Low-intensity ultrasound enhances the antimicrobial activity of neutral peptide TGH2 against *Escherichia coli*

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**A R T I C L E  I N F O**

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**A B S T R A C T**

In recent years, foodborne diseases caused by *Escherichia coli* are a major threat to the food industry and consumers. Antimicrobial peptides (AMPs) and ultrasound both have good inhibitory effects on *E. coli*. In this work, the mechanism of action and synergistic effect of an *in silico* predicted AMP, designated as TGH2 (AEFL-REKLGDKCTDRHV), from the C-terminal sequence of Tegillarca granosa hemoglobin, combined with low-intensity ultrasound was explored. The minimal inhibitory concentration (MIC) of TGH2 on *E. coli* decreased by 4-fold to 31.25 μg/mL under 0.3 W/cm² ultrasound treatment, while the time kill curve analysis showed that low-intensity ultrasound combined with peptide TGH2 had an enhanced synergistic bactericidal effect after 0.5 h. The permeability on *E. coli* cell membrane increased progressively during combined treatment with peptide TGH2 and low-intensity ultrasound, resulting in the leakage of intracellular solutes, as shown by transmission electron microscopy (TEM). Structural analysis using circular dichroism (CD) revealed that peptide TGH2 has an α-helical structure, showing a slight untwisting effect under 0.3 W/cm² ultrasound treatment for 0.5 h. The findings here provide new insight into the potential application of ultrasound and AMPs combination in food preservation.

1. Introduction

Human health is threatened by foodborne pathogens found in food raw materials, processing, and storage, with foodborne diseases estimated to affect 600 million people causing 420,000 deaths annually, 40% of which are children under 5 years old [1]. *Escherichia coli* are Gram-negative bacteria, the common cause of gastrointestinal tract or urethra infections in humans and animals. Many foodborne disease outbreaks have been caused by *E. coli*, including the 2009 Godstone Farm outbreak in England, 2011 sprouted foods outbreak in Germany, and the 2018 romaine lettuce outbreak in the United States of America [2].

Ultrasounds, which are acoustic waves with a frequency greater than 20 kHz, are vibration energy with good directivity, penetrability, and reflectivity, currently being used as a front-line antimicrobial technique in the food industry [3]. Compared with traditional thermal sterilization technology, ultrasound has the advantages of shortening sterilization time, energy saving, improving food quality, and high automation [4]. Under controlled ultrasonic waves, the cell membrane absorbs acoustic field energy and transforms into nano-scale vibration and deformation. The separation and closing of lipid bilayer disturbs the orderly arrangement of cell membrane molecules to instantaneously produce holes in the cell membrane, which increases bacteria cell membrane permeability to inhibit bacteria growth [5]. Ultrasound has been shown to inhibit *E. coli* O157:H7 and *Listeria monocytogenes* in almond milk and increase the shelf life during refrigeration [6]. Similarly, ultrasound has the same antimicrobial effect on these two bacteria on lettuce [7]. However, when the ultrasonic wave intensity is lower than the bacterial tolerance threshold, ultrasound alone cannot inhibit microbial growth, which increases public health risks [8]. Therefore, novel complementary antimicrobial methods are required to enhance the antimicrobial activity of ultrasound on bacteria.

Antimicrobial peptides (AMPs) are mostly cationic, amphipathic peptides produced by diverse species against invading pathogenic
Although numerous studies had explored the synergistic effect of ultrasound and antimicrobial agents [3], few studies have examined ultrasound and antimicrobial peptides against E. coli. Under high-intensity ultrasound treatment, the structure of peptides could be damaged, decreasing the antimicrobial activity of AMPs. Therefore, this study aimed to investigate whether low-intensity ultrasound synergistically enhance the antimicrobial activity of net-naturally charged peptide TGH2 against E. coli and explore the mechanisms involved. This approach would provide a new method in the combined use of ultrasound/antimicrobial peptides to control foodborne pathogens.

2. Materials and methods

2.1. Microorganism and reagents

The bacteria (E. coli, Vibrio parahaemolyticus, and Vibrio alginolyticus) used are stocks stored in the Guangdong Provincial key Laboratory of Marine Biotechnology, Shantou University, Shantou, China. Bacteria were cultured at 37 °C for 24 h in nutrient broth (NB) medium. O-nitropheryl-β-D-galactopyranoside (ONPG) was purchased from Sigma-Aldrich (Shanghai, China).

