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Antibacterial activities and membrane permeability actions of glycinin basic peptide against *Escherichia coli*

Ying-Qiu Li [a,*], Xiu-Xiu Sun [a], Jian-Ling Feng [a,b], Hai-Zhen Mo [c]

[a] School of Food Science & Engineering, Qilu University of Technology, No. 3501 University Road of Changqing District, 250353, Jinan, Shandong Province, China
[b] Weihai Supervision and Inspection Institute of Product Quality, Weihai, 264209, China
[c] School of Food Science, Henan Institute of Science and Technology, Xinxiang, 453003, China

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ABSTRACT

The objective of this study was to determine the antibacterial characteristics of glycinin basic peptide (GBP) and its effects on the cell membrane of *Escherichia coli* (*E. coli*). The antibacterial activities of GBP increased with increasing GBP concentrations and treatment times. Atomic force microscope analysis showed that GBP damaged the morphology of *E. coli* cells. GBP significantly ($p < 0.05$) increased the permeability of the outer membrane of *E. coli* cells treated with 80 μg/ml GBP, thereby enhancing the sensitivity of *E. coli* cells to erythromycin and rifampicin. Moreover, O-nitrophenyl-β-D-galactopyranoside (ONPG) entered into bacterial cells and immediately reacted with β-galactosidase in the cells due to the destruction of the inner membrane of *E. coli*. The damage to the bacterial membrane caused by GBP resulted in Ca^{2+}, K^{+}, and Mg^{2+} leakage from the cells. SDS–PAGE of the membrane proteins further demonstrated that GBP significantly destroyed the cell membrane and promoted the extraction of membrane proteins in the presence of Triton X-114.

Industrial relevance: In recent years, chemical synthetic preservatives have aroused wide public concern because of various negative effects on human health. Novel natural food preservatives have received increasing interest from the food industry and researchers. The results of this study indicate that the antibacterial actions of GBP against *E. coli* occurred via interacting with destroying the bacterial membrane structure. Therefore, GBP may be a potential natural food preservative used in the food industry.

1. Introduction

The consistent use of chemical synthetic preservatives in the food industry may cause various hazards to human health (Ho, Ishizaki, & Tanaka, 2000). Therefore, safe natural food preservatives have garnered interest and have become a priority for the food industries and public consumers because of their safety (Najjar, Kashtanov, & Chikindas, 2007).

Antimicrobial peptides (AMPs), particularly natural antimicrobial peptides, play a crucial role in food preservatives. Several studies demonstrated that many natural AMPs have broad-spectrum antimicrobial activities against Gram-positive and Gram-negative bacteria (García-Olmedo, Molina, Alamillo, & Rodríguez-Palenzuéla, 1998; Sitohy, Mahgoub, & Osman, 2012). Li, Han, Feng, Tian, and Mo (2014) showed that ε-poly-lysine (ε-PL) had significant antibacterial activity against *Escherichia coli* and *Staphylococcus aureus*. The antibacterial activities of AMPs are mediated by various characteristics of the peptides, including their cationic nature, low molecular weight, and hydrophobicity. The bacterial cell membrane is a primary target for the action of AMPs (Tang, Zhang, Wang, & Qian, 2013). Cationic AMPs bind to the negatively charged phospholipid membranes of bacteria to form transmembrane pores or ion channels in the cellular membrane, leading to membrane permeability and, eventually, cell death (Lee et al., 2002). Pellegrini and Fellenberg (1999) elucidated that the hydrophobic interaction between the peptide and bacterial membrane is likely the most important feature of the bactericidal mechanism of the antibiotic peptides. Wu, Di, and Matthews (2013) found that a 20-mer pAMP purified from the seeds of *Impatiens balsamina*, Ib-AMP1 could permeate cell membranes and form pores of a sufficient size to allow the efflux of K^{+}, ATP, and large molecules. Additionally, Tang et al. (2013) reported that Opep2, an antimicrobial peptide isolated from ovalbumin hydrolysate, could interact with the *E. coli* cell membrane to cause potassium efflux.

