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Prokaryotic expression and purification of porcine Sox6

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Abstract: Sox6, a member of the Sox (*Sry*-related HMG box) family of transcription factors, plays an important role in embryonic muscle development and adult fast myofiber maintenance. Here we report the expression and purification of a His-tagged version of recombinant porcine Sox6 (pSox6) in *Escherichia coli*. The recombinant pSox6 was expressed as a C-terminal fusion protein with His6 tag in the *E. coli* Rosetta (DE3) host strain. The protein was purified by Ni affinity chromatography, yielding approximately 5 μg/mL. The protein was identified by western blot analysis and confirmed by matrix-assisted laser desorption/ionization time-of-flight/time-of-flight (MALDI-TOF/TOF) mass spectrometer analysis. In vitro biological activity assay demonstrated that the refolded purified recombinant pSox6 downregulated *MyHC I*, *Tnnt1*, *Tnnc1*, and *Tnni1* mRNA expressions in porcine myotubes, suggesting that it was active. The present study provides a reliable technique for the recombinant expression and purification of pSox6 protein.

Key words: Porcine Sox6, expression and purification, *Escherichia coli*, identification, in vitro assay

1. Introduction

Domesticated pigs are raised throughout the world as a meat production animal and pork is the world's most popular meat protein. At present, consumers are looking for high-quality pork and pork products. Unfortunately, the long-term selection for high growth rate and lean percentage led to a significant decline in pork quality. Thus, it is necessary to identify candidate genes associated with the quality of pork.

Sox6, a member of the Sox [*Sry*-related high mobility group (HMG) box] family of transcription factors, was first isolated from an adult mouse testis cDNA library (Connor et al., 1995). Sox6 is expressed at a significantly higher level in fast-twitch muscles than in slow-twitch muscles (Cohen-Barak et al., 2001; Hagiwara et al., 2005). In Sox6 mutant mice skeletal muscle, slow-twitch fiber isoform genes are expressed at significantly higher levels, whereas fast-twitch fiber isoform genes are expressed at distinctly lower levels than in the wild-type (Hagiwara et al., 2005). In Sox6 null-*p100H* mutant mice, all fetal muscle fibers have slow-twitch fiber characteristics, and the slow-twitch myosin heavy chain is increasingly expressed (Hagiwara et al., 2007). The knockdown of Sox6 in skeletal muscle causes a dramatic increase in slow-twitch fibers and a significant decrease in fast-twitch fibers in fetal as well as in adult mice (Hagiwara et al., 2007; An et al., 2011; Quiat et al., 2011). These findings indicate that Sox6 plays a critical role in embryonic muscle development and adult fast myofiber maintenance. It is well established that muscle fiber characteristics are closely related to meat quality (Henckel et al., 1997; Maltin et al., 1997; Joo et al., 2013). Therefore, Sox6 still needs to be studied further as a candidate gene that may contribute to pork quality traits.

In our previous study, we reported the cloning and functional characterization of porcine Sox6 (pSox6) (Wen et al., 2016). In the present study, the full-length *pSox6* gene was subcloned into the prokaryotic expression vector pET-30a(+). Its expression in *Escherichia coli*, purification, and effect on expressions of slow fiber-specific genes in porcine myotubes were also investigated.

2. Materials and methods

2.1. Materials

E. coli DH5α (Tiangen Biotech, Beijing, China) was used for routine plasmid amplification and *E. coli* Rosetta (DE3) (Tiangen Biotech) was used for protein expression. The

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pET-30a(+) plasmid kept by our laboratory was used to construct the expression vector. LA Taq DNA polymerase, restriction enzymes, and DNA ligation kit were purchased from TaKaRa (Dalian, China). Kanamycin, isopropyl β-D-thiogalactopyranoside (IPTG), and UNIQ-10 Spin column DNA gel extraction kit were purchased from Sangon Corporation (Shanghai, China). The HisTrap HP column (1 mL, 7 × 25 mm) prepacked with Ni Sepharose High Performance Resin was from GE Healthcare Bio-Sciences Corporation (Pittsburgh, PA, USA). The protein molecular weight marker (low) was from Tiangen Biotech. All other reagents were of analytical grade.