2.2. Screening and synthesis of AMPs

Hemoglobin-derived AMPs from Tegillarca granosa were predicted using two online software, antibacterial peptides (AntiBP) server and collection of anti-microbial peptides (CAMP) server, as previously described [15]. The AntiBP server was used to search for AMPs from hemoglobin sequences, whereas the CAMP server was used to evaluate the reliability of the predicted AMPs. On the other hand, the hydrophobicity and net charge of AMPs were calculated with the antimicrobial peptide calculator and predictor (APD3) server.

The predicted peptide was synthesized and purified by a commercial company, Sclight Biotechnology Co, as previously described [16]. More than 99% peptide purity could be attained using HPLC with an Agela C18 column. The purity and molecular mass of purified synthetic peptide was determined by liquid chromatography coupled to mass spectrometry (LC-MS/ESI).

2.3. Antibacterial assay

2.3.1. The effect of ultrasound on bacterial growth

The growth of E. coli was observed with a JY92-1HDN ultrasound cell disruptor (Sciente, China) under low-intensity ultrasound. Briefly, NB liquid medium was inoculated with 10^5 CFU/mL E. coli, cultured at 37 °C, followed by treatment with 0.3 W/cm² ultrasound for 0.5 h. Next, 20 μL samples were taken at 0, 1, 2, 3, 4, 5, 6, 7, and 8 h for plate counting. Bacterial suspension without ultrasound treatment was used as control [17].

2.3.2. Determination of the minimum inhibitory concentration (MIC)

E. coli suspension was cultured in nutrient broth (NB) at 37 °C for 16 h, before being diluted to a concentration of 10^8 CFU/mL with sterile 0.01 M phosphate buffer saline (PBS). Next, peptide TGH2 dissolved in sterile PBS was added to an equal volume of the bacterial suspension in sterile 96-well microplates under 0.3 W/cm² ultrasound treatment. Plates were incubated at 37 °C for 16 h. The MIC was defined as the lowest concentration that visibly prevented growth after 16 h incubation at 37 °C [10].

2.3.3. Time-kill analysis of TGH2

To assess the antimicrobial activity of peptide TGH2, time-kill kinetics analysis was carried out as previously described with some modifications [18]. Briefly, peptide TGH2 was added into logarithmic-phase cultures (10^3–5 CFU/mL) to a final concentration of 31.25 μg/mL (1 × MIC). Next, the bacterial suspension was treated with 0.3 W/cm² ultrasound for 0.5 h, followed by incubation at 37 °C. At 0.5, 1, 1.5, 2, 2.5 and 3 h, 20 μL of bacterial suspensions were taken, the colonies counted after being cultured on NB plates at 37 °C for 16 h. Bacterial suspension without ultrasound treatment was used as control.

2.4. Determination of electrical conductivity of the bacterial suspension

The inner membrane permeability of peptide TGH2 under ultrasound treatment was determined by measuring the level of O-nitrophenol produced by cytoplasmic β-galactosidase from E. coli cells as described previously [20]. Briefly, exponential phase bacteria were collected by centrifugation at 2700 × g for 10 min before being incubated in M9 medium with lactose as the sole carbon source. Next, samples were cultured at 37 °C until an OD_420 of 0.4 was reached, after which various concentrations of peptide TGH2 (i.e., 1/2 × MIC and 1 × MIC) and 0.5 mg/mL ONPG were added. The bacterial suspension was incubated under 0.3 W/cm² ultrasound treatment for 0.5 h at 37 °C, whereas control samples were cultured without ultrasound treatment. The production of O-nitrophenol over time was measured at 420 nm using a multimode plate reader (PerkinElmer, USA).

2.6. Transmission electron microscopy (TEM)

To determine the antimicrobial mechanism of peptide TGH2 under low-intensity ultrasound treatment, E. coli was cultured to exponential phase before being resuspended in NB broth to 10^6–7 CFU/mL. Next, peptide TGH2 was added to the bacterial suspension to a final concentration of 1 × MIC and then incubated at 37 °C with 0.3 W/cm² ultrasound treatment for 0.5 h. The bacterial suspensions were centrifuged at 2700 × g for 10 min, followed by fixing cells with 10 mg/mL osmic acid and dehydrated with ethanol. Samples were embedded by roasting at 70 °C for 24 h, after which 70–90 nm thin slides were prepared on copper grids and stained with lead citrate and uranyl acetate. The ultrastructure of E. coli was observed with an H-7650 transmission electron microscope (Hitachi, Japan).

