Purification and Properties of Acridone Synthase from Cell Suspension Cultures of *Ruta graveolens* L.

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Acridone synthase has been purified from cell suspension cultures of *Ruta graveolens* using a combination of gel filtration and ion exchange chromatography. The purified enzyme has an apparent molecular weight of 69 kDa on gel filtration and a subunit structure on SDS-PAGE of 40 kDa. The apparent K_m-values are 10.64 μM and 32.8 μM for N-methylanthraniloyl-CoA and malonyl-CoA, respectively. Tryptic digestion of the homogeneous acridone synthase was performed. Seven of the peptides were chosen for microsequencing. The homology of the amino acid sequences from this particular polypeptide and corresponding peptides from chalcone synthase 3 from garden pea amounted to 76%.

Introduction

Acridone alkaloids comprise a relatively small group of alkaloids which are solely found in some genera of the Rutaceae plant family. About 100 members of this particular alkaloid type showing a remarkable variety in structure are known. Besides monomeric acridones acrimarines were isolated from *Citrus* plants. These are the first naturally occurring acridone-coumarin dimers [1]. Also binary acridone alkaloids containing a carbon-carbon linkage have been described [2, 3].

In 1955 Robinson has postulated in his famous essay [4] that monomeric acridones are biosynthetically formed from anthranilic acid (**I**) and acetate *via* a polyketo acid. Later on it was unequivocally demonstrated by using [^{13}C]acetate that ring C of the acridone nucleus is acetate-derived [5].

Cell-free extracts of *Ruta graveolens* cell cultures were found to catalyze the condensation of N-methylanthranilic acid (**II**) and malonyl-CoA (**IV**) in the presence of ATP and Mg^{2+}. As reaction product 1,3-dihydroxy-N-methylacridone (**V**) was identified, the key intermediate in the pathway which leads to more complex acridones. The mechanism of activation of N-methylanthranilate was not clarified at that time. As "activated" anthranilic acid the CoA thiol ester of this particular aminobenzoic acid (**III**) has been postulated [6]. This hypothesis was proved when N-methylanthraniloyl-CoA became available [7]. Acridone synthase, catalyzing the formation of **V** from N-methylanthraniloyl-CoA and malonyl-CoA (Fig. 1) was detected in crude extracts of *Ruta graveolens* cells [7, 8]. We now present a more detailed report on this enzyme system which was purified to apparent homogeneity. Cell suspension cultures of *Ruta graveolens* were used as enzyme source.

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Materials and Methods

Chemicals

[2-^{14}C]Malonyl-CoA was from Amersham Buchler (Braunschweig), malonyl-CoA came from Serva (Heidelberg), N-methylanthraniloyl-CoA and 1,3-dihydroxy-N-methylacridone were prepared according to [7] and [9] respectively. All other chemicals were of analytical grade.

Plant material

Cell suspension cultures of the common rue (*Ruta graveolens* strain R-20) were propagated and harvested as described [10].

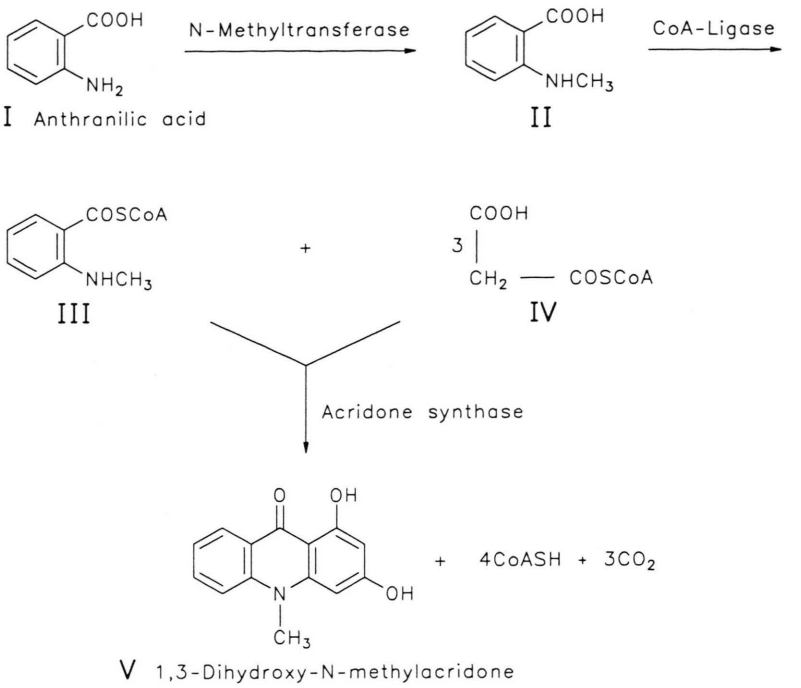

Fig. 1. Biosynthetic pathway from anthranilic acid to 1,3-dihydroxy-N-methylacridone.

