ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Mar. 1985, p. 380–387
0066-4804/85/030380-08$02.00/0

Vol. 27, No. 3

Isopenicillin N Synthetase of *Penicillium chrysogenum*, an Enzyme That Converts δ-(L-α-Aminoadipyl)-L-Cysteinyl-D-Valine to Isopenicillin N

FILOMENA R. RAMOS, MANUEL J. LÓPEZ-NIETO, AND JUAN F. MARTÍN*

Departamento de Microbiología, Facultad de Biología, Universidad de León, León, Spain

Received 7 August 1984/Accepted 7 December 1984

The tripeptide δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine, an intermediate in the penicillin biosynthetic pathway, is converted to isopenicillin N by isopenicillin N synthetase (cyclase) of *Penicillium chrysogenum*. The cyclization required dithiothreitol and was stimulated by ferrous ions and ascorbate. Co^{2+} and Mn^{2+} completely inhibited enzyme activity. Optimal temperature and pH were 25°C and 7.8, respectively. The reaction required O_2 and was stimulated by increasing the dissolved oxygen concentration of the reaction mixture. Purification of the enzyme to a single major band in polyacrylamide gel electrophoresis was achieved by protamine sulfate precipitation, ammonium sulfate fractionation (50 to 80% of saturation), DEAE-Sephacel chromatography, and gel filtration on Sephacryl S-200. The estimated molecular weight was 39,000 ± 1,000. The apparent K_m of isopenicillin N synthetase for δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine was 0.13 mM. The enzyme activity was strongly inhibited by glutathione, which acts as a competitive inhibitor. A good correlation was observed between the isopenicillin N synthetase activity in extracts of four different strains of *P. chrysogenum* (with widely different penicillin-producing capability) and the amount of penicillin production by these strains.

The enzymes carrying out the conversion of the precursor amino acids (α-aminoadipic acid, cysteine, and valine) into penicillin G have not been purified and characterized in spite of the industrial importance of this antibiotic (6). The three component amino acids are linked to form the tripeptide intermediate δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV) in the initial step(s) of the pathway (1, 5; M. J. López-Nieto, Ph.D. thesis, University of Salamanca, Salamanca, Spain, 1984). In the biosynthetic pathway of cephalosporins the ACV tripeptide is converted to isopenicillin N by cyclization (11, 21) (see Fig. 1). The same cyclization process appears to occur in *Penicillium chrysogenum* (19).

Purification of isopenicillin N synthetase (cyclase) of the cephalosporin producer *Cephalosporium acremonium* (syn. *Acremonium chrysogenum*) has been reported recently (8). This enzyme, which carries out in vitro the cyclization of ACV to isopenicillin N, has also been reported in extracts of *Streptomyces clavuligerus* (9) and *Nocardia lactamdurans* (5a).

The isopenicillin N synthetase of *P. chrysogenum* has not been purified and characterized so far. Preliminary evidence of the cyclization of the ACV tripeptide into an isopenicillin-like compound was reported by Meesschaert et al. (19). However, the existence of a monocyclic intermediate proposed by these authors has not been confirmed (6).

Good knowledge of the enzymes involved in the penicillin biosynthetic pathway is necessary to advance the field of genetic manipulation of penicillin-producing microorganisms, something which so far has been approached in a purely empirical way (17). This contribution reports the isolation and characterization of the isopenicillin N synthetase of *P. chrysogenum*.

* Corresponding author.

MATERIALS AND METHODS

Microorganisms. Strains of *P. chrysogenum* used in this work are listed in Table 1. Purification and characterization of isopenicillin N synthetase was done by using cell-free extracts of the high-penicillin-producing strain *P. chrysogenum* AS-P-78 (16). Other strains of *P. chrysogenum* were used in comparative studies. *Bacillus subtilis* ATCC 6633, *Micrococcus luteus* ATCC 9341, and *Escherichia coli* Ess 22-31 (Table 1) were used for the routine determination of penicillin G and isopenicillin N. Strains were kept lyophilized.

Media and culture conditions. Inoculum of *P. chrysogenum* was developed in complex seed medium, and 2.5 ml was used to inoculate 50 ml of complex production medium in 500-ml flasks as described previously (15, 16). *Penicillium* cultures were incubated for 120 h at 25°C.

Penicillin G was measured in the culture broths after centrifugation at 15,000 × *g* for 10 min. Bioassays and colorimetric determination of penicillin G were carried out as described before (16).

Cell-free extracts. Mycelium of *P. chrysogenum* cultures in complex production medium was collected at 24 h of cultivation, except in the isopenicillin N synthetase time-course experiments. The mycelium (100 ml of culture) was collected by filtration, washed four times with NaCl (9 g · $liter^{-1}$) to remove penicillin G that adhered to the cell walls, and resuspended in 0.03 M Tris-hydrochloride buffer (pH 8.0) containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and 0.1 mM dithiothreitol (DTT; Sigma Chemical Co.) (TPD buffer) to a final density of 1 g (wet weight) of mycelium per 10 ml of buffer.

The mycelium was disrupted by either sonication or

TABLE 1. Characteristics of strains used

Strains	Characteristics	Reference(s)
P. chrysogenum AS-P-78	High penicillin producer	16, 20
P. chrysogenum Wis 54-1255	Intermediate penicillin producer	15, 16
P. chrysogenum Wis 49-2105A	Low penicillin producer; Leu^- $PABA^-$	2
P. chrysogenum Wis 49-2105B	Low penicillin producer; Leu^- Met^-	2
B. subtilis ATCC 6633	Standard test strain for penicillin determination	16
M. luteus ATCC 9341	Very sensitive to isopenicillin N; test strain for isopenicillin N determination	9, 12
E. coli ESS 22-31	Supersensitive mutant to penicillins and cephalosporins	11, 23

mechanical disintegration with glass beads. Sonication was carried out in an ice bath with a Branson Sonifier B-12 (setting no. 8) during 20-s periods at intervals of 1 min. Optimal total sonication times were 5 min for strain AS-P-78 and 3 min for strains Wis 54-1255 and Wis 49-2105. Glass bead (0.25-mm diameter; Ballotini no. 12) disruption was carried out in a Braun MSK mechanical disintegrator by using liquid CO_2 refrigeration during 3 periods of 20 s at 1-min intervals. Wet mycelium and glass beads were mixed in a proportion of 2:1 (vol/vol).

