Journal of Industrial Microbiology, (1995) 14, 35–40

Production of δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine by entrapped ACV-synthetase from *Streptomyces clavuligerus*

Tenshuk A. Kadima, Susan E. Jensen and Michael A. Pickard

Department of Microbiology, M330 Biological Sciences Building, University of Alberta, Edmonton, Alberta T6G 2E9, Canada

(Received 12 April 1994; accepted 21 October 1994)

Key words: Streptomyces clavuligerus; Entrapped enzyme; δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine synthesis; ACV; ACV-synthetase

SUMMARY

δ-(L-α-Aminoadipyl)-L-cysteinyl-D-valine (ACV)-synthetase from *Streptomyces clavuligerus* was studied under conditions that enabled the reuse of the enzyme. Coupling of ACV-synthetase to DEAE-Trisacryl and aminopropyl-glass resulted in an immobilized enzyme product of little or no catalytic activity. However, an enzyme reactor was designed by physical confinement of partially-purified ACV-synthetase in an ultrafiltration cell. This system was stimulated by phosphoenolpyruvate at lower concentrations of ATP, an effect not observed with purified enzyme. Up to 30% conversion of the limiting substrate, cysteine, to ACV occurred under semi-continuous conditions. Reaction products were investigated as potential inhibitors: AMP was the most inhibitory, but only when used at concentrations in excess of those produced in reaction mixtures. Under a nitrogen atmosphere, both product and enzyme stabilities were greatly improved and the enzyme retained 45–65% of its initial activity after five uses at room temperature during a 24-h period. Extrapolations based on these data suggest that 1.3 g partially purified enzyme (0.13 U g^{-1}) would be capable of producing 411 mg of ACV in a 1-L reaction mixture in this period.

INTRODUCTION

Antibiotics with the penam and cephem structures are formed by the same biosynthetic pathway which, at an early stage, assembles the three amino acids: L-α-aminoadipic acid, L-cysteine, and L-valine into the tripeptide ACV [3,12]. This reaction is catalyzed by ACV-synthetase (ACVS), a large multifunctional enzyme with a molecular weight ranging from 405 to 425 kDa, as determined from the nucleotide sequence of the gene from different microorganisms [2,9,24].

The broad substrate specificity of ACVS permits the use of this enzyme in vitro for the synthesis of unnatural precursors of penicillins and cephalosporins from substrate analogues [3,4]. This process cannot be carried out with whole cells because of permeability and toxicity problems [26]. The immobilization of five enzymes involved in the biosynthesis of penicillin and cephalosporin β-lactam antibiotics in *Streptomyces clavuligerus*, including ACVS, onto DEAE-Trisacryl resin has previously been demonstrated by Jensen and coworkers [13,14,16]. However, these systems needed improvements in both productivity and operational stability. Significant progress has since been made on studies of the stability and biochemical properties of ACVS from *S. clavuligerus*, and the kinetics of enzyme production [15,18,20,29,30]. This progress has made possible the development of an efficient enzymic process for the in vitro production of ACV. In this study we report on further investigations on the entrapment and use of ACVS from *S. clavuligerus*, as a potential alternative to chemical synthesis of ACV.

Correspondence to: M.A. Pickard, Dept of Microbiology, M330 Biological Sciences Building, University of Alberta, Edmonton, Alberta T6G 2E9, Canada.

MATERIALS AND METHODS

Bacterial strain and culture conditions

Streptomyces clavuligerus NRRL 3585 from the Northern Regional Research Laboratories (Peoria, IL, USA) was maintained as lyophilized spores or spore suspensions in 20% glycerol at −75 °C. The organism was grown from a seed culture in a Trypticase Soy broth medium supplemented with 1% (w/v) soluble starch, as described previously [20].

Isolation of ACVS for immobilization

The mycelia from 1 L of a 38-h culture of *S. clavuligerus* were collected by filtration through a Whatman No. 1 filter paper; washed with 50 mM Tris/HCl buffer, pH 7.5, containing 50 mM KCl; resuspended in 40 ml of MKG lysis buffer (100 mM MOPS-KOH buffer, pH 7.5, 50 mM KCl, 5 mM $MgCl_2$, 20 mM EDTA, and 50% glycerol) supplemented with β-mercaptoethanol (30 mM); and disrupted by sonication to make a crude cell-free extract. The crude cell-free extract was clarified by centrifugation for 30 min at 17 000 × *g*, and crude ACVS purified by a combination of salt precipitation, ultrafiltration, and anion-exchange chromatography as described by Jensen et al. [17]. Partially purified ACVS was obtained following ammonium sulfate fractionation of the cell-free extract, in the 35–45% saturation ammonium sulfate fraction, and resuspended in MDKG buffer (MDK buffer containing 20% glycerol (v/v)).

