

## Purification and Characteristics of 3-Deoxy-D-arabino-heptulosonate-7-phosphate Synthetase from *Streptomyces caespitosus*

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3-Deoxy-D-arabino-heptulosonate-7-phosphate(DAHP) synthetase, the first enzyme of the shikimate pathway was purified to near homogeneity from *Streptomyces caespitosus*. It had a molecular weight of approximately 56,000 daltons on SDS-PAGE and Sephadex G-150 gel filtration. The  $K_m$  values for phosphoenolpyruvate (PEP) and D-erythrose-4-phosphate(E-4-P) were 0.43 mM and 0.22 mM, respectively. The pH and temperature for maximum enzyme activity were 7.2 and 37°C. Treatment with ethylenediamine-tetraacetic acid(EDTA) removed 80% of the activity of the enzyme. This activity was restored upon the addition of  $\text{Co}^{2+}$  ion and partially restored upon the addition of  $\text{Zn}^{2+}$  or  $\text{Mn}^{2+}$  ion. But,  $\text{Ni}^{2+}$ ,  $\text{Fe}^{2+}$ , and  $\text{Ca}^{2+}$  ions inhibited activity of the DAHP synthetase from *S. caespitosus*.

KEY WORDS □ *Streptomyces caespitosus*, DAHP synthetase

*Streptomyces caespitosus* produces mitomycin C, an antibiotic belonging to the quinone group which contains a biosynthetically unique moiety, called an mC<sub>7</sub>N unit, consisting of a six-membered carbocyclic ring carrying an extra carbon and a nitrogen in a meta arrangement (2, 11, 13). Extensive tracer and genetic experiments have demonstrated the shikimate pathway origin of this mC<sub>7</sub>N unit and have identified 3-amino-5-hydroxybenzoic acid (AHBA) as its proximate precursor (15). Also, antibiotics belonging to the ansamycin group which involves many commercially important antibiotics such as rifamycin B contain an mC<sub>7</sub>N unit, synthesized from a common aromatic precursor 3-amino-5-hydroxybenzoic acid deriving from the shikimate pathway (7, 8).

3-Deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) synthetase is the first enzyme of shikimate pathway, and it catalyzes the condensation of phosphoenolpyruvate(PEP) and D-erythrose-4-phosphate(E-4-P) to form DAHP. DAHP synthetase which had several isoenzymes or a single enzyme occupies a key position in the regulation of biosynthetic pathway of aromatic amino acids and has been well-studied in many microorganisms (1, 4, 7, 9, 10, 14, 17, 18).

Also, this enzyme participates in the regulation of biosynthesis of some mC<sub>7</sub>N units containing antibiotics from antibiotics-producing microor-

ganisms. In *Amycolatopsis mediterranei*, rifamycin B producer, DAHP synthetase which was composed of a single enzyme exerts important regulatory roles in rifamycin B and aromatic amino acids biosynthesis (19, 20). So, it will be a valuable work that investigates the regulatory roles of DAHP synthetase on mitomycin and aromatic amino acids biosynthetic pathways of the mitomycin C producer, *S. caespitosus*. In this paper, the purification and some characteristics of DAHP synthetase from *S. caespitosus* are reported.

### MATERIALS AND METHODS

#### Chemicals

E-4-P, PEP, Sephadex G-150 and DEAE-cellulose were purchased from Sigma. Other chemicals purchased from commercial sources were of the highest available purity.

#### Organism and culture condition

*Streptomyces caespitosus* KCTC 9096 was inoculated in *Streptomyces* minimal medium (12) supplemented with 0.01% casamino acid and 0.5% yeast extract and cultivated on rotary shaker at 27°C. Mycelia were harvested in the early exponential phase of growth.

