred LSH strain of Syrian hamsters (*4*) were relatively resistant to activated macrophage-mediated lysis compared to Ad2HE1, but they expressed progressively increasing cytolytic susceptibility when infected with increasing multiplicities of Ad2 for 18 to 24 hours (Fig. 1). No significant viral replication appeared [less than 0.2 $\log_{10}$ plaque-forming units (PFU) per milliliter above the inoculum] as detected by production of viral progeny in one-step growth curve assays until approximately 30 hours after infection. The viral specificity of the induction of cytolytic susceptibility was confirmed by the abrogation of the effect with Ad2-specific neutralizing antiserum (Table 1) (*8*). In this same series of experiments the macrophage-resistant, SV40-transformed LSH hamster cell line SV40HE1 (*5, 9*) also exhibited increased susceptibility to macrophage-mediated lysis 18 to 24 hours after infection with Ad2.

In kinetic studies with a multiplicity of infection of 10 PFU per cell, an association between the induction of cytolytic susceptibility in Ad2-infected HEC's and the accumulation of Ad2 T antigens in target cells was shown (Fig. 2). A second method to evaluate the role of early Ad2 polypeptides in the induction of cytolytic susceptibility was to block viral DNA synthesis and thus the expression of late viral polypeptides by infecting cells and performing cytolysis assays in the pres-