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Expression of Antimicrobial Peptide Dybowskin-2CAMa in *Pichia pastoris* and Characterization of its Antibacterial Activity

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Abstract: In this study we used a yeast expression system to express a new antimicrobial peptide dybowskin-2CAMa from the skin cDNA library of Rana amurenisis. The entire coding region of the dybowskin-2CAMa was cloned into the plasmid *pPICZα-A* and then transformed into competent *P. pastoris* X33. The expressed dybowskin-2CAMa was purified from the culture supernatant by Sephadex G-25 and YMC*GEL ODS-A chromatography followed by C18 reverse phased HPLC. The purified peptide exhibited a single band of about 2 kDa when resolved by Tricine-SDS-PAGE. Its exact molecular weight was 2456.46 Da which was consistent with the value predicted from its deduced amino acid sequence. Antimicrobial activity assay showed that the recombinant dybowskin-2CAMa could inhibit the growth of a broad spectrum of bacteria, while displaying very low level of hemolytic activity (≤4% relative to Triton X-100), even at concentration of up to 500 μg/mL.

Keywords: Antimicrobial peptide, antibacterial activity, dybowskin-2CAMa, expression

INTRODUCTION

Antimicrobial Peptides (AMPs) are important components of the host innate immune system and they are present in various organisms and feature a broad-spectrum antimicrobial activity. Such peptides have attracted increasing attention due to their inhibitory activity against bacteria, fungus, virus and other pathogenic microorganisms, providing protection against microbial invasion (Zasloff, 2002).

Over use of antibiotics has led to a growing number of antibiotic-resistant bacteria and currently, there seems to be a lack of antimicrobial peptides available for dealing with these antibiotic-resistant bacteria (Chan *et al*., 2006). Recently, antibacterial peptides have become a hot field in biology, medicine and pharmacy research. At least 1,500 kinds of AMPs have been discovered, which not only can kill gram-positive bacteria, gram-negative bacteria, parasite and fungus, but also play an important role in immune cell recruitment, enhancing innate immune system, promoting wound healing and acting as anti-tumor agent (Ahmad *et al*., 2012; Guaní-Guerra *et al*., 2010). Globally, there are so far more than 10 antimicrobial peptide-based medicines being approved or in the stage of clinical trials (Fang *et al*., 2010).

A new kind of antibacterial peptides called Amurin-7AM was recently cloned from the skin cDNA library of *Rana amurensis* by Xia R, *et al*. from northeast forestry university of China and our laboratory (GenBank number: AEP84582.1). The mature peptide consists of 20 amino acids with the sequence SLGRFQGRFGRR THRKHFVN. Analysis of the amino acid sequences using ExPASy (http://www.cbs.dtu.dk/service/SignalP) showed the peptide has a pI value of about 12.6, an overall charge of +8 and the highest similarity with Dybowskin-2CDYa, a peptide that we isolated and identified from *Rana dybowskii* (Jin *et al*., 2009a). This new peptide was considered a member of the Dybowskin-2 family and therefore it was named dybowskin-2CAMa according to the antibacterial peptides nomenclature. Dybowskin-2 family is a novel kind of antibacterial peptides with little resemblance to others reported frog AMPs, both in composition and amino acid sequence. These peptides have broad antibacterial spectrum, high antibacterial activity and low haemolytic activity (Jin *et al*., 2009a, 2009b).

Direct purification of these peptides from the animals is not only difficult and result in low yield, but also requires sophisticated equipments. On the other hand, chemical synthesis of these peptides is expensive and the synthesized peptides usually have unstable activity. Expression of these peptides in a suitable host such as *E. coli*, appears to offer a cost effective mean for the production of these peptides (Micheelsen *et al*., 2008).

In this study, we describe the cloning of *dybowskin-2CAMa* gene and its expression in the yeast *Pichia pastoris*. The recombinant Dybowskin-2CAMa was also purified and its antibacterial activity analyzed. The result obtained lays a foundation for further study into the structure and function of dybowskin-2CAMa.

