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RESEARCH ARTICLE

# Antibacterial Activity of Recombinant Porcine β-Defensin 2

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**A B S T R A C T**

To express and purify the recombinant porcine β-defensin 2 (rpBD-2) using yeast *Pichia Pastoris* expression system and in vitro characterization of it's antibacterial activity. rpBD-2 was expressed and then purified using methylotrophic yeast *Pichia Pastoris* expression system. To assess its antimicrobial activity against *Escherichia coli, Salmonella paratyphi A, Pseudomonas aeruginosa, Staphylococcus epidermidis, Staphylococcus aureus, Bacillus pumilus,* and *Bacillus subtilis,* liquid growth inhibition assay was used. The antibacterial activity was further kinetically evaluated against representative bacterial strains *E. coli* and *S. aureus.* The hemolysis activity of rpBD-2 was also checked against porcine erythrocytes. rpBD-2 was expressed and purified successfully using yeast *Pichia Pastoris* expression system. The yield of final product was 9 mg per liter of the harvested supernatant. rpBD-2 possessed antimicrobial activity against *E. coli, S. aureus, S. epidermidis, B. subtilis,* and *B. pumilus* at 33.3-66.6 μM and killed all *E. coli* and *S. aureus* within 30 min. Negligible hemolytic activity was observed against porcine erythrocytes. We have successfully developed rpBD-2 as an effective antibacterial peptide against both gram-positive and negative bacteria. rpBD-2 will be a potential novel therapeutic agent against bacterial infection.



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## INTRODUCTION

Defensins, a kind of antimicrobial peptides (AMPs), having a crucial role in the mammals immune systems that provides protection against potential pathogens. Classic mammalian defensins contains 29 to 45 amino acids, three disulphide bonds that formed by the presence of six cysteine residues and a β-sheet structure (Mcdermott, 2004). Considering the spatial distribution of the six cysteine residues and their disulfide bonds pairing, mammalian defensins are divided into α-, β- and θ-defensins (Selsted and Ouellette, 2005). The defensins have a wide antimicrobial spectrum, with the capacity to repel the assault of diverse infectious agents including viruses, bacteria, parasites and fungi (Peng *et al*., 2016). Their bactericidal activity depends on the disruption of the bacterial membrane and increase the permeability by forming non-specific electrostatic interactions with the membrane lipid components (Masuda *et al*., 2018). Moreover, defensins can interact with neutrophils, T cells, macrophages, and epithelial cells that induces inflammatory mediators production such as IL-8, IL-6 and IL-1β, which are involved in the inflammatory reaction (Fusco *et al*., 2017).

β-defensins, the evolutionarily oldest defensins, consist of 30-45 residues of amino acid that includes 5-12 positively charged residues such as arginine and lysine. Typical structure of β defensins composed of an alpha helix with three beta sheets (Huang *et al*., 2015). The β-defensins are either constitutively expressed or induced in the skin and the mucosal surfaces of airways, digestive tract, and urogenital tract for inflammatory and infectious diseases (Elahi *et al*., 2006; Sang *et al*., 2006). Host defence peptides, enhances the total immune response including both innate as well as adaptive immunity, enhances chemoattraction of both antigen presenting and phagocytic cells, regulate host cytokine response, effect the function of both B and T lymphocytes including activation of the B-cells and production of antibodies, and function of the cytotoxic T-cell, natural killer cells and T helper cells (Allaker, 2008).

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So far, β-defensins have been reported for pigs only (Sang *et al*., 2006; Sang and Blecha, 2009), and 29 β-defensin gene-like sequences were identified in the porcine genome (Min-Kyeung *et al*., 2012). β-defensin 2, as a member of the β-defensin family, is expressed in the porcine intestines and is speculated to be the porcine orthologue of human β-defensins 1 (Sang *et al*., 2006; Veldhuizen *et al*., 2007). The porcine β-defensin 2 shows preferentially attack gram-positive and negative as well as multi-resistant bacteria, which indicate that β-defensin 2 has a crucial role in the innate immune response of porcine intestine (Veldhuizen *et al*., 2008). It has been shown that, administration of β-defensin 2 ameliorated signs and symptoms of inflammation, maintained the integrity of the intestinal mucosal barrier (Han *et al*., 2015), promoted the piglet growth, increased average daily feed intake and in the duodenum and jejunum increase in the intestinal villus height that causes gain in their average daily and overall body weight and reduced diarrhea incidence of the piglets (Peng *et al*., 2016; Xu *et al*., 2016). In addition, the porcine β-defensin 2 could prevent the infection of bacteria from pigs (Yang *et al*., 2015; Tang *et al*., 2016). These results demonstrate that β-defensin 2 could be an alternative to the traditional antibiotic feed or novel therapeutic drugs for intestinal infections of pigs.