2.2. Construction of the expression plasmid

For the expression of pSox6 in *E. coli*, the entire open reading frame (ORF) of *pSox6* was PCR amplified using specific primers (pET-30a(+)-pSox6-F: 5'-CCC AATTCCATATGTCTTCCAAGCAAGCCACCTCTC-3' and pET-30a(+)-pSox6-R: 5'-AAACTCGAGGTTGG CACTGACAGCCTCTGG-3') and the plasmid pMD19-T-pSox6 (Wen et al., 2016) as a template. Primer pET-30a(+)-pSox6-F introduced an *Nde*I site (underlined), and primer pET-30a(+)-pSox6-R contained an *Xho*I site (underlined). After digestion with *Nde*I and *Xho*I, the PCR product was cloned into the pET-30(+) plasmid. The resulting recombinant plasmid was transformed into *E. coli* DH5α. Proper construction was confirmed by restriction endonuclease analysis and DNA sequencing and was defined as pET-30(+)-pSox6. Theoretically, the pET-30(+)-pSox6 vector can express a fusion protein corresponding to pSox6 carrying the extra C-terminal sequence LEHHHHHH.

2.3. Expression of recombinant pSox6

The pSox6 fusion protein was expressed in *E. coli* Rosetta (DE3) transformed with pET-30(+)-pSox6. The transformants were cultured in Luria-Bertani (LB) medium containing kanamycin (50 μg/mL) overnight at 37 °C with rotary shaking at 250 rpm. The resulting seed culture, diluted 1:100, was used to inoculate 15 mL of fresh LB medium containing kanamycin (50 μg/mL) in a 50-mL flask. Strains were grown at 37 °C with rotary shaking at 280 rpm to an OD_{600} of 0.4–0.6, and protein expression was subsequently induced at 30 °C by the addition of IPTG (1 mmol/L) for different times (0, 1, 3, 4, 5 , 6, and 8 h). One milliliter of the bacterial culture was then harvested and centrifuged at 8000 rpm for 5 min at 4 °C. The bacterial pellets were resuspended in 50 μL of 1X SDS reducing sample buffer (20 mM Tris-HCl (pH 6.8), 1% SDS, 5% glycerol, 2.5% beta-mercaptoethanol, and 0.05% bromophenol blue) and boiled for 5 min before loading. Equivalent amounts of the denatured samples were subjected to 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) followed by staining with Coomassie Brilliant Blue R-250.

2.4. Solubility testing

E. coli Rosetta (DE3) harboring pET-30(+)-pSox6 was grown in 50 mL of LB medium containing kanamycin (50 μg/mL) at 37 °C with rotary shaking at 280 rpm until OD_{600} reached 0.4–0.6, at which point IPTG was added to a final concentration of 1 mM and the culture temperature was reduced to 30 °C. After 5 h, the induced cells were harvested by centrifugation at 8000 rpm for 5 min at 4 °C, resuspended in 8 mL of cell lysis buffer (50 mM Tris-HCl (pH 8.0), 0.1% Triton X-100, 5 mM EDTA, 1 mM phenylmethanesulfonyl fluoride (PMSF)), and disrupted by mild sonication on ice. After centrifugation at 12,000 rpm for 20 min at 4 °C, the supernatant (soluble fraction) and pellet (insoluble fraction) were collected and analyzed on 12% SDS-PAGE followed by Coomassie Brilliant Blue R-250 staining.

2.5. Purification of recombinant pSox6

The *E. coli* pellet obtained from a 50-mL shake flask was resuspended in 3 mL of Ni-Denature-GuHCl buffer (100 mM NaH_2PO_4, 300 mM NaCl, 6 M GuHCl, pH 8.0), followed by centrifugation at 12,000 rpm for 20 min at 4 °C. The resulting supernatant was filtered through a 0.45-μm membrane filter and loaded onto a 1-mL HisTrap HP column prepacked with Ni Sepharose High Performance Resin. The HisTrap HP affinity column was equilibrated with 10 column volumes of binding buffer (20 mM sodium phosphate, 0.5 M NaCl, 8 M urea, 20 mM imidazole, 0.2% Triton X-100, pH 7.4). Fifteen column volumes of binding buffer were applied to wash unbound impurities, followed by elution with 5 column volumes of elution buffer (20 mM sodium phosphate, 0.5 M NaCl, 8 M urea, 500 mM imidazole, 0.2% Triton X-100, pH 7.4) at 1 mL/min flow rate. The purity of the collected samples was verified with 12% SDS-PAGE, followed by Coomassie Brilliant Blue R-250 staining. The protein concentration was determined using the BCA protein assay kit (Pierce, Rockford, IL, USA).