The extracts (after separation of the glass beads in the second method) were ultracentrifuged at 100,000 × *g* for 1 h at 4°C (Beckman L8-70 ultracentrifuge). This ultracentrifuged crude extract was stored frozen at −20°C.

Isopenicillin N synthetase reaction. The reaction mixture contained crude or purified (see below) cell-free extract (2 mg of protein · ml^{-1}, unless otherwise stated), 150 μl; ACV substrate (dimer form), 0.2 mM; $FeSO_4$, 0.1 mM; ascorbic acid (Sigma), 1 mM; DTT, 2 mM in a total volume of 250 μl of 0.03 M Tris-hydrochloride buffer (pH 8.0). Concentrations of ACV or protein were changed in experiments to study the effect of substrate or enzyme in the reaction. The reaction mixture was incubated for 30 min at 25°C and stopped by addition of an equal volume (250 μl) of methanol. In the experiments on the enzyme kinetics and on the effect of temperature on enzyme activity, the samples were incubated for only 15 min. The reaction mixture was not agitated, except in experiments to measure the oxygen requirement. Control reactions were always carried out without substrate or with heat-inactivated enzyme. Protein was measured by the method of Lowry et al. (14). Enzyme activity is given as nanokatals (nanomoles of isopenicillin N formed per second) per gram of protein.

Determination of isopenicillin N. After the reaction was stopped with methanol, triplicate (50-μl) samples were applied to wells 7 mm in diameter in plates containing 15 ml of tryptic soy agar (Difco Laboratories) medium inoculated with *M. luteus* (0.5 ml of a culture with an optical density of 1). Controls of pure isopenicillin N (Gist-Brocades, Delft, The Netherlands) (10 to 200 μg · ml^{-1}) were treated with methanol like the reaction samples. The samples in the assay plates were kept at 4°C for 3 h to allow diffusion and then incubated at 30°C for 12 h. A very good correlation was obtained in the bioassay between the diameters of the inhibition zones and the logarithm of isopenicillin N concentration (see Fig. 2, insert).

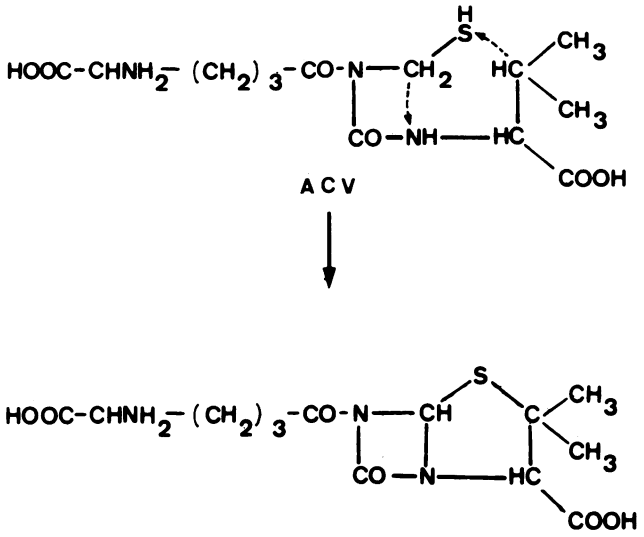
A sample of the reaction product was treated with penicillinase (cephalosporinase free) of *Bacillus cereus* UL1 (10 μg/100 μl of reaction sample incubated at 35°C for 1 h) (23). The product of the reaction was destroyed by the penicillinase treatment. The nature of isopenicillin N formed was established by high-pressure liquid chromatography by using a Varian 5000 chromatograph with a micro-Pak CH-10 30-cm column. Methanol-precipitated samples were centrifuged (16,000 × *g*, 10 min) to remove precipitated protein before injection. Isopenicillin N from the reaction mixture eluted simultaneously with an authentic sample of isopenicillin N under the conditions described by Jensen et al. (10), with a retention time of 5.2 min.

Purification of isopenicillin N synthetase. Ultracentrifuged crude extract (100 ml) (7.5 mg of protein · ml^{-1}) was mixed in an ice bath with 10 ml of a concentrated solution of protamine sulfate (20 g · $liter^{-1}$) in TPD buffer with agitation to precipitate nucleic acids. The mixture was kept in ice without agitation for 15 min and then centrifuged at 20,000 × *g* for 30 min at 4°C. The pellet was discarded, and the supernatant was precipitated with ammonium sulfate (enzyme grade). The fraction precipitated between 50 and 80% saturation was collected, dissolved in 4 ml of the same buffer, and centrifuged again at 20,000 × *g* for 20 min at 4°C to remove the residual pellet. Excess ammonium sulfate was removed by dialysis at 4°C against 1,000 volumes of the same buffer for 12 h, changing the buffer twice.

The supernatant was then applied to a DEAE-Sephacel (Pharmacia) column (15 by 2.5 cm) equilibrated with TPD buffer. The column was eluted with a Tris-hydrochloride gradient from 0.03 to 1 M (pH 8.0) supplemented with 0.1 mM DTT and 1 mM PMSF with a flow rate of 28 ml · h^{-1}. Fractions (5 ml) were collected, and aliquots were assayed directly for isopenicillin N synthetase. All the process was carried out at 2°C, and the fractions were immediately frozen at −20°C.