#### Enzyme assay

DAHP synthetase was assayed as described by Sprinson *et al.* (18). The reaction mixture contained phosphate buffer (100 mM), PEP (0.8 mM),

E-4-P (0.8 mM) and enzyme solution. The total volume of the reaction mixture was made up to 0.25 ml with distilled water. The reaction was initiated by the addition of enzyme, and after 10 min of incubation at 37°C, it was stopped by the addition of 0.1 ml of 10% trichloroacetic acid. The mixture was centrifuged and 0.25 ml of supernatant was added to 0.25 ml periodic acid (0.025 M). After 45 min at room temperature, 0.5 ml of sodium arsenite (2%) and 2 ml of thiobarbiturate (0.3%) are added, and boiled for 5 min. After cooling in a water bath at 40°C, the pink color is measured at 549 nm. One unit (U) of enzyme activity was defined as the amount that catalyzed the formation of 1  $\mu$ M of DAHP in 10 minutes at 37°C unless stated otherwise.

#### Protein determination

The protein concentration was determined by the procedure of Bradford (5), with bovine serum albumin (Sigma) as a standard.

#### Purification of DAHP synthetase

Preliminary experiments with crude extracts suggested that DAHP synthetase is most stable in the presence of 1 mM dithiothreitol (DTT). Therefore, 40 mM potassium phosphate buffer (pH 7.0) containing 1 mM DTT was used in the purification procedures, and all the centrifugations were done at 12,000 $\times$ g for 20 min, unless stated otherwise. All operations were carried out at a temperature below 4°C.

**Step 1—Preparation of crude extracts:** Mycelia were harvested by centrifugation (12,000 $\times$ g, 20 min) and washed twice in phosphate buffer (40 mM, pH 7.0). Harvested mycelia (50 g, wet weight) were suspended in 100 ml phosphate buffer (40 mM, pH 7.0) containing 1 mM dithiothreitol (DTT) and sonicated in an ice bath, and mycelial debris was removed by centrifugation.

**Step 2—Precipitation of nucleic acids:** To 130 ml of the crude extracts, 13 ml of streptomycin sulfate (25% in phosphate buffer) was added dropwise. Stirring was continued for a further 20 min, and the precipitate was removed by centrifugation.

**Step 3—The first ammonium sulfate fractionation:** Ammonium sulfate powder was added to the supernatant from step 2 until 30% saturation was reached. The suspension was stirred for a further 20 min, and the precipitate was removed by centrifugation. More ammonium sulfate was added to supernatant till 50% saturation was reached, and the precipitate was collected by centrifugation and dissolved in 8 ml phosphate buffer and dialyzed against the same buffer.

**Step 4—Chromatography on Sephadex G-150:** A portion of 10 ml enzyme solution was loaded onto a Sephadex G-150 column (2.5 $\times$ 78 cm), eluted with phosphate buffer at a flow rate of 10 ml/hour, and fractions (5 ml) were collected. A single

peak of enzyme activity was eluted and fractions containing this activity were pooled.

**Step 5—The first chromatography on DEAE-cellulose:** 20 ml enzyme solution of step 4 was directly loaded onto DEAE-cellulose column (2 $\times$ 15 cm), washed with phosphate buffer containing 0.15 M KCl and eluted with a gradient of KCl (100 ml of phosphate buffer containing 0.15 M KCl and 100 ml of same buffer containing 0.55 M KCl) at a flow rate of 6 ml/hour, and 2 ml fractions were collected. Fractions containing enzyme activity were pooled.

**Step 6—The second ammonium sulfate fractionation:** Ammonium sulfate was added to 24 ml of the enzyme solution from step 5 until 38% saturation was reached. The precipitate was removed by centrifugation and more ammonium sulfate was added to supernatant till 47% saturation was reached. The precipitate was collected by centrifugation and dissolved in 1 ml phosphate buffer and dialyzed against phosphate buffer containing 0.4 M KCl.

**Step 7—The second chromatography on DEAE-cellulose:** 1 ml enzyme solution of step 6 was loaded onto DEAE-cellulose column (1.1 $\times$ 75 cm) pre-equilibrated with phosphate buffer containing 0.4 M KCl, eluted with phosphate buffer containing 0.4 M KCl at a flow rate of 4.5 ml/hour, and 1.1 ml fractions were collected. The active enzyme fractions were pooled and dialyzed thoroughly against phosphate buffer (40 mM, pH 7.0).

