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Short Communication

Expression of antimicrobial peptide Cecropin P1 in *Saccharomyces cerevisiae* and its antibacterial and antiviral activity *in vitro*

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ABSTRACT

Background: Cecropin P1, acting as an antimicrobial, has a broad-spectrum antibacterial activity with some antiviral and antifungal properties. It is a promising natural alternative to antibiotics which is originally isolated from the pig intestinal parasitic nematode *Ascaris suum*. Many studies have shown that Cecropin P1 is helpful for the prevention or treatment of clinical diseases. Therefore, it is very necessary to establish a safe, nontoxic, and efficient expression method of Cecropin P1.

Results: The results indicated that the recombinant protein was about 5.5 kDa showed by Tricine–SDS–PAGE and Western blot. And Cecropin P1 was efficiently secreted and expressed after 12 h of induction, with an increasing yield over the course of the induction. Its maximum concentration was 7.83 mg/L after concentration and purification. In addition, *in vitro* experiments demonstrated that Cecropin P1 not only exerted a strong inhibitory effect on *Escherichia coli*, *Salmonella* sp., *Shigella* sp., and *Pasteurella* sp., but also displayed an antiviral activity against PRRSV NADC30-Like strain.

Conclusions: Collectively, the strategy of expressing Cecropin P1 in *Saccharomyces cerevisiae* is harmless, efficient, and safe for cells. In addition, the expressed Cecropin P1 has antiviral and antibacterial properties concurrently.

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1. Introduction

Overreliance on antibiotics to treat different diseases has led to the development of general drug resistance in clinical setups. Antimicrobial peptides (AMPs) have a unique rupture mechanism, which could be considered an ideal antibiotic substitute for the treatment of clinically resistant bacteria strains [1]. Moreover, AMPs play an important role in host innate immunity, suggesting that they may be developed as novel antivirals [2,3,4] The antibacterial Cecropin P1, a positively charged α-helical peptide [5], is an AMP originally isolated from the pig intestinal parasitic nematode *Ascaris suum*. It is composed of 31 amino acid residues, with a molecular weight of 3339 Da [6], and has a strong alkaline N-terminal and highly hydrophobic C-terminus [7]. The secondary structure, helix–curl–helix, can inhibit and kill most gram-negative bacteria and some gram-positive bacteria [8,9], which makes it suitable for clinical treatment, transgenic research, and as a feed additive [10].

Recombinant expression of genes is the most economical and effective means of obtaining AMPs *in vitro*, including prokaryotic expression systems represented by *Escherichia coli* [11] and eukaryotic expression systems, such as yeast [12,13,14,15]. However, as prokaryotic expression systems do not result in posttranslational modifications and folding, thus a natural secondary structure is not formed that would ensure antibacterial activity. Therefore, the *Saccharomyces cerevisiae* expression system has become one of the most widely used foreign protein expression systems and a major methodology in the biotechnology, food, and pharmaceutical industries [16,17] *S. cerevisiae* is a probiotic with galactose as an inducer, which has the characteristics of safety, fast reproduction, short growth cycle (generally, one

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generation per 1.5~2 h), vigorous metabolism, and abundant nutrition. In addition, its own metabolites, such as amino acids, enhance growth performance and immunity of livestock and poultry [18,19]. Because of this, *S. cerevisiae* has been metabolically engineered to produce target proteins and study the biochemical and biological functions of these proteins [20]. Currently, there are some reports on the expression of AMPs in *S. cerevisiae* [21,22] but no reports on the expression of Cecropin P1 in *S. cerevisiae*. So it is very necessary to establish a safe, nontoxic, and efficient expression method of Cecropin P1.

