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In Vitro Stabilization of ACV Synthetase Activity from *Streptomyces clavuligerus*

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ABSTRACT

ACV synthetase (ACVS) from *Streptomyces clavuligerus* is very labile. The study and in vitro application of this important enzyme for cephamycin biosynthesis requires a relatively stable preparation. The stability of the crude enzyme was substantially increased by dithiothreitol and the cofactor, magnesium (Mg^{2+}). The purified enzyme was also unstable and especially sensitive to moderate to high temperature. Addition of the substrate L-valine (L-val) along with the cofactors (ATP and MG^{2+}) raised the thermal inactivation temperature, and increased the stability of the enzyme at low temperature. Amino acids capable of replacing L-val as ACVS substrate generally stabilized the enzyme. The ACVS level remained high during fermentation in a complex medium containing high concentrations of amino acids, in contrast to the situation in chemically-defined medium.

Index Entries: *Streptomyces clavuligerus;* cephamycin; ACV synthetase; stability; stabilization; L-valine; fermentation.

INTRODUCTION

δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV) synthetase (ACVS) is the first enzyme in the biosynthesis of all natural penicillins and cephalosporins from amino acids precursors by both eukaryotes and prokaryotes (*1*). Its activity catalyzes the probable rate-limiting step in the biosynthetic processes (2). The stability of ACVS at least partially determines the

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longevity of antibiotic production during fermentations, and a stable preparation is a prerequisite for the in vitro application of this important enzyme.

ACVS is well known for its extreme instability in vitro. For example, the enzyme from *Cephalosporium acremonium* has a half-life of only 2 h at 25°C and only 10 h at 4°C (*3*). This low stability severely impeded the study of this enzyme until recently when certain means of stabilization were found. Banko et al. (*4,5*) succeeded in establishing a reliable cell-free assay for ACVS by incorporating a high concentration (50%) of glycerol into the buffer during preparation and storage of *C. acremonium* extracts. Similarly, Jensen et al. (*6*) were able to detect ACVS activity in *Streptomyces clavuligerus* cell-free extracts containing glycerol. However this high concentration of glycerol made purification by chromatographic methods almost impossible. We found that a combination of 5 m*M* of the reducing agent dithiothreitol (DTT), 5 m*M* of the cofactor Mg^{2+} and a narrow range ammonium sulfate fractionation substantially increased the stability of the crude *C. acremonium* ACVS (*7*). By virtue of these findings, ACVS's from both *C. acremonium* and *S. clavuligerus* were purified in the absence of glycerol more than 100-fold to apparent electrophoretic homogenity (*3,7*). In the presence of 1 m*M* DTT and 20% glycerol, the *S. clavuligerus* ACVS was also purified by Jensen et al. (*8*) to near homogeneity, but the increase in specific activity was only 12-fold.

This report presents studies on the stability and the stabilization of *S. clavuligerus* ACVS under various conditions.

MATERIALS AND METHODS

Microorganisms

The filamentous bacterium *Streptomyces clavuligerus* NRRL 3585 (ATCC 27064), a typical prokaryotic producer of cephamycin C, was used in this research.

Chemicals

All chemicals used were purchased commercially and were of the highest purity available. The major source for the biochemicals was Sigma Chemical Company.

Medium Compositions

The seed medium of Aharonowitz and Demain (*9*) was used. For fermentations, the following media were employed: trypticase soy broth (TSB)-based complex medium (3% TSB, 1% soluble starch, 100 m*M* MOPS, initial pH 7) (modified from Jensen et al. (*10*), and chemically-defined medium [0.06% $MgSO_4 \cdot 7H_2O$, 0.35% K_2HPO_4, 0.1% (v/v) trace

salts solution, 100 m*M* MOPS, 15 m*M* L-asparagine, 10 m*M* L-lysine, 1% glycerol, initial pH 6.8] (modified from Aharonowitz and Demain) (*9*).

Cultivation Conditions

Liquid cultivations were conducted at 30°C on a 250 rpm rotary shaker. Fifty mL of seed medium in 250 mL baffled flasks was inoculated with a spore suspension, and incubated for 2 d. With an inoculum of 4%, fermentations in 50 mL complex medium were carried out in 500 mL unbaffled flasks, and in chemically-defined medium in 500 mL baffled flasks. Fermentations were complete within 6 d in chemically-defined media and 3 d in complex medium.

