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

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SUMMARY

δ-(L-α-Aminoacidipyl)-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 phospho-enol-pyruvate at lower concentrations of ATP, an effect not observed with purified enzyme. Up to 30% conversion of the limiting substrate, cysteine, to ACV occurred 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-aminoacidipic 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 co-workers [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.

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 20% (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 MgCl₂, 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), MgCl₂ (6 mM), dithiothreitol (DTT, 5 mM), phosphoenolpyruvate (PEP, 5 mM), L-α-aminoacidipic 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 (CPG: 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 MgCl₂, 5 mM L-α-aminoacidipate, 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

Monohromobimane (Thiolyte MB) was from Calbiochem (San Diego, CA, USA). ACV was from Incell Corporation (Milwaukee, WI, USA). CPG (240-Å pore size and 200–400 mesh size) was from Sigma Chemical Co., St Louis, MO, USA. The resins DEAE-Trisacyl and CM-Trisacyl 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