Our previous study has proved that porcine β-defensins-2 over-expressed in lungs and intestine of pigs infected by Porcine respiratory and reproductive syndrome virus. The aim of this study is to establish a *Pichia Pastoris* expression system for producing the recombinant porcine β-defensins-2 (rpBD-2), to investigate the antibacterial effect and hemolytic action of rpBD-2 *in vitro*.

## MATERIALS AND METHODS

**Plasmid construction:** The pBD-2 mRNA sequence was obtained from the National Center for Biotechnology Information (Accession: AY506573.1). The Invitrogen (Shanghai, China), synthesized the *Xho*I and *Eco*RI sites carrying codon-optimized pBD-2 DNA. At the C-terminus Six histidines (6× His tag) were added, for facilitation of downstream purification. Using *Xho*I+*Eco*RI and Gel Extraction Kit (OMEGA bio-tek, Georgia, USA) to cut the synthesized pBD-2 and extract it respectively. The extracted product was cloned into digested pwPICZalpha vector (*Xho*I- pBD-2 -*Eco*RI).

**Expression and Purification of protein:** The detailed protocols used for the Expression and Protein Purification was as previously described by Fan *et al*. (2016).

**Western blotting:** The purified rpBD-2 was treated with non-reducing and reducing loading buffer, separated by electrophoresis through SDS-PAGE, Commassie Blue Fast Staining and electro-transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore, Massachusetts, USA) at 60 V. Using nonfat dry milk (5%) in Tris-buffered saline (1×) and 0.02% Tween 20 (TBST) with shaking for 1 h to block the membrane and then at room temperature, incubated the membrane for 2 h with shaking using mouse anti-His monoclonal antibody (1:500) (BGI, Beijing, China). Washed three times with TBST, the membrane was again incubated for 1 h and at 37°C with shaking using rabbit anti-mouse immunoglobulin G (IgG)-horseradish peroxidase (HRP) (1:10,000) (CWBIO, Beijing, China). Following the manufacturer's instruction, electrochemiluminescence (ECL) (CWBIO, Beijing, China) was used for the detection of proteins.

**Antimicrobial activity assay:** The rpBD-2 antimicrobial activity was assessed against a panel of microorganisms including *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella paratyphi A*, *Staphylococcus epidermidis* and *aureus*, *Micrococcus luteus*, *Bacillus pumilus* and *subtilis.* Liquid growth inhibition assays were used to determine the minimal bactericidal concentration (MBC) and minimal growth inhibition concentration (MIC) as previously described (Peng *et al*., 2010; Haidong *et al*., 2013). Shortly, using PBS (pH 7.4) to dilute the bacteria in to $1 \times 10^4$ CFU. Composition of the assay mixture was, diluted bacterial suspension (30 µL), diluted purified peptide (50 µL) and culture media (20 µL). After incubation at 30°C and for 24 h, MIC was calculated as the lowest peptide concentration yielding no detectable growth. At 37°C the aliquots mixtures were then plated for 24 h on the corresponding agar plates and the MBC value was determined as the lowest concentration that killed more than 99.9% of bacteria. Every assay was performed in triplicate.

**Time-kill curve studies:** As previously described (Haidong *et al*., 2013), using *E. coli* and *S. aureus* bactericidal or bacteriostatic effect was determined and as mentioned above, 66.65 µM rpBD-2 was incubated with the bacteria. From the mixture, 5 µL at various time points was taken, diluted serially using PBS (pH 7.4), plated on nutrition broth agar, at 37°C incubated for 24 h and then counted the colonies. Relative to the CFU obtained in the control (100% CFU at 0 min), the % CFU was calculated.

