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Han-Ning Huang
Marine Research Station, Institute of Cellular and Organismic Biology, Academia Sinica, Ilan, Taiwan, R.O.C.

Chang-Jer Wu
Department of Food Science, National Taiwan Ocean University, Keelung, Taiwan, R.O.C.

Jyh-Yih Chen
Marine Research Station, Institute of Cellular and Organismic Biology, Academia Sinica, Ilan, Taiwan, R.O.C., zoocjy@gate.sinica.edu.tw

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THE EFFECTS OF GAMMA IRRADIATION STERILIZATION, TEMPERATURE, AND PH ON THE ANTIMICROBIAL ACTIVITY OF EPINECIDIN-1

Han-Ning Huang[1], Chang-Jer Wu[2], and Jyh-Yih Chen[1]

Key words: gamma irradiation, antimicrobial peptide, epinecidin-1, methicillin-resistant *Staphylococcus aureus* (MRSA), antibacterial activity.

ABSTRACT

This study examined how different processing conditions affected the antibacterial activity of epinecidin-1 alone or in combination with deionized distilled water or KY jelly and evaluated its activity against methicillin resistant *Staphylococcus aureus* (MRSA) in a product development pipeline. High temperature and γ-irradiation (25 kGy) decreased antimicrobial activity of epinecidin-1, as determined by measuring minimal inhibitory concentrations (MICs) and minimal bactericidal concentrations (MBCs). However, epinecidin-1 exhibited high antimicrobial activity under low pH conditions. The pH stability and the wide spectrum of action against methicillin resistant *Staphylococcus aureus* (MRSA) may make epinecidin-1 peptide a suitable drug candidate. This research demonstrates the effects of gamma irradiation, temperature, and pH on the antimicrobial activity of epinecidin-1 in a product development pipeline. Its stability at low pH and wide spectrum of action against MRSA make the epinecidin-1 peptide an excellent drug candidate. Epinecidin-1 is effective at repressing the growth of MRSA, and it could be used as a lead for developing new antimicrobial agents against MRSA and related organisms.

I. INTRODUCTION

The antimicrobial peptides (AMPs) of fish are gene-encoded natural antibiotics of ancient defense systems (Pan et al., 2007; Pan et al., 2008). Fish AMPs not only directly have antimicrobial activity but are also multifunctional mediators of the immune system (Rajanbabu et al., 2010; Rajanbabu et al., 2011; Pan et al., 2012). The mechanisms of action of fish AMPs (such as TH2-3) have been investigated using mammalian cells, revealing that they inhibit TNF-α and other proinflammatory cytokines through COX-2-, PDE4D-, and pERK1/2-dependent mechanisms (Rajanbabu et al., 2010). Additionally, epinecidin-1, a fish AMP, can destroy the bacterial membrane by inducing saddle-splay curvature, causing membrane vesicular budding or blebbing. Membrane disruption by extensive nonzero curvature tension destabilizes membrane integrity, and the resulting release of cellular contents causes death in *Helicobacter pylori* (Narayana et al., 2015b). The amphipathicity, cationic charge, and molecular size of AMPs allow them to attach to and insert into bacterial membranes, ultimately damaging the bacterial membrane (Zhang and Falla, 2006). Several AMPs have entered clinical development as potentially safe (Narayana and Chen, 2015a), efficient, easy-to-use, and inexpensive drugs (Aoki and Ueda, 2013).

We previously isolated the gene encoding epinecidin-1 from a grouper (*Epinephelus coioides*) cDNA and genomic DNA library. Epinecidin-1 exhibits antimicrobial activity against Gram-positive bacterial strains, Gram-negative bacterial strains, fungi, and viruses (Pan et al., 2007; Pan et al., 2010). In addition, epinecidin-1 can protect against MRSA infection in mice with skin injuries, can serve as an active ingredient in cleaning solutions against pathogens, and can be used as the basis of inactivated vaccines (Pan et al., 2009; Rajanbabu et al., 2010; Huang et al., 2011; Huang et al., 2013; Narayana et al., 2015b). However, the molecular mechanisms underlying epinecidin-1-mediated enhancement of healing and induction of host cell immune responses are not clear. Recently, a cost-effective experimental method for using *E. coli* to produce recombinant epinecidin-1 with antimicrobial activity was reported (Pan et al., 2012). Thus, epinecidin-1 may be suitable as an inexpensive therapeutic material.

