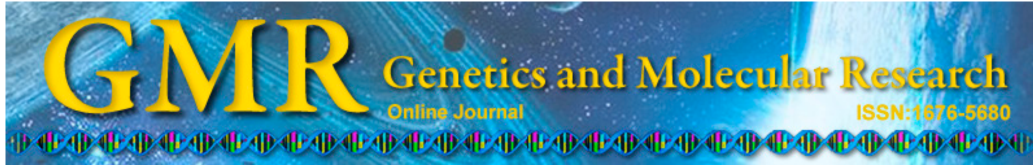

Codon optimization enhances the expression of porcine β-defensin-2 in *Escherichia coli*

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ABSTRACT. Porcine β-defensin-2 (pBD2) is a cationic antimicrobial peptide that has therapeutic potential. The amount of pBD2 in nature is limited, and the expression of pBD2 in *Escherichia coli* is low, probably because a different gene codon is used by prokaryotic organisms to that used by eukaryotes. Codon preference optimization is one of the ways to increase heterologous expression of pBD2. To achieve high expression of pBD2, the *pBD2* gene was redesigned according to the preferred codon in *E. coli* without altering the amino acid sequence. The optimized gene was inserted into expression vector pET-30a and transformed into *E. coli* BL21 (DE3) plysS. Our results showed that pBD2 was expressed as His-Tag fusion protein at a level that was approximately 4-6 times greater than from the native gene, based on total protein expression. Expressed fusion pBD2 showed antimicrobial activity against both *E. coli* and *Staphylococcus aureus*. Moreover, pBD2 showed weak hemolytic activity and strong heat resistance. These results indicate that fusion pBD2 is functional and has similar properties to those of pBD2 from the native gene. Our current study demonstrated that codon optimization could enhance pBD2 expression in *E. coli* without altering its function. Therefore, the expression of

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pBD2 after codon optimization in heterologous host cells might be useful and is worthy of further research.

Key words: Codon optimization; Porcine β-defensin-2; Heat resistance; Antimicrobial activity analysis; Recombinant expression; Hemolytic activity

INTRODUCTION

Defensins are a family of small, positively charged peptides secreted by organisms. They play an important role in the immune response to pathogens that include bacteria, viruses, and fungi (Selsted and Ouellette, 2005; Peschel and Sahl, 2006), and have a particular antimicrobial mechanism that makes it difficult for those pathogens to resist them (Hancock, 2001; Sang et al., 2006).

With the exception of porcine β-defensin-1 (pBD1), 12 known kinds of porcine β-defensins have been discovered by genomic sequence analysis. However, the expression and function of porcine defensins are less well studied compared to other species (Shi et al., 1999; Zhang et al., 2000; Sang et al., 2009). pBD2 is highly expressed in the kidney, liver, and tongue (Chen et al., 2010). Moreover, pBD2 shows high antimicrobial activity against bacteria, including multi-resistant bacteria, which makes it a good candidate for antibiotics (Zhang et al., 2000; Veldhuizen et al., 2008; Li et al., 2013).

Owing to its potential as an antibiotic, pBD2 production using heterologous expression systems has been reported (Zhang et al., 2010; Hu et al., 2011; Li et al., 2013). However, the production of pBD2 by eukaryotic systems suffers from low growth of the strain and low yield of the active products (Zhang et al., 2010; Hu et al., 2011). Expression of pBD2 by bacterial systems might overcome these problems. However, pBD2 expression in *Escherichia coli* is also low, probably because a different gene codon is used by prokaryotic organisms to that used by eukaryotes (Li et al., 2013). Indeed, it has been reported that rare codons in genes affect protein expression, including slowing down protein translation, and even leading to translational errors or no translation (Kane, 1995; Zahn, 1996; Hannig and Makrides, 1998; Roche and Sauer, 1999; Gustafsson et al., 2004; Sørensen and Mortensen, 2005; Plotkin and Kudla, 2010). Therefore, codon optimization could achieve higher protein expression in *E. coli* systems (Gvritishvili et al., 2010; Tang et al., 2011; Wang et al., 2010, 2012; Dai et al., 2013; Park et al., 2014).

The natural *pBD2* gene contains rare codons such as AGG for Arg (at codon 22), GGG for Gly (at codon 25), TGT for Cys (at 27), and ATA for Ile (at 4) (Kane, 1995). In this study, the *pBD2* gene was optimized by eliminating rare codons and simultaneously minimizing the secondary structure of the mRNA, with the aim of achieving its higher expression in *E. coli*. Our results show that the codon-optimized *pBD2* gene leads to increased expression of recombinant pBD2 protein, which has similar functional properties to pBD2 from the native gene, such as antimicrobial activity, hemolytic activity, and heat stability.

