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ORIGINAL ARTICLE

The Synergistic Antibacterial Properties of Glycinin Basic Peptide against Bacteria via Membrane Damage and Inactivation of Enzymes

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Abstract

This study investigated the antibacterial properties of glycinin basic peptide (GBP), a natural antibacterial component from soybean protein, against *Staphylococcus aureus* (*S. aureus*). The minimum inhibitory and bactericidal concentrations of GBP against *S. aureus* were 0.2 mg/mL and 0.8 mg/mL, respectively. Flow cytometry analysis manifested that GBP decreased the number of intact and normal cells. Higher concentrations of GBP induced more severe damage of the bacterial membrane; the maximal percentage of injured and dead cells was 93.8% with 0.8 mg/mL GBP. Electron microscopy imaging visually showed the morphological damage of *S. aureus* by GBP. Intracellular K^+ leakage and the membrane depolarization of *S. aureus* further verified that GBP could destroy the bacterial membrane. Moreover, GBP decreased the activity of nonspecific esterase and ATPase of *S. aureus* in a concentration-dependent manner. These results demonstrated that GBP exhibited antibacterial properties against *S. aureus* via synergistic actions of damage to the cell membrane and inactivation of metabolic enzymes.

Keywords Glycinin basic peptide · *Staphylococcus aureus* · Flow cytometry · Cell membrane damage · Inactivation of enzymes

Introduction

Biological hazards, especially bacteria, and food-borne diseases caused by bacterial contaminated food, have become a major public concern in the world. In 2015, a total of 4362 food-borne diseases broke out in 32 European countries, for which bacteria were the predominant detected causative agents, followed by bacterial toxins [1]. Among these bacteria involved in food-borne diseases, *Staphylococcus aureus* (*S. aureus*) has been spotlighted widely as it is a leading cause of gastroenteritis. Food safety incidents associated with *S. aureus* have been frequently reported in recent years at home and abroad. For example, seven outbreaks of food-borne disease induced by *S. aureus* occurred in Xi'an (the capital of Northwest China's Shaanxi province) between 2006 and 2013 [2]. An annual estimate of 241,000 illnesses are caused by consumption of food contaminated by *S. aureus* in the United States [3].

S. aureus is a siccostabile and salt-tolerant bacterium with the ability to grow in a wide range of temperatures (7 to 48 °C) and pH (4 to 10) [4]. These characteristics favor the growth of *S. aureus* in many food products, causing contamination of food products during food preparation and processing [5]. The pathogenicity of *S. aureus* is mainly related to the enterotoxins produced by this bacterium during the growth phase [6]. People consuming the contaminated food by *S. aureus* usually experience hyper salivation, nausea, vomit, and abdominal cramp with or without diarrhea [7]. Various types of foods serve as a useful growth medium and/or transmission vehicle for *S. aureus* and/or its toxins. The foods contaminated by *S. aureus* usually include meat and meat products, chicken, milk and dairy products, fermented food items, vegetables, fish products, and salted food products [8, 9]. The control of *S. aureus* in food production and processing is critical to prevent it from contaminating the food items. The application of

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preservatives is an effective method to inhibit the growth and reproduction of *S. aureus* in food items [10]. At present, chemical synthetic preservatives, such as potassium sorbate, nitrite and paraben, have been used widely in beverages, meat products and condiments due to their low price and good preservative effect. However, increasing number of studies have revealed the potential risks of the long-term use of synthetic preservatives on human health [11–13]. Mamur et al. evaluated the genotoxic potential of potassium sorbate in cultured and isolated human lymphocytes, and the result demonstrated that potassium sorbate exhibited genotoxic effects on the human peripheral blood lymphocytes [14]. It has also been reported that the migratory and invasive activities of human breast cancer cells in vitro increased after being exposed for a year to parabens at the concentration of maximal proliferative response [15]. Nitrite combined with certain amines or amides could potentially form carcinogens [16]. Therefore, natural food preservatives have received increasing interest from the food industry and researchers on account of their safety and nontoxicity [17].

Glycinin basic peptide (GBP), which is derived from soybean glycinin, is a cationic peptide without color and odor. GBP has excellent heat stability (withstanding 20 min at 121 °C) and solubility in water, especially in an alkaline environment [18, 19]. Our previous studies demonstrated that GBP exhibits antimicrobial activities against *Escherichia coli* (*E. coli*), *Aspergillus niger* and *Penicillium* [20–22]. Cytotoxicity assays have shown that GBP exhibits no cytotoxicity on human embryonic kidney cells [22].

However, there have been few studies regarding the inhibitory effect of GBP on *S. aureus*. The purpose of this study was to investigate the antibacterial properties of GBP against *S. aureus*. The antibacterial activity of GBP was detected by the conventional broth microdilution assay. The number of damaged cells, the activity of nonspecific esterase (NSE) and the morphological changes induced in *S. aureus* by GBP were assessed via flow cytometry and microscopy. Leakage of K^+ ions and membrane depolarization of *S. aureus* upon treatment with GBP were measured using an inductive coupled plasma emission spectrometer and a fluorescence spectrophotometer.

Materials and Methods

Reagents and Chemicals

Peptone, beef extract and agar for bacterial cultivation were purchased from Solarbio Life Sciences Ltd. Co. (Beijing, China). Glutaraldehyde, osmium tetroxide, epoxy resin, uranyl acetate and lead citrate for microscopy were obtained from Zhongxingbairui Technology Ltd. Co. (Beijing, China). Carboxyfluorescein Diacetate (CFDA), propidium iodide (PI), rhodamine (Rh) 123 and 2, 3, 5-triphenyltetrazolium chloride were also obtained from Solarbio Life Sciences Ltd. Co. The total ATPase determination kit was bought from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). All other reagents and chemicals were of analytical grade.