Measurement of Growth, Cephalosporins, and Proteins

Growth in fermentations was determined by absorbance using the Klett Summerson coliometer with a red filter. When the measurement was made in the range of 50–150 Klett units, a dry cell weight (DCW) of 1 mg/mL is equivalent to 260 Klett U for *S. clavuligerus* in defined medium (*9*). Growth in complex medium was expressed directly as Klett units.

Production of cephalosporins was followed by bioassay using cephalosporin C as standard, and *Escherichia coli* ESS as test organism in the presence of 10,000 U of narrow-spectrum penicillinase (Difco)/mL agar. Strain ESS responds to cephalosporin C, deacetoxycephalosporin C, deacetylcephalosporin C, and cephamycin C.

Protein contents in cell-free extracts were measured by the method of Bradford (*11*) with the Bio-Rad dye reagent, using bovine serum albumin as standard.

Preparation of Cell-Free Extracts and Purification of ACVS

The enzyme preparations used for stabilization studies were made from mycelia grown ~2 d in complex medium. Preparation of extracts and purification of ACVS were done according to the procedures described earlier (*7*). The enzyme activity in cell-free extracts is referred to as "crude activity." The cell-free extracts and the purified preparations were stored at −80°C until used. Immediately before enzyme reactions, the extracts were depleted of small molecules by passage through a Sephadex G-25 column, with 100 m*M* MOPS/pH 7.5 as the desalting buffer. The purified enzymes were in 100 m*M* MOPS/5 m*M* DTT/5 m*M* $MgCl_2$/pH 7.5.

ACV Synthetase Assay

ACVS activity was measured by HPLC estimation of the ACV formed in the cell-free enzyme reactions, with ATP and Mg^{2+} as cofactors and

L-α-aminoadipate (L-aaa), L-cysteine (L-cys), and L-valine (L-val) as substrates. The method was derived from Banko et al. (*4,5*) and was described in detail in our previous publication (*12*). One unit of enzyme activity was defined as the amount producing 1 μmole of ACV/min; specific activity was expressed as milliunits (munits) per mg of protein.

Investigation of Stability and Stabilization of ACVS Activity

The stability of the enzyme in crude cell-free extracts, desalted with 100 m*M* MOPS/pH 7.5, was examined by preincubating the preparations at 4°C in the presence or absence of different concentrations of potential stabilizing compounds. After various lengths of time, enzyme reactions were carried out to determine the residual activity. When the activity was higher than the control, the compound added was considered to have stabilized the crude enzyme.

For the investigation of stability and stabilization of the purified ACVS activity, the enzyme preparations (in purification buffer: 100 m*M* MOPS/5 m*M* DTT/5 m*M* $MgCl_2$/pH 7.5), in the presence or absence of additives, were incubated at the given temperature for specified times. Then fresh cofactors and substrates were added and the enzyme reactions were carried out to determine the residual activities, expressed as percent of the controls. It should be noted that there was some product formation during the preincubation when all cofactors and substrates were present; this was taken into consideration in calculating the results.

RESULTS AND DISCUSSION

Stability and Stabilization of the Crude ACVS Activity

The crude *S. clavuligerus* ACVS was found to be very unstable, having a half-life of about 25 h at 4°C in 100 m*M* MOPS/pH 7.5 (Fig. 1). However, this stability was higher than that of the crude *C. acremonium* enzyme (*3*).

