



Mechanism of antimicrobial peptide NP-6 from Sichuan pepper seeds against *E. coli* and effects of different environmental factors on its activity

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Abstract

A novel antimicrobial peptide named NP-6 was identified in our previous work. Here, the mechanisms of the peptide against *Escherichia coli* (*E. coli*) were further investigated, as well as the peptide's resistance to temperature, pH, salinity, and enzymes. The transmission electron microscopy (TEM), confocal laser scanning microscopy (CLSM), and flow cytometric (FCM) analysis, combined with measurement of released K⁺, were performed to evaluate the effect of NP-6 *E. coli* cell membrane. The influence of NP-6 on bacterial DNA/RNA and enzyme was also investigated. The leakage of K⁺ demonstrated that NP-6 could increase the permeability of *E. coli* cell membrane. The ATP leakage, FCM, and CLSM assays suggested that NP-6 caused the disintegration of bacterial cell membrane. The TEM observation indicated that NP-6 could cause the formation of empty cells and debris. Besides, the DNA-binding assay indicated that NP-6 could bind with bacterial genomic DNA in a way that ethidium bromide (EB) did, and suppress the migration of DNA/RNA in gel retardation. Additionally, NP-6 could also affect the activity of β-galactosidase. Finally, the effect of different surroundings such as heating, pH, ions, and protease on the antimicrobial activity of NP-6 against *E. coli* was also investigated. Results showed that the peptide was heat stable in the range of 60–100 °C and performed well at pH 6.0–8.0. However, the antimicrobial activity of NP-6 decreased significantly in the presence of Mg²⁺/Ca²⁺, and after incubation with trypsin/proteinase K. The results will provide a theoretical support in the further application of NP-6.

Keywords Antimicrobial peptide · *Zanthoxylum bungeanum* Maxim seeds · Mechanisms · Antimicrobial activity · Surroundings effects

Introduction

Antimicrobial peptides (AMPs) play an important role in the immune system of all living organisms, and have attracted a great deal of attention due to the emergence of bacterial resistance. Nowadays, more than 2,500 natural AMPs have been isolated from animals, plants, bacteria, and fungi (Wang et al. 2016). Generally, they share characteristics such as hydrophobic and always positively charged (Drin and Antonny 2010), which in turn associates with their activity (Hancock and Sahl

2006). Other characteristics including size, primary sequence, conformation, and structure are also essential for antimicrobial activity and action mode of AMPs (Guilhelmelli et al. 2013).

The mechanism is affected by the varying composition and structure of AMPs (Li et al. 2014). The classic, namely, membrane damage effect, has been widely accepted as key point (Nguyen et al. 2011; Hancock 2011; Malmsten 2016). It is generally believed that the positively charged AMPs firstly bind with the bacterial cell membrane which is composed of negatively charged lipids, leading to penetration of the cell membrane (Li et al. 2012). However, a large number of papers' results indicated that AMPs have intracellular targets (Aerts et al. 2008; Guilhelmelli et al. 2013; Mahlapuu et al. 2016). In this case, the death-inducing mechanisms may involve in the interaction with DNA/RNA, then negatively impact protein synthesis and activity of intracellular enzyme, or inhibit the formation of cell wall/membrane (Ageitos et al. 2017).

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Sichuan pepper (*Zanthoxylum bungeanum* Maxim) is an aromatic tree and shrub plant belonging to the genus *Zanthoxylum* of the family Rutaceae (Tang et al. 2014a). The pericarps of *Z. bungeanum* Maxim are normally used as a spice in Chinese kitchen, as well as a folk medicine for treatment of vomiting, toothache, stomachache, abdominal pain, and infected wounds (Xia et al. 2011). So far, more than 140 compounds have been identified from the plant, and some biological activities such as anti-inflammatory, antioxidant, and antimicrobial effects have been proved (Zhang et al. 2017). However, being a by-product of *Z. bungeanum* Maxim production, *Z. bungeanum* Maxim seeds which are rich in oil, protein, melanin, and dietary fiber are often discarded. To the best of our knowledge, reports referring to the bioactive compounds in *Z. bungeanum* Maxim seeds are limited. Besides, the topics of these reports often focus on *Z. bungeanum* Maxim seed oil which is rich in polyunsaturated fatty acids (Tang et al. 2014a; Yang et al. 2014; Xia et al. 2011). Actually, *Z. bungeanum* Maxim seeds are also a good resource of protein. Previous report had proven that the hydrolysate of *Z. bungeanum* Maxim seed protein exhibited inhibitory activity against angiotensin-I-converting enzyme (ACE) (Wu et al. 2016). Jiang et al. (2015) reported that two antimicrobial fractions of *Z. bungeanum* Maxim seed protein hydrolysate were active against *E. coli* and *S. aureus*. So more investigation should be conducted to fully explore the bioactive compounds in *Z. bungeanum* Maxim seeds. More recently, there have been literatures reporting the medicinal characters of *Z. bungeanum* Maxim seed oil (Tang et al. 2014a; Yang et al. 2014), but limited attention has been paid to the protein/peptides.

In our previous work, it was found that a peptide (NP-6) being a fragment of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) of *Z. bungeanum* Maxim seed protein exhibited antimicrobial activity (Hou et al. 2019). In the present work, the mechanisms of NP-6 against *Escherichia coli* were investigated, which were involved in damage effects of bacteria cell membrane, interaction with genomic DNA/RNA, and effects on activities of intracellular enzymes. Meanwhile, peptide resistance to temperature, pH, salinity, and enzymes was determined by monitoring the changes in antimicrobial activity against *E. coli* after being heated at various temperature, exposed in solutions with different pH and monovalent/divalent cations, and incubated with different protease. Results of the work will help a lot in clarifying the action mode of NP-6 and considering the application of the peptide in a suitable way.