Soybean glycinin consists of six subunits. Each subunit is composed of acidic peptides (MW = 37–42 kDa) and basic peptides (MW = 17–20 kDa) that are linked by disulfide bridges (Shin, Park, Park, & Kim, 2007). Several studies demonstrated that glycinin basic peptide (GBP) has antimicrobial activities against Gram-negative and Gram-positive bacteria (Sitohy et al., 2012; Sitohy & Osman, 2010). Dhatwalia, Sati, Tripathi, and Kumar (2009) reported that basic proteins or peptides had antimicrobial activities. Therefore, glycinin and glycinin basic peptides may react with bacteria cell membrane because of their cationic and hydrophobic nature. Sitohy et al. (2012) also demonstrated that glycinin and glycinin basic peptides provided antibacterial activities

* Corresponding author.
E-mail addresses: lyq@qlu.edu.cn, sdililyq@126.com (Y.-Q. Li).

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against *Listeria monocytogenes* and *Salmonella enteritidis*. In addition, glycinin and glycinin basic peptides were used in pasteurized milk against contaminating bacteria, as well as against artificially inoculated bacterial pathogens, enhancing the quality of milk during storage at 4 °C (Osman, Mahgoub, & Sitohy, 2013).

However, currently, there have been few reports of the possible inhibitory actions of GBP against specific bacteria. The purpose of this study was to investigate the antibacterial activities and actions of GBP against *E. coli*. The antibacterial characteristics of GBP against *E. coli* were measured using total bacterial counts and atomic force microscopy. The antibacterial actions of GBP against *E. coli* were elucidated by evaluating the leakage of Ca^{2+}, K^{+}, and Mg^{2+}; the permeability of the inner and outer membranes of *E. coli* cells; and the changes in the bacterial cell membrane proteins.

2. Materials and methods

2.1. Bacteria cultures

In present, pathogenic and spoilage *E. coli* is regarded widely as an indicator bacterium in the fields of food microbiology. *E. coli* ATCC 8793 is an enterohemorrhagic serotype of *E. coli* and can cause illness to human being through contaminating food. So *E. coli* ATCC 8793 was chosen as indicator bacterium *E. coli*, which was obtained from the Culture Collection, Qilu University of Technology. Freeze-dried bacteria were activated according to the ATCC guidelines. The activated bacteria were inoculated into 100 ml of beef extract peptone (BEP) medium (0.3% beef extract, 1% peptone, 0.5% NaCl, 2% agar and 96.2% distilled water) and cultivated with shaking (130 rpm) in a 300-ml flask at 37 °C for 10 h to yield a final cell concentration of 10^8–10^9 CFU/ml, which was used for further study.

2.2. Preparation and purification of GBP

Defatted soybean flakes (Scents Holding Co., Ltd., Jinan, Shandong) were finely ground to pass through a sieve (1 mm^2), and the obtained powder was used for the isolation of glycinin according to the method reported by Nagano, Hirotsuka, Mori, Kohyama, and Nishinari (1992). Glycinin was dissolved in 30 mM Tris buffer (pH 8.0, containing 15 mM β-mercaptoethanol) to a final concentration of 2% (*w*/*v*). The protein solution was heated to 90 °C for 30 min and then centrifuged (11,000 *g*) for 15 min at 4 °C. The precipitate (crude GBP) was washed three times with 30 mM Tris buffer (pH 8.0), dispersed into distilled water and freeze-dried. The crude GBP was dissolved in phosphate buffer (10 mM, pH 7.2) to a final concentration of 1 mg/ml and then eluted with the same phosphate buffer (pH 7.2) from Sephadex G-150 at 2 ml/min. The eluent containing purified GBP was collected and freeze-dried to powder for the experiments.

2.3. Measurement of GBP antibacterial activities against E. coli cells

The antibacterial activities of GBP were measured using the colony-counting method (Chang, Lu, Park, & Kang, 2010). Aliquots (10 ml) of *E. coli* cells were collected by centrifugation at 4500 *g* for 15 min. The bacteria cells were washed three times with 1 ml of sterile saline and were then resuspended in 9 ml of sterile saline. Aliquots (1 ml) of different concentrations of GBP were added to the bacterial suspensions to final concentrations of 0, 50, 100, 150, 200, 250, 300, 350, and 400 μg/ml. The total bacterial counts were calculated using the colony-counting method after incubation at 37 °C for 5 h in a SHP-150 incubator (Jing Hong Experimental Equipment Co., Ltd., Shanghai, China).