Materials

The GBP powder was prepared in the laboratory following the method described in our previous report [23]. GBP was added to distilled water to obtain dispersions of different concentrations and autoclaved at 121 °C for 20 min before use in further experiments.

Test Strain and Inoculum Preparation

The microorganism *S. aureus* (ATCC 6538) was obtained from the Culture Collection, Qilu University of Technology. The freeze-dried bacteria was activated according to the ATCC guidelines. Aliquots (2 mL) of sterilized liquid beef extract peptone (BEP) medium (0.3 g beef extract, 1.0 g peptone and 0.5 g NaCl were dissolved in 100 mL of distilled water and boiled) were mixed with the freeze-dried *S. aureus*. The mixed *S. aureus* was transferred into the slants of solid BEP medium (0.3 g beef extract, 1.0 g peptone, 0.5 g NaCl and 2% agar were dissolved in 100 mL of distilled water and boiled) and cultivated at 37 °C until they grew single colonies. Next, one single colony of *S. aureus* was inoculated into 100 mL of liquid BEP medium and cultivated at 37 °C (130 rpm) for approximately 10 h to yield the logarithmic phase (approximately 10^7–10^8 CFU/mL) for further study.

Antibacterial Activity Assay

The antibacterial activity of GBP against *S. aureus* was measured by the broth microdilution assay with some modifications [24]. *S. aureus* was inoculated into 100 mL of BEP medium, and cultivated at 37 °C (130 rpm) for approximately 10 h to yield the logarithmic phase. Aliquots (0.1 mL) of the obtained bacteria solutions were mixed with 4.9 mL of sterile BEP liquid medium. GBP was added to the mixed bacterial suspensions to obtain 0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1.0 mg/mL GBP, respectively. These bacterial suspensions were incubated at 37 °C for 24 h to obtain the minimum inhibitory concentration (MIC) of GBP against *S. aureus*. MIC refers to the lowest concentration of GBP that inhibits the visible growth of the *S. aureus* cells. Subsequently, 0.1 mL of the incubated bacterial suspensions were plated on nutrient agar plates and incubated at 37 °C for 48 h to obtain the minimum bactericidal concentration (MBC) of GBP against *S. aureus*. MBC refers to the lowest concentration of GBP that allows no growth of the *S. aureus* cells on plates.

Flow Cytometry

The *S. aureus* cells from the logarithmic phase were treated with GBP (0, 0.2, 0.4 and 0.8 mg/mL) at 37 °C for 4 h. The treated cells were washed and resuspended in PBS (10 mM, pH 7.4). The resuspended cells were initially stained with 50 μg/mL CFDA at 25 °C for 15 min to allow intracellular NSE to convert CFDA into carboxyfluorescein (CF), followed by staining with 50 μg/mL PI at 25 °C for 5 min. The stained cells were kept in the dark for no more than 1.0 h until flow cytometry analysis was performed. The stained cells were analyzed on a flow cytometer (Imagestream^X Mark II, Merck Millipore Inc., Darmstadt, Germany) that was equipped with a fully functional double laser and six detectors. The forward scatter, side scatter, green (FL2) and red fluorescence (FL4) of each cell were measured, amplified, and converted into digital signals for further analysis. The CF emitted FL2 at 533 ± 35 nm following excitation with laser light at 488 nm, whereas FL4 at 610 ± 30 nm was emitted by PI-stained cells. Moreover, the cells were illuminated with 488 nm excitation light, and the FSC (size) and SSC (granularity) of the cells were measured [25].

TEM Observation of *S. aureus* Cells

The *S. aureus* cells from the logarithmic period (approximately 10^8 CFU/mL) in 5 mL of BEP were incubated with 0, 0.2 and 0.4 mg/mL GBP at 37 °C with shaking (130 rpm) on a thermostatic oscillator (DHZ-C, Shanghai Fuma Laboratory Instrument Co. Ltd., Shanghai, China) for 4 h. The incubated cells were washed twice with PBS (0.1 M, pH 7.4) and fixed with glutaraldehyde (2.5% in 0.1 M PBS, pH 7.4) at 4 °C overnight. The fixed cells were postfixed with 1% osmium tetroxide at 4 °C for 3 h and washed thrice with the same buffer. After postfixation, the cells were dehydrated by a graded series of acetone solutions (30%, 50%, 70%, 90%, and 100%). Next, the dehydrated cells were embedded in epoxy resin and dried at 70 °C for 18 h to form specimen blocks. The ultramicrotome (Leica EM UC7, Wetzlar, Germany) was applied to slice the specimen blocks into thin sections (approximately 60 nm). The thin sections were placed on copper grids (300 mesh) and stained with uranylacetate for 30 min, followed by lead citrate (10 min). Ultimately, the stained sections were photographed via TEM (Jeol-Jem-1200 EX, Tokyo, Japan).

Measurement of K^+ Ion Leakage

The leakage of K^+ ions of the *S. aureus* cells were determined via an inductive coupled plasma emission spectrometer (Optima 2000DV, Waltham, United States). The *S. aureus* cells from the logarithmic period were harvested by centrifugation at 4500 g for 5 min. The obtained cells were washed thrice and adjusted to obtain 10^8 CFU/mL bacterial suspensions with sterile physiological saline. GBP was added to the bacterial suspensions to obtain 0, 0.4, 0.8 mg/mL GBP and incubated at 37 °C (130 rpm). Aliquots (6 mL) of the bacterial suspensions were centrifuged (4500 g for 5 min) every 30 min to obtain cell-free supernatants. Aliquots of 5 mL of the supernatants, 5 mL of nitric acid (14.5 M), and 1 mL of perchloric acid (18.4 M) were mixed in a nitrated cup and then heated in a thermostat water bath (J-HH-6A, Shanghai LNB Instrument Co., Ltd., Shanghai, China) at 90 °C until approximately 1 mL of liquid remained in the cup. The mixture (5:1) of nitric acid (14.5 M) and perchloric acid (18.4 M) was then added constantly to the remaining liquid with agitation until the mixture became transparent. Next, the mixtures were transferred into a test tube and diluted with 25 mL of double-distilled water. The K^+ ions in the dilutions were tested on an inductive coupled plasma emission spectrometer.