NADC30-Like is a new mutant strain of porcine reproductive and respiratory syndrome virus (PRRSV). Studies have shown that NADC30-like strains have become the main causative agents of PRRS in China and current commercial PRRSV vaccines cannot provide complete protection to the infection [23,24]. So there is an urgent need for a drug to treat or prevent it. In 2018, one NADC30-like PRRSV strain caused about 60% of sows to miscarry in a pig farm in Sichuan, bringing huge losses to the pig industry. Then, this strain from Sichuan province was successfully isolated on Marc-145. Since Cecropin P1 has antiviral effect, it was used to treat NADC30-like PRRSV strain to fill the gap in prevention and treatment. *E. coli*, *Salmonella* sp., *Shigella* sp., and *Pasteurella* sp. are common bacterial diseases that can infect a variety of animals. The abuse of antibiotics leads to worse and worse effective treatment of these diseases. Cecropin P1 is a natural drug that has inhibitory effect on most gram-negative bacteria. Therefore, studying the expression and antibacterial effect of Cecropin P1 can provide new ideas for clinical bacterial diseases. This study aimed to effectively express the antibacterial Cecropin P1 mature peptide gene in *S. cerevisiae.* While investigating the antibacterial effect of Cecropin P1, we also preliminarily tested whether it has antiviral activity against NADC30-Like PRRSV strain. It laid the foundation for the safe production of Cecropin P1 and the diversity of Cecropin P1 activities and also provided a theoretical basis for the development of new antibacterial and antiviral drugs.

2. Materials and methods

2.1. Strains and chemicals

All strains (*E. coli*, *Salmonella* sp., *Shigella* sp., *Pasteurella* sp., and PRRSV NADC30-Like) were isolated from infected animals and kept in the Animal Quarantine Laboratory of Sichuan Agricultural University, Sichuan, China. Restriction enzymes *Xho* I, *Xba* I, and *Not* I were purchased from TaKaRa Co, Beijing, China. pYES2/CT-α factor was purchased from Changsha Yingrun Biotechnology Co, Changsha China. *S. cerevisiae* was purchased from Invitrogen Co. Ni-NTA-Sefinose Column was purchased from Sangon Biotech Co, Shanghai, China. All other chemicals were purchased from Solarbio Co, Beijing China.

2.2. Codon optimization and synthesis of Cecropin P1 mature peptide gene

S. cerevisiae was used as the expression host [25]. We used the full-length coding sequence of the Cecropin P1 mature peptide gene as a reference from GenBank (GenBank: AB186032.1), and used the GenScript OptimumGene Codon Optimization Analysis online software for codon optimization, including codon adaptation index, frequency of optimal codons, and genomic GC content. The optimized Cecropin P1 gene was synthesized by Suzhou Jinweizhi Biotechnology Co., Ltd, Suzhou, China. A *Xho* I cleavage site, an α-signal peptide, and Kex2 cleavage site were added to the 5′ end of the sequence, and an *Xba* I cleavage site was added to the 3′ end (Fig. 1). The plasmid was named pUC57-CP1.

2.3. Construction of pYES2/CT-α factor-CP1 eukaryotic expression vector

pUC57-CP1 and the expression vector pYES2/CT-α factor were digested by *Xho* I and *Xba* I, respectively, at 37°C for 2 h. The products were predyed with 10× loading buffer then added into 1% agarose gel, and identified by electrophoresis for 15 min, 120 V. The molar ratio of vector to fragment for ligation overnight by T4 ligase was 1:3. Recombinant plasmid was transformed into *E. coli* DH5α, and positive clones were confirmed by polymerase chain reaction (PCR). Positive clones were then sent to Huada Technology Co., Ltd, Wuhan, China for nucleotide sequencing and plasmid extraction for identification by restriction enzyme digestion.

2.4. Transformation of pYES2/CT-α factor-CP1

pYES2/CT-α factor-CP1 and pYES2/CT-α factor were transformed into *S. cerevisiae* INVSc1 competent cells and positive clones were screened using synthetic complete uracil-deficient (SC-Ura) selection medium. DNA templates were prepared by the boil-freeze-cooking method, with positive clones being sent to Huada Technology Co., Ltd. for nucleotide sequencing.

