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Production kinetics and stability properties of ∂(L-α-aminoadipyl)-L-cysteinyl-D-valine synthetase from *Streptomyces clavuligerus*

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SUMMARY

∂-(L-α-Aminoadipyl)-L-cysteinyl-D-valine (ACV)-synthetase is a key enzyme that channels primary metabolites to a tripeptide common to cephalosporin and cephamycin biosynthesis in *Streptomyces clavuligerus*. Time-course studies indicated that the *S. clavuligerus* ACV-synthetase was stable during the cephamycin C fermentation: the enzyme was produced early in the growth phase and its activity remained high up to 96 h of growth. The detection of crude ACV-synthetase activity in older cultures was best achieved with an assay medium supplemented with 5 mM phosphoenolpyruvate, at lower ATP concentrations. During storage at 4°C, a progressive decrease in the stability of crude ACV-synthetase was observed with increasing culture age. Although a proteinolytic activity with a pH optimum at 8.2 was detected in crude cell-free extracts, no significant variation was observed in its activity with increasing culture age to account for the instability of ACV-synthetase in vitro. Addition of proteinase inhibitors did not improve the stability of the enzyme. However, a stabilization cocktail containing dithiothreitol, $MgCl_2$, the three substrate amino acids, and glycerol increased the stability of the enzyme isolated from cultures grown for 30–40 h, which was shortly after the appearance of antibiotics in the culture fluid. This stabilized enzyme retained half of its initial activity after 6 days at 4°C.

INTRODUCTION

∂-(L-α-Aminoadipyl)-L-cysteinyl-D-valine (ACV)-synthetase is the enzyme in fungi and bacteria that channels primary metabolites towards the production of β-lactam antibiotics. This peptide synthetase catalyses the non-ribosomal formation of ACV, in an ATP-dependent multi-step condensation of three amino acids [5]. ACV-synthetase from *Streptomyces clavuligerus* is a very large single protein that has been purified and partially characterized [4,15,22,32]. Formation of the enzyme is repressed by ammonium and phosphate, but not by easily utilized carbon sources [27].

Peptide antibiotic synthetases are unstable and rapidly inactivated [25]. Gramicidin S synthetases from *Bacillus brevis* [26], bacitracin synthetase from *Bacillus licheniformis* [16], and enniatin synthetase from *Fusarium scirpi* [7] are labile and only transitorily formed during the growth cycle. It is therefore difficult to harvest large quantities of these enzymes. Destabilizing mechanisms have been evoked as a cause of inactivation such as increased oxygen tension and intracellular redox state of the culture [2,9], and induction of synthetase specific proteinases [10,18] during the idiophase. Maintenance of high dissolved oxygen levels throughout the fermentation of *S. clavuligerus* did not affect the formation of ACV-synthetase but reduced the in vivo stability of the enzyme [21]. In vitro, ACV-synthetase is a relatively unstable multifunctional enzyme, and requires additives to retard inactivation [6,32].

We are interested in developing an enzymatic process for the production of ACV, as an alternative to chemical synthesis, using a partially purified ACV-synthetase as a reusable biocatalyst. Since the inactivation kinetics of gramicidin S synthetase from *B. brevis* and the degree of its amino acid-mediated stabilization in vivo were shown to be strongly dependent on the growth stage of the culture [23], the in vitro stability of S. clavuligerus ACV-synthetase may be similarly dependent on the physiology of the culture from which it is obtained. Therefore, the optimum time for harvesting cultures must be defined to obtain the most stable enzyme preparation for immobilization. Enhancement of enzyme storage and operational stability in vitro also would be of benefit for the successful use of ACV-synthetase in enzyme biotechnology.

