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CELL-FREE SYNTHESIS OF δ-(L-α-AMINOADIPYL)-L-CYSTEINE, THE FIRST INTERMEDIATE OF PENICILLIN AND CEPHALOSPORIN BIOSYNTHESIS

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Summary: δ-(L-α-aminoadipyl)-L-cysteine synthetase (LL-AC synthetase) activity has been found in extracts of Cephalosporium acremonium C-10. The enzyme extract carries out a linear synthesis of LL-AC from its constituent amino acids for at least 6 hours. The reaction is dependent on active enzyme, time, L-α-aminoadipate, L-cysteine, ATP and Mn^{2+} or Mg^{2+}. The activity is stabilized by glycerol.

Many years of study have been devoted to the biosynthesis of β-lactam antibiotics (for reviews, see Demain, 1983; Wolfe et al., 1984). Virtually all steps of both penicillin and cephalosporin biosynthesis have been demonstrated in cell-free extracts but only little is known about the initial reaction which is common to both types of antibiotic. This reaction involves the synthesis of the dipeptide δ-(L-α-aminoadipyl)-L-cysteine (LL-AC) from its constituent amino acids by an enzyme that can be termed LL-AC synthetase. In this communication, we report the detection of LL-AC synthetase activity in extracts of Cephalosporium acremonium.

MATERIALS AND METHODS

Organism and culture conditions. Cephalosporium acremonium C-10 (Acremonium chrysogenum ATCC 48272)was maintained on slants and grown in seed medium no. 1 as described by Shen et al., (1986). Fermentations were carried out in a medium modified from Fujisawa et al. (1975) which contained 3% sucrose, 3.2% soy bean meal, 0.3% DL-methionine and 0.15% $CaCO_3$ (pH 6.8). One hundred ml of medium was used in 500 ml Erlenmeyer flasks. Four ml of the seed culture was used as inoculum for each flask. The fermentations were performed at 25°C on a 250 rpm rotary shaker. Mycelia were harvested by centrifugation (1400xg

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for 10 min) when the cephalosporin titer reached 1000 to 1500 µg per ml. The cephalosporin titer was determined by the colorimetric method of Lübbe et al. (1986.)

Preparation of cell free extract. Mycelia were washed twice with cold 50 mM Tris/HCl buffer (pH 7.2) containing 100 mM KCl. After centrifugation, the pellet was resuspended in a volume of cold 100 mM MOPS/KOH buffer (pH 7.2) equal to twice the pellet volume; the buffer also contained 50 mM KCl, 30 mM 2-mercaptoethanol, 20 mM EDTA and 50% glycerol (v/v). The cells were disrupted in a French Press (Aminco) at 8000 psi and the debris removed by centrifugation (30,000xg, 15 min). The clear yellow supernatant fluid was stored at -20°C or immediately desalted by passage through a Sephadex G-15 column using an elution buffer of 100 mM MOPS/KOH (pH 7.2) containing 50 mM KCl and 20% glycerol (v/v). The resulting solution contained 10 to 20 mg protein per ml and was used immediately for the cell-free reaction.

Cell-free reaction. The reaction was conducted in a test tube of 5 ml capacity containing a final volume of 600 µl. The final mixture contained cell-free extract (3 to 6 mg protein), 5 mM L-α-aminoadipate, 5 mM L-cysteine, 15 mM ATP, 20 mM $MnCl_2$, 5 mM EDTA and 120 µg cycloheximide (to prevent protein synthesis) in 100 mM MOPS/KOH buffer (pH 7.2) containing 50 mM KCl and 20% glycerol (v/v). The reaction was initiated by addition of the amino acids and transfer of the tube from ice to a water bath shaker (25°C, 200 rpm). Incubation was for periods of 60 to 360 min. The reaction was stopped by addition of 150 µl of trichloroacetic acid (20% w/v). After removal of precipitated protein by centrifugation (2 min at 15,000xg), the supernatant fluid ("reaction fluid") was maintained at -20°C until the contents could be analyzed.