2.5. Induced expression of recombinant Cecropin P1

Positive clones of pYES2/CT-α factor-CP1 and pYES2/CT-α factor were inoculated into 15 mL SC-Ura selection medium (containing 2% glucose), and incubated in a 30°C water bath with shaking for 24 h. Resulting cells were collected by centrifugation and cultured for 2 h in carbon-free SC-Ura selection medium. The cells collected by centrifugation were then transferred to 50 mL yeast extract peptone dextrose (YPD) induction medium for an initial OD_{600nm} value of 0.4 [26,27]. After induction with 2% galactose [28] for 0, 12, 24, 36, and 72 h, respectively, the induction cultures were collected. The supernatant was centrifuged at 12,000× *g* for 20 min at 4°C, filtered through a sterile 0.22 μm millipore membrane filter, and stored at −80°C.

2.6. Purification of recombinant protein and Western blot analysis

The recombinant protein was concentrated with Amicon-Ultra-15 (Millipore). According to the protocol of Ni-NTA-Sefinose Column, the recombinant protein was purified and then transferred to nitrocellulose (NC) membrane after Tricine–SDS–PAGE electrophoresis. Identification by western blot analysis used primary antibodies (3000 times dilution) to the His-tag, and horseradish peroxidase (HRP)-labeled goat anti-mouse antibody IgG (5000 times dilution) as the secondary antibody [29,30]. Recombinant protein concentration was determined with NanoDrop 2000 Spectrophotometer.

2.7. Determination of antibacterial activity of recombinant Cecropin P1

In order to investigate the antibacterial activity of Cecropin P1 *in vitro*, the minimum inhibitory concentrations (MIC) of Cecropin P1 against *E. coli*, *Salmonella* sp., *Shigella* sp., and *Pasteurella* sp. were determined by microdilution test according to Clinical and Laboratory Standards Institute [31]. In addition, four antibiotics, gentamicin, kanamycin, streptomycin, and tetracycline were selected for the same experiment as listed in Table S1. The results of antibiotic experiments were compared with Cecropin P1 to further verify its antibacterial activity *in vitro*.

