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Molecular cloning and expression of the porcine *S14R* gene in *Escherichia coli*

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ABSTRACT. We amplified S14R protein gene cDNA of porcine, cloned it into a prokaryotic expression plasmid, and expressed it in *Escherichia coli*. A pair of primers was designed based on the cDNA sequence of the porcine *S14R* gene in GenBank. The target gene fragment from porcine liver tissue was amplified by RT-PCR. Confirmed by auto-sequencing, the target gene fragment was subcloned into an expression vector of pET28a. The pET28a-*S14R* construct was subsequently transformed into *E. coli* BL21 (DE3). This construct was verified by restriction endonuclease digestion and sequencing. Using isopropyl β-D-1-thiogalactopyranoside induction, a new recombinant protein with the expected relative molecular mass of 24 kDa appeared. The result was identified by SDS-PAGE electrophoresis. Porcine *S14R* includes 549bp (GenBank No. JN793537), with an open reading frame of 549 bp coding 182 amino acids.

Key words: *MID1IP1*; Gene cloning; Prokaryotic expression; Porcine

INTRODUCTION

The thyroid hormone-responsive spot14 (THRSP) (or Spot14, S14) responds to thyroid hormone stimulation (Seelig et al., 1981; Liaw and Towle, 1984; Jump et al., 1984). S14 is a small acidic nuclear protein that is a transcription factor involved in the transcriptional control of lipogenic enzymes (Brown et al., 1997). Some publications have reported that S14 plays important roles in the regulation of lipogenesis (Kinlaw et al., 1995; Zhu et al., 2001, 2005). The S14-related gene (*S14R*) is a paralog of *S14* (Tsatsos et al., 2008). In 2004, using a yeast two-hybrid screening approach, Berti et al. identified the *S14R* gene, whose protein product interacted with midline1 (Mid1) to stabilize microtubules during embryonic development. *S14R* has therefore also been called Mid1-interacting G12-like protein (*MIG12*); however, its official name is now Mid1 interacting protein (*MID1IP1*). *MID1IP1* is located on the X chromosome (Zhu et al., 2005), but *S14* on autosomal chromosomes (Grillasca et al., 1997). MID1IP1 is a cytoplasmic protein (Kim et al., 2010), while S14 is a nuclear protein. In addition, *S14* is predominately expressed in liver, adipose tissues and lactating mammary gland (Jump and Oppenheimer, 1985; Freake and Oppenheimer, 1987), while *MID1IP1* is widely expressed in multiple tissues but not in mammary gland. Just like with *S14* function, recent research (Aipoalani et al., 2010) has demonstrated that *MID1IP1* has essential roles in the regulation of lipogenesis.

Lipid metabolism is very important for the improvement of meat quality. Pork is an important food, and its quality directly correlates with human health concerns, such as obesity and cardiovascular disease. As an important factor in regulating lipid metabolism, *MID1IP1* has previously been studied, but the function of porcine *MID1IP1* requires further investigation. In this study, we extracted total RNA from porcine liver and cloned an *MID1IP1* cDNA by RT-PCR. We then cloned *MID1IP1* into a prokaryotic expression vector, pET28a, and expressed *MID1IP1* in *Escherichia coli* at high levels. This provides a basis for studying the function of porcine *MID1IP1*.

MATERIAL AND METHODS

Animal and sample collection

Four pigs (averaging 45 kg) of the landrace were collected from Yongkang pig breeding farm in Kaifeng of Henan Province. Liver tissue obtained from pigs was immediately dissected, frozen in liquid nitrogen, and stored at -80°C.

Total RNA isolation and cDNA synthesis

Total RNA was isolated from frozen porcine liver tissue using Trizol (Gibco-BRL, Germany) according to the manufacturer instructions. All extracted RNA samples were dissolved in RNase-free water. The purity and quantity of total RNA were measured with an ultraviolet/visible spectrophotometer (Nanodrop 2000/2000C, USA). First strand cDNA was synthesized using MMLV reverse transcriptase (Promega, Madison, WI, USA), ~3 µg RNA, and an oligo $(dT)_{18}$ primer.