For molecular weight determination the active fractions obtained from ion-exchange chromatography were concentrated to 1 ml in a dialysis bag by using polyethylene glycol 20,000 (Sigma) and applied to a Sephacryl S-200 superfine (Pharmacia) column (52 by 1.5 cm) equilibrated previously with TPD buffer. The column was eluted with the same buffer with a flow rate of 13.5 ml · h^{-1}. The protein profile of the fractions was followed directly with a UV protein monitor (Pharmacia). Fractions (4.5 ml) were collected, assayed for isopenicillin N synthetase, and frozen immediately. Blue dextran (molecular weight, 2,000), RNase A (molecular weight, 13,700), chymotrypsinogen A (molecular weight, 25,000), ovalbumin (molecular weight, 43,000), and albumin (molecular weight, 67,000) (Sigma) were used to calibrate the column.

PAGE. Polyacrylamide gel electrophoresis (PAGE) was carried out by using 5 mM Tris-hydrochloride–30 mM glycine (pH 8.3) buffer, supplemented with 0.1 mM DTT and 1 mM PMSF as developing buffer (22). Proteins were stained with brillant blue R (Sigma).



ISOPENICILLIN N

FIG. 1. Cyclization of ACV to isopenicillin N by isopenicillin N synthetase. The dotted arrows indicate the C—N and C—S bonds that are formed during the reaction.

Chemicals. ACV (dimer form) was kindly provided by E. P. Abraham and J. E. Baldwin, Oxford University, Oxford England. Isopenicillin N was a gift of P. van Dijck, and P. van Dijck, Gist Brocades, Delft, The Netherlands. All other chemicals were reagent quality.

RESULTS

Cyclization of ACV tripeptide to isopenicillin N. Cyclization of ACV to isopenicillin N (Fig. 1) was shown by the accumulation of an antibiotic substance with the characteristic antimicrobial spectrum of isopenicillin N (i.e., the antibiotic activity against *M. luteus* was much higher than that against *E. coli* Ess 22-31) (9, 11, 12) and showing the same retention time as an authentic sample of isopenicillin N in high-pressure liquid chromatography. The product of the reaction was completely inactivated by penicillinase of *B. cereus* UL1. The isopenicillin N formed was linearly dependent on the concentration of protein up to 1 mg · ml^{-1} (Fig. 2a). Formation of isopenicillin N was linear up to 15 min, when the reaction was stopped by addition of 50% methanol (Fig. 2b). When methanol was not added, the reaction proceeded during the bioassay even at low temperature.

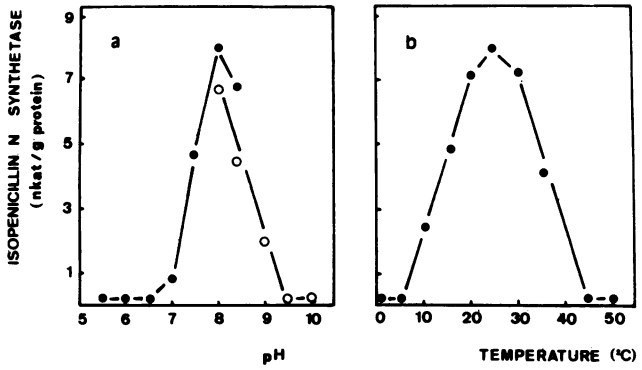


FIG. 3. Effect of pH (●, 0.03 M MOPS [morpholinepropanesulfonic acid] buffer; ○, 0.03 M Tris-hydrochloride buffer) (a) and temperature (b) on the activity of isopenicillin N synthetase. Incubations in these experiments were carried out for 15 min. The enzyme extract used was as described in the legend to Fig. 2.

Optimal pH and temperature. Optimal pH for the isopenicillin N synthetase was between 7.6 and 8.0 (Fig. 3a). The enzyme showed little activity below pH 6 or above pH 9.

Optimal temperature for the enzyme (determined in the linear range of the reaction after 15 min of incubation) was 25°C, but the enzyme worked below 10°C and even above 35°C (Fig. 3b).

Effect of DTT, ferrous ions, and ascorbate. DTT reduced the dimer of the ACV tripeptide, i.e., bis-ACV-disulfide, to the monomer. The cyclization was strictly dependent on the addition of DTT (Fig. 4a). When bis-ACV was used as substrate in the absence of DTT, no isopenicillin N was formed. Cyclase activity markedly increased with increasing DTT concentration up to 0.5 mM.

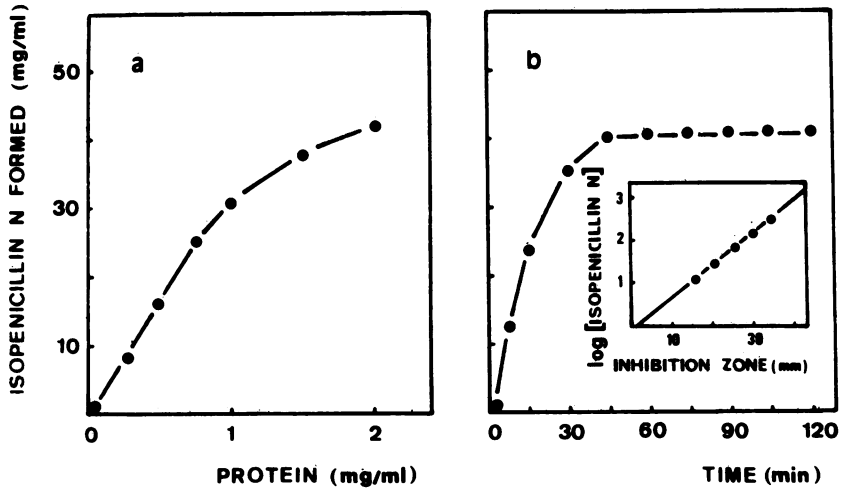


FIG. 2. Effect of increasing concentrations of protein in the extract (a) and time of incubation (b) on the formation of isopenicillin N. Insert, Correlation of the logarithm of isopenicillin N concentration in the bioassay with the diameter of the inhibition zone formed. A dialyzed protamine sulfate precipitate was used as the enzyme extract.

