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Heterologous expression and characterization of soluble recombinant 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase from *Actinosynnema pretiosum* ssp. *auranticum* ATCC31565 through co-expression with Chaperones in *Escherichia coli*

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ABSTRACT

3-Deoxy-D-arabino-heptulosonate-7-phosphate synthase (DAHPS), (EC 2.5.1.54) catalyzes the first step of the shikimate pathway, the route for the biosynthesis of aromatic compounds in plants and microbes. In *Actinosynnema pretiosum*, the *aro*F gene (GenBank: AF056968.1) encodes DAHPS to condensate phosphoenolpyruvate (PEP) and D-erythrose 4-phosphate (E4P) to generate DAHP. In this study, a recombinant pET28a-*aro*F plasmid was constructed and *A. pretiosum* DAHPS was successfully expressed in soluble form by co-expression with chaperonins GroEL/GroES in *Escherichia coli*. The purification and kinetic characterization of the expressed protein were then investigated. The DAHPS originated from *A. pretiosum* demonstrated a pronounced substrate inhibition by PEP but was not sensitive to E4P. The purified enzyme was completely inactivated by EDTA but potently activated by several bivalent metal ions, especially Mn^{2+} and Co^{2+}.

Introduction

Ansamitocins, one type of the maytansines which are used to inhibit tumors including leukemia and lung cancer in clinic test, are produced by *Actinosynnema pretiosum* [1]. Ansamitocins are series of complex aromatic compounds including AP-0, AP-2, AP-3, AP-3′, and AP-4, among which AP-3 is confirmed to be the active ingredient. Results of many experiments suggested that during Ansamitocins synthesis, 3-amino-5-hydroxybenzonic acid (AHBA)[1] is first formed through the amino-shikimate pathway [2]. AHBA then serves as the starter unit for the assembly of a polyketide which eventually links back to the amino group of AHBA to form the macrolactam ring [3].

3-Deoxy-D-*arabino*-heptulosonate-7-phosphate synthase (DAHPS) (EC 2.5.1.54) catalyzes the condensation of phosphoenolpyruvate (PEP) and erythrose 4-phosphate (E4P) to produce DAHP and inorganic phosphate. There are seven steps in the shikimate pathway and its end product of chorismate is the biosynthetic precursor of aromatic amino acids, vitamin K, folic acid, ubiquinone and other secondary metabolites [4]. DAHPS is essential to plants, microorganisms and the parasites, but does not exist in animals and Homo sapiens [4,5]. So DAHPS is a potential herbicide and antiparasite. The researches on DAHPS originated in bacteria such as *Neurospora crassa*, *Bacillus subtilis*, *Salmonella*, *Escherichia coli* and *Brevibacterium flavum* [6–10], and some eukaryotic microorganisms especially *Saccharomyces cerevisiae* [11]. In 1974, Huisman and Kosuge separated the DAHPS from cauliflower for the first time [12] and from then on more studies on DAHPS were springing up for various plants including carrot root, potato tuber, tomato pericarp, tobacco and arabidopsis [13–17]. It is not surprising to find that DAHP synthase activity is regulated somewhat differently in plants, because there is less than 20% sequence identity between the bacterial and the plant enzyme. Two distinct classes of DAHPS from microorganisms have been identified before, a type I DAHPS having an *E. coli*-like sequence with a monofunctional subunit M_r of around 39 kDa and a type II DAHPS having a plant-like sequence with a monofunctional subunit M_r of around 54 kDa [18,19]. Several type II DAHP synthases purified from *Streptomyces coelicolor* A3 (2), *Streptomyces rimosus* and *N. crassa* were characterized and the sequences of DAHPS were found similar to plant DAHP synthase [18].

Since *E. coli* does not produce AHBA, studies on the DAHPS can provide more information about the shikimate pathway in *A. pretiosum*. A heterologously expressed *A. pretiosum* DAHPS might be investigated to see if it yields aminoDAHP by supplying with proper nitrogenous precursor such as nitrogen monomer or

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[1] *Abbreviations used:* DAHPS, 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase; AHBA, 3-amino-5-hydroxybenzonic acid; E4P, erythrose 4-phosphate; HRP, horseradish peroxidase.

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polypeptides. At present, there is no report to identify if the DAHP synthase from the shikimate pathway could produce a precursor for ansamycin or if a completely separate enzyme catalyzes the formation of aminoDAHP. The same puzzle exists in the rifamycin biosynthesis [4]. The identification and characterization of enzymes involved in AHBA synthesis by heterologous expression will be very meaningful for the synthetic biology and generation of genetically engineered microorganisms that can produce clinically useful antibiotic variants with AHBA as the precursor.

The gene *aro*F from *A. pretiosum* encoding DAHP synthase were found out of the *asm* cluster. To characterize this gene function, this study focused on how to heterologously express this gene in a soluble form in *E. coli* and how to characterize the corresponding putative DAHPS. However, several experiment results indicated that it was tough to obtain heterologously expressed target protein. Co-expression is an efficient strategy to prevent the expression of target protein in the formation of inclusion bodies as the molecular chaperones are involved in the protein folding process [20]. In this study, the recombinant protein *Ap*-DAHPS (3-deoxy-D-arabino-heptulosonate-7-phosphate synthase from *A. pretiosum*) was obtained with the construction of chaperone plasmid pGro7 and the properties of this enzyme was characterized in detail.