ACVS assay

ACVS activity was measured in standard reaction mixtures (0.1 ml) consisting of ATP (2 mM), $MgCl_2$ (6 mM), dithiothreitol (DTT, 5 mM), phosphoenolpyruvate (PEP, 5 mM), L-α-aminoadipic acid (5 mM), L-cysteine (1 mM) and L-valine (5 mM) in 100 mM Tris/HCl, pH 8.5 buffer [20]. Assays were incubated for 1 h at 22–24 °C, terminated with 0.025 ml of 20% trichloroacetic acid (TCA), and precipitated protein was removed by centrifugation. Kinetic characteristics of purified enzyme were determined in the standard reaction mixtures, without PEP, by holding two of the substrate amino acids constant while varying the other. ACV in the reaction mixtures was derivatized with the fluorescent thiol reagent Thiolyte MB, and quantitated by High Performance Liquid Chromatography as described previously [15], except that the solvent system consisted of solvent A (10% methanol: 0.025% (v/v) acetic acid adjusted to pH 5.0 with NaOH) and solvent B (90% methanol: 0.025% (v/v) acetic acid adjusted to pH 5.0 with NaOH). One unit of ACVS activity was defined as the amount producing 1 μmol of ACV per min, and specific activity was expressed as mU mg^{-1} protein, assayed by the protein dye-binding method [6] using bovine serum albumin as a standard. Quantitation of ACVS chemically coupled to an insoluble carrier was done as described previously [16].

Derivatization of controlled-pore glass

Controlled-pore glass (CGP: 240-Å pore size, 200–400 mesh) was aminated as described before [19]. CPG beads (1 g) were acid-washed by heating for 1 h in 10% nitric acid at 80–90 °C, extensively washed with water, and then heated in 20 ml of 10% aqueous aminopropyltriethoxysilane solution, pH 3.4 [27], for 3 h at 70 °C. The aminopropyl-CPG was washed with water on a sintered glass filter, air dried, then dried overnight at 95–100 °C. The aminopropyl-CPG was washed with water again before crosslinking with glutaraldehyde or being used as an ionic binding matrix.

Immobilization of ACVS

During the immobilization procedures described below, all washing and enzyme storage buffers were supplemented with ACVS stabilization cocktail (20% glycerol, 5 mM DTT, 3 mM $MgCL_2$, 5 mM L-α-aminoadipate, 1 mM cysteine and 5 mM valine) [20].

(a) Covalent immobilization by the Weetall method [27]. This was carried out by incubating partially purified enzyme (7.8 mg ml^{-1} protein, 0.13 mU mg^{-1}) with glutaraldehyde-treated aminopropyl-CPG (0.1 mg dry weight) for 6 h, at 4 °C. The bound enzyme was then washed with high ionic strength buffer (0.1 M MDG buffer, pH 7.5 containing 0.5 M NaCl) to remove non-covalently bound protein.

(b) Covalent immobilization by the modified Weetall method [19]. This was carried out with the same amounts of partially purified enzyme and aminopropyl-CPG as in (a) but not treated with glutaraldehyde. The mixture was incubated at 4 °C for 6 h, washed with a low ionic strength solution (0.02 M MDG buffer, pH 7.5), and then treated with glutaraldehyde (1 ml, 2.5%) for 1 h at room temperature to crosslink the ionically-bound protein to the aminopropyl-CPG. The preparation was subsequently washed with high ionic strength buffer and stored at 4 °C until use.

(c) Ionic binding. Ionic binding to DEAE-Trisacryl resin was carried out as described by Jensen et al. [16], and to aminopropyl-CPG according to the procedure of Kadima and Pickard [19]. A Mono Q-purified ACVS preparation (11.1 mg protein, 0.64 mU mg^{-1}) was desalted by ultrafiltration using a 300-kDa membrane [16] in 20 mM MOPS buffer, pH 7.5, containing the ACVS stabilization cocktail to reduce the ionic strength prior to immobilization. Half of the sample (5.5 mg protein in 2 ml) was loaded on 0.5 g of damp DEAE-Trisacryl resin in a 1-ml syringe and the other half used for immobilization on 0.1 g of aminopropyl-CPG, also in a 1-ml syringe. After the 2-ml protein sample was circulated three times through the carrier, unbound protein was removed by washing with low ionic strength stabilization buffer (4 ml), and measured to estimate protein loading by difference.