MATERIAL AND METHODS

E. coli TG1 for gene cloning and BL21 (DE3) plysS for expression were maintained in our laboratory. *E. coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 for testing the antimicrobial activity of pBD2 were purchased from the Beijing Ordinary Microbiology Strain Store Center (Beijing, China).

Construction of the expression vector

According to the amino acid sequence of pBD2 (GenBank accession No. AY506573.1), the nucleotide sequence was optimized and redesigned to the preferred codon usage in *E. coli* (Grosjean and Fiers, 1982). For insertion into the expression vector, an *Nco*I site was introduced to the 5'-end while two stop codons and an *Hin*dIII site were introduced to the 3'-end of the *pBD2* gene. The optimized codons are listed in Table 1. The optimized *pBD2* gene was synthesized by Shanghai Sangon Biotechnology Co., Ltd. (Shanghai, China), and was cloned into the pMD18-T vector. After double-enzyme digestion by *Nco*I and *Hin*dIII, the sequence was purified and cloned into a pET-30a expression vector (Novagen). The correct insertion was identified by polymerase chain reaction (PCR) and double-enzyme digestion, and the correct plasmid pET-*pBD2* was transformed into *E. coli* BL21 (DE3) pLysS cells for expression and conformation by sequencing.

Table 1. Sequence comparison between optimized and non-optimized codons of the *pBD2* gene.

non-op	GAC	CAC	TAC	ATA	TGT	GCC	AAG	AAA	GGG	GGG	ACC	TGC	AAC	TTC	TCC	CCC	TGC	CCG	CTC
op	GAC	CAC	TAC	ATC	TGC	GCT	AAA	AAA	GGT	GGT	ACC	TGC	AAC	TTC	TCT	CCG	TGC	CCG	CTG
aa	D	H	Y	I	C	A	K	K	G	G	T	C	N	F	S	P	C	P	L
non-op	TTC	AAC	AGG	ATT	GAA	GGG	ACC	TGT	TAC	AGT	GGC	AAG	GCC	AAG	TGC	TGC	ATC	CGC	
op	TTC	AAC	CGT	ATC	GAA	GGT	ACC	TGC	TAC	TCT	GGT	AAA	GCT	AAA	TGC	TGC	ATC	CGT	
aa	F	N	R	I	E	G	T	C	Y	S	G	K	A	K	C	C	I	R	

non-op = non-optimized codons; op = optimized codons; aa = amino acid.

Expression and purification of pBD2

When grown to mid-log phase, the expression strain BL-pET-*pBD2* (optimized) was induced by addition of isopropylthio-D-galactoside to a final concentration of 1 mM. The engineered strain containing the native *pBD2* gene was also induced at almost the same phase. Finally, the cells were collected by centrifugation at 8000 *g* for 5 min after cultivation for 4-6 h.

The *E. coli* cells were re-suspended in 20 mM sodium phosphate buffer, pH 7.4, with 40 mM imidazole and 0.5 M NaCl. Then, the cells were cracked by ultra-sonication for 50 min (at 300 W, 5-s bursts with 5-s intervals). The supernatant was obtained by centrifugation and was subjected to purification on an Ni-NTA affinity column. The protein of interest was collected as a 1-mL fraction from the column with an elution of the same buffer containing 150 mM imidazole, pH 7.4. The protein was dialyzed at 4°C against sodium phosphate buffer, pH 7.4, overnight and enriched with polyethylene glycol 8000. The total purified protein was quantified by the value of ultraviolet absorption at 280 and 260 nm by spectrophotometer (2800 UV/VIS, UNICO Instruments Co. Ltd., China).

Analysis of antimicrobial activity

The activity of pBD2 was estimated by the 96-well turbidimetry method (Huang et al., 2009). The purified pBD2 proteins were diluted to different concentrations (0-80 µg/mL) and mixed with 100 µL diluted bacteria (10^5-10^6 CFU/mL, *E. coli* or *S. aureus*) in a polypropylene 96-well microtiter plate to test the antimicrobial activity. The optical density (OD) at 630 nm (reference 405 nm) was measured at different time points on a microplate reader (Stat

Fax 2100, Awareness Technology Inc., USA). The lowest concentration of pBD2 at which no bacterial growth was observed overnight was defined as the minimal inhibitory concentration (MIC). All assays were implemented three times. Data are reported as means ± standard deviation (SD).

Analysis of hemolytic activity

The hemolytic activity of pBD2 was tested as described previously (Veldhuizen et al., 2008; Li et al., 2013). In short, we centrifuged the fresh porcine blood at 1800 *g* for 10 min to collect erythrocytes, which were then washed three times with saline solution. Subsequently, 100-µL aliquots were introduced to a 96-well microtiter plate and made up to 100 µL by mixing with the pBD2 solutions (final concentrations ranged from 0 to 80 µg/mL). The mixtures were centrifuged after cultivation at 37°C for 1 h. Then, 100-µL aliquots of the supernatant were investigated at 450 nm (reference 630 nm) using a plate reader. The percentage of hemolysis was counted by comparison with the controls without peptide and with 1% Tween-20 (Schulz et al., 2005; Bai et al., 2009). All assays were implemented three times. Data are reported as means ± SD.