Amplification of the *MID1IP1* gene by RT-PCR

The primers were designed on the basis of the human and rat *MID1IP1* mRNA (NM_001098791.1 and NM_206950.1) published in GenBank: forward primer (containing *Eco*RI restriction enzyme site), 5'-CGGAATTCATGATGCAAATTTGCGACAC-3', and reverse primer (containing *Hin*dIII restriction enzyme site), 5'-CCCAAGCTTTCAGTGCCC CCAATTGCTG-3'. RT-PCR was performed using the cDNA above. The 25-µL reaction solution contained 1.0 µL cDNA, 1.0 µL 20 pM forward primer, 1.0 µL 20 pM reverse primer, 12.5 µL 2X Master mix (Shanghai Wonhon Biotechnology Company), and 9.5 µL sterile water. The PCR reaction was started with pre-denaturation at 94°C for 4 min, followed by 30 cycles of denaturation at 94°C for 40 s, annealing at 58°C for 40 s and extension at 72°C for 50 s, completed with a final extension at 72°C for 10 min. Ten microliters of PCR reaction were electrophoresed on a 1.0% agarose gel. The DNA band of the expected size was purified using a QIAquick Gel Extraction kit (Qiagen, Germany), ligated into pMD19-T simple vector (Takara, Dalian, China), and then sequenced (Sangon, Shanghai, China). The resulting sequences were deposited in GenBank and submitted to the BLAST program.

Sequence analysis

The molecular weight (MW) and the theoretical isoelectric point (pI) were calculated by ExPaSy (http://expasy.ch/tools/protparam.html). Sequence analysis of porcine *MID1IP1* was carried out using the BLAST program at the National Center for Biotechnology Information (NCBI) server (http://www.nibi.nlm.nih.gov/BLAST). The prediction of signal peptides was carried out using SignalP 3.0 (http://www.cbs.dtu.dk/services/Signalp). The protein secondary structure was predicted by SABLE (http://sable.cchmc.org).

Construction of a recombinant *MID1IP1* expression vector

Both pMD19-T-*MID1IP1* and pET28a (Takara) plasmids were digested with *Hin*dIII and *Eco*RI endonucleases. The fragments of *MID1IP1* and pET28a were gel-purified (Qiagen), and were then linked in the presence of T4 DNA ligase (Takara, Dalian, China). The reaction system contained 2 µL buffer, 10 µL *MID1IP1* fragments, 5 µL pET28a vector fragments, 1 µL T4 DNA ligase and 2 µL ultrapure water. After 12 h at 16°C, the recombinant plasmid pET28-*MID1IP1* was transformed into *E. coli* BL21 (DE3). The pET28-*MID1IP1* recombinant plasmid was confirmed by colony PCR and double digestion with *Hin*dIII and *Eco*RI.

Protein expression in *E. coli* BL21 (DE3)

Single colonies of pET28-*MID1IP1* in *E. coli* BL21 (DE3) were added to LB medium containing 50 µg/mL ampicillin and cultured at 37°C overnight. Fresh LB was then inoculated 1:100 with the overnight culture and cells grown until an OD_{600} of 0.4-0.6. IPTG at a final concentration of 1 mM was then added to induce expression, and cultures were incubated for another 8 h. Cells were then pelleted at 12,000 rpm for 1 min and 100 µL SDS loading buffer per 1.5 ml cells added. SDS-PAGE was carried out using the Bio-Rad Mini-protein system (USA). A 12% SDS-PAGE was used to analyze the recombinant protein. The protein bands were stained with Coomassie Brilliant Blue R-250 for visualization, after electrophoresis.

RESULTS

Molecular cloning of *MID1IP1*

The *MID1IP1* RT-PCR products were analyzed by agarose gel electrophoresis. A fragment of the expected size, ~549 bp, was detected (Figure 1), isolated, purified and cloned into pMD19-T. After transformation and overnight culture, purified pMD19-T-*MID1IP1* plasmids were analyzed by PCR (Figure 2).

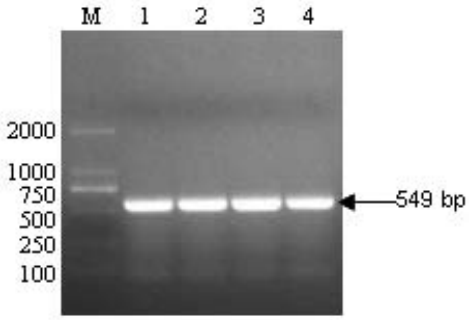

Figure 1. Gel electrophoresis of RT-PCR products. *Lane M* = Marker DL2000; *lanes 1-4* = expected PCR product of ~549 bp.

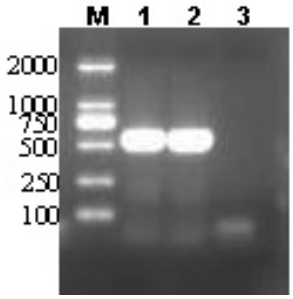

Figure 2. PCR identification of pMD19-T-*S14R* by gene-specific (GS) primers of *S14R*. *Lane M* = Marker DL2000; *lanes 1-3* = PCR of pMD19-T-*S14R* by GS primers of *S14R*.

