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Identification of BPI protein produced in different expression system and its association with *Escherichia coli* F18 susceptibility

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ABSTRACT. The super antibiotic bactericidal/permeability-increasing (BPI) protein is a member of a new generation of proteins that have been implicated as endotoxin-neutralizing agents. In this study, recombinant porcine BPI protein was obtained by generating porcine BPI encoding prokaryotic, eukaryotic, and yeast expression vectors. Recombinant protein expression was detected in yeast GS115, *Escherichia coli*, and 293-6E cells by gel electrophoresis and Western blotting. *Escherichia coli* F18 is the primary Gram-negative bacteria in the gut and the main pathogen leading to diarrhea and edema disease in weaning piglets. Therefore, *E. coli* F18-resistant and -sensitive Sutai piglets were used to test differential expression of BPI protein by Western blotting and to investigate the potential correlation between BPI protein expression and *E. coli* F18-susceptibility. Recombinant porcine BPI protein expression was not detected in the prokaryotic and yeast expression systems; however, soluble protein was detected in the eukaryotic expression system. These data indicate the strong bacterio-

static action of the BPI protein and confirm the feasibility of obtaining large amounts of recombinant porcine BPI recombinant protein using this eukaryotic expression system. In addition, the BPI protein expression levels in the *E. coli* F18-resistant group were significantly higher than those in the sensitive group, indicating that high BPI protein expression is associated with resistance to *E. coli* F18. Our findings provide a basis for further investigations into the development of a drug designed to confer resistance to *E. coli* F18 in weaning piglets.

Key words: Bactericidal/permeability-increasing protein; Porcine; *Escherichia coli* F18; Expression system

INTRODUCTION

Bactericidal/permeability-increasing protein (BPI) is a 55-kDa endogenous cationic protein found in humans and other mammals. It is primarily present in the aniline blue particles of polymorphonuclear leukocytes, as well as on the surface of mononuclear leucocytes (Iovine et al., 1997; Canny and Levy, 2008). BPI not only kills Gram-negative bacteria (GNB) and neutralizes endotoxins (Akin et al., 2011), but also promotes complement activation and opsonization for increased phagocytosis, inhibits angiogenesis, inhibits the release of inflammatory mediators, and inhibits infection by fungi and protozoa. The function of BPI is related to its structure, which includes a cationic, lysine-rich N-terminus with antibacterial and lipopolysaccharide (endotoxin)-neutralizing activities (Iovine et al., 1997) and a C-terminus that contributes to the stability and opsonic activity (Ooi et al., 1991). BPI also plays an important role in natural defense mechanisms (Weiss et al., 1978; Iovine et al., 1997; Elsbach, 1998).

Previous studies have demonstrated that porcine *BPI* gene polymorphisms affect disease resistance and susceptibility, and *BPI* has been identified as a candidate gene for disease resistance breeding programs (Tuggle et al., 2003). The successful extraction and purification of porcine BPI protein from pig neutrophils was confirmed in both *in vivo* and *in vitro* biological activity experiments (Zhou et al., 1992, 1999). Furthermore, BPI protein was also shown to mediate endotoxin neutralization and Gram-negative antibacterial functions. Further studies showed that anti-human BPI protein antibodies enhanced the biological activity of porcine BPI protein *in vitro* (Zhou et al., 2002). Moreover, studies revealed the importance of BPI protein in the resistance to fungal and toxoplasma infections (Gavit and Better, 2000), indicating that this protein can be used to treat various diseases.

In this study, we constructed expression vectors encoding porcine BPI in prokaryotic, eukaryotic, and yeast expression vectors. The expression levels of the recombinant protein were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting. The purified protein was used to investigate BPI function and evaluate methods for preparing porcine antimicrobial proteins on a large-scale. *Escherichia coli* F18 is the primary GNB in the gut and the primary pathogen leading to diarrhea and edema disease in weaned piglets. In previous studies, based on the *FUT1* genetic marker, we established *E. coli* F18-resistant and -susceptible resource populations in Sutai pigs. Differential expression of *BPI* between enterotoxigenic *E. coli* F18 (ETEC F18)-resistant and ETEC F18-sensitive piglets was analyzed in our preliminary study, and we determined that expression of *BPI* is significantly high only in the duodenum and jejunum of pigs, suggesting that increased

expression of *BPI* in the intestines is related to the resistance to ETEC F18 (Ye et al., 2011). Moreover, Zhu et al. (2013) observed significant differences between 35-day expression and expression during other development stages in the duodenum by analyzing the age-dependent tissue expression of porcine *BPI* in Sutai pigs (Zhu et al., 2013). The greatest susceptibility to *E. coli* F18 infection was observed at 35 days of age, with predominant *E. coli* F18 colonization in the duodenum. Because previous studies have indicated that the *BPI* gene is associated with *E. coli* F18, we investigated the role of recombinant porcine BPI protein in the resistance and susceptibility to *E. coli F*18 infections in Sutai pigs.