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MATERIALS AND METHODS

Escherichia Coli JM109, *Pichia pastoris X33*, the yeast expression vector *pPICZa-A*, restriction enzyme*XhoI*, *XbaI*, *SacI*, T4 DNA ligase and DNA marker were all purchased from TAKARA (Dalian, Liaoning, China). SDS, Tricine, Acrylamide, Bisacrylamide and Low Molecular Weight Standard Protein were obtained from Sigma Chemical Company. Zerocin was from Invitrogen. Sephadex^{TM} G-25 Fine and YMC*GEL ODS-A were obtained from GE Co., Ltd. All other bacterial strains were provided by the National Institute on Drug Abuse of China. Clinical Drug-Resistant Strains were given by the second affiliated hospital of China Medical University. All other chemicals used were of analytical grades.

Methods: Cloning of the gene *dybowskin*-2*CAMa* and construction of *pPICZa-A-D.* Four primers, Fsa1(51 bp), Fsa2 (45 bp), Fsa11 and Fsa21 were designed and synthesized according to the cDNA sequence of the mature peptide dybowskin-2CAMa. The sequences of the primers, the engineered restriction site, complementary sequence and termination codon are shown in Table 1. The gene of dybowskin-2CAMa was cloned by SOE (Splicing by Overlap Extension) PCR using the four primers shown in Table 1. The PCR-amplified *dybowskin*-2*CAMa* gene was digested with *Xho*I and *Xba*I and then inserted into *Xho*I-*Xba*I cut *pPICZa-A* to yield the construct *pPICZa-A-D*. The plasmid was transformed into JM 109 and positive transformants were obtained by on the basis of Zeocin-resistance. The plasmid was purified and the presence of the *dybowskin*-2*CAMa* gene was confirmed by restriction enzyme digestion and DNA sequencing.

Transformation *P. pastoris X*33 with *pPICZa-A-D*. *pPICZa-A-D* was linearized by digestion with *Sac* (which cut at the *AOX1* promoter region) and transformed into *P. pastoris X*33 using the Invitrogen Easy Select Pichia Expression Kit. The empty vector *pPICZa-A* was used as negative control. The transformed *P. pastoris* X33 was plated on YPD agar (yeast extract 1%, peptone 2%, dextrose 2%, 1M glycitol, 1.5% agar) plate containing 100 μg Zeocin/mL and cultured for 2-3 days at 28°C.

Selection and identification of transform ed *P.pastoris* X33. Positive *P. pastoris* X33 transformants were inoculated into YPDS containing 2000 μg Zeocin/mL to select for high-resistant clones. To prove whether the whole *dybowskin*-2*CAMa* gene had been integrated into the yeast chromosome, total DNA of the recombinant *P. pastoris* X33 was extracted and used as template for the amplification using the universal primers for 5' α-factor (5'-GAC TGG TTC CAA TTG ACAAGC) and 3'AOX1 (5′-GCAAATGGCA

Table 1: The primer sequence of antibacterial peptide dybowskin-2CAMa gene clone

Primer name	Primer sequence (5′–3′)
Fsa1	5'CCGCTCGAGAAAAGATCTTTGGGTAGA TTTCAAGGTAGATTTGGTAGAAGA3'
Fsa2	5'ATTTACAAAATGTTTTCTATGAGTTCTT CTACCAAATCTACCTTG3'
Fsa11	5'CCGCTCGAGAAAAGATCTTTGGGTAG3'
Fsa21	5'CTAGTCTAGA*AA*TCATCAATTTACAAA ATGTTTTCT3'

Restriction Enzyme cutting site are shown in bold; double underlined bases are complementary sequences of the two primers; single underlined bases show the restriction site of KEX2; bases with wavy line represent the overlap section of primer Fsa2 and Fsa21; grey highlighted bases show the position of the termination codon; the two italic *'AA'* in Fsa21 were added to maintain correct reading frame

TTCTGACATCC). The resulting amplified DNA fragment was digested with *XhoI* and *XbaI* to confirm the presence of *dybowskin*-2*CAMa* gene insert.

Expression of dybowskin-2CAMa in *P. pastoris X*33*.* Initial expression of dybowskin-2CAMa was carried out in small scale using flask fermentation. Single clones of *P. pastoris* X33 harbouring the *dybowskin*-2*CAMa* gene were each cultured in YPD. The culture supernatant was subjected to Tricine-SDS-PAGE analysis to detect the presence of the peptide. The clone that yielded the highest level of expression was used for large scale expression. For large scale expression, a seed culture was first prepared by culturing a single clone of *P. pastoris* X33 harbouring the *dybowskin*-2*CAMa* gene in YPD and 500 mL of this culture was used to inoculate 5000 mL of fresh YPD in a 10 L fermentor. Fermentation was carried out at 28°C and pH 6, with 30-40% dissolved oxygen. Fermentation was terminated after four days and the fermentation broth was separated from the cells by centrifugation at 12,000× g/4°C for 10 min. The recombinant peptide was purified from the broth as described below.

