Journal of Animal and Veterinary Advances 13 (4): 251-256, 2014
ISSN: 1680-5593

Construction of Recombinant pPIC9K-AvBD2 and Expression of Chicken AvBD2 Mature Peptide in *Pichia pastoris*

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Abstract: Avian β-Defensin-2 (AvBD2) is an antimicrobial peptide that plays a significant role in the innate immunity of chickens. In this research, the expression vector pPIC9K-AvBD2 was established and expressed in *Pichia pastoris*. AvBD2 cDNA was cloned from total RNA of chicken lung by Transcriptase-Polymerase Chain Reaction (RT-PCR). The AvBD2 mature peptide gene was amplified from and cloned into a pPIC9K plasmid. Then the recombinant expression vector pPIC9K-AvBD2 was transformed into *P. pastoris* GS115 to express the fusion protein. The result showed that a recombinant secretion expression vector pPIC9K-AvBD2 was successfully constructed and a fusion protein about 3.9 kDa chicken AvBD2 was expressed. Densitometric scanning analysis demonstrated that the expressed protein exaccounted for about 63.7% of the total secreted bacterial protein. The expression products had antimicrobial activity to *Escherichia coli* and *Staphylococcus aureus* in the Assay of Agar Diffusion Method.

Key words: AvBD2 mature peptide, *P. pastoris*, cloning, expression, antibacterial activity

INTRODUCTION

Defensins are a type of Antimicrobial Peptides (AMPs), constitute a large family of small, cysteine-rich, cationic peptides that are capable of killing a broad spectrum of pathogens including various bacteria, fungi and certain enveloped viruses (Ganz, 2003; Milona *et al*., 2007; Cederlund *et al*., 2011). These peptides play a critical role in host defense and disease resistance by protecting the hosts against infections (Lynn *et al*., 2007).

AvBD (Avian β-defensin) is a group beta-defensins that coded by the third chromosome of chicken (Milona *et al*., 2007). All chicken beta-defensins have now been assigned gene names using the term Avian Beta-Defensins (AvBDs). Until now, the sequences of 14 different *AvBDs* genes have been reported. Lynn named Avian beta-defensin as Avian beta-defensin-1~14 (AvBD1~14) according to the named order of NCBI RefSeq Database (Lynn *et al*., 2007). They have a broad spectrum of antimicrobial activity against bacterial, fungi and even some enveloped viruses. They show chemotaxis and cellular factor secretion which may clearly accelerate bacteria eradication speed (Chen and Cao, 2009; Wu and Zhang, 2005). AvBD2 is widely distributed in tissue such as bone marrow, spleen, larynx trachea, tongue, skin, liver, kidneys, pancreas, lungs, bursa of Fabricius and so on (Zhang *et al*., 2004). Evans *et al*. (1994) has studied that AvBD2 can kill *Escherichia coli*, *Salmonella typhimurium*, white suspended animation yeast and *Campylobacter jejuni in vitro*. *P. pastoris* expression system is a new type of yeast expression system of being eveloped in the early 1980s and developed. In recent years, *P. pastoris* expression system was attentionec by more and more people and used widely.

In order to make better use of genetic engineering technology to develop recombinant chicken AvBD2 preparations, this study has selected cDNA sequence of AvBD2 mature peptide as the object to express the target product with a secretion plasmid of *P. pastoris* which will provide an effective way for mass production of defensin and lay a foundation for research in the next step and application in future.

MATERIALS AND METHODS

Bacterial strains, enzymes, plasmid and main chemical reagents: *E. coli* strain DH5α was used as the host for gene manipulation. The yeast strain *P. pastoris* GS115

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was used as the host for heterogeneous expression. *Escherichia coli* (CMCC44102) and *Staphylococcus aureus* (ATCC25923) were used for the antimicrobial assay. The *E. coli* strains DH5α and BL21 (DE3) were purchased from Invitrogen (Beijing, China). The pMD18-T plasmid purchased from Takara Biotech Co., Ltd. (Dalian, China) was used as the cloning vector. The pPIC9K plasmid purchased from Invitrogen (USA) was used to construct expression vectors. All the restriction enzymes, T4 DNA ligase and enterokinase were purchased from Takara Biotech Co., Ltd. (Dalian, China). AMV avian source reverse transcriptase and rTapDNA polymerase were purchased from Shanghai Sangon Biotech Service Co., Ltd. (Shanghai, China). RNAiso Reagent was purchased from Takara Biotech Co., Ltd. (Dalian, China). Gel Extraction Mini Kits, plasmid extraction kits and Protein molecular markers were purchased from Shanghai Sangon Biotech Service Co., Ltd. (Shanghai, China).