MATERIAL AND METHODS

Experimental materials

DH5α competent, BL21 (DE3) pLysS, and Rosetta cells were purchased from Invitrogen (Carlsbad, CA, USA). The pMD-18T vector was purchased from TaKaRa Biotechnology (Dalian, China). The pET22B, pPIC9, and pTT5 vectors, HEK293 cells, and Pichia GSll5 were stored in our laboratory. Polymerase chain reaction (PCR) products were extracted using a gel extraction kit (BBI, Canada). Restriction enzymes (*Nco*I, *Xho*I, *Avr*II, and *Not*I) and T4 DNA ligase were purchased from TaKaRa Biotechnology. Platimum *Pfx* Polymerase and platinum HIFI *Taq* Polymerase were purchased from Invitrogen. The primers were synthesized by Shanghai Biological Engineering Technology (Shanghai, China). PCR products were sequenced using the ABI PRISM 377 DNA auto sequencer (Applied Biosystems, Foster City, CA, USA).

Prokaryotic expression of the porcine *BPI* gene

Prokaryotic expression vector construction

Single-stranded oligos for amplification of the porcine *BPI* gene were designed and synthesized according to the coding sequence available in the GenBank database (Accession No.: EF436278). The complete coding sequence was obtained by PCR amplification and ligated into the pMD-18T vector, which was then used to transform competent *E. coli* DH5α cells. Moreover, mutation sites in the gene sequence were repaired by overlapping PCR. Positive clones were screened by sequencing and the resulting plasmid that included the correct objective sequence was designated as pMD-18T-BPI.

The pMD-18T-BPI plasmid was digested by *Nco*I and *Xho*I to yield a fragment of approximately 1450-base pair fragment that was then ligated into the pET22B vector overnight at 16°C using T4 DNA ligase. The plasmid was then used to transform competent *E. coli* DH5α cells, which were cultivated for 45 min prior to inoculation of Amp^+ LB solid culture plates containing ampicillin. Plates were cultured overnight at 37°C. Finally, monoclonal colonies were verified by restriction enzyme digestion and sequencing; the correct vector was designated as pET22B-BPI1450.

Optimization of conditions for porcine BPI gene expression in E. coli

Following sequencing verification, pET22B-BPI1450 was used to transform *E. coli* BL21 and Rosetta cells. A monoclonal colony was used to inoculate 3 mL LB liquid medium

containing 50 μg/mL Amp+. The culture was incubated overnight at 37°C with shaking at 230 rpm. Initial culture (3 mL) was diluted 1:100 and transferred to a new culture medium, and then cells were cultured for 110 min to an optical density $(OD)_{600}$ value of 0.6-0.7. Next, 1 mL bacterial culture was used as a negative control, and protein expression was induced in the remaining culture by the addition of 1 mM isopropyl β-D-1-thiogalactopyranoside and incubation for 4 h at 37°C and 16 h at 25°C, respectively. Following centrifugation and removal of the supernatant, the bacterial pellet was resuspended in 50 μL phosphate-buffered saline (PBS) and analyzed by SDS-PAGE.

Eukaryotic expression of porcine *BPI* gene

Plasmid preparation

The cDNA sequence of the porcine *BPI* gene was synthesized for codon optimization for mammalian expression in 293 6E cells. The target DNA sequence containing an N-terminal Kozak sequence and a C-terminal 6X His tag was subcloned into the mammalian expression vector pTT5. Transfection grade bulk preparations of the plasmid were prepared (sterile, highly supercoiled, and endotoxin <5 EU/mg).

Transient transfection and purification

Cell cultures were harvested and centrifuged at 4000 rpm (3220 *g*) for 40 min. The supernatant was collected and cells were stored at -80°C. The pH of the supernatant was adjusted to 8.0. Approximately 1.5 mL Ni Sepharose™ 6 fast flow affinity chromatography resin was fully equilibrated with buffer A, added to the cell culture supernatant, and gently mixed for 5 h. The resin was then collected in a syringe column and the target protein was step-gradient eluted with 50, 100, 250, and 500 mM and 1 M imidazole solution. The target protein was mainly observed in the 500 mM imidazole fraction.