Purification and identification of expressed *dybowskin*-2*CAMa.* The broth was first filtered through a 0.45-µm nitrocellulose filter and then boiled at 100°C for 3 min followed by cooling in an ice bath for 10 min. It was centrifuged at 4°C for 12000×*g* for 10 min and the supernatant was filtered through a 0.22-µm nitrocellulose filter. EDTA solution was then added to the filtrate to a final concentration of 10 µM to inhibit the degradation of the peptide by metal-dependent proteases. The supernatant was diluted with distilled water (containing 0.02% NaN_3) to a protein concentration of 1mg/mL. It was then loaded onto the Sephadex G-25 (GE. Healthcare) column (2.6×28 cm) pre-equilibrated with distilled water containing 0.02% NaN_3. The column was then eluted with the same buffer at a flow rate of 4 mL/min. The eluent was monitored by absorbance 280 nm. Fraction exhibiting antibacterial activity was concentrated under vacuum and diluted with distilled water containing 0.02% NaN_3 to a protein concentration of 1 mg/mL and then loaded onto a octyldecyl silane chromatography column (1.2×18 cm) packed with YMC*GEL ODS-A. The column was washed with the same buffer and eluted by linear gradient of methanol (0-100%) at a flow rate of 3 mL/min. The eluent was monitored by absorbance at 214 nm. Peak fraction exhibiting antibacterial activity was lyophilized and dissolved in distilled water (containing 0.02% NaN_3) to a protein concentration of 0.5 mg/mL and 200 □ of this material was then applied to a RP-HPLC semi-preparative C18 column (10 by 150mm, Beckman USA). The column was eluted with the following condition at a flow rate of 4 mL/min: 0-10 min, 100%A {0.1% (v/v) Trifluoroacetic Acid (TFA) in distill water}; 10-20 min, 0-100% B (methanol); 20-30 min, 100% B. The eluent was monitored by absorbance at 214 nm. The peak fraction with antibacterial activity was lyophilized and its purity was examined by Tricine-SDS-PAGE whereas its molecular weight was determined by MALDE-TOF-MS.

Antimicrobial activity and hemolysis activity of dybowskin-2CAMa. Bacteria were grown at 37°C in LB medium, harvested while in exponential phase (OD600 nm: 0.6-0.8), centrifuged (8×10^3 g for 10 min), washed with saline (0.15 M NaCl), resuspended in Muller Hinton (MH) broth at the concentration of approximately 2×10^6 CFU/mL and distributed, in triplicate, into 96 well plates (100 μL/well), mixed with increasing concentrations of dybowskin-2CAMa dissolved in sterile distilled water (5-400 μg/mL, 100 μL/well) and incubated at 37°C for 20 h. The minimal peptide concentration at which 100% inhibition of microbial growth was observed, is defined as MIC and determined by measuring the absorbance at 540 nm (BIO-RAD imark14530, US).

Healthy human blood (5 mL) was prepared and the red blood cells were separated from the plasma by centrifugation at 700×g for 10 min and then washed with sterile 0.9% NaCl and centrifuged as before. This washing step was repeated two more times and after the last wash, the all trace of supernatant was removed and the cells were resuspended in 0.9% NaCl solution to yield a 2% (v/v) erythrocyte suspension. Aliquots 100 μL of the cell suspension were dispensed onto a 96-well plate. Working solutions of dybowskin-2CAMa were prepared by two-fold serial dilutions with physiological saline and 100 μL was dispensed into each well containing the cell suspension. For positive control, the cells were treated with 0.1% TritonX-100, whereas for blank control, the cells were treated with 0.9% NaCl solution. The plate was incubated for 1h at 37°C and then centrifuged to pellet the cells. The supernatant from each well was transferred to a new plate and the absorbance of the samples was read at 540 nm using a microtiter plate reader.