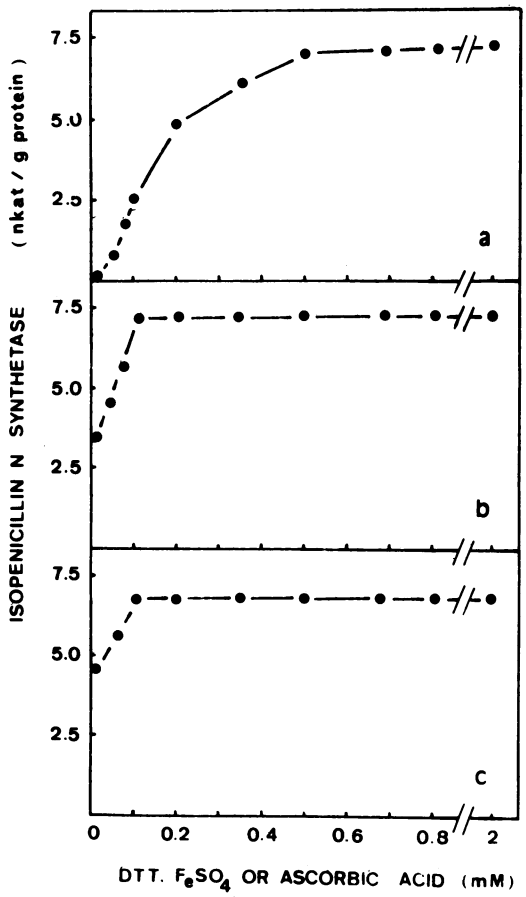


FIG. 4. Effect of DTT (a), $FeSO_4$ (b), and ascorbic acid (c) on isopenicillin N synthetase activity. The enzyme extract used was as described in the legend to Fig. 2.

Addition of ferrous sulfate up to 0.1 mM doubled the activity of isopenicillin N synthetase (Fig. 4b). Addition of ferric instead of ferrous ions did not exert any stimulatory effect at any concentration. Ascorbate (up to 0.1 mM) had a moderate activation effect (Fig. 4c). α-Ketoglutarate had no effect on the cyclase activity. Membrane solubilization by addition of 0.5 mM Triton X-100 to the crude extracts (before centrifugation) did not increase the cyclase activity. ATP was not required for the cyclization process, nor did it exert any inhibitory effect.

TABLE 2. Effect of different ions on isopenicillin N synthetase activity[a]

Addition (0.5 mM)	Isopenicillin N synthetase (nkat · g of protein^{-1})	% Inhibition
None (control)	65.3	0
K^+	65.3	0
Na^+	65.3	0
Ca^{2+}	65.3	0
Co^{2+}	0	100
Cu^{2+}	40.6	37
Cd^{2+}	65.3	0
Hg^{2+}	65.3	0
Mg^{2+}	65.3	0
Mn^{2+}	0	100
Sn^{2+}	8.0	87
Zn^{2+}	40.9	38

[a] All ions were added as chlorides at the concentration indicated to a Fe^{2+}-containing (0.1 mM) reaction mixture by using a dialyzed extract after protamine sulfate precipitation.

TABLE 3. Oxygen requirement for isopenicillin N synthetase activity[a]

Expt condition	Sp act (nkat · g of protein^{-1})	% Inhibition or activation
Control	67.5	
Under nitrogen atmosphere	7.2	89 (inhibition)
Aerated (bubbling air)	112.5	66.6 (activation)
Oxygenated (bubbling O_2)	72.7	7.7 (activation)

[a] A dialyzed cell-free extract after protamine sulfate precipitation was used.

Effect of other ions. The effect of several monovalent and divalent ions at 0.5 mM on enzyme activity is shown in Table 2. Co^{2+} and Mn^{2+} exerted 100% inhibition of enzyme activity, probably due to competition with Fe^{2+}, whereas Sn^{2+}, Zn^{2+}, and Cu^{2+} produced inhibitions of 87, 38, and 37%, respectively. The other ions tested did not affect the enzyme activity.

Requirement for oxygen. The cyclization reaction in vivo is an oxidative process and requires molecular oxygen (3, 4). To test for this requirement in vitro, the reaction was carried out under a nitrogen atmosphere, or the reaction mixture was saturated with oxygen by bubbling air or pure oxygen through it. The results (Table 3) indicate that oxygen is required for the reaction to take place. Almost no enzyme activity was detected in the absence of oxygen (nitrogen atmosphere). When the dissolved oxygen concentration of the reaction mixture was increased by passing air through the mixture, the activity of the enzyme increased by 66%. Even enzyme preparations with little residual activity experienced the same activation when aerated. When pure oxygen was bubbled through the reaction mixture, there was also some degree of stimulation, although the activation was smaller than that with air, probably because excess oxygen oxidizes the reduced substrate (ACV) and cofactors (DTT and Fe^{2+}) required for the reaction.

Purification of isopenicillin N synthetase. Crude extracts were purified as indicated in Table 4, always keeping the extract in presence of 1 mM PMSF and 0.1 mM DTT. A fivefold purification was achieved by precipitation of nucleic acids with protamine sulfate. Most of the contaminantproteins were removed during fractionation (50 to 80%) with ammonium sulfate. However, enzyme inactivation occurred during removal of ammonium sulfate. Isopenicillin N synthetase was retained by filtration in DEAE-Sephacel and eluted with a gradient (0.03 to 1 M) of Tris-hydrochloride buffer (pH 8) containing 0.1 mM DTT and 1 mM PMSF. The

TABLE 4. Purification of isopenicillin N synthetase

Treatment	Vol (ml)	Protein (mg · ml^{-1})	Total enzyme (nkat)	Yield (%)	Sp act (nkat · g of protein^{-1})
Crude extract	100	7.5	11.0	100	14.6
Protamine sulfate precipitation	40	4.2	10.8	98.0	65.3
Ammonium sulfate precipitation and dialysis	15	0.5	0.9	8.3	122.0
DEAE-Sephacel filtration	3	0.88	0.5	4.9	205.3

enzyme activity eluted at 0.25 M Tris-hydrochloride, resulting in an additional twofold purification. The enzyme was rapidly inactivated during purification. However, a good degree of purification could be achieved, as determined by PAGE (see below).