Analysis of thermal stability

The pBD2 was purified as described and treated at different temperatures (60°, 80°, and 100°C) in a water bath for different times (10 and 30 min). The supernatants were collected after centrifugation and analyzed. The activity of purified pBD2 was measured after heating at 80°C for 30 min as described above.

Statistical analysis

Analysis of variance was implemented by SPSS 17.0. Means were compared by least significant difference or the Dunnett test. Significance was defined at $P < 0.05$.

RESULTS

Construction of expression vector of the *pBD2* gene

According to the sequence of *pBD2* and the preferred codons for *E. coli*, the nucleotide sequences of *pBD2* were optimized. By optimization, rare codons in *pBD2* were replaced by the preferred codons for *E. coli*, and the GC content was reduced from 56.7 to 50.4%. In addition, the mRNA secondary structure was minimized by analysis. The optimized *pBD2* gene was inserted into a pET-30a vector between the restriction sites for *Nco*I and *Hin*dIII. Recombinant plasmid pET-*pBD2* was identified by PCR and double-enzyme digestion with *Nco*I/*Hin*dIII (Figure 1). The sequencing further confirmed that the *pBD2* gene was in accordance with design and was 129 bp in length (data not shown). The constructed expression vector of pET-*pBD2* was introduced into *E. coli* BL21 (DE3) plysS, which resulted in the expression strain BL-pET-*pBD2*.

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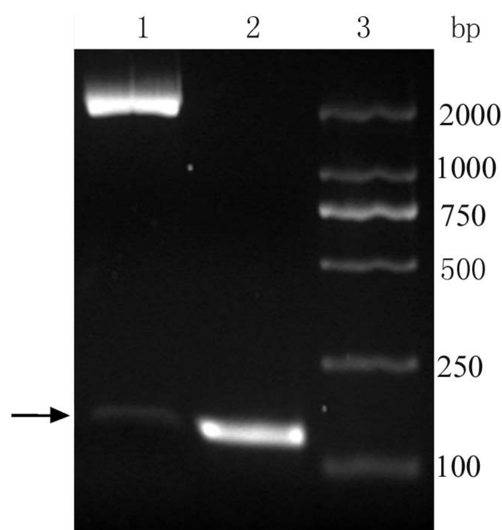

Figure 1. Identification of the pET-pBD2 by polymerase chain reaction (PCR) and double-enzyme digestion. *Lane 1* shows the product after pET-pBD2 was digested by *Nco*I and *Hin*dIII; *lane 2* shows the PCR product from pET-pBD2; *lane 3* shows the DNA marker.

Expression and purification of the recombinant pBD2

Both the optimized and native *pBD2* genes were induced by 1 mM isopropylthio-D-galactoside in the mid-exponential phase [OD measured at a wavelength of 600 nm (OD_{600}) = 0.6] at 37°C. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis showed that the optimized gene had higher expression than the native gene (Figure 2A). According to the analysis by Gel-ProAnalyzer (3.1), the peptide expressed by the optimized gene accounted for 22.55, 29.09, and 29.01% of total strain protein at 2, 4, and 6 h, respectively, while 4.79, 4.87, and 6.82% of the total protein was expressed by the native gene at 2, 4, and 6 h, respectively. The results indicated that the amount of fusion protein expressed by the optimized gene was about 4-6 times that of the native gene. Moreover, the optimized gene was expressed faster than the native gene, indicating that codon optimization makes the translation more efficient.

The expressed protein was purified using the His-Tag affinity column. A 1-mL fraction from the column was obtained and analyzed by SDS-PAGE (Figure 2B). The results showed that the protein that was eluted first contained other proteins. After 6 mL (or 6 fractions) of purification, the eluted protein showed a purity of above 95%.

Analysis of antimicrobial activity

The antibacterial activity of the expressed pBD2 was determined by the turbidimetry method. The OD_{630} (reference 405 nm) was measured at different times (Figure 3). The results showed that the bacteria cannot grow within 18 h at high pBD2 concentrations (40 µg/mL or above). However, the bacteria grew at an exponential rate within 8 h at lower concentrations of pBD2. The OD was significantly lower at high pBD2 concentrations than at low concentra-

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tions at the same time points (P < 0.01). Moreover, there were significant differences between the control and pBD2 treatment for both *E. coli* and *S. aureus* (P < 0.01). The results revealed that the MIC of recombinant pBD2 is 20 μg/mL (about 2 μM) for both *E. coli* and *S. aureus*. These data indicate that pBD2 shows high antimicrobial activity against *E. coli* and *S. aureus*. The antimicrobial activity of optimized pBD2 was similar to that of the native pBD2, as reported in previous studies (Li et al., 2013).

