Cloning and prokaryotic expression of the porcine lipasin gene

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ABSTRACT. Lipasin has recently been demonstrated to be involved in lipid metabolism. In this study, two specific primers were used to amplify the lipasin open reading frame from porcine liver tissue. The polymerase chain reaction product was cloned to a pGEM®-T Easy Vector, digested by *Sal*I and *Not*I, and sequenced. The lipasin fragment was then cloned to a pET21(b) vector and digested by the same restriction enzyme. The recombinant plasmid was transferred to *Escherichia coli* (BL21), and the lipasin protein was induced with isopropyl-β-D-thiogalactopyranoside. The protein obtained was identified by sodium dodecyl sulfate polyacrylamide gel electrophoresis and western blotting. A pET-lipasin prokaryotic recombinant expression vector was successfully constructed, and a 25.2-kDa protein was obtained. This study provides a basis for further research on the biological function of porcine lipasin.

Key words: Porcine; Lipasin cloning; Prokaryotic expression

INTRODUCTION

Angiopoietin-like proteins (ANGPTLs) are glycoproteins that were discovered by polymerase chain reaction (PCR) amplification according to the amino acid homology of Ang1 and Ang2 (Kim et al., 1999). The seven ANGPTLs (ANGPTL1 to ANGPTL7) are encoded by seven different genes (Mattijssen and Kersten, 2012), contain a coiled-coil domain, a linker region, and a fibrinogen-like domain, and are structurally similar to angiopoietins (Miida and Hirayama, 2010). Most ANGPTLs are multi-functional and are involved in lipid metabolism (Kersten, 2005), inflammation (Tabata et al., 2009), hematopoietic stem cell activity (Zhang et al., 2006), and cancer cell invasion (Galaup et al., 2006; Kuo et al., 2013).

ANGPTL3 and ANGPTL4 are highly homologous and regulate plasma triglycerides. Shimizugawa et al. (2002) found that Angptl3-null mice have low plasma levels of high-density lipoprotein, which can be increased by injecting ANGPTL3 via an adenovirus. Yoshida et al. (2002) demonstrated that overexpression of ANGPTL4 in mice significantly increases triglyceride levels, and Gm6484-null mice have low-plasma triglyceride levels (Tang et al., 2010). Gm6484 is also known as betatrophin (Yi et al., 2013), RIRF (Ren et al., 2012), ANGPTL8 (Quagliarini et al., 2012), TD26, and lipasin (Zhang, 2012), which is how it will be referred to henceforth. Lipasin is a paralog of ANGPTL3, but does not have a fibrinogen-like domain and appears to lack a coiled-coil domain. It is also homologous with ANGPTL4's N-terminal domain, which mediates lipoprotein lipase binding. The three proteins share functional motifs and act in the same metabolic pathways, confirming that lipasin plays a vital role in lipid metabolism.

Meat quality is dependent on normal lipid metabolism. Pork is an important food source, and low-quality pork is related to health problems such as obesity and cardiovascular disease. Therefore, lipid metabolism in pigs is an important issue, and the function of porcine lipasin requires investigation. In this study, we aimed to construct a prokaryotic expression vector and create a fusion protein.

MATERIAL AND METHODS

Animals

Four Landrace pigs (averaging 45 kg in weight) from Yongkang pig-breeding farm in Kaifeng, Henan Province, China, were used. Freshly harvested liver tissue was protected by liquid nitrogen and then stored at -80°C until use.

Total RNA isolation and cDNA synthesis

Total RNA was isolated from frozen porcine liver tissue using TRIzol (Gibco-BRL, Gel Company, San Francisco, CA, USA), according to manufacturer instructions. The cDNA was obtained using a Reverse Transcriptase M-MLV kit (TaKaRa, Dalian, China), according to the manufacturer instructions.

Cloning the open reading frame (ORF) of the lipasin gene

A pair of primers (F, 5'-gtcgacatgtccacgctcatgctgtg-3'; R, 5'-gcggccgcggccgggagtgc

tgccatg-3') containing the *Sal*I and *Not*I restriction sites (TaKaRa) was designed according to the electronic cloning sequence of the porcine lipasin gene. The PCR (Biometra, Göttingen, Germany) was conducted in a 50-µL total volume that contained 0.5 µL LA *Taq*™ polymerase (TaKaRa) (5 U/µL), 1 µL cDNA (100 ng/µL), 8 µL dNTPs (2.5 mM/µL), 0.5 µL of each primer (20 mM), 25 µL 2X GC buffer I, and 14.5 µL ddH_2O. The template was denatured at 95°C for 5 min, which was followed by 30 cycles of amplification (95°C for 30 s, 60°C for 30 s, and 72°C for 42 s) and a final cycle for 10 min at 72°C. The PCR products were confirmed by 1% agarose gel electrophoresis.