Gamma irradiation is used to eliminate bacteria from food (Ndoti-Nembe et al., 2015). While it may be possible to develop a cream or ointment formulation of epinecidin-1 for burn healing or as an active ingredient in cleaning solutions against pathogens, its response to gamma irradiation sterilization, temperature, and pH need to be determined first. It is also important to develop

Paper submitted 03/01/16; revised 11/09/16; accepted 12/29/16. Author for correspondence: Jyh-Yih Chen (e-mail: zoocjy@gate.sinica.edu.tw).
[1]Marine Research Station, Institute of Cellular and Organismic Biology, Academia Sinica, Ilan, Taiwan, R.O.C.
[2]Department of Food Science, National Taiwan Ocean University, Keelung, Taiwan, R.O.C.

methods that improve the shelf life and enhance the microbial safety of drugs. Control of the growth of bacterial strains during storage would increase the shelf life of epinecidin-1 products. Therefore, we evaluated the antimicrobial effects of epinecidin-1 against methicillin resistant *Staphylococcus aureus* (MRSA) following treatment of the AMP with gamma irradiation (25 kGy), different temperatures, or different pH values.

II. MATERIALS AND METHODS

1. Synthesis of Epinecidin-1 Peptides and Sample Preparation

Epinecidin-1 was obtained from a grouper (*Epinephelus coioides*). The peptide possessed the sequence H-GFIFHIIKGLFHAGKMIHGLV-OH and was synthesized by GL Biochem (Shanghai, China) using a solid-phase procedure involving Fmoc chemistry. Crude peptides were extracted, lyophilized, and purified by reverse phase high-performance liquid chromatography (HPLC). The molecular masses and purity of the fractionated peptide were verified by mass spectrometry and HPLC, respectively. The obtained synthetic peptide was > 95% pure, and the peptide was freshly reconstituted in deionized distilled water to generate working stocks prior to each experiment.

2. Sample Preparation.

1) Preparation of Samples for Gamma Irradiation

Five milligrams of epinecidin-1 were soaked in 1 ml of deionized distilled water or KY jelly (Johnson & Johnson; NJ, USA). These mixtures were vortexed at maximum speed for 60 s at room temperature, and the samples were then irradiated. A second experimental group involved direct irradiation of epinecidin-1 powder.

2) Preparation of Samples at Different Temperatures

Epinecidin-1 (2.5 mg) was dissolved in 1 ml deionized distilled water and incubated for 5 minutes at 25°C, 40°C, 60°C, 80°C, or 100°C.

3) Preparation of Samples at Different pH

Deionized distilled water was adjusted to different pH values (2, 4, 6, 8, 10, and 12) by adding sodium hydroxide or hydrochloric acid. Epinecidin-1 peptide was dissolved in each pH solution and mixed by vortexing at maximum speed for 60 s at room temperature to generate working stocks (2.5 mg/ml) prior to each experiment.

4) Irradiation

Samples were sealed in polyethylene bags ensuring full protection against recontamination during the storage period. The samples were irradiated with 25 kGy using a ^{60}Co γ-ray at the China Biotech Corporation. After irradiation, samples were stored at room temperature prior to antimicrobial experiments.

3. Antibacterial Activity

The antibacterial properties of the peptides were evaluated by the microdilution broth method against Gram-positive pathogenic bacteria, as described in the NCCLS guidelines (M26-A) (http://www.nccls.org). A clinical isolate of methicillin resistant *Staphylococcus aureus* (MRSA) used in this study was obtained from the Taipei City Hospital (Heping Fuyou branch); it is multidrug resistant. For MIC assessment, epinecidin-1 peptide was diluted to final concentrations of 1250.000, 625.000, 312.500, 156.250, 78.125, 39.062, 19.531, or 9.765 μg/ml. Fifty microliters of each epinecidin-1 dilution was mixed in a microtiter plate well with 50 μl of the appropriate bacterial indicator suspension and 50 μl of TSB with *S. aureus* to a total volume of 200 μl. Three replicates were examined for each *S. aureus* strain, epinecidin-1 peptide condition, and concentration. Controls contained TSB without bacterial suspensions, and negative controls contained bacterial suspensions without epinecidin-1 peptide. Microbial growth was automatically determined by optical density measurements at 600 nm using a Molecular Devices SpectraMax® i3 (Molecular Devices, CA, USA). Microplates (Corning, NY, USA) were incubated at 37°C. Absorbance readings were taken at hourly intervals over a 24-hour period, and the plates were shaken for 20 seconds before each measurement. The experiment was repeated twice. The lowest compound concentration that resulted in zero growth by the end of the experiment was taken as the minimum inhibitory concentration (MIC). Minimum bactericidal concentration (MBC) was determined by taking a portion of liquid (10 μl) from each well that exhibited no growth, and then incubating it at 37°C for 24 hours. The lowest concentration that revealed no visible bacterial growth after sub-culturing was taken as the MBC. Positive and negative cultures were also prepared.