Buffers

Buffer A, 0.1 M sodium phosphate, pH 7.5, containing 10% glycerol, 20 mM sodium ascorbate and 0.5 mM EDTA. The buffer was degassed before addition of ascorbate; buffer B, 0.1 M sodium phosphate, pH 7.5, containing 10% glycerol and 0.5 mM EDTA; buffer C, 0.01 M sodium phosphate, pH 7.5, containing 10% glycerol, and 0.5 mM EDTA; buffer D, 0.01 M sodium phosphate, pH 7.5, containing 10% glycerol 0.5 mM EDTA and 1.5 M $(NH_4)_2SO_4$.

Preparation of cell-free extracts

All operations were carried out between 0–4 °C. Lyophilized cells (150 g) were intensively ground in a mortar with dry ice in the presence of 50 g Polyclar AT and subsequently suspendend in 1.8 l buffer A. After mixing for 30 min the homogenate was centrifuged at 20000 × *g* for 10 min. To the enzyme containing solution various amounts of solid $(NH_4)_2SO_4$ were added whilst stirring. The protein fraction which precipitated between 55 and 80% saturation was dissolved in 40 ml buffer C and used for further experiments.

Purification of acridone synthase

Column chromatography was carried out with an FPLC system (Pharmacia).

The protein solution (40 ml in buffer C) was desalted by passing through a column of Sephadex G-25 equilibrated with buffer C. The protein solution was applied in two separate runs to a Q-Sepharose 16/10 column (Pharmacia) which had been equilibrated with buffer C. Unbound protein was washed off with the same buffer (300 ml). The elution of bound protein was performed by applying a gradient by mixing of buffer C and buffer B (flow rate 3 ml/min). 5 ml fractions were collected. Acridone synthase was detected in fractions 28–80 (60–100% buffer B).

The combined enzyme containing fractions were concentrated by using an Amicon ultrafiltration cell, Model 8050. The resulting protein solution (6 ml) was applied in two separate runs to a Superdex 200 16/60 column (Pharmacia) which had been equilibrated with buffer C. Acridone synthase was eluted with buffer C (2 ml fractions, flow rate 1 ml/min). Enzyme containing fractions [32–42] were combined and concentrated by ultrafiltration (Centriprep-10, Amicon). Subsequently buffer C

was replaced by buffer D and the enzyme solution was applied to a Phenyl Superose 5/5 column (Pharmacia) after equilibration with buffer D. Acridone synthase was eluted with a linear gradient by mixing of buffer D and buffer C at a flow rate of 0.5 ml/min (1 ml fractions were collected). Fraction 26–38 were combined and concentrated by ultrafiltration using a Centriprep cartridge. The protein solution was desalted by passing through a PD-10 column (Pharmacia), which had been equilibrated with buffer C.

The desalted protein was applied on a Mono Q HR 5/5 column (Pharmacia). Acridone synthase was eluted with a linear gradient by mixing of buffer C and buffer B. Enzyme activity was detected in fractions 24–27 which were concentrated by ultrafiltration. Subsequently the enzyme solution was applied in five different runs (each 200 µl) to a Superose 12 HR 10/30 column (Pharmacia). Acridone synthase was eluted with buffer C and concentrated with a Centricon cartridge (Amicon).

Enzyme assay

The acridone synthase assay was slightly modified according to [8]. The reaction mixture contained in a total volume of 200 µl: 6 nmol N-methylanthraniloyl-CoA, 12 nmol [2-^{14}C]malonyl-CoA (1.1×10^5 dpm), 5–100 µg protein and buffer A. Incubations were carried out at 32 °C for 30 min. The reaction was stopped by adding 100 µg 1,3-dihydroxy-N-methylacridone in 1 ml ethanol. After extraction with $CHCl_3$ the alkaloid fraction was chromatographed on silica gel PF_{254} plates (Merck), solvent system: benzene/EtOAc (6:4). After elution of the alkaloid zone an aliquot was used for quantitation by measuring the absorption of light at 400 nm and another aliquot was used for scintillation counting.