Measurement of Membrane Depolarization of *S. aureus*

The depolarization of the *S. aureus* cell membrane by GBP was determined by changes in Rh 123 fluorescence intensity in the cells. The *S. aureus* cells from the logarithmic phase were harvested by centrifugation at 4500 g for 5 min and resuspended in PBS (10 mM, pH 7.4). The harvested cells were treated with 0, 0.2, 0.4, 0.6, 0.8 and 1.0 mg/mL GBP at 37 °C for 4 h (130 rpm). The treated cells were stained with Rh 123 (10 mg/mL) at 37 °C for 30 min in the dark. The stained cells were washed thrice and resuspended in PBS. The fluorescence intensity of Rh 123 in cells was examined on a fluorescence spectrophotometer (Cary Eclipse EN55011, Agilent Technologies Inc., California, United States).

Measurement of ATPase Activity of *S. aureus*

The ATPase activity of GBP-treated *S. aureus* cells was measured with a total ATPase determination kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The *S. aureus* cells were shaken (130 rpm) at 37 °C overnight and collected at the logarithmic phase. The collected cells were treated with different concentrations of GBP (0, 0.2, 0.4, 0.6, 0.8 and 1.0 mg/mL) at 37 °C for 4 h. The treated cells were resuspended in physiological saline (approximately 10^8 CFU/mL). An aliquot (1 mL) of the cell suspensions was used for determining the content of bacterial protein by the Folin-phenol method [26]. Aliquots (100 μL) of the treated bacterial suspensions were added to a reaction mixture containing 100 mM NaCl, 10 mM KCl, 6 mM $MgCl_2$ and 3 mM ATP in 25 mM Tris (pH 7.4), and incubated at 37 °C for 10 min. The released inorganic phosphate was measured spectrophotometrically at 636 nm and the activity of ATPase was expressed as U/mg prot (Pi released/min/mg of protein).

Statistical Analysis

Every experiment was performed in triplicate and the results were expressed as the average values with standard error. The software IDEAS Analysis 5.0 (Merck Millipore Inc., Darmstadt, Germany) was applied for flow cytometric data analyses. Quantitative assessment of each bacterial sample was performed by counting the number of events of the selected regions. Additionally, graphs were produced using Origin 8.0, and regression analysis was used to determine significant differences with 95% confidence ($P < 0.05$).

Results and Discussion

Antibacterial Activity of GBP against *S. aureus*

MIC is the lowest concentration of an antibacterial agent that inhibits bacterial growth of a bacterium. MBC is the lowest concentration of an antibacterial agent that prevents the growth of a bacterium after inoculation in an antibiotic-free media. The results from the broth microdilution assay demonstrated that 0.2 mg/mL GBP inhibited the visible growth of *S. aureus*, and 0.8 mg/mL GBP did not allow the growth of *S. aureus* on plates; this indicated that the MIC and MBC of GBP against *S. aureus* cells were 0.2 mg/mL and 0.8 mg/mL, respectively.

Effect of GBP on Cell Damage to *S. aureus*

CFDA is a nonfluorescent precursor that can easily permeate intact cell membrane. Inside the cell, CFDA is hydrolyzed by intracellular NSE into CF, which is a membrane-impermeant green fluorescent compound. Thus, the retention of CF in the cell can indicate the activity of NSE to assess bacterial viability [27]. PI, a nucleic acid dye, can permeate the damaged cell membrane and bind with DNA to form a stable complex with red fluorescence. PI-stained cells include compromised or damaged membranes cells, which are considered injured or dead. After double staining with CFDA and PI, three subpopulations are observed, according to the degree of cell damage. They are CF-positive (R2), CF- and PI-positive (R3), and PI-positive (R4) cells, which represent intact, injured and dead cells, respectively.

The subpopulations of *S. aureus* cells treated with GBP are depicted in Fig. 1a, b, c, and d, respectively. The subpopulation rates of R2 (intact) treated with 0, 0.2, 0.4 and 0.8 mg/mL GBP were 75.9%, 20%, 15.2% and 5.8%, respectively, indicating that the number of intact cells decreased with increase in GBP concentration. The subpopulation rates 61.4% and 50.5% for R3 (injured cells) with 0.2 and 0.4 mg/mL GBP treatment were higher than those for the control (13.3%) and 0.8 mg/mL GBP (4.1%). Meanwhile, the subpopulation rate 89.7% for R4 (dead) with 0.8 mg/mL GBP treatment was higher than that for the control (4.9%), 0.2 mg/mL GBP (14.6%) and 0.4 mg/mL GBP (29.3%). These results showed that a low concentration of GBP could damage *S. aureus* cells, and a high concentration of GBP led to a greater extent of bacterial cell damage, and inactivation.

The damage induced by graphene oxide to *Pseudomonas putida* was explored via CFDA/PI double staining by flow cytometry, and the result indicated that the number of damaged and dead cells increased with the concentration of graphene oxide [28]. Similarly, Paparella et al. [29] and Liu et al. [30] applied CFDA/PI double staining to evaluate the damage to *Listeria monocytogenes* and *Enterobacter cloacae* by oregano and thyme essential oils and phenyllactic acid, respectively. They concluded that high concentrations of the antibacterial agents exerted a more severe damage to the bacterial cells than the low concentrations. Moreover, our previous studies demonstrated that GBP could cause cell damage in *E. coli, Aspergillus niger and Penicillium* in a dose-dependent manner, as determined by microscopy, ion leakage, mycelial growth and spore germination [20–22].