2.6. Protein identification by MALDI-TOF/TOF mass spectrometer analysis

In-gel digestion of the gel bands was done as described by Chen et al. (2014). The purified recombinant protein on Coomassie Blue-stained gel was sliced from the gel and destained with 50 mM NH_4HCO_3 in 50% acetonitrile. The dried gel pieces were digested with sequencing-grade modified trypsin (Promega, Madison, WI, USA) and subjected to matrix-assisted laser desorption/ionization time-of-flight/time-of-flight (MALDI-TOF/TOF) mass spectrometer analysis using an AB SCIEX MALDI-TOF/TOF 5800 Analyzer (AB SCIEX, Foster City, CA, USA). The sample spectra acquired was processed using TOF/TOF Explorer Software (AB SCIEX) in default mode. The result was searched using GPS Explorer (Version 3.6, Applied Biosystems, Foster City, CA, USA) with the Mascot search engine (Version 2.3).

2.7. Western blot analysis

Western blot analysis was performed as previously described by Huang et al. (2007), with minor modifications. The proteins were subjected to 12% SDS-PAGE, transferred to nitrocellulose membranes, and incubated overnight at 4 °C with the monoclonal anti-His (C-term) antibody (Invitrogen, Carlsbad, CA, USA) (1:2000). Bolts were then incubated with horseradish peroxidase-conjugated secondary antibodies (1:5000) for 1 h at room temperature. The signals were visualized with SuperSignal West Pico Chemiluminescent Substrate (Pierce) and a ChemiDoc XRS Imager System (Bio-Rad, Hercules, CA, USA).

2.8. Cell culture

Porcine myoblasts isolated from the longissimus lumborum muscle of 3-day-old male Duroc × Landrace × Yorkshire pigs (Yang et al., 2014) were maintained in Dulbecco modified Eagle medium (DMEM) + Ham's F-12 (1:1) medium (Invitrogen) supplemented with 20% fetal bovine serum (Invitrogen) and antibiotics (100 U/mL penicillin and 100 µg/L streptomycin; Invitrogen) at 37 °C and 5% CO_2 in a saturated humid atmosphere. To induce differentiation, the medium was replaced with a medium consisting of DMEM + Ham's F-12 (1:1) with 2% horse serum when the cells reached approximately 80% confluence. The medium was then renewed every 24 h.

2.9. Real-time quantitative PCR

Total RNA was extracted from the adherent cultured cells using TRIzol reagent (Invitrogen) according to the manufacturer's directions. The total RNA concentration was determined spectrophotometrically using a Beckman Coulter DU 730 spectrophotometer (Beckman Coulter, Fullerton, CA, USA). cDNA was synthesized with 1 µg of total RNA from each sample using a PrimeScript RT Reagent Kit with gDNA Eraser (TaKaRa) according to the manufacturer's instructions. Real-time quantitative PCR was done in a 7900HT real-time PCR system (384-cell standard block; Applied Biosystems) using SYBR Select Master Mix (Applied Biosystems) in a final volume of 10 µL. The gene specific primers used were as follows: porcine *MyHC I* (forward) 5'-CGTGGACTACAACATCATAGGC-3' and (reverse) 5'-CCTTCTCAACAGGTGTGTCG-3'; porcine *Tnnc1* (forward) 5'-GGCACAGTGGACTTCGATGA-3' and (reverse) 5'-CTCTGTGATGGTCTCGCCTG-3'; porcine *Tnnt1* (forward) 5'-GCAGAGAGAGC TGAGCAACA-3' and (reverse) 5'-CTTCTCCTCCGCCAGCTTAG-3'; porcine *Tnni1* (forward) 5'-CCTGCTGGGCTCTAAACACA-3' and (reverse) 5′-TGGCCTCGACGTT CTTTCTC-3′; porcine *β-actin* (forward) 5'-CCACGAAACTACCTTCAACTCC-3' and (reverse) 5'-GTGATCTCCTTCTGCATCCTGT-3'. The PCR cycling conditions used were 45 cycles at 95 °C for 15 s and 60 °C for 30 s. A single sharp peak was detected in the melting curve. Relative expression of the target gene was calculated using the comparative Ct method (Livak and Schmittgen, 2001) with *β-actin* as an endogenous control.