Electroblotting and preparation of tryptic peptides for Edman degradation

For final purification acridone synthase was separated on a 10% SDS/Polyacrylamide gel prepared from electrophoretically pure reagents from Biorad loading 10 µg per lane. After electrophoresis the gel was electroblotted to Immobilon membrane (Millipore) according to [11] with some modifications. The transfer membrane was first washed with methanol (30 min) and then equilibrated with the transfer buffer (50 mM boric acid containing 15% CH_3OH adjusted to pH 8.5 with 1 M NaOH) for at least 30 min. Filterpapers (Whatman 3 MM) were washed three times (15 min) in transfer buffer. A sandwich was assembled from three sheets of paper, the gel (briefly rinsed with transfer buffer), the membrane and another three sheets of paper. Electroblotting was done in a semi-dry blot chamber at 1.5 mA/cm^2 for 2 h. The membrane was stained with Coomassie blue and the bands cut out and either used for N-terminal sequence analysis or for proteolytic digestion.

Tryptic digestion was done essentially as described [12]. Briefly the spots were destained with 80% ethanol for 30 min, reactive sites were blocked with 0.5% PVP 40 dissolved in 0.1 M CH_3COOH at 37 °C for 30 min and washed five times with water to remove excess reagent. The pieces of membranes were submerged in 200 µl 0.1 M TRIS buffer, pH 8, containing 10% acetonitrile and digested with 2 µg trypsin at 37 °C over night. After removing the blot pieces the solution was directly used for HPLC analysis. Peptides were separated on a Vydac 218 TP 5 column (250 × 2.1 mm) using the standard trifluoroacetic acid/acetonitrile system.

Automated Edman degradation was performed on an Applied Biosystems model 477 A amino acid sequencer with on-line PTH amino acid analyser model 120 A.

Analytical methods

SDS-PAGE gel electrophoresis was carried out according to Laemmli [13] in 10% separation and 5% stacking gels using a LKB 2050 Midget electrophoresis unit (Pharmacia).

Protein concentrations were determined according to Bradford [14] using bovine serum albumine as standard.

Results

Acridone synthase was isolated from lyophilized cells of *Ruta graveolens* cell-line R-20, which accumulates mainly rutacridone.

Previously it was shown [8] that metal ions are not required as cofactors but that the buffer composition strongly influenced enzyme activity. Moreover sodium ascorbate as oxidation protec-

Table I. Purification procedure for acridone synthase from *Ruta graveolens* cell cultures.

Purification step		Protein [mg]	Specific activity [μkat kg^{-1} protein]	Purification (-fold)	Recovery (%)
1	Crude extract	14470	3.52	1	100
2	Ammonium sulfate (55–80%)	2560	17.05	4.8	85
3	Q Sepharose 16/10	192	32	9.1	12.1
4	Superdex 200 16/60	74.3	50	14.2	7.2
5	Phenyl Superose HR 5/5	27	92.8	26.3	4.9
6	Mono Q HR 5/5	5.6	101.7	28.8	1.0
7	Superose 12 10/30	2.7	100.6	28.5	0.5

tant has been found to have an advantageous effect on acridone synthase activity. Therefore we applied buffer A for protein extraction and used the protein fraction which precipitated between 55–80% $(NH_4)_2SO_4$ saturation for enzyme purification. The temperature optimum for the reaction was 38 °C. Acridone synthase was purified to apparent homogeneity by a 7-step procedure which is summarized in Table I. This was done by a combination of gel filtration and ion exchange chromatography. Acridone synthase was nearly homogeneous after step 5 (Table I). Traces of protein impurities were separated by chromatography on Mono Q and Superose 12. Hydroxyapatite was not suited for purification because the enzyme protein was not bound to the matrix. Most of the enzyme activity from crude cell extracts was lost during purification (< 99%). Our main interest in this report was to obtain a homogeneous protein.

A molecular mass of 69 ± 1 kDa was repeatedly determined for the native acridone synthase on a calibrated column of Superose 12. SDS-PAGE of the purified enzyme revealed only one band after staining with Coomassie blue (Fig. 2) corresponding to M_r 40 ± 1 kDa. These data are indicative that acridone synthase from *Ruta graveolens* is composed of two subunits.

The protein obtained after chromatography on Q-Sepharose was stable over a period of at least four weeks, if stored at −70 °C. This partially purified enzyme preparation was used for K_m-value determinations. Acridone synthase showed normal Michaelis-Menten kinetics for both substrates. Double reciprocal plots of velocity against substrate concentration gave a linear relationship for N-methylanthraniloyl-CoA over a range of 1.0 μM to 50 μM. The apparent K_m-value was 10.64 μM and V_{max} was 0.18 pkat. The apparent K_m-value of malonyl-CoA amounted to 32.8 μM. Substrate inhibition was observed for malonyl-CoA (> 100 μM) and for N-methylanthraniloyl-CoA (> 250 μM).

