

Expression and purification of porcine Akirin2 in *Escherichia coli*

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Abstract: Akirin2 is a recently discovered gene related to immune responses that also plays an important role in skeletal myogenesis. In this study, in order to scale up the production of recombinant porcine Akirin2 (pAkirin2), we report the expression and purification of a His-tagged version of recombinant pAkirin2 in *Escherichia coli*. The pAkirin2 is a polypeptide of 203 amino acids containing 42 rare codons. The recombinant pAkirin2 was expressed as an N-terminal fusion protein with His6 tag in *E. coli* Rosetta (DE3) host strain. The protein was purified by Ni-IDA affinity chromatography, yielding over 90% highly purified recombinant pAkirin2, with a considerable yield of 4 mg/L. The purified recombinant pAkirin2 was confirmed by matrix-assisted laser desorption/ionization time-of-flight/time-of-flight (MALDI-TOF/TOF) mass spectrometer analysis. The refolded purified recombinant pAkirin2 significantly promoted the proliferation of C2C12 cells, indicating that it was active. This report provides a reliable technique for recombinant expression and purification of pAkirin2 protein.

Key words: porcine Akirin2, expression and purification, *Escherichia coli*, C2C12 cells

1. Introduction

Akirins are recently reported important nuclear cofactors regulating the NF- κ B-dependent gene expression of *Drosophila melanogaster* and mice (Goto et al., 2008). The Akirins have at least 2 highly conserved homologues, Akirin1 and Akirin2 (Goto et al., 2008). In mammals, Akirin2 is considered to be closely related to innate immune response and embryonic development (Goto et al., 2008; Macqueen and Johnston, 2009; Beutler and Moresco, 2008). There is a body of recent evidence suggesting that Akirin2 plays a crucial role in skeletal myogenesis (Macqueen and Johnston, 2009; Macqueen et al., 2010; Chen et al., 2013), and the *Akirin2* gene is regarded as a positional functional candidate for the gene responsible for marbling (Sasaki et al., 2009; Watanabe et al., 2011; Kim et al., 2013).

To date, very little research has been conducted on the function of porcine Akirin2 (pAkirin2). Porcine Akirin2 contains a nucleotide of 612 bp and encodes a protein of 204 amino acids with a molecular mass of 22,493 Da (Chen et al., 2012). Real-time quantitative PCR analysis showed a unique pattern of gene expression of *Akirin2* in various porcine tissues and found that the *pAkirin2* transcript was most abundant in the lung, followed by the skeletal muscle, heart, liver, fat, thymus, lymph node,

small intestine, kidney, and spleen (Chen et al., 2012). The expression pattern of pAkirin2 raises the question about its possible role in skeletal myogenesis, but the details remain unknown at present.

In this study, the full-length *pAkirin2* gene was sub-cloned into prokaryotic expression vector pET28a(+). Its expression in *E. coli*, purification, and proliferative effect on C2C12 myoblasts were also investigated.

2. Materials and methods

2.1. Materials

The *E. coli* DH5 α (TIANGEN Biotech, Beijing, China) was used as the host-vector system. The *E. coli* BL21 (DE3), BL21 (DE3) pLysS, and Rosetta (DE3) strains were used as the hosts for protein expression. pET-28a(+) plasmid was used to construct the expression vector. Kanamycin sulfate, UNIQ-10 Spin Column DNA Gel Extraction Kit, and Ni-IDA purification system were purchased from Sangon Corp. (Shanghai, China). The restriction enzymes *Eco*RI and *Hind*III and DNA ligation kit were obtained from TaKaRa (Dalian, China). The DNA marker, 2X Taq PCR master mix, and protein molecular weight marker (low) were purchased from TIANGEN Biotech (Beijing, China). All primers used in this study were synthesized by Sangon Corp. (Shanghai, China).

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2.2. Plasmid construction for *E. coli* expression

The *pAkin2* gene (GenBank accession no. JN227885) was amplified by PCR with the specific primers ATe-F (5'-CCGGAATTCATGGCGTGCGGAGC-3') and ATH-R (5'-CCCAAGCTTCATGAAACATAACTAGC-3') (the restriction enzyme sites *EcoRI* and *HindIII* are underlined). The plasmid pcDNA3.1(+)-*pAkin2* kept by our laboratory, which contains the *pAkin2* cDNA, was used as a template (Chen et al., 2012).