4. Statistical Analysis

Two replicates were performed for all experiments. Data were analyzed using PASW Statistics Base 18.0 software (SPSS Inc., IL, USA). Statistical significance was determined using Student's t-test. A p-value of < 0.05 was considered to be statistically significant.

III. RESULTS AND DISSCUSSION

1. Effect of Irradiation on the Antimicrobial Activity of Epinecidin-1 in Different Solvents

AMPs are potential novel antimicrobial agents against human and animal pathogens. We used KY jelly to dissolve epinecidin-1 sterilized by gamma irradiation (25 kGy) and evaluated its activity against MRSA. KY jelly was used as it is a potential topical ointment for female genitalia, which can become infected with MRSA. Table 1 shows the effect of γ-irradiation on epinecidin-1 in different solvents. Irradiation of epinecidin-1 powder prior to dissolution in ddH_2O decreased antimicrobial activity (MIC of 39.060 μg/ml and MBC of 78.125 μg/ml) compared to the unirradiated control (MIC of 9.765 μg/ml and MBC of 19.531

Table 1. MIC and MBC under gamma irradiation.

Sample preparation	Non-Gamma sterilization	Gamma sterilization	Average[a]	MIC (μg/ml)[b]	MBC (μg/ml)[c]
Epi-1	+	-	0.039 ± 0.043	9.765	19.530
Epi-1	-	+	0.043 ± 0.004	39.060	78.120
Epi-1 was dissolved in water	-	+	0.089 ± 0.011	156.250	312.500
KY	+	-	0.052 ± 0.035	NE[d]	NE
Epi-1 was dissolved in KY	+	-	0.044 ± 0.039	9.765	19.530
Epi-1 was dissolved in KY	-	+	0.067 ± 0.018	78.120	156.250

[a] Average absorbance for OD_{600}; [b] The minimal inhibitory concentration; [c] The minimal bactericidal concentration; [d] NE: No effect

Table 2. MIC and MBC after pre-treatment at different temperatures.

Temperature	Average[a]	MIC (μg/ml)[b]	MBC (μg/ml)[c]
25°C	0.040 ± 0.029	9.765	19.530
40°C	0.024 ± 0.020	312.500	312.500
60°C	0.061 ± 0.022	312.500	625.000
80°C	0.278 ± 0.100	> 625.000	NE[d]
100°C	0.229 ± 0.071	> 625.000	NE

[a] Average absorbance for OD_{600}; [b] The minimal inhibitory concentration; [c] The minimal bactericidal concentration; [d] NE: No effect

Table 3. MIC and MBC at the indicated pH values.

pH	Average[a]	MIC (μg/ml)[b]	MBC (μg/ml)[c]
2	0.063 ± 0.027	9.765	19.530
4	0.058 ± 0.058	19.531	39.062
6	0.062 ± 0.017	19.531	39.062
8	0.064 ± 0.009	78.125	156.250
10	0.084 ± 0.013	78.125	312.500
12	0.600 ± 0.204	> 625.000	NE[d]

[a] Average absorbance for OD_{600}; [b] The minimal inhibitory concentration; [c]The minimal bactericidal concentration; [d] NE: No effect

μg/ml). If epinecidin-1 was irradiated after being dissolved, the MIC was 156.250 μg/ml and the MBC was 312.500 μg/ml. Finally, γ-irradiation of epinecidin-1 dissolved in KY jelly also reduced activity (MIC of 78.125 μg/ml and MBC of 156.250 μg/ml) compared to the control (MIC of 9.765 μg/ml and MBC of 19.531 μg/ml).