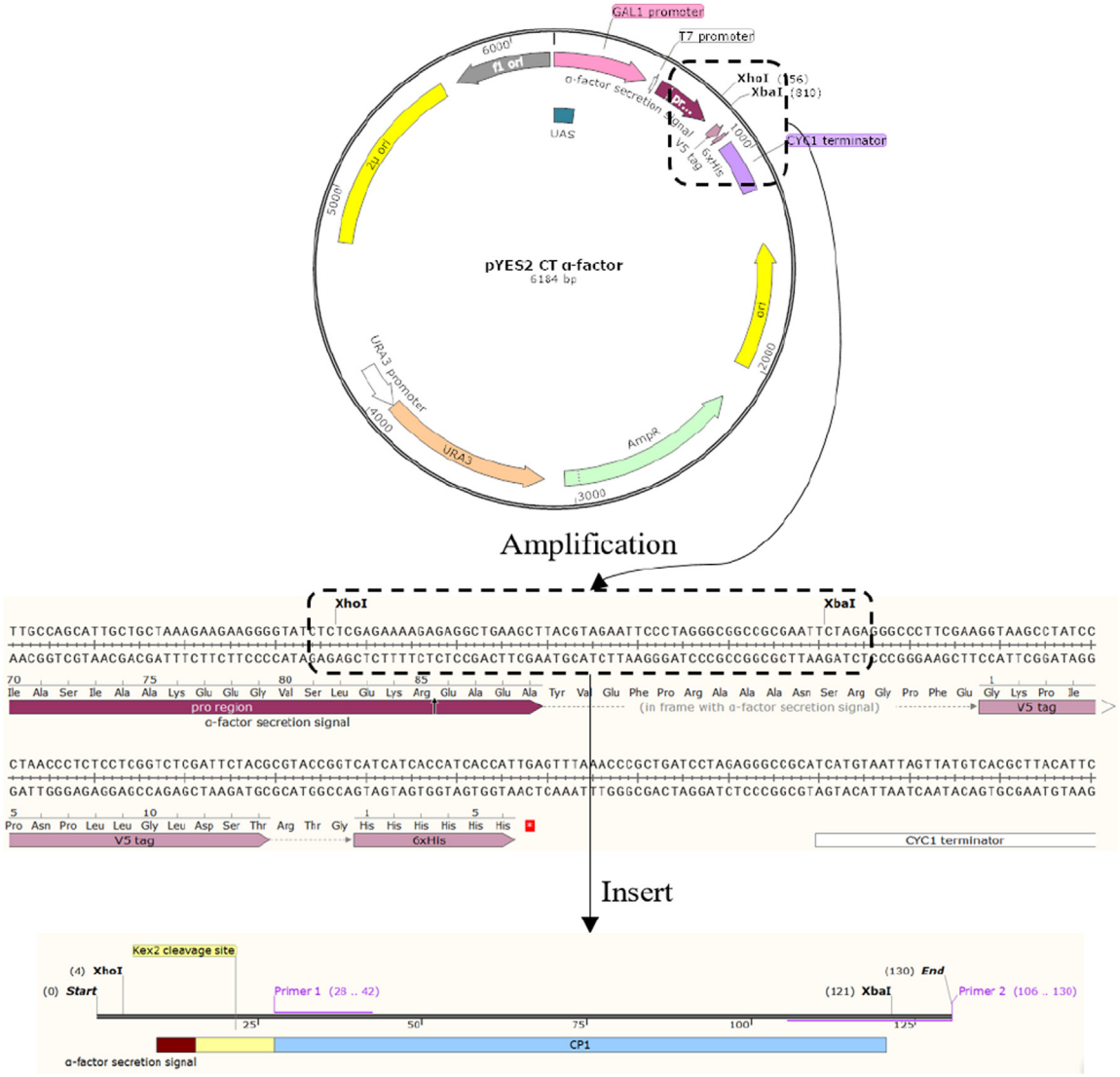

Fig. 1. Construction of recombinant plasmid pYES2/CT-α factor-CP1.

2.8. Determination of 50% tissue culture infective dose ($TCID_{50}$)

Marc-145 cells with a concentration about 1.5 × 10^5/mL were incubated in 96-well cell culture plate and cultured with DMEM containing 5% fetal bovine serum (FBS). Sixteen wells were selected as the negative control group, and at the same time, PRRSV NADC30-Like dilution gradients (from 10^{-1} to 10^{-8}) were set as the experimental groups, with 20 repeats for each dilution gradients. When the cells grew to about 80% of the wells, experimental groups were inoculated with virus and cultured at 37°C in 5% CO_2. After 5 d of incubation, $TCID_{50}$ was calculated by Reed–Muench method [32,33] and recorded. Then PRRSV NADC30-Like virus was diluted to the concentration of $TCID_{50}$ and stored at −80°C for reserve.

2.9. Preliminary study on Cecropin P1 cytotoxicity.

Marc-145 cells were seeded with DMEM containing 5% FBS on a 96-well cell culture plate and incubated at 37°C. When the cells covered 80~90% of the wells, the culture medium was discarded and the wells were washed 3 times with sterile physiological saline solution. Four different Cecropin P1 concentrations were added to cells, each concentration was repeated 3 times [34] (C1 = 15.6 μg/mL, C2 = 3.9 μg/mL, C3 = 0.977 μg/mL, and C4 = 0.244 μg/mL). A cell control group (DMEM containing 2% FBS) and a virus control group (PRRSV NADC30-Like diluent equal to the concentration of $TCID_{50}$) were set at the same time. After incubation for 2 h at 37°C, fresh maintenance solution was replenished. When 80% of the cells in the positive control produced cytopathic effect (CPE), 15 μL 50 μM MTT was added to each well and incubated 37°C for 3~4 h [35,36]. Then, the liquid was aspirated and 200 μL pure DMSO was added. The OD_{490nm} value was measured with Nano-Drop 2000 Spectrophotometer and recorded. Then, the cell survival rate was calculated. [Cell survival rate = (average OD_{490nm} value of the antibacterial peptide group ÷ average OD_{490nm} value of the cell control group) × 100%].