The catalytical activity of the enzyme was inhibited by various sulfhydryl reagents in the following concentrations (% inhibition) viz. *p*-chloromercuribenzoate: 0.05, 0.1 and 0.5 mM (55, 75, 95%); N-ethylmaleimide: 0.2 and 1 mM (95, 100%); iodoacetamide 0.1 and 0.5 mM (85, 95%).

Acridone synthase obtained after the Superose 12-step was subjected to electrophoresis and electroblotted onto a Immobilon membrane according to [11]. The blotted protein was visualized by staining with Coomassie blue, cut out and used either for N-terminal sequence analysis or for tryptic digestion. Direct tryptic digestion of the protein on

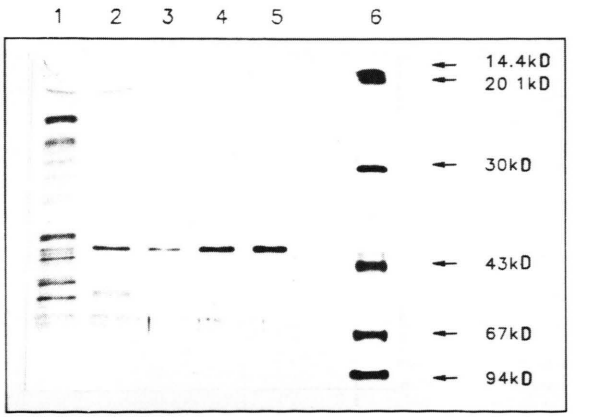

Fig. 2. SDS-gel of different purification steps of acridone synthase (Table II). (1) Desalted 55–80% $(NH_4)_2SO_4$ fraction; (2) Q Sepharose fraction; (3) Phenyl Superose fraction; (4) Mono Q fraction; (5) Superose 12 fraction; (6) marker proteins.

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P.1    A I K E W G Q P K
       | | | | | | | | |
       A I K E W G Q P K  (pos. 113-121)

P.17   MIXTURE OF PEPTIDES

P.36   a)H V M S E Y(G)N M S(S)X X V L F
          |   | | | | | | | |     | | |
         E V L S E Y G N M S S A C V L F  (pos.329-344)

       b)K A Q M S E G P A A I L A I G T  (cf. P.42)

P.42   K A Q M S E G P A A I L A I G T A T P D N V F M
       | | |     | | | |   | | | | | | | | | |   |
       K A Q R A E G P A T I L A I G T A T P A N C V E
       (pos.9-32)

P.43   A Q M S E G P A A I L A I G T A T P D N V F M Q A D Y P D Y Y F
       | |     | | | |   | | | | | | | | | |   |       |     | | | . | |
       A Q R A E G P A T I L A I G T A T P A N C V E Q S T Y P D F Y F
       (pos. 10-41)

P.44   G P S P D A V D S L V G Q A L F A D G A A A L V V G A D P
       | | |       | | | | | | | | | |   | | | | | | |   | |   | |
       G P S D T H L D S L V G Q A L F G D G A A A L I V G S D P
       (pos. 200-228)

P.48  (S)T T G E G L D(W)G V L F G F G P G L T V E T I V(L)
         | | | | | | | | | | | | | | | | | | | |   | |   | |
       K T T G E G L D W G V L F G F G P G L T I E T V V L
       (pos. 359-384)

P.50  (D)V P A L F S A N I D(T)P L V E A F K
       | | |       |   | |       | | | | |
       D V P G I V S K N I N K A L V E A F Q  (pos: 270-288)
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Table II. Amino acid sequences of tryptic peptides from acridone synthase and alignment with corresponding peptides from chalcone synthase 3 from garden pea [15]. The location of the amino acid residues within the sequence is given in parentheses. Peak 17 contained two peptides in approximately equal amounts. The assignment was possible since one of each peptide was obtained in pure form from peaks 42 and 43.

the PVDF membrane was performed as described by [12]. Individual peptides were separated from the digest by reversed phase HPLC (Fig. 3). Seven of the peptides were chosen for microsequencing and six out of those yielded sequences of at least ten amino acids (Table II). Edman degradation analysis of the blotted and also the chromatographically purified polypeptide failed, indicating that the N-terminus was blocked. Totally 132 amino acid residues were obtained from all parts of the protein amounting roughly to one third of the enzyme. Comparison of these sequences with those available in protein sequence databases viz. 100 residues out of 132 sequenced amino acids displayed a homology as high as 76% with chalcone synthase 3 from garden pea [15].