2. Effect of Pretreatment at Different Temperatures on Antimicrobial Activity

Previous research has shown epinecidin-1 antibacterial activity against various microorganisms *in vitro* and *in vivo*. This research has shown how temperature affects the antibacterial properties and stability of epinecidin-1 antibacterial activity at different temperatures. Therefore, we used the same procedure to understand its potential in product development (ex, medical equipment and drugs) and as a natural antibiotic. First, we proceeded by examining the effects of temperature on the MIC and MBC. Antimicrobial activity was greatest after pretreatment at 25°C (MIC of 9.765 μg/ml and MBC of 19.531 μg/ml), and steadily decreased with the pretreatment temperature (Table 2). Next, we examined bacterial numbers after pretreatment of freshly prepared solutions containing epinecidin-1 at various temperatures for 5 minutes (Fig. 1). Increased bacterial numbers (based on OD_{600}) were observed at pretreatment temperatures of 60°C, 80°C, and 100°C. After pretreatment at 25°C, the minimum peptide concentration was 4.882 μg/ml, which was significantly greater than that of MRSA alone. However, pretreatment of epinecidin-1 at 100°C reduced this to 312.500 μg/ml. Thus, this AMP cannot be heated without losing its activity.

3. Effect of pH on the Antimicrobial Activity of Epinecidin-1

Antibacterial properties against microorganisms are dependent on various parameters. These include the effects of pH. This part of the experiment mainly demonstrated the antibacterial effects of epinecidin-1 in various pH conditions against MRSA. As shown in Table 3, we examined the effects of acidic and basic environments on the antimicrobial activity of epinecidin-1. Activity was greatest at a pH of 2 (MIC of 9.765 μg/ml and MBC of 19.531 μg/ml), with a successive decrease in activity as the

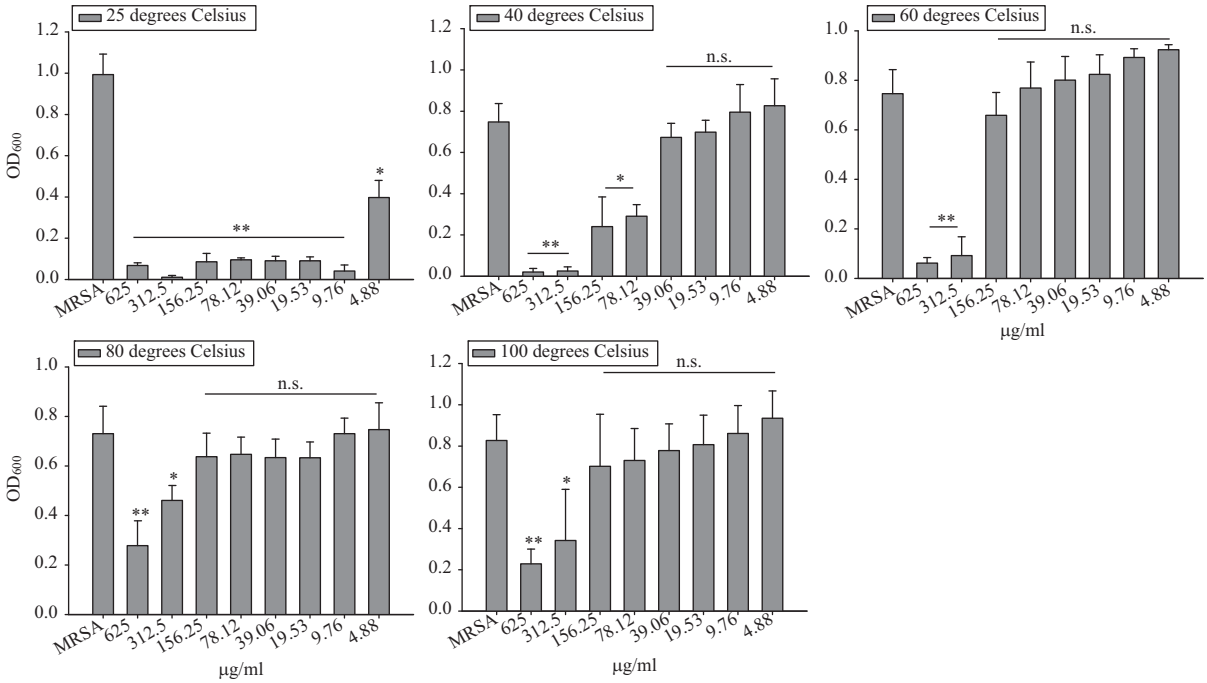

Fig 1. The effect of temperature on the antibacterial activity of epinecidin-1.