Primer design and total RNA extraction: Two pair primers were designed according to the reported *cDNA* gene sequence of AvBD2 (NM-204992) (Han *et al*., 2009) from Genbank. The mature peptide sequence primers (P1: 5'-CGGAATTCCTGTTCTGTAAAGGAGGGTC-3') and P2: 5'-ATAAGAATGCGGCCGCTTATGCATTCC AAGGCCA-3') containing restriction enzyme sites with EcoRI and NotI, respectively and the coding sequence primers (P3: 5'-ATGAGGATTCTTTA CCTGCTTT-3' and P4: 5'-TTATGCATTCCAAGGCCA-3') were synthetized in Shanghai Sangon Biotech Service Co,. Ltd. (Shanghai, China). Then RNAiso Reagent Kits instruction provided by Takara Biotech Co., Ltd. was based to extract the total RNA.

RT-PCR, cloning of AvBD2 cDNA and identification: First chain of cDNA was got using AMV avian source reverse transcriptase. Reverse transcription reaction system of 20 μL was adopted as follows: 10 μL RNA, 2 μL Universal primers OligodT (10 mmol L^{-1}). The mixture was incubated in water at 70°C for 5 min and then chilled on ice for 2 min. Then, the following substances were added into that mixture by order which were 2 μL dNTP (10 mmol L^{-1}), 4 μL 5×Buffer, 1 μL RNases enzyme inhibitor (40 U L^{-1}), 1 μL M-MLV reverse transcriptase (200 U L^{-1}), blended and incubated at 42°C in water for 1 h. Using RT-PCR products as the template, the full length gene was generated by PCR with primers p3 and p4 in the 50 μL PCR System. After it was pre-denatured 5 min at 94°C, the PCR reaction was carried out for 35 cycles, each cycle consisting of denature 50 sec at 94°C, annealing 40 sec at 53°C and extension 50 sec at 72°C reactions finally extended at 72°C for 10 min.

PCR products were linked to pMD18-T clone vector after A tail was added to it and gel extraction then it was transformed into *E. coli* DH5α. A single white colony was picked up in LB plate containing Amp and selected as the template to amplify AvBD2 cDNA. Plasmid of the positive colony was extracted and sent to Shanghai Sangon Biotech Service Co., Ltd. to sequencing for the further identification. The clone vector constructed named as pMD18-T-AvBD2.

Construction of expression vector: Using pMD18-T-AvBD2 as the template, the mature peptide gene of AvBD2 was generated by nested-PCR technique with primers p1 and p2. Then, the mature peptide gene was linked to pPIC9K vector under the action of T4 DNA ligase. After the transformation, filter and verification, *E. coli* containing pPIC9K-AvBD2 recombinant plasmid was obtained. The expression vector constructed named as pPIC9K-AvBD2.

Electroporation and screening of positive yeast recombinant transformants: According to the *P. pastoris* yeast operations manual provided by Invitrogen, yeast competent cells were prepared (Wu and Letchworth, 2004). DNA (5-20 μg) linearized by SacI enzyme and 80 μL competent cells were mixed and tansformed into GS115 by electroporation. The 200 μL transformation liquid was coated on MD plate without histidine. Then, the MD plate was placed in an incubator to culture at 30°C until single colony appeared. Using the single colony as the template, positive yeast recombinant transformants was identified by PCR with primers (5'AOX1: 5'-GACTG GT TC CA AT TGACAAGC-3' and 3'AOX1: 5'-GCAAATGGCATTC TGACATCC-3').

Inducible expression and tricine-SDS-PAGE analysis: A positive yeast recombinant transformant was inoculated to a 250 mL flask containing 50 mL BMGY medium, cultured at 30°C/250 rpm to OD600 = 2-6 (16-18 h) and centrifugated for 5 min at 1500-3000×g to collect thallus at room temperature. Then, the thallus was suspended using 100 mL BMMY medium, induction cultured continuously in flask at 28°C for 5 days. In the induction process, methanol was added into the culture medium after 1 mL sample was taken out to 1.5 mL centrifuge tube every 24 h and concentration of anhydrous methanol was maintained in culture medium for 0.5%. Sample was centrifuged at 12000 rpm for 3 min to collect the expression supernate and thallus precipitation. Finally, Tricine-SDS-PAGE was done to test the expression of recombinant proteins (Schagger, 2006).

