Chapter 15
Cloning of ATP-Citrate Lyase (*acl1*) from *Aspergillus niger* and its Expression in *Escherichia coli*

Fang Sun, Hong Chen, Xihong He and Hao Liu

Abstract *Aspergillus niger* is an important strain used for industrial fermentation of citrate. The production of citrate is closely related to the growth and metabolism of *A. niger*. ATP-citrate lyase (ACL) is responsible for catalyzing the conversion of citrate into oxaloacetate and acetyl-CoA, which is a bridge between glucose metabolism and fatty acid synthesis. In *A. niger*, tandem divergently transcribed genes (*acl1* and *acl2*) encode the subunits of ACL, whose physiological function is unclear. In this study, *acl1* was obtained from *A. niger* by RT-PCR. The sequencing result was consistent with the sequence of genome database. We constructed the expression vector pET28a^{+}-*acl1-his6* which was suitable for the efficient expression in *Escherichia coli* BL21. After purification with Ni^{2+} chelating chromatography column, SDS-PAGE analysis showed that the molecular mass of the ACL1 was 66 KDa. The result laid the foundation for further research about protease characteristics and physiological functions of ACL1 in *A. niger*.

Keywords *Aspergillus niger* · ATP-citrate lyase · Acetyl-CoA · Protein expression · RT-PCR

15.1 Introduction

Aspergillus niger is an important industrial workhorse with extensive application in the sectors of industrial enzymes, heterogeneous proteins, organic acids, and so on [1]. *A. niger* is economically important as a fermentation organism used for the

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production of citric acid. Industrial citric acid production by *A. niger* represents one of the most efficient, highest yield bioprocesses in use currently by industry. At present, the annual output of citric acid is more than 1.5 million tons in the world, of which 99 % citric acid is fermented from *A. niger*. The production of citric acid is closely related to the growth and metabolism of *A. niger* [2–4]. For a long time, researchers have focused on the genes related to the metabolism of *A. niger*. The researches have clarified the significant roles of the glycolytic pathway, the tricarboxylic acid cycle, and the glyoxylate cycle in the citric acid metabolism and revealed that pyruvate dehydrogenase, pyruvate carboxylase, and citrate synthase directly involved in the function of key enzyme encoding the genes in the citric acid metabolism [5, 6]. The disclosure of the genomic sequence to the public brought the study of *A. niger* into the post-genomic era [7, 8]. The genome of *A. niger* ATCC 1015, historic strain was used in research that resulted in the first patented citric acid process, that was accepted for sequencing through the US Department of Energy Microbial Genome Program. Acetyl coenzyme A (CoA) is an important intermediate involved in both intermediary carbon and energy metabolism as well as in biosynthetic pathways [9]. Acetyl-CoA generated through the degradation of lipids, carbohydrates, and amino acids is used for the synthesis of several cellular components, and nucleocytosolic acetyl-CoA is particularly important in the acetylation of histones [10]. Acetyl-CoA also serves as an important precursor for several metabolites, such as polyketides, terpenes and lipids [11]. ATP-citrate lyase is considered to be an essential cytoplasmic enzyme of the carbon dioxide-fixing reductive tricarboxylic acid cycle, since it catalyzes the formation of oxaloacetate and acetyl-CoA from the cleavage of citrate. A survey of a wide variety of oil-producing microorganisms has found a correlation between those that accumulate high levels of lipids and the presence of ACL activity. Another important function of the ACL is the regulation of gene expression. The study found that the ACL activity is required to link growth factor-induced increases nutrient metabolism to the regulation of histone acetylation and gene expression [12]. ACL is present in fungi, plants, animals, and some prokaryotes [10]. The ACL polypeptides of animal have a molecular mass of 110–120 kDa and are encoded by a single gene [13]. ACL of mammalian positively regulates the glycolytic pathway by regulating transcriptional activation through histone acetylation and by inhibiting glycolysis during hypoxic conditions [14]. In filamentous fungi, ACL is thought to consist of two different subunits of 70 and 55 kDa, respectively [15, 16]. It was reported that in filamentous fungi two different subunits of ACL are encoded by two separate genes adjacent on a chromosome, and this is in contrast to animals where ACL is encoded by a single gene. The 66 kDa ACL1 polypeptide of *A. niger* shows significant homology to the C-terminal parts of animal ACL polypeptides. *A. niger* has been shown to have two adjacent genes (*acl1* and *acl2*) for different subunits of ACL separated by 2.6 kb, and these are divergently transcribed. The catalytic center is conserved in both the rat ACL and the fungal ACL1 polypeptides [17]. Analysis of sequenced fungal genomes indicates that predicted ACL1 encoding genes are present widely in fungi and have high similarity (Fig. 15.1). The catalytic enter of *A. niger* ACL1