2.10. Cecropin P1 pretreatment antiviral test

In order to investigate whether Cecropin P1 has antiviral effect, 100 μL Cecropin P1 (C1~C4) was added to 70–80% confluent Marc-145 cells for 2 h. Cells were then infected with PRRSV NADC30-Like (equal to the concentration of $TCID_{50}$), and their cytopathic conditions were recorded every 12 h. When the positive control cytopathy was stable, the cell survival rate was calculated according to 2.8.

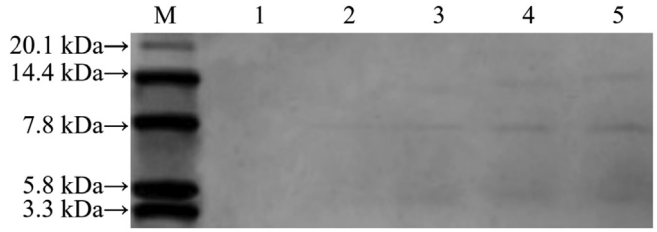

Fig. 2. Recombinant protein Tricine–SDS–PAGE detection. M: Protein Marker (3.3 kDa–20.1 kDa). 1–5: Induced supernatant for 0, 12, 24, 36, and 72 h, respectively.

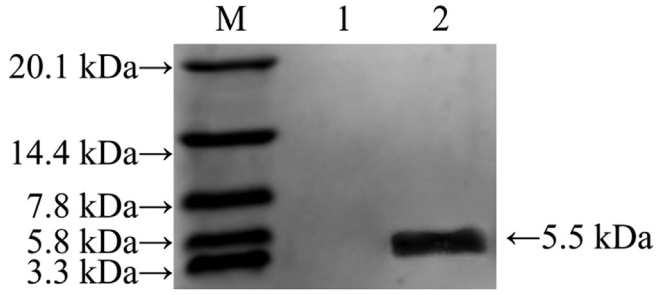

Fig. 3. Western blot analysis of recombinant protein. M: Protein Marker (3.3 kDa–20.1 kDa). 1: Western blot analysis of recombinant pYES2/CT/α factor-CP1 without inducing by galactose. 2: Purified recombinant pYES2/CT/α factor – CP1 that has been induced by galactose.

3. Results

3.1. Codon optimization, and synthetic and identification of the Cecropin P1 mature peptide gene

The Cecropin P1 mature peptide gene was optimized using OptimumGene Codon Optimization Analysis online software (Fig. S1a). Codon adaptation index and frequency of optimal codons were increased from 0.58 to 0.90 and from 40 to 70%, respectively, while genomic GC content was decreased from 48.12 to 36.51%. After codon optimization, the Cecropin P1 gene was synthesized and cloned as pUC57-CP1. After restriction enzyme digestion, one band of 125 bp was obtained for Cecropin P1 (Fig. S1b), which was consistent with the expected result, and proved that the recombinant plasmid was successfully constructed.

3.2. Identification and induced expression of recombinant protein

The Cecropin P1 insert in pUC57-CP1 was ligated into pYES2/CT-α factor to construct the eukaryotic expression vector pYES2/CT-α factor-CP1, which was transformed into *S. cerevisiae* after confirmation by restriction enzyme digestion. Only positive clones were selected for inductive expression. Tricine-SDS-PAGE used to identify recombinant proteins showed a major band at approximately 5.5 kDa after 12 h of induction, with an increasing yield over the course of the induction (Fig. 2). This indicated the successful expression of Cecropin P1.

Table 2
$TCID_{50}$ test results.