Materials and methods

Materials

The peptide NP-6 was chemically synthesized according to the standard fluorenylmethyloxycarbonyl (Fmoc) methodology at GL

Biochemistry Corporation (Shanghai, China), followed by further purification with RP-HPLC system and characterization with ESI-MS (Waters, Madrid, Spain), yielding final purity of the peptide as more than 95%. Propidium iodide (PI) was purchased from Sigma (St. Louis, MO, USA). SYTO 9 (S34854) was purchased from Molecular Probes (Invitrogen, Barcelona, Spain). Pepsin (25000 U/mg), trypsin (2500 U/mg), proteinase K (40 U/mg), ethidium bromide (EB), and o-nitrophenyl- β -D-galactopyranoside (ONPG) were purchased from Sangon Biotech Co., Ltd. (Shanghai, China). DNA extraction kit was purchased from TransGen Biotech Co., Ltd. (Beijing, China). One-stop RNA electrophoresis pack was purchased from Shanghai Biya Biotechnology Co., Ltd. (Shanghai, China). The T-ATPase was purchased from Jiancheng Bioengineering Institute (Nanjing, China). Enhanced ATP Assay Kit and BCA protein assay kit were obtained from Beyotime Biotechnology (Shanghai, China). All other chemicals were of analytical grade.

Strains and growth conditions

Escherichia coli (*E. coli*, ATCC 25922) were obtained from China Center of Industrial Culture Collection (Beijing, China), cultured in sterilized nutrient broth (NB, peptone, 10 g/l, yeast extract, 5 g/l, NaCl, 10 g/l) at 37 °C.

Leakage of K⁺

E. coli was cultured in NB media at 37 °C for 16–18 h; the cells were centrifuged, washed twice with deionized water, and diluted to 10⁶ CFU/ml. NP-6 solution was added to a final concentration of 1 × MIC (64 µg/ml) and the mixture were incubated at 37 °C for various times, with deionized water-treated cells as control. Samples were centrifuged at 13400×g for 3 min and the amount of released K⁺ in the supernatants was measured on an atomic absorption spectrometer (Spectra AA 220 FS, Varian, USA) according to a previous description (Lu et al. 2011).

ATP leakage

The assessment of ATP leakage was performed according to Gottschalk (2017). The bacterial strain *E. coli* was incubated in NB overnight at 37 °C and grown to mid-exponential phase. Five milliliters of bacterial cells was harvested by centrifugation at 2000×g for 10 min. After being washed twice in 50 mM HEPES buffer, bacterial cells were resuspended in the same buffer (1 ml) and then energized with 0.2% (w/v) glucose at 37 °C for 10 min. Cell suspension was treated with NP-6 (final concentration 64 µg/ml and 1 mg/ml) for 0, 15, 30, 45, and 60 min. The samples were then centrifuged at 2000×g for 10 min, and the supernatant was collected for estimating the leakage of ATP from the cells by using Beyotime Enhanced ATP Assay Kit.

Flow cytometry

Log-phased cells of *E. coli* were harvested, washed, and re-suspended in PBS. Solutions of NP-6 were added to a final concentration of 1.0 mg/ml, and the mixture was incubated at 37 °C for 1 h, with PBS treatment as a control. Then cells were centrifuged and washed by PBS, followed by being incubated with PI (50 µg/ml solution in dd H₂O) at 4 °C for 30 min in darkness (Sik et al. 2006). The samples were centrifuged at 13400×g for 3 min, washed three times with PBS, and then resuspended in the same buffer. Analysis was performed using a FACSCalibur flow cytometer (Becton Dickinson, USA).

Confocal laser scanning microscopy

The assay was performed using a method according to Qian et al. (2011) with some modification. A working solution containing two dyes was employed to stain *E. coli* in the study. 1.5 µl SYTO 9 (5 mM solution in DMSO) was mixed with the same volume of PI (50 µg/ml solution in ddH₂O), then 1 ml PBS was added to the mixture and the solution was prepared fresh daily. *E. coli* cells treated by NP-6 (1.0 mg/ml) were incubated with the working solution for 30 min in the dark at 4 °C; cells without treatment of NP-6 were set as control. The stained *E. coli* was observed under a SP2 confocal microscope (Leica, German) after washing three times with PBS. Images were collected at an excitation/emission wavelength of 485/498 nm for SYTO 9 signal and 535/617 nm for PI signal.

Transmission electron microscopy

Log-phased cells of *E. coli* were treated by NP-6 (1.0 mg/ml) for 1 h at 37 °C, followed by being centrifuged and wash twice with phosphate-buffered saline (PBS, 0.01 M, pH 7.4). PBS-treated cells were set as the control. The collected cells were firstly fixed with 2.5% (v/v) glutaraldehyde for 3 h, then post-fixed with 1% osmium tetroxide for another 2 h; both were conducted at 4 °C (Shi et al. 2016). Cells were then dehydrated by a series of ethanol and embedded in resin. The cut sections of samples were stained with uranyl acetate and lead citrate. Analysis were conducted by a transmission electron microscopy system (Tecnai G2 F20 S-TWIN, USA).

Extraction of bacterial DNA/RNA

The DNA of *E. coli* was prepared using a DNA extraction kit (TransGen Biotech, Beijing). Total RNA was purified using a one-stop RNA electrophoresis pack (Biya Biotech, Shanghai).

Competitive binding of NP-6 and EB with bacterial DNA

The assay was performed as described by Li et al. (2013) with little modification. DNA of *E. coli* was firstly dissolved in TE buffer (10 mM, pH 8.0) to a final concentration of 50 µg/ml, then 15 µl EB solution was added and the mixture was incubated at 37 °C for 10 min in the dark. Various concentrations of NP-6 (0, 1.0, 2.0, and 4.0 mg/ml) were added to the mixture. After being incubated in the dark at 37 °C for 30 min, the fluorescence of the solution was measured using a Lumina Fluorescence Spectrophotometer (Thermo Fisher Scientific, MA, USA). The excitation wavelength was set at 535 nm, and the spectra were recorded at 550–750 nm.