(d) Entrapment. Physical confinement of ACVS was carried out in a 50-ml ultrafiltration cell (Amicon Corp model 52, Amicon Corp., Beverly, MA, USA) containing a YM-30 membrane (molecular exclusion 30 kDa), which retained the enzyme and permitted its reuse in a semi-continuous operation. To the enzyme (7.8 mg ml^{-1} protein, 0.13 mU mg^{-1}) was added ACVS assay mixture (5 ml) and incubation proceeded with gentle mixing at 24 °C, under an oxygen or a nitrogen atmosphere, at an operating pressure of 15 p.s.i. but with the outlet clamped. Samples (0.5 ml) were collected from the outlet from which duplicate 100-μl volumes were removed for analysis, and the unused portions were returned to the reactor. In testing the reusability of the immobilized enzyme, most of the reaction mixture was removed by ultrafiltration under pressure, and the enzyme washed twice with 5 ml of 20 mM Tris/HCl buffer, pH 8.5 containing 2 mM DTT and 10% glycerol, before replenishment of the reactor with fresh reagents.

Chemicals and matrices

Monobromobimane (Thiolyte MB) was from Calbiochem (San Diego, CA, USA). ACV was from Incell Corporation (Millwaukee, WI, USA). CPG (240-Å pore size and 200–400 mesh size) was from Sigma Chemical Co., St Louis, MO, USA. The resins DEAE-Trisacryl and CM-Trisacryl were from LKB Instruments, Rockville, MD, USA. All other chemicals were of reagent grade.

RESULTS

Covalent binding of ACVS to aminopropyl controlled-pore glass

Initially, attempts were made to immobilize ACV-synthetase covalently on porous glass beads (240-Å pore size, 200–400 mesh) using two related methods, that of Weetall [27] and a modification of that method [19]. Since the loading of protein to aminopropyl-CPG is pH-dependent, the effect of pH on enzyme stability and the coupling of protein to the carrier

was also investigated. During an overnight incubation at 4 °C, ACVS was found to be most stable at pH 7.5, losing activity at pH 5 and at pH 9.0. Subsequent studies were carried out at pH 7.5. At this pH, a loading capacity of 25–31 mg protein g^{-1} of aminopropyl-CPG was estimated from the amount of unbound protein. The two immobilized enzyme preparations were analyzed for activity, but in both cases no detectable catalytic activity was observed.

Ionic binding of ACVS to aminopropyl controlled-pore glass and DEAE-Trisacryl

Since the immobilization of ACVS by ionic linkage to DEAE-Trisacryl resin provided an active preparation [16] it was decided to determine the efficiency of this method and compare it to ionic binding on aminopropyl-CPG [19].

Purified enzyme (5.5 mg protein, 0.64 mU mg^{-1}), prepared in a low ionic strength MOPS buffer supplemented with ACVS stabilization cocktail (20% glycerol, 5 mM DTT, 3 mM $MgCl_2$, 5 mM L-α-aminoadipate, 1 mM cysteine and 5 mM valine) [20], was loaded in a 1-ml syringe containing 0.5 g of damp DEAE-Trisacryl resin or 0.1 g of aminopropyl-CPG. Most of the protein bound to DEAE-Trisacryl at 11 mg g^{-1} resin, which corresponded to only a fraction of the estimated maximum loading capacity of 81–94 mg g^{-1} for this support [16]. Protein bound to aminopropyl-CPG at 31 mg g^{-1} glass. In both cases the resulting immobilized enzyme products were catalytically active but their specific activities were low compared to the specific activity of the purified soluble enzyme. The enzyme immobilized on DEAE-Trisacryl was most active but catalyzed the formation of ACV at only 4.4% of the rate of the soluble enzyme, whereas the aminopropyl-CPG-bound enzyme had only 1.7% of the specific activity of the soluble enzyme. Analysis of the kinetics of ACV formation by the DEAE-Trisacryl-bound enzyme showed a linear rate for about 1.5 h before decreasing markedly. Supplementation with cysteine produced only a slight improvement in the production level (Fig. 1). Activity was highest when reactions were shaken, presumably due to accelerated mass transfer in the system but the increasing aeration decreased the enzyme stability (data not shown) which has implications for a continuous process.

Entrapment of ACVS in an ultrafiltration cell

Since physical or chemical coupling of the enzyme to insoluble carriers resulted in low catalytic activity, physical confinement was investigated as a system for the reuse of the enzyme. Because of the large size of ACVS, confinement of the enzyme in an ultrafiltration unit fitted with a 30- or 50-kDa cutoff membrane enabled reuse of the enzyme, the removal of products and unused substrates, and the replenishment with fresh reagents in a semi-continuous operation.