Effect of GBP on Size and Granularity of *S. aureus*

The impact of GBP on size and granularity of the *S. aureus* cells is indicated by the forward scatter (FSC) and side scatter (SSC) characteristics (Fig. 2). FSC (x-axis) refers to cell size, and SSC (y-axis) refers to cell granularity. Cells in the R area exhibited intact and plump morphologies. As shown in Fig. 2a, b, c, and d, there were clear decreases in the FSC and SSC of cells with GBP than the control, which indicated that GBP treatment changed the cell size and granularity. The percentage of *S. aureus* cells (R area) decreased from 82.3% to 34.8% with GBP treatment from 0 to 0.8 mg/mL, which indicated that the number of intact and plump cells decreased with increase in GBP concentration. These data suggested that GBP could significantly destroy cell morphology and cause shrinkage of the bacterial cell volume.

Similarly, the FSC/SSC assay clearly showed that perillaldehyde decreased the size of *Aspergillus flavus* cells and increased the granularity, indicating that perillaldehyde obviously changed the size and morphology of cells [31]. It has also been reported that lactoferricin B-like peptide decreased the cell size and caused shrinkage of *Candida albicans*, as determined by the decrease in FSC values [32]. Additionally, flow cytometry analyses have demonstrated shrinkage of the cell surface and complication of cell granularity of *E. coli* with GBP treatment [22].

Effect of GBP on Microscopic Intracellular Changes of *S. aureus*

TEM was used to observe microscopic intracellular changes in the *S. aureus* cells exposed to GBP (Fig. 3). The untreated

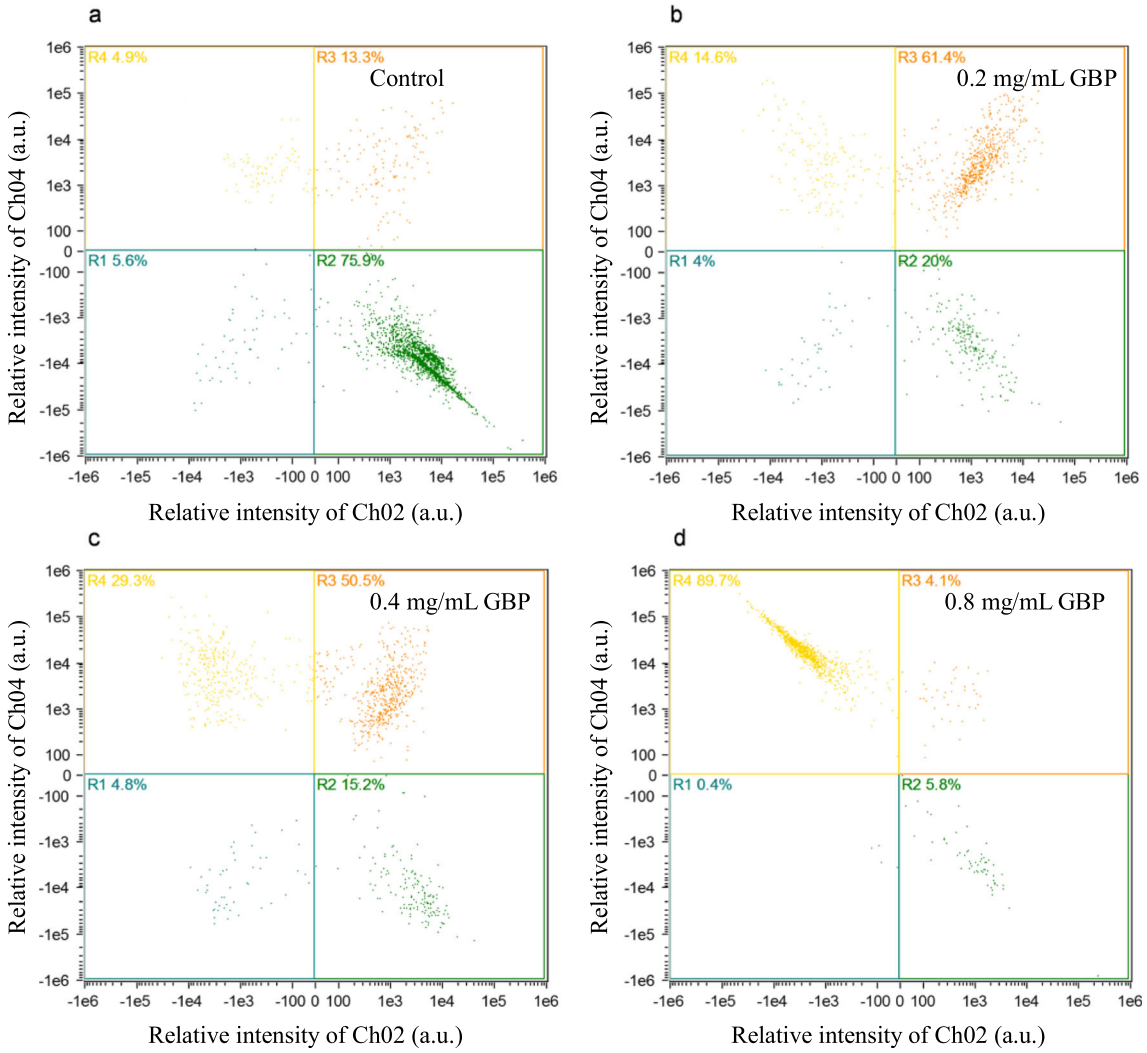

Fig. 1 Various distribution patterns (%) of the subpopulation cells, as obtained by CFDA/PI double staining of *S. aureus* after 4 h treatment with GBP. (**a**) Control, (**b**) 0.2 mg/mL GBP, (**c**) 0.4 mg/mL GBP, and (**d**) 0.8 mg/mL GBP