**Hemolytic assay:** Using heparinized tube, collect Porcine fresh blood, centrifuged for 10 min at 800 *g*, using cold PBS (pH 7.4), gently washed the pellet three times, resuspended and adjusted the concentration to 8%. Using 96-well microtiter plate, erythrocyte suspension (100 µL) was added along with different concentrations of rpBD-2 solution to each well and incubated for 1 h at 37°C. Triton-X 100 (0.2%) and PBS were used as positive and negative controls respectively. After centrifugation (for 10 min at 1000 *g*), the released supernatant hemoglobin was measured by microplate reader (ELx808; Gene Co., Ltd., Hong Kong, China) at 490 nm (Yan *et al*., 2012).

## RESULTS

**Expression and purification of the rpBD-2:** rpBD-2 was cloned and expressed in yeast *Pichia Pastoris* X33 strain. For the first step of purification, ProteinIso™ Ni-NTA resin was used. As shown in Fig. 1, most of the target protein was eluted with 500 mM imidazole. Based on the calculated PI=9.10, a strong cation exchange resin Poros® 50 HS was chosen for the 2nd step of purification. The pure soluble rpBD-2 was eluted with 200 mM sodium

borate (Fig. 2). SDS-PAGE analysis revealed the dispersed bands under non-reducing condition. A single band was observed under the reducing condition with both SDS gel and Western blot analysis (Fig. 3). Taken together, using yeast *Pichia Pastoris* expression system, rpBD-2 was successfully expressed. More than 95% of purified rpBD-2 was obtained following two steps purifications. The final yield was 9 mg per liter of the original harvested supernatant. The data demonstrated that the disulfide bonds were formed between the rpBD-2 molecules.

**Antimicrobial activity analysis of the rpBD-2:** Minimum bactericidal concentration (MBC) and Minimal inhibitory concentration (MIC) assays were performed to assess the antimicrobial activity of the rpBD-2 against five Gram-positive bacterial strains (*S. epidermidis, S. aureus, B. subtilis, B. pumilus* and *M. luteus*) and eight strains of bacteria including three Gram-negative bacterial strains (*E. coli, P. aeruginosa* and *S. paratyphi A*) As shown in Table 1, Antimicrobial activity of rpBD-2 was observed against one Gram-negative bacterial strain, *E. coli* (MIC 33.3-66.6 µM, MBC >66.6 µM) and four Gram-positive bacterial strains including *S. aureus* (MIC 33.3-66.6 µM, MBC 33.3-66.6 µM), *S. epidermidis* (MIC 33.3-66.6 µM, MBC 33.3-66.6 µM), *B. subtilis* (MIC 33.3-66.6 µM, MBC 33.3-66.6 µM) and *B. pumilus* (MIC 33.3-66.6 µM, MBC >66.6 µM). rpBD-2 is highly effective against Gram-positive bacteria compared to Gram-negative one.

The bactericidal activity of rpBD-2A was further kinetically evaluated using a highly sensitive Gram-positive bacterial strain *S. aureus* and *a* Gram-negative bacterial strain *E. coli.* To assess the bactericidal activity of this peptide, plating cultures and after incubation at 37°C for 24 h, CFUs were counted that show that approximately 35, 59 and 100% *E. coli* and approximately 30, 77 and 100% *S. aureus* were killed by purified rpBD-2 after 10, 20 and 30 min of incubation respectively (Fig. 4).

**Hemolytic activity of the rpBD-2:** The hemolytic activity of rpBD-2 was assessed by the level of breakdown of porcine erythrocytes. In Fig. 5, porcine erythrocytes weak hemolysis was observed at 33.33 µM. Hemolysis was less than 10% at 66.65 µM, at which it exhibited potent antibacterial activity. rpBD-2, hence had very low hemolytic activity and suitable to be use as an antibiotic.

## DISCUSSION

Defensins are cysteine-rich cationic AMPs with a molecular weight between 2 to 6 kDa, having a broad spectrum antimicrobial activity that kills bacteria, viruses and fungi. Furthermore, defensins have the ability of linking innate and adaptive immune responses in higher organisms. It acts as a signaling molecule in the immune system and also as a chemotactic agent for T lymphocytes and immature dendritic cells. So, defensins have both anti-microbial and immunomodulatory effect (Choi *et al*., 2012).