2.10. Statistical analysis

Data are presented as mean ± SE. One-way ANOVA followed by Tukey's tests (SPSS Inc., Chicago, IL, USA) was 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-30a(+)-pSox6 plasmid, the *pSox6* gene from the pMD19-T-pSox6 plasmid was subcloned into the pET-30a(+) plasmid. The recombinant plasmid construction is illustrated in Figure 1. The proper construction was confirmed by restriction endonuclease analysis (data not shown) as well as DNA sequencing. No mutation was found in the nucleotide sequence of the inserted fragment after DNA sequencing (data not shown), suggesting that the target fragment was successfully cloned into pET30a(+).

3.2. Expression of recombinant pSox6

As shown in Figure 2, a clear protein band of about 90 kDa (the addition of target protein 88.8 kDa and histidine marker 1.1 kDa) was observed from cell lysates of *E. coli* Rosetta (DE3) harboring pET-30(+)-pSox6, which was consistent with the theoretical value. The target protein, as determined from the SDS-polyacrylamide gel image, was approximately 22.4%, 26.1%, 33.8%, 34.6%, 32.9%, and 30.7% of the total cellular protein at 1, 3, 4, 5, 6, and

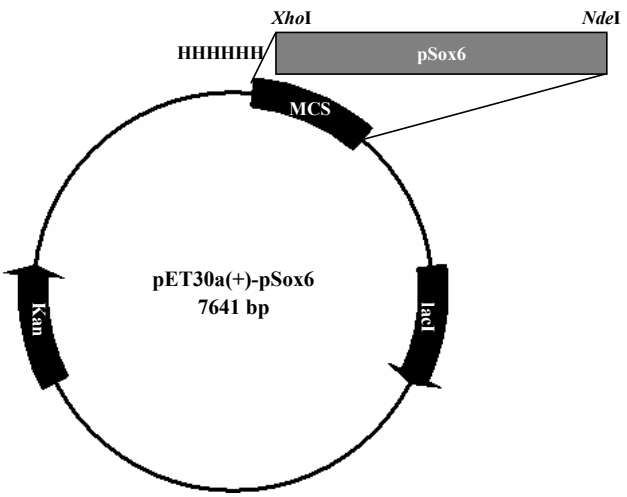


Figure 1. Map of the pET30a(+)-pSox6 expression vector. The *pSox6* gene was cloned into the pET30a(+) vector between *Nde*I and *Xho*I restriction sites. This vector allows C-terminal fusion of recombinant protein with a His6 tag and contains a kanamycin-resistant gene under the control of a T7 promoter.

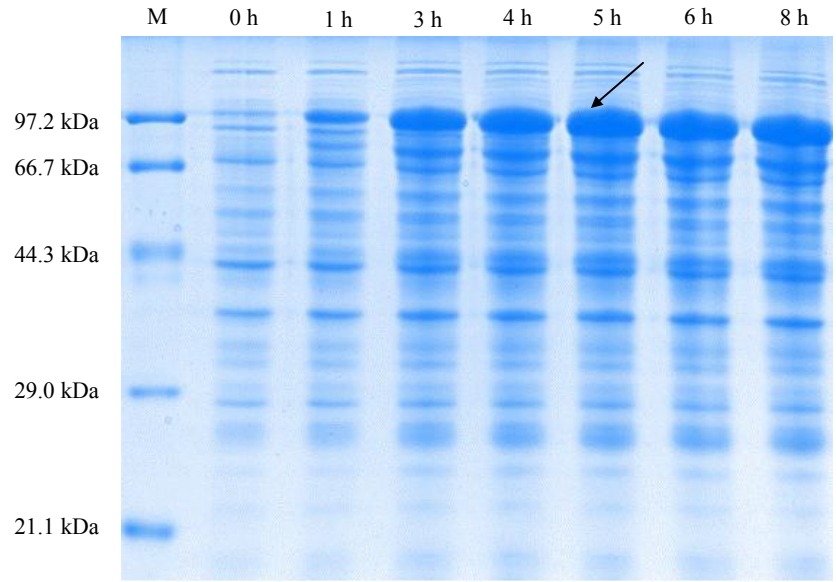


Figure 2. SDS-PAGE analysis of pSox6 fusion proteins in *E. coli* Rosetta (DE3) at different induction times. The bacterial culture harboring the pET30a(+)-pSox6 expression plasmid was induced in 1 mM IPTG and incubated for 0, 1, 3, 4, 5, 6, and 8 h at 30 °C, respectively. M: Protein size markers. The arrow indicates the position of the fusion protein.