MATERIALS AND METHODS

Strain and culture conditions

Streptomyces clavuligerus NRRL 3585 from the Northern Regional Research Laboratories, Peoria, Illinois, was maintained as lyophilized spores or spore suspensions in 20% glycerol at −75°C. Seed culture was prepared from 0.5 ml of a glycerol spore stock in 25 ml of Trypticase Soy broth supplemented with 1% (w/v) soluble starch (TCS/S) medium incubated at 28°C for 48 h [12]. Production flasks were inoculated with 2% (v/v) of *S. clavuligerus* seed culture into TCS/S and grown at 28°C.

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Cell free extract preparation

For time-course studies, 100-ml amounts of culture were harvested in duplicate at different times of growth. The separate mycelia were collected by filtration through Whatman No. 1 filter paper, and the filtrate saved for antibiotic assay. Mycelia from each flask were then washed with 50 mM Tris/HCl buffer, pH 7.5 containing 50 mM KCl, resuspended in 4 ml of lysis buffer consisting of 100 mM MOPS-KOH buffer, pH 7.5, containing 50 mM KCl, 5 mM $MgCl_2$, 30 mM 2-mercaptoethanol, 20 mM EDTA, and 50% glycerol, and disrupted by sonication to make a crude cell free extract (CFE) as described previously [12]. For proteinase analysis, $MgCl_2$ and EDTA were omitted from the lysis buffer. The CFE was centrifuged for 30 min at 17 000 × *g* and the clarified CFE was then stored at −75°C until use. Time-course experiments were carried out three times.

Purification of ACV-synthetase

ACV-synthetase was purified by a combination of salt precipitation, ultrafiltration, and anion-exchange chromatography as described by Jensen et al. [15]. Partial purification was achieved by treatment of the CFE, diluted four-fold in MKD buffer (100 mM MOPS-KOH, pH 7.5, 50 mM KCl, 1 mM dithiothreitol (DTT)), with streptomycin sulfate (1% w/v), followed by ammonium sulfate fractionation. ACV-synthetase was obtained in the 35–45% saturation ammonium sulfate precipitate, and resuspended in MKDG buffer (MDK buffer containing 20% glycerol (v/v)).

Antibiotic assay

The antibiotic content in the culture fluid was determined by bioassay using the super-sensitive *Escherichia coli* strain Ess, and the concentration estimated from cephalosporin C standards [13].

Protein assay

Protein was assayed according to the protein dye-binding method of Bradford [8] (BioRad, Richmond, CA, USA), using bovine serum albumin as a standard.

Proteinase assay

The proteinolytic activity of *S. clavuligerus* crude CFE was measured using a modified assay based on the proteinolytic digestion of azocasein [20]. The CFE was preincubated at 35°C for 2 min, and 0.1 ml of the extract was added to a reaction mixture containing 0.3 ml 100 mM Tris/HCl buffer, and 0.3 ml 2% azocasein (Sigma, St Louis, MO, USA), at 35°C. The reaction was allowed to proceed for 1 h, and stopped with 0.7 ml 15% trichloacetic acid (TCA). Controls consisted of heat-treated CFE (20 min, at 100°C), or reaction mixture without the CFE. The colored TCA soluble material released from the digestion of azocasein was quantitated spectrophotometrically. One unit of proteinase activity was defined as the amount producing 1 absorbance unit per minute at 335 nm, and specific activity was expressed as munits mg protein^{-1}. The pH activity profile of proteinases in the CFE was determined with the following buffer systems: 100 mM glycine/HCl (pH 3.0–5.0), 100 mM KH_2PO_4/K_2HPO_4 (pH 5.0–7.5), 100 mM Tris/HCl (pH 7.5–8.5), and 100 mM triethanolamine (pH 8.5–9.5).

Proteinase inhibitor study

The proteinase inhibitors: phenanthroline, phenylmethylsulfonyl fluoride, and thioacetamide were dissolved to give concentrated solutions in ethanol; and ethylenediaminetetraacetic acid and *p*-chloromercuriphenylsulfonic acid were dissolved in 100 mM Tris/HCl pH 8.2. Small volumes of each inhibitor solution were added to the CFE, pre-incubated for 10 min at 35°C, and residual proteinolytic activity was then determined with azocasein for 1 h at 35°C.