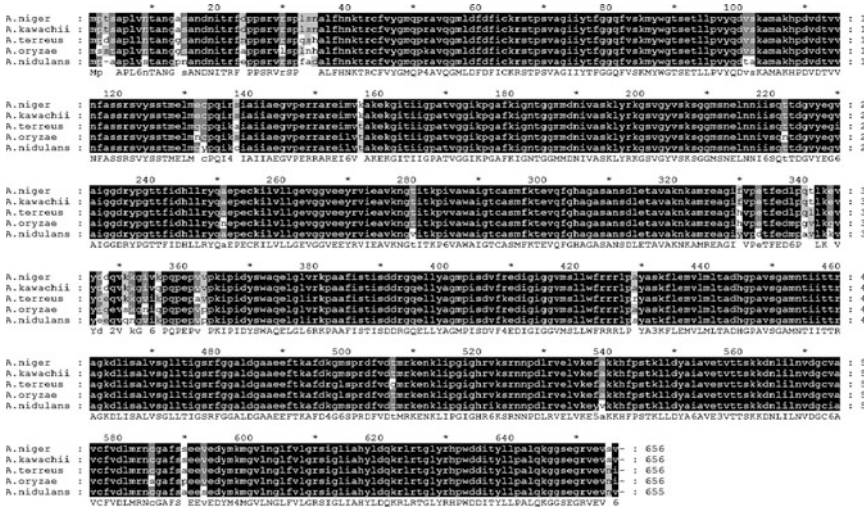


Fig. 15.1 Alignment of ACL1 polypeptide sequences from filamentous fungi. Amino acid residues conserved in all sequences are shown in *black*. The following sequences were used for comparison: *A. niger* (Accession no. xp_001394055); *A. kawachii* (Accession no. GAA84137); *A. terreus* (Accession no. xp_001211553); *A. oryzae* (Accession no. xp_001820781); *A. nidulans* (Accession no. xp_660040)

protein also has a conserved histidine. It is unknown whether autophosphorylation occurs in the catalytic process.

In this study, we reported cloning and sequence analysis of cDNA that encode the *A. niger* ACL1. Moreover, the recombinant fusion protein was expressed and purified using Ni^{2+} chelating chromatography column successfully. The result laid the foundation for further research of protease characteristics and physiological functions of ACL1 in *A. niger*.

15.2 Materials and Methods

15.2.1 Strains

The *Aspergillus niger* strain ATCC1015 used in this study was held in our laboratory collection and propagated on potato dextrose agar. The *E.coli* JM109 and BL21 used for cloning and protein expression were both held in our laboratory collection.

15.2.2 Chemicals and Reagents

KOD FX polymerase, all PCR reagents, enzymes for molecular cloning and PrimeScript RT reagent Kit were purchased from Takara. Gel Extraction Kit and the rapid plasmid DNA mini-prep kit were from Solaribio. Trizol were purchased from Invitrogen. His-select Ni-Chelating affinity gel column used for protein purification was purchased from Novagen.

15.2.3 RNA Isolation and cDNA Synthesis of acl1

Mycelia used for genomic DNA and RNA extraction were harvested from cultures grown in liquid potato dextrose medium at 30 °C for 2 days. Total RNA of *A. niger* was extracted using the Trizol method, and the initial mycelia amount was 500 mg [18]. DNA was removed from RNA by scavenger of DNA. Genomic DNA of *A. niger* was extracted using the CTAB protocol [19]. The extraction of total RNA was identified and isolated by agarose gel electrophoresis to check the integrity.