HPLC determination of LL-AC. The reaction fluid (40 µl) was adjusted to pH 8.0 with 4 µl of 3 M KOH. Four µl of 2 M ammonium bicarbonate (pH 8.0) and 2 µl of 0.25 M dithiothreitol (DTT) were added and the mixture was incubated at room temperature for 20 min. The resulting solution was subjected to derivatization with 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) according to the procedure of Reeve et al., (1980) using 7 µl of 0.4 M DTNB (pH 8.0) per sample. Prior to injection, the pH was lowered with 5 µl of glacial acetic acid. Thus, sulfhydryl compounds were converted to their mixed disulfides, which were separated on a µBondapak reversed phase (RP) C-18 HPLC column (3.9 mm x 30 cm) and monitored at 328 nm. The HPLC system was a Waters Liquid Chromatograph with a model 6000A solvent delivery system, Model 660 solvent programmer, model U6K injector and model 481 Lambda-Max LC spectrophotometer. Two mobile phases were used: A, 960 mg 1-pentanesulfonic acid, 10 ml glacial acetic acid, and 1 liter of water; B, 960 mg 1-pentanesulfonic acid, 10 ml glacial acetic acid, 40 ml water, 200 ml acetonitrile and 800 ml methanol. Elution was carried out with a linear gradient over 120 min (1.5 ml per min) from 7% B in A to 100% B. Under these conditions, authentic LL-AC eluted with a retention time of 26.0 min. Estimation of LL-AC concentrations in reaction mixtures was based on comparison of peak heights with those of LL-AC standards.

Synthesis of LL-AC. This peptide was generated in situ by reduction of the dimeric disulfide $(AC)_2$ with DTT. The synthesis of $(AC)_2$ followed the general strategy described by Wolfe and Jokinen (1979) and Jensen et al. (1984). Benzhydryl S-trityl-L-cysteinate was coupled with α-benzhydryl-N-t-butoxycarbonyl-L-α-aminoadipate, in ethyl acetate solvent, using 1-ethoxycarbonyl-2-ethoxy-1,2-dihydro quinoline (EEDQ). The fully protected dipeptide was detritylated and converted to the protected disulfide with iodine (2 equiv) in methanol, containing pyridine (4 equiv and this compound was deprotected to $(AC)_2$ with formic acid. Upon TLC on silica gel, the $(AC)_2$ showed Rf = 0.14 (methyl ethyl ketone-water-acetic acid, 3:1:1, red with ninhydrin); ^{1}Hmr (400 MHz, D_2O, δ): 1.215 (4H, m, adipyl CH_2), 1.399 (4H, m, adipyl CH_2), 1.886 (4H, t, adipyl CH_2CO), 2.475 (2H, q, 9.0, 13.5 Hz, cysteinyl β-CH), 2.798 (2H, q, 3.9, 13.5 Hz, cysteinyl β-CH), 3.308 (2H, t, 5.4 Hz, aminoadipyl α-CH), 4.154 (2H, q, 3.9, 9.0 Hz, cysteinyl β-CH).

RESULTS

HPLC of reaction fluid. Under the conditions described above for DTNB derivatization and HPLC analysis, the reaction fluid showed a small peak at 26.0 min. When the reaction fluid was supplemented with authentic LL-AC prior to derivatization, the height of the 26.0 min peak increased. The amount of LL-AC produced by different extracts over a period of 9 months ranged from 200 to 700 ng/ml of reaction fluid.

Identity of LL-AC peak. Three methods were used to confirm that the peak at 26.0 min could be attributed to LL-AC produced in the reaction. The first procedure employed derivatization of the reaction fluid with o-phthaldialdehyde (Hill et al., 1979) following a preliminary conversion of thiols to their S-carboxymethyl derivatives (Gurd, 1967). HPLC was conducted according to Hill et al., (1979). A peak was found with a retention time identical to that of the authentic LL-AC derivative (7.0 min) and an addition of LL-AC prior to conversion to the S-carboxymethyl derivative increased the size of this peak. The amount of LL-AC produced was 250 ng/ml of reaction fluid, comparable to the amount (295 ng/ml)found by the DTNB method on the same sample.

The second procedure involved derivatization of thiols with monobromobimane (Newton et al., 1981). The reaction fluid (40 µl) was pretreated as described for DTNB derivatization. Seven µl of 0.4 M monobromobimane in acetonitrile was added and the solution was placed in the dark for 20 min at room temperature. The pH was lowered by addition of 10 µl of 1 M citrate/HCl buffer (pH 2.2). Two mobile phases were used: A, 960 mg 1-pentanesulfonic acid, 2.5 ml glacial acetic acid, 50 ml acetonitrile and 950 ml water (adjusted to pH 3.9 with 6 M NaOH); B, 960 mg 1-pentanesulfonic acid, 2.5 ml glacial acetic acid, 50 ml water and 950 ml acetonitrile. Elution was performed with a linear gradient over 120 min (1.5 ml per min) from 100% A to 100% B. The reaction fluid was found to give an HPLC peak with a retention time of 12 min, identical to that of authentic LL-AC treated in the same way. Addition of LL-AC before derivatization and chromatography increased the size of the peak. The amount of