Optimization of the enzymic production of ACV in vitro

To ensure optimum overall substrate conversion level, the effect of substrates on ACVS activity was studied with purified enzyme and the Michaelis constants determined. L-Cysteine was inhibitory to the enzyme at higher concentrations, and a similar observation was made with ATP, implying that careful

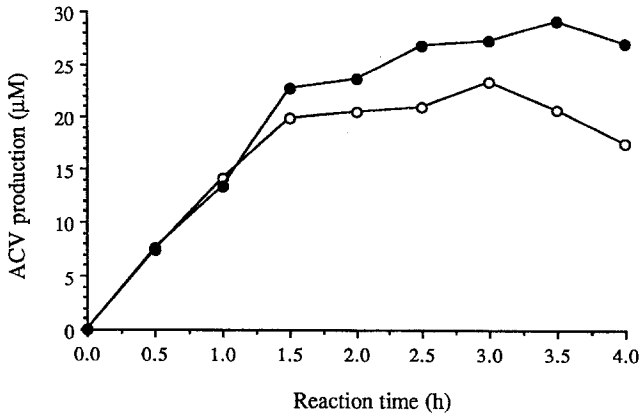

Fig. 1. Effect of cysteine supplementation on ACV production by ACV-synthetase immobilized on DEAE-Trisacryl. Purified ACV-synthetase (11.1 mg protein, 0.64 mU mg^{-1}) was divided into two portions and each bound to damp DEAE-Trisacryl resin (0.5 g), loaded in 1-ml syringe. The immobilized enzyme was resuspended in a 10-ml vial and reacted with 1 ml of the standard assay mixture with shaking at 188 r.p.m. at room temperature for 4 h without (○) or with (●) incremental supplements of 20 mM L-cysteine (5 μl) to a final concentration of 0.1 mM at 30-min intervals. At each addition 20-μl aliquots of the reaction mixtures were removed and assayed for ACV production.

supply of these two components was necessary to improve the efficiency of ACV production by the immobilized enzyme system. The K_m values for the three substrate amino acids were: 6.25, 0.43, and 3.75 × 10^{-4} M for L-α-aminoadipate, L-cysteine and L-valine, respectively. The highest affinity of ACVS for L-cysteine was consistent with previous reports [5,15,30], but some variations were observed with the reported K_m values, as shown in Table 1. The K_m for ATP was 7.58 × 10^{-4} M. Analysis of the rate of catalysis over a temperature range of 5–24 °C was also carried out for a possible use of the enzyme at low temperature to prolong enzyme stability. The activity profile showed a linear increase with temperature, and a Q_{10} value of 2 was estimated. However, the slow rate of catalysis at lower temperatures obviated the use of the enzyme at such temperatures, though the stability of the enzyme was significantly higher as reported previously [20].

Semi-continuous production of ACV by entrapped ACVS

Preparation of pure ACVS is labour-intensive and results in only low enzyme recovery [17], thus the reuse of entrapped ACVS was studied with partially purified enzyme. In this way advantage was taken of the stimulatory effect of phosphoenolpyruvate on ACV formation in the presence of an endogenous ATP-regenerating system [20]. As shown in Fig. 2, the kinetics of ACV formation by partially purified ACVS were greatly improved by PEP at low ATP concentration. This effect was not observed with purified enzyme.

ACVS may be inactivated by oxidation of its essential sulfhydryl groups [25,30], and its activity may be affected by conversion of cysteine to cysteic acid. Therefore, the effect of replacing an air atmosphere in the reactor with a nitrogen atmosphere was investigated to improve enzyme stability during operation at room temperature. The results in Table 2 show

TABLE 1

Michaelis constants for ACV-synthetase substrates

Organism	L-AAA[a]	L-cysteine	L-valine	Reference
C. acremonium (crude)	1.7×10^{-4} M	2.6×10^{-5} M	3.4×10^{-4} M	[5]
S. clavuligerus (crude)	5.6×10^{-4} M	7.0×10^{-5} M	1.1×10^{-3} M	[14]
S. clavuligerus (pure)	6.3×10^{-4} M	1.2×10^{-4} M	3.0×10^{-4} M	[29]
S. clavuligerus (pure)	6.3×10^{-4} M	4.3×10^{-5} M	3.8×10^{-4} M	this work

[a]L-AAA: L-α-aminoadipate.