SDS-PAGE

Prior to loading, 20 μL sample was mixed with 5 μL 5X loading buffer and heated at 90°C for 5 min. The sample mixture was then loaded into the wells of a 12% SDS-PAGE gel (GenScript, Piscataway, NJ, USA). A pre-stained protein marker was loaded alongside the samples to identify the weight of protein bands in each sample. SDS-PAGE was performed using a Mini-PROTEAN Tetra system (Bio-Rad, Hercules, CA, USA). The gel was run at 120 V for 1500 s followed by 180 V for 2400 s. The gel was then stained with 150 mL Coomassie brilliant blue R250 overnight at room temperature on a shaking table. Subsequently, the gel was washed and destaining until the background gel was clear.

Western blot assay

For Western blotting, the stacking gel was removed and the resolving gel was soaked in 1 L protein transfer buffer for 5 min. Hybond-P polyvinylidene fluoride membranes and the filter paper were soaked in 100% (v/v) methanol for 10 s and equilibrated in the protein transfer buffer for 5 min. Band-transfer was carried out using a Trans-blot Electrophoretic Transfer

Cell (Bio-Rad) at 100 V for 1 h at room temperature. Following transfer, non-specific binding sites on the membrane were blocked by incubation in 5% dried skim milk in PBS containing Tween-20 for 1.5 h. Membranes were then incubated with 0.2 μg/mL mouse-anti-His primary monoclonal antibody for 1.5 h at room temperature followed by incubation with the goat anti-mouse-horseradish peroxidase detection antibody (diluted 1:2500 in 5% dried skim milk in PBS containing Tween-20) for 1 h at room temperature. The blot was rinsed 3 times with PBS containing Tween-20, incubated with luminescence substrate for 3 min and exposed to X-ray film for 60 s.

Yeast expression of the porcine *BPI* gene

Construction of the yeast expression vector

The protein structure of the porcine *BPI* gene (accession No.: EF436278) was analyzed to identify the signal peptide sequence using the SignalP 3.0 software (http://www.cbs.dtu.dk/services/SignalP 3.0). Primers were designed for PCR amplification of the BPI cDNA sequence minus the signal sequence. The pMD-18T/BPI construct containing the *BPI* cDNA sequence was stored in our laboratory. The *BPI* sequence incorporating the AvrII/NotI restriction enzyme sites and a 6XHis tag was amplified using pMD-18T/BPI as a template with the following primers designed using the Primer Premier 5.0 software (Premier Biosoft, Palo Alto, CA, USA): F: 5'-TCCGCCTAGGATGGCCAGGGGCGCTGACAAC-3', R: 5'-ATAAGAATGCGGCCGCTCAGTGGTGGTGGTGGTGGTGCTCGA-3'. The 50-μL PCR reaction system consisted of 5 μL 10X Accuprime Pfx Buffer mix, 5 μL 10X Amplification enhancer, 1 μL each 10 μM primer, 0.5 μL Accuprime *Pfx* polymerase, 1 μL pMD-18T/BPI template, and 37.5 μL ddH_2O. The PCR amplification program consisted of 1 cycle of predenaturation at 95°C for 3 min, 25 cycles of denaturation at 95°C for 30 s, annealing 60°C for 30 s, and extension at 68°C for 80 s; a final cycle of 68°C for 5 min. PCR products were evaluated on 1% agarose gels stained with ethidium bromide. PCR products were extracted using a gel extraction kit (Bio Basic, Inc., Ontario, Canada). The complete *Avr*II-BPI-6XHis-*Not*I target fragment was digested with *Avr*II and *Not*I, ligated into the pPIC9 vector, and transformed into competent DH5α cells. Positive colonies were picked and recombinant pPIC9-BPI-6XHis was extracted by alkaline lysis. The construct was verified by PCR amplification, enzyme digestion, and sequencing (Shanghai Biotechnology, Shanghai, China).

Electroporation of GS115

Monoclonal GS115 colonies were picked and cultivated at 30°C in 20 mL Yeast peptone dextrose medium. At an OD value of approximately 1.0, competent GS115 were prepared. The pPIC9-BPI-6XHis plasmid (200 ng/μL; 50 μL) was digested with 5 μL *Not*I, 50 μL 10X H Buffer, 50 μL 0.1% bovine serum albumin, 50 μL 0.1% Triton X-100, and 295 μL ddH_2O overnight at 37°C. The linearized plasmid was isolated by ethanol precipitation and dissolved in 20 μL ddH_2O and added to 200 μL competent GS115 on ice for 10 min. Finally, the pPIC9-BPI-6XHis plasmid was transfected into competent DH5α cells by electroporation (1680 V).