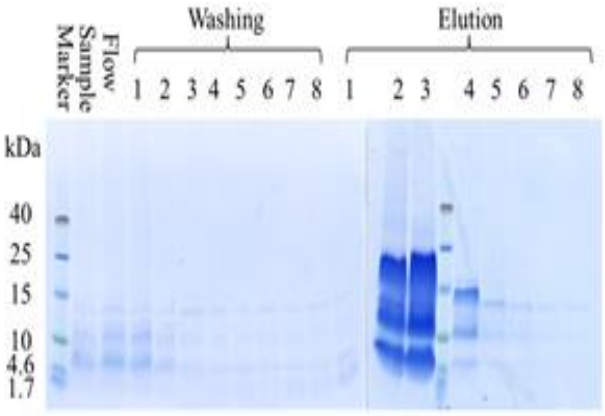


**Fig. 1:** First step purification using ProteinIso™ Ni-NTA resin.

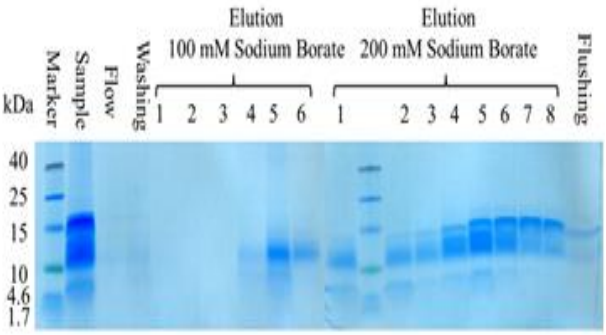


**Fig. 2:** Second step purification using strong cation exchange resin Poros® 50 HS.

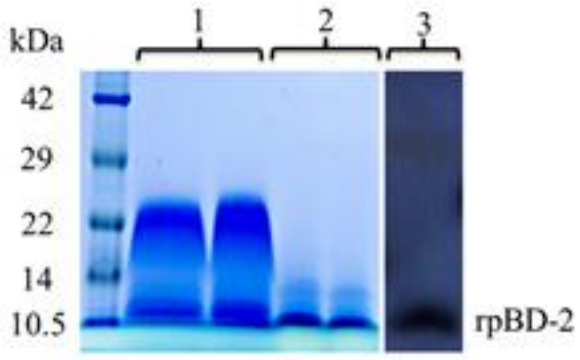


**Fig. 3:** SDS-PAGE and western blot analysis of the rpBD-2 final product. Lane 1: SDS-PAGE analysis of the non-reducing loading buffer treated rpBD-2; Lane 2: SDS-PAGE analysis of the reducing loading buffer treated rpBD-2; Lane 3: western blot analysis of the rpBD-2 using anti-6×His tag mAb.

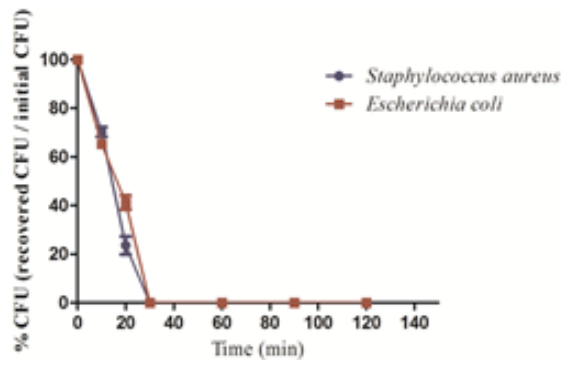


**Fig. 4:** Kinetics of killing *E. coli* or *S. aureus* by the rpBD-2. The percentage of CFU was defined relative to the CFU obtained in the control (100 % at 0 min).

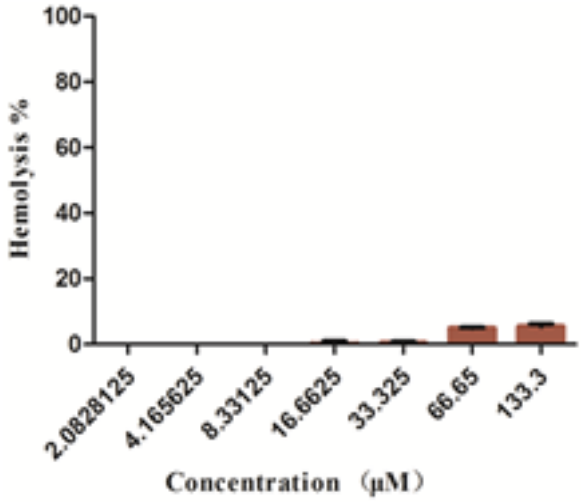


**Fig. 5:** The hemolytic activity of the rpBD-2 was measured by the release of hemoglobin from porcine erythrocytes. The erythrocytes were incubated with different concentrations of rpBD-2 at 37°C for 60 min. 0.2% Triton-X 100 and PBS were used as positive and negative controls, respectively.