Dilution multiple	CPE wells	No CPE wells	Accumulated numbers		Percentage of CPE wells
			CPE wells	No CPE wells	
10^{-1}	20	0	74	0	100% (74/74)
10^{-2}	20	0	54	0	100% (54/54)
10^{-3}	20	0	34	0	100% (34/34)
10^{-4}	12	8	14	8	**64% (14/22)**
10^{-5}	2	18	2	26	**7% (2/28)**
10^{-6}	0	20	0	46	0 (0/46)
10^{-7}	0	20	0	66	0 (0/66)
10^{-8}	0	20	0	86	0 (0/86)

3.3. Purification of recombinant protein and western blot analysis.

After induced expression for 0, 12, 24, 36, and 72 h, the secreted protein was concentrated and purified. The concentration of Cecropin P1 was 0.57, 1.33, 2.26, 5.79, and 7.83 mg/L at different induction time, respectively. The time point with the highest protein expression was selected. Western blot analysis showed that recombinant protein as a result of induction for 72 h specifically reacts with the primary antibody, showing good reactogenicity (Fig. 3).

3.4. Antibacterial activity of recombinant Cecropin P1

In order to assess the inhibitory effect of recombinant Cecropin P1 on bacteria, common pathogenic strains were selected for antibacterial activity determination. As listed in Table 1, Cecropin P1 had antibacterial activity against all tested gram-negative bacteria. In addition, Cecropin P1 showed better antibacterial activity compared to some other antibiotics, suggesting that Cecropin P1 is promising to replace antibiotics for clinical treatment.

3.5. Determination of $TCID_{50}$

With reference to the Reed−Muench method, the results are shown in the following Table 2. The $TCID_{50}$ of PRRSV

Table 1
Antibacterial activity test results.

Pathogenic strains	MIC (μg/mL)				
	Cecropin P1	Gentamicin	Kanamycin	Streptomycin	Tetracycline
Salmonella(Swine)	**2**	2	8	16	256
Salmonella (Cow)	**4**	2	16	16	128
Salmonella (Moschus berezovskii)	**4**	4	8	32	128
Salmonella (Moschus berezovskii)	**4**	2	2	16	64
E. coli (Swine)	**2**	1	4	8	2
E. coli (Avium)	**4**	2	4	8	4
E. coli (Cow)	**4**	2	4	8	8
E. coli (Cow)	**8**	2	8	512	8
Pasteurella (Rabbit)	**8**	1	32	16	4
Pasteurella (Goose)	**>8**	16	64	32	2
Pasteurella (Cow)	**4**	32	128	8	32
Shigella (Swine)	**4**	256	512	128	64
Shigella (Swine)	**2**	128	256	256	8

NADC30-Like was calculated to be $10^{-4.25}$/0.1 mL. The PRRSV NADC30-Like virus was diluted to the concentration of $TCID_{50}$ for use in subsequent experiments.

3.6. Cecropin P1 cytotoxidcity test

The results of MTT assay showed that the survival rates of C1, C2, C3, and C4 were 89.93 ± 0.64, 93.12 ± 1.37, 95.02 ± 0.33, and 94.74 ± 0.91%, respectively ($P < 0.05$). In other words, all dilution concentrations were not sufficient to reach 50% cytotoxic concentration, indicating that the expressed protein Cecropin P1 was safe for Marc-145 cells (Fig. S2).

3.7. Cecropin P1 pretreatment antiviral test

Cells were treated with Cecropin P1 for 2 h and then infected with PRRSV NADC30-Like. The results of MTT assay showed that the survival rates of C1, C2, C3, and C4 were 92.26 ± 1.40, 88.68 ± 2.62, 83.71 ± 0.72, and 68.84 ± 0.57% at 72 h ($P < 0.05$), suggesting Cecropin P1 exerted a significant inhibitory effect on PRRSV