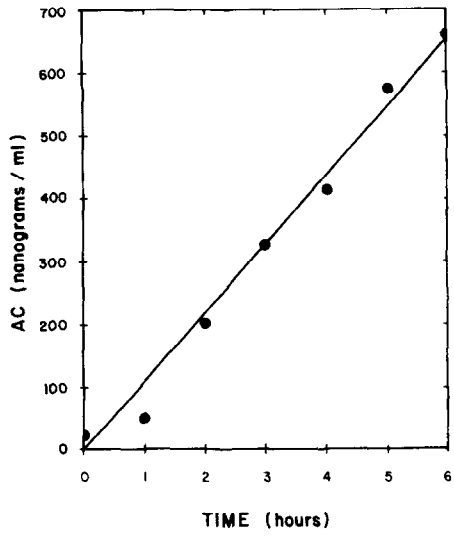


Fig. 1 : Dependence of LL-AC formation on incubation time.

LL-AC found by the monobromobimane method was 245 ng/ml of reaction fluid, comparable to that found (210 ng/ml) by the DTNB method with the same fluid.

The final method involved isolation of the material from the 26.0 min HPLC peak from the DTNB method, lyophilization, redissolution in 50 mM Tris/HCl buffer (pH 8.0) and rederivatization with DTNB. Pretreatment was limited to addition of DTT. The solution was rechromatographed, this time using 50 mM ammonium formate (pH 7.0) as the initial mobile phase and a linear gradient over 30 min from 0 to 100% acetonitrile, with a flow rate of 1.5 ml per min. Under these conditions, the biosynthetic peak had a retention time of 4.3 min, identical to that obtained with derivatized authentic LL-AC. Co-chromatography of the reaction fluid with authentic LL-AC revealed a single peak at 4.3 min.

Time course of the reaction. The time course of the reaction can be seen in Figs. 1 and 2. The production of LL-AC is linear for the 6 hour duration of the experiment. Although the extent of conversion is low, it is significant, reproducible and LL-AC production was observed in all experiments.

Dependence on reaction mixture components. The reaction is dependent on α-aminoadipate, cysteine, ATP, Mn^{2+} and active enzyme extract. Table 1 shows that omission of either amino acid prevents LL-AC formation. Furthermore,

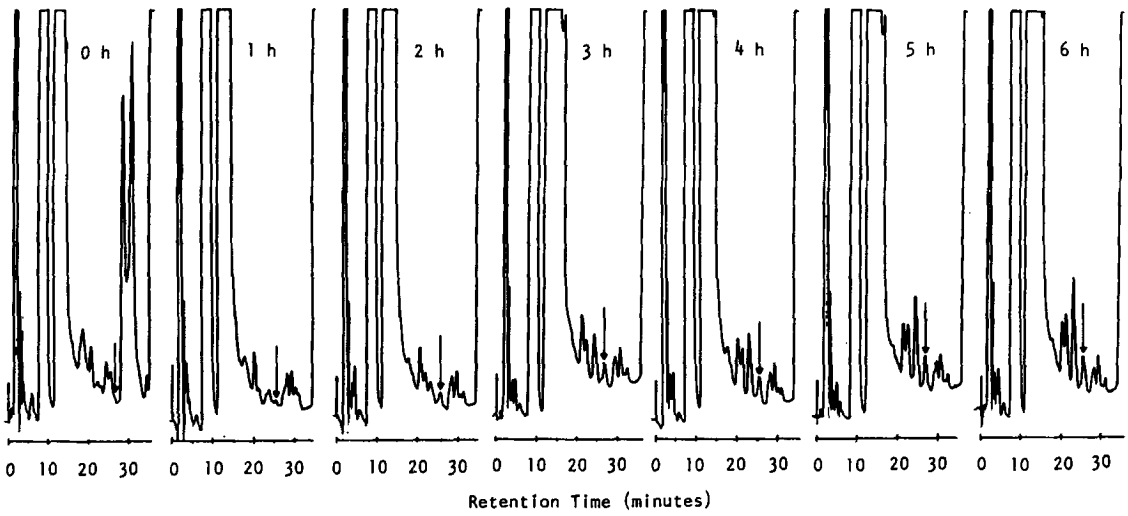


Fig. 2 : Course of reaction of Fig. 1 as determined by HPLC of DTNB-derivatized reaction fluids. Arrows indicate position of LL-AC-derivative.