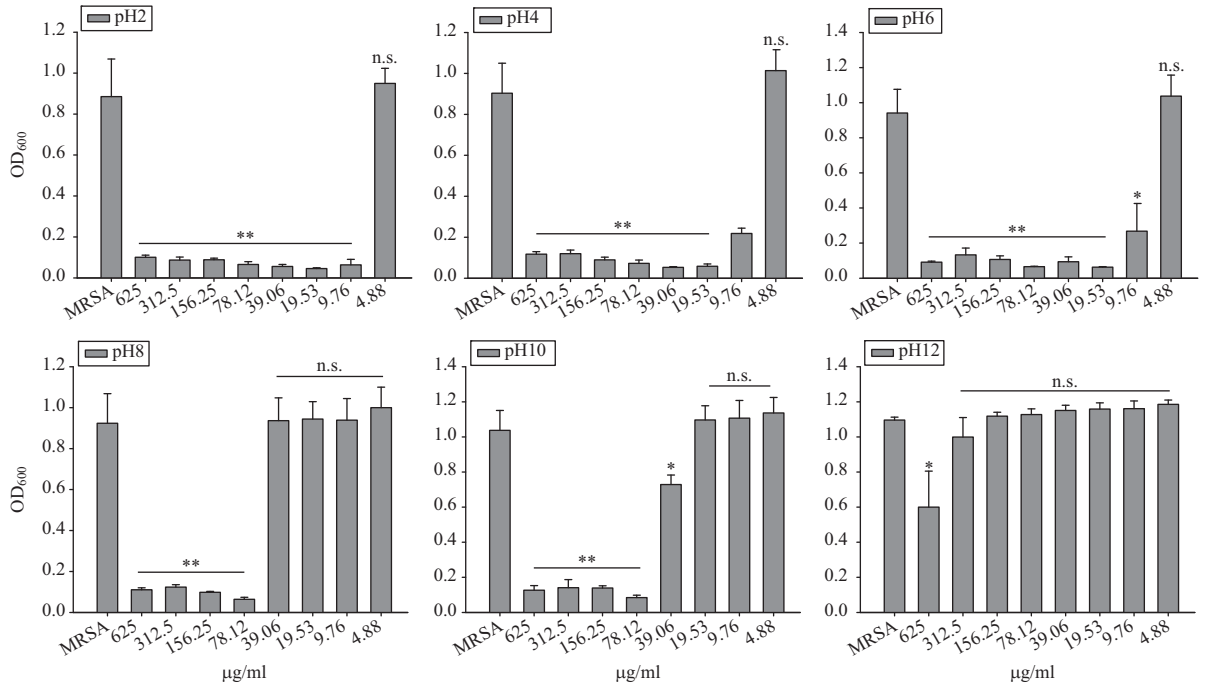

Fig 2. The effect of different pH values on the antibacterial activity of epinecidin-1.

pH increased. This indicates that epinecidin-1 antimicrobial activity is greatest under acidic conditions. To assess the microbicidal activities of solutions containing epinecidin-1 over a broad range of relevant acidic and basic environments, we determined bacterial numbers (based on OD_{600}) at different pH values. Epinecidin-1 in pH 2 solution had a minimum peptide concentration of 9.765 μg/ml, which was significantly different to that of MRSA alone. At pH 8 and pH 10, the minimum peptide concentration was 78.125 μg/ml, and at pH 12 solution, it was 625.000 μg/ml, indicating that activity decreases with pH, and was lost at a pH of 12 (Fig. 2). These results suggest that epinecidin-1 possesses antimicrobial activity in aqueous environments at pH 2, 4, and 6.