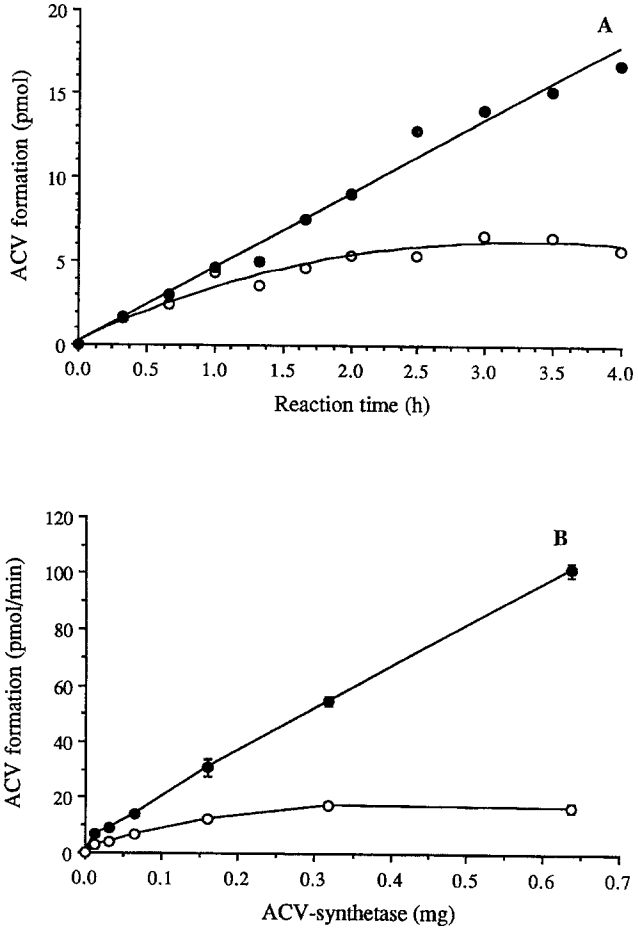

Fig. 2. Effect of PEP on the kinetics of ACV formation by partially purified ACV-synthetase. Effects of reaction time (A) and enzyme amounts (B) determined with the standard assay system (•) or with the standard assay system without PEP (○).

that during the first two uses both reactors were equally efficient in producing ACV up to a concentration of 0.26 mM, representing a 26% conversion of cysteine in 3 h. Occasionally conversion levels of 30% were achieved. However, as the number of uses increased, the enzyme under nitrogen atmosphere was more stable than the enzyme under air, in which case the ACV production level was reduced after the fourth use to a quarter or less of the original. However under a nitrogen atmosphere, although the production levels began to decrease after the fourth use, the enzyme still retained a half or more of its initial operational activity after 24 h. Other results showed that the product was much more stable under nitrogen atmosphere, and that disappearance of ACV from the reaction mixture after overnight incubation in air could be reversed by the addition of DTT to the reaction mixture at the end of the incubation, but not to the same level as that produced under nitrogen. This showed that both ACV and ACVS were subject to air oxidation, and that a nitrogen atmosphere enhanced the stability of both product and enzyme.

TABLE 2

Effect of repeated use of ACV-synthetase on ACV formation under nitrogen or air atmosphere

Number of use #[a]	Total time elapsed[c] (h)	Conversion of 1 mM Cys (%)[d]	
		Nitrogen	Air
1	3.0	24.6	25.4
2	6.5	25.9	25.3
3	10.0	22.0	19.0
4[b]	14.0 (20.0)[b]	11.0 (25.9)	4.9 (7.0)
5	23.0	16.0	5.9

[a]Each normal use represents a 3-h reaction period, except
[b]for use #4, the conversion values in brackets correspond to the values estimated after an additional 6-h period of operation to the normal 3-h use, resulting in a total reaction time of 9 h before reaction mixture was replaced with fresh reagents.
[c]Total time elapsed from first use to the fifth use, including washings.
[d]Conversion is estimated on the basis of the amount of ACV produced (μmol) from the initial amount of cysteine (5 μmol) in 5 ml of the standard reaction mixture.

Analysis of factors affecting conversion yields

Although the conversion of cysteine into ACV occurred at up to 30% yield, there was still a large excess of unconverted substrate, and attempts to increase the yield above 30% were unsuccessful. No product inhibition of ACVS by ACV occurred at concentrations up to 137 μM ACV. Higher concentrations of ACV increased the background to levels of ACV that prevented an accurate determination of product formed from catalytic activity alone.