Construction of the pGEM®-lipasin clone vector

The amplified PCR products were gel-purified using a gel extraction kit (Qiagen, Venlo, Netherlands), and then ligated into a pGEM®-T Easy Vector (TaKaRa) in the presence of T4 DNA ligase (TaKaRa) according to the manufacturer instructions. They were then transferred to *Escherichia coli* DH5a cells, spread onto a lysogeny broth (LB) medium with 5-bromo-4-chloro-3-indolyl-beta-D-galacto-pyranoside and isopropyl-β-D-thiogalactopyranoside (IPTG), and blue-white screened for ampicillin resistance. The resulting recombinant plasmid (pGEM®-T-lipasin) was confirmed by restriction analysis and sequencing, and the resulting sequences were deposited in GenBank and subjected to the BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Sequence analysis

Sequence similarity was ascertained based on the alignment between porcine lipasin and other types of lipasin using BLAST. Lipasin sequence characteristics, such as molecular weight (MW), theoretical isoelectric point (pI), and the conserved domain, were calculated using ExPASy (http://expasy.org). The protein's secondary structure was predicted using SABLE (http://sable.cchmc.org).

Construction of the pET-lipasin expression vector

The pGEM®-T-lipasin recombinant plasmid was double-digested with *Sal*I and *Not*I, and the lipasin was then cloned into the expression vector pET-21(b) and digested by the same restriction enzyme. The recombinant plasmid was then introduced into *E. coli* BL21(DE3), spread on an ampicillin-resistant LB plate, and cultured at 37°C overnight until the appearance of a single white colony. The single colony was cultured in 2 mL LB liquid medium that contained ampicillin (100 mg/mL). The plasmid obtained was verified by restriction analysis and sequencing, and the recombinant prokaryotic expression vector was named pET-lipasin.

Recombinant protein expression in *E. coli*

The *E. coli* BL21(DE3) cells that harbored the pET-sts plasmid were induced and cultured overnight at 37°C in LB liquid medium containing ampicillin (100 mg/mL), transferred to fresh medium, and incubated for another 2 h until the optical density of the cultured cells was 0.5. Expression of the objective protein was induced with 1 mM IPTG at 16°C overnight, and 0.2 mL of the strain that harbored pET-lipasin was harvested by centrifugation at 9000 *g*

for 10 min. The precipitates were suspended in 30 µL 1X phosphate-buffered saline, and the remaining pellet was resuspended in 30 µL lysis buffer. The pellet was then analyzed by 14% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently stained with Coomassie Brilliant Blue R-250 for visualization.

Detection of the lipasin protein by western blot analysis

Western blotting was performed according to the standard procedure (Sambrook et al.,1989). The cell lysates were subjected to SDS-PAGE and the proteins were transferred to a nitrocellulose membrane. The membrane was incubated for 1 h in non-fat powdered milk (5% bovine serum albumin in TBST buffer) at room temperature (RT). The membrane was then incubated with the primary antibody, Anti-HIS-FLAG (1:3000, Sigma-Aldrich, St. Louis, MO, USA), overnight at 4°C before being incubated again for 2 h with the secondary antibody, goat anti-rabbit IgG (1:4000, Sigma-Aldrich), at RT after washing with TBST. After being washed three times (5 min each time) with TBST buffer, the membrane was analyzed using an ECL assay kit (Beyotime, Nantong, China) and exposed to X-ray film (Eastman Kodak Co., Rochester, NY, USA) for 2 min.

RESULTS

Cloning the lipasin gene ORF

The gene-specific primers were designed and synthesized to isolate the coding DNA sequence (CDS) region of the lipasin gene by PCR. The resulting sequences were deposited in GenBank (No. KF017598). A 601-bp PCR product was obtained, as expected (Figure 1).