8 h of induction by adding 1 mM IPTG, respectively. In subsequent studies the induction time used was 5 h.

3.3. Solubility testing

As shown in Figure 3, the target protein existed in the supernatant, which means it was expressed in a soluble form.

3.4. Purification and identification of recombinant pSox6

As shown in Figure 4A, a protein band at about 90 kDa was observed. The purity of the recombinant protein was greater than 90% and about 5 mg of the recombinant protein was purified from 1 L of culture medium by one-step affinity chromatography. Western blot analysis revealed that the purified protein was a His-tagged fusion protein because it was specifically recognized by the monoclonal anti-His (C-term) antibody (Figure 4B). To further identify the His-tagged fusion protein as recombinant porcine Sox6, the purified protein was digested with trypsin and the resulting peptides were analyzed by MALDI-TOF/TOF mass spectrometer analysis. As shown in Figure 4C, a clear match between the mass of 11 generated peptides and the peptides derived from porcine Sox6 amino acids sequence was observed.

3.5. In vitro assay

As shown in Figure 5, compared with the control, the refolded purified recombinant pSox6 significantly downregulated the expression of *MyHC I*, *Tnnt1*, *Tnnc1*, and *Tnni1* mRNA ($P < 0.001$).

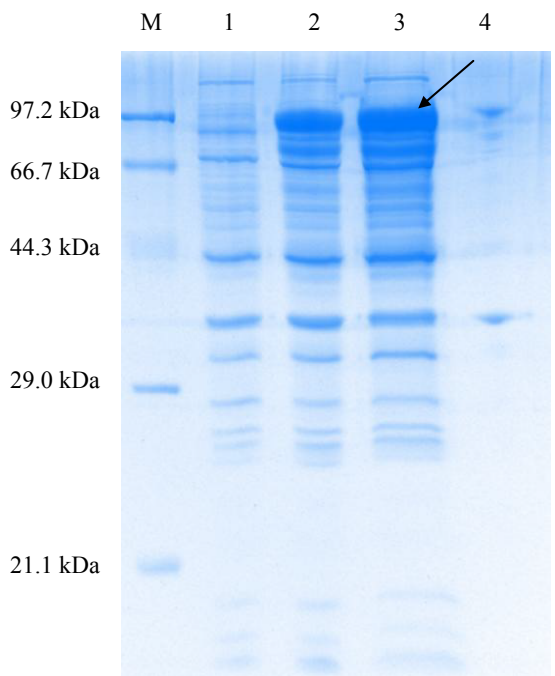
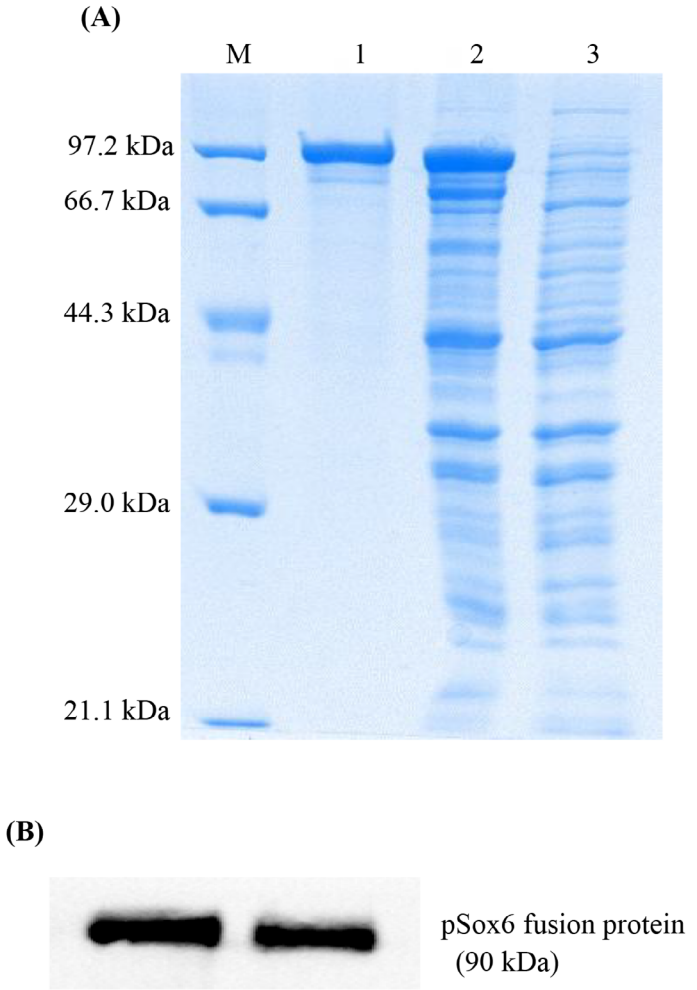