#### Molecular weight determination

Molecular weight of native enzyme was determined by Sephadex G-150 column (1.45 $\times$ 78 cm) chromatography based on the method of Andrews (3). Alcohol dehydrogenase, bovine serum albumin, and carbonic anhydrase were used as standards, whereas the void volume was estimated by using blue dextran. Molecular weight of denatured enzyme was determined by SDS-polyacrylamide gel electrophoresis with 10% resolving and 4% stacking gel containing 0.1% SDS, based on the method of Laemmli (16). Alcohol dehydrogenase (150,000 Da), bovine serum albumin (66,000 Da), egg albumin (45,000 Da), carbonic anhydrase (29,000 Da) and cytochrome C (14,500 Da) were used as standards.

#### Kinetic studies

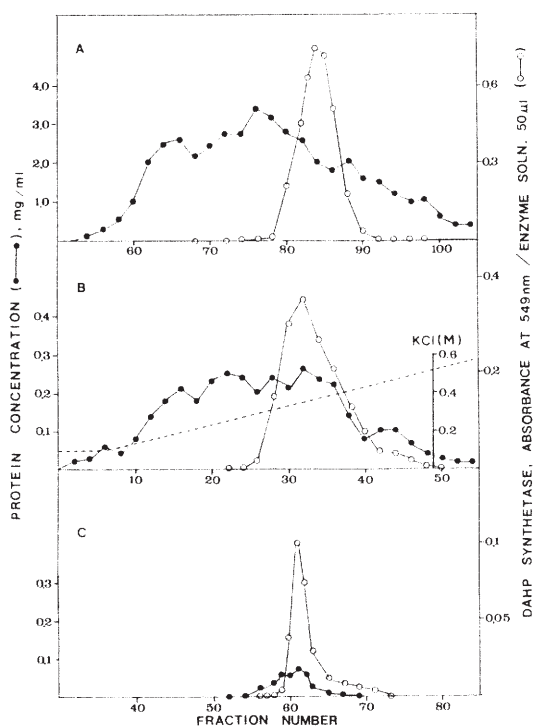
For the determination of  $K_m$  values of the purified enzyme for substrates, the enzyme activity was assayed at various concentrations of the substrates.  $K_m$  values were determined from the Lineweaver-Burk plot.

#### Effect of EDTA and divalent cations

Purified enzyme solution was preincubated with 0.2 mM EDTA at 0°C for 30 min. After 30 min, various divalent cations were added to the enzyme reaction mixture.

**Table 1.** Purification of DAHP synthetase from *S. caespitosus* KCTC 9096.

Purification step	Total activity(U)	Total protein(mg)	Spec. act.(U/mg)	Recovery (%)	Purification (fold)
Crude extracts	84	1040	0.08	100	1
1st Ammonium sulfate	83	405	0.21	99	2.6
Sephadex G-150	47.5	58.5	0.82	57	10
1st DEAE-cellulose	32.8	5.3	6.25	39	78
2nd Ammonium sulfate	18.7	0.84	22.26	22	278
2nd DEAE-cellulose	3.4	0.29	11.72	4.0	147

**Fig. 1.** Purification of DAHP synthetase from *S. caespitosus*.

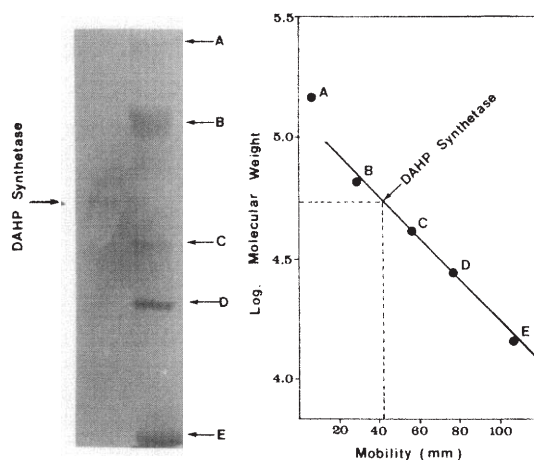
A, Sephadex G-150 column chromatography; B, First DEAE-cellulose column chromatography; C, Second DEAE-cellulose column chromatography.

