Process Biochemistry 50 (2015) 1349–1356

Contents lists available at ScienceDirect

Process Biochemistry

journal homepage: www.elsevier.com/locate/procbio

Cloning, expression, and characterization of an acetolactate synthase (ALS) gene from *Anabaena azotica*

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ARTICLE INFO

Article history:
Received 7 October 2014
Received in revised form 29 May 2015
Accepted 30 May 2015
Available online 11 June 2015

Keywords:
Acetolactate synthase
Cloning
Expression
Structure

ABSTRACT

A novel acetolactate synthase (ALS) gene was cloned from *Anabaena azotica*, which was isolated from China and is regarded as a vital and beneficial photosynthetic microorganism that can contribute to soil fertility and maintain ecosystem stability. The full-length gene consists of an open reading frame (ORF) of 1644 bp and encodes a 59.2 kDa 547 amino acid protein. Sequence alignment using the BLAST similarity search in the GenBank database revealed that the amino acid sequence was similar to ALS derived from *Anabaena* sp. PCC 7120. The ALS gene was subcloned into the plasmid pET28a (+) and expressed in *Escherichia coli* BL21 (DE3) by IPTG induction. The recombinant protein was purified and subsequently characterized. The properties of ALS from the *E. coli* transformant are similar to that of an enzyme from the original *A. azotica*. Conserved amino acid sequence and the predicted structure of the active site were consistent with those observed for the bacterial enzyme *Klebsiella pneumoniae* ALS in similar experiments. Our results represent a significant functional demonstration of the existence of an algae ALS subunit and provide a solid basis for further studies on the structure, function and properties of ALS, as well as its response mechanism to inhibitors.

1. Introduction

Acetolactate synthase (ALS; EC 2.2.1.6), also known as aceto-hydroxy acid synthase (AHAS), catalyzes the first committed step in branched-chain amino acid biosynthesis; it converts 2 mol of pyruvate to acetolactate plus CO_2, using thiamine diphosphate (ThDP) as a cofactor [1,2]. AHAS is found in plants, fungi, and bacteria and requires non-catalytic FAD, whereas ALS is found only in some bacteria and does not need FAD [3,4]. The enzyme is known to be the site of action for several structurally diverse classes of herbicides including sulfonylureas [5–7], imidazolinones [8], triazolopyrimidines, pyrimidinylthiobenzoates [9] and [10], and sulfonylamino-carbonyl-triazolinones.

As the enzyme is present in microorganisms and plants but not in animals, these herbicides are widely used in agriculture due to their specificity and efficacy at low application rates against weeds with low mammalian toxicities. Unfortunately, the rapid use of ALS-inhibiting herbicides all over the world has caused a series of environmental pollution hazards during the past 20 years, e.g., jeopardizing the survival of cyanobacteria that are one of the largest and most important groups of prokaryotes on earth [11]. In particular, several beneficial nitrogen-fixing cyanobacteria are vital photosynthetic microorganisms that contribute to soil fertility by fixing atmospheric nitrogen and maintaining ecosystem stability [12]. However, as they have many characteristics of higher plants [13], they are also quite sensitive to herbicides, especially to ALS inhibitors.

The encoding gene, expression method, and regulation of this enzyme have been studied in some detail in Bacteria and Eucarya [14–16]. A few ALS structures from some species, such as yeast, *Arabidopsis thaliana*, and *Escherichia coli*, have been determined and well characterized [2–4]. However, the biochemical and molecular properties of this enzyme are not well characterized in cyanobacteria, even though a gene annotated as encoding ALS has been identified from an analysis of the complete genome sequence of *Anabaena* sp. PCC 7120. To date and in the context of the bacterial enzyme, little is known about the specific roles of ALS-inhibiting herbicides in the field of non-target nitrogen-fixing cyanobacteria, what components in ALS gene sequence of *A. azotica* are significant, or whether the cyanobacteria holoenzyme is composed of both large and small subunits.

Anabaena azotica is one of the several beneficial nitrogen-fixing cyanobacteria that are commonly found in the rice fields of China [17]. In this study, we report the discovery of a DNA clone that encodes a subunit of algae ALS based on homology of the encoded peptide with various bacterial ALS and hope that this gene will

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http://dx.doi.org/10.1016/j.procbio.2015.05.027
1359-5113/

provide clues about the effects of inhibitors on mutations in the ALS active site. The gene was expressed in *E. coli* and the purified recombinant enzyme was characterized. We attempted to predict the three dimensional structure of ALS to investigate the active site and its natural role. This achievement will contribute to our understanding of the evolution of this enzyme family. It will also be of great value for research on herbicide-resistant cyanobacteria or developing new herbicides with a high degree of selectivity between beneficial nitrogen-fixing cyanobacteria and weed seedlings in the future.

2. Materials and methods

2.1. Bacterial strains, plasmids, enzymes, and fine chemicals

The algae *A. azotica* (FACHB-686) were provided by the Institute of Hydrobiology of the Chinese Academy of Sciences in Wuhan, China. The pMD18-T Simple vector used as the plasmid recombinant and for ALS gene cloning was purchased from TaKaRa (Japan). The *E. coli* strains DH5-α and BL21 (DE3) and the pET28 (a)+ expression vector were obtained from Dr K. X. Tang (Shanghai Jiaotong University). *E. coli* DH5-α was used as a host in standard cloning, and *E. coli* BL21 (DE3) was used for expression of the pET28 (a)+ vector. The DNA purification kit was purchased from Qiagen (Germany). T4 DNA ligase, restriction endonucleases, and Taq DNA polymerase were purchased from TaKaRa.