DNA/RNA gel retardation

Gel retardation assays were conducted according to a previous literature (Shi et al. 2016) with some modification. Varying concentrations of NP-6 (0, 0.1, 0.2, 0.4, 0.8, 1.0, 2.0, and 4.0 mg/ml) were added to the DNA solution, and the mixture was incubated at 37 °C for 30 min. After that, 2 µl binding buffer (5% glycerol, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1 mM DL-dithiothreitol, 20 mM KCl, and 50 µg/ml albumin) was added and the reaction mixture was subjected to gel electrophoresis on a 1% agarose gel. For RNA binding, various concentrations of NP-6 (0, 0.1, 0.2, 0.4, 0.8, 1.0, 2.0, and 4.0 mg/ml, dissolved in diethyl pyrocarbonate (DEPC)-treated water) were incubated with bacterial RNA for 10 min, and the formaldehyde denaturing gel electrophoresis was conducted using an one-stop RNA electrophoresis kit according to the manufacturer's recommendations.

Effect of NP-6 on the activity of T-ATPase

The assay was performed using a previous method (Shi et al. 2016) with minor modification. *E. coli* was incubated with NP-6 (0.5, 1.0, and 2.0 mg/ml) at 37 °C for 1 h, with PBS as a control. The culture was centrifuged at 13400×g for 5 min, washed three times with PBS and resuspended in the same buffer. Then samples were disrupted by sonication at 300 W for 5 min in an ice bath. The bacterial total protein content was determined using a BCA protein assay kit. The T-ATPase level was measured using a commercial assay kit according to the recommendations of the manufacturer. The samples were scanned by a microplate reader (Dinex Technologies Inc., Chantilly, USA) at 636 nm.

Effect of NP-6 on the activity of β-galactosidase

Lactose can be decomposed into one glucose and one galactose and is used as substrate for β-galactosidase determination. ONPG and lactose have similar structures,

and also can be decomposed by β -galactosidase into yellowish O-nitrophenol (ONP). The amount of ONP released can be measured to determine the activity of β -galactosidase. Log-phased cells of *E. coli* were collected, centrifuged, washed, and resuspended in M9 media, incubated for 8 h at 37 °C. Then cells were centrifuged at 13400 $\times g$ for 5 min, washed three times with PBS, and resuspended in the same buffer. After that, NP-6 was added to a various concentrations of 0.5, 1.0, and 2.0 mg/ml, followed by being incubated at 37 °C for 1 h, with PBS-treated cells as the control. The culture was then centrifuged, washed, resuspended in PBS, and finally disrupted by sonication at 300 W for 5 min in an ice bath. Samples were further centrifuged and 20 μ l of the bacterial suspension mixed with 80 μ l ONPG (5 mM) was added to the well of a standard 96-well plate. The plates were incubated at 37 °C for 10 min and the reaction was stopped by adding 100 μ l 0.5 M cold Na₂CO₃ and scanned by a microplate reader (Dinex Technologies Inc., Chantilly, USA) at 420 nm (Kragol et al. 2001). Determination of protein content was done using BCA protein assay kit.

One unit of β -galactosidase was defined as the hydrolysis to produce 1 μ mol ONP per minute and labeled as 1 U.

Resistance of NP-6 to temperature, pH, salinity, and enzymes

The surrounding sensitivity analysis was performed as described (Tang et al. 2015) with minor modification. The recovered antimicrobial activities after treatment with different conditions (heat, pH, ions, and enzymes) were measured by monitoring the total numbers of colony. For the heating treatment, NP-6 was dissolved in distilled deionized water (dd H₂O) to a final concentration of 1.0 mg/ml, then heated at 60, 70, 80, 90, and 100 °C for 30 min in a water bath. For the acid/alkali treatment, NP-6 was dissolved in dd H₂O with different pH values (2.0, 4.0, 6.0, 8.0, and 10.0) for 15 min and then applied to pH 7.0. For ion sensitivity analysis, the peptide was exposed to different concentrations (50 mM, 100 mM, and 150 mM) of K⁺, Na⁺, Mg²⁺, and Ca²⁺. For enzyme sensitivity test, NP-6 was exposed in pepsin, trypsin, and proteinase K for 15, 30, and 60 min, followed by being heated at 100 °C for 5 min to terminate the reactions. NP-6 without any treatment was set as a control. The inhibition rate was calculated as follows:

$$\text{Inhibition rate (\%)} = \frac{N_{\text{control}} - N_{\text{treated}}}{N_{\text{control}}} \times 100 \quad (1)$$

where N_{control} is the number of bacteria in untreated samples, N_{treated} is the number of bacteria in treated samples.

Statistical analysis

All the tests were repeated at least three times. Data analysis was performed using the software of SPSS (version 19.0, IBM SPSS, USA). Results were expressed as the means \pm standard deviation. The significance of the differences between the treatments and the respective controls were determined based on Student's *t* test, and a value of $p < 0.05$ was considered statistically significant.

Results

Potassium efflux

In order to characterize the effect of NP-6 on permeability of *E. coli* cell membrane, the amount of released potassium from peptide-treated cells were determined, with untreated cells as a control. As shown in Fig. 1, after 1 h of treatment with 1 \times MIC NP-6, the efflux of potassium was immediate. By comparison, the amount of released K⁺ of untreated *E. coli* cells was kept in a low level. The result could be the evidence that NP-6 increased the permeability of *E. coli* cell membrane.

Detection of ATP leakage

Extracellular ATP levels were determined as indexes of cell lysis (shown in Fig. 2). In the presence of 1 \times MIC NP-6, the concentration of extracellular ATP was maintained at a low level, but the increase of released ATP was observed after 2 h. Meanwhile, treatment with NP-6 (1 mg/ml) caused a significant increase in the concentration of extracellular ATP after 15 min. In contrast, concentration of extracellular ATP in untreated cells was maintained at approximately 9 nM.

