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Purification and Partial Characterization of δ-(L-α-Aminoadipyl)-L-Cysteinyl-D-Valine Synthetase from *Streptomyces clavuligerus*

SUSAN E. JENSEN,* A. WONG, M. J. ROLLINS,† AND D. W. S. WESTLAKE

Department of Microbiology, University of Alberta, Edmonton, Alberta T6G 2E9, Canada

Received 26 March 1990/Accepted 11 September 1990

δ-(L-α-Aminoadipyl)-L-cysteinyl-D-valine synthetase (ACVS) was purified from *Streptomyces clavuligerus* by a combination of salt precipitation, ultrafiltration, and anion-exchange chromatography. The final purified material gave two protein bands with molecular weights of 283,000 and 32,000 by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Electrophoresis in nondenaturing gels gave a single protein band with an estimated molecular weight of 560,000. These results suggest that ACVS is a multimer composed of nonidentical subunits.

The biosynthetic pathway leading to penicillin and cephalosporin antibiotics in *Streptomyces clavuligerus* begins with the enzyme δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine synthetase (ACVS). The enzyme carries out an ATP-dependent multistep condensation of three amino acids, L-α-aminoadipic acid, L-cysteine, and L-valine, into the linear tripeptide δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine. ACVS was first detected in *Cephalosporium acremonium* (1, 2), and subsequently, enzymes with similar characteristics in *S. clavuligerus* (8, 9) and *Aspergillus nidulans* (14) were described. ACVS resembles the nonribosomal peptide synthetases, which synthesize primary metabolites such as glutathione (6, 13) and secondary metabolites such as gramicidin S, tyrocidine, and bacitracin (7, 10, 11). Peptide synthetases are complex enzymes which carry out multistep reactions and can involve the interaction of multienzyme systems. In addition, the large size of the multienzyme complexes and the lability of the enzyme activities can make enzyme isolation and purification difficult. This study reports the purification and partial characterization of ACVS from *S. clavuligerus*.

Crude cell extract of *S. clavuligerus* NRRL 3585 was prepared as described previously (8). A 10-ml portion of the extract was diluted to 40 ml with 0.1 M MOPS (morpholinepropane sulfonic acid)-KOH buffer (pH 7.5) containing 1 mM dithiothreitol and 0.05 M KCl, and then streptomycin sulfate (0.4 g) was added and the mixture was stirred for 15 min at 4°C and centrifuged for 15 min at 17,000 × g. The supernatant was then subjected to ammonium sulfate precipitation. The optimum concentration of ammonium sulfate for sedimentation of ACVS was determined by increasing the level of saturation in 5% increments from 30 to 50%. The material sedimenting between 35 and 45% saturation was used for further purification of ACVS. This material was resuspended in 40 ml of 0.1 M MOPS-KOH buffer (pH 7.5) containing 1 mM dithiothreitol, 0.05 M KCl, and 20% glycerol (0.1 M MDKG buffer) and then concentrated by ultrafiltration. In initial studies, a range of membranes (Amicon Corp., Danvers, Md.) with molecular weight cutoffs ranging from 10,000 (PM-10) to 300,000 (XM-300) were used to estimate the molecular weight of ACVS. All of the membranes gave complete retention of ACVS activity (data not shown), and so XM-300 membranes were used in all subsequent steps. The ammonium sulfate fraction was concentrated to 4 ml by ultrafiltration at 4°C, diluted to 40 ml with 0.1 M MDKG buffer, and concentrated again to a final volume of 2 ml. The enzyme concentrate was clarified by centrifugation at 13,000 × g for 5 min and applied in 0.5-ml aliquots to a Mono Q HR 5/5 column (Pharmacia Inc., Uppsala, Sweden). The column was equilibrated with 0.1 M MDKG buffer and eluted under the following mobile phase conditions: solvent A, 0.1 M MDKG buffer; solvent B, 0.5 M MDKG buffer (0.5 M with respect to MOPS-KOH, all other components unchanged); flow rate, 0.5 ml/min; gradient, 5 min at 0% B, linear gradient over 30 min to 100% B, 15 min at 100% B, and linear gradient over 5 min to 0% B. Fractions were monitored for protein (A_{280}) and ACVS activity by using the standard ACVS assay described previously (8). The high-pressure liquid chromatography procedure for analysis of ACVS reaction mixtures was modified from that used previously, on the basis of the studies by Fahey and Newton (4). Fluorescent derivatives were formed by mixing 0.01 ml of centrifuged acidified reaction mixture with 0.03 ml of 0.2 M *N*-2-hydroxyethylpiperazine-*N'*-3-propanesulfonic acid (EPPS) buffer (pH 8.0) containing 0.005 M diethylenetriaminepentaacetic acid (DTPA) and 0.003 M monobromobimane. The derivatization mixtures were held for 10 min at 22°C and then acidified by the addition of 0.005 ml of 1.0 M methanesulfonic acid. Samples (0.01 ml) of the derivatization mixtures were analyzed by high-pressure liquid chromatography as described previously (8).