S. aureus cells exhibited uniform cytoplasmic appearance and well-defined walls and membranes (Fig. 3a). However, bacterial cytoplasm treated with 0.2 mg/mL GBP exhibited aggregation, and lysis of the cell wall was observed (Fig. 3b). Furthermore, the morphological changes of bacterial cells exposed to 0.4 mg/mL GBP were more evident than those with 0.2 mg/mL GBP. The cells exposed to 0.4 mg/mL GBP were not uniform in size and shape, with broken cell walls of partial cells, and some cellular debris and effluent cytoplasm were observed in the surrounding environment of the cells (Fig. 3c). These phenomena demonstrated that GBP damaged the cell walls and membranes, which resulted in the loss of cellular structure and outflow of the cytoplasm.

GBP-induced cell lysis and outflow of cytoplasm have also been observed in *E. coli* cells by TEM [22]. According to TEM, *Listeria monocytogenes* and *Salmonella enteritidis* cells that were treated with glycinin basic subunit exhibited uneven cytoplasm and changed cellular structure compared with the control, which exhibited intact membranes and uniform cytoplasmic appearance [33]. These results showed that GBP exhibited the same destructive effects on *E. coli* and *S. aureus* as on *Listeria monocytogenes* and *Salmonella enteritidis*.

Effect of GBP on K^+ Ion Leakage in *S. aureus*

In normal cells, the dynamic equilibrium of intracellular ions is important for maintaining physiological functions and signal transmissions. The intracellular ions will leak out once the bacterial membranes are destroyed, which will disrupt cellular homeostasis and cause cell death [34].

Figure 4 presents the leakage of intracellular K^+ in *S. aureus* cells with GBP treatment. The extracellular amounts of K^+ ions of the control were almost stable (8.50 mg/L) over a period of 2.5 h. Nevertheless, there was an increment in the leakage of cellular K^+ ions upon treatment with 0.4 mg/mL GBP from 8.09 to 16.13 mg/L during the same period. In the

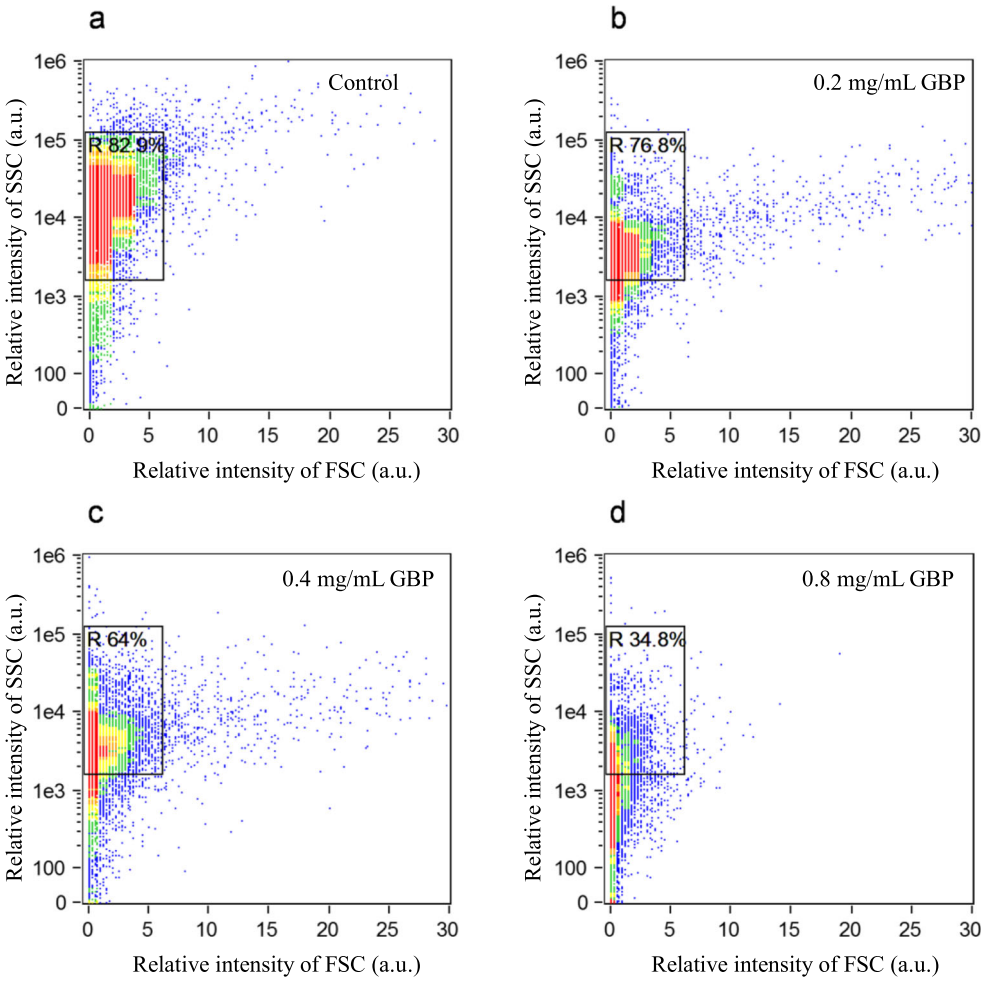

Fig. 2 The size and granularity of *S. aureus* treated with GBP. (**a**) Control, (**b**) 0.2 mg/mL GBP, (**c**) 0.4 mg/mL GBP, and (**d**) 0.8 mg/mL GBP

0.8 mg/mL GBP group, the leakage of K^+ ions in *S. aureus* cells increased rapidly from 8.09 to 18.25 mg/L over 1.5 h and then hardly changed after 1.5 h. These data indicated that a high concentration of GBP (0.8 mg/mL) could greatly damage the bacterial membrane in a short period to induce K^+ release from the intracells. The massive leakage of K^+ from the intracells led to an imbalance in the cellular homeostasis, which led to cell damage and even death. These phenomena were in accord with the results from CFDA/PI double staining, in which treatment with 0.8 mg/mL GBP treatment resulted in 89.7% injured and dead *S. aureus* cells.