One of the possible mechanisms of inactivation of ACVS could be covalent modification of its active site, such as disulfide bond formation or oxidation of cysteine residues owing to exposure to a high oxygen tension in the cell-free state (*13*). Indeed, the reducing agent, DTT, was found to substantially enhance enzyme stability (Fig. 1), suggesting the presence of sulfhydryl groups important to the enzyme's catalytic abilities. Loss of cofactors at the catalytic site could be another cause of enzyme inactivation (*13*). We found that addition of the cofactor Mg^{2+} further enhanced enzyme stability when added to the preparation containing DTT (Fig. 1). The positive effect of Mg^{2+} could also be due to the "ligand-induced stabilization" phenomenon as described by Goldberg and Dice

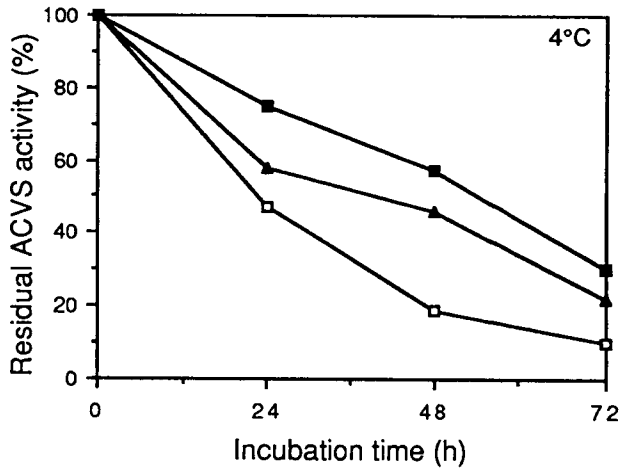

Fig. 1. In vitro stability at 4°C and stabilization of the crude ACVS activity by DTT and Mg^{2+}. □, extract incubated in 100 m*M* MOPS/pH 7.5 (control) ▲, extract incubated in 100 m*M* MOPS/pH 7.5/5 m*M* DTT; ■, extract incubated in 100 m*M* MOPS/pH 7.5/5 m*M* DTT/5 m*M* $MgCl_2$.

(*14*). In the absence of DTT, Mg^{2+} did not increase enzyme stability (data not shown). The presence of 5 m*M* DTT and 5 m*M* Mg^{2+} increased the enzyme's half-life at 4°C to about 65 h (Fig. 1). This increased ACVS stability facilitated the purification of the active enzyme (*7*).

Stability and Thermal Stability of the Purified ACVS Activity

A relatively stable preparation is important for the study and eventual in vitro application of purified ACVS. In the presence of 5 m*M* DTT and 5 m*M* Mg^{2+}, the purified enzyme preparation was incubated at 25°C and 4°C for various times, and the residual activities were assayed. As shown in Fig. 2, the purified enzyme was still labile, losing half of its activity in about 8 h at 25°C and about 70 h at 4°C. The stability at 4°C under these conditions was similar to that of the crude enzyme (Fig. 1), indicating that the major cause of enzyme instability was not the presence of inactivating agents (such as proteases) in crude extracts.

Incubating the purified enzyme at the indicated temperature in the presence of DTT and Mg^{2+} for 10 min followed by assaying the remaining activity revealed that the purified ACVS activity is quite thermally unstable (Fig. 3, "original"), with 50% inactivation occurring at only 32°C. Below 28°C, the enzyme was stable, but at 34°C the activity was almost completely lost in 10 min. The result was quite surprising since we knew that nearly 90% of the activity was retained at 32°C during a 90 min enzyme reaction; even at 34°C, the 90 min reaction still gave 50% activity (data not shown).

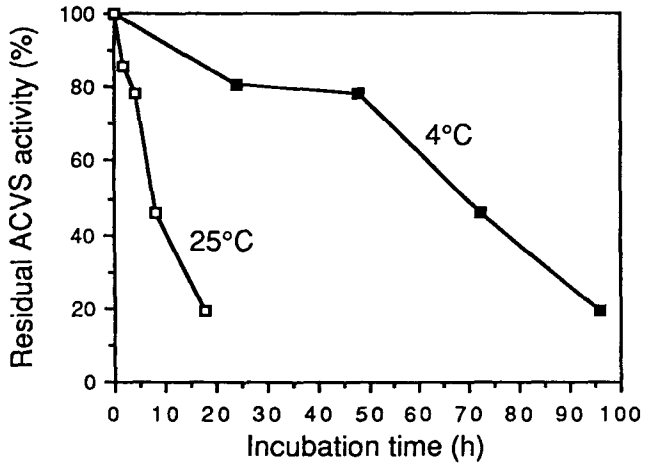

Fig. 2. Stability of the purified ACVS activity at 25°C and 4°C in the presence of DTT and Mg^{2+}. The enzyme was incubated in 100 m*M* MOPS/5 m*M* DTT/5 m*M* $MgCl_2$/pH 7.5 at 25°C and 4°C.