ACVS activity was found to coincide with the largest protein peak in the Mono Q elution profile (Fig. 1A). Active fractions were pooled, diluted with an equal volume of 0.1 M MDKG buffer to reduce the ionic strength, and concentrated by ultrafiltration to 2 ml. The ACVS concentrate was then reapplied to the Mono Q column by using the mobile phase conditions described above except with a gradient starting at 40% B and going to 100% B over 30 min. Fractions were monitored for protein and ACVS activity as described above. A single major protein peak was observed (Fig. 1B), which coincided with ACVS activity.

The specific activity of the ACVS preparation increased, but only modestly (12.3-fold), during the course of the purification procedure, because of the lability of the ACVS activity or possibly because of the removal of some essential component (Table 1). Similarly, the overall recovery of

* Corresponding author.
† Present address: Biostar Inc., Saskatoon, Saskatchewan S7N 0W0, Cananda.

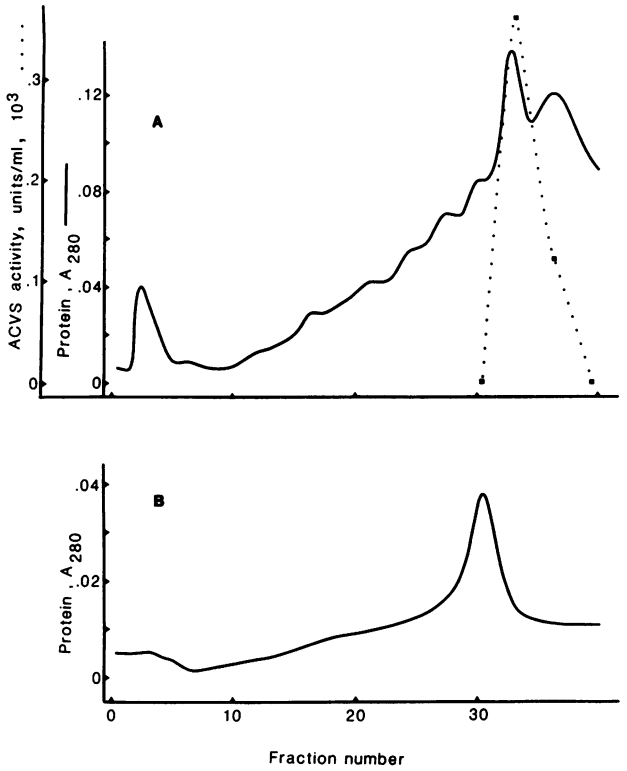

FIG. 1. Anion-exchange chromatography of ACVS on a Mono Q HR 5/5 column. (A) A 0.5-ml portion of ACVS enzyme concentrate which had been partially purified by salt precipitation and ultrafiltration was applied to a Mono Q HR 5/5 anion-exchange column and eluted with a linear gradient varying from 0.1 to 0.5 M MDKG buffer as described in Materials and Methods. Fractions were monitored for protein (A_{280}) and ACVS activity. (B) The fractions containing ACVS activity from the first Mono Q column were pooled, diluted in half with 0.1 M MDKG buffer, concentrated by ultrafiltration, and reapplied to the Mono Q HR 5/5 column. The column was eluted with a linear gradient varying from 0.2 to 0.5 M MDKG as described in Materials and Methods.

activity was very low (0.79%). Nonetheless, the purification procedure did give extensive purification of ACVS from contaminating proteins.