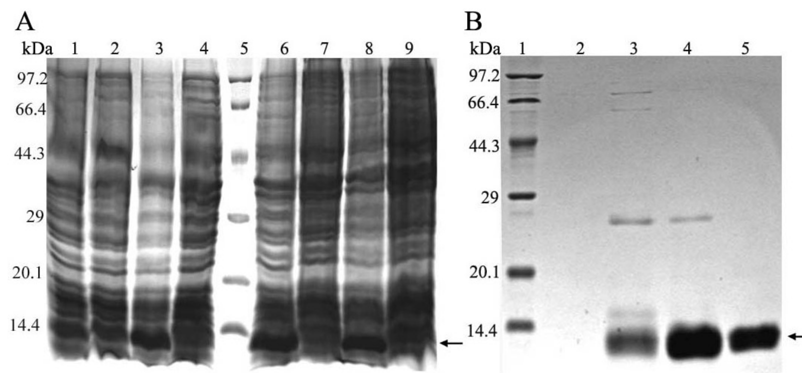

Figure 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of expressed recombinant and purified pBD2. **A.** SDS-PAGE analysis of expressed recombinant pBD2. *Lanes 1*, *3*, *6*, and *8* show the pBD2 expressed by the optimized gene after induction for 0, 2, 4, and 6 h, respectively; *lane 5* shows the protein marker; *lanes 2*, *4*, *7*, and *9* show the pBD2 expressed by the native gene after induction for 0, 2, 4, and 6 h, respectively; the arrow indicates the target protein. **B.** SDS-PAGE analysis of the purified pBD2. *Lane 1* shows the protein marker; *lanes 2-5* show the different fractions from Ni-agarose beads with the elution buffer containing 150 mM imidazole (*lanes 2-5* show elution fractions 1, 4, 6, and 10). The arrow indicates the target protein.

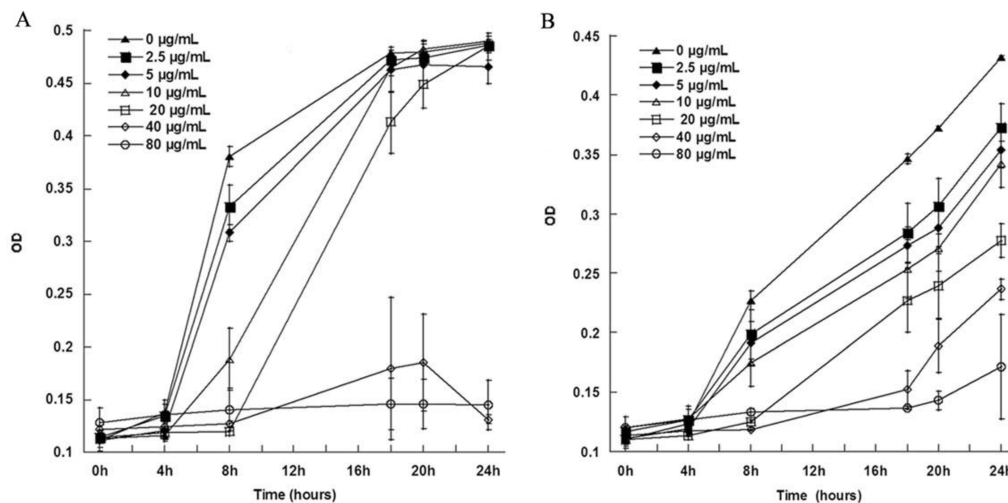

Figure 3. Antimicrobial activity of pBD2 against *Escherichia coli* (**A**) and *Staphylococcus aureus* (**B**). Bacteria were diluted to 10^5-10^6 CFU/mL, then treated with different concentrations of pBD2 (0-80 μg/mL) on a 96-well plate. Growth of bacteria was measured by optical density at 630 nm (reference 405 nm) by a microplate reader after incubation for different times.

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Analysis of hemolytic activity

Different concentrations of pBD2 (0-80 μg/mL) were mixed with erythrocytes from fresh porcine blood and incubated at 37°C for 1 h. Hemolysis was less than 10% at all concentrations (Figure 4). No significant differences were observed at different concentrations (P > 0.05). These results were consistent with previous reports (Veldhuizen et al., 2008; Li et al., 2013), indicating that the fusion recombinant pBD2 had very low hemolytic activity. Therefore, recombinant pBD2 is suitable for use as an antibiotic.