Induced protein expression

Positive clones were cultured in 10 mL BMGY medium overnight at 30°C with shak-

ing. Subsequently, the culture was centrifuged at 2500 rpm for 5 min at 4°C. After centrifugation, the thallus was used for heavy suspension culture in 10 mL BMGY medium. Protein expression was induced for 3 days by adding 100 μL methanol into the medium at 24 h intervals. Expression of the target protein was detected by SDS-PAGE and Western blotting as described above.

Differential expression of porcine *BPI* gene in *E. coli* F18-resistant and susceptible piglets

This study was conducted in 3 *E. coli* F18-resistant piglets and 3 *E. coli* F18-susceptible piglets (Bao et al., 2012). All experiments were conducted in the Animal Hospital of Yangzhou University according to the regulations of the Administration of Affairs Concerning Experimental Animals (Ministry of Science and Technology, China, revised in June 2012) and approved by experimental animal using permit with No. SYXK (Su) 2012-0029. The animals were allowed access to feed and water *ad libitum* under normal conditions and humanely sacrificed as necessary to ameliorate suffering. Fifteen centimeters of the duodenum were obtained according to the method described by Alwan et al. (1998) to isolate and prepare intestinal epithelial cells. For nuclear translocation experiments, nuclear and cytosolic fractions were obtained using the NE-PER kit (Pierce, Rockland, IL, USA) according to the manufacturer protocol. Protein levels were normalized using the BCA kit (Pierce). SDS-PAGE was performed with 10 μL protein separated on a 10% gel at 160 V for 70 min. For Western blotting analysis proteins were transferred to polyvinylidene fluoride membranes and immunoblotted with antibodies to BPI (1:500, ABmart, Shanghai, China), β-actin, and anti-rabbit (1:3,000) IgG.

RESULTS

Prokaryotic expression analysis

SDS-PAGE analysis of bacterial samples following induction for 4 h at 37°C revealed a recombinant protein of approximately 57 kDa that was absent in the pre-induction culture (Figure 1). Expression of the 57-kDa protein was also absent from cultures induced for 16 h at 25°C (Figures 2).

Yeast expression analysis

Successful construction of pPIC9-BPI-6XHis was confirmed by sequencing. However, inducible expression of the target protein was not detected by SDS-PAGE and Western blotting analyses (Figures 3 and 4).

Eukaryotic expression analysis

The recombinant eukaryotic expression vector encoding BPI was transiently transfected into HEK293 cells and the target protein was isolated from the cell culture supernatant by 1-step affinity chromatography. Analysis of the eluted fractions by SDS-PAGE and Western blot revealed the presence of the BPI protein with an apparent molecular weight of 62 kDa

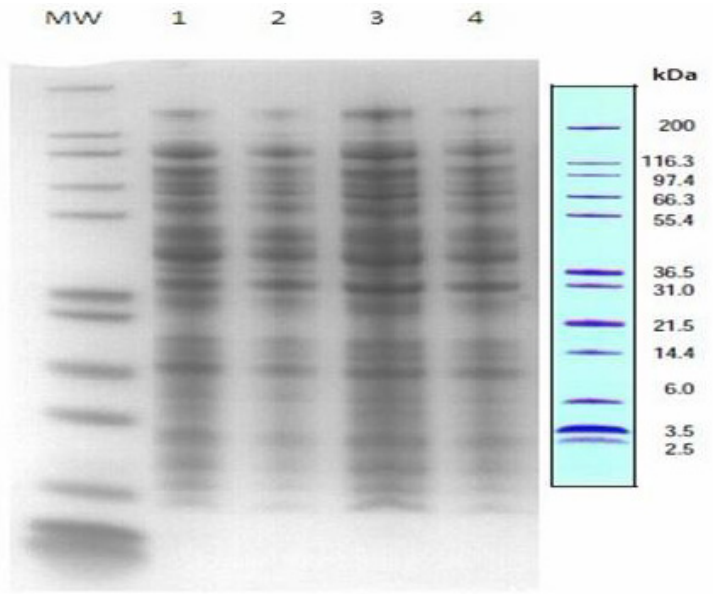

Figure 1. SDS-PAGE analysis of bacterial samples following induction for 4 h at 37°C. *Lane MW*: molecular weight; *lane 1*: pre-induction with BL21(DE3)plysS; *lane 2*: post-induction with BL21(DE3)plysS; *lane 3* pre-induction with Rosetta(DE3); *lane 4* represents post-induction with Rosetta(DE3).