Other reaction products were tested as potential inhibitors of ACVS. The enzyme activity was inhibited by AMP, PP_i, and ADP, at 5 mM each, by 90, 62 and 40%, respectively.

AMP inhibition was strongest, but only at concentrations in excess of those formed in reaction mixtures. Yield was not improved above 30% by increasing the enzyme concentration or by increasing the enzyme concentration and adding cysteine incrementally.

DISCUSSION

Immobilized ACVS is a useful alternative to complex syntheses of ACV (see references in ref. [11]) and ACV analogues [11]. Synthesis of ACV analogues by ACVS by providing analogues of L-α-amino adipate, L-cysteine and L-valine has been proposed as a means of developing novel antibiotics [3,22,28]. Many ACV analogues have been prepared by laborious chemical techniques and exposed to IPNS to make novel β-lactam antibiotics [11]. Immobilized ACVS offers the chemist an opportunity to focus on making substrate analogues and provides a simple reagent for condensing them into the tripeptide structure. Although the use of ACVS in this manner would necessarily limit the scope of novel compounds by the existing specificity of ACVS, the benefits probably outweigh this limitation.

Thus, as a continuation of the work reported by Jensen et al. [16] on the immobilization of ACVS from *S. clavuligerus*, an enzymic process for ACV synthesis has been developed which shows improved stability of the catalyst and product yield. In the previous study, ACVS was immobilized on the anion-exchange resin DEAE-Trisacryl and shown to carry out repeated synthesis of ACV. However, after six 1-h uses the enzyme had lost 70% of the initial activity. The amount of product formed was also small, representing only 2.5% conversion of the limiting substrate cysteine after 4 h of operation. Following immobilization, the estimated specific activity of the enzyme drastically decreased from 94×10^{-3} to 4.6×10^{-3} mU mg^{-1} protein, corresponding to a 95% reduction in activity. This study has confirmed that enzyme bound ionically onto DEAE-Trisacryl had only 4.4% the specific activity of the soluble enzyme. This decrease in activity was likely due in part to mass transfer limitation, since a slight improvement in the catalytic rate was observed with increase in the agitation rate. Conformational and steric effects [21] were probably also a factor, since ACVS is a large multifunctional enzyme and binding to the support could interfere with its proper functioning, either from occlusion of the active site or restriction of enzyme flexibility. These features were not a problem with the smaller and more stable fungal enzyme chloroperoxidase which was successfully covalently immobilized onto aminopropyl-glass by methods similar to those used here [19].

Entrapment of ACVS in an ultrafiltration cell was found to be an effective method to achieve the enzymic production of ACV. This procedure presented a number of advantages over the other methods investigated. The large size of ACVS permitted its repeated use within the cell using readily available ultrafiltration membranes, and the removal by filtration of the low molecular weight products and unused substrates in a semi-continuous fashion. In using partially purified enzyme, the system exploited the stimulatory effect of the endogenous cellular ATP-regenerating components and also minimized loss and handling of the enzyme associated with extensive purification procedures. Also, enzymic activity was not affected by mass transfer limitations and loss of activity due to rigid coupling to an insoluble support. Finally, control of the ambient atmosphere minimized oxidation of both enzyme and product by air.

Entrapped ACVS was used repeatedly and produced ACV to amounts corresponding to up to 25–30% of cysteine added to the reactor, with all other components being in excess. Immobilization of a cephalosporin acetylesterase from *Bacillus subtilis* by containment within an ultrafiltration device had also been reported [1]. This enzyme which deacylates 7-aminocephalosporanic acid solutions containing 4–24 mg ml^{-1} of this compound was reused 20 times over an 11-day span. In this study, entrapped ACVS was reused five times, and the enzyme retained 45–65% of its initial operational activity after 24 h of continuous incubation at room temperature under a nitrogen atmosphere. Thus, incubation of the enzyme in the presence of its substrates and less oxidizing environment provided a significant improvement in the stability of this labile enzyme [4]. A similar improvement in the stability of gramicidin S synthetases by substrates was observed, resulting in the persistence of gramicidin S production for several hours at 37 °C [8,10]. The loss of activity of physically-confined ACVS may have been due to inactivation or adsorption of the enzyme to the membrane and walls of the reactor vessel, since similar loss of enzymic activity due to binding of the enzyme to ultrafiltration membranes had been observed during the purification of ACVS by Schwecke et al. [23].