Fist strand cDNA synthesis was carried out with a PrimeScript RT reagent Kit Perfect Real Time using Oligo (dT) primers and total RNA as the template. Reaction condition was as follows: 65 °C 5 min, then chilling on ice 5 min, 37 °C 15 min, 42 °C 15 min, 50 °C 15 min, 85 °C 5 s. The PCR specific primers for *acl1* were designed according to *acl1* sequence of *A. niger* published in GenBank. The two specific primers used were as follow: *acl1*-F: ATGCC TAC-CTCTGCTCCCCTCGTC; *acl1*-R: TTAGACGCTGACCTCGACACGACCC. PCR amplification was performed in 20μL reaction mixture containing 1.6 μL of cDNA, 10 μL of 2 × PCR buffer, 2 μL of 2 mM dNTPs, 0.4 μL each of 10 μM forward and reverse primers, 0.4 μL of KOD FX. The temperature gradients (55–72 °C) were used to determine the optimum annealing temperature. The reaction conditions comprised an initial denaturation of 2 min at 94 °C, followed by 42 cycles of 98 °C for 10 s, 68 °C for 30 s, and 68 °C for 2 min, with a final extension at 68 °C for 10 min. After checking the product on an analytical 1 % agarose gel, the 1.9 kb band was then extracted from the gel by Gel Extraction Kit.

15.2.4 Cloning and Sequencing of the PCR Products

The purified PCR product was then ligated into vector pIJ2925 at 16 °C overnight and transformed. The pIJ2925-*acl1* vector was transformed into competent JM109 *E. coli* cells and plated on LB-ampicillin/IPTG/X-gal plates followed by incubation at 37 °C for about 20 h. (We prepared plates containing X-Gal and IPTG, 50 μL of 50 mg/mL X-Gal and 100 μL of 0.1 M IPTG onto previously prepared LB plates

containing ampicillin. Allow these components to absorb for at least 30 min at 37 °C prior to plating cells). The resulting colonies were screened by Colony PCR using the gene specific oligonucleotide primers. The plasmid DNA was purified from overnight culture using the rapid plasmid DNA mini-prep kit and the presence of insert was verified by *Eco*R I and *Pst* I, *Sac* I, *Xho* I, *Mun* I restriction digestion of purified recombinant plasmid, respectively. One clone was selected and sequenced. The sequence of cloned fragment was analyzed using public domain database of NCBI http://blast.ncbi.nlm.nih.gov/Blast.cgi.

15.2.5 Sub-cloning of acl1 *into Expression Vector pET28a*$^+$

The *A. niger acl1* gene was amplified from pIJ2925-*acl1* vector using the gene specific forward and reverse primers: *acl1-Nde* I: GGGAATTCCATATGCCTAC CTCTGCTCCCCTCG; *acl1-Eco*R I: CGGAATTCTTAGACGCTG ACCTCGA-CACGAC. The primers containing the restriction enzyme sites in order to generate the *Nde* I and *Eco*R I sites in the PCR product. The PCR amplified *acl1* gene and the pET28a$^+$ expression vector was restriction digested, gel purified, and the digested *acl1* gene product was ligated into the *Nde* I and *Eco*R I sites of pET28a$^+$ expression vector by incubation at 16 °C overnight and the transformation was carried out by mixing 10 μL of ligation mix to 100 μL of competent BL21 *E. coli* cells following the standard procedure. The transformed cells were plated on LB-agar plates containing 100 $\mu g \cdot mL^{-1}$ of kanamycin, and incubated at 37 °C for about 20 h. The positive clones were selected by Colony PCR and confirmed by restriction digestion.

15.2.6 Expression and Purification of ACL1 in E. coli

For analytical studies, induction was done at different IPTG concentrations (0.1, 0.4, 1 mM), different temperatures (20, 28, 37 °C), and different time intervals (4, 6, 8, 12 h). After optimizing the expression conditions, a starter culture was set up by inoculating 3 mL of LB broth containing 100 $\mu g \cdot mL^{-1}$ of kanamycin with single bacterial colony of pET28a$^+$-*acl1-his6* plasmid, and the culture was grown overnight in 37 °C orbital shaker at 200 rpm. The 4 mL of LB broth supplemented with kanamycin was inoculated with 5 % of the starter culture and allowed to grow at 37 °C with shaking at 200 rpm till OD_{600} reached 0.5–0.6, then induction was done with 0.1 mM IPTG at 20 °C for 12 h. The cultures were chilled on ice and the cell pellet harvested by centrifugation at 12000 rpm for 10 min at 4 °C, was suspended in 1 × bind buffer (50 mM NaH_2PO_4, 500 mM NaCl, pH 8.9). The cell suspension was sonicated (4 s each with 6 s cooling between successive bursts in 20 min). The resulting lysate was centrifuged at 12000 rpm for 20 min at 4 °C.