ACV-synthetase assay

ACV-synthetase activity was measured as described by Jensen et al. [14] with some modifications. Reaction mixtures (0.1 ml) contained: ATP (2 mM), $MgCl_2$ (6 mM), DTT (5 mM), phosphoenolpyruvate (PEP, 5 mM), L-α-aminoadipic acid (5 mM), cysteine (1 mM) and valine (5 mM), in 100 mM Tris/HCl, pH 8.5 buffer. When the effect of ATP concentration was studied, $MgCl_2$ was kept constant at 10 mM. Controls without added amino acids, $MgCl_2$ and ATP were set up to correct for endogenous cellular ACV, plus the amount of ACV that could possibly arise from enodgenous precursors during the enzyme incubation period. This parameter was defined as intracellular ACV and was subtracted from the ACV-synthetase activity profile. Assays were incubated at 22–24°C, terminated with 0.025 ml of 20% TCA, and precipitated protein was removed by centrifugation. ACV in the mixtures was derivatized with Thiolyte MB reagent and quantitated by HPLC as described previously [11,14]. One unit of ACV-synthetase activity was defined as the amount producing 1 μmol of ACV per minute, and specific activity was expressed as munits mg protein^{-1}.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE)

SDS–PAGE was carried out using 5% polyacrylamide gels by the procedure of Laemmli [17], and gels were stained with Coomassie Brilliant Blue R-250.

Stability studies

Studies of the stability of ACV-synthetase during storage were carried out with filter sterilized CFEs from different culture ages, prepared in the lysis buffer. The effect of additives on enzyme stability was studied using partially purified ACV-synthetase. Additives (DTT, $MgCl_2$, L-α-aminoadipic acid, cysteine, and valine), were added at final concentrations found in the enzyme assay, and glycerol at 20% (v/v), in one-quarter strength MDKG buffer.

Materials

Monobromobimane (Thiolyte MB) was from Calbiochem (San Diego, CA, USA), L-α-aminoadipic acid was from Sigma (St Louis, MO, USA), ACV was from Incell

Corporation (Millwaukee, WI, USA), and all other chemicals were of reagent grade.

RESULTS

Dynamics of cephamycin C fermentation

Time-course of ACV-synthetase activity was measured by assaying enzyme activity in crude CFE of *S. clavuligerus* growing in TCS/S medium, at 28°C. Since an established seed culture (48 h) was used as inoculum, the onset of the culture growth was almost immediate, reaching stationary phase around 40 h (Fig. 1). The growth profiles, as measured by optical density at 600 nm and CFE protein, were very similar (Fig. 1A), indicating that either method was suitable for estimating growth of *S. clavuligerus*. ACV-synthetase activity and intracellular ACV were observed 8 h into the fermentation, and both increased up until 38–40 h (Fig. 1B). Intracellular ACV levels dropped, coinciding with the appearance of cephamycin C in culture fluids. β-Lactam production reached a maximum around 60 h and leveled off thereafter. However, ACV-synthetase activity remained high up to 96 h of fermentation, and analysis of the CFEs by SDS–PAGE also showed the presence of ACV-synthetase protein in younger as well as in older cultures.