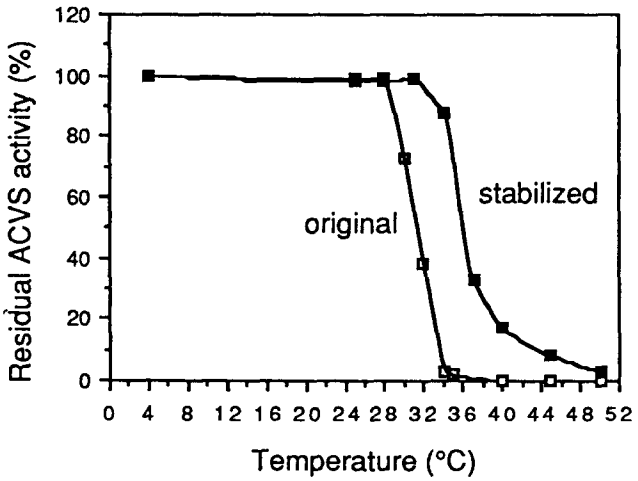

Fig. 3. Thermal stability of the purified ACVS activity and stabilization by L-val in presence of cofactors. The enzyme was incubated at different temperatures for 10 min, followed by determination of the remaining activity. □, incubated in 100 m*M* MOPS/pH 5 m*M* DTT/5 m*M* $MgCl_2$/pH 7.5; ■, incubated as above plus 5 m*M* L-val and cofactors (5 m*M* ATP and 5 m*M* Mg^{2+}).

Protection Against Thermal Inactivation by Substrates in Presence of Cofactors

The discrepancy between thermal stability and stability under the reaction conditions pointed to the existence of stabilization effects by certain components of the ACVS reaction mixture. Possible candidates, alone or in combination, were then added to the preparation before the 10

Table 1
Effect of Reaction Components on the Thermal Stability of Purified ACVS[a]

Additive, m*M*	34°C incubation time, min	Residual ACVS activity, %
None	0	100
None	10	3
DTT (5)	10	1
Mg^{2+} (5)	10	7
ATP/Mg^{2+} (5/5)	10	33
A/C/V/DTT (5/1/5/3)	10	3
ATP/Mg^{2+}/A/C/V/DTT (5/5/5/1/5/3)	10	91

[a]The enzyme preparations were in purification buffer (100 m*M* MOPS/5 m*M* DTT/5 m*M* $MgCl_2$/pH 7.5) before addition of additives. A = L-aaa, C = L-cys, V = L-val.

min incubation at 34°C (Table 1). The purification buffer already contained the known stabilizers, Mg^{2+} and DTT at 5 m*M* each. Doubling their concentration did not protect the purified enzyme activity from thermal inactivation, indicating that such inactivation was not likely owing to the loss of bound Mg^{2+} or acceleration of thiol group oxidation under elevated temperature. A mixture of cofactors (ATP/Mg^{2+}) protected the enzyme activity to some extent, but the substrates (L-aaa, L-cys, and L-val) themselves failed to show any stabilization. It was the combination of cofactors and substrates that almost totally prevented the thermal inactivation of the enzyme at 34°C (Table 1).

It has been reported that the bacitracin-synthesizing enzyme complex is protected by substrate amino acids, ATP, and Mg^{2+} against dissociation into its component enzymes (*15*). However, in the case of *S. clavuligerus* ACVS, some other mechanism is probably involved in substrate stabilization in the presence of cofactors, because our data indicated that the enzyme is most likely a monomer (*7*). Binding of substrates to the enzyme should be important in this effect. The failure of substrates alone to exert protection is possibly owing to the inability of substrate amino acids to bind to the enzyme in the absence of cofactors (ATP and Mg^{2+}), i.e., an ordered reaction mechanism might exist. The prevention of thermal inactivation by substrate binding suggests that the inactivation of ACVS activity at the fairly low temperature of 34°C is a conformational rather than a covalent process.