RESULTS

Cloning of *dybowskin*-2*CAMa* gene. The entire coding region of the dybowskin-2CAMa was obtained by PCR amplification using the four primers shown in Table 1. The amplified DNA was subsequently cloned into the yeast expression vector *pPICZα-A* and the presence of the *dybowskin*- 2*CAMa* gene in the resulting construct was confirmed by restriction enzyme digestion, whereas the sequence of the gene was confirmed by DNA sequencing.

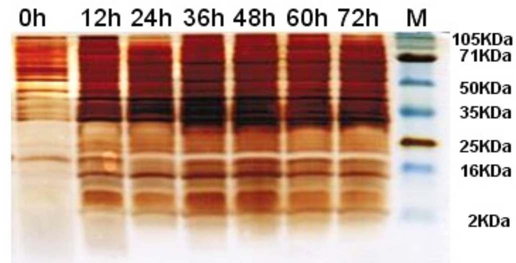

Fig. 1: Tricine-SDS-PAGE analysis of the supernatant from fermentation supernatant of recombinant *P. pastoris* Samples were collected at 12 h intervals after methanol induction, analysed by 16.5% Tricine-SDS-PAGE and stained with Protein sliver staining. 2 KD expression product was detected after 12 h methanol induction M: low molecular weight marker; 0 h-72 h: fermentation time of recombinant *P. pastoris*

Expression of *dybowskin*-2*CAMa*. Expression of *dybowskin*-2*CAMa* was investigated by detecting for the presence of the recombinant peptide in the supernatant of the culture of *P. pastoris* that had the *dybowskin*-2*CAMa* gene integrated into its chromosome. Small scale expression showed that a band of about 2 kDa was present in the supernatant of *P. pastoris* carrying the *dybowskin*-2*CAMa* gene (Fig. 1). One of the clones that successfully expressed the target peptide was used in large scale fermentation to express the peptide for purification.

Identification and purification of recombinant *dybowskin*-2*CAMa* the culture supernatant was subjected to a heat pretreatment step to remove the heat sensitive proteins, thereby enriching the presence of dybowskin-2CAMa. The material was subjected to size-exclusion chromatography using SephadexG-25 column and four peaks were resolved (Fig. 2A). Among these, antibacterial activity was observed for material collected from peak 2 (Fig. 2B). Peak 2 was further chromatographed on a MC*GEL ODS-A column, which resolved four main peaks (Fig. 3a) and only peak 1 exhibited antibacterial activity (Fig. 3b). This peak was further resolved by RP-HPLC using a semi-preparative C18 column. Three peaks were eluted in the water phase (Fig 4A) and only peak 2 exhbitied antibacterial activity (Fig 4B). Tricine-SDS-PAGE analysis of peak 2 revealed a single band of about 2 kD (Fig. 5). The purified peptide was therefore considered to be purified recombinant dybowskin-2CAMa and

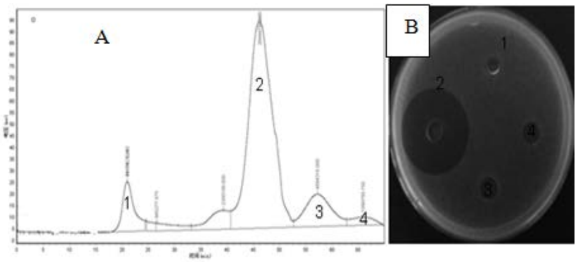

Fig. 2: A: Purification of dybowskin-2CAMa by Sephadex G-25 chromatography; B: Antibacterial activity of the four peak fractions shown in A the processed fermentation broth was loaded onto Sephadex G-25 (GE. Healthcare) column (2.6×28 cm) pre-equilibrated with distilled water containing 0.02% NaN_3. The column was then eluted with the same buffer at a fl T rate of 4 mL/min. The eluent was monitored by absorbance 280 nm and separated to four peaks (A). Among those, antibacterial activity was observed for material collected from peak 2 (B)