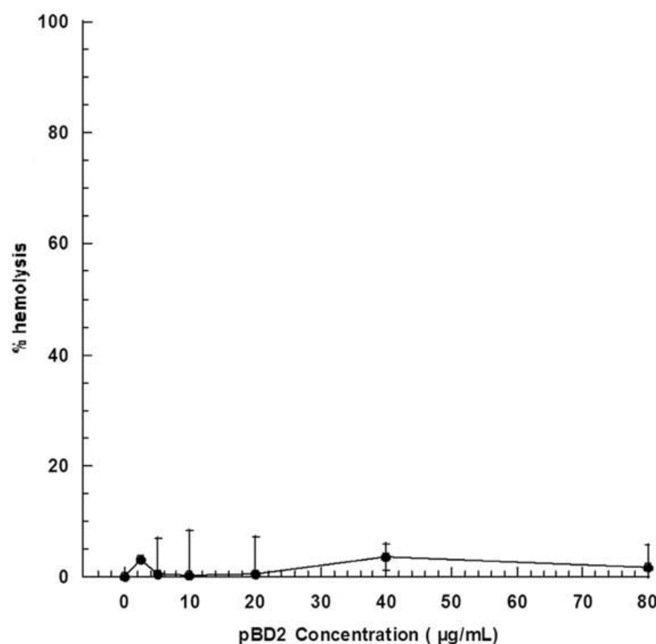

Figure 4. Hemolytic activity of pBD2. The erythrocytes from fresh porcine blood were incubated with different pBD2 concentrations (0-80 μg/mL) on a 96-well microtiter plate (200 μL total volume). The plate was centrifuged after incubation at 37°C for 1 h, then 100 μL supernatant from each well was separately transferred to a new 96-well microtiter plate and the optical density was determined at 450 nm (reference 630 nm) using a microplate reader. The percentage of hemolysis was calculated by comparison to no peptide or 1% Tween-20.

Thermal stability of recombinant pBD2

After different heat treatments, the concentrations of purified pBD2 were measured (Figure 5). The samples had no significant differences except for those treated at 100°C (P < 0.05). These results imply that pBD2 is heat resistant.

We then studied the antimicrobial activities of the heated recombinant pBD2. After heating at 80°C for 30 min, the purified recombinant pBD2 (80 μg/mL) was cultivated with *S. aureus* and *E. coli* cells, as described above. The bacteria showed no growth within 24 h (Figure 6). The samples showed no significant difference whether heated or not (P > 0.05). These data demonstrate that the recombinant pBD2 protein is thermo-stable, as is the pBD2 expressed by the native gene (Li et al., 2013).

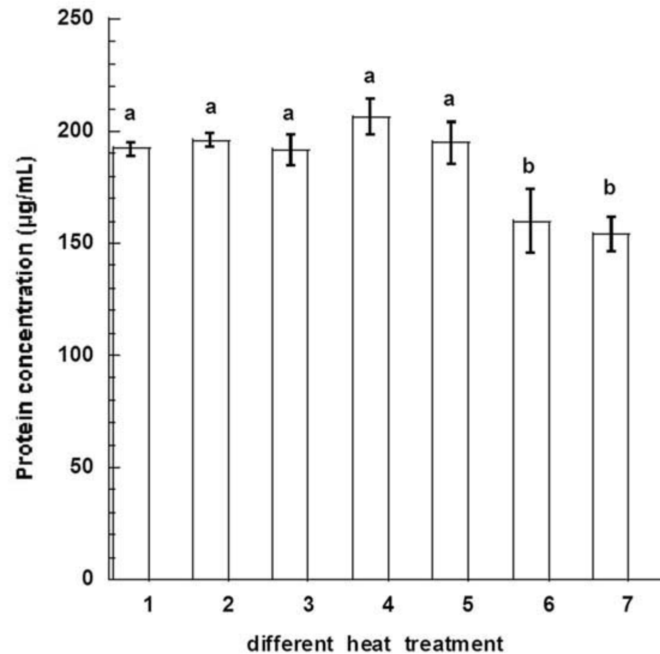

Figure 5. Concentrations of purified pBD2 after different heat treatments. Column 1 shows the samples without heat treatment as control; columns 2-7 show the samples after different heat treatments: 60°C for 10 min, 60°C for 30 min, 80°C for 10 min, 80°C for 30 min, 100°C for 10 min, and 100°C for 30 min, respectively. The letter 'a' at the top of the columns indicates that there was no significant difference to the control (without heat treatment) ($P > 0.05$). The letter 'b' at the top of columns indicates that there was a significant difference to the control ($P < 0.05$).