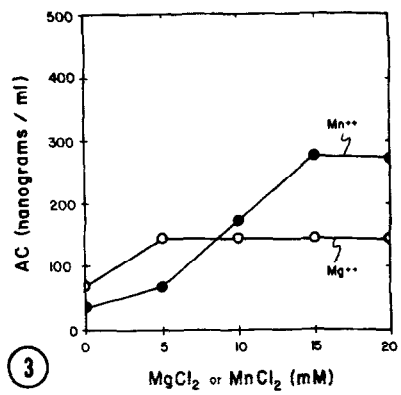
boiling of the extract eliminates the conversion.

A comparison of Mn^{2+} and Mg^{2+} is shown in Fig. 3. $MgCl_2$ shows its maximum activity at 5 mM whereas $MnCl_2$ shows increasing activity up to a maximum at 15 mM. The overall production of LL-AC is about twice as high with Mn^{2+} as with Mg^{2+}. In this experiment, ATP concentration was maintained at a constant 10 mM.

The dependence of the reaction on ATP concentration is shown in Fig. 4. The experiment was conducted using molar concentrations of $MnCl_2$ which

Table 1 Dependence of LL-AC formation on L-α-aminoadipic acid, L-cysteine and cell-free extract

Enzyme source	L-α-amino-adipate	L-cysteine	LL-AC formed (nanograms/ml)
cell-free extract	+	+	280
cell-free extract	-	+	<30
cell-free extract	+	-	<30
boiled cell-free extract	+	+	<30



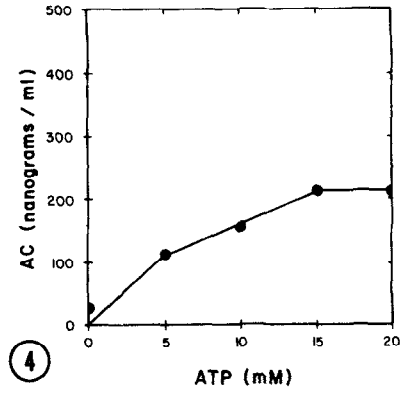


Fig. 3 : Effect of $MgCl_2$ and $MnCl_2$ on LL-AC synthesis.

Fig. 4 : Effect of ATP on LL-AC synthesis. An excess of 5 mM $MnCl_2$ over ATP concentration was used in the presence of 5 mM EDTA.

were 5 mM in excess of the ATP concentration. ATP showed its best stimulation when it was at 15 mM and $MnCl_2$ was at 20 mM.

The optimum temperature of the reaction is 25-30°C. At 20°C, the rate is 80% and at 37°C, about 50% as fast as at 25 or 30°C (measured after 3 hours of incubation.)

Other additives. A number of other additives were tested with negative results. Ferrous ion, ascorbic acid and DTT, which are required for many of the subsequent reactions of cephalosporin biosynthesis, are inactive. Bovine serum albumin (4 mg/ml) has no effect. Cystathionine (5 mM) (an intermediate in the reverse transsulfuration pathway between methionine and cysteine (Treichler et al., 1979))fails to show any activity as a cysteine replacement or as an additive to cysteine-containing reaction mixtures. Lysine addition (5 mM) has no effect on the reaction.

Effect of time of harvest. In order to detect LL-AC formation, it was necessary to harvest mycelia during the early phase of cephalosporin production, i.e. when about 1000-1500 µg of cephalosporins per ml had been produced. Mycelia taken at earlier times or at the end of the fermentation were inactive. Under the conditions employed, a useful time for harvesting was usually 60-65 hours. The fermentation yields about 3000 µg of cephalosporins per ml in this medium at 120 hours.

Stability of activity. As stated in Materials and Methods, the extract after cell disruption could be stored in the freezer for later use or immediately desalted and used as the enzyme source. In the frozen state, activity has been found to be fully stable for at least one month. This long-term stability is dependent upon the presence of glycerol in the storage buffer. Glycerol must also be present during the preparation of the extract. When this step is deleted, 80% of the activity is lost within one hour.

DISCUSSION

The first description of LL-AC synthetase activity in cell-free extracts was made by Lara et al. (1982) with Penicillium chrysogenum. Adlington et al. (1983) reported on the conversion of labeled α-aminoadipate, cysteine and valine into LLD-ACV by soluble extracts of C. acremonium. Incorporation of the amino acids into the tripeptide amounted to 0.1 to 0.4%. They further noted that the labels (0.03 to 0.15%) were also found in the dipeptide, LL-AC. The present work confirms the latter observation and establishes the dependence of the reaction on time, substrates, cofactors and active enzyme. Unfortunately, the conversion based on substrate added is low. Our calculations show the following conversions: 0.07% based on 5 mM cysteine or 5 mM α-aminoadipate added; 0.6% based on 0.1 mM cysteine and 2.1% based on 0.1 mM α-aminoadipate added.

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