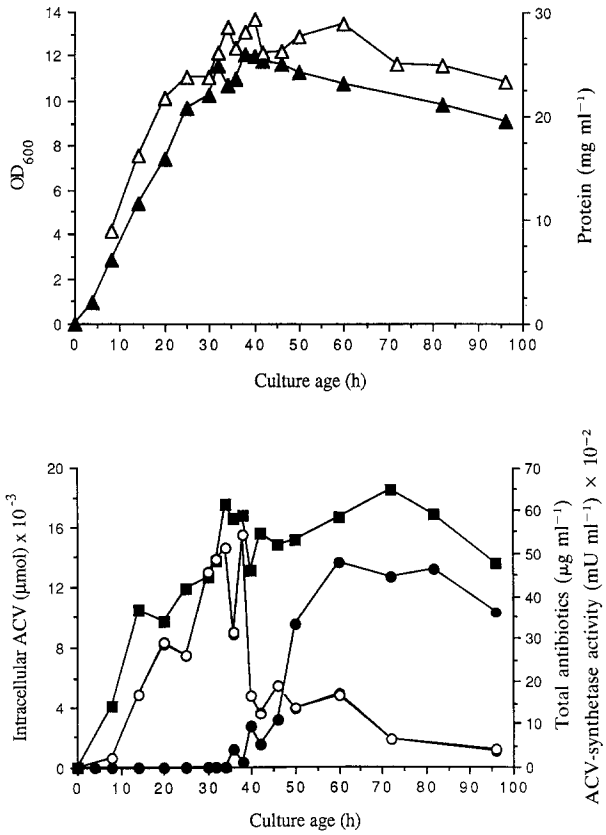

Fig. 1. Dynamics of cephamycin C fermentation by *S. clavuligerus*. (A) Growth profile determined by OD_{600} of the culture (▲) and protein of crude cell-free extract (△). (B) Time-course profiles of ACV-synthetase activity (■), ACV (○) and total antibiotics (●) production.

Improvement of estimation of ACV-synthetase in crude CFE by phosphoenolpyruvate

In this study the appearance of ACV-synthetase during the growth of *S. clavuligerus* was not transient: the enzyme was present during both trophophase and idiophase. This finding was in contrast to enzyme activity profiles reported previously by Demain and co-workers [30,31], where ACV-synthetase activity was quantitated by essentially the same HPLC assay, except that the reaction mixture contained 10 mM ATP [30,31], rather than 2 mM ATP in the presence of 5 mM PEP (this study). To determine whether the variation was due to differences in the assay systems, the enzyme activity in crude CFEs of different ages was analyzed in the presence of ATP at 2 mM, 10 mM, and 2 mM in the presence of 5 mM PEP (Fig. 2). The results showed significant increases in the activity of ACV-synthetase with 2 mM ATP plus 5 mM PEP when compared with ATP alone at 2 or 10 mM. Increasing the ATP concentration from 2 to 10 mM increased the detectable activity but still did not reach the level of activity obtained with the ATP/PEP system. Higher ATP concentrations were found to be inhibitory. The activity profiles obtained with the three assay systems also differed, with significant improvement in the detection of ACV-synthetase activity in older cultures when PEP was present, in contrast to an apparent decay in ACV-synthetase activity in older cultures when assayed without PEP.

Dependence of the PEP effect on the purity of ACV-synthetase

Enzyme preparations of different purity were used to analyze the stimulatory effect of PEP. Increasing the purity of ACV-synthetase reduced this effect, so that with pure enzyme preparation, obtained after two passages on an anion-exchange column, PEP no longer had any stimulatory effect on enzyme activity (Table 1). The dependence of the stimulatory effect of PEP on enzyme purity suggested that this effect was mediated by contaminating components

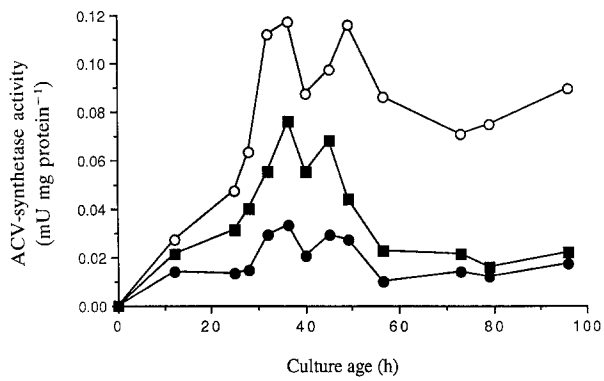

Fig. 2. Improvement of estimation of ACV-synthetase activity in crude extracts by PEP. ACV-synthetase activity in cell-free extracts prepared from cultures harvested at different times of the growth was measured in the enzyme assay mixtures containing 2 mM ATP (●), 10 mM ATP (■), and 2 mM ATP supplemented with 5 mM PEP (○).