When samples of ACVS from throughout the purification procedure were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (PAGE) (3) on 5% gels, a single major protein band with an estimated molecular weight of 283,000 was seen in the purified material (Fig. 2A). Proteins with molecular weights less than 65,000 are not resolved by 5% gels and run at the dye front. When purified ACVS was analyzed on a 10% gel to detect lower-molecular-

TABLE 1. Purification of ACVS

Purification stage	Total activity (U, 10^4)	Sp act (U/mg of protein, 10^4)	% Recovery	Purification (fold)
Cell extract	184	0.442	100	1.0
Ammonium sulfate fraction	87.2	1.41	47.4	3.2
XM 300 concentrate	29.3	1.17	15.9	2.6
First Mono Q pool	16.1	4.38	8.8	9.9
Final Mono Q pool	1.45	5.45	0.79	12.3

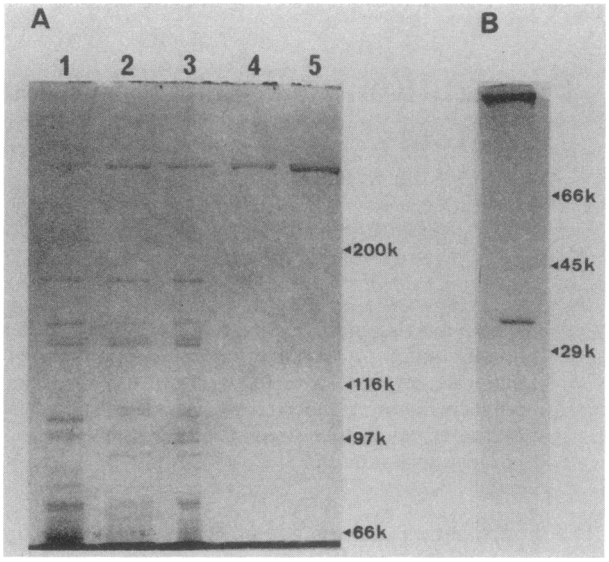

FIG. 2. Analysis of purified ACVS by sodium dodecyl sulfate-PAGE. (A) 5% polyacrylamide gel. Lane 1, Crude cell extract, 25 μg of protein; lane 2, streptomycin sulfate-ammonium sulfate-precipitated cell extract, 15 μg of protein; lane 3, XM-300 concentrate, 15 μg of protein; lane 4, ACVS after the first Mono Q column, 1.5 μg of protein; lane 5, purified ACVS after the second Mono Q column, 1.5 μg of protein. (B) 10% polyacrylamide gel. Purified ACVS after the second Mono Q column, 3.0 μg of protein. Arrows indicate the location of the appropriate molecular weight marker proteins.

weight components, a second protein band was apparent, but the high concentration of MOPS-KOH buffer in the sample caused distortion of the band (data not shown). Attempts to desalt the purified ACVS by dialysis and ultrafiltration resulted in severe losses of material, and so the purified ACVS was desalted by chloroform-methanol precipitation (15). When this sample was examined on a 10% gel, the lower-molecular-weight component was clearly evident as a band with a molecular weight of 32,000 (Fig. 2B), while the high-molecular-weight component appeared near the top of the gel. Since the ACVS had been subjected to three rounds of ultrafiltration with an XM-300 membrane during the purification procedure, the 32,000-molecular-weight component was presumed to be part of a larger complex.