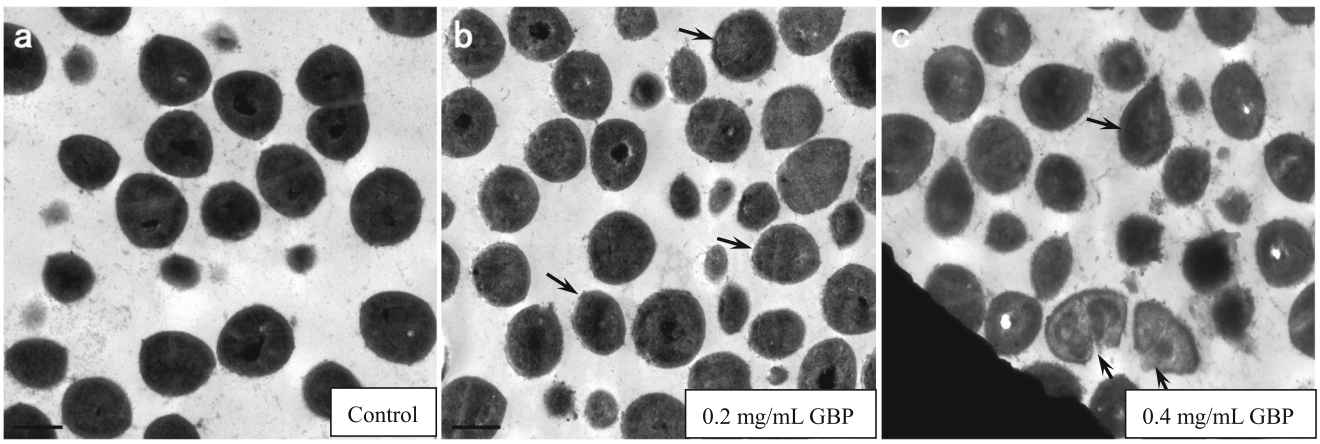

Fig. 3 TEM images of the GBP-treated *S. aureus*. (**a**) Control, (**b**) 0.2 mg/mL GBP, and (**c**) 0.4 mg/mL GBP. The arrows indicate cytoplasm aggregation and cell-wall damage in *S. aureus*

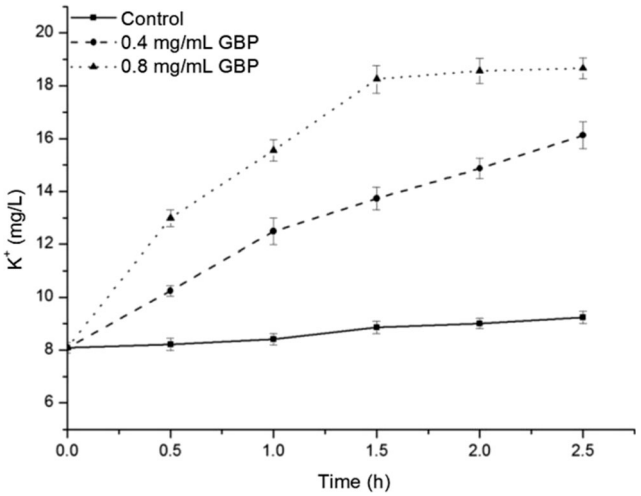

Fig. 4 The leakage of K^+ ions in *S. aureus* cells treated with GBP. (■) Control, (●) 0.4 mg/mL GBP, and (▲) 0.8 mg/mL GBP

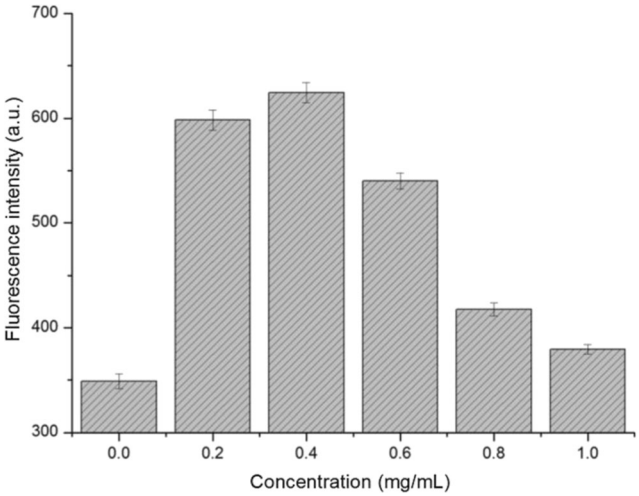

Fig. 5 The depolarization of *S. aureus* cell membrane when treated with GBP at 0, 0.2, 0.4, 0.6, 0.8 and 1.0 mg/mL

It has been reported that Peptide F1 [35] and tea-tree oil [36] could damage the membrane of *S. aureus* cells by determining the leakage of K^+. In this study, *S. aureus* cells treated with 0.4 mg/mL GBP exhibited a K^+ leakage that was 75% higher than that in the cells without GBP treatment. Shen et al. measured the leakage of K^+ into the supernatant of *S. aureus* cells with 5 mg/mL AgO nanoparticles; a higher K^+ leakage of approximately 24% was observed in the treated cells than in the control, suggesting that the destructive effects of GBP on the *S. aureus* cell membrane was stronger than that of AgO nanoparticles [37].