The supernatant and the pellet obtained from the uninduced and induced cells were analyzed on SDS-PAGE.

To purify the ACL1 protein, the large amount of pellet obtained as described above, was resuspended in 1 × Ni-NTA Buffer (50 mM NaH_2PO_4, 500 mM NaCl, 5 mM imidazole, 10 % glycerin, pH 8.9). The cell suspension was sonicated as described above. One milliliters of the supernatant was applied onto a column filled with His-select Ni-Chelating affinity gel preconditioned with 1 × Ni-NTA Buffer. The elution was performed with 5 mL of 1 × bind buffer containing 50 mM imidazole and then 5 mL of 1 × bind buffer containing 100 mM imidazole and then 5 mL of 1 × bind buffer containing 300 mM imidazole and then 5 mL of 1 × bind buffer containing 500 mM imidazole. The purity of recombinant ACL1 was analyzed on SDS-PAGE.

15.3 Results and Discussion

15.3.1 Isolation and Sequencing of acl1 *in* A. niger

It is important to obtain total RNA of high quality and integrity for the further PCR amplification. The total RNA of *A. niger* was pure without genome DNA pollution (Fig. 15.2). The full length of *acl1* cDNA was amplified from total RNA of *A. niger* by RT-PCR using gene specific primers corresponding to the *acl1* cDNA. The PCR amplification of gene coding for ACL1 using a temperature gradient from 55 to 72 °C showed maximum amplification of 1.9 kb PCR product at 68 °C. The amplified *acl1* cDNA showed an electrophoretic mobility on agarose gel corresponding to about of *acl1* cDNA fragment was smaller than the size of *acl1* DNA, which confirmed that the total RNA was not polluted by genome DNA (Fig. 15.3).

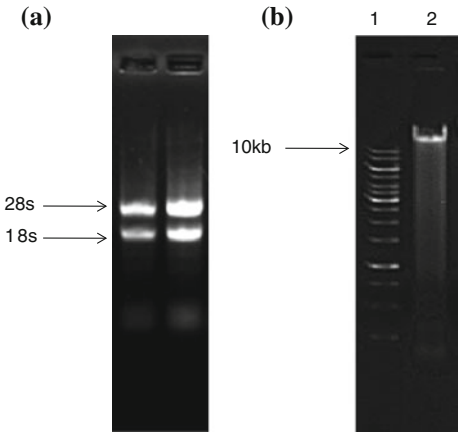


Fig. 15.2 Isolation of total RNA and genome DNA from *A. niger*, **a** total RNA of *A. niger*, **b** genome DNA of *A. niger*: Lane *1*: 1 kb DNA ladder, Lane *2*: Product of Genome DNA from *A. niger*

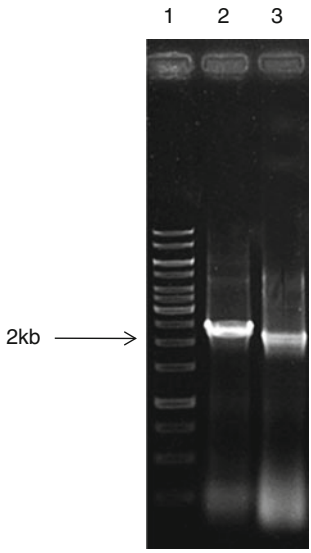


Fig. 15.3 RT-PCR amplification of *acl1* from *A. niger*. Lane *1*: 1 kb DNA ladder, Lane *2*: PCR product of *acl1* genome DNA generates fragment of 2139 bp, Lane *3*: RT-PCR product of *acl1* cDNA generates fragment of 1971 bp

The PCR product was cloned into pIJ2925 vector and its restriction digestion is shown in Fig. 15.4. The presence of *acl1* cDNA fragment was verified by *Eco*R I and *Pst* I restriction digestion. The pIJ2925-*acl1* vector has two *Sac* I restriction sites, one is in the *acl1*, and the other is in the multiple cloning site. So the direction of ligation was verified by *Sac* I restriction digestion. The full length of pIJ2925-*acl1* vector was verified by *Xho* I single restriction digestion. The *Mun* I restriction digestion confirmed *acl1* cDNA without genome DNA pollution.