TABLE 1

Dependence of the PEP effect on the purity of ACV-synthetase

Purification step[a]	Relative ACV-synthetase activity (%)		
	2 mM ATP	10 mM ATP	2 mM ATP/5 mM PEP
Crude cell extract	17	60	100
Ammonium sulfate (35–45%)	45	75	100
Ultrafiltration (XM 300)	53	77	100
Mono Q (2nd run)	103	109	100

[a] The activity of ACV-synthetase of different purity levels, obtained following the purification scheme previously described by Jensen et al. [15], was measured in three assay systems and expressed as a % relative to the activity obtained with the assay system containing both ATP (2 mM) and 5 mM PEP (5 mM).

present in the CFE, rather than a direct effect on ACV-synthetase activity.

Dependence of the storage stability of ACV-synthetase on the culture age

Crude CFEs prepared from cultures grown for different time periods were analyzed for the stability of ACV-synthetase activity at 4°C. ACV-synthetase stability profiles obtained after 1, 2 and 4 days of storage differed significantly from each other and from the initial enzyme activity profile, obtained from freshly thawed CFEs (no storage) as shown in Fig. 3. The enzyme from mycelia harvested before 36–38 h of fermentation was most stable and retained most of its initial activity after 2 days of storage. A significant drop in residual activity after 4 days of storage was observed for CFEs from cultures up to 38 h of growth, and no activity remained in CFEs from older cultures.

Proteinolytic activity profile during cephamycin C fermentation

Instability of peptide synthetases has been suggested to reflect intracellular proteinases, so crude CFEs were also examined for proteinolytic activity on azocasein. A proteinase with an optimum activity at pH 8.2 was detected. This proteinolytic activity increased with biomass, but no drastic change in specific activity was observed in the idiophase that correlated with the observed instability of ACV-synthetase in CFEs from older cultures which do not contain intracellular ACV. To see whether the proteinase activity of CFEs changed during storage, the crude CFEs from cultures grown at various times were incubated for 3 days at 4°C and the proteinolytic activity profile examined again. A nearly two-fold increase in proteinolytic activity was observed (Fig. 4), presumably as a result of activation of pre-proteinases during the storage period. However, this effect was not localized to a particular growth period, for it occurred at all culture ages.

Inhibition of the CFE proteinolytic activity

To determine the type of proteinase present and whether the observed azocaseinolytic activity was a composite of overlapping proteinase activities, a variety of proteinase inhibitors was used. No strong inhibitory effect was observed with a range of specific inhibitors up to 2 mM final concentration, indicating that activity was not a serine-, metallo- or thiol-proteinase. Nevertheless, the partial purification of ACV-synthetase by ammonium sulfate fractionation resulted in the removal of most of the azocaseinolytic activity from the enzyme preparation, yet without resulting in the enhancement of ACV-synthetase storage stability. Therefore, no direct effect of proteinases on the stability of ACV-