Stabilization by Substrate L-val in Presence of Cofactors

It would be advantageous if the stabilization of ACVS by substrates did not require the presence of all three amino acids, because the reaction occurs in this case, complicating the study or application. Thus the individual effect of the amino acids was investigated by adding each amino

Table 2
Protection of the Purified ACVS Activity Against Thermal Inactivation by Substrate Amino Acids in Presence of Cofactors[a]

Additives, m*M*	34°C incubation time, min	Residual ACVS activity, %
None	0	100
None	10	2
ATP/Mg^{2+} (5/5)	10	31
ATP/Mg^{2+}/A (5/5/5)	10	44
ATP/Mg^{2+}/C/DTT (5/5/1/3)	10	57
ATP/Mg^{2+}/C/DTT (5/5/5/15)	10	40
ATP/Mg^{2+}/V (5/5/5)	10	92

[a]The enzyme preparations were in purification buffer (100 m*M* MOPS/5 m*M* DTT/5 m*M* $MgCl_2$/pH 7.5) before addition of additives. A = L-aaa, C = L-cys, V = L-val.

acid separately, together with cofactors, before incubation at 34°C (Table 2). In the presence of cofactors, L-val alone almost fully protected the enzyme against thermal inactivation at 34°C during the 10 min incubation, while L-aaa or L-cys displayed much lower stabilizing effects. These results suggest that the binding of each substrate amino acid is independent of each other.

Figure 3 (''stabilized'') shows the stabilization of ACVS activity against thermal inactivation by L-val in the presence of cofactors. In a 10 min incubation, more than 90% activity was retained at 34°C, and the temperature required for 50% inactivation of the enzyme increased by ~5 degrees to about 36°C. Some activity even remained after 10 min incubation at 45°C. The thermal stability of ACVS was thus significantly enhanced.

As shown above (Figs. 1 and 2), ACVS is labile even at 4°C. It is possible that one of the mechanisms for this instability involves a conformational change of the protein molecule, although the rate of unfolding, i.e., of inactivation of the enzyme activity, would be much lower at low temperature. The very large size of *S. clavuligerus* ACVS (~400 kDa) (*7*) would make it more vulnerable to the change in conformation than the majority of smaller proteins. It would thus tend to unfold and become inactive under nonphysiological conditions. If so, addition of L-val to the enzyme preparation might also increase enzyme stability at 4°C. Indeed, as shown in Fig. 4, the presence of 5 or 10 m*M* L-val, along with cofactors, dramatically enhanced the stability of ACVS at 4°C, with only 15% activity lost in 4 d and 30% in 9 d. The time required for loss of 50% activity was increased from less than 3 d to possibly 15 d. Cofactors (ATP and Mg^{2+}) did not show any stabilizing effect on ACVS activity at 4°C (Fig. 4), unlike the situation at 34°C (Tables 1 and 2). The reason for the effectiveness of cofactors at 34°C and ineffectiveness at 4°C is unknown.

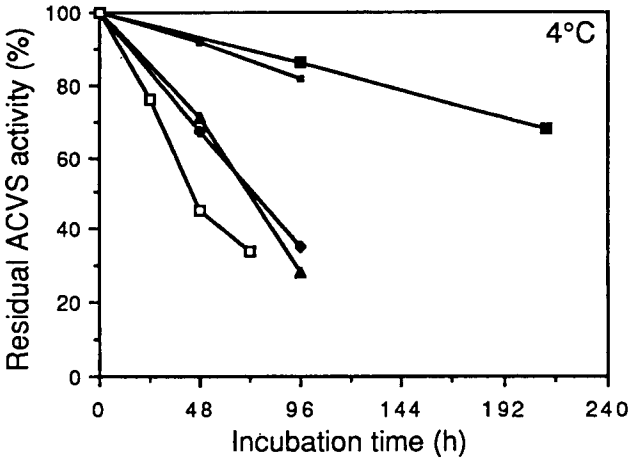

Fig. 4. Stabilization of the purified ACVS activity at low temperature (4°C) by L-val in presence of cofactors. □, incubated in 100 m*M* MOPS/pH 7.5; ▲, incubated in 100 m*M* MOPS/pH 7.5/5 m*M* DTT/5 m*M* $MgCl_2$; ◆, incubated in 100 m*M* MOPS/pH 7.5/5 m*M* DTT/10 m*M* $MgCl_2$/5 m*M* ATP; ■, incubated in 100 m*M* MOPS/pH 7.5/5 m*M* DTT/10 m*M* $MgCl_2$/5 m*M* ATP/5 m*M* L-val; ■, incubated in 100 m*M* MOPS/pH 7.5/5 m*M* DTT/10 m*M* $MgCl_2$/5 m*M* ATP/10 m*M* L-val.