The *acl1* cDNA inserted was then sequenced. Nucleotide sequence corresponding 1971 bp was then compared with the nucleotide sequences deposited in the GenBank database using the BLAST program on the NCBI Blast server. The isolated *acl1* showed that no mutations were found in the cDNA compared to the deposited *acl1* sequence from *A. niger*. The full length cDNA of *A. niger acl1* was deposited in GenBank under accession number XM_001394018. The open reading frame of *A. niger* ACL1 consisted of coding region of 1971 nucleotides and the deduced amino acid sequence represents 657 amino acid residues with a calculated molecular weight of 66 KDa.

15.3.2 Cloning of acl1 *into Expression Vector*

The isolated DNA was amplified with forward and reverse primers containing the restriction sites *Nde* I at 5′ and *Eco*R I at 3′ (see Materials and methods) for inserting it in the pET28a$^+$ vector which has been previously used for the expression of proteins [20]. The presence of *acl1* cDNA fragment was verified by *Nde* I and *Eco*R I restriction digestion (Fig. 15.5). The recombinant pET28a$^+$-*acl1*

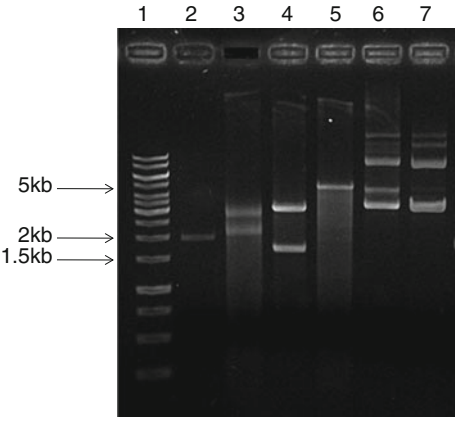
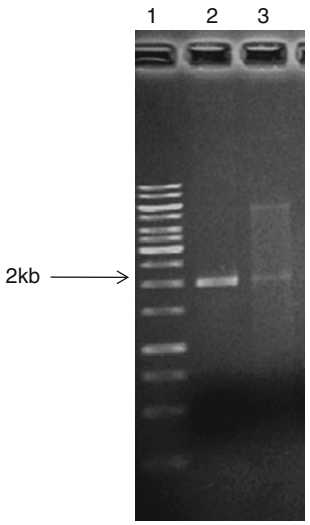


Fig. 15.4 Analysis of pIJ2925-*acl1* vector by restriction enzyme digestion. Lane *1*: 1 kb DNA ladder, Lane *2*: PCR amplification of *acl1* cDNA generates fragment of 1971 bp, Lane *3*: Digestion of pIJ2925-*acl1* with *Eco*R I and *Pst* I generates fragments of 1971 and 2706 bp, Lane *4*: Digestion of pIJ2925-*acl1* with *Sac* I generates fragments of 1597 and 3080 bp, Lane *5*: Digestion of pIJ2925-*acl1* with *Xho* I generates fragment of 4677 bp, Lane *6*: Digestion of pIJ2925-*acl1* with *Mun* I generates the same fragments as pIJ2925-*acl1* vector, Lane *7*: Undigested pIJ2925-*acl1* vector

Fig. 15.5 Analysis of pET28a^{+}-*acl1-his6* by double digestion. Lane *1*: 1 kb DNA ladder, Lane *2*: PCR amplification of *acl1* cDNA generates fragment of 1971 bp, Lane *3*: Digestion of pET28a^{+}-*acl1-his6* with *Nde* I and *Eco*R I generates fragments of 1971 and 5371 bp



plasmid, encoding ACL1 fused with the 34 amino acid extra N-terminal sequence MGSSHHHH HHSSGLVPRGSHMASMTGGQQMGRGS, containing a *his6* tag, was used for heterologous expression and purification of the protein in the *E. coli* BL21 by Ni^{2+} chelate chromatograph column.