2.7. Circular dichroism (CD) spectrophotometry

Circular dichroism (CD) was used to evaluate the effect of ultrasound on the secondary structure of peptide TGH2. For the CD analysis, peptide TGH2 was treated with ultrasound for 0.5 h before being analyzed using a spectropolarimeter (Jasco 810, Tokyo) at 25 °C with 100 nm/min scanning speed [21]. Peptide TGH2 was dissolved in 25 mM sodium dodecyl sulfate (SDS) to a final concentration of 0.20 mg/mL under 0.3 W/cm² ultrasound treatment for 0.5 h. Next, 0.2 mg/mL of peptide TGH2 in SDS solution was loaded into a 1-mm quartz cuvettes, and the data recorded from 180 to 250 nm.
3. Results and discussion

3.1. Antimicrobial peptides prediction and screening

The hemoglobin of *T. granosa* (152 amino acids) has immune-related functions and can generate antimicrobial peptides (AMPs) to clear pathogenic microorganisms [22]. Our previous study revealed that an in silico predicted AMP, TGH1, from the hemoglobin sequence of *T. granosa*, possessed strong antimicrobial activity against *Vibrio parahaemolyticus* [15]. Unlike peptide TGH1, which was predicted from the N-terminal of hemoglobin sequence, we also predicted and screened two peptide sequences, i.e., H1 (FLREKLGDKCTDRHV) and H2 (AEFLREKLGDKCTDR) on the C-terminal Table 1. Given that peptides H1 and H2 are very similar in sequence, we decided to combine them to form a new sequence, i.e., AELREKLGDKCTDRHV, designated as TGH2. Peptide TGH2 has a net neutral charge, different from most AMPs, with net positive charges from +2 to +9 [23]. It is generally difficult to adsorb neutral peptides onto the negatively charged bacteria cell membranes to destroy or exhibit antibacterial activity [24]. Interestingly, TGH2 could damage bacterial cell membranes under low-intensity ultrasound treatment. Therefore, we explored the synergistic mechanism between ultrasound and peptide TGH2 in this study, which could provide further insight into the combined application of ultrasound techniques and AMPs in food processing and preservations.

3.2. Antimicrobial activity of peptide TGH2 with and without ultrasound treatment

As a nonthermal technique, ultrasound has been widely used to inactivate microbes in food industry [3]. Therefore, before determining whether the antimicrobial activity of peptide TGH2 could be enhanced by ultrasound treatment, the effects of ultrasound on *E. coli* was examined. Interestingly, under the condition of 0.3 W/cm² ultrasound treatment, there was no significant difference in the number of *E. coli* compare with the control group after 8 h (Fig. 1). Similar observations have previously been reported, where low-intensity ultrasound alone could not efficiently kill bacteria, with bacteria survival improved in some cases because the low-intensity ultrasound activated the bacterial stress response system [25]. To assess the synergistic antimicrobial effect of ultrasound and peptide TGH2, the MIC of synthetic TGH2 against three pathogenic bacteria was determined. Peptide TGH2 had no antimicrobial effect on *V. parahaemolyticus* and *V. alginolyticus* but had good antimicrobial activity against *E. coli*. Under the condition of 0.3 W/cm² ultrasound treatment, the MIC of peptide TGH2 against *E. coli* decreased by 4-fold, from 125 μg/mL to 31.25 μg/mL (Table 2). In the time-kill curve analysis, the peptide and ultrasound group was much better than the peptide only treatment group after 0.5 h (Fig. 2). These results are consistent with previous studies that reported that ultrasound could enhance the inhibitory effect of antimicrobial agents. For instance, Yang et al. revealed that low-intensity ultrasound could trigger a synergistic effect with amphotericin B-loaded nanoparticles to produce a more effective antifungal activity against *Candida Albicans* [26]. A synergistic action between Erythrosin B and low-frequency ultrasound was shown to enhance the inactivation rate of *Listeria innocua* [27]. Although various studies have thus far shown that ultrasound assists antimicrobial agents to enhance their antimicrobial activity, few have explored this synergistic effect with AMPs, especially given that the characteristic secondary structure of AMPs could be damaged by ultrasound, thereby attenuating their antibacterial activity [28]. Therefore, it is necessary to study the synergistic effects and mechanism of ultrasound and AMPs.