Materials and methods

Chemicals, enzymes and plasmids

PEP and E4P were purchased from Sigma (Sigma Chemical Co., St. Louis, MO). All metal salts were purchased from Shanghai Lingfeng Chemical Reagent Co., Ltd. (Shanghai, China). Aromatic acids, glycine, IPTG, agar, acrylamide, *N*,*N*′-methylene bis-acrylamide, chloramphenicol, kanamycin, ampicillin, coomassie brilliant blue R250 and β-mercaptoethanol were purchased from Shanghai Majorbio Technologies Co., Ltd. (Shanghai, China). Imidazole, potassium periodate, ethanol absolute, EDTA and trichloroacetic acid were purchased from Sinopharm Chemical reagent Co., Ltd. (Shanghai, China). Tryptone and yeast extract were purchased from Oxoid Ltd. (Cambridge, UK). Sodium dodecyl sulfate (SDS) and the Sepharose were obtained from Genebase Gene-Teach Co., Ltd. and Invitrogen (Carlsbad, CA), respectively.

All the restriction endonucleases, modifying enzymes, ligation enzymes, pGMT-vector and pGro7 plasmid were purchased from TaKaRa (TaKaRa Biotechnology Co., Ltd., Dalian, China) and Fermentas (Fermentas Intl. Inc., Burlington, Canada).

Strains and heterologous expression

A. pretiosum ssp. *auranticum* ATCC31365 was cultured in YMG medium (4 g/L yeast extract, 10 g/L malt extract and 4 g/L glucose) at 28 °C for 5 days. Genomic DNA of *A. pretiosum* was isolated from cells grown at the mid-exponential phase with a TIANamp bacteria DNA Kit (Tiangen Biotech, Beijing, China) and used as template. The construction of recombinants was similar to that described below where the pET28a-aroF construction was briefly shown. DNA corresponding to the ORF of *A. pretiosum* ssp. *auranticum* DAHP synthase gene (*aro*F, GenBank: AF056968.1) was amplified from genomic DNA using TaKaRa LA Taq polymerase (TaKaRa Biotechnology) with the designed primers 5′-TCACATATGAACTGGACCGTG GACGT-3′ (forward) incorporating an *Nde* I restriction site and 5′-TATGAATTCTCAGGAGCGGAGCATCTCCGCCACC-3′ (reverse) incorporating an *Eco*R I restriction site. The PCR product (*ca*. 1.4 kbp) was purified by an EZ Spin Column PCR Product Purification Kit (Sangon Biotech, Shanghai, China) and then ligated into pGMT-Vector using T4 DNA ligase. Recombinant plasmid was digested with *Nde* I and *Eco*R I, followed by gel purification. The fragment was then ligated using T4 DNA ligase into plasmid pET28a (Novagen) previously digested with the same endonucleases to obtain pET28a-*aro*F. After verification, pET28a-*aro*F was transformed into the competent *E. coli* BL21 (DE3) containing the pGro7 plasmid. For the expression of recombinant DAHPS, BL21 (DE3)/pGro7/pET28a-*aro*F was growing at 37 °C in Luria–Bertani broth containing L-arabinose (2 mg/mL), kanamycin (100 μg mL^{-1}) and choramphenicol (25 μg mL^{-1}) until the mid-exponential phase. IPTG was then added to a final concentration of 1 mM and the culture temperature was lowered to 30 °C. After 8 h of post induction, cells were harvested (10,000 rpm for 1 min) and stored at −40 °C for further analysis. Similar procedure applied to the construction of all other recombinant plasmids containing the *aroF* gene (Table 1).

SDS–PAGE and Western blot analysis

SDS–PAGE was performed by a Mini-PROTQN II Electrophoresis Cell (Bio-Rad Laboratories, Inc., Hercules, CA) based on the method of Laemmli [21] to determine the fusion expression system and the degree of purification. The whole cell lysate, sediment and supernatant were treated with a 2×SDS sample buffer, vortexed and heated to 100 °C for 5 min. Samples ranging from 5 to 10 μl were loaded onto SDS–12% polyacrylamide gels. SM1881 was used as the protein marker to determine the molecular weight of proteins.

Table 1
Strains and plasmids used in this study.

Strains/plasmids	Properties or product	Source
Strains		
A. pretiosum ssp. *auranticum*	Wild type to provide genomic DNA	ATCC31565
E. coli JM109	A good strain for bearing plasmids and as expression strain for pSE380-his-aroF	Invitrogen
E. coli BL21 (DE3)	A high level expression strain for pET28a-aroF	Invitrogen
Plasmids		
pET28a	Carrying an N-terminal His-Tag/thrombin/T7-Tag configuration plus an optional C-terminal His-Tag sequence.	Novagen
pET28a-aroF	Nde I-EcoR I aroF gene fragment cloned into pET28a to expression DAHPS with high-expression	This study
pSE380	Ampicillin^r	Invitrogen
pSE380-his-aroF	Nco I -EcoR I aroF gene fragment containing his_6-tag sequence cloned into pSE380 to expression DAHPS with low-expression	This study
pCold	Designed for fusion expression with a translation enhance effect at low temperature	Invitrogen
pCold-aroF	Nde I - EcoR I aroF gene fragment cloned into pCold to expression DAHPS at low temperature	Invitrogen
pET43.1a	Designed for cloning and high-level expression of peptide sequences fused with the 491 aa Nus·Tag™ protein.	Novagen company
pET43-aroF	BamH I-EcoR I aroF gene fragment cloned into pET43.1a to fusion expression DAHPS together with NusA-Tag in high level-expression	This study
pGEX	A fusion vector with the GST-tag	Yueke biotech
pGEX-aroF	BamH I-EcoR I aroF gene fragment cloned into pGEX to fusion expression DAHPS together with GST-Tag in high level-expression	This study