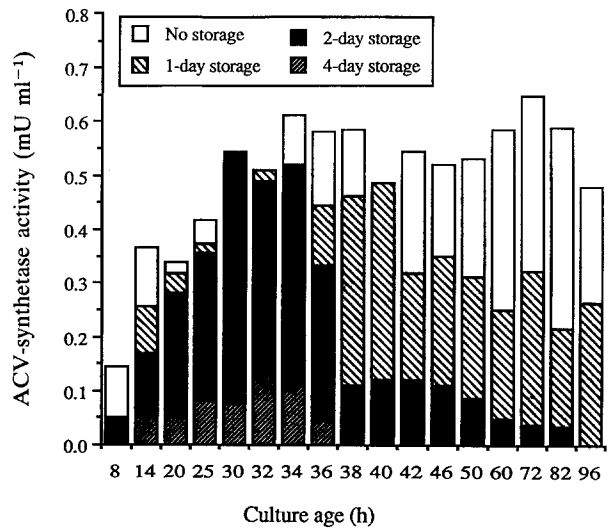

Fig. 3. Dependence of storage stability of ACV-synthetase on culture age. Initial ACV-synthetase activity of crude cell-free extracts from different age cultures was measured before storage of the cell-free extracts at 4°C, then residual ACV-synthetase activity was measured after 1, 2 and 4 days of storage.

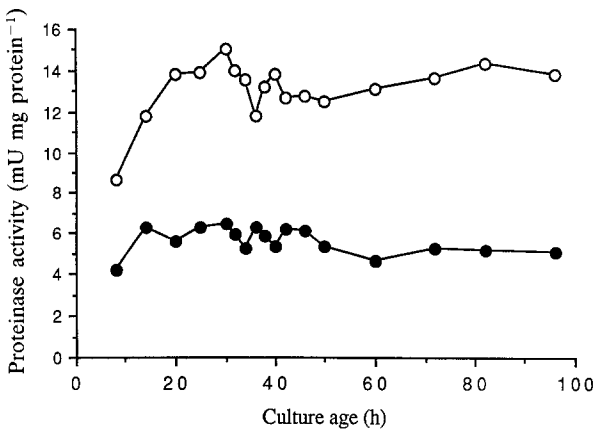

Fig. 4. Time-course profile of intracellular proteinase. Freshly prepared crude cell-free extracts obtained from cultures grown for different periods of time were analyzed for proteinase activity (●), then the cell-free extracts were incubated at 4°C for a period of 70 h and proteinolytic activity was assayed again (○) after storage.

synthetase could be established from these studies. These observations also suggested that factors other than proteinases were involved in the inactivation of ACV-synthetase during storage.

Effects of additives on the stability of ACV-synthetase during storage

The stability of partially purified ACV-synthetase was analyzed by following loss of initial enzyme activity during storage in the presence of various additives, to define a stabilization cocktail that will enhance the lifetime of the enzyme during storage and operation. DTT was the most important of the additives tested in stabilizing the enzyme (Table 2). The most effective stabilizing mixture contained glycerol, $MgCl_2$, and the three substrate amino acids, in addition to DTT, and enabled the enzyme to retain full activity after 48 h of storage at 4°C, when the control had lost 75% of its initial activity. At room temperature, the stability of the enzyme was poor, with significant loss in activity occurring within a few hours. Slight improvement in stability at room temperature was achieved in the presence of the six additives mentioned above. A combination of the optimal cell harvest time and the stabilization cocktail produced an enzyme preparation which retained more than 50% of its initial activity after 6.5 days of storage at 4°C.

DISCUSSION

The dynamics of cephamycin C fermentation by *S. clavuligerus* revealed two distinct phases. The first involved the channelling of the amino acids towards the formation of ACV, and its intracellular accumulation. The second phase corresponded to the period in which this ACV pool was drawn upon for cephamycin C production, and the antibiotic accumulated in the culture fluid. ACV-synthetase was found in the mycelia during both phases, even in older cultures when the rate of cephamycin C formation had become negligible. However, from the viewpoint of cellular economy, ACV-synthetase should represent an ideal major regulatory site for β-lactam biosynthesis: the appearance of this enzyme should indicate the onset of antibiotic biosynthesis, and its disappearance indicate the curtailment of antibiotic production. In this study, evidence suggested that after the onset of antibiotic biosynthesis, the activity of ACV-synthetase was primarily regulated at the enzyme level, since the enzyme was physically present throughout the course of the fermentation regardless of variations in the rate of cephamycin C production. The fact that the activity profile of the enzyme in vitro could be altered by PEP, presumably through the activation of a PEP-dependent ATP-regenerating system, suggested that the activity of this enzyme in vivo may be affected in similar ways. Substrate and ATP