The stabilization effect at 4°C by L-val in the presence of ATP and Mg^{2+} was also observed in the absence of DTT, with both purified and crude *S. clavuligerus* ACVS's (Fig. 5); however, the enzyme was much more stable when DTT, L-val, and cofactors were present (Fig. 4).

A study of L-val dosage on the stability of ACVS activity (Table 3) showed that a saturating L-val concentration ($\geq$5 m*M*) is required for the maximum stabilization effect, similar to the dependence of enzyme activity on L-val concentration (*12*). This supports the possibility that stabilization requires the binding of L-valine to the enzyme. As postulated above, such binding might help maintain the enzyme in its native conformation. The cofactors Mg^{2+} and ATP must be present for activation and subsequent binding of the substrate amino acid to the enzyme according to the non-ribosomal peptide synthesis mechanism.

The significantly increased stability of ACVS activity should certainly benefit its application, such as in vitro production of ACV and unnatural tripeptides, and total enzymatic synthesis of novel β-lactam antibiotics (*16*).

General Stabilization Effect by L-val Replacements

The above results indicated that L-val is the primary stabilizer among the three substrates. We were interested in determining whether amino

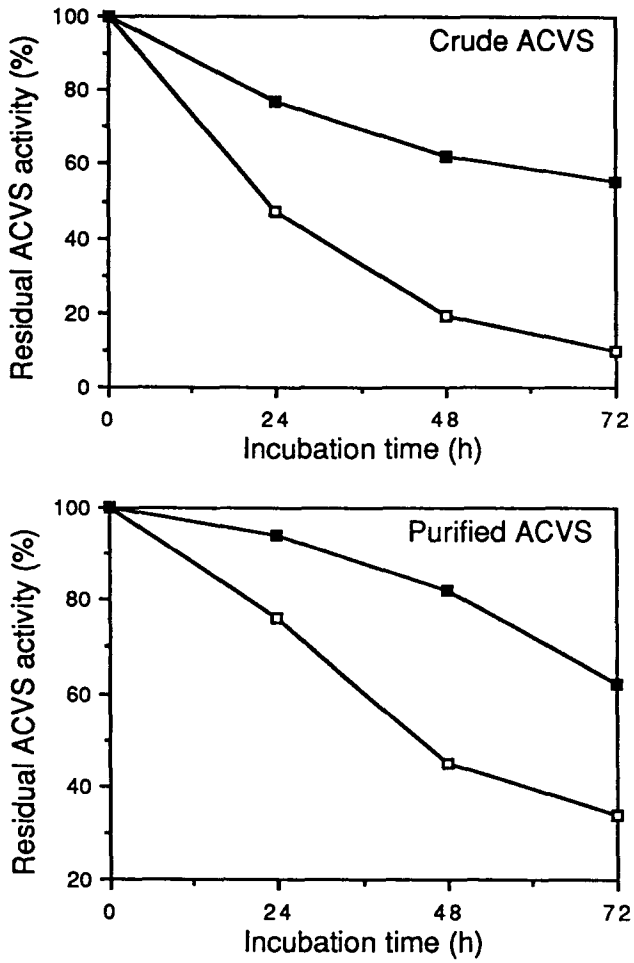

Fig. 5. Stabilizing effect of L-val plus cofactors on the crude and the purified ACVS activities in the absence of DTT at 4°C. Enzyme preparations were incubated in 100 m*M* MOPS/pH 7.5 at 4°C. □, control; ■, added 5 m*M* L-val plus 5 m*M* ATP and 5 m*M* $MgCl_2$.