Production of ACV to levels greater than 0.25–0.30 mM could not be achieved and factors that affected ACV production levels, other than enzyme stability, were analyzed. The reaction products resulting from ATP utilization inhibited the enzyme to varying degrees. Similar observations have been made by Zhang et al. [30] from the analysis of the effects of a number of compounds on ACVS activity. In this study AMP was most inhibitory but only at non-physiological concentrations. The addition of enzymes to further metabolize the reaction products, AMP and pyrophosphate, might have pushed the reaction further to completion. However addition of adenylate kinase, adenylic acid deaminase, or inorganic pyrophosphatase to the reaction mixture did not improve the production of ACV, suggesting that AMP and pyrophosphate were not the primary limitation to substrate conversion. Similarly, the lack of stimulation by increments of cysteine showed that the system was not substrate-limited and some other factor is limiting ACV synthesis. Nevertheless, on the basis of the conversion levels shown in Table 2, under a nitrogen atmosphere, a 1-L reaction mixture could produce 411 mg ACV in 24 h using the same batch of enzyme.

As a result of recent intensive studies of the biosynthetic pathways of cephamycin and cephalosporin, the total synthesis of these classical β-lactam antibiotics in vitro is now possible since most of the enzymes, substrates and cofactors involved in the pathway are known and available [3,7,12]. The next logical step would be to attempt the total synthesis of these antibiotics with immobilized or entrapped enzymes, and initial

efforts towards these goals have been made by Jensen and co-workers with the enzymes from *S. clavuligerus* [13].

ACKNOWLEDGEMENTS

This research was supported by grants from the Natural Sciences and Engineering Council of Canada to M.A.P. and S.E.J.

REFERENCES

1 Abbott, B.J., B. Cerimele and D.S. Fukuda. 1976. Immobilization of a cephalosporin acetylesterase by containment within an ultrafiltration device. Biotechnol. Bioeng. 18: 1033–1042.

2 Aharonowitz, Y., J. Bergmeyer, J.M. Cantoral, G. Cohen, A.L. Demain, U. Fink, J. Kinghorn, H. Kleinkauf, A. MacCabe, H. Palissa, E. Pfeifer, T. Schwecke, H. van Liempt, H. von Döhren, S. Wolfe and J. Zhang. 1993. δ-(L-α-aminoadipyl)-L-cysteine-D-valine synthetase, the multienzyme integrating the four primary reactions in β-lactam biosynthesis, as a model peptide synthetase. Bio/Technology 11: 807–810.

3 Baldwin, J.E. and C. Schofield. 1992. The biosynthesis of β-lactams. In: The Chemistry of β-Lactams (Page, M.I., ed.), pp. 1–79, Blakie Academic & Professional, London.

4 Banko, G., A.L. Demain and S. Wolfe. 1986. δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine synthetase (ACV synthetase): a multifunctional enzyme with broad substrate specificity for the synthesis of penicillin and cephalosporin precursors. J. Amer. Chem. Soc. 109: 2858–2860.

5 Banko, G., S. Wolfe and A.L. Demain. 1987. Cell-free synthesis of δ-(L-α-aminoadipyl)-L-cysteine, the first intermediate of penicillin and cephalosporin biosynthesis. Biochem. Biophys. Res. Commun. 137: 528–535.

6 Bradford, M. 1976. A rapid, sensitive method for the quantitation of microgram quantities of protein utilizing the principal of protein-dye binding. Anal. Biochem. 72: 248–254.

7 Demain, A.L. and J.M. Piret. 1991. Cephamycin production by *Streptomyces clavuligerus*.. In: Genetics and Product Formation in *Streptomyces* (Baumberg, S., H. Krugel and D. Noack, eds), pp. 87–103, Plenum Press, New York.

8 Demain, A.L. and D.I.C. Wang. 1976. Enzymatic synthesis of gramidicin S. In: Second International Symposium on the Genetics of Industrial Microorganisms (Macdonald, K.D., ed.), pp. 115–128, Academic Press, London.

9 Díez, B., S. Gutiérrez, J.L. Barredo, P. Solingen, L.H.M. van der Voort and J.F. Martín. 1990. The cluster of penicillin biosynthetic genes: identification of the *pcbAB* gene encoding the α-aminoadipyl-L-cysteinyl-D-valine synthetase and linkage to the *pcbC* and *penDE* genes. J. Biol. Chem. 265: 16358–16365.

10 Hamilton, B.K., J.P. Montgomery and D.I.C. Wang. 1973. Enzyme reaction for preparative scale synthesis. In: Enzyme Engineering, Vol. II (Pye, E.K. and L.B. Wingard Jr, eds), pp. 153–159, Plenum Press, New York.