Sequence analysis

Porcine *MID1IP1* showed an open reading frame of 549 bp, encoding 182 coding amino acids. From the deduced amino acid sequence, the MW and pI of porcine MID1IP1 are 20186.6 and 5.51, respectively. The cDNA nucleotide sequence analysis running the BLAST program showed that the porcine *MID1IP1* had high homology with the human (95%), mouse (90%), rat (90%), and cattle (95%) protein. It was submitted to the GenBank database (JN793537). The prediction of signal peptide by SignalP indicated that the porcine MID1IP1 protein had no signal peptide. SABLE on-line analysis software was applied and full-automated mode was selected to conduct predictive parsing for protein sequence secondary structure. The porcine MID1IP1 protein contained three alpha-helix, one β-sheet and random coils (Figure 3).

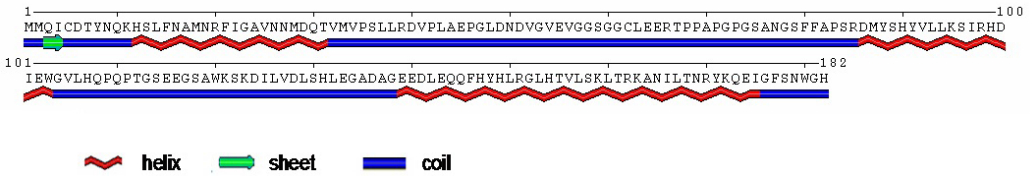

Figure 3. The secondary structure of porcine S14R protein was constructed by SABLE.

Construction of recombinant MID1IP1 expression vector

After digestion of pMD19-T-*MID1IP1* and pET28a (Takara) with *Hin*dIII and *Eco*RI endonucleases, the resulting DNA fragments were gel-purified. A ~549-bp restriction fragment from the sequenced pMD19-T-*MID1IP1* plasmid was subcloned into the *Hin*dIII and *Eco*RI sites of pET28a (Takara) in frame with the N-terminal polyhistidine (6xHis) tag. The recombinant plasmid pET28-*MID1IP1* was then transformed into *E. coli* BL21 (DE3). After transformation and overnight culture, the pET28-*MID1IP1* recombinant plasmid was isolated and confirmed by colony PCR (Figure 4) and restriction enzyme digestion analysis (Figure 5). Sequence analysis of pET28-*MID1IP1* showed that the insert orientation and position of *MID1IP1* were correct, which indicated that the cloned porcine *MID1IP1* gene could be induced to express protein.

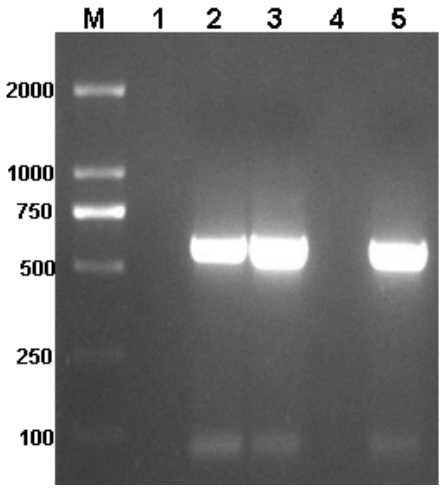

Figure 4. PCR identification of pET28-*S14R* by gene-specific (GS) primers of *S14R*. *Lane M* = Marker DL2000; *lanes 1-5* = PCR of pET28-*S14R* by GS primers of *S14R*.

MID1IP1 gene expressed in *E. coli*

The pET28-*MID1IP1* was transformed into *E. coli* BL21 (DE3) and was induced with IPTG to express MID1IP1. The cell lysates were separated by 12% SDS-PAGE electrophoresis, which showed that a protein with a molecular weight of about 24 kD was expressed, with no such band in the non-IPTG-induced control sample, indicating that the 24-kD protein band was the expression product of MID1IP1 (Figure 6).

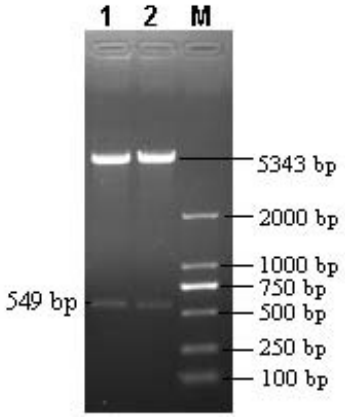

Figure 5. *Hin*dIII/ *Eco*RI digestion analysis of pET28-*S14R* recombinants. *Lane M* = Marker DL2000; *lanes 1* and *2* = pET28-*S14R* candidates digested by *Hin*dIII/*Eco*RI.