## RESULTS AND DISCUSSION

### Purification of DAHP synthetase

A summary of the purification procedure is presented in Table 1.

Enzyme solution after the second DEAE-cellulose step possessed a specific activity of DAHP synthetase which was 147-fold higher than that of the crude extracts. It can be seen from Table 1 that the yield was very low, about 4.0%. The instability of this enzyme was apparent

**Fig. 2.** Determination of the molecular weight of DAHP synthetase by SDS-PAGE (10%).

A, Alcohol dehydrogenase (150,000 Da); B, Bovine serum albumin (66,000 Da); C, Egg albumin (45,000 Da); D, Carbonic anhydrase (29,000 Da); E, Cytochrome C (14,500 Da).

during step 7. One major protein-stained band was detected when the purified enzyme was tested by SDS-polyacrylamide gel electrophoresis (Fig. 2).

### Characteristics of DAHP synthetase

The purification procedures of DAHP synthetase from *S. caespitosus* indicated that it had only a single enzyme protein (Fig. 1). This result was different from those of *Escherichia coli*, *Salmonella typhimurium* and *Neurospora crassa*, in which DAHP synthetases had three isoenzymes, each was inhibited by phenylalanine, tyrosine and tryptophan, respectively (21). But it was similar with DAHP synthetases of *Amycolatopsis mediterranei*, *Streptomyces aureofaciens*, and *Pseudomonas aeruginosa* which were composed of a single enzyme (10, 20, 21). DAHP synthetase from *S. caespitosus* had a molecular weight of approximately 56,000 as determined by Sephadex G-150 gel filtration (Fig. 3) and the molecular weight of denatured enzyme assayed by SDS-polyacryl-

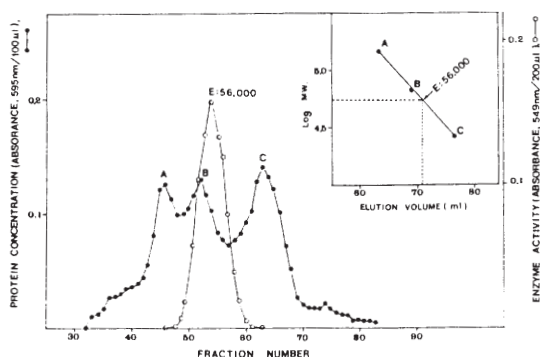


Fig. 3. Determination of the molecular weight of DAHP synthetase by Sephadex G-150 gel filtration.

A, Alcohol dehydrogenase (150,000 Da); B, Bovine serum albumin (66,000 Da); C, Carbonic anhydrase (29,000 Da); E, DAHP synthetase

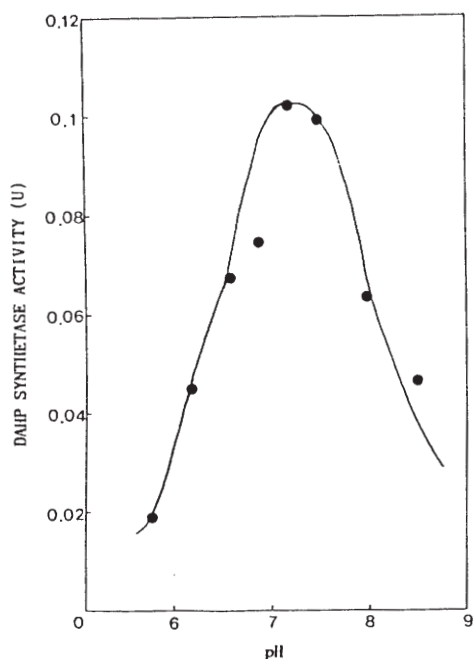


Fig. 4. Effect of pH on DAHP synthetase activity in potassium phosphate buffer (100 mM).

amide gel electrophoresis was 56,000 (Fig. 2). Thus, the enzyme contained a single subunit. This result is different from the report in *A. mediterranei* where DAHP synthetase contained four subunits, each had same molecular weight, 35,000 (21). Molecular weight of DAHP synthetase from *S. caespitosus* was similar to the native DAHP