Antibacterial activity test of expression supernate: Expression supernatant was placed in the vacuum freeze drying machine for concentrated treatment and the

concentrated products were dissolved in sterile 0.1 M PBS for stand-by. Then, 5×10^5 cfu mL^{-1} indicative bacterial was added into LB medium melted for about 50°C, mixed and the mixture was quickly poured into a plate of 9 cm diameter. Thickness of plate containing bacteria was about 3 mm and there had 4 holes on the plate was laid evenly by the sterile puncher. Then, the AvBD2 expression supernate, empty vector expression supernate, Amp and PBS was added into the hole on plate, respectively 30 μL $hole^{-1}$. The plate was placed in an incubator to incubate at 37°C for 12 h till the bacteriostatic circle appeared.

RESULTS AND DISCUSSION

RT-PCR of AVBD2: Total RNA was extracted from the chicken and the first strand of cDNA was synthesized with reverse transcription. *AvBD2* gene fragment was amplified from cDNA by PCR with specific primers P1/P2 and the PCR product was detected by 1% agarose gel electrophoresis. Finally, a DNA band about 195 bp was observed which was in accordance with the expected result. The result was showed in Fig. 1.

Identification of cloning vector: Positive clone transformant screened was identified by PCR amplification and sequencing identification. As a result was showed in Fig. 2, a DNA band about 195 bp was observed. The homology of sequencing result and Genebank on display was 100% after sequence alignment.

Construction and identification of expression vector: After PCR amplification products of mature peptide were purified, pPIC9K and the purification products were digested with restriction endonuclease EcoRI and NotI, then recombinant plasmid pPIC9K-AvBD2 was constructed using T4 DNA ligase. The construction process was shown in Fig. 3. White single colony grown on plate was picked out to use as a template for PCR identification. Finally, a DNA band about 108 bp shown in Fig. 4 was observed and sequencing result from Shanghai Sangon Biotech Service Co., Ltd. was demonstrated that *AvBD2* mature peptide gene was successfully inserted into the clone sites of the expression vector.

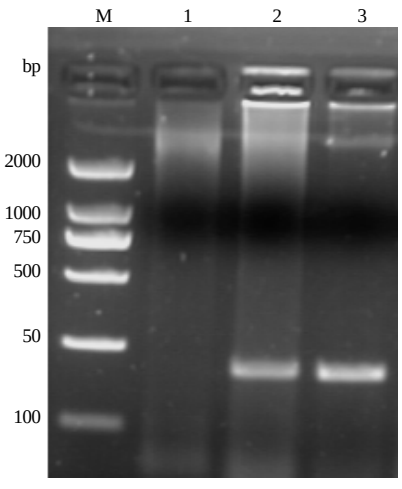

Fig. 1: Identification of PCR-amplified cDNA of AvBD2; M: DL 2000 DNA Marker; 1: Negative control; 2 and 3: PCR-amplified product

Screening and identification of positive yeast recombinant transformants: Positive yeast recombinant transformants was identified by PCR with primers 5'AOX1 and 3'AOX1. The result shown in Fig. 5 indicated that two DNA bands about 2200 and 600 bp were observed when yeast containing AvBD2 mature peptide gene was used as the temperate, two DNA bands about 2200 and 500 bp

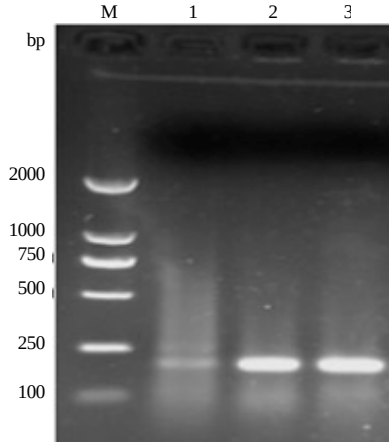

Fig. 2: PCR identification of cloning plasmid; M: DL 2000 DNA Marker; 1: Negative control; 2 and 3: PCR-amplified product