15.3.3 Expression and Purification of ACL1

To optimize the expression of ACL1, the time after induction by IPTG and growth temperature were varied. The cell lysates obtained under the different conditions were separated in the soluble and the insoluble fractions by centrifugation. The whole extracts, soluble and insoluble fractions of cell lysates were analyzed by SDS-PAGE. To optimize the expression of ACL1 in *E. coli* BL21 transfected with the pET28a^{+}-*acl1-his6* construct, the IPTG concentration was varied from 0.1 to 1 mM. The best level of expression was obtained at 0.1 mM of the inducer IPTG. As shown in Fig. 15.6, based on the molecular weight of ACL1 protein, the expression was notably visible on SDS-PAGE following IPTG induction and reached its maximum level after 12 h incubation at 20 °C. The apparent molecular mass was determined on the basis of the molecular mass of marker proteins (Fig. 15.6 lane 1). Its value was 66 KDa.

The purification of the recombinant *A. niger* ACL1 was achieved in a single chromatographic step on Ni^{2+} chelate chromatograph column with gradient elution of 100 mM imidazole, 300 mM imidazole and 500 mM imidazole. Fractions from the affinity column containing purified ACL1 were pooled together and analyzed by SDS-PAGE (Fig. 15.6).

SDS-PAGE analyses showed a single protein band of 66 kDa that represent the molecular weight of the *A. niger* ACL1 fused to vector specific fusion histidine tag peptide. The calculated ACL1 molecular weight was in agreement with the reported molecular weight value for *Aspergillus nidulans* [21].

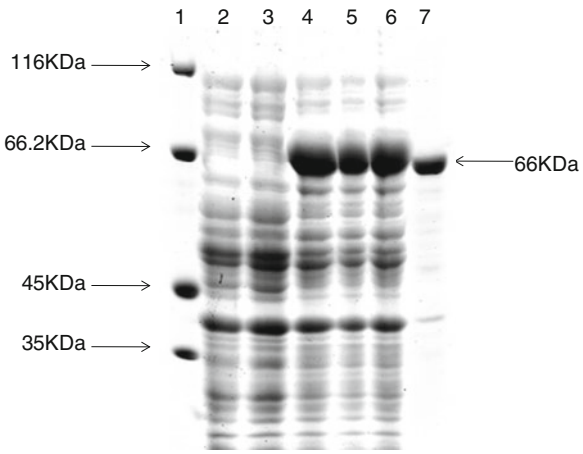


Fig. 15.6 Expression of recombinant pET28a^{+}-*acl1-his6* in *E. coli* BL21. Lane *1*: Protein molecular weight marker, Lane *2*: *E.coli* BL21 transformed with pET28a^{+}, Lane *3*: *E.coli* BL21 transformed with pET28a^{+}-*acl1-his6* without induction, Lane *4-6*: *E.coli* BL21 transformed with pET28a^{+}-*acl1-his6* whole extracts, soluble fractions and insoluble fractions, respectively, induced at 20 °C by 0.1 mM IPTG for 12 h, Lane *7*: The protein purified with Ni^{2+} chelating chromatography column generates fragments of 66 kDa

15.4 Conclusion

In the present study, we have amplified and cloned the *acl1* from total RNA of *A. niger* using the primer pairs based on the *acl1* cDNA sequence. The sequencing and characterization of pIJ2925-*acl1* vector confirmed the open reading frame of *acl1* gene was the same as the sequence in GenBank under accession number XM_001394018. We have cloned *acl1* cDNA into pET28a$^+$ expression vector to construct pET28a$^+$-*acl1-his6*. The ACL1 fusion protein was expressed and highly purified in *E. coli* with IPTG induction. The expression and purity of fusion protein was analyzed by SDS-PAGE and stained with Coomassie Brilliant Blue. The *acl1* gene of *A. niger* was homologous to the ACL gene of mammalian, suggesting that it has ACL activity. However, we need further experimental verification to confirm whether ACL1 protein has the ACL activity. This research has cloned and expressed *acl1* gene in E. coli to obtain the high purity ACL1 protein. The result laid the foundation for the activity of ACL1 protein analysis in vitro, to determine ACL and kinetic characteristics of ACL1 protein.

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