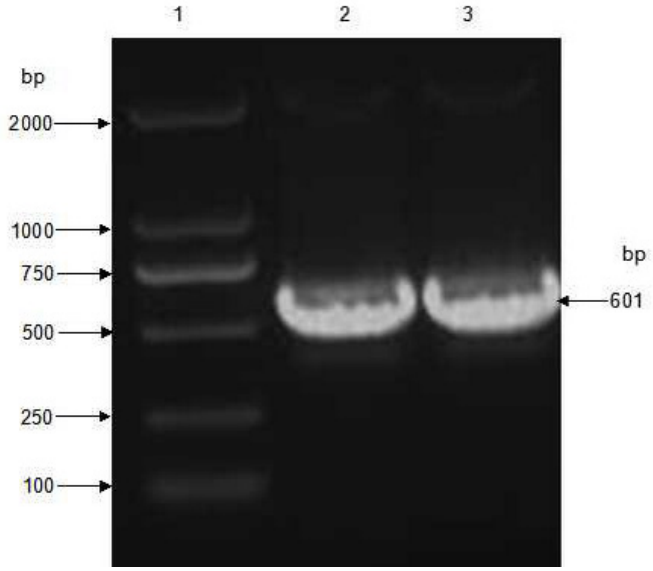

Figure 1. Gel electrophoresis of the polymerase chain reaction products. *Lane 1*, marker DL2000; *lanes 2* and *3*, PCR products of 601 bp in length.

Sequence analysis

The lipasin CDS was 594 bp long and encoded 198 amino acids. The lipasin protein's MW and pI were 21.99 kDa and 6.79, respectively. Sequence homology analysis indicated that it shared 72% identity with the human lipasin protein and 68% with the mouse lipasin protein. The protein's secondary structure is illustrated in Figure 2.

Figure 2. Secondary structure of the porcine lipasin protein as predicted by SABLE.

Identification of the pGEM®-lipasin clone vector

The pGEM®-lipasin plasmid was digested with two restriction enzymes, *Sal*I and *Not*I. The results of the agarose gel electrophoresis indicated that the enzyme fragments were in accord with the size of the known objective and empty vector (Figure 3).

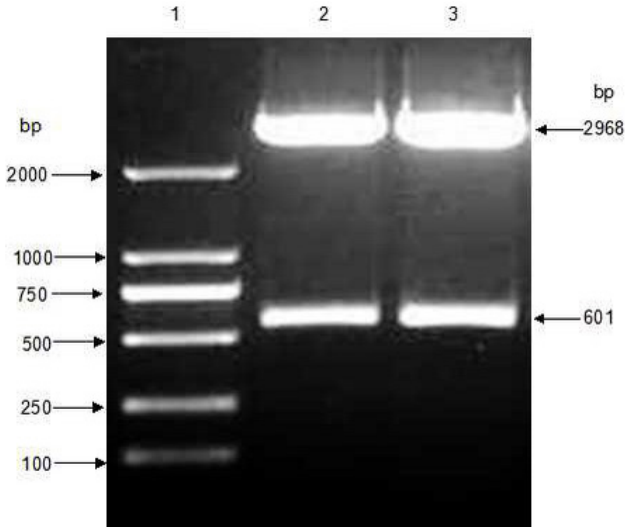

Figure 3. Identification of the pGEM®-lipasin recombinant plasmid by restriction analysis. *Lane 1*, marker DL2000; *lanes 2* and *3*, pGEM®-4T-T14/*Sal*I and *Not*I.

Identification of the pGEM®-lipasin expression vector

The results of the agarose gel electrophoresis confirmed that the constructed expression vector was correct. In addition, the sequencing results also indicated that it was correct (Figure 4).

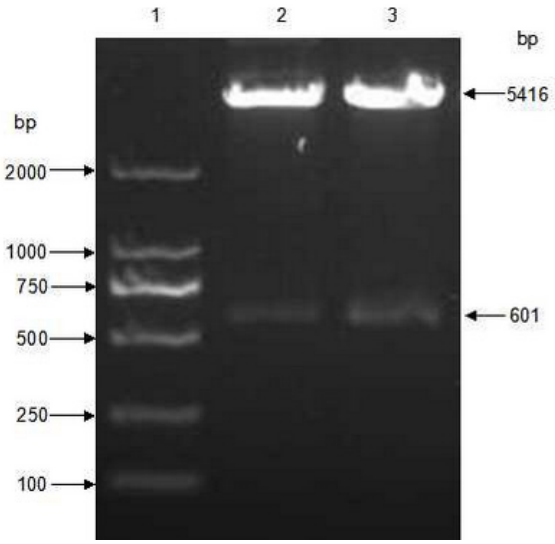

Figure 4. Identification of the pET-lipasin recombinant plasmid by restriction analysis. *Lane 1*, marker DL2000; *lanes 2* and *3*, pET-lipasin/*Sal*I and *Not*I.