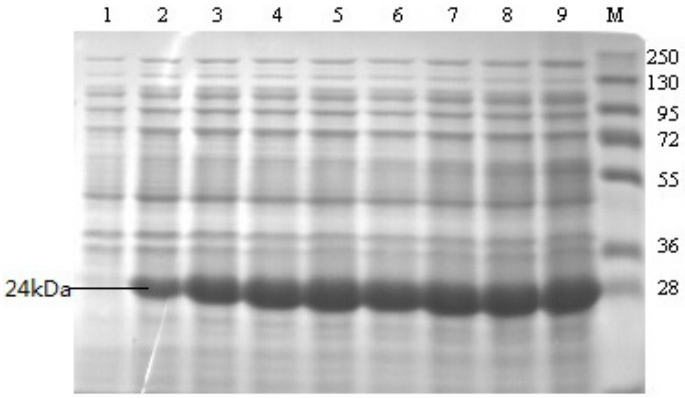

Figure 6. SDS-PAGE analysis of pET28-*MID1IP1* expression in *Escherichia coli* BL21. *Lane 1* = BL21/pET28-*MID1IP1* without IPTG induction; *lanes 2-9* = induced with IPTG for 1, 2, 3, 4, 5, 6, 7, 8 h respectively. Arrow indicates the expressed MID1IP1 fusion protein; *lane M* = protein marker

DISCUSSION

Although MID1IP1 shared a low amino acid homology with S14, as the paralog of S14, some studies (Kim et al., 2010; Colbert et al., 2010) have reported that it plays important roles in lipid metabolism. As we all know, acetyl-coenzyme A carboxylase (ACC) is a rate-limiting enzyme for de novo fatty acid synthesis. Recent studies have shown that MID1IP1 is an ACC-binding protein (Kim et al., 2010). MID1IP1 can activate acetyl-coenzyme A carboxylase. Another recent report has revealed that MID1IP1 is a new liver X receptor (LXR) target (Inoue et al., 2011). In this paper, the open reading frame (ORF) of porcine *MID1IP1* was isolated. It contains 549 nucleotides encoding 182 amino acids. These data will provide the molecular basis for porcine *MID1IP1* analysis of nucleotide sequence and deduced amino acid sequence. BLAST analysis of the deduced amino acid sequence demonstrated that porcine MID1IP1 had high homology with the human (95%), mouse (90%), rat (90%), and bovine

(95%) molecule. Signal peptide analysis showed that the MID1IP1 protein is a non-secretory protein. MID1IP1 is a cytoplasmic protein, which has been confirmed in humans and mice (Berit et al., 2004; Kim et al., 2010). To further investigate the detailed structure of porcine MID1IP1, SABLE on-line analysis software was employed to determine secondary structure. Our results indicate that the secondary structure of porcine MID1IP1 protein was the mixed type, including alpha-helix, β-sheet and random coils.

To study the function of porcine MID1IP1 and S14, we had previously completed the cloning, expression and affinity purification of porcine S14 (Wang et al., 2012). On this basis, we first obtained the full length coding sequence of porcine *MID1IP1*. Prokaryotic expression can produce large quantities of recombinant proteins with biological activity, which can be used for further functional studies. After *Eco*RI and *Hin*dIII digestion, the full length gene encoding porcine *MID1IP1* was inserted into prokaryotic expression plasmid pET28a. The recombination plasmid pET28-*MID1IP1* was transformed into *E.coli* BL21 (DE3), and expressed upon IPTG induction. Different expression conditions were explored and a 4-h period of IPTG induction at a final concentration of 1 mM was found to be optimal for high level expression of the MID1IP1-His_6 fusion protein, with a molecular weight of about 24 kD. The pET28a prokaryotic expression vector incorporates a 6 x His tag. This enables large quantities of the recombinant MID1IP1-His_6 fusion protein to be purified by the standard procedure of nickel-based affinity chromatography.

To our knowledge, this study is the first to report the cloning and expression in *E. coli* of porcine MID1IP1. The recombinant prokaryotic expression vector pET28-*MID1IP1* was constructed, and MID1IP1-His_6 protein was successfully expressed in *E. coli* BL21 (DE3). This provides a valuable source of porcine MID1IP1 for further experimentation into *MID1IP1* function for further investigation of meat quality in swine.

ACKNOWLEDGMENTS

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