Determination of molecular weight. The molecular weight determined by gel filtration on Sephacryl-S200 was 39,000 ± 1,000 (Fig. 5).

The purified enzyme showed a main band in PAGE that correlated with the mobility of a protein with a molecular weight of 39,000 and traces of other minor bands (Fig. 6).

Crude extracts were stable and could be maintained at −20°C for more than 6 months in 0.03 M Tris-hydrochloride buffer (pH 8.0) even in absence of PMSF and DTT. Preparations of the enzyme purified by gel filtration were very unstable and lost activity at 4°C, although they could be stored frozen at −20°C for 48 h. When the purified extracts were supplemented with 1 mM PMSF and 0.1 mM DTT, the enzyme activity was maintained at −20°C for at least 4 months.

Substrate kinetics. The effect of substrate concentration was studied by using a DEAE-Sephacel- and Sephacryl-S200-purified enzyme (Fig. 7). Increasing concentrations of ACV up to 0.6 mM stimulated the velocity of the reaction. The calculated K_m for ACV was 0.13 mM.

Inhibition of the cyclase by glutathione. Glutathione (L-γ-glutamyl-L-cysteinyl-L-glycine) is a structural analog of ACV (see below). Glutathione did not appear to function as a substrate of the cyclization reaction (no antibiotic activity was formed), but it strongly inhibited the reaction with ACV as substrate. The inhibition was higher with increasing concentrations of glutathione (Fig. 8). By using the Dixon plot, an apparent K_i of 8.9 mM for glutathione was obtained. The inhibition appears to be competitive.

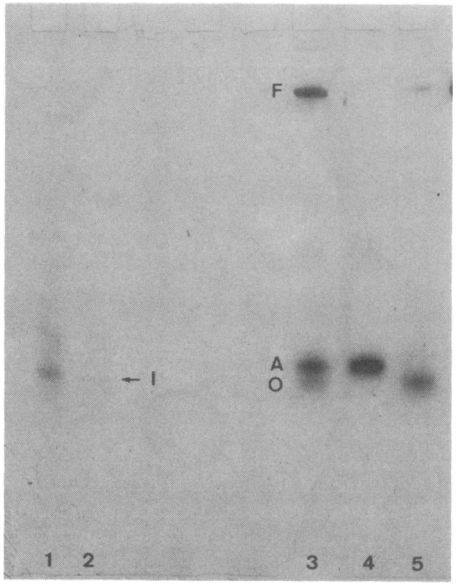


FIG. 6. PAGE of isopenicillin N synthetase after gel filtration by Sephacryl S-200. Tracks 1 and 2, Isopenicillin N synthetase (62 and 25 μg, respectively); track 3, mixture of ferritin (F), ovalbumin (O), and albumin (A); track 4, albumin; track 5, ovalbumin.

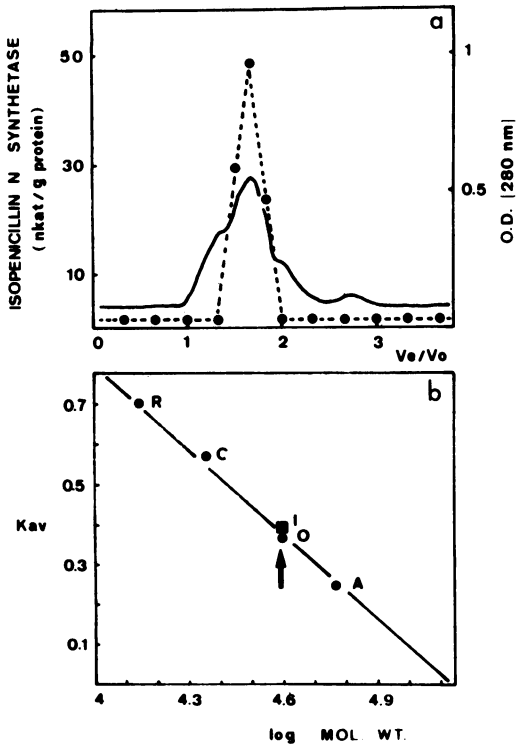


FIG. 5. Purification of isopenicillin N synthetase by filtration on Sephacryl S-200. (a) Elution pattern of isopenicillin N synthetase (dotted line) and protein (solid line). (b) Correlation of the logarithm of the molecular weight with the K_{av} [$K_{av} = (V_e - V_0)/(V_t - V_0)$] of known standards: R, RNase; C, chymotrypsinogen; O, ovalbumin; and A, bovine albumin. I, Isopenicillin N synthetase.