### Table 1

Predicted antimicrobial peptides from hemoglobin sequence of *T. granosa*.

| NO. | Peptide sequences | MW (Da) | Start position | Predicted score |
|-----|------------------|---------|----------------|-----------------|
| H1  | FLREKLGDKCTDRHV | 1817    | 121            | 1.378           |
| H2  | AEFLREKLGDKCTDR | 1781    | 119            | 0.615           |
| H3  | RHVESWGLKIDVIRA | 1779    | 133            | 0.492           |
| H4  | GWIKAPLAEFLREKL | 1771    | 112            | 0.133           |
| H5  | GDKCTDRHVESWGLK | 1731    | 127            | 0.122           |

### Table 2

Antibacterial activity of peptide TGH2 against three pathogenic bacteria.

| Microorganism | MIC (μg/mL) | Ultrasound treatment | Control |
|---------------|-------------|----------------------|---------|
| *Escherichia coli* | 31.25 | 125 | |
| *Vibrio parahaemolyticus* | >500 | >500 | |
| *Vibrio alginolyticus* | >500 | >500 | |

![Fig. 1. Growth curve of *E. coli* under different treatments.](image1)

![Fig. 2. Time-kill curve of peptide TGH2 against *E. coli*. Peptide TGH2 concentration was 31.25 μg/mL, ultrasonic intensity was 0.3 W/cm².](image2)
3.3. Antimicrobial mechanism of peptide TGH2 with low-intensity ultrasound treatment

3.3.1. Changes in bacteria inner membrane permeability

When cell membrane permeability increases, the intracellular conductive materials (i.e. H⁺, K⁺ and inorganic etc.) will flow out through the cell membrane, which increases the conductivity of the bacterial culture medium [19]. The initial conductivity of E. coli was very low, however, there was a marked increase with TGH2 and ultrasound treatment after 1.5 h, which indicates that TGH2 and ultrasound could change the membrane permeability of bacteria (Fig. 3).

The level of O-endorphin, a degradation product of ONPG in the cytoplasm, was measured to ascertain the changes in bacterial (E. coli) membrane permeability due to peptide TGH2 and ultrasound treatment [18]. The level of O-endorphin remained constant after 0.5 h in the control group, while the OD_{420} value increased significantly with combined TGH2 and ultrasound treatment, indicating cell membrane damage after 4 h. The O-endorphin values for only ultrasound treatment and only 1 × MIC peptide TGH2 treatment were the same, which was much lower than combined TGH2 peptide and ultrasound treatment for 7 h. These results indicate that combined ultrasound and peptide TGH2 treatment could enhance E. coli permeability more effectively than individual treatment (Fig. 4).

The electric conductivity of E. coli increased significantly after 1.5 h, while o-endorphin levels increased after 4 h. Thus, it can be speculated that during the initial 1.5 h treatment, TGH2 and ultrasound modified the bacteria membrane permeability by forming some pores that allow small ions (such as H⁺, K⁺ and inorganic phosphates) to pass through the cell membrane but not large compounds (e.g., ONPG enzymes). On the other hand, the increase in O-endorphin levels after 4 h results from damage to the bacterial membrane structure, allowing the release of ONPG enzyme. Therefore, the destruction of the bacterial cell membrane was a progressive process during the combined treatment with peptide TGH2 and ultrasound.

Generally, ultrasound assist antibacterial agents to enhance their antibacterial effect mainly through acoustic cavitation, sonoporation, and sonochemistry mechanisms [3]. Acoustic cavitation occurs when bubbles in liquid irradiated with ultrasound form and collapse violently to generate strong physical forces (i.e., liquid shear force, shock wave, micro-jets, turbulence, etc.) [29]. Given that these strong forces can cause series of physical damages including perforation of membranes and increasing cell membrane permeability (i.e., sonoporation), this could be the reason for the obvious cell damage observed under low-intensity ultrasound [30]. On the contrary, sonochemistry is considered the most chemically active in the range of 100–1000 kHz, which maximizes the production of free radicals and causes oxidative damage to bacteria [31]. On this basis, sonoporation might be the mechanism by which the antimicrobial effect of TGH2 is enhanced by low-intensity ultrasound. In particular, low-intensity ultrasound first induces the formation of pores on the bacterial cell membrane, allowing the outflow of small molecules and the entry of peptide TGH2 through these pores to destroy and kill bacteria. Sonoporation was first used to explain the phenomenon of ultrasound-induced permeability changes to temporal lobe cells [32], which is related to the mechanical effect caused by collapse of acoustic cavitation bubbles [33]. Moreover, sonoporation has been shown to generate holes on the bacterial cell membrane for many antimicrobial agents to pass through rapidly without changing the characteristics of bacterial cell membrane. This might explain the enhancement of the synergistic antimicrobial activity of ultrasound when used with other antibacterial agents [25].