The molecular weight of native ACVS was estimated by using Ferguson plots (5) as described in the *Nondenatured Protein Molecular Weight Marker Kit* technical bulletin (Sigma Chemical Co., St. Louis, Mo.). Purified ACVS was analyzed together with molecular weight marker proteins by nondenaturing PAGE on gels containing 4.5, 5, 5.5, 6, 7, and 9% polyacrylamide. The change in the mobility of the proteins in response to increasing polyacrylamide concentration was related to the known molecular weights of standard proteins and used to estimate the molecular weight of ACVS (Fig. 3). This analysis indicated that native ACVS has a molecular weight of 560,000. The simplest explanation for these disparate results is that ACVS is actually a multimeric protein composed of nonidentical subunits, probably two large subunits and one small subunit per complex. This composition differs from that of the ACVS of *A. nidulans*, in which both native and denatured enzyme had a molecular weight of about 230,000 (14).

Peptide synthetases which catalyze similar reactions in other species have been found to be multienzyme complexes with very large subunits (gramicidin synthetase 2, molecular

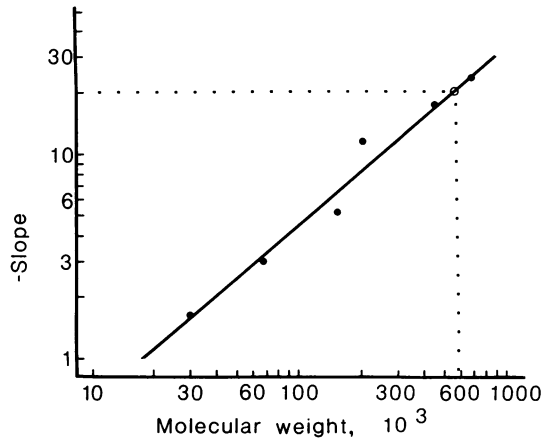

FIG. 3. Estimation of the molecular weight of ACVS by nondenaturing PAGE. ACVS and molecular weight marker proteins were electrophoresed on a series of polyacrylamide gels with polyacrylamide concentrations ranging from 4.5 to 9%. For each molecular weight marker, the R_f of the protein was determined at each gel concentration and $100[\log(R_f \times 100)]$ was plotted as a function of the percent polyacrylamide. The slopes of the resulting graphs were then calculated, and the negative slope was plotted on a logarithmic scale as a function of the logarithm of the molecular weight of the protein. The molecular weight marker proteins used were thyroglobulin, 669,000; apoferritin, 443,000; β-amylase, 200,000; alcohol dehydrogenase, 150,000; bovine serum albumin, 66,000; and carbonic anhydrase, 29,000.

weight of 350,000; tyrocidine synthetase 3, molecular weight of 450,000; and bacitracin synthetase 3, molecular weight of mol. wt. 380,000) (11). In addition, the protein-thiotemplate mechanism proposed for synthesis of peptide antibiotics requires a thioesterase activity to release the newly synthesized peptide. Recent studies on the enzymes involved in gramicidin S biosynthesis have shown that the biosynthetic operon includes a gene encoding a 29.1-kDa protein homologous to known thioesterases (12). ACVS from *S. clavuligerus* appears to be a single large multisubunit enzyme which catalyzes the condensation of three amino acids with racemization of one of the amino acids. The high-molecular-weight subunit of the complex could represent the peptide-forming component, while the low-molecular-weight component of the complex could represent the thioesterase required to release the completed ACV peptide from the enzyme complex. No low-molecular-weight component was seen in purified ACVS from *A. nidulans*, but the requirement for a thioesterase activity was recognized (14).

Although the overall recovery of purified ACVS was low, this purification procedure has nonetheless provided purified protein suitable for further analyses aimed at determining the N-terminal sequence of the components or fragments of the components. This sequence information will be used to design oligonucleotide probes for the isolation of the genes encoding ACVS. Studies to this end are currently in progress.

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