acid replacements of L-val are also effective in enzyme stabilization. The prerequisites are that they must be accepted by ACVS (i.e., they must bind to the enzyme) and possess properties close to L-val. Fortunately, ACVS has a rather broad substrate specificity with respect to L-val (*17*), enabling the examination of a number of replacements for their ability to stabilize ACVS against thermal inactivation (34°C, 10 min) (Table 4). Indeed, most of the L-val substitutes stabilized the enzyme to some extent. L-*allo*-isoleucine, the best substitute for ACVS activity, was the most stabilizing replacement; L-norvaline, L-isoleucine, and L-aminobutyrate

Table 3
Effect of L-val Dosage on the Stability
of the Purified ACVS Activity in Presence of Cofactors[a]

Additives, m*M*	Preincubation temp (°C)	Preincubation time	Residual ACVS activity, %
None	34	0 min	100
None	34	10 min	3
ATP/Mg^{2+}/V (5/5/2.5)	34	10 min	81
ATP/Mg^{2+}/V (5/5/5)	34	10 min	93
ATP/Mg^{2+}/V (5/5/10)	34	10 min	93
None	4	0 h	100
None	4	96 h	27
ATP/Mg^{2+}/V (5/5/2.5)	4	96 h	78
ATP/Mg^{2+}/V (5/5/5)	4	96 h	84
ATP/Mg^{2+}/V (5/5/10)	4	96 h	86

[a]The enzyme preparations were in purification buffer (100 m*M* MOPS/5 m*M* DTT/5 m*M* $MgCl_2$ pH 7.5) before addition of additives. V = L-val.

Table 4
Effect of L-val Replacements on the Thermal Stability
of the Purified ACVS Activity in Presence of Cofactors

Amino acid added, 5 m*M*	Tripeptide formation rate (%)[17]	ATP/Mg^{2+} addition, 5 m*M*/5 m*M*	34°C incubation time, min	Residual ACVS activity, %
		—	0	100
None		—	10	3
		+	10	31
L-valine	100	+	10	91
L-*allo*-isoleucine	44	+	10	67
L-isoleucine	11	+	10	52
L-aminobutyrate	41	+	10	50
L-norvaline	27	+	10	43
L-norleucine	8	+	10	41
L-leucine	13	+	10	38
Glycine	10	+	10	28
D-valine	0	+	10	31

were also good stabilizers. Glycine and D-val were not effective for stabilization. Being the natural substrate, L-val probably has the highest affinity to the enzyme, binds to the enzyme most efficiently and fits the "pocket" best. It is thus not surprising that L-val exerted the highest stabilizing effect.

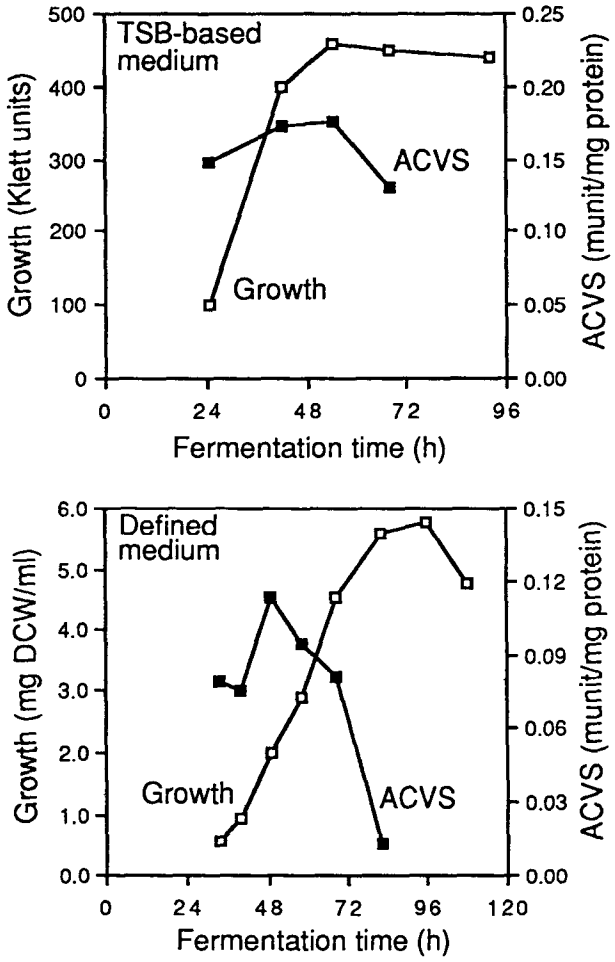

Fig. 6. *S. clavuligerus* ACVS profiles during fermentations in defined medium and trypticase soy broth-based medium.