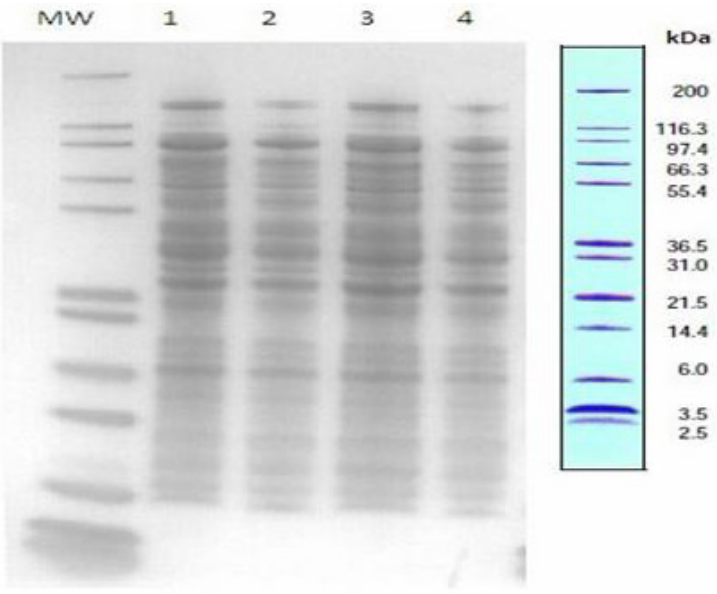

Figure 2. SDS-PAGE analysis of bacterial samples following induction for 16 h at 25°C. *Lane MW*: molecular weight; *lane 1*: pre-induction with BL21(DE3)plysS; *lane 2*: post-induction with BL21(DE3)plysS; *lane 3* pre-induction with Rosetta(DE3); *lane 4* represents post-induction with Rosetta(DE3).

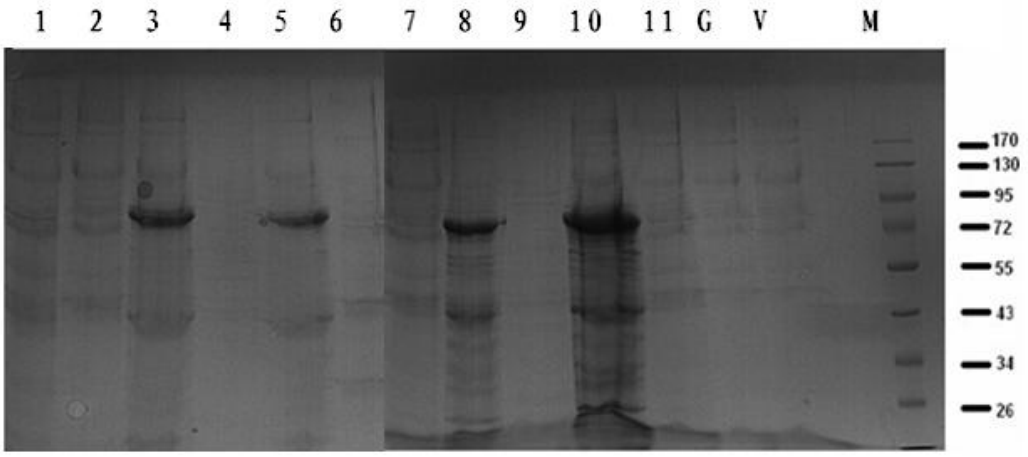

Figure 3. SDS-PAGE analysis of porcine BPI expression in yeast GS115. *Lane M* represents protein marker; *lanes 1-11* represents recombinant porcine BPI protein; G represents yeast GS115; V represents pPIC vector.