We observed that irradiation at 25 kGy was sufficient to re-

duce the antimicrobial activity of both epinecidin-1 powder and epinecidin-1 dissolved in KY jelly against MRSA. γ-irradiation is widely used for sterilization of medical devices, such as alginate foams or gels. However, γ-irradiation can cause dose-dependent degradation of many polysaccharides and alginates in the dry state, in solution, and in the gel state, as radiation can induce both cross-linking and degradation via radical reactions (Nagasawa et al., 2000; Lee et al., 2003; Andersen et al., 2012; Ulset et al., 2014). For example, it was previously reported that type I collagen and gelatin undergo simultaneous cross-linking and degradation in response to γ-irradiation (Hsiong et al., 2008; Hara et al., 2010; Vacharathit et al., 2011). Our finding that epinecidin-1 activity is reduced by γ-irradiation may be due to the formation of hydroxyl radicals by radiolysis of water (Huq et al., 2012). It was reported that irradiation at 1.0 kGy was also effective at reducing *Listeria monocytogenes* in carrots (Ndoti-Nembe et al., 2015). Earlier analysis of alginate-based biomaterials revealed that alginate chains degrade randomly in a dose-dependent manner after irradiation, which alters material features. Thus, it is reasonable to suggest that irradiation can significantly decrease the antimicrobial activity of epinecidin-1 by inducing degradation of the peptide.

We addressed the significant challenge of distributing antimicrobial peptides in developing countries with a hot climate and limited opportunities for refrigeration by examining the effect of high temperature on antimicrobial activity. We found that epinecidin-1 is not thermostable above 40°C. Using peptide self-assemblies based on ESAT651-70, it was found that the immunogenicity of ESAT651-70-Q11 stored as a dry powder or as aqueous nanofibers was undiminished even when stored as long as six months at 45°C (Sun et al., 2015). These results suggest that peptide self-assemblies may possess attractive thermal stability properties. The self-aggregation of α-crystallin induced by denaturants at higher temperature can be controlled and even partially reversed, suggesting that self-aggregation plays an important role in thermostability (Villari et al., 2014). The effects of protein quaternary structure on thermal stability have been theoretically considered, and experimentally validated for several proteins with allosteric factors (Brandts et al., 1989; Milardi et al., 1996; Lisi et al., 2014). Epinecidin-1 has not been studied in this regard to the extent that one would expect, and as such, there remains a need to study the thermodynamic consequences of inter-amino acid interactions and changes in the secondary and tertiary structure of the amino acids by CD measurements.

AcAMP, a 6.0-kDa antimicrobial peptide from *Aspergillus clavatus* ES1, is a particularly interesting peptide that is sensitive to proteolytic enzymes, stable between pH 5 and 10, and heat resistant (15 minutes at 100°C) (Hajji et al., 2010). Bacteriocins from 13 strains of *Bifidobacteria* are active at pH values ranging from 2.0 to 10.0, unlike the AcAMP peptide, which has an optimum pH of 4.8 and maximum inhibitory activity only from pH 4.8 to 5.5 (Hajji et al., 2010). Epinecidin-1 has an amphipathic helix structure, with one side composed of clustered hydrophobic side chains and the other composed of the helix and hydrophilic side chains. Epinecidin-1 has high antimicrobial activity in a pH 2 solution, possibly due to amphipathic helix structural changes and the formation of dimers and transmembrane pores. This result confirms the earlier results with *H. pylori* (Narayana et al., 2015b). At pH 12, on the other hand, epinecidin-1 assumed an entirely unknown structure that could not be assigned and did not exhibit bactericidal activity. Antimicrobial peptides often interact or react with bacterial membranes in neutral to low pH environments in a normal eukaryotic host (Misiewicz et al., 2015) The amenability to low pH may make AMPs suitable for survival in the stomach after oral administration. Many structure-activity relationship studies have shown that increasing α-helical components can enhance the membrane-lytic activity of antimicrobial peptides (Dathe and Wieprecht 1999). For example, changes of structure and membrane binding tendency of AMPs at different pH values have been shown to play an important role in pH-dependent antitumor activity (Song et al., 2013).

IV. CONCLUSIONS

This study demonstrates how different processing conditions affected the antibacterial activity of epinecidin-1 in powder or reconstituted in deionized distilled water or KY jelly. We evaluated its activity against methicillin resistant *Staphylococcus aureus* (MRSA) in a product development pipeline. The epinecidin-1 peptide possesses the features of small, basic, cysteine-rich antimicrobial peptides from fish. Its stability at low pH and its wide spectrum of action against methicillin resistant *Staphylococcus aureus* (MRSA) make epinecidin-1 peptide an excellent drug candidate.

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