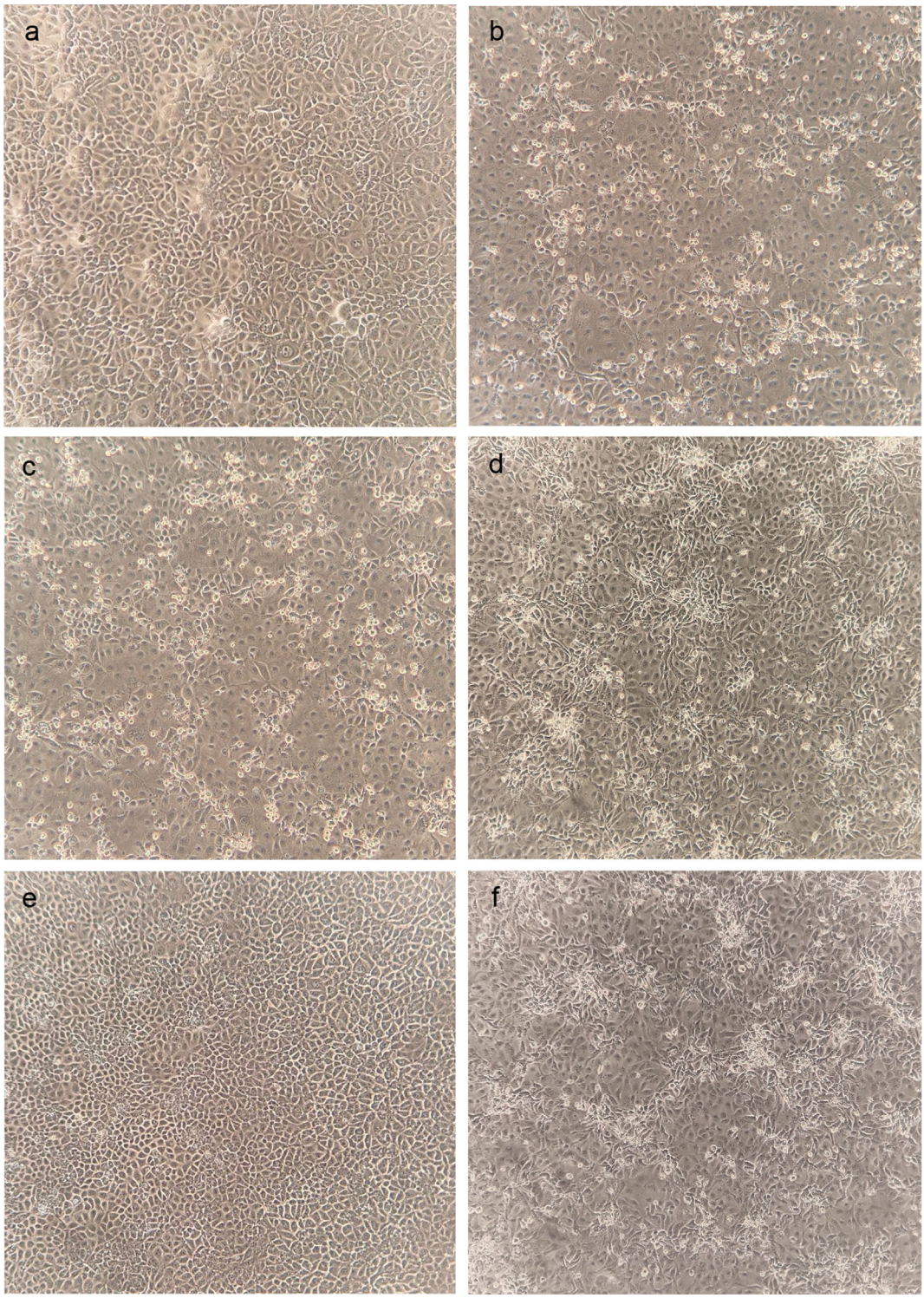

Fig. 4. Cell status of infected virus for 72 h (100×). (a)~(d): C1, C2, C3, C4 were added to Marc-145 cells for pretreatment, respectively. (e) Negative control. (f) Positive control.

NADC30-Like in Marc-145 cells. Cells treated with lower concentration of Cecropin P1 showed CPE, which was characterized by aggregation, shedding, irregular shape, and strong refractive property. In addition, the CPE became more obvious with the decrease of Cecropin P1 concentration (Fig. 4).