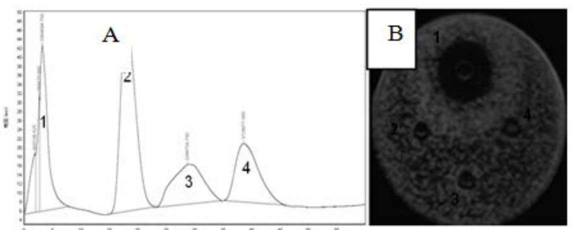

Fig. 3: A: Purification of dybowskin-2CAMa by YMC*GEL ODS-A chromatography; B: Antibacterial activity of the four peak fractions shown in A. Peak 2 collection of Sephadex G-25 chromatography was concentrated under vacuum and diluted with distilled water containing 0.02% NaN_3 to a protein concentration of 1mg/mL and then loaded onto a octyldecyl silane chromatography column (1.2×18 cm) packed with YMC*GEL ODS-A. The column was washed with the same buffer and eluted by linear gradient of methanol (0-100%) at a fl8 rate of 3 mL/min. The eluent was monitored by absorbance at 214 nm and resolved four main peaks (A), and only peak 1 exhibited antibacterial activity (B)

further analysis by MALDE-TOF-MS gave a mass of 2456.46 Da, which is the same as the value calculated from its deduced amino acid sequence.

Antimicrobial activity of recombinant *dybowskin*-2 *CAMa.* The MIC of dybowskin-2 CAMa against different bacteria is shown in Table 2. The peptide was inhibitory against a broad spectrum of bacteria and at the microgram level. The potency of the peptide was similar across the different species of bacteria, although it was most inhibitory against *Shigella enterobacter and least inhibitory against Cedecea* V.

Hemolysis of recombinant dybowskin-2CAMa. Purified dybowskin- 2CAMa displayed little (<4%

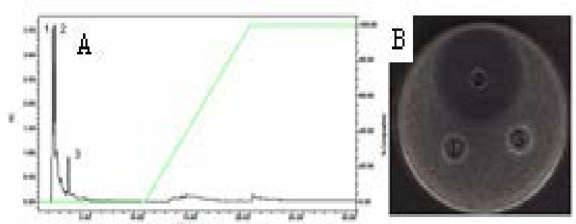

Fig. 4: A: Purification of dybowskin-2CAMa by RP-HPLC with a semi-preparative C18 column chromatography; B: Antibacteria activity of the three peak fractions shown in A Peak 1 collection of YMC*GEL ODS-A chromatography was lyophilized and dissolved in distilled water (containing 0.02% NaN_3) to a protein concentration of 0.5mg/ml and 200 □ of this material was then applied to a RP-HPLC semi-preparative C18 column (10 by 150 mm, Beckman USA). The column was eluted with the following condition at a flow rate of 4 mL/min: 0-10 min, 100%A (0.1% (v/v) Trifluoroacetic Acid (TFA) in distill water); 10-20 min, 0-100% B (methanol); 20-30 min, 100% B. The eluent was monitored by absorbance at 214 nm and three peaks were eluted in the water phase (A) and only peak 2 exhbitied antibacterial activities (B)

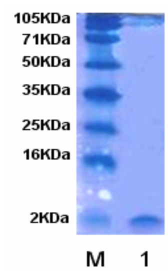

Fig. 5: Tricine-SDS-PAGE analysis of purified recombinant dybowskin-2CAMa Peak 2 collection of RP-HPLC chromatography was lyophilized, dissolved in distilled water and subjected to Tricine-SDS-PAGE analysis. A single band of about 2 kD was shown in the gel. M, low protein marker: 1, purified recombinant dybowskin-2CAMa

Table 2: MIC of dybowskin-2CAMa against different bacteria

Tested strains:	MIC(μmL)
Staphyloccocus aureus	5±0.20
Bacillus subtilis	10±0.25
Staphylococcus auricularis	5±0.34
Bacillus cereus	10±0.12
Bacillus thuringiensis	10±0.80
Bacillus magaterium	20±0.16
Corynebacterium parvum	20±0.37
Corynebacterium crenatum[a]	10±0.59
Bacillus licheniformis	10±0.42
E. coli O157[b]	20±0.38
Enterobacter aerogenes	20±0.36
Cedecea V	40±0.57
Shigella enterobacter	5±0.42
Acinetobacter haemolyticus	30±0.36
Enterobacter sakazakii	20±0.25
Aeromonas hydrophila	20±0.14
Staphyloccocus aureus Mrsa+W	30±0.56
E. coli EBSL	30±0.23
Pseudomonas aeruginosa Z	30±0.74

MIC is the minimal peptide concentration at which 100% inhibition of microbial growth. a, Actinomycetes b, Hemolytic E. coli O157

Table 3: Hemolysis test of recombinant dybowskin-2CAMa

Test sample	Hemolysis (% relative to Triton-X100)
Dybowskin-2CAMa (500μg/mL)	4.17±0.015
Dybowskin-2CAMa (250μg/mL)	3.92±0.009
Dybowskin-2CAMa (125μg/mL)	3.51±0.021
Dybowskin-2CAMa (62.5μg/mL)	2.75±0.021
Dybowskin-2CAMa (31.25μg/mL)	2.4±0.017
Dybowskin-2CAMa (15.6μg/mL)	2.2±0.011
Physiological saline	1.72±0.007

relative to Triton X-100) hemolytic activity at concentrations below 500 μg/mL, with very little increase even at 500 μg/mL (Table 3).