The amplified *pAkin2* gene was purified and ligated into the *EcoRI/HindIII* sites of the pET-28a(+) expression vector and the resulting recombinant plasmid, defined as pET-28a(+)-*pAkin2*, was transformed into *E. coli* DH5a. The sequence of the positive colony was confirmed by colony PCR and DNA sequencing analysis. Theoretically, the pET-28a(+)-*pAkin2* vector can express a fusion protein corresponding to the *pAkin2* carrying the extra N-terminal sequence MGSSHHHHHSSGLVPRGSHMASMTGGQQMGRGSEF.

2.3. Expression of *pAkin2* protein in *E. coli*

To obtain the recombinant *pAkin2* protein, the recombinant plasmid pET-28a(+)-*pAkin2* was transformed into the expression host *E. coli* BL21 (DE3), *E. coli* BL21 (DE3) pLysS, and *E. coli* Rosetta (DE3), respectively. The empty plasmid pET-28a(+) was used as a control. Selection of the transformed colony was performed on LB-agar plates containing 50 µg/mL kanamycin. Strains carrying the recombinant plasmid were inoculated in 5 mL of LB medium supplemented with 50 µg/mL kanamycin for *E. coli* BL21 (DE3)/pET-28a(+)-*pAkin2*, *E. coli* BL21 (DE3) pLysS/pET-28a(+)-*pAkin2*, and *E. coli* Rosetta (DE3)/pET-28a(+)-*pAkin2*, and cultured overnight at 37 °C with rotary shaking (250 rpm). The resulting seed culture was then transferred into 50 mL fresh LB medium supplemented with 50 µg/mL kanamycin in a 250-mL flask. When the strains grew to approximately $OD_{600} = 0.4-0.6$, protein production was induced by the addition of isopropyl β-D-thiogalactopyranoside (IPTG) (0.5 mM). Every 1 h, the strains were harvested by centrifugation at 5000 rpm for 5 min at 4 °C, and the bacterial pellet was resuspended in 5 mL of 1X PBS (0.01 M, pH 7.4) supplemented with 1% Triton X-100 and 1 mM phenylmethanesulfonyl fluoride (PMSF). Strains were disrupted by mild sonication on ice for 10 min, pulses of 5 s sonication, and 5 s intermission. The supernatant and pellet fractions were fractionated by centrifugation at 12,000 rpm for 30 min at 4 °C and analyzed using 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The purity of target proteins was assessed according to Image Lab software (Version 4.1, Bio-Rad).

2.4. Purification of *pAkin2* fusion protein

For initial purification of recombinant *pAkin2* from the 50-mL shake flask culture, the strains were harvested by centrifugation at 5000 rpm for 10 min at 4 °C. The pellet fractions were dissolved with Ni-Denature-GuHCl buffer (100 mM NaH₂PO₄, 300 mM NaCl, 6 M GuHCl, pH 8.0) and centrifuged at 12,000 rpm for 20 min at 4 °C. Then the supernatants were filtrated with a 0.45-µm filter membrane and loaded onto a 1-mL pre-packed Ni-IDA column. The column was then equilibrated with 10 column volumes of Ni-Denature-urea buffer (100 mM NaH₂PO₄, 300 mM NaCl, 8 M urea, pH 8.0). The bound His-tagged fusion protein was eluted with 5 times the column volume of Ni-Native-250 buffer (100 mM NaH₂PO₄, 300 mM NaCl, 250 mM imidazole, 8 M urea, pH 8.0). The purity of the collected samples was verified by 12% SDS-PAGE followed by staining with Coomassie brilliant blue R250. The concentration of the protein was determined using the BCA protein assay kit (Pierce, Rockford, IL, USA).

2.5. In-gel digestion and MALDI-TOF-MS identification of recombinant *pAkin2*

The purified recombinant protein on Coomassie-blue-stained gel was sliced and plated into a 96-well microtiter plate. Excised slices were first destained twice with 60 µL of 50 mM NH₄HCO₃ and 50% acetonitrile, and then dried twice with 60 µL of acetonitrile. After that, the dried pieces of gel were incubated in ice-cold digestion solution (12.5 ng/µL trypsin and 20 mM NH₄HCO₃) for 20 min, and then transferred into a 37 °C incubator for digestion overnight. Finally, peptides in the supernatant were collected and subjected to tandem mass spectrometry (MS) analysis using the AB SCIEX MALDI-TOF/TOF 5800 Analyzer. All acquired spectra of samples were processed using TOF/TOF Explorer Software (AB SCIEX) in default mode. Data were searched using GPS Explorer (Version 3.6, Applied Biosystems, Foster City, CA, USA) with the Mascot search engine (Version 2.3).