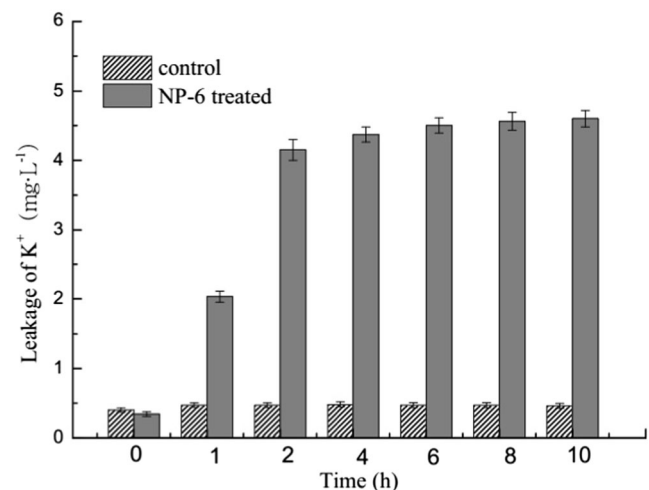


Fig. 1 Leakage of K⁺ from the peptide-treated *E. coli* cells at various times. The control was processed without NP-6

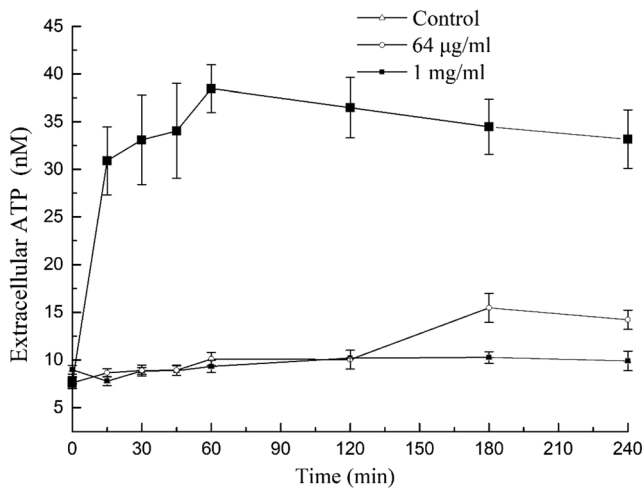


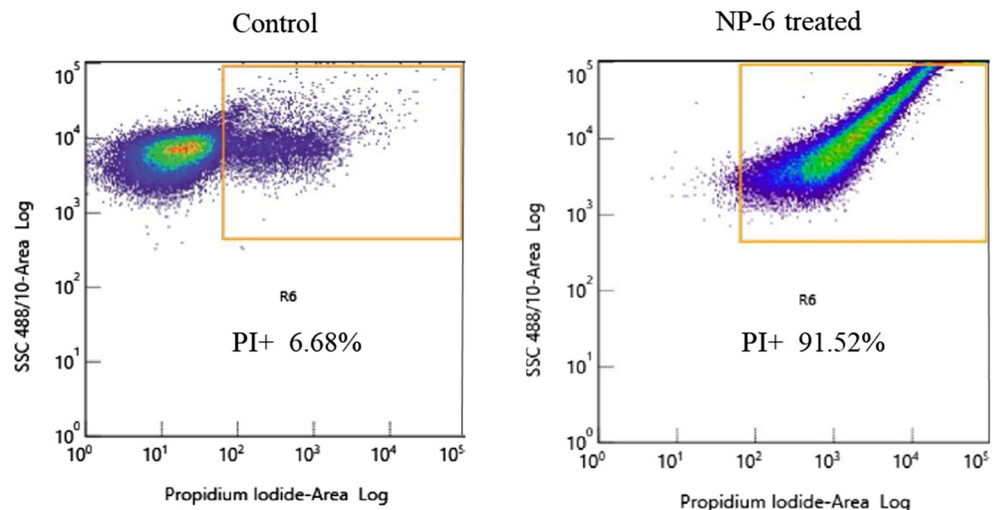
Fig. 2 Concentration of the released ATP from the peptide-treated *E. coli* cells at various times. The control was processed without NP-6

These results clearly indicated that NP-6 could disrupt the integrality of bacterial cell membrane and cause the release of large molecules.

Flow cytometric analysis

The effects of NP-6 on *E. coli* cell membrane were further investigated by flow cytometer with PI as a probe. PI is a DNA-intercalating fluorescent dye that can penetrate into impaired cells, but does not enter into intact cells. It is often used as a probe to investigate the integrity of cell membrane. This fluorescent probe will enter into the cytoplasm if the bacterial cell membrane is damaged. As shown in Fig. 3, only 6.68% of untreated *E. coli* cells showed PI fluorescence signal. By contrast, 91.52% peptide-treated cells were labeled fluorescence, indicating that the bacterial cell membrane integrity was impaired.

Fig. 3 The membrane damage effect of NP-6 on *E. coli* cells by flow cytometry analysis, as measured by an increase of fluorescent intensity of PI (50 µg/ml). The control was processed without NP-6



CLSM images

The above results were confirmed by confocal laser scanning microscopy (CLSM) with SYTO 9 and PI as fluorescent probes. SYTO 9 is a kind of green fluorescent nuclear acid stain; it was proved to stain cells whether alive or dead. While PI is a red-fluorescent nuclear dyes, it is commonly used to detect dead cells due to its non-penetrability to alive cells. As was observed in Fig. 4, green dots on the left are all bacterial cells including alive and dead ones, red dots in the middle are dead cells, yellow dots on the right are dead cells, and green dots are alive ones. It was obvious that most of the untreated cells were alive while an overwhelming fraction of the peptide-treated *E. coli* cells were dead. The CLSM images were another evidence for the disintegrality of bacterial cell membrane.

TEM observation

Transmission electron microscopy (TEM) was performed to investigate the ultrastructural changes of *E. coli* cells. As was illustrated in Fig. 5, in the absence of NP-6, the *E. coli* cells maintained an intact, smooth cell membrane and presented a dense internal organization. Upon exposure to NP-6 for 1 h, cell wall and membrane disruption were clearly observed and the leaked cytoplasmic materials were found around the cells. The result of cell membrane disruption followed by the treatment of NP-6 was consistent with the results obtained from CLSM and flow cytometry (FCM).