2.4. Atomic force microscope (AFM) analysis of E. coli cells

To further observe the morphological changes of *E. coli* cells, AFM analysis was performed as described previously (Meincken, Holroyd, & Rautenbach, 2005). Samples containing *E. coli* (approximately 10^8 CFU/ml) in 4 ml of BEP with 0 and 400 μg/ml GBP were incubated at 37 °C with gentle shaking for 4 h and 8 h, respectively. Bacterial cells were collected by centrifugation at 4500 *g* for 15 min, washed with 4 ml of BEP, and resuspended in 4 ml of 2.5% glutaraldehyde at 4 °C for 12 h. The resuspended bacterial cells were collected again by centrifugation at 4500 *g* for 15 min and resuspended in 4 ml of double-distilled water. Aliquots (1 ml) of the bacterial suspension were dripped onto a glass slide (1 cm × 1 cm). The glass slides were dried in a thermostatic drier box (DHG-9140A, Jing Hong Experimental Equipment Co., Ltd., Shanghai, China) at 37 °C for 12 h and then observed by AFM (Mutimode 8 Nanoscope V system, Bruker Corporation, USA).

2.5. Measurement of Ca^{2+}, K^{+}, and Mg^{2+} leakage

The leakage of Ca^{2+}, K^{+}, and Mg^{2+} was determined by using an inductive coupled plasma emission spectrometer as described previously (Soylak, Narin, Bezerra, & Ferreira, 2005). *E. coli* cells were collected by centrifugation at 4500 *g* for 15 min and dispersed into 20 ml of sterile saline (0.85%, *w*/*v*). Aliquots (1 ml) of double-distilled water and GBP solution were added to 9 ml of a bacterial suspension to final concentrations of 0 and 400 μg/ml. The treated bacterial suspensions were centrifuged at 6500 *g* for 6 min after cultivation in a thermostatic oscillator (DHZ-C, Forma Experimental Equipment Co., Ltd., Shanghai, China) for 0.5, 1, and 1.5 h at 37 °C. Aliquots of 6 ml of the supernatant, 5 ml of nitricacid (purity 99%), and 1 ml of perchloric acid (purity 99%) were combined in a nitrated cup (50 ml) and then heated in a thermostat water bath at 90 °C (DK-98-IIA, Jin Nan instrument plant, Jintan, China) until approximately 1 ml of liquid remained in the cup. Aliquots of 5 ml of nitricacid and 1 ml of perchloric acid were then added to the remaining liquid with agitation. The mixtures were incubated at 90 °C until they became transparent, and then, the mixtures were transferred into a test tube (50 ml). Aliquots of 25 ml of double-distilled water were added to the test tube with agitation. The concentration of Ca^{2+}, K^{+}, and Mg^{2+} in the *E. coli* cells was then determined using an inductive coupled plasma emission spectrometer (Optima 2000DV, U.S. PE firm, U.S.).

2.6. Determination of outer membrane permeability

The permeability of the *E. coli* outer member was measured as described by Viljanen, Matsunaga, Kimura, and Vaara (1990), with slight modifications. Erythromycin and rifampicin (two hydrophobic reagents, Tianjin Damao Chemical Reagent, Tianjin, China) were diluted serially to 0.7, 1.5, 3, 6, 25, 100 μg/ml and 0.6, 1.25, 2.5, 5, 10, 20 μg/ml, respectively. Aliquots (250 μl) of different concentrations of erythromycin were combined with 250 μl of GBP (80 μg/ml) and 5 ml of an *E. coli* culture solution (approximately 10^8–10^9 CFU/ml) as erythromycin + GBP groups. Moreover, aliquots (250 μl) of these concentrations of erythromycin were replaced by different concentrations of rifampicin in the rifampicin + GBP groups. Two corresponding controls were prepared with 250 μl of sterile water instead of 250 μl of the GBP solution (80 μg/ml). The outer membrane permeability of the *E. coli* cells was determined by measuring the OD_{600} of all of the samples incubated at 37 °C for 5 h in a shaker (130 rpm), using a spectrophotometer (V-1100, Meipuda Instrument Co. Ltd., Shanghai, China).

In order to assess possible synergistic or additive activity, the fractional inhibitory concentration (FIC) index was calculated according to the formula:

$$\text{FIC index} = \text{FIC}_X + \text{FIC}_{GBP} = [X]/\text{MIC}_X + [GBP]/\text{MIC}_{GBP} \quad (1)$$

Respectively, [X] and [GBP] indicated the concentrations of erythromycin or rifampicin and GBP used for partial inhibition; MIC_x and MIC_{GBP} indicated the minimum inhibitory concentrations of erythromycin or rifampicin and GBP. The resulting FIC index of <0.5 indicated synergy,