2.6 EdU proliferation assay

The purified recombinant *pAkin2* protein was step-by-step dialyzed against 0.1 M of PBS (pH 7.4) containing 6, 4, 2, and 0 mol/L of urea at 4 °C for 24 h. Mouse C2C12 myoblasts (ATCC CRL-1772) were seeded in a 24-well plate at a density of 1×10^4 cells/well and cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen) at 37 °C in a 5% CO₂ humidified atmosphere. After 48 h, the medium was removed and replaced with DMEM/0.5% FBS supplemented with different concentrations (0–1 µg/mL) of refolded purified recombinant *pAkin2*. After 36 h of recombinant *pAkin2* treatment, proliferating C2C12 cells were evaluated using the Click-iT EdU Alexa Fluor 594 Imaging Kit (Invitrogen) following the manufacturer's

instructions. EdU (5-ethynyl-2'-deoxyuridine) is a thymidine nucleoside analog that is incorporated into DNA during active DNA synthesis only by proliferating cells. Data are presented as mean \pm SE. One-way ANOVA and Tukey's tests (SPSS Inc., Chicago, IL, USA) were performed to assess the statistical significance between treatments. Statistical significance was set at $P < 0.05$.

3. Results

3.1. Construction of the expression plasmid

For the construction of the pET-28a(+)-pAkirin2 recombinant plasmid, the *pAkirin2* gene from pcDNA3.1(+)-pAkirin2 plasmid was subcloned into the pET-28a(+) vector (Figure 1). The proper construction was confirmed by colony PCR and DNA sequencing. No mutation was found in the nucleotide sequence of the inserted fragment after sequencing (data not shown), indicating that the target fragment was successfully cloned into pET28a(+).

3.2. Expression of pAkirin2 fusion protein in different *E. coli* hosts

At the beginning, we tried to express the pAkirin2 protein in the *E. coli* BL21 (DE3) and *E. coli* BL21 (DE3) pLysS strains. We found that the *pAkirin2* gene cannot be induced to express in these 2 *E. coli* strains (Figure 2). Therefore, we analyzed the codon usage bias difference of *pAkirin2* gene by *E. coli* codon usage analyzer, and found that the mature

pAkirin2 contains 42 rare codons (Table). Hence, we used the modified BL21 (DE3) *E. coli*, named Rosetta (DE3), which was supplemented with 6 tRNAs for Leu, Arg, Pro, and Ile compared to the BL21 (DE3). After induction with IPTG for 5 h, the strains were harvested and the bacterial pellets were analyzed with 12% SDS-PAGE. As shown in Figure 2, the pAkirin2 was expressed at high levels in the Rosetta (DE3) strain. The molecular mass of the recombinant pAkirin2 was about 27 kDa (Figure 2), which was consistent with the size of the predicted pAkirin2 fusion protein (203 amino acid residues of pAkirin2 and 36 amino acid residues of N-terminal fusion segment contained a His-tag). Apparently, *E. coli* Rosetta (DE3) was much more efficient for the expression of pAkirin2, and thus was used for the optimization of expression conditions in the following experiments.

3.3. Effect of induction time on the expression of pAkirin2 fusion protein

The optimal induction time was determined by analyzing induced strain samples taken every 1 h until 6-h induction at 28 °C was achieved. The expression levels were analyzed by SDS-PAGE, and the results showed that the target fusion protein accounted for 19.1%, 21.5%, 27.3%, 29.5%, 31.6%, and 31.8% of total bacterial protein at 1-, 2-, 3-, 4-, 5-, and 6-h induction by adding 0.5 mM IPTG, respectively (Figure 3). Thus, the preferred induction time was set at 5 h.