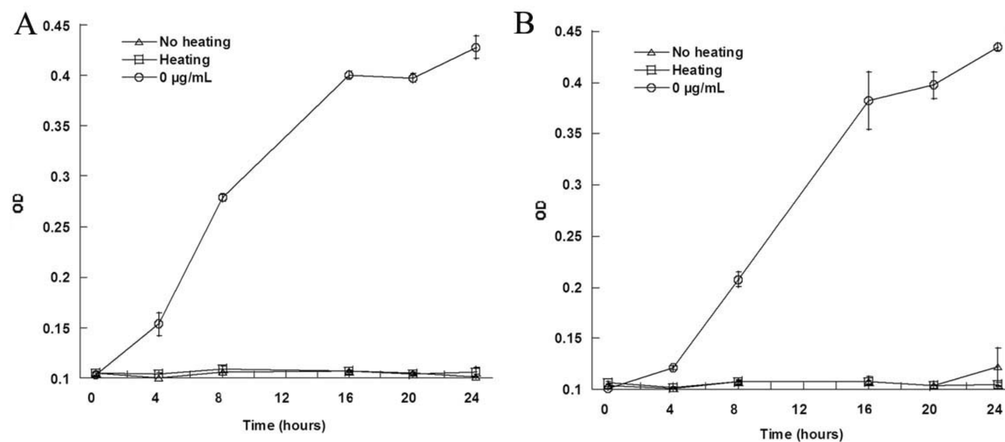

Figure 6. Antibacterial activity of the purified pBD2 against *Escherichia coli* (**A**) and *Staphylococcus aureus* (**B**) after heat treatment. The purified pBD2 (80 µg/mL) was heated at 80°C for 30 min and cultivated with both *E. coli* and *S. aureus* for 24 h. Absence of peptide and purified pBD2 without heat treatment were used as controls. The growth of bacteria was measured by optical density at 630 nm (reference 405 nm) using a microplate reader after incubation for different times. All assays were carried out in triplicate.

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DISCUSSION

Multi-resistant bacteria pose a serious global problem and affect the morbidity and mortality of infected pigs. Studies have shown that pBD2 has high antimicrobial activity against bacteria including multi-resistant bacteria isolated from sick foul (Li et al., 2013). These studies suggest that pBD2 is a promising candidate for antibiotics. The practical application of pBD2, however, is dependent on its availability in sufficient quantities and the natural resource of pBD2 is limited. Therefore, it is important to develop methods to increase the yield of pBD2 expression.

In recent years, some antimicrobial peptides have been successfully cloned and expressed in heterologous host cells, especially in *E. coli*. *E. coli* possesses superior characteristics for expressing target genes, including fast, high-density cultivation, ease of purification, and cost-effectiveness. Despite its many advantages, the efficient expression of different genes in *E. coli* is not trivial. For example, it is known that some eukaryotic genes are poorly expressed in *E. coli* (Zahn, 1996; Hannig and Makrides, 1998; Roche and Sauer, 1999). It is predicted that more than 40% of human genes would express poorly when transformed into *E. coli* (Plotkin and Kudla, 2010). Their poor expression is due to rare codons that affect protein expression level, decrease translational efficiency (Hannig and Makrides, 1998; Sørensen and Mortensen, 2005; Plotkin and Kudla, 2010), and even lead to translational errors or inhibit protein synthesis and cell growth (Zahn, 1996; Sørensen and Mortensen, 2005).

In the current study, the codon was optimized by the “one code-one amino acid” method to obtain high expression of pBD2 in *E. coli*. By optimization, rare codons in *pBD2* were replaced by preferred codons for *E. coli*, the GC content was reduced, and the mRNA secondary structure was minimized. In addition, the optimized *pBD2* gene was successfully cloned and expressed in *E. coli*, and the protein expression level was about 4-6 times higher than that from the native gene. Our results are consistent with previous reports showing that codon optimization can enhance expression and make translation proceed more quickly and efficiently (Gvritishvili et al., 2010; Tang et al., 2011; Wang et al., 2010, 2012; Dai et al., 2013). High expression of pBD2 would be valuable for its further research and application, and would compensate for its limited natural abundance. In this study, the His-Tag at the N-terminal of pBD2 was not removed by enterokinase. It has been reported that the fusion defensin with the His-Tag shows similar antimicrobial activities to native defensin (Li et al., 2005; Zhao and Cao, 2012), and that defensin with tags including the His-Tag have antibacterial activity (Ma et al., 2008, 2009; Wu et al., 2011). Leaving the His-Tag intact would reduce the costs because the steps of cleavage and re-purification would be eliminated.

There are two ways to optimize the codon. One is the “one code-one amino acid” method, which has been used extensively in previous studies. The other is the “systematic optimization” or “codon randomization” method, which has been used recently (Menzella, 2011; Liu et al., 2012). Both methods are based on synonymous codon substitution in which rare codes are replaced with bias codes in the host. The “systematic optimization” method designs genes using software comprising a suite of integrated tools, and it has the advantage of being capable of designing long genes (even more than 5 kb) (Jayaraj et al., 2005). Since pBD2 only contains 37 amino acids (111 bp in length for DNA), the “one code-one amino acid” method was chosen in this study. In addition to codon optimization, other methods have been used to make the translation more efficient, such as increasing the content of rare tRNAs and development of new vectors or hosts. However, codon optimization was employed in the current study

because it is a cost-effective method of increasing pBD2 expression.