TABLE 2

Effects of additives on the stability of ACV-synthetase during storage at 4°C

Additive(s)[a]	% Residual ACV-synthetase activity[b]					
	0 h	24 h	48 h	96 h	120 h	156 h
Control	100.0	69.1	24.9	2.5	0	0
AAA	101.1	59.8	27.1	0	0	0
Cys	99.4	66.5	22.8	1.2	0	0
Val	100.2	70.1	30.4	2.5	0	0
AAA+cys+val	112.1	32.4	12.4	0	0	0
$MgCl_2$	98.2	35.5	1.2	0	0	0
$MgCl_2$+AAA+cys+val	106.5	27.1	2.1	0	0	0
Glycerol	100.4	75.6	45.2	20.5	1.2	0
Glycerol+AAA+cys+val	109.7	41.9	3.4	0	0	0
Glycerol+$MgCl_2$+AAA+cys+val	101.8	35.2	9.2	5.5	0	0
DTT	102.5	107.4	87.6	45.3	12.5	0
DTT+AAA+cys+val	105.2	95.7	83.2	56.2	29.1	2.2
DTT+glycerol+$MgCl_2$+AAA+cys+val	108.5	113.2	98.3	67.2	53.1	48.9

[a] The ammonium sulfate fraction of ACV-synthetase from a 44-h culture was preincubated at 4°C with one or more of the following additives: AAA (aminoadipate at 5 mM), cys (cysteine at 1 mM), val (valine at 5 mM), DTT (dithiothreitol at 2 mM), $MgCl_2$ at 3 mM, and glycerol at 20% (v/v) in 80 mM MOPS/KOH buffer pH 7.5.

[b] ACV-synthetase activity remaining after the indicated storage period was determined, and the activity was expressed relative to the initial activity of the control at time 0 h. Data points are averages of duplicate activity values from two independent experiments.

limitations, brought about by a drop in the intracellular levels of these compounds or an increase in competing reactions, may cause an apparent decline in ACV-synthetase activity in CFEs from older cultures. The inhibition of this enzyme by easily utilized carbon sources has been reported to be due to competition for ATP in crude cell extracts [27]. In this study, results suggested that the increase in the competition for ATP by cellular ATP-utilizing enzymes may be one of the mechanisms of regulating the activity of this enzyme during the idiophase, possibly in combination with substrate amino acid limitations.

The activity profile of ACV-synthetase was not transient, as has been observed for other non-ribosomal peptide synthetases such as gramicidin S synthetase [26], bacitracin synthetase [16], and enniatin synthetase [7]. In previous reports of ACV-synthetase activity profiles [30,31], the enzyme activity decayed during the antibiotic production phase. However, as shown from this study, the apparent decay in ACV-synthetase activity during this phase was primarily due to an effect related to ATP availability, which could be overcome with the addition of PEP. On this basis, the kinetics of production of ACV-synthetase from *S. clavuligerus* are similar to those of the same enzyme from *Penicillium chrysogenum* [19]. The time-course of ACV-forming activity during the fermentation of *P. chrysogenum* showed no decay in ACV-synthetase activity when measured in vivo by blocking protein synthesis at different times and determining the rate of incorporation of labelled substrate amino acid into ACV by preexisting ACV-synthetase. The *P. chrysogenum* enzyme reached maximum activity at 48 h, and also remained constant for up to 120 h of fermentation.