2.2. Cloning of the A. azotica ALS gene

Axenic cultures were grown in a liquid sterilized medium as described by Shen et al. [17] at 30 ± 2 °C under constant fluorescent light at an intensity of 36.2 μmol/m^2/s (pH 7.2). For isolation of the genomic DNA, the cells were harvested and immediately treated with lysozyme (5 mg/ml for 1 h). The genomic DNA was isolated according to the method of Cai and Wolk [18] and then the ALS gene was PCR amplified. The amplifications were carried out using the corresponding forward and reverse primers (P_1 and P_2) which introduced *EcoR* I and *Xhol* I restriction sites at the 5′ ends, respectively (Table 1). The cycling conditions were as follows: (1) 94 °C for 3 min, (2) 30 cycles of 94 °C for 45 s, 49 °C for 1 min, and 72 °C for 2 min, and (3) a final extension at 72 °C for 10 min. The resulting PCR-amplified fragments were ligated into the pMD18-T Simple vector to generate the pMD18-T-ALS plasmid. Next, the pMD18-T-ALS plasmid was transformed into DH5-α cells. Plasmids from individual antibiotic-resistant colonies were purified according to the alkaline lysis method; transformation of *E. coli* was carried out using a standard $CaCl_2$ transformation protocol as described previously [19]. The correct insert was identified by restriction enzyme analysis and PCR and verified by DNA sequencing.

2.3. Gene analysis and phylogenetic tree construction

Sequence assembly was performed with programs from the DNAMAN 6.0 software (Lynnon, Co., Ltd.). Alignments of the DNA and protein sequences were conducted with the blastn and blastp programs in GenBank, respectively (http://www.ncbi.nlm.nih.gov/BLAST/) [20]. The nucleotide sequence of the ALS gene has been submitted to the Genbank databases under accession No. HQ631428.

Table 1
The sequences of primers and oligonucleotides for PCR amplification.

Primers	Sequences (5′–3′)	T_m (°C)
P_1	CCG **GAA TTC** ATG AAT ACA GCA GAA CTG TTAG	69.7
P_2	CCG **CTC GAG** CTA AAC AGA ACA ACT TAAC	67.5
P_3	CCG **CTC GAG** AAC AGA ACA ACT TAA CTC AC	66.2

The isoelectric point, molecular weight, and hydropathy profile were determined with the Lasergene Protean. The phylogenetic tree was constructed with the MEGA 4.1 program [21] using the neighbor-joining method. Bootstrap values were calculated on 500 replications. The ALS sequences of different species were retrieved from Genbank (http://www.ncbi.nlm.nih.gov/Genbank/index.html). We predicted the three-dimensional structure of the ALS protein and assessment space structure with the SWISS-MODEL service platform. The structure was viewed with the SwissPDBviewer (http://www.expasy.ch/spdbv) programs, PyMOL 0.99 software, and Raswin (version 2.7.2.1).

2.4. Construction of the pET-ALS expression plasmid

The pET-ALS expression vector was constructed based on the pET28(a)+ commercial expression vector, which contains a leader sequence with a six-histidine affinity tag. To fuse expression with the N-terminal His-tag, which allowed convenient purification of the native protein directly from crude cell extracts, we designed a new reverse primers P_3 that deleted the stop codon (Table 1). The 1.6-kb purified PCR product was inserted into the pMD18-T Simple vector using a cloning strategy described previously [22]. Then, the ALS gene was excised from the pMD18-T-ALS vector with *EcoR* I and *Xho* I, isolated from a 1% agarose gel, and cloned into the of pET28 (a)+ *EcoR* I and *Xho* I sites. Plasmid pET-ALS was then transformed into *E. coli* BL21 (DE3) competent cells to obtain recombinant *E. coli* BL21 (DE3)/pET-ALS which would be grown in LB supplemented with 50 μg kanamycin/ml.

2.5. Expression and SDS-PAGE of ALS

The BL21 (DE3)/pET-ALS cells were grown at 37 °C in LB medium, and expression of ALS was induced by adding IPTG from 0.2 to 1 mM when the OD_{600} reached 0.6 to identify the best IPTG inducing concentration. The cultivation was continued for 1 to 5 h to determine the best IPTG inducing time and the cells were harvested by centrifugation at 12,000 × *g* for 1 min. The cells were resuspended in 8 M urea, maintained at room temperature for 0.5 h, and then combined with the same volume of SDS-PAGE loading buffer. Then, the mixture was boiled for 3 min, cooled to room temperature, and loaded for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under conditions according to Laemmli [23]. Protein bands were visualized by Coomassie blue staining. The gels were calibrated using molecular weight markers. Protein concentration was measured according to the Bradford method with bovine serum albumin as the standard.

2.6. Purification of the His6-tagged ALS

Cells transformed with the recombinant pET-ALS plasmid were induced with IPTG at the best expression condition and then collected by centrifugation at 7500 × *g* for 10 min. The bacterial pellet was resuspended in extraction buffer (1 × PBS, pH 7.4), lysed by repeated freezing and thawing, and centrifuged again as described previously. The supernatant was transferred into another tube and precipitated by progressively adding solid ammonium sulfate to 60% saturation. The main components of centrifugal sedimentation, the inclusion bodies, were harvested at the same time and purified by five cycles of sonication (constant pulse for 2 min at 0 °C), each followed by centrifugation at 3700 × *g* for 30 min. Then, the inclusion bodies were washed twice in cell lysis buffer (50 mmol/L Tris–HCl pH 8.0, 10 mmol/L EDTA, 50 mmol/L NaCl, and 0.5% Triton-X 100) and deionized water in turn, and stored at −20 °C. The ALS content difference in the inclusion bodies and the supernatant were analyzed by SDS-PAGE before and after the cell lysis.