Possible In Vivo Stabilization of ACVS by Amino Acids

Since a number of amino acids, especially L-val, are able to stabilize ACVS in the presence of cofactors, it is possible that the relatively stable *S. clavuligerus* ACVS level within the fermentation cycle in TSB-based complex medium (Fig. 6) is partly due to the presence of high concentrations of amino acids in tripticase soy broth. This might result in higher intracellular concentrations of various amino acids which could stabilize the enzyme in vivo. In contrast, there was rapid decay of the ACVS level in chemically-defined medium (containing L-asparagine as sole amino acid) even though growth was continuing, possibly owing to the lack of in vivo

stabilization. Cephamycin production in TSB-based medium is more than twice that in the defined medium (data not shown). Agathos and Demain (*18*) observed that with *Bacillus brevis,* the addition of the five constituent amino acids of gramicidin S stabilized in vivo the gramicidin S synthetase complex; L-leucine and L-ornithine played the major role in this amino acid-mediated in vivo stabilization.

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REFERENCES

1. Demain, A. L. and Wolfe, S. (1987), *Devel. Ind. Microbiol.* **27,** 175–182.
2. Malmberg, L. H. and Hu, W.-S. (1990), in *Abstr. 200th ACS National Meeting. Division of Biochemical Technology,* American Chemical Society, Washington, DC.
3. Zhang, J. and Demain, A. L. (1990), *Biochem. Biophys. Res. Commun.* **169,** 1145–1152.
4. Banko, G., Wolfe, S., and Demain, A. L. (1986), *Biochem. Biophys. Res. Commun.* **137,** 528–535.
5. Banko, G., Demain, A. L., and Wolfe, S. (1987), *J. Amer. Chem. Soc.* **109,** 2858–2860.
6. Jensen, S. E., Westlake, D. W. S., and Wolfe, S. (1988), *FEMS Microbiol. Lett.* **49,** 213–218.
7. Zhang, J. and Demain, A. L. (1990), *Biotechnol. Lett.* **12,** 649–654.
8. Jensen, S. E., Wong, A., Rollins, M. J., and Westlake, D. W. S. (1990), *J. Bacteriol.* **172,** 7269–7271.
9. Aharonowitz, Y. and Demain, A. L. (1979), *Can. J. Microbiol.* **25,** 61–67.
10. Jensen, S. E., Leskiw, B. K., Vining, L. C., Aharonowitz, Y., and Westlake, D. W. S. (1986), *Can. J. Microbiol.* **32,** 953–958.

11. Bradford, M. M. (1976), *Analyt. Biochem.* **12,** 248–254.
12. Zhang, J. and Demain, A. L. (1992), *Arch. Microbiol.* **158,** in press.
13. Scopes, R. K. (1987), *Protein Purification, Principles and Practice,* Springer-Verlag, New York, pp. 246–252.
14. Goldberg, A. L. and Dice, J. F. (1974), *Annu. Rev. Biochem.* **43,** 835–869.
15. Vitkovic, L. and Pfaender, P. (1982), in *Peptide Antibiotics, Biosynthesis and Functions,* Kleinkauf, H., and von Dohren, H., eds., Walter de Gruyter, Berlin, pp. 297–306.
16. Wolfe, S., Demain, A. L. Jensen, S. E., and Westlake, D. W. S. (1984), *Science* **226,** 1386–1392.
17. Zhang, J. (1991) *ACV synthetase in cephalosporin biosynthesis,* PhD. Thesis, Massachusetts Institute of Technology, Cambridge, MA 02139.
18. Agathos, S. N. and Demain, A. L. (1986), *Enzyme Microb. Technol.* **8,** 465–468.