DNA/RNA binding

The result of competitive binding of NP-6 and EB with bacterial genomic DNA is shown in Fig. 6a. It was found

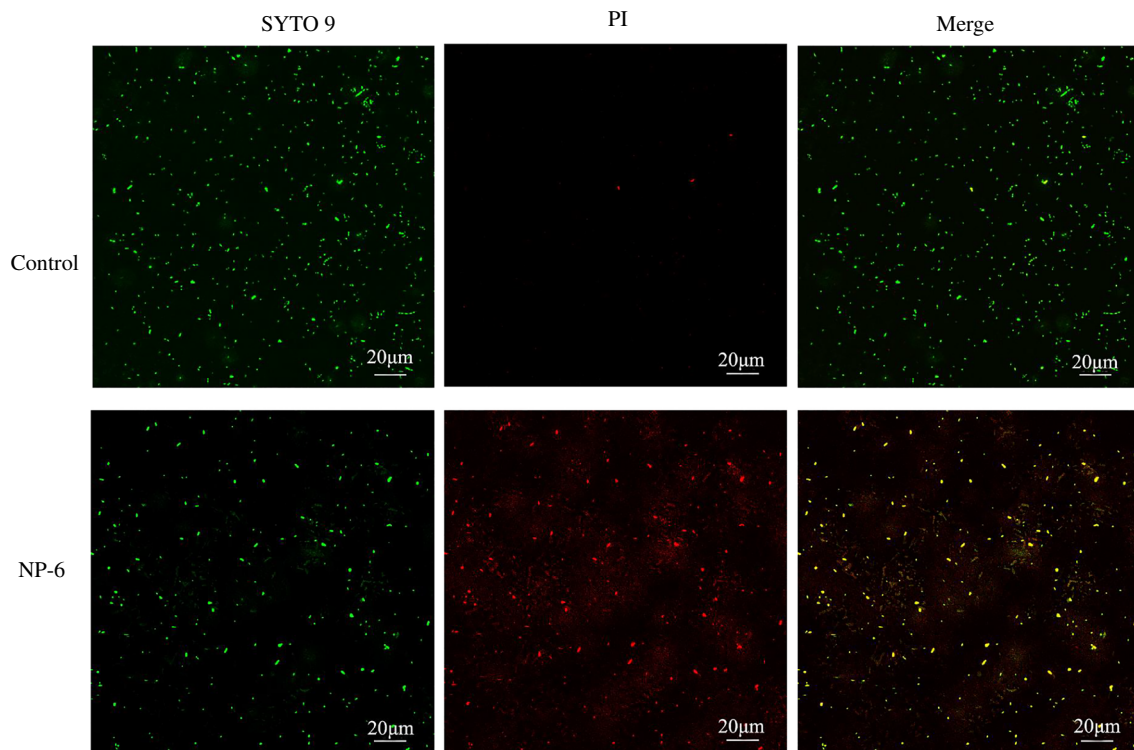


Fig. 4 Confocal images of *E. coli* treated and untreated by NP-6. The control was processed without NP-6

that the fluorescence of the free EB is the lowest, while bacterial DNA without treatment of NP-6 showed the highest fluorescence. With the increasing concentration of NP-6, a remarkable fluorescence decline of the EB-DNA system was observed. Lepecq and Paoletti (1967) pointed that the fluorescence could be quenched when there was a reagent that executed a similar reaction. The result indicated that NP-6 could bound to *E. coli* genomic DNA. In addition, EB molecules inserting into the bacterial DNA were replaced by NP-6, suggesting a stronger binding affinity of NP-6 than EB.

A gel retardation assay was performed to further determine the DNA-binding activity of NP-6 (shown in Fig. 6b). It was observed that the lower the peptide concentration, the brighter the band of the peptide-DNA system. The band was almost trapped in the spotting hole when the peptide concentration was 0.2 mg/ml. The observations suggested that NP-6 had DNA-binding activity.

A gel retardation assay was conducted to estimate the RNA-binding activity of NP-6. As shown in Fig. 6c, the bacterial RNA was suppressed by NP-6 in a concentration-dependent manner. Meanwhile, a degradation of 23S rRNA was observed, and all the peptide-treated samples only had a band of 16S rRNA. The migration of RNA was almost completely suppressed when the concentration of NP-6 was 1.0 mg/ml. The results demonstrated that NP-6 had the ability to interact with *E. coli* RNA.

Effect of NP-6 on the activities of T-ATPase and β -galactosidase

In order to learn whether NP-6 can inhibit the activity of bacterial intracellular enzyme, the activity of T-ATPase and β -galactosidase in bacterial cells treated/untreated by NP-6 was determined. The results are shown in Fig. 7. It was observed that NP-6 did not significantly inhibit the T-ATPase in the range of 0–2.0 mg/ml, indicating that NP-6 had no effect on the T-ATPase of *E. coli*. However, it was also observed that NP-6 strongly inhibited the β -galactosidase activity of *E. coli* in a dose-dependent manner (8.72–1.24 U/mg). By the concentration of 2.0 mg/ml, the β -galactosidase activity of *E. coli* had been suppressed in a very low level compared with the samples without treatment of NP-6.

Resistance of NP-6 to temperature, pH, salinity, and enzymes

The surrounding sensitivity assays were performed to investigate the potential value of NP-6 as a natural preservative agent used in food. As shown in Fig. 8a, heating treatment in the range of 60–100 °C had little effect on the antimicrobial activity of NP-6 against *E. coli*. After heating for 30 min at 100 °C, the retained activity of NP-6 was more than 90%, indicating that the peptide was heat stable at 60–100 °C.

When the pH was 6.0 and 8.0, the antimicrobial activity of NP-6 against *E. coli* was more than 90%, but it decreased when