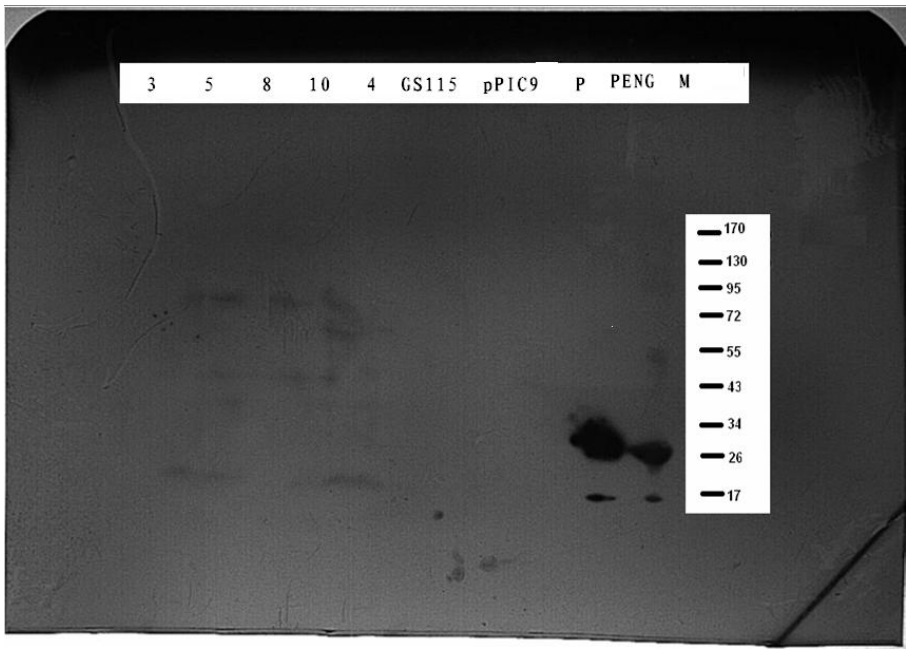

Figure 4. Western blot analysis of porcine BPI expression in yeast GS115. *Lane M* represents protein marker; *lanes 3*, *5*, *8*, *10*, *4* represent recombinant porcine BPI protein; P represents positive control.

(calculated MW: approximately 52 kDa) (Figure 5). Similar analysis of cell lysates and supernatants were performed at day 6 post-transfection (Figure 6). Approximately 10 μg protein was obtained from 100 mL cell culture.

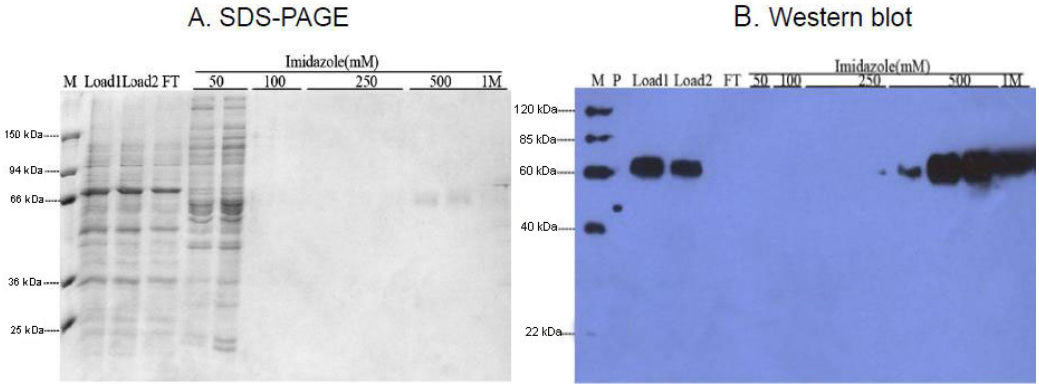

Figure 5. SDS-PAGE and Western blot analysis of BPI in HEK293 cells. *Lane M*: Protein marker; *lane 1*: Cultured medium after centrifugation; *lane 2*: Cultured medium after filtration; *lane FT*: Flow through; *lane P*: Multiple-tag protein (GenScript, Cat.No.M0101), as a positive control.