It has been reported that synonymous codon substitutions influence not only gene expression but also protein structure and function (Hu et al., 2013; Zhou et al., 2013). For example, Zhou et al. (2013) reported that codon optimization increases FREQUENCY protein (FRQ) expression, alters FRQ phosphorylation and stability, and impairs functions in the circadian feedback loops. Hu et al. (2013) also reported that 342 codon variants of scFv antibody differed significantly in protein solubility and functionality without amino acid substitution. In this study, the pBD2 protein expressed by the optimized gene had antimicrobial activity against Gram-positive *S. aureus* and Gram-negative *E. coli*. Therefore, the function of fused pBD2 protein is similar to that of pBD2 produced from the native gene (Li et al., 2013). The results also show that pBD2 has low hemolytic activity and high thermal stability, similar to those of pBD2 from the native gene (Li et al., 2013). Together, our data imply that codon optimization does not alter the function of pBD2.

In conclusion, the sequence encoding pBD2 was optimized and expressed in *E. coli*. The expressed recombinant pBD2 protein level was about 4-6 times greater than that from the native gene, based on total protein expression. The expressed pBD2 protein showed antimicrobial activity against *E. coli* and *S. aureus*, weak hemolytic activity, and high thermal stability, which were similar to those of pBD2 produced from the native gene. These results suggest that codon optimization can enhance pBD2 expression in *E. coli* without altering its function.

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REFERENCES

Bai Y, Liu S, Jiang P, Zhou L, et al. (2009). Structure-dependent charge density as a determinant of antimicrobial activity of peptide analogus of defensin. *Biochemistry* 48: 7229-7239.

Chen J, Qi S, Guo R, Yu B, et al. (2010). Different messenger RNA expression for the antimicrobial peptides beta-defensins between Meishan and crossbred pigs. *Mol. Biol. Rep*. 37: 1633-1639.

Dai Z, Chen Y, Qi W, Huang L, et al. (2013). Codon optimization increases human kallistatin expression in *Escherichia coli*. *Prep. Biochem. Biotechnol.* 43: 123-136.

Grosjean H and Fiers W (1982). Preferential codon usage in prokaryotic genes: the optimal codon-anticodon interaction energy and the selective codon usage in efficiently expressed genes. *Gene* 18: 199-209.

Gustafsson C, Govindarajan S and Minshull J (2004). Codon bias and heterologous protein expression. *Trends Biotechnol.* 22: 346-353.

Gvritishvili AG, Leung KW and Tombran-Tink J (2010). Codon preference optimization increases heterologous PEDF expression. *PLoS One* 5: e15056.

Hancock RE (2001). Cationic peptides: effectors in innate immunity and novel antimicrobials. *Lancet Infect. Dis.* 1: 156-164.

Hannig G and Makrides SC (1998). Strategies for optimizing heterologous protein expression in *Escherichia coli*. *Trends Biotechnol.* 16: 54-60.

Hu H, Yu B and He Q (2011). Expressing of porcine beta-defensin-2 mature peptide in the yeast. *Wei Sheng Wu Xue Bao* 51: 704-709.

Hu S, Wang M, Cai G and He M (2013). Genetic code-guided protein synthesis and folding in *Escherichia coli*. *J. Biol. Chem.* 288: 30855-30861.

Huang L, Leong SS and Jiang R (2009). Soluble fusion expression and characterization of bioactive human beta-defensin 26 and 27. *Appl. Microbiol. Biotechnol.* 84: 301-308.

Jayaraj S, Reid R and Santi DV (2005). GeMS: an advanced software package for designing synthetic genes. *Nucleic Acids*

Res. 33: 3011-3016.

Kane JF (1995). Effects of rare codon clusters on high-level expression of heterologous proteins in *Escherichia coli*. *Curr. Opin. Biotechnol.* 6: 494-500.

Li C, Ruan H, Chen Z, Chen Q, et al. (2005). Expression of recombinant human β defensin 3 in *E. coli* and its antimicrobial activity analysis. *Chin. J. Biochem. Mol. Bio.* 21: 705-708.

Li CL, Zhao YC, Song XY, Huang XX, et al. (2013). Molecular cloning, expression and characterization of the porcine beta defensin 2 in *E. coli*. *Protein Pept. Lett.* 20: 715-723.

Liu H, Li J, Du G, Zhou J, et al. (2012). Enhanced production of α-cyclodextringlycosyltransferase in *Escherichia coli* by systematic codon usage optimization. *J. Ind. Microbiol. Biotechnol.* 39: 1841-1849.