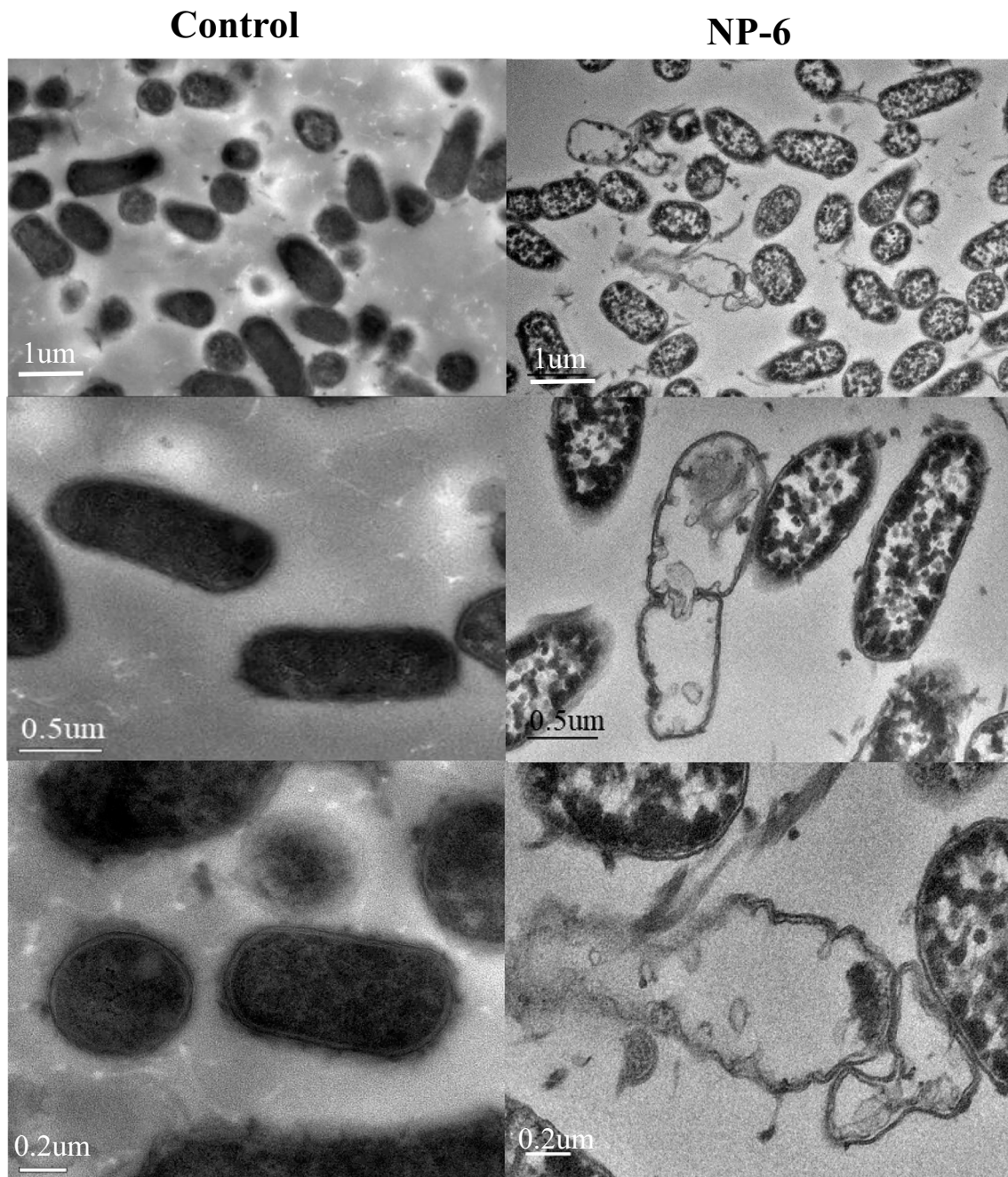


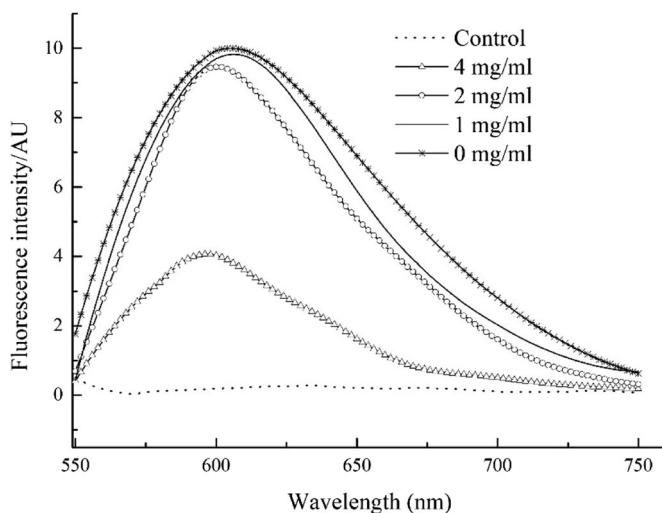
Fig. 5 Transmission electron microscopy images of *E. coli* treated and untreated by NP-6. The control was processed without NP-6

the pH was lower than 6.0 or higher than 8.0 (Fig. 8b). As the pH decreased to 2.0, the antimicrobial activity of NP-6 against *E. coli* reduced to 34.25%, and it reduced to 44.85% when the pH increased to 10.0. The results suggested that NP-6 had the strongest antimicrobial activity at physical pH, and acidic and basic conditions could inhibit its activity against *E. coli*.

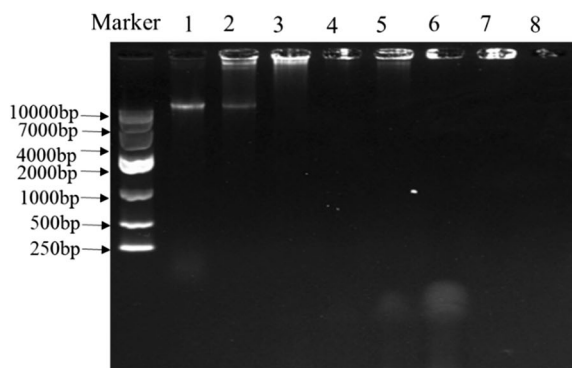
As shown in Fig. 8c, the antimicrobial activity of NP-6 against *E. coli* was not significantly affected by Na^+ and K^+ , but it was greatly suppressed in the presence of Mg^{2+} and Ca^{2+} . The residual activity decreased to 15.96% and 20.85% when the concentration of Mg^{2+} and Ca^{2+} was 150 mM, and it

almost lost its antimicrobial activity against *E. coli*. The results indicated that divalent ions had a stronger inhibitory effect on the antimicrobial activity of NP-6 than the monovalent ones.