Isopenicillin N synthetase in strains with different penicillin-producing capability. To test whether penicillin-producing ability of different strains of *P. chrysogenum* is related to the cyclase activity level, cell-free extracts of four different strains that produced widely different levels of penicillin were prepared (Table 5). Penicillin production by the two mutants derived from Wis 49-2105 was 70 and 90 μg · ml^{-1}, respectively, whereas strain Wis 54-1255 produced 810

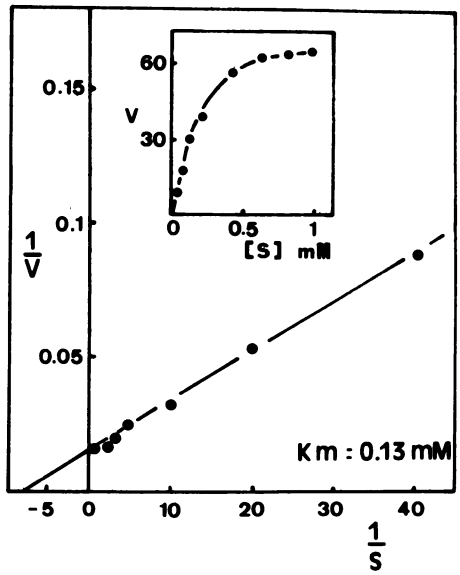


FIG. 7. Kinetics of isopenicillin N synthetase activity versus substrate (ACV) concentration. An enzyme purified by Sephacryl S-200 gel filtration was used.

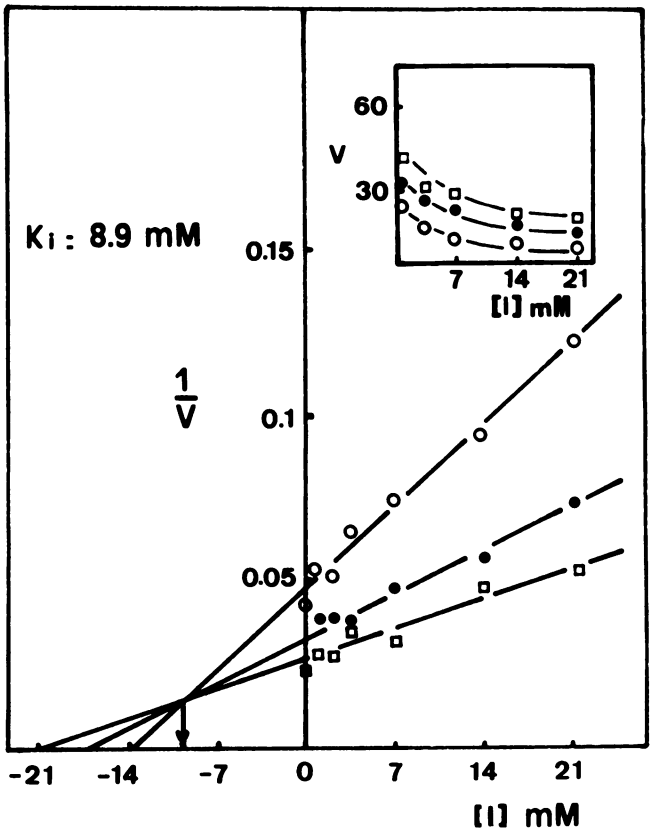


FIG. 8. Kinetics of isopenicillin N synthetase activity versus glutathione (inhibitor) concentration, with ACV as substrate (○, 0.1 mM ACV; ●, 0.2 mM ACV; □, 0.4 mM ACV).

$\mu g \cdot ml^{-1}$, and the high-producing AS-P-78 strain reached 4,520 $mg \cdot ml^{-1}$ in the same experiment. Strains 49-2105 A and B had only barely detectable traces of cyclase activity, whereas *P. chrysogenum* 54-1255 contained ca. 16% of the activity of the high-producing strain AS-P-78.

Time-course of isopenicillin N-synthetase. The isopenicillin N synthetase activity during the course of penicillin fermentations was determined in extracts of the high-producing strain AS-P-78 (Fig. 9). Isopenicillin N synthetase was not formed in the first 12 h of incubation, during which fast growth occurred. After a downshift in growth rate, cyclase activity was rapidly formed, reaching peak levels between 18 and 24 h. No penicillin was yet synthesized at 24 h. Thereafter, the level of isopenicillin N synthetase decreased slowly, keeping an intermediate value up to 120 h, and penicillin G was accumulated (see below).

TABLE 5. Isopenicillin N synthetase activity of strains with different penicillin-producing ability

Strain	Penicillin produced ($\mu g \cdot ml^{-1}$)	Isopenicillin N synthetase[a]	
		Sp act (nkat · g of protein^{-1})	% Activity
P. chrysogenum AS-P-78	4,500	121.7	100
P. chrysogenum Wis 54-1255	810	18.3	16.2
P. chrysogenum Wis 49-2105A (Leu$^-$ PABA$^-$)	70	Traces	<1.0
P. chrysogenum Wis 49-2105B (Leu$^-$ Met$^-$)	90	Traces	<1.0

[a] In the 50 to 80% fraction of ammonium sulfate saturation.

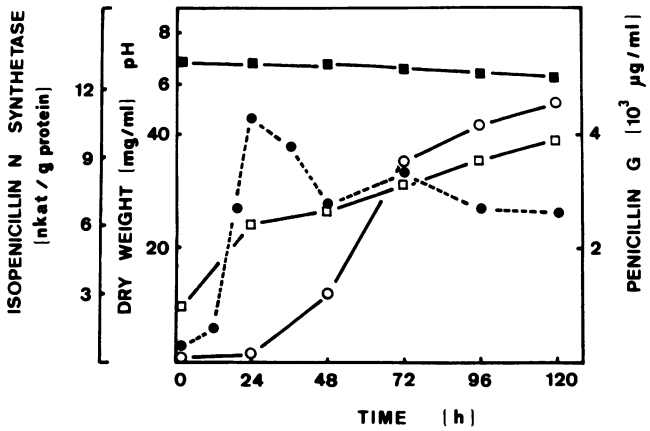


FIG. 9. Time-course of growth (cell dry weight [□], isopenicillin N synthetase activity [●], and penicillin G formation [○]) during a long-term culture of *P. chrysogenum* in complex production medium for penicillin production. ■, pH.