11 Huffman, G.W., P.D. Gesellchen, J.R. Turner, R.B. Rothenberger, H.E. Osborn, F.D. Miller, J.L. Chapman and S.W. Queener. 1992. Substrate specificity of isopencillin N synthetase. J. Medicinal Chem. 35: 1897–1914.

12 Jensen, S.E. and A.L. Demain. 1994. Beta-Lactams. In: Genetics and Biochemistry of Antibiotic Production (Vining, L.C. and C. Stuttard, eds), pp. 239–268, Butterworth-Heinemann, Stoneham, MA.

13 Jensen, S.E. and D.W.S. Westlake. 1989. Immobilization of β-lactam synthesizing enzymes. Developments in Industrial Microbiology 30: 113–119. J. Cooney and O. Sebek (eds), Society for Industrial Microbiology.

14 Jensen, S.E., D.W.S. Westlake and S. Wolfe. 1984. Production of penicillins and cephalosporins in an immobilized enzyme reactor. Appl. Microbiol. Biotechnol. 20: 155–160.

15 Jensen, S.E., D.W.S. Westlake and S. Wolfe. 1988. Production of the penicillin precursor δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV) by cell free extracts from *Streptomyces clavuligerus*. FEMS Microbiol. Lett. 49: 213–218.

16 Jensen, S.E., S. Wolfe and D.W.S. Westlake. 1989. Synthesis of the penicillin precursor, δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV), by an immobilized enzyme preparation. Appl. Microbiol. Biotechnol. 30: 111–114.

17 Jensen, S.E., A. Wong, M.J. Rollins and D.W.S. Westlake. 1990. Purification and characterization of δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine synthetase from *Streptomyces clavuligerus*. J. Bacteriol. 172: 7269–7271.

18 Kadima, T.A. 1993. Production, stabilization, and immobilization of δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV) synthetase from *Streptomyces clavuligerus*; refolding and characterization of the domain-1 polypeptide. Ph.D. thesis, University of Alberta, Edmonton, Alberta, Canada.

19 Kadima, T.A. and M.A. Pickard. 1990. Immobilization of chloroperoxidase on aminopropyl-glass. Appl. Environ. Microbiol. 56: 3473–3477.

20 Kadima, T.A., S.E. Jensen and M.A. Pickard. 1993. Production kinetics and stability properties of δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine synthetase from *Streptomyces clavuligerus*. J. Ind. Microbiol. 12: 58–65.

21 Kennedy, J.F. and J.M.S. Cabral. 1983. Immobilized enzymes. In: Solid Phase Biochemistry (Scouten, W.H., ed.), pp. 253–391, John Wiley & Sons, New York.

22 Martín, J.F. and P. Liras. 1989. Enzymes involved in penicillins, cephalosporin and cephamycin biosynthesis. Adv. Biochem. Eng. 39: 154–187.

23 Schwecke, T., Y. Aharonowitz, H. Palissa, H. von Döhren, H. Kleinkauf and H. van Liempt. 1992. Enzymatic characterization of δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine synthetase from *Streptomyces clavuligerus*. Eur. J. Biochem. 205: 687–694.

24 Smith, D.J., A.J. Earl and G. Turner. 1990. The multifunctional peptide synthetase performing the first step of penicillin biosynthesis in *Penicillium chrysogenum* is a 421 073 dalton protein similar to the *Bacillus brevis* peptide antibiotic synthetases. EMBO J. 9: 2743–2750.

25 van Liempt, H., H. von Döhren and H. Kleinkauf. 1989. δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine synthetase from *Aspergillus nidulans*. J. Biol. Chem. 264: 3680–3684.

26 Vandamme, E.J. 1990. Biochemical, genetical and biotechnical aspects of antibiotic production via immobilized biocatalysts. In: Biochemistry of Peptide Antibiotics (Kleinkauf, H. and H. von Döhren, eds), pp. 379–410, Walter de Gruyter, Berlin.

27 Weetall, H.H. 1976. Covalent coupling methods for inorganic support materials. Methods Enzymol. 44: 134–148.

28 Wolfe, S., A.L. Demain, S.E. Jensen and D.W.S. Westlake. 1984. Enzymatic approach to syntheses of unnatural β-lactams. Science 226: 1386–1392.

29 Zhang, J. and A.L. Demain. 1992. ACV-synthetase. Crit. Rev. Biotechnol. 12: 245–260.

30 Zhang, J., S. Wolfe and A.L. Demain. 1992. Biochemical studies on the activity of δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine synthetase from *Streptomyces clavuligerus*. Biochem. J. 283: 691–698.