3.3.2. Changes in morphology

The most visible effect of the antimicrobial mechanism of AMPs and ultrasound is the damage to bacteria cell membrane and internal structures [10,25]. The ultrastructural changes induced by peptide TGH2 and ultrasound in E. coli were observed by TEM. Control samples showed uniformly distributed tissues, with no leakage and smooth cell membranes and walls (Fig. 5A). However, with peptide TGH2 treatment, there were some membrane blurring and irregular structures with homogeneous electron density in the cytoplasm (Fig. 5B). When bacteria were treated with both TGH2 and ultrasound, there was internal solute leakage and complete vacuolation in E. coli cells. Bacteria cell membranes and walls were however continuous and smooth, further confirming that low-intensity ultrasound enhanced the antimicrobial activity of peptide TGH2 through the mechanism of sonoporation (Fig. 5C).

Similar studies on the perforation effect on bacterial cells by antimicrobials enhanced by ultrasound have recently been reported [34]. These perforation effect induced pore formation on the cell walls, increased bacteria cell membrane permeability, triggered intracellular uptake of chemicals to damage proteins, enzymes, and DNA, resulting in internal solute leakage [35]. For instance, electron microscopy examination of the synergetic inactivation effects of ultrasound and the ovotransferrin (OVT) on E. coli and S. aureus revealed that the acoustic cavitation could contribute to the penetration of OVT into bacterial cells by weakening cell walls, breaking chemical bonds in cell membrane and increasing cell permeability [36].

3.3.3. Changes in the secondary structure of peptide TGH2

Given that the secondary structure of peptides can be damaged by
ultrasound treatment to decrease the antimicrobial activity of peptides [16,28], changes to the secondary structure of peptide TGH2 was examined using circular dichroism. Low-intensity ultrasound had little effect on the random structure of peptide TGH2 in PBS buffer. However, the secondary structure of TGH2 was transformed to an α-helical structure (one positive peak at 191 nm and two negative peaks at 207 nm and 220 nm) from a random structure (one negative peak at 200 nm) in a membrane mimicking environment. Meanwhile, the peak value of the α-helical structure decreased at 191 nm, 207 nm and 220 nm, which indicated a slight untwisting effect on peptide TGH2 after 0.5 h of low-intensity ultrasound treatment (Fig. 6).

Antimicrobial peptides represent promising alternatives to conventional antibiotics, which could solve the increasing incidence of bacterial resistance to available antibiotics [37]. With diverse structures, i.e., α-helical and β-sheet, AMPs play a bacteriostatic role by nonspecific membrane interactions or specific targets [38]. For α-helical peptides, the toroidal model is the primary antimicrobial mechanisms by which AMPs such as magainins, melittin, and protegrins act on bacteria cell membranes [39]. In this model, transmembrane pores induced by AMPs, leading to membrane dysfunction, leakage of cellular content, and cell death [40]. Peptide TGH2 exhibits random structure in PBS solution and adopts an α-helical structure in membrane mimicking environment to enhance interaction with the cell membrane [21]. This transformation of peptide TGH2 structure did not change under low-intensity ultrasound treatment, although the secondary structure of peptides could be destroyed by high-intensity ultrasound [41]. Under the condition of 0.3 W/cm² ultrasound, the helical structure of peptide TGH2 still had a slight untwisting. Therefore, these results suggest that controlled ultrasound intensity, when combined with antimicrobial peptides, could significantly enhance the effectiveness of antimicrobial agents.

4. Conclusion

Collectively, the current study indicates that ultrasound can accelerate the formation of transmembrane pores on bacteria cell membranes through the action of acoustic cavitatation, which results in the outflow of cellular components and allowing easy entry by peptide TGH2 into bacteria cells, thereby increasing its antibacterial activity. Moreover, low-intensity ultrasound does not cause significant damage to the secondary structure of TGH2. These results indicate that the combination of low-intensity ultrasound and AMPs is a promising method for enhancing antimicrobial activity.

CRediT authorship contribution statement

Shen Yang: Writing - original draft. Zijin Yuan: Software, Validation. Jude Juventus Aweya: Methodology, Writing - review & editing. Shiyng Huang: Methodology. Shanggui Deng: Visualization. Linfan Shi: . Mingqing Zheng: . Yueling Zhang: Methodology. Guangming Liu: Supervision, Resources, Writing - review & editing.

Fig. 5. TEM images of E. coli under different treatments. (A) Control; (B) TGH2; (C) TGH2 and ultrasound.

Fig. 6. CD spectra of TGH2. SDS concentration was 25 mM, PBS concentration was 10 mM, ultrasonic intensity was 0.3 W/cm².

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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