These studies raise some questions about the cellular function of ACV-synthetase. Microorganisms are known to remove proteins that are no longer useful. Since ACV-synthetase remains active longer than needed for antibiotic production, it could be speculated that this enzyme may be playing another role in the cell. Unlike the *Bacillus* nonribosomal peptide antibiotic synthetases which direct primary metabolites to an active product, ACV-synthetase produces an intermediate in antibiotic synthesis, not a product. There have been reports of excretion of ACV into the culture fluid of *P. chrysogenum* [1,19], suggesting that in this organism activity is not tightly controlled. Recent work by Aharonowitz [3] suggests that ACV and a disulfide reductase may, in the absence of glutathione, be responsible in part for the thiol-disulfide redox balance in *S. clavuligerus*. Such a function could explain the persistence of ACV-synthetase in the cell beyond the end of the antibiotic production phase.

The variations in the estimation of ACV-synthetase activity with the ATP level in the assay mixtures and the purity level of the enzyme point out some of the difficulties that have affected the development of a reliable assay for ACV-synthetase, suitable for both crude and purified enzyme preparations. Since the original conditions for a cell-free ACV synthesis system were defined by Banko et al. [6], ATP concentrations in assay mixtures have varied considerably, with 1 mM [14], 5 mM [24], 10 mM [29,30], and 15 mM [5,6,29] concentrations being used, in an attempt to optimize ACV production. With crude enzyme, Jensen et al. [14] found 1 mM ATP with a regenerating system gave better results than higher ATP concentrations alone. A systematic analysis of this assay showed that higher initial ATP concentrations were inhibitory, and lower concentrations were limiting, unless a cellular PEP-dependent ATP-regenerating system was included. We also found that pyruvate kinase, previously included as a component of the ATP-regenerating system [14], had no positive effect on the activity of either crude or purified ACV-synthetase preparations. The stimulatory effect observed was due to the exogenous PEP combined with the cellular regenerating system in the crude extract, not to the exogenous pyruvate kinase. As the enzyme purification level increased, PEP no longer showed a stimulatory effect. These observations stressed the problems associated with the estimation of enzyme recovery during a purification scheme, due to the underestimation of the specific activity of ACV-synthetase in crude enzyme preparations. The standard reaction mixture supplemented with PEP represents the optimal system for assay of ACV-synthetase activity during purification procedures, since it supports optimal activity in crude enzyme preparations and has no deleterious effects on purified ACV-synthetase.

Although ACV-synthetase was present at most culture ages, the enzyme isolated during the period when ACV was accumulating in the mycelia was the most stable during storage. No direct evidence of enzyme inactivation by proteinase was observed, though an intracellular proteinase with a pH optimum of pH 8.2 was detected at all culture ages. However, this does not exclude the possibility that other proteinlytic activities not detectable with azocasein may be involved in the inactivation of ACV-synthetase in vitro.

The enhanced stability of the enzyme from mycelia harvested before the decrease in the intracellular ACV level could be due to stabilization by low molecular weight molecules, such as the enzyme substrates. On the other hand, the effect may be simply due to an intrinsic property of the enzyme, so that after ACV has disappeared from the mycelia, the enzyme is affected in some way to become more susceptible to inactivation in vitro. Evidence of the important role of reducing agents and substrates in the stabilization of the enzyme was demonstrated in this study. DTT and glycerol provided the greatest stabilization of the enzyme and the addition of $MgCl_2$ plus the three substrate amino acids enhanced the stability of partially purified ACV-synthetase. From an independent study by Zhang and Demain [28,32] with purified ACV-synthetase, these additives were also found to improve the stability of the enzyme, and among the three substrate amino acids, valine was the only substrate found to enhance the enzyme stability in vitro. These results emphasize the importance of substrate production of the enzyme, presumably through the protection of the active site or by forcing the enzyme to remain in the native conformation. Maintaining a reduced environment appeared to be an essential requirement for stability of

ACV-synthetase. Improvement of the stability of the enzyme by substrates and DTT provides a convenient means of achieving good operational stability of a partially purified ACV-synthetase for use in studies on the production of ACV.

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