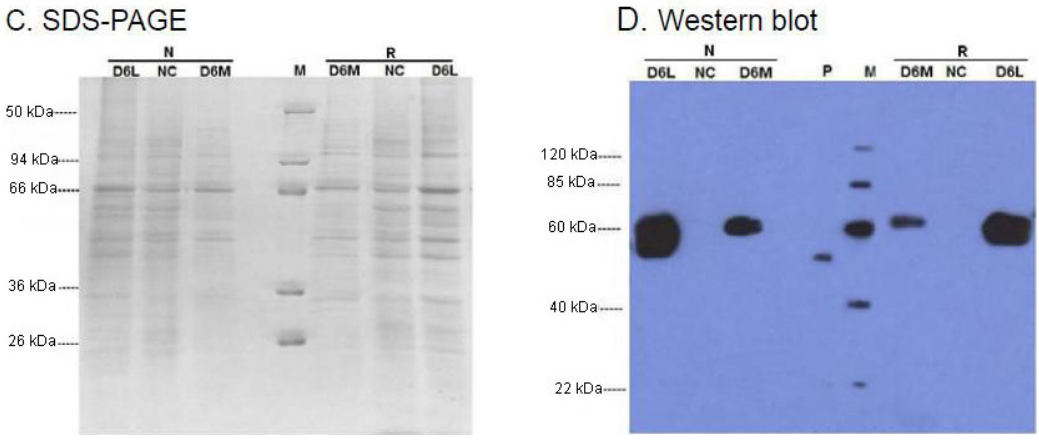

Figure 6. SDS-PAGE and Western blot analysis of BPI protein expression in HEK293 cells at day 6 post-transfection. *Lane M*: protein marker; *lane NC*: untransfected cell lysate; *lane N/D6L*: cultured lysate at day 6 post-transfection under non-reduced conditions; *lane N/D6M*: cell culture medium at day 6 post-transfection under non-reduced conditions; *lane R/D6L*: cultured lysate from day 6 post-transfection under reduced conditions; *lane R/D6M*: cell culture medium from day 6 post-transfection under reduced conditions; *lane P*: multiple-tag protein (GenScript, Cat.No.M0101), as positive control.

Expression of BPI protein in *E. coli* F18-resistant and susceptible individuals

Expression of the recombinant porcine BPI protein was detected as a band of approximately 53 kDa in the duodenum of *E. coli F*18-resistant and susceptible Sutai pigs by Western blot analysis. The results showed markedly higher expression of the BPI protein in *E. coli* F18-resistant group compared with that detected in the *E. coli* F18-susceptible group (Figure 7).

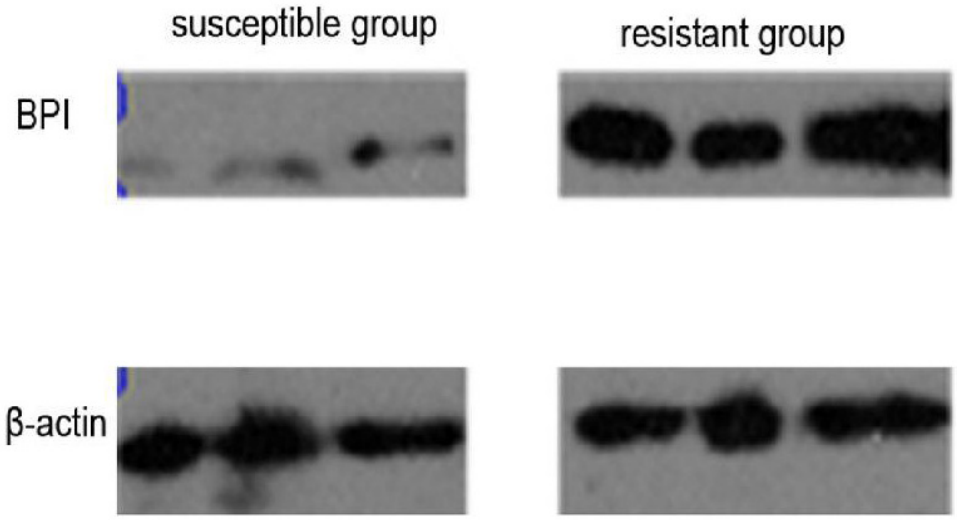

Figure 7. Western blot analysis of the differential expression of BPI protein in the duodenum of *Escherichia coli* F18-resistant and susceptible piglets (N = 3 per group).

DISCUSSION

BPI protein is a type of cationic antimicrobial glycoprotein secreted by neutrophilic granulocytes. BPI protein possesses bacteriostatic capacity against GNB, such as *E. coli*, *Salmonella*, *Pseudomonas aeruginosa*, *Proteus vulgaris*, *Shigella dysenteriae*, and *Neisseria*, although there are differences in the sensitivity to BPI among different bacterial strains. Furthermore, high concentrations of BPI can damage Gram-positive bacteria and eukaryotic cells (Elsbach, 1994). In addition, BPI neutralizes endotoxins and possesses antifungal activity. Little et al. (1994) reported that BPI combined with and neutralized heparin, indicates its capacity to inhibit angiogenesis. Their study also identified 3 structural domains of rBPI23 that were all related to biological activity, with the third domain (residues 148-161) possessing strong antifungal activity *in vivo* (Appenzeller et al., 1996). BPI had been identified as a new generation of protein drugs with the capacity for highly efficient sterilization and endotoxin neutralization, indicating its potential for applications involved in the resistance to microbial infection.