Discussion

A key enzyme of acridone alkaloid biosynthesis catalyzing the condensation of N-methylanthraniloyl-CoA and malonyl-CoA leading to 1,3-dihydroxy-N-methylacridone was obtained (Fig. 1). A remarkable loss of enzyme activity during purification was observed. On the other hand for the first time acridone synthase was purified to apparent homogeneity. SDS-PAGE of acridone synthase gave a single band with a M_r of 40 kDa, apparently a subunit of the enzyme.

It has been now unambiguously shown [10] that the activation of N-methylanthranilic acid proceeds *via* the corresponding CoA-ester. Obviously acridone synthase catalyzes the chain elongation of a primer molecule viz. N-methylanthraniloyl-CoA by acetate *via* malonyl-CoA. The antibiotic cerulenin which inhibits specifically the 2-keto-acyl-ACP synthase, the formation of acetate-derived secondary metabolites and the chalcone synthase reaction [16] inhibits also acridone synthase. Apparently the formation of the acridone nucleus proceeds like the mechanism observed in the flavonoid and stilbene biosynthesis [17]. In the latter cases 4-coumaroyl-CoA and cinnamoyl-CoA serve as acyl acceptors. Interestingly to note that acridone synthase, chalcone and stilbene synthases are rather similar regarding some molecular properties *e.g.* molecular weight, number of subunits and K_m-values. Another remarkable aspect is the homology of the amino acid sequences from acridone synthase and corresponding peptides from chalcone synthase 3 from garden pea (Table II) which amounted to 76%. For comparison

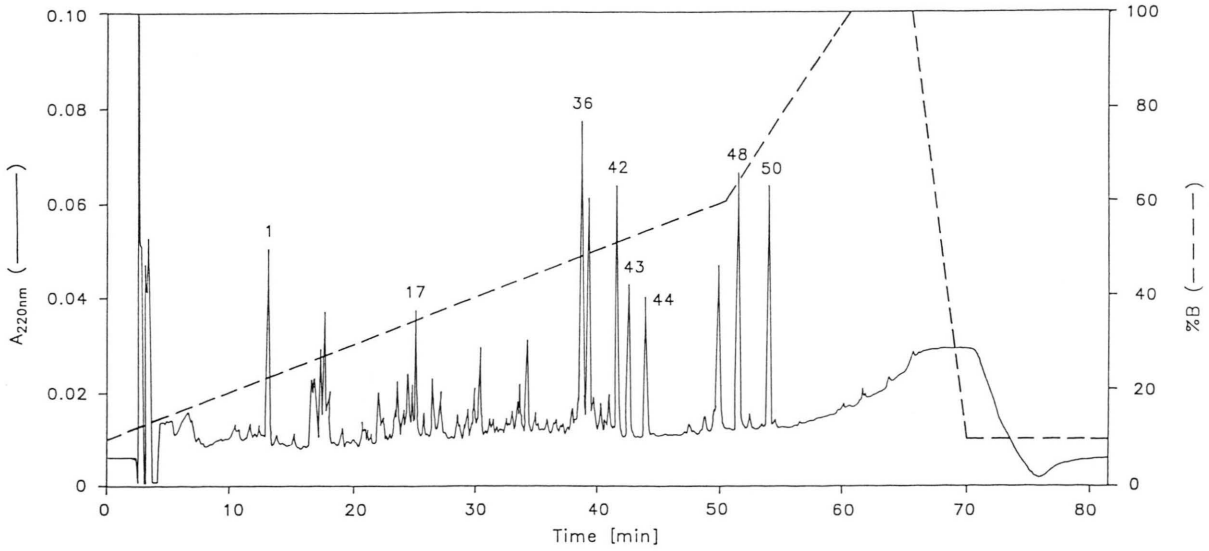

Fig. 3. Separation of tryptic peptides of acridone synthase. The separation was done at 55 °C on a Vydac C 18 column (250 × 21 mm), equilibrated with 90% solvent A (0.12% trifluoroacetic acid) and 10% solvent B (70% acetonitril in 0.1% trifluoroacetic acid). The peptides were eluted by a linear gradient of 10–60% B within 50 min. (B = – – –). The flow rate was 0.3 ml/min. All peaks were collected, those numbered were selected for sequencing. The amino acid sequences obtained are listed in Table II.

resveratrol synthase sequences revealed 70–75% identity on the protein level with anyone of the known chalcone synthases [17, 18]. At present it is not obvious if a gene family of acridone synthases does exist like in the case of chalcone synthases.

Further informations on acridone synthase will be obtained after production of antibodies against this particular polypeptide and the isolation of cDNA clones and genes encoding for this enzyme.

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