DISCUSSION

Isopenicillin N synthetase (cyclase) of *P. chrysogenum* converts the tripeptide intermediate ACV into isopenicillin N. The efficiency of conversion under the best experimental conditions was between 80 and 95% in different experiments. The optimal pH (7.6 to 8.0) and temperature (25°C) for the cyclase (Fig. 2 and 3) are consistent with the pH (6.5 to 7.5) and temperature (25°C) used for penicillin production. The broad range of temperature shown by the cyclase activity suggests that the temperature is not a critical parameter for the cyclization step of penicillin biosynthesis.

The enzyme requires O_2 (Table 3) and ferrous ions and is stimulated by ascorbate (Fig. 4), all of which are cofactors of oxygenases. Cyclization, therefore, appears to proceed by oxygen-mediated removal of hydrogen atoms during formation of the C—N and C—S bonds of the β-lactam and thiazolidine rings (Fig. 1). Similar requirements have been described for the isopenicillin N synthetase of *C. acremonium* (11, 21), *S. clavuligerus* (9), and *N. lactamdurans* (5a). ATP and α-ketoglutarate were not required for the reaction.

The role of DTT in the reaction is complex. The dimer ACV is reduced to the monomer by DTT (10), and this could explain the requirement of DTT for the reaction. Our results suggest that the monomer form of ACV is the true substrate of the reaction. Moreover, DTT has been found to play an important role in stabilizing the enzyme against inactivation during storage, indicating that thiol groups of the enzyme are essential for the reaction. The role of DTT appears therefore to be dual: (i) protecting the enzyme against inactivation by oxygen (itself required in the reaction) and (ii) keeping the substrate in the adequate monomer form.

The strong inhibition of Co^{2+} and Mn^{2+} may be explained on the basis of competition with Fe^{2+}. Other divalent (but not monovalent) cations also exerted a partial inhibitory effect, supporting this hypothesis.

The cyclase appears to be soluble since it is easily released by sonication (or by glass bead disruption), and enzyme activity is not increased by membrane solubilization with Triton X-100. A 15-fold purification of the cyclase was obtained by a combination of protamine sulfate precipitation and ammonium sulfate fractionation, followed by dialysis, ion-exchange chromatography on DEAE-Sephacel, and gel

filtration on Sephacryl S-200. However, a significant inactivation of the enzyme was observed during dialysis after ammonium sulfate fractionation and also during gel filtration. Instability of the cyclase of *C. acremonium* has also been reported (8).

The estimated molecular weight by gel filtration was 39,000 ± 1,000 (Fig. 5a), which is similar to the reported molecular weight of the isopenicillin N synthetase of *C. acremonium*, initially estimated as 31,000 (12) and recently revised to 40,000 to 42,000 (8). The estimated molecular weight is consistent with the mobility of the band in PAGE (Fig. 6).

The purified enzyme showed an apparent K_m for ACV of 0.13 mM, which is lower than the reported value for *C. acremonium* (K_m = 0.3 mM) (12), suggesting that the enzyme of *P. chrysogenum* AS-P-78 may have a slightly higher substrate affinity that the enzyme of *C. acremonium*.

The cyclase activity is inhibited by glutathione (γ-glutamylcysteinyl-glycine), which acts as a competitive inhibitor of ACV. The relationship between both peptides in relation to penicillin biosynthesis is interesting. Glutathione is always present in large amounts in extracts of *P. chrysogenum* (López-Nieto, Ph.D. thesis). It is likely, therefore, that glutathione may act as an intracellular inhibitor of penicillin biosynthesis at the isopenicillin N synthetase level. Glutathione was not converted into an antibiotic derivative. Similarly, γ-L-glutamyl-L-cysteinyl-D-valine is not cyclized by the isopenicillin N synthetase of *C. acremonium*, although the adipyl derivative was converted into an antibiotic substance (12).

P. chrysogenum strains with different abilities to produce penicillin differ greatly in isopenicillin N synthetase activity. Strain Wis 54-1255 originated from strain Wis 49-2166 (a sister strain of Wis 49-2105) by eight nitrosoguanidine and UV mutageneses and clone screening steps (7). These mutations have resulted in a drastic increase in isopenicillin N synthetase (Table 5) that correlates with an increase in penicillin titers from less than 100 $\mu g \cdot ml^{-1}$ in Wis 49-2105 (13) to ca. 800 $\mu g \cdot ml^{-1}$ in Wis 54-1255. The high-producing strain AS-P-78 (producing ca. 4,500 $\mu g \cdot ml^{-1}$ under the same conditions) contained a sixfold-higher isopenicillin N synthetase activity than that of Wis 54-1255, thus correlating well with the five- to sixfold increase in penicillin production observed.

Isopenicillin N was formed after the initial rapid growth phase of the culture (Fig. 9). This sequential formation of isopenicillin N synthetase (18 h after growth) and penicillin G (24 to 36 h after growth) is characteristic of fungal secondary metabolites (18). Formation of isopenicillin N synthetase (21), onset of penicillin N, and conversion of penicillin N to cephalosporin C (23) are also known to be delayed with respect to growth in *C. acremonium*. A high level of cyclase activity was present throughout the penicillin fermentation, in contrast to the rapid disappearance of the cyclase in *C. acremonium* and *S. clavuligerus* (9, 21). This might account for the continued synthesis of penicillin during at least 120 h by the high-penicillin-producing strain *P. chrysogenum* AS-P-78.

ACKNOWLEDGMENTS

This research was supported by grants from the Comisión Asesora de Investigación Científica y Técnica, Madrid, and Antibióticos, S.A. León, Spain.

We are greatly indebted to E. P. Abraham and J. E. Baldwin (Oxford University, Oxford, England) for ACV and to P. van Dijck (Gist-Brocades, Delft, Holland) for isopenicillin N and ACV.