To date, systematic theoretical and applied studies on native and recombinant forms of BPI protein have been carried out. In addition to applications for treating GNB infectious diseases, series of BPI-derived peptides (such as antifungal drugs, anti-angiogenesis drugs, etc.) have been developed. BPI has been shown to be secreted in both *in vitro* and *in vivo* models using an adenoviral system for the delivery of BPI (Alexander et al., 2004). Secretory BPI protein effectively neutralizes lipopolysaccharide and reduces the generation of the inflammatory cytokine tumor necrosis factor-α and macrophage inflammatory protein-2. Zheng et al. (1999) investigated the molecular design, selection, and evaluation of the BPI structure/function simulation peptide, resulting in the identification of a simulated peptide molecule possessing strong sterilization and endotoxin neutralization functions (Zheng et al., 1999). Currently, recombinant BPI-21 and BPI-23 have been approved for phase III clinical trials

by the United States Food and Drug Administration. Further studies confirmed that rBPI2l, rBPI23, and BPI-derived peptides all possess anti-angiogenic activity.

Recombinant BPI protein shows promise for clinical application. Therefore, in this study, BPI prokaryotic, eukaryotic, and yeast expression vectors were successfully constructed to identify a suitable system for the expression of recombinant porcine BPI. Expression of the target protein was not detected in the prokaryotic and yeast expression systems despite optimization of the expression conditions in *E. coli*. However, expression of the protein was detected in 293-6E cell culture supernatants in the eukaryotic expression system. Expression of porcine BPI has also been reported in Chinese hamster ovary cells (Xu et al., 2001a,b; Ma et al., 2001). These observations indicate the feasibility of using this eukaryotic expression system for bulk preparation of recombinant porcine BPI protein.

E. coli F18 is the primary GNB in the gut and the primary pathogen leading to diarrhea and edema disease in weaned piglets. In previous studies, Sutai pig resource populations were established carrying either the ETEC F18-resistant AA genotype or the ETEC F18-sensitive AG or GG genotypes. The display of functional adhesin through the type V secretion system was combined with receptor binding experiments to further analyze and verify the resistance and sensitivity to the ETEC F18 strain among these pig resource populations (Wu et al., 2007). Using these populations, we analyzed the expression level of the porcine BPI gene *in vivo*. Ye et al. (2011) analyzed the differential expression between *E. coli* F18-resistant and sensitive piglets using real-time PCR. The results showed that expression of *BPI* gene was extremely low or undetectable in various tissues, including the heart, liver, spleen, lung, kidney, stomach, muscle, thymus, and lymph nodes. In contrast, significantly high levels of BPI mRNA expression were detected in the duodenum and jejunum, with significant higher levels in resistant animals compared to in sensitive individuals. Zhu et al. (2011) also used real-time PCR to analyze the expression of the *BPI* gene in post-weaning Yorkshire, Sutai (*E. coli* F18-resistant population) and Meishan piglets. The results of this study showed that expression of the BPI gene in the duodenum and jejunum of Sutai piglets was significantly higher than that in Yorkshire and Meishan piglets. In a previous study, we analyzed *BPI* gene expression in Sutai piglets over a time period ranging from newborns to post-weaning days 8, 18, 30, and 35 (Zhu et al., 2013). There were significant differences between 35-day expression and expression at other developmental stages in the duodenum, with the greatest vulnerability to *E. coli* F18 infection detected at 35 days of age. Therefore, previous studies revealed an association between the expression level of the porcine *BPI* gene and susceptibility to *E. coli* F18 strains. However, previous studies have mainly analyzed mRNA expression using real-time PCR, which does not determine the level of BPI protein expression. In this study, we further analyzed the correlation between BPI protein expression and *E. coli* F18 sensitivity by Western blotting. The expression level of the BPI protein in the *E. coli* F18-resistant group was significantly higher than that in the sensitive group. This study not only confirmed the conclusions of previous studies, but also suggested that *BPI* protein expression is associated with the susceptibility or resistance to intestinal *E. coli* F18 infection, with increased intestinal *BPI* protein expression associated with resistance to *E. coli* F18 infection.

CONCLUSIONS

In this study, we successfully constructed prokaryotic, eukaryotic, and yeast expression vectors for the porcine BPI protein. While the prokaryotic and yeast expression sys-

tems failed to produce the target protein, recombinant porcine BPI protein was detected in the culture supernatant of the 293-6E cell eukaryotic expression system. These observations indicated strong bacteriostatic activity of the BPI protein, in addition to the potential for using the eukaryotic expression system for large-scale production of recombinant porcine BPI recombinant protein.

In addition, using *E. coli* F18-resistant and -sensitive piglets as an experimental model, we observed significantly higher expression of the BPI protein in the *E. coli* F18-resistant group compared with that in the *E. coli* F18-sensitive group. This indicates that higher expression of BPI is associated with the resistance to *E. coli* F18 infection. The findings of this study provide a basis for further investigations into the development of a drug designed to confer resistance to *E. coli* F18 in weaning piglets.

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