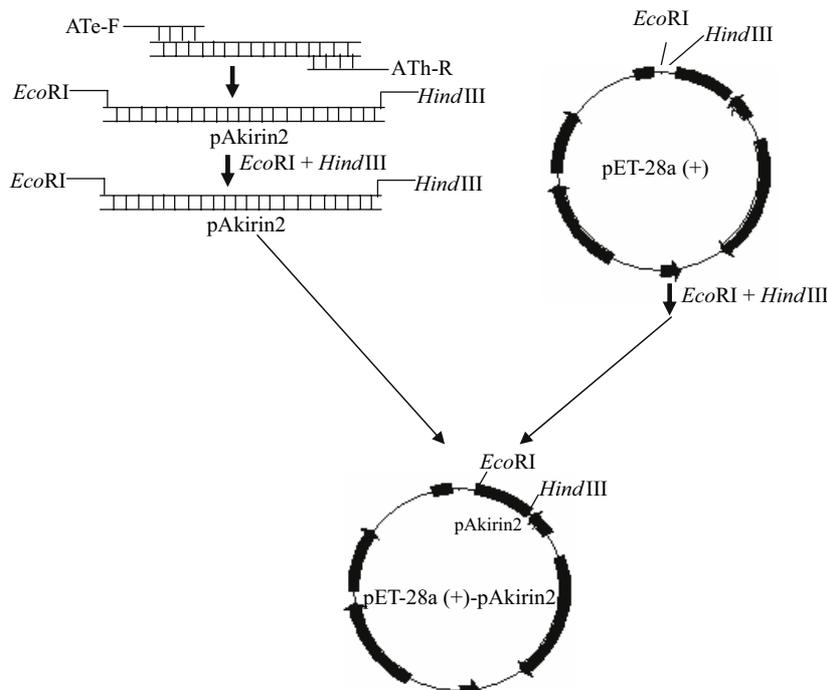


Figure 1. Schematic illustration of the construction of expression vector of pET-28a(+)-pAkirin2.

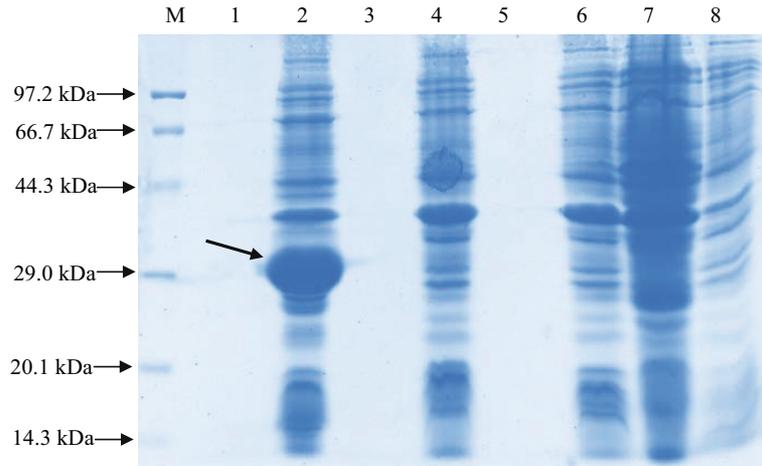


Figure 2. SDS-PAGE analysis of pAkin2 expression in different strains. Lane M: protein molecular mass marker. Lane 1: the supernatant of Rosetta (DE3) containing pET-28a(+)-pAkin2 induced with IPTG for 5 h; Lane 2: the precipitation of Rosetta (DE3) containing pET-28a(+)-pAkin2 induced with IPTG for 5 h; Lane 3: the supernatant of BL21 (DE3) pLySs containing pET-28a(+)-pAkin2 induced with IPTG for 5 h; Lane 4: the precipitation of BL21 (DE3) pLySs containing pET-28a(+)-pAkin2 induced with IPTG for 5 h; Lane 5: the supernatant of BL21 (DE3) containing pET-28a(+)-pAkin2 induced with IPTG for 5 h; Lane 6: the precipitation of BL21 (DE3) containing pET-28a(+)-pAkin2 induced with IPTG for 5 h; Lane 7: total proteins of pET-28a(+)-pAkin2 transformant without IPTG induction; Lane 8: control (-) pET-28a(+) induced for 5 h; the arrow indicates the target protein.

Table. Rare *E. coli* codons found in the mature pAkin2 gene by *E. coli* codon usage analyzer.

Rare <i>E. coli</i> codons	Amino acid	Residue no.	No. of rare codons in mature pAkin2 gene
UGC	Cys	3, 108	2
GCU	Ala	5, 36, 124, 179	4
ACU	Thr	6, 10, 100, 110, 128	5
AGG	Arg	9, 25	2
CGG	Arg	24, 146	2
CGA	Arg	26, 63, 164, 192, 193	5
AGA	Arg	95, 96	2
UGU	Cys	27, 109, 152	3
CCC	Pro	33, 68, 141	3
CCU	Pro	198	1
GUC	Val	72	1
AGU	Ser	101, 121, 200	3
GGA	Gly	4, 122, 195	3
GGG	Gly	127, 149	2
AUA	Ile	85, 170, 190	3
CUA	Leu	145	1
Total			42