Ma D, Wang R, Liao W, Han Z, et al. (2009). Identification and characterization of a novel antibacterial peptide, avian beta-defensin 2 from ducks. *J. Microbiol.* 47: 610-618.

Ma DY, Liu SW, Han ZX, Li YJ, et al. (2008). Expression and characterization of recombinant gallinacin-9 and gallinacin-8 in *Escherichia coli*. *Protein Expr. Purif.* 58: 284-291.

Menzella HG (2011). Comparison of two codon optimization strategies to enhance recombinant protein production in *Escherichia coli*. *Microb. Cell Fact.* 10:15.

Park SY, Kim B, Lee S, Oh M, et al. (2014). Increased 2,3-butanediol production by changing codon usages in *Escherichia coli*. *Biotechnol. Appl. Biochem.* doi:10.1002/bab.1216.

Peschel A and Sahl HG (2006). The co-evolution of host cationic antimicrobial peptides and microbial resistance. *Nat. Rev. Microbiol.* 4: 529-536.

Plotkin JB and Kudla G (2010). Synonymous but not the same: the causes and consequences of codon bias. *Nat. Rev. Genet.* 12: 32-42.

Roche ED and Sauer RT (1999). SsrA-mediated peptide tagging caused by rare codons and tRNA scarcity. *EMBO J.* 18: 4579-4589.

Sang Y, Patil AA, Zhang G, Ross CR, et al. (2006). Bioinformatic and expression analysis of novel porcine beta-defensins. *Mamm. Genome* 17: 332-339.

Sang Y, Ruchala P, Lehrer RI, Ross CR, et al. (2009). Antimicrobial host defense peptides in an arteriviral infection: differential peptide expression and virus inactivation. *Viral Immunol*. 22: 235-242.

Schulz A, Klüver E, Schulz-Maronde S and Adermann K (2005). Engineering disulfide bonds of the novel human beta-defensins hBD-27 and hBD-28: differences in disulfide formation and biological activity among human beta-defensins. *Biopolymers* 80: 34-49.

Selsted ME and Ouellette AJ (2005). Mammalian defensins in the antimicrobial immune response. *Nat. Immunol.* 6: 551-557.

Shi J, Zhang G, Wu H, Ross C, et al. (1999). Porcine epithelial beta-defensin 1 is expressed in the dorsal tongue at antimicrobial concentrations. *Infect. Immun.* 67: 3121-3127.

Sørensen HP and Mortensen KK (2005). Advanced genetic strategies for recombinant protein expression in *Escherichia coli*. *J. Biotechnol.* 115: 113-128.

Tang L, Jiang R, Zheng K and Zhu X (2011). Enhancing the recombinant protein expression of halohydrin dehalogenase HheA in *Escherichia coli* by applying a codon optimization strategy. *Enzyme Microb. Technol.* 49: 395-401.

Veldhuizen EJ, Rijnders M, Claassen EA, van DijkA, et al. (2008). Porcine beta-defensin 2 displays broad antimicrobial activity against pathogenic intestinal bacteria. *Mol. Immunol*. 45: 386-394.

Wang Q, Mei C, Zhen H and Zhu J (2012). Codon preference optimization increases prokaryotic cystatin C expression. *J. Biomed. Biotechnol.* doi: 10.1155/2012/732017.

Wang X, Li X, Zhang Z, Shen X, et al. (2010). Codon optimization enhances secretory expression of *Pseudomonas aeruginosa* exotoxin A in *E. coli*. *Protein Expr. Purif.* 72: 101-106.

Wu J, Wang C, He H, Hu G, et al. (2011). Molecular analysis and recombinant expression of bovine neutrophil beta-defensin 12 and its antimicrobial activity. *Mol. Biol. Rep.* 38: 429-436.

Zahn K (1996). Overexpression of an mRNA dependent on rare codons inhibits protein synthesis and cell growth. *J. Bacteriol.* 178: 2926-2933.

Zhang D, Sun L, Yang L and Liu W (2010). Fusion expression and bioactivity comparison of porcine beta-defensin-2 and porcine interferon-gamma in *Pichia pastoris*. *Sheng Wu Gong Cheng Xue Bao* 26: 1652-1659.

Zhang G, Ross CR and Blecha F (2000). Porcine antimicrobial peptides: new prospects for ancient molecules of host defense. *Vet. Res.* 31: 277-296.

Zhao P and Cao G (2012). Production of bioactive sheep beta-defensin-1 in *Pichia pastoris*. *J. Ind. Microbiol. Biotechnol.* 39: 11-17.

Zhou M, Guo J, Cha J, Chae M, et al. (2013). Non-optimal codon usage affects expression, structure and function of clock protein FRQ. *Nature* 495: 111-115.