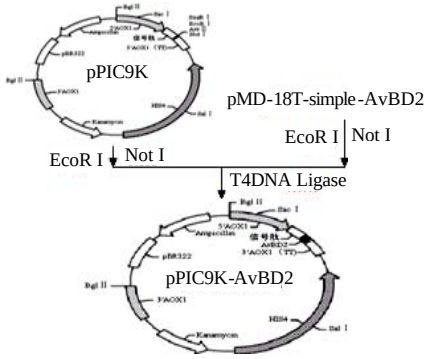

Fig. 3: Scheme for construction of expression plasmid pPIC9K-AvBD2

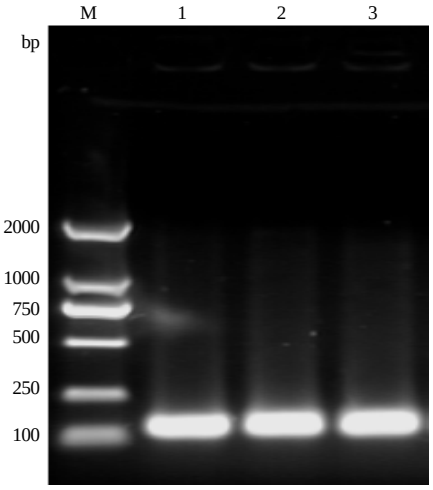

Fig. 4: PCR identification of expression plasmid; M: DL 2000 DNA Marker; 1-3: PCR-amplified product

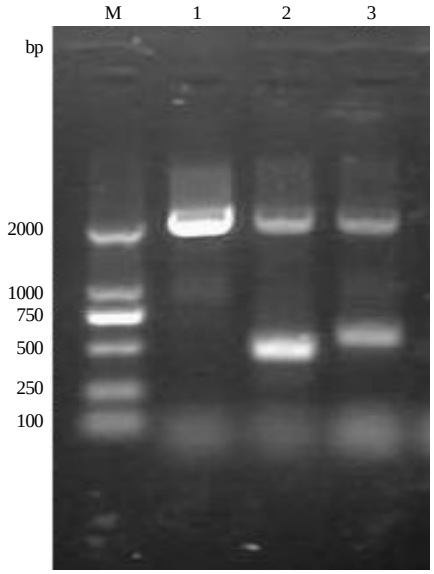

Fig. 5: Identification of *Pichia pastoris* GS115 recombinant by PCR; M: DL 2000 DNA Marker; 1: yeast without any plasmid; 2: yeast containing blank plasmid pPIC9K 3) yeast containing *AVBD2* mature peptide gene

were observed when yeast containing blank plasmid pPIC9K was used as the temperate and one DNA band about 2200 bp was observed when yeast without any plasmid was used as the temperate which was in accordance with the expected result and demonstrated that plasmid was restructured in *P. pastoris* by homologous recombination. This positive yeast recombinant transformant was Mut^+ phenotype from lane 3 PCR product suggested in Fig. 5.

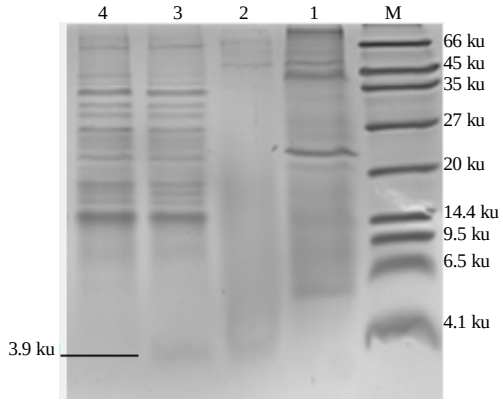

Fig. 6: Tricine-SDS-PAGE analysis of the AVBD2 protein expressed in *Pichia pastoris*; M: Low molecular weight protein Marker; 1: Negative control of supernatant; 2: Product from pPIC9K-AVBD2 in GS115 induction supernatant; 3: Product from pPIC9K-AVBD2 in GS115 induction precipitation; 4: Product from pPIC9K in GS115 induction precipitation

Tricine-SDS-PAGE analysis: After the positive yeast recombinant was induced by methanol, the expressed supernate was concentrated by trichloroacetic acid and detected by Tricine-SDS-PAGE. The result shown in Fig. 6 revealed that the protein molecular weight of AvBD2 mature peptide was about 3.9 kDa, the AvBD2 mature peptide existed in both supernate and precipitation and the optimal induction time was 48 h. Densitometric scanning analysis demonstrated that the expressed protein exaccounted for about 63.7% of the total secreted bacterial protein.