Recombinant protein expression in *E. coli*

Samples of BL21 that contained pET-lipasin were analyzed by SDS-PAGE. After electrophoresis, the protein bands were stained for visualization and a pET-lipasin protein with a MW of 25.2 kDa was observed (Figure 5).

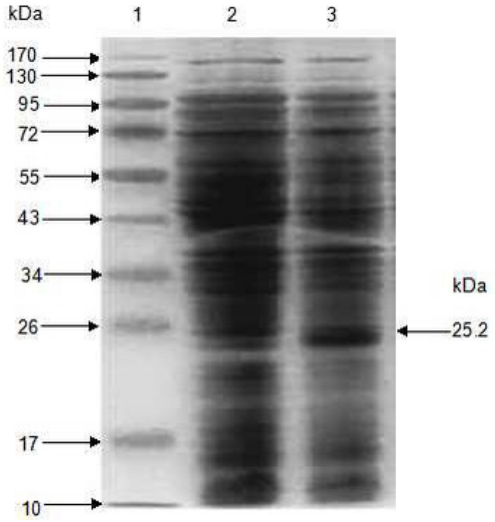

Figure 5. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of pET-lipasin expression in *Escherichia coli* BL21. *Lane 1*, protein marker; *lane 2*, BL21/pET-lipasin without isopropyl β-D-1-thiogalactopyranoside (IPTG) induction; *lane 3*, induced with IPTG for 12 h at 16°C overnight. The arrow indicates the lipasin fusion protein expressed.

Detection of the lipasin protein using western blot analysis

Western blotting revealed that the expressed protein had a MW of 26 kDa and exhibited a specific reaction with the HIS-Tag antibody (Figure 6).

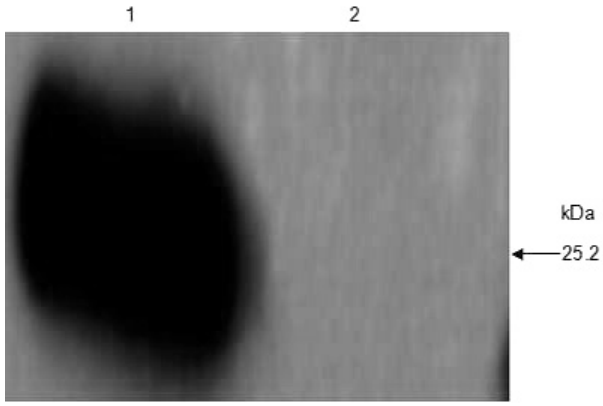

Figure 6. Western blotting of pET-lipasin expression in *Escherichia coli* BL21. *Lane 1*, BL21/pET-lipasin with isopropyl β-D-1-thiogalactopyranoside (IPTG) induction; *lane 2*, induced without IPTG for 12 h at 16°C overnight.

DISCUSSION

Lipasin proteins are present in human and mice, but until now, they have not been shown to be present in pigs. In our study, we found that lipasin mRNA is highly expressed in the porcine liver, as it is in the human liver and plasma. In mice, it is found in the liver and in adipose tissue, particularly brown adipose tissue. Lipasin can activate ANGPTL3 by promoting its cleavage (Ono et al., 2003; Quagliarini et al., 2012); ANGPTL3 is closely associated with triacylglycerol and cholesterol levels in mice (Musunuru et al., 2010) and humans (Koishi et al., 2002), and our results suggest that it plays a role in porcine metabolism.

In this study, we successfully cloned the lipasin CDS and conducted sequence homology. The CDS was 594 bp long and encoded 188 amino acids. Sequence homology analysis indicated that it shared 72% identity with the human lipasin gene (C19orf80) and 68% with the mouse lipasin gene (Gm6484). The SABLE results revealed that the lipasin protein has a helix and coil structure. Quagliarini et al. (2012) performed immunoblotting with polyclonal antibodies raised against full-length recombinant ANGPTL8, and found a 22-kDa protein in human plasma, which is consistent with the results obtained in the present study. In our study, we cloned the lipasin gene into a pET21 vector at the relevant sites after digestion by *Sal*I and *Not*I; the resulting recombinant plasmid was named pET-lipasin. We then transferred it to BL21(DE3). Lipasin proteins were expressed after IPTG induction at 16°C overnight. The SDS-PAGE and western blotting analyses revealed that the MW of the lipasin protein was approximately 26 kDa, as we expected, indicating that we successfully constructed a recombinant prokaryotic expression vector that was named pET-lipasin. The lipasin proteins obtained should provide a strong foundation for the further study of lipasin at the protein level with regard to lipid metabolism in pigs, which will improve the quality of pork.

Conflicts of interest

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

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