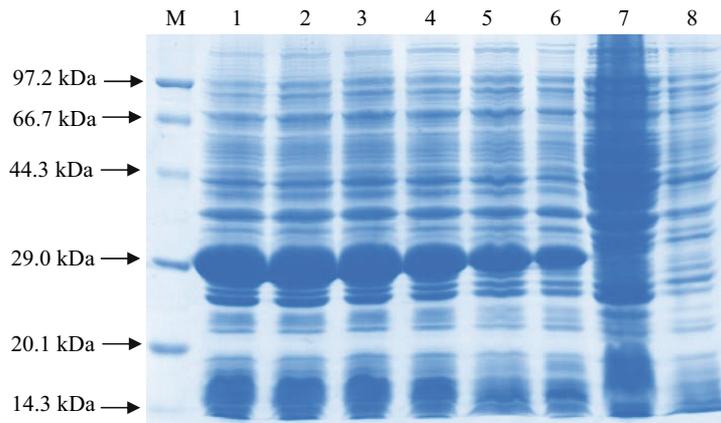


Figure 3. SDS-PAGE analysis of the target protein expression in different induction time at 28 °C. Lanes 1–7 are the corresponding induction time (6 h, 5 h, 4 h, 3 h, 2 h, 1 h, and 0 h); Lane 8: control (-) pET-28a(+).

3.4. Purification of the recombinant pAkirin2

The target fusion protein was purified from insoluble fraction through Ni²⁺-affinity chromatography. To elute the target fusion protein after the resin binding step, 250 mM imidazole elution buffer was used. The collected fractions were analyzed by SDS-PAGE, and a single homogeneous band was observed (Figure 4). The resulting purity of the fusion protein was greater than 90%, with a molecular mass of about 27 kDa as predicted, and approximately 4 mg/L of pure recombinant pAkirin2 was obtained. The highly purified recombinant pAkirin2 was used in the following studies.

3.5. Mass spectrometry analysis of recombinant pAkirin2

The MALDI-TOF mass spectrum of tryptic digestion of the gel band of pAkirin2 is shown in Figure 5. The acquired peptide masses were searched using the Mascot search engine with a peptide mass tolerance of 100 ppm. Eight independent peptide fragments matched the predicted sequence for a 239 amino acid protein, and the sequence coverage was 44%. These results confirmed that the enzymatic hydrolysis of peptides belonged to the recombinant pAkirin2.

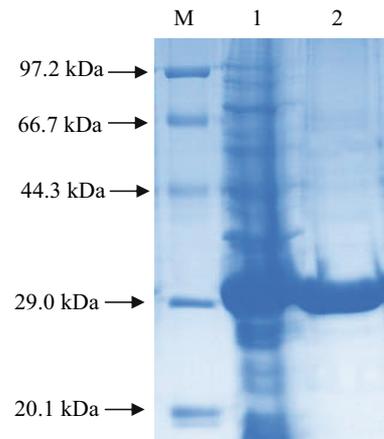


Figure 4. Affinity chromatographic purification of recombinant pAkirin2. Recombinant pAkirin2 was purified by Ni-IDA column and the purified protein was separated on SDS-PAGE and stained with Coomassie blue R250. Lane M: protein molecular mass marker; Lane 1: the precipitation of Rosetta (DE3) containing pET-28a(+)-pAkirin2 induced with IPTG; Lane 2: the purified pAkirin2 fusion protein.

MGSSHHHHHSSGLVPRGSHMASMTGGQQMGRGSEFMACGATLKRTLDFD
PLLNPASPKRRRCAPLSAPTSAAASPSSAAAATAASFSAASPQKYLRM
EPSPFQDVSSRLTTEQILYNIKQEYKRMQKRRHLETSFQQTDPCCSDAQ
 PHAFLLSGPASPGTPSATSSPLKKEQPLFTLRQVGMICERLLKEREKVR
EEYEEILNTKLAEQYDAFVKFTHDQIMRRYGEQPASYVS

Figure 5. MALDI-TOF mass spectrum of the trypsin-digested recombinant pAkirin2 protein. The tryptic peptides obtained by mass spectrometry are underlined.

3.6. Effect of recombinant pAkirin2 on myoblast proliferation

To investigate the effect of recombinant pAkirin2 on myoblast proliferation, C2C12 cells were supplemented with different concentrations of refolded purified recombinant pAkirin2, and EdU incorporation experiments were performed to assess its proliferation. As shown in Figure 6, the refolded purified recombinant pAkirin2 significantly promoted C2C12 myoblast proliferation ($P < 0.001$) when compared with the control group.