**Table 1** Antimicrobial activity of the rpBD-2

| Microorganisms | CMCC no.[a] | MIC (b-c μM) | MBC (d-e μM) |
|---|---|---|---|
| *Escherichia coli* | 44102 | 33.3-66.6 | 33.3-66.6 |
| *Salmonella paratyphi A* | (B) 50001 | NT[f] | NT |
| *Pseudomonas aeruginosa* | (B) 10104 | NT | NT |
| *Staphylococcus aureus* | 26003 | 33.3-66.6 | 33.3-66.6 |
| *Staphylococcus epidermidis* | (B) 26069 | 33.3-66.6 | 33.3-66.6 |
| *Bacillus subtilis* | 63501 | 33.3-66.6 | 33.3-66.6 |
| *Bacillus pumilus* | (B) 63202 | 33.3-66.6 | NT |
| *Micrococcus luteus* | (B) 28001 | NT | NT |

a, National Center For Modical Culture Collections number. b, c, MIC values are expressed as the interval of concentration b-c μM, where b is the highest concentration tested at which microbial growth can be observed, and c is the lowest concentration yielding no detectable microbial growth (n= 3). d, e, MBC values are expressed as the interval of concentration d-e μM, where d is the same mean as the b,and e is the lowest concentration tested that inhibited microorganism growth or that killed more than 99.9% of the microorganisms (n=3). f, Not tested.

In the recent study, we expressed porcine β-defensin 2 using *Pichia Pastoris* expression system and tested its antibacterial and hemolytic activity *in vitro*, successfully. It was demonstrated that the recombinant porcine β-defensin 2 can not only inhibit the growth of bacteria (Table 1) but also has low hemolytic activity to porcine erythrocytes (Fig. 5) that make it very suitable to be used as an antibiotic in future.

Previous studies have shown that porcine β-defensin 2 can be expressed in *Pichia Pastoris* but only an inhibitory effect on *Salmonella choleraesuis* A500 strain has been studied (Hu *et al*., 2011), while porcine β-defensin 2, which was expressed in fusion with porcine IFN-γ, shows no antibacterial activity (Zhang *et al*., 2010). (Li *et al*., 2013) obtained a histidine-tagged β-defensin 2 using an *E. coli* expression system, which has a bacteriolytic effect on *Escherichia coli* and *Staphylococcus aureus*. In this study, the histidine-tagged β-defensin 2 was obtained with the *Pichia Pastoris* expression system (Fig. 3). The purified recombinant β-defensin 2 has been tested for the antibacterial and hemolytic activity. The results showed that it has not only an inhibitory effect on the growth of *Escherichia coli* and *Staphylococcus aureus* but can also inhibit the growth of *Staphylococcus epidermidis*, *Bacillus subtilis* and *Bacillus pumilus* (Table 1) which showed that recombinant β-defensin 2 has a wide range antibacterial activity.

β-defensin 2 performs antimicrobial actions by inserting its C-terminal into the cell membrane. Long peptide chains of the cell membrane connected with the C-terminal, that affect the formation of correct spatial structure of the cell membrane, thereby affecting the broad function and structure of the membrane (Zhang *et al*., 2010). Since β-defensin 2 has not been purified from pigs to date, whether the histidine tag affects the antibacterial activity of recombinant β-defensin 2, needs to be explored in future.

**Conclusions:** rpBD was expressed and purified successfully using yeast *Pichia Pastoris* expression system. rpBD-2 is an effective antibiotic peptide against all tested Gram-positive and Gram-negative bacteria. Only weak hemolytic activity was observed.

**Authors contribution:** KF designed the experiment, done the expression and purification part of protein. YA did the antimicrobial and hemolytic activity analysis. AK helps in the write up and editing of the manuscript. All authors statistically analyzed, discussed, critically revised the contents and approved the final manuscript.

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