Figure 3. Solubility analysis of recombinant pSox6. M: Protein size markers; lane 1: total protein of the bacterial pellets without IPTG induction; lane 2: total protein of bacterial pellets with IPTG induction; lane 3: soluble fraction after cell disruption by sonication; lane 4: insoluble fraction (inclusion bodies) after cell disruption by sonication. The arrow indicates the position of the fusion protein.



(C)

MSSKQATSPFACAADGEEAMTQDLTSREKEEGSDQHVASHLPLHPIMHNKPHSEELPTLVSTIQQDAD
WDSVLSSQQRMGSENNK**LCSLYSFR**NTSTSPHKPDEGSRDREIMTSVTFGTPERR**KGSLADVVDTLK**Q
K**KLEEMTR**TEQEDSSCMEKLLSKDWKEKMERLNTSELLGEIKGTPESLAEKER**QLSTMITQLISLR**EQ
LLAAHDEQKKLAASQIEKQR**QQMDLARQQQEQIAR**QQQQLLQQQHKINLLQQQIQVQGHMPPLMIPIF
PHDQRTLAAAAAAQQGFLFPPGITYKPGDNYPVQFIPSTMAAAAASGLSPLQLQQLYAAQLASMQVSP
GAKMPSTPQPPNTAGAVSPTGIKNEKRGTSPVTQVKDEAAAQPLNLSSRPKTAEPVKSPTSPTQTLFP
ASKTSPVSLPNK**SSIPSPIGGSLGR**GSSLGKWKGQHQEETYELDILSSLNSPALFGDQDTVMKAIQEA
RKMREQIQREQQQQQPHGVDGK**LSTMNNMGLNNCR**NEKER**TRFENLGPQLTGK**SSEDGKLGPGVIDLT
RPEDAEGSKAMNGSAAKLQQYYCWPTGGATVAEARVYRDARGRASSEPHIK**RPMNAFMVWAK**DERRKI
LQAFPDMHNSNISKILGSRWKSMSNQEK**QPYYEEQAR**LSKIHLEKYPNHKYKPRPKRTCIVDGKKLRI
GEYKQLMRSR**RQEMR**QFFTVGQQPQIPITTGTGVVYPGAITMATTTPSPQMTSDCSSTSASPEPSLPV
IQSTYGMKTDSGSLAGNEMINGEDEMEMYDDYEDDPKSDYSSENEAPEAVSANLEHHHHHH

Figure 4. Purification and identification of the recombinant pSox6. (A) Affinity chromatographic purification of pSox6 fusion protein. Lane M: Protein size markers; lane 1: purified pSox6 fusion protein; lane 2: total protein of the bacterial pellets with IPTG induction; lane 3: total protein of the bacterial pellets without IPTG induction. (B) Purified pSox6 fusion protein was detected by western blot analysis using monoclonal anti-his (C-term) antibody. (C) MALDI-TOF/TOF mass spectrometer analysis of the trypsin-digested purified pSox6 fusion protein. The tryptic peptides obtained by mass spectrometry are bold red and underlined.