Result of antibacterial activity test: Induced supernatant was tested by antibacterial experiment with pPIC9K/GS115 strains to be used as the blank control group. Results showed that the expressed supernatant had bacteriostatic circle for several target indicating bacteria against the blank control group, as shown in Fig. 7.

P. pastoris has many advantages as a protein expression system, for example, low training costs, high output, high cell density fermentation, ease purification of products and so on. People are increasingly using it as an expression system of foreign genes for the production of proteins and successfully express a variety of exogenous protein (Basanta *et al*., 2010; Long *et al*., 2005).

This study selects pPIC9K plasmid to be the expression vector because it has a α-factor signal peptide sequence and methanol inducible powerful promote AOX1, the former can move secreted proteins out of the cell which is good for purification and detection of

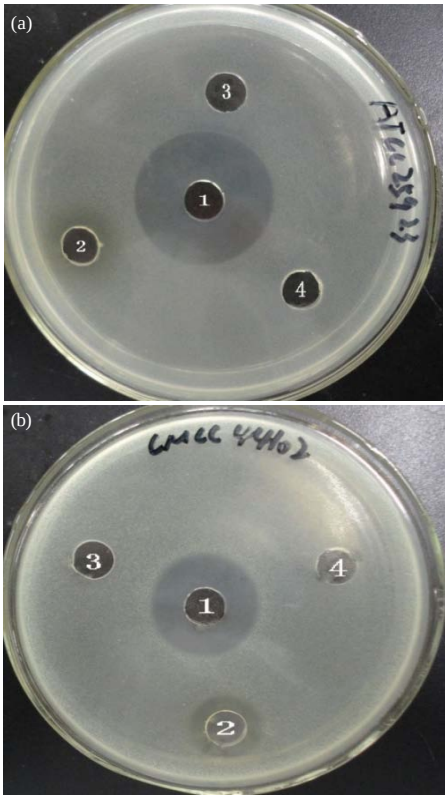

Fig. 7: Bacteriostatic activity detection of expressed supernatant; a) *Staphylococcus aureus* ATCC25923; b) *E. coli* CMCC44102; 1: Amp; 2: AVBD2 mature peptide supernatant; 3: Empty vector control; 4: PBS

products. However, the secreted expression supernatant using this expression system has low protein content in general. Thus, expressed products are concentrated by trichloroacetic acid and tested using protein electrophoresis to get a clear objective band. In addition, expressed products are not concentrated by trichloroacetic acid but by the vacuum freeze drying machine when doing the antibacterial activity test because trichloroacetic acid damage protein activity while freeze drying maintain its activity. The latter AOX1 promote is a powerful induction promote which can strengthen expression of foreign gene (Cereghino and Cregg, 2000; Macauley-Patrick *et al*., 2005). Plasmid can not live in methyl nutritional yeast so, carriers must recombine with homologous region of the yeast genome and then expression of foreign protein stability can be achieved (Wang *et al*., 2008). SacI enzyme is used in this experiment to linearize the expression vector. Under the circumstances *AOX1* gene remains so linear DNA fragment consolidation to the yeast chromosomes by single exchange and the phenotype of generated transformant is Mut^+. However, the AOX1 promoter should be induced by methanol which is flammable and is not apply to large-scale fermentation tank production so you can try construction of yeast expression vectors containing GAP promoter (Menendez *et al*., 2004; Wang *et al*., 2006).

CONCLUSION

The recombinant pPIC9K-AvBD2 is constructed successfully and expression supernatant containing AvBD2 mature peptide shows a better bacteriostatic activity to both Gram-positive and Gram-negative bacteria. The result was according with the report of Sugiarto and Van Dijk (Sugiarto and Yu, 2004; Van Dijk *et al*., 2008) and laid a certain foundation for the next evaluation studies.

ACKNOWLEDGEMENTS

This research was supported by a grant from the National High Technology Research and Development Program of China (863 Program; No. 2006AA10Z320) and Natural Science Foundation of Anhui Province China (11040606M91).

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