LITERATURE CITED

1. **Adriaens, P., B. Meesschaert, W. Wuyts, H. Vanderhaeghe, and H. Eyssen.** 1975. Presence of δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine in fermentations of *Penicillium chrysogenum*. Antimicrob. Agents Chemother. **8**:638–642.
2. **Anne, J., and J. F. Peberdy.** 1976. Induced fusion of fungal protoplasts following treatment with polyethylene gycol. J. Gen. Microbiol. **92**:413–417.
3. **Bahadur, G., J. E. Baldwin, T. Wan, M. Jung, E. P. Abraham, J. A. Huddleston, and R. L. White.** 1981. On the proposed intermediacy of β-hydroxyvaline and thiazepinone-containing peptides in penicillin biosynthesis. J. Chem. Soc. Chem. Commun. **1981**:1146–1147.
4. **Baldwin, J. E., A. P. Davis, and L. D. Field.** 1982. The stereochemistry of cyclisation of *N-t*-butyldihydrocinnamamide to a β-lactam. Tetrahedron **38**:2777–2782.
5. **Chan, J. A., F. C. Huang, and C. J. Sih.** 1976. The absolute configuration of the amino acids in δ-(α-aminoadipyl)-cysteinyl-valine from *Penicillium chrysogenum*. Biochemistry **15**:177–180.

5a. **Cortes, J., J. M. Castro, P. Liras, and J. F. Martin.** 1984. Enzymes involved in carbon catabolite regulation of β-*lactam biosynthesis in streptomyces*, p. 21–28. *In* reprints "Third European Congress of Biotechnology," vol. I. Verlag-Chemie, Weinheim, Federal Republic of Germany.

6. **Demain, A. L.** 1983. Biosynthesis of β-lactam antibiotics, p. 189–228. *In* A. L. Demain and N. A. Solomon (ed.), Antibiotics containing the β-lactam structure, vol. I. Springer Verlag, Berlin.
7. **Elander, R. P.** 1983. Strain improvement and preservation of β-lactam producing microorganisms, p. 97–146. *In* A. L. Demain and N. A. Solomon (ed.), Antibiotics containing the β-lactam structure, vol. I. Springer Verlag, Berlin.
8. **Hollander, I. J., Y. Q. Shen, J. Heim, A. L. Demain, and S. Wolfe.** 1984. A pure enzyme catalyzing penicillin biosynthesis. Science **224**:610–612.
9. **Jensen, S. E., D. W. Westlake, and S. Wolfe.** 1982. Cyclization of δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine to penicillins by cell-free extracts of *Streptomyces clavuligerus*. J. Antibiot. **35**:483–490.
10. **Jensen, S. E., D. W. S. Westlake, and S. Wolfe.** 1982. High performance liquid chromatographic assay of cyclization activity in cell-free systems from *Streptomyces clavuligerus*. J. Antibiot. **35**:1026–1032.
11. **Konomi, T., S. Herchen, J. E. Baldwin, M. Yoshida, N. A. Hunt, and A. L. Demain.** 1979. Cell-free conversion of δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine into an antibiotic with the properties of isopenicillin N in *Cephalosporium acremonium*. Biochem. J. **184**:427–430.
12. **Kupka, J., Y. Q. Shen, S. Wolfe, and A. L. Demain.** 1983. Studies on the ring cyclization and ring expansion enzymes of β-lactam biosynthesis in *Cephalosporium acremonium*. Can. J. Microbiol. **29**:488–496.
13. **López-Nieto, M. J., I. García-Acha, and J. F. Martín.** 1980. Penicillin production by recombinant strains of *Penicillium chrysogenum* obtained by fusion of protoplasts, p. 57–61. *In* C. Vezina and K. Singh (ed.), Advances in biotechnology. Vol III. Fermentation products. Pergamon Press, Ltd., Toronto.
14. **Lowry, O. H., N. J. Rosbrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
15. **Luengo, J. M., G. Revilla, M. J. López, J. R. Villanueva, and J. F. Martín.** 1980. Inhibition and repression of homocitrate synthase by lysine in *Penicillium chrysogenum*. J. Bacteriol. **144**:869–876.
16. **Luengo, J. M., G. Revilla, J. R. Villanueva, and J. F. Martin.** 1979. Lysine regulation of penicillin biosynthesis in low-producing and industrial strains of *Penicillium chrysogenum*. J. Gen. Microbiol. **115**:207–211.
17. **Martín, J. F., and Y. Aharonowitz.** 1983. Regulation of biosynthesis of β-lactam antibiotics, p. 229–254. *In* A. L. Demain and N. A. Solomon (ed.), Antibiotics containing the β-lactam structure, vol. I. Springer Verlag, Berlin.
18. **Martín, J. F., and A. L. Demain.** 1978. Fungal development and

metabolite formation, p. 426–450. *In* J. E. Smith and D. R. Berry (ed.), The filamentous fungi, vol 3. Edward Arnold, London.

19. **Meesschaert, B., P. Adriaens, and H. Eyssen.** 1980. Studies on the biosynthesis of isopenicillin N with a cell-free preparation of *Penicillium chrysogenum*. J. Antibiot. **33**:722–730.
20. **Revilla, G., M. J. López-Nieto, J. M. Luengo, and J. F. Martín.** 1984. Carbon catabolite repression of penicillin biosynthesis by *Penicillium chrysogenum*. J. Antibiot. **37**:27–35.
21. **Sawada, Y., J. E. Baldwin, P. D. Singh, N. A. Solomon, and A. L. Demain.** 1980. Cell-free cyclization of δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine to isopenicillin N. Antimicrob. Agents Chemother. **18**:465–470.
22. **Shuster, L.** 1971. Preparative acrylamide gel electrophoresis: continuous and disc techniques. Methods Enzymol. **22**:412–433.
23. **Zanca, D. M., and J. F. Martín.** 1983. Carbon catabolite regulation of the conversion of penicillin N into cephalosporin C. J. Antibiot. **36**:700–708.