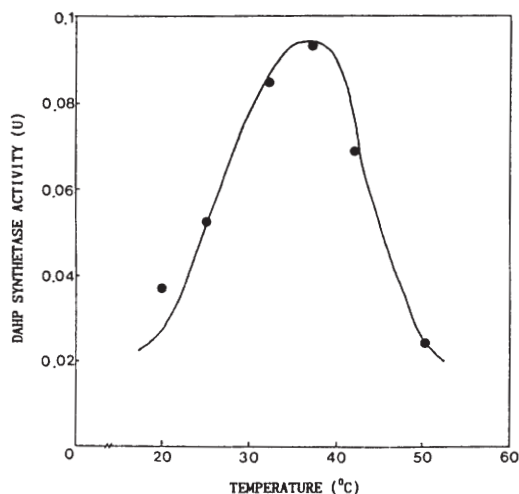


Fig. 5. Effect of temperature on DAHP synthetase activity.

Table 2. Effect of EDTA<sup>a</sup> and divalent metal cations<sup>b</sup> on DAHP synthetase<sup>c</sup> from *S. caespitosus*.

Addition		Remaining
Preincubation	Enzyme assay	Activity(%)
No preincubation		100
EDTA	none	20
EDTA	Zn <sup>2+</sup>	60
EDTA	Mn <sup>2+</sup>	75
EDTA	Co <sup>2+</sup>	120
EDTA	Mg <sup>2+</sup>	23
EDTA	Ni <sup>2+</sup>	18
EDTA	Fe <sup>2+</sup>	18
EDTA	Ca <sup>2+</sup>	17
No preincubation	EDTA	38
No preincubation	Co <sup>2+</sup>	110
No preincubation	EDTA + Co <sup>2+</sup>	105

<sup>a</sup>0.2 mM of EDTA was used.

<sup>b</sup>Metal ions were added at each 1 mM.

<sup>c</sup>Enzyme was preincubated with phosphate buffer (40 mM, pH 7.2) containing 0.2 mM EDTA at 0°C for 30 min.

Enzyme reaction was initiated by addition of 0.8 mM PEP and E-4-P.

synthetase(Trp) from *E. coli* where its molecular weight was 52,000 (6).

As shown in Fig. 4, optimal pH of DAHP synthetase in phosphate buffer was 7.0~7.2, and there was an abrupt decrease above pH 7.6 and below pH 6.4. Optimal temperature of DAHP synthetase was 37°C and more than 75% of maximal activity was observed in the range of 28~42°C (Fig. 5). This result is similar to that



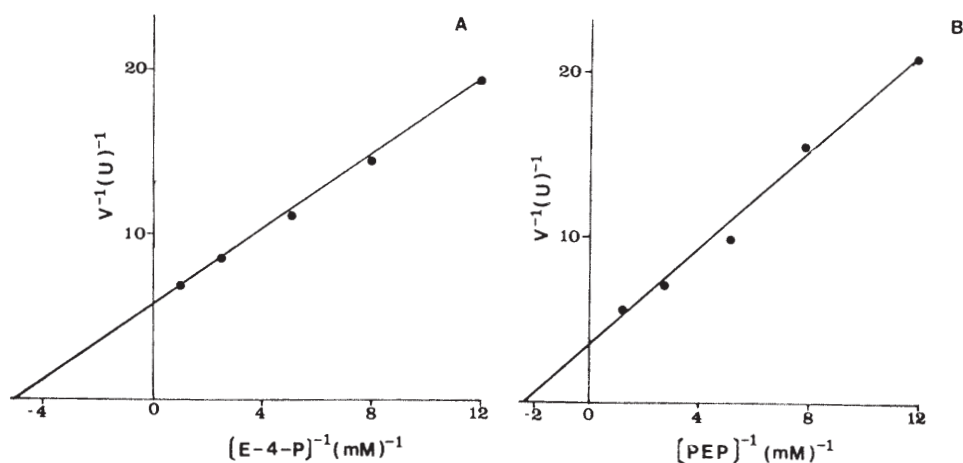


Fig. 6. Lineweaver-Burk plots of DAHP synthetase reaction rate against the substrate concentration. Fixed concentration of PEP(A) or E-4-P(B) was 0.8 mM.