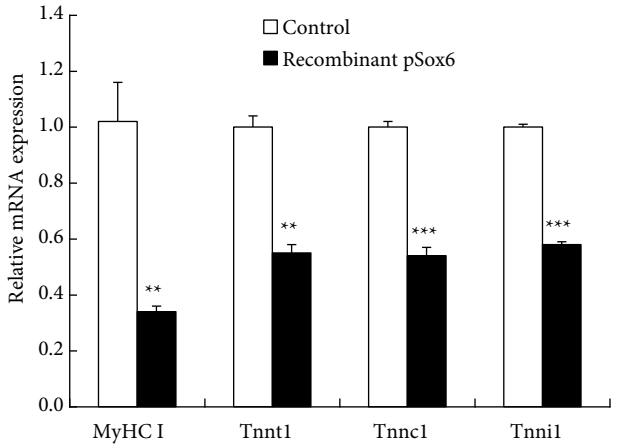


Figure 5. Effect of recombinant pSox6 on expressions of slow fiber specific genes in porcine myotubes. Approximately 80% confluent porcine myoblasts were cultured in differentiation medium (DMEM/F12, 2% horse serum) for 24 h and then stimulated with 0.5 μg/mL recombinant pSox6 for another 72 h. The mRNA levels of *MyHC I*, *Tnnt1*, *Tnnc1*, and *Tnni1* were determined using real-time quantitative PCR. Samples were performed in duplicate. The amounts of *MyHC I*, *Tnnt1*, *Tnnc1*, and *Tnni1* mRNA were normalized to the amount of *β-actin* mRNA. Data are presented as means ± SE (n = 3). **P < 0.01 and ***P < 0.001 as compared with control.

4. Discussion

In the present study, the pET-30a(+) vector is used for T7 promoter-driven expression of the pSox6 protein, and *E. coli* is used as the host for pSox6 protein expression because the *E. coli* and T7 promoter-driven expression system has the characteristics of fast growth rate, high yield of target protein, and easy operation and purification procedure (Chen et al., 2014). The *pSox6* gene was successfully cloned into the pET30a(+) vector to construct a recombinant expression plasmid containing a His6 tag fusion partner in the C-terminal, which would be useful for purification of target protein (Esposito and Chatterjee, 2006).

In this study, the recombinant pSox6 yield was significantly improved through optimization of the induction conditions with 1 mM IPTG for 5 h at 30 °C. Solubility testing showed that the fusion protein was predominantly expressed in a soluble form. However, when we tried to purify the target protein in nondenaturation conditions, the target protein could not bond to the affinity column and could not be collected (data not shown), which may be due to an insufficient exposure to the His6 tag. Thus, we purified the pSox6 protein by resolubilizing it with strong denaturants of 6 M guanidine hydrochloride and 8 M urea (Middelberg, 2002). The purified pSox6 protein was then identified by western blot analysis using the monoclonal anti-His (C-term) antibody and confirmed by MALDI-TOF/TOF mass spectrometer analysis, suggesting that the purified recombinant protein was the pSox6 protein.

Mammalian skeletal muscle comprises muscle fibers that are generally classified as fast- or slow-twitch subtypes (Schiaffino and Reggiani, 2011). The formation of different muscle fiber types requires the expression of specific isoforms of sarcomeric proteins, such as myosin heavy chain (MyHC) and troponins (von Hofsten et al., 2008). MyHC I is a slow-twitch MyHC isoform, while Tnnt1, Tnnc1, and Tnni1 are isoforms of slow-twitch troponins (Gan et al., 2013). Sox6 has been well defined as a suppressor of slow-twitch fiber specific genes (Hagiwara et al., 2005, 2007; Quiat et al., 2011). In order to determine whether the recombinant pSox6 was functionally active, we evaluated the effect of recombinant pSox6 on expressions of slow fiber-specific genes in porcine myotubes. Here we showed that the refolded purified recombinant pSox6 decreased the expression levels of *MyHC I*, *Tnnt1*, *Tnnc1*, and *Tnni1*, indicating that the refolded purified recombinant pSox6 was active. Consistent with this, overexpression of pSox6 also led to downregulation of *MyHC I* expression (Wen et al., 2016). Taken together, these findings suggest that pSox6 may play an important role in fast myofiber maintenance in pigs.

In conclusion, we expressed the *pSox6* gene in *E. coli* for the first time. The recombinant pSox6 was purified by Ni affinity chromatography and identified by western blot and MALDI-TOF/TOF mass spectrometer analysis. The expression, purification, and refolding procedures in this study provide a simple and efficient method for yielding sufficient quantities of pure active recombinant pSox6. In the future, it will be interesting to investigate the effect of active immunization against pSox6 on meat quality in pigs.

Acknowledgments

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