Effect of GBP on Depolarization of *S. aureus* Cell Membrane

Rh 123 is a voltage-sensitive cationic dye that is electrophoretically taken up into bacteria by the trans-membrane electrochemical potential of the plasma membrane [38, 39]. The fluorescence intensity of Rh 123 changes with the cell membrane potential. The changes in the cell membrane potential demonstrate the membrane depolarization of the cells. Therefore, the fluorescence intensity of the Rh 123 responds to depolarization of the cell membrane [40].

As displayed in Fig. 5, the fluorescence intensity of Rh 123 in cells increased from 348.72 to 624.64 a.u. with GBP concentrations from 0 to 0.4 mg/mL, while that of Rh 123 decreased from 624.64 to 379.38 a.u. with GBP treatment at a concentration range of 0.4–1.0 mg/mL. This showed that low-concentration GBP could lead to depolarization of the *S. aureus* cell membrane, and GBP concentration greater than 0.4 mg/mL effectively destroyed the bacterial membranes and resulted in the leakage of Rh 123, which conforms to the results for the K^+ ion leakage.

Similarly, depolarization of the cell membrane of *S. aureus* induced by nanoconjugated vancomycin was measured via a rapid increase of Rh 123 fluorescence intensity [40]. A decrease in the fluorescence intensity of Rh 123 in *S. aureus* cells treated with the tri-hybrid antimicrobial peptide LHP7 demonstrated that LHP7 could induce the bacterial membrane to leak and eventually rupture [41].

Effect of GBP on NSE and Cell Membrane

Figure 6 shows the effects of GBP on NSE and the cell membrane. Areas of A1 (CF-stained cells) and A2 (PI-stained cells), represented cells with NSE activity and cells with membrane damage, respectively. The percentages of *S. aureus* cells with NSE activity (A1) were 89%, 66.3%, 51.7% and 0.4% with 0, 0.2, 0.4 and 0.8 mg/mL GBP, respectively; this demonstrated that the number of *S. aureus* cells with NSE activity markedly decreased with increase in GBP concentration. Meanwhile, the percentages of *S. aureus* cells with membrane damage (A2) increased from 9.9% to 98.7% with GBP concentrations from 0 to 0.8 mg/mL. This suggested that the decrease in NSE activity was responsive to the severity of membrane damage with GBP.

These results were consistent with the finding of Hong et al., who reported that the percentages of *E. coli* cells with NSE activity decreased from 90.48 to 3.09%, and those of cells with membrane damage increased from 2.47 to 98.36% with tachyplesin I concentrations from 0 to 40 μg/mL by CFDA and PI staining [42]. After being exposed to *Zingiber officinale*, 70.06% *Pseudomonas aeruginosa* cells exhibited membrane damage via PI staining [43]. By CFDA staining, there was a drastic decrease in the NSE activity of pathogenic yeast and *Candida albicans* cells treated with plantaricin peptides compared with that in untreated cells [44]. Additionally, previous studies indicated that GBP exhibited strong antibacterial activities against *E. coli* with an MIC of 0.2 mg/mL. GBP severely damaged bacterial cells and inactivated NSE