Table 3. Effect of divalent metal cations on DAHP synthetase from *S. caespitosus*.

Metal ions <sup>a</sup>	Remaining activity(%)
none	100
Zn <sup>2+</sup>	81
Mn <sup>2+</sup>	88
Co <sup>2+</sup>	112
Mg <sup>2+</sup>	107
Ni <sup>2+</sup>	16
Fe <sup>2+</sup>	40
Ca <sup>2+</sup>	46

<sup>a</sup>Divalent metal ions were added to enzyme reaction mixture at each 2 mM.

reported for *A. mediterranei* (21).

$K_m$  values of DAHP synthetase for PEP and E-4-P, calculated from the Lineweaver-Burk plots of the data, were 0.43 and 0.22 mM (Fig. 6).

#### Effect of EDTA and divalent cations

After 30 min of preincubation of enzyme with 0.2 mM EDTA, 80% of activity was lost (Table 2). This activity was restored upon the addition of Co<sup>2+</sup> ions and partially restored by Mn<sup>2+</sup> or Co<sup>2+</sup> ions, but it was not restored upon the addition of Ni<sup>2+</sup>, Fe<sup>2+</sup>, Mg<sup>2+</sup>, and Ca<sup>2+</sup> ions (Table 2). When 1 mM of Co<sup>2+</sup> ions was present at the same time with EDTA, no activity was lost. With DAHP synthetase (Trp) from *E. coli*, Camakaris and Pittard (6) reported that Co<sup>2+</sup> and Mn<sup>2+</sup> could restore this activity and Ni<sup>2+</sup> and Ca<sup>2+</sup> could partially restore this activity, but Fe<sup>2+</sup> and Zn<sup>2+</sup> ions could not restore lost enzyme activity. And they suggested that Co<sup>2+</sup> or Mn<sup>2+</sup> is required for normal activity of DAHP synthetase(Trp) from *E. coli*. Considering the above facts, it appears

likely that Co<sup>2+</sup> ion is essential for normal activity of the DAHP synthetase from *S. caespitosus*. Ni<sup>2+</sup>, Fe<sup>2+</sup>, and Ca<sup>2+</sup> ions not only could not restore activity that was lost by EDTA but also decreased the enzyme activity when they were added to standard enzyme reaction mixture in which enzyme was not preincubated with EDTA (Table 3). So, it appears that they inhibit the activity of the DAHP synthetase from *S. caespitosus*. Meanwhile, there is a slightly positive effect on enzyme activity when Mg<sup>2+</sup> or Co<sup>2+</sup> ion is added to standard assay condition (Table 3).

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# 초 록: *Streptomyces caespitosus*의 3-Deoxy-D-arabino-heptulosonate-7-phosphate synthetase 정제 및 특성연구

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Mitomycin C 생산균주인 *Streptomyces caespitosus* KCTC 9096의 균사체로부터 3-deoxy-D-arabino-heptulosonate-7-phosphate synthetase를 분리 정제하고 특성을 조사하였다. 본 효소는 분자량 56,000달톤의 단일 subunit로 이루어졌으며, isoenzymes으로의 존재양상은 확인되지 않았다. 효소의 반응에 있어 최적 pH는 7.2이었으며, 최적온도는 37°C이었다. Phosphoenolpyruvate와 erythrose-4-phosphate에 대한 본 효소의 K<sub>m</sub>값은 각각 0.43 mM과 0.22 mM이었다. 본 효소는 EDTA에 의하여 80%의 효소활성이 소실 되었으며, 소실된 효소의 활성은 Co<sup>2+</sup> 이온의 첨가에 의하여 정상으로 회복되었고, Mn<sup>2+</sup>과 Zn<sup>2+</sup> 이온에 의하여는 일부의 효소 활성만이 회복되었다. Ni<sup>2+</sup>, Fe<sup>2+</sup> 그리고 Ca<sup>2+</sup>는 본 효소의 활성을 저해하였다.