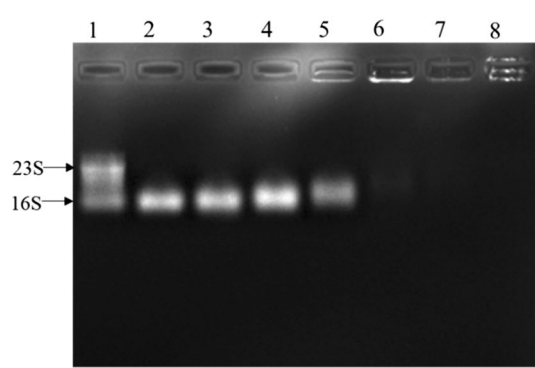
Figure 8d showed the changes in antimicrobial activity of NP-6 after incubation with different enzymes in a time series. It was observed that all the three enzymes could cause decrease in antimicrobial activity of NP-6. The decrease caused by pepsin was slight, and the residual activity of NP-6 against *E. coli* was more than 70% after incubation for 60 min with pepsin. However, the antimicrobial activity of NP-6 was greatly suppressed after incubation with trypsin and proteinase K, and it



(a)



(b)



(c)

Fig. 6 Effect of NP-6 on bacterial nucleic acid. **a** Competitive binding of NP-6 and EB with *E. coli* genomic DNA, and the fluorescence spectra were measured from 550 to 750 nm. **b** A gel retardation assay was conducted after various concentrations of NP-6 were incubated with *E. coli* genomic DNA. Bands 1–8 were for the concentrations of 0, 0.1, 0.2, 0.4,

0.8, 1.0, 2.0, and 4.0 mg/ml, respectively. **c** A gel retardation analysis of the binding activity of NP-6 to *E. coli* RNA. Various concentrations of NP-6 were incubated with RNA. Bands 1–8 were for the concentrations of 0, 0.1, 0.2, 0.4, 0.8, 1.0, 2.0, and 4.0 mg/ml, respectively.

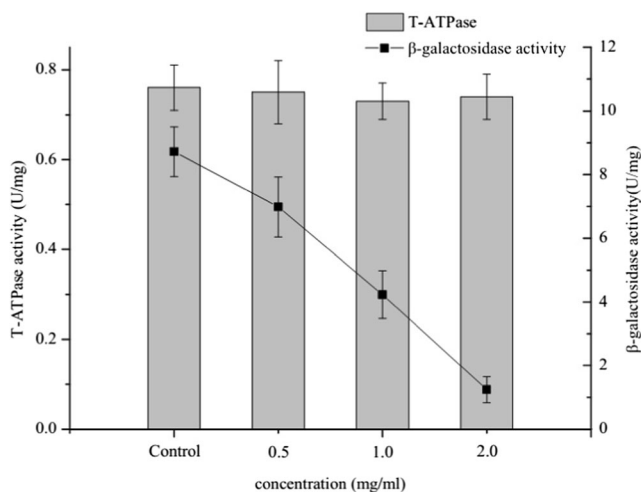


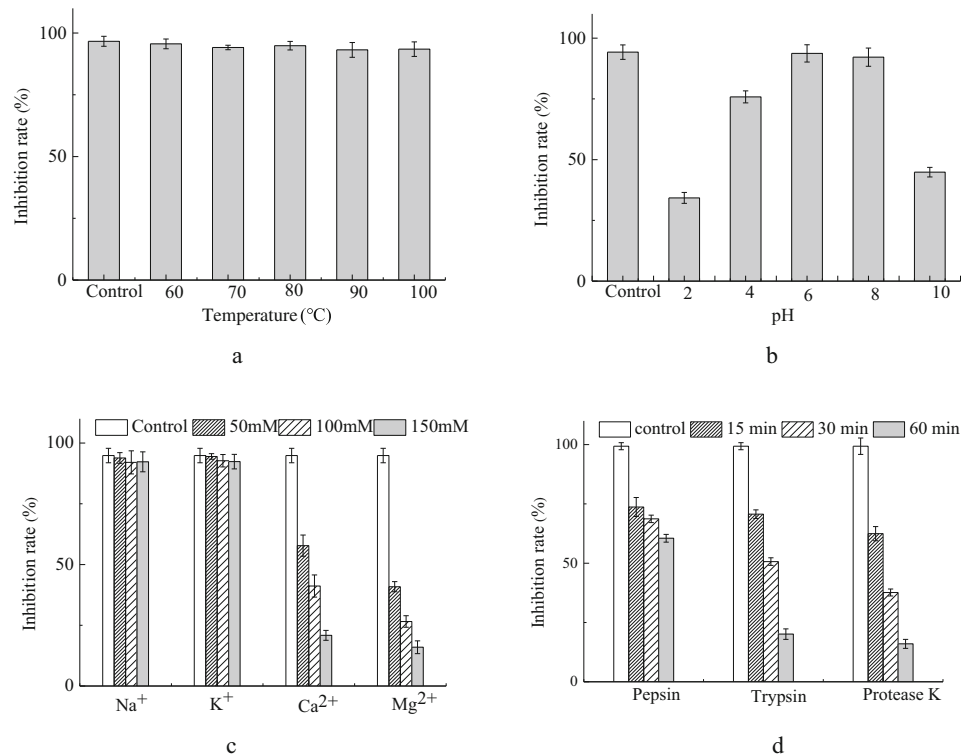
Fig. 7 Effect of NP-6 on the activity of intracellular T-ATPase and β -galactosidase of *E. coli*

was almost lost after long-time hydrolysis with the two enzymes. The antimicrobial activity of NP-6 reduced to 16.00% after incubation for 60 min with proteinase K, while it was 20.10% after incubation with trypsin for the same time. The results suggested that NP-6 was sensitive to the hydrolysis of trypsin and proteinase K, but was resistant to pepsin in a certain extent.

Discussion

We had previously reported that a novel antimicrobial peptide NP-6 derived from pepsin hydrolysis of *Z. bungeanum* Maxim seeds protein was active against some Gram-positive and Gram-negative bacteria (Hou et al. 2019). In the present work, the mechanisms of NP-6 against *E. coli* were further investigated, as well as the resistance of the peptide to temperature, pH, salinity, and enzymes. All the results will help a

Fig. 8 Recovered antimicrobial activity of NP-6 against *E. coli* after treatment by different conditions. **a** Heated at 60, 70, 80, 90, and 100 °C for 15 min; **b** exposed to solutions with different pH values; **c** in the presence of K^+ , Na^+ , Mg^{2+} , and Ca^{2+} ; **d** incubation with pepsin, trypsin, and proteinase K for different times



lot in the further application of NP-6 whether as a bio-preservative in food industry or as a therapy agent in medical science. It was said that the negatively charged bacterial cell surface was supposed to be the receptors of the positively charged AMPs (Hirakura et al. 2002).