4. Discussion

The development of AMPs is a key research area all over the world and has potentially broad applications in drug research and development [37], in the transgenic field [38], and practical production [39,40]. Compared with antibiotics, they are generally nontoxic, showing only hemolytic activity. They are also less susceptible to drug resistance and produce less residue and pollution [41,42]. Many AMPs, such as Cecropin P1, kill or inhibit the growth and reproduction of pathogenic microorganisms by interacting with their cell membranes [43]. Currently, there are many hypotheses to explain this phenomenon, including the barrel stave model, the carpet model, and the toroidal pore model [44]. The main biological function of Cecropin P1 is antibacterial activity; however, they also have inhibitory and killing effects on fungi, parasites and viruses [45,46]. In addition, they can regulate immunity [47], protect against necrotic skin infection [48], promote wound healing [49], and induce apoptosis. Therefore, Cecropin P1 has great potential for the prevention of unknown diseases and has become a prominent topic of research in the area of antibiotic alternatives.

For the large-scale production of AMPs, neither natural extraction nor synthetic ones are beneficial. Rather, genetic engineering is a cost-effective method to address this area. In previous studies, many AMPs were expressed in *Pichia pastoris* [12,13,14,15]. However, the *P. pastoris* expression system uses methanol as an inducer, which needs to be removed during production as it poses a safety hazard. By contrast, *S. cerevisiae* with galactose as an inducer is safer. In this expression system, organisms are easy to culture with fast growth rates, and the expressed proteins have natural structure for optimal biological activity. In recent years, there have been few cases of using *S. cerevisiae* to express AMPs. Shen et al., [50] took 4 d to express gene *Crustin* in *S. cerevisiae* S78. Compared with this study, high concentration of Cecropin P1 can be obtained within 36 h of induced expression, which is more efficient. Wu et al., [51] successfully expressed the AMPs mytilin and myticin in *S. cerevisiae* S78, but they did not study whether mytilin or myticin had antibacterial activity *in vitro*. In this study, Cecropin P1 was verified to have a strong inhibitory ability against gram-negative bacteria, which has certain guiding significance for clinical medication. However, it had no inhibitory effect on *Staphylococcus aureus*, contrary to previous reports [17,52]. A possible reason for this is the concentration of Cecropin P1, which was too low to inhibit the growth of *S. aureus*. Alternatively, different serotypes of *S. aureus* with different virulence could have different sensitivities to Cecropin P1. In addition, deletion of some genes in *S. aureus* has been shown to reduce antibacterial resistance to peptides [47,53].

In addition to studying the antibacterial activity of Cecropin P1, we have further explored its antiviral activity. Some studies have shown that Cecropin P1 has antiviral activity against PRRSV [4,54]. PRRSV is very susceptible to mutation, and currently, there is no vaccine on the market that can completely prevent it. So we further explore whether Cecropin P1 has a certain therapeutic and preventive effect on PRRSV mutant strains. Such treatments are more widely used and less restrictive than vaccines, which can fill the gap in preventing PRRSV. Finally, it has been verified Cecropin P1 was preliminarily verified to have the activity of preventing cells from infecting the PRRSV virulent strain NADC30-Like *in vitro*. And this is the first study to report that Cecropin P1 has a preventive effect on PRRSV mutants, which may help the late-term sows reduce abortions, early farrowings, fetal death, and the birth of weak.

At present, no one successfully exploited *S. cerevisiae* for secretory expression of Cecropin P1. So it is very necessary to establish an expression method of Cecropin P1. This study provided a reliable and simple method for the preparation of large amounts of Cecropin P1 by recombinant expression. Judging from the results of this study, both the safety and efficiency of *S. cerevisiae* expression system, and the broad-spectrum antibacterial effect of Cecropin P1 provides new ideas for disease treatment. What is more, it also provided a new idea for the treatment of virus and laid the foundation for the development of new drugs.

Conflict of interest

The authors declare no competitive interest.

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Supplementary material

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