4. Discussion

Compared with the yeast expression system, the T7-based pET expression system is by far the most commonly used system in the production of recombinant proteins because of its fast growth rate, high yield target protein, easy purification procedure, and relatively low cost. In the present study, the *pAkirin2* gene was amplified and successfully expressed in *E. coli*. However, our results showed that the pAkirin2 was expressed mainly as insoluble inclusion bodies in *E. coli*.

It was observed that pAkirin2 fusion protein was not expressed in *E. coli* BL21 (DE3) and *E. coli* BL21 (DE3) pLySs, which may be due to the rare codons. Results from sequence analyses using an *E. coli* codon usage analyzer (Table) showed that the mature pAkirin2 gene contains 42 rare codons (20.69% of the total of 203 codons), 17 of which include UGC, GCU, ACU, AGG, CGG, CGA, UGU, CCC, GUC, AGA, AGU, GGA, GGG, CCU, AUA,

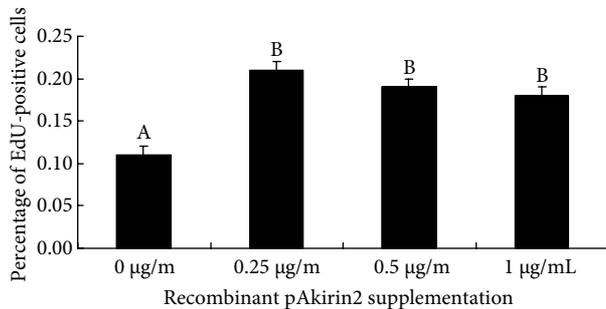


Figure 6. Effect of recombinant pAkirin2 on myoblast proliferation. C2C12 cells were cultured in DMEM/10% FBS medium for 48 h and subjected to treatment in DMEM/0.5% FBS media by adding 0 µg/mL (control), 0.25 µg/mL, 0.5 µg/mL, or 1 µg/mL of recombinant pAkirin2. Cell proliferation was evaluated by EdU proliferation assay after 36 h of recombinant pAkirin2 treatment. The percentage of EdU-positive C2C12 cells were quantified. Results are presented as mean ± SE ($n = 6$). Bar with different letter indicates significant difference ($P < 0.001$).

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CUA, and ACU, a feature that could lead to the dramatic reduction of target protein expression. Rosetta (DE3) host strain supplied tRNAs for 6 rare codons (AGG, AGA, AUA, CUA, CCC, and GGA) on a compatible chloramphenicol-resistant plasmid (Grosjean and Fiers, 1982), and it has the ability to increase the expression of eukaryotic proteins that contain the rare codons used in *E. coli* (Kane, 1995; Yin et al., 2007). In order to improve the yield of recombinant pAkirin2 protein, we selected Rosetta (DE3) as the expression host strain, which harbored 6 of the rare tRNAs (underlined) from the pAkirin2. In this study, the pAkirin2 fusion protein was successfully expressed when *E. coli* Rosetta (DE3) was applied as the host, providing an easy and inexpensive way to improve the expression level of heterologous proteins with rare codons in *E. coli*.

After systematic optimization of the expression conditions, the expression level of recombinant pAkirin2 was greatly improved when the culture was effectively induced with 0.5 mM IPTG for 5 h at 28 °C. The fusion protein was purified efficiently by Ni-IDA affinity chromatography with greater than 90% purity. The purified recombinant pAkirin2 was confirmed by MALDI-TOF/TOF mass spectrometer analysis.

Skeletal myogenesis is a complex and tightly regulated process that commences with the commitment of multipotent precursor cells to myoblasts, followed by proliferation, irreversible withdrawal from the cell cycle, differentiation, and fusion into multinuclear myotubes and then myofibers (Buckingham, 2001, 2006). It is well established that Akirin2 plays an important role in skeletal myogenesis (Macqueen and Johnston, 2009; Macqueen et al., 2010; Chen et al., 2013). In this study, we showed that the refolded purified recombinant pAkirin2 promoted the proliferation of C2C12 cells. Our data suggested that the refolded purified recombinant pAkirin2 was active.

In summary, we successfully expressed *pAkirin2* gene in *E. coli*. The recombinant pAkirin2 protein was purified by Ni-IDA affinity chromatography and identified by using MALDI-TOF/TOF mass spectrometer analysis. The expression, purification, and refolding procedures in the present study provide a simple and efficient method for yielding pure active recombinant pAkirin2 protein.

Acknowledgments

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