Many AMPs exhibit their antimicrobial activities by targeting bacterial cell membrane firstly (da Costa et al. 2015, Nguyen et al. 2011, Anderson et al. 2004). Researchers have employed numerous methodology to investigate the effect of AMPs on bacterial cell membrane (Tao et al. 2011; Tang et al. 2014b; Rončević et al. 2019). Determining the amount of K^+ released from the peptide-treated cells is a classical method for investigating the permeability of AMPs to bacterial cell membranes (Du et al. 2018). The amount of K^+ released to the medium from peptide-treated cells was drastically increased after 1 h and remained stable in 2 h. This might be the evidence that NP-6 increased the permeability of the *E. coli* cell membrane. Similar results were obtained for bifidocin A (Liu et al. 2016), peptide F1 (Miao et al. 2016), and APP (Li et al. 2016). ATP levels in the medium were measured as indexes of cell lysis. When the peptide was in low concentration ($1 \times \text{MIC}$, 64 $\mu\text{g/ml}$), the extracellular ATP contents of peptide-treated cells increased after 2 h. However, significant increase in K^+ efflux ($p < 0.05$) was observed after only 1 h. The result suggested that the later release of ATP might be due to the early efflux of K^+ as the cells consume available ATP in a futile attempt to re-accumulate K^+ (Auliffe et al. 1998; Liu et al. 2016). Additionally, significant increase in the amount of ATP ($p <$

0.05) was observed within 15 min after incubation with NP-6 at a concentration of 1 mg/ml. These results suggested that NP-6 could increase the permeability of *E. coli* cell membrane at low concentration, thus induced the efflux of K^+ . However, it could also cause the bacterial cell membrane disintegration in high concentration followed by the release of large molecules such as ATP. The FCM, CLSM, and TEM results were also evidence for the point that NP-6 could cause the disruption of *E. coli* cell membrane.

Like many other AMPs, despite membrane-disruption effect, NP-6 have intracellular targets as well. In our study, the DNA/RNA-binding assays suggested that the peptide could interact with bacterial DNA/RNA and suppress its migration in vitro. Moreover, the decrease in fluorescence of the EB-DNA system indicated that NP-6 could compete with EB in binding to bacterial DNA, and replace some EB molecular embedded into the bacterial DNA. A similar result was found by Li et al. (2013). In fact, many other AMPs, such as melittin, indolicidin, and NKLP 27, are reported to have the ability to interact with bacterial genomic DNA (Hsu et al. 2005; Zhang et al. 2014; Shi et al. 2016). Besides, it was found that NP-6 could also inhibit the activities of β -galactosidase. Kragol et al. (2001) found that the antimicrobial peptide pyrrolicorin strongly inhibited the ATPase and β -galactosidase of DnaK, thus prevented the chaperone-assisted protein folding. All these results suggested that NP-6 might exhibit multiple action modes to exert its antimicrobial activity.

Besides, NP-6 was a fragment of glycerol-3-phosphate dehydrogenase (GAPDH) through the protein that is generally

associated with its glycolytic role (Branco et al. 2017). Actually, GAPDH also plays roles in many other different sub-cellular locations, apoptosis, and nuclear pathways (Silva et al. 2011; Nakajima et al. 2009). Besides, there have been a few publications reporting the antifungal/antibacterial activity of fragments of GAPDH. One was found secreted by *Saccharomyces cerevisiae* (Branco et al. 2017), and the other three were identified from the skin of yellow tuna (Seo et al. 2012), skipjack tuna (Seo et al. 2014), and human placental tissue (Wagener et al. 2013). All these observations indicated that GAPDH plays a significant role in defense system, further investigations are supposed to be carried out, to explore its extra function. Moreover, GAPDH-derived AMPs had not been identified except one.

It appeared that NP-6 was heat stable in the range of 60–100 °C. The reason that NP-6 exhibits extreme properties may be attributed to the small molecule weight and simple secondary structure. That is to say extreme environmental conditions such as temperature have little effect on the peptide's structure because it is too simple, or it is easy for the peptide to recover its original structure in the absence of extreme conditions. The resistance of AMPs to high temperature has been reported by many literatures (Du et al. 2018; Kumar et al. 2018; Mahlapuu et al. 2016). This feature also makes AMPs have a wider range of applications. Meanwhile, the peptide performed well in a pH range of 6.0–8.0, and the antimicrobial activity decreased significantly when the pH value was higher than 8.0 or lower than 6.0. This may attribute to the different charged state of the peptide in solutions with different pH values, which would further have an effect on the interaction between NP-6 and *E. coli* cell membrane (Tang et al. 2015). Meanwhile, the antimicrobial activity of NP-6 was significantly decreased in the presence of Mg^{2+} and Ca^{2+} . Tang et al. (2015) also declared that the divalent ions had a stronger inhibitory effect than the monovalent ones on the antimicrobial activity. Finally, the protease digestion assay indicated that the antimicrobial activity of NP-6 was significantly affected by trypsin and proteinase K, indicating that the peptide was sensitive to the hydrolysis of protease. A similar case was declared by Seo et al. (2014) who found that SJGAP almost lost all of its activity after treatment of trypsin.

In conclusion, all these results suggested that NP-6 could increase the permeability of *E. coli* cell membrane, cause the disruption of the bacterial cell membrane, bind with bacterial DNA/RNA, and inhibit the activity of β -galactosidase. But it is still unclear which way is the leading role and more assays should be conducted to further investigate it. What's more, NP-6 was heat stable in the range of 60–100 °C and performed well in a pH range of 6.0–8.0. However, the peptide was sensitive to Mg^{2+} and Ca^{2+} , as well as trypsin and proteinase K. All these results suggested that NP-6 could be a potential biological preservative for the agricultural and food industry.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals by any of the authors.

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