DISCUSSION

Antimicrobial peptides have attracted widespread attention because of their small molecular weight, broad spectrum of antimicrobial activity and inhibition of microbes that does not lead to resistance against the peptides. Antimicrobial peptides have also been used in feed additive, cultivation of transgenic plants and animals, as well as in drug research (Mangoni *et al*., 2008). However, the popularity and application of antimicrobial peptides also face much difficulty because natural antimicrobial peptides are rare and the process of direct extraction of the peptides from the organisms that produce them is complicated and expensive. Production of the peptide by chemical synthesis provide an alternative to large scale production of these peptides, but the activity of the synthesized peptides is difficult to guarantee (Zhao *et al*., 2010). Therefore, finding an efficient and more economical way to produce antimicrobial peptides in large quantity, while maintain a high level of activity and low level of cell toxicity has become the focus of antimicrobial peptide research. Although *Escherichia coli* is the first choice when considering the host for expressing antimicrobial peptides, the low molecular weights of these peptides and their potential toxicity to the host mean that high level of expression is difficult to achieve (Lee *et al*., 2011; Niu *et al*. 2008). Recently, the successful expression of antimicrobial peptides at high level using *P. pastoris* expression system has made it possible to express these peptides in large-scale (Jin *et al*., 2006).

The expression of heterologous proteins in *P. pastoris* can be affected by many factors. These factors include gene-dose, integration site, presence of the signal peptide, transcription level and translation efficiency as well as culture medium, fermentation condition and protein procession and modification in the host strain (Li *et al*., 2007). In this study *P. pastoris* was used as the host for expressing dybowskin-2CAMa. The *dybowskin-2CAMa* gene was first cloned into the vector pPICZa-A and the resulting construct was transformed into *P. pasotris*, which then integrated the *dybowskin-2CAMa* gene into its chromosomal DNA. As the methanol inducible *AOXI* promoter of the vector was also integrated along with the *dybowskin-2CAMa* gene, the expression of the peptide could then be induced with methanol, presenting greater control over its expression. The optimum condition for the expression of *dybowskin-2CAMa* was induction by 1% methanol at 28°C for 48 h. There is a linear relationship between the exogenous gene dose and expression levels (Boettner *et al*., 2007). *AOXI* expression in *P. pastoris* can account for up to 5% of the total mRNA and the content of enzymes expressed under the control of the *AOXI* promoter could reach more than 30% of the total protein (Micheelsen *et al*., 2008). In our case, the expression of *dybowskin-2CAMa* accounted for about 5% of the total proteins produced by *P. pastoris.*

The purification of the recombinant dybowskin-2CAMa was achieved using a combination of different chromatographic media, including gel filtration and reversed phase HPLC. Since *dybowskin-2CAMa* was expressed as a secretory peptide in the fermentation broth, this also made it easier to purify the peptide. Not only was *dybowskin-2CAMa* purified to a homogenous stage, but that the purified peptide still maintained a good level of antimicrobial activity and against a wide spectrum of bacteria. Thus successful purification coupled with maintenance of antimicrobial activity is vital to the production of antimicrobial peptides.

In order to be considered as useful antimicrobial peptide, the peptide must either be non-toxic or exhibit only very low level of toxicity when apply to animals. Hemolysis test has been used as a standard test to assess the toxicity of antimicrobial peptides and dybowskin-2CAMa displayed very low level of hemolysis even at concentration as high as 500 μg/mL (Table 3), making it comparable to *Rana dybowskii*, but superior to brevinin-1and japovin-1 family antimicrobial peptides (Jin *et al*., 2009b). Our study therefore lays a foundation for further research on the antimicrobial mechanism and clinic application of dybowskin-2CAMa.

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