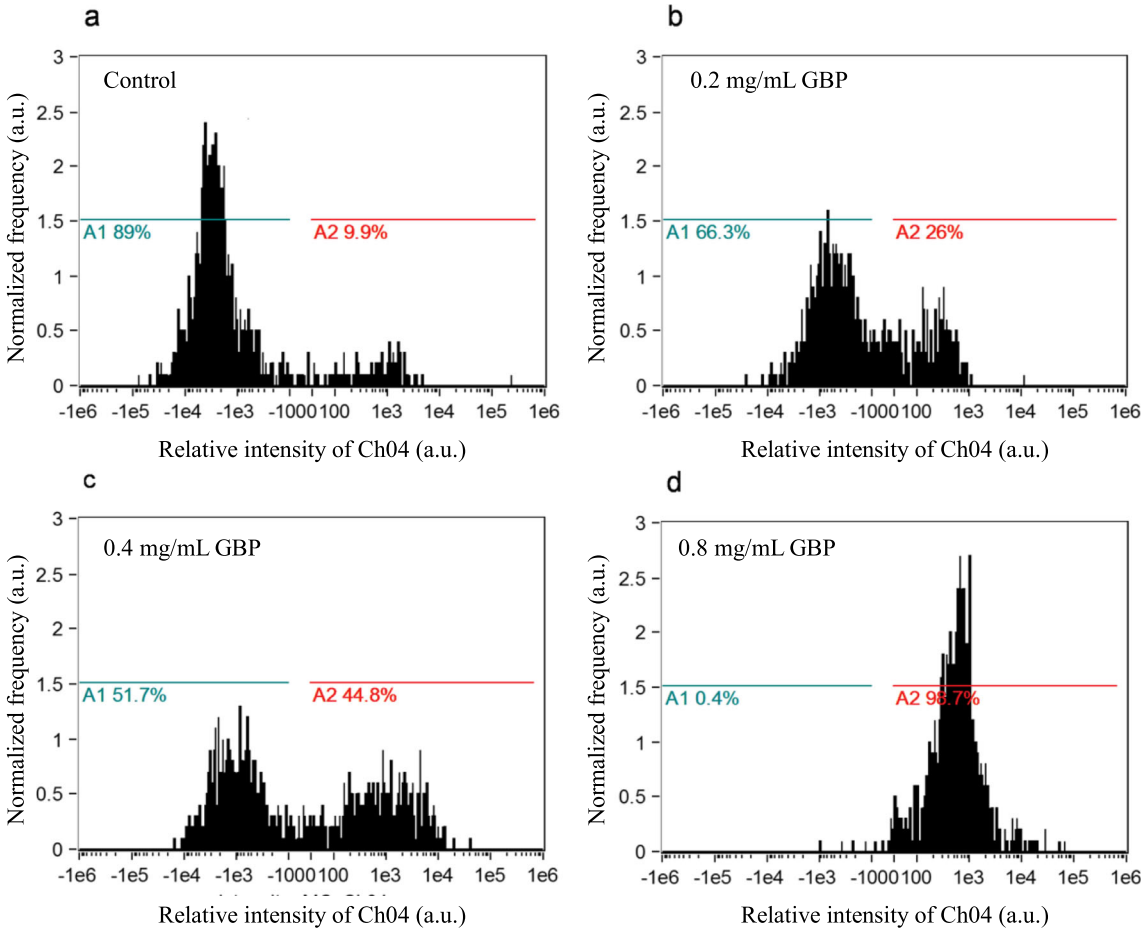

Fig. 6 The effect of GBP on NSE and the cell membrane of *S. aureus*. (a) Control, (b) 0.2 mg/mL GBP, (c) 0.4 mg/mL GBP, and (d) 0.8 mg/mL GBP

in *E. coli*, as determined through CFDA/PI dual staining by flow cytometry. GBP severely disrupted the bacterial membrane integrity as observed by microscopy [20, 22]. It has also been reported that GBP exhibited strong antibacterial activities against *Bacillus subtilis* and *Listeria monocytogenes* with an MIC of 0.05 mg/mL. According to microscopy analysis, the bacterial cell morphologies were significantly destroyed after GBP treatment [33].

Effect of GBP on ATPase of *S. aureus*

ATPase catalyzes the decomposition of ATP into ADP and inorganic phosphorus with the release of energy. The activity of ATPase could be determined by measuring the release of inorganic phosphorus [45]. Figure 7 describes the ATPase activity of *S. aureus* cells treated with different GBP concentrations. The ATPase activities of *S. aureus* cells with 0, 0.2, 0.4, 0.6, 0.8 and 1.0 mg/mL GBP were 1.43, 1.22, 1.11, 0.77, 0.43 and 0.38 U/mg prot, respectively, which indicated the decrease in ATPase activities of *S. aureus* cells with increase in GBP concentrations.

ATPase is broadly functional in the biological membrane, participating in ATP hydrolysis, trans-membrane transportation, cell signal transduction, and cell homeostasis [46]. The inactivation of ATPase will lead to a decrease in these physiological functions of bacteria, or even loss, which will minimize bacterial growth and, ultimately, cause cell death. The decrease in ATPase activity by GBP indicated that

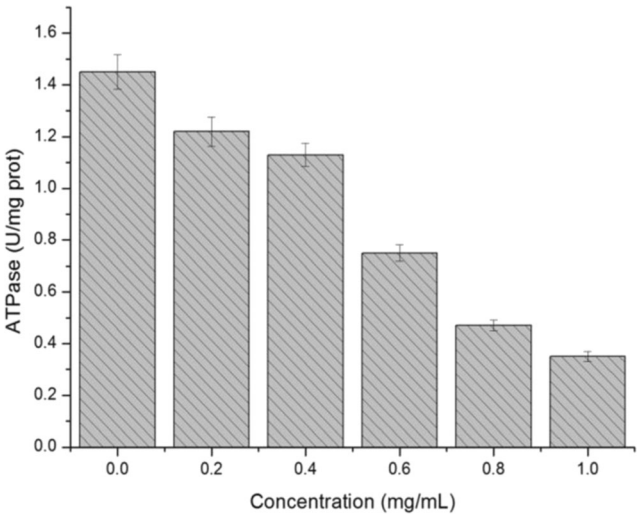

Fig. 7 The ATPase activity in *S. aureus* treated with GBP at 0, 0.2, 0.4, 0.6, 0.8 and 1.0 mg/mL

the antibacterial action of GBP might be achieved through destroying the ATPase in *S. aureus*. It has been reported that magainin-I analog peptide [47] and LL37-analogous peptide LLAP [48] could inhibit the ATPase activity of *mycobacterial plasma* membrane by measuring the inorganic phosphorus release. The ATPase activity of *S. aureus* treated with 1.0 mg/mL GBP was 26.6% of the control. The ATPase activities for *S. aureus* treated with 1.2 mg/mL p-coumaric, ferulic and caffeic acid were 35.1%, 73.0% and 80.3% of the control, respectively. These results suggested that the inhibitory effect of GBP on the ATPase activity of *S. aureus* was stronger than that of p-coumaric, ferulic and caffeic acid [49].

Conclusions

GBP exhibited a strong antibacterial activity against *S. aureus*. GBP severely damaged the membrane of *S. aureus*, resulting in leakage and disruption of the bacterial membrane. Meanwhile, phenomena of cell deformation, cytoplasm aggregation and cell wall lysis were observed in GBP-treated *S. aureus* cells, which demonstrated that GBP destroyed the bacterial cell structure. The changes in Rh 123 fluorescence intensity and the K^+ ion leakage in *S. aureus* cells further showed that GBP induced depolarization of the *S. aureus* cell membrane and leakage and rupture of the bacterial membrane, eventually resulting in cell death. Moreover, inactivation of NSE and ATPase by GBP inhibited bacterial growth and propagation. Thus, it is possible to use GBP as a natural food preservative in the food industry.

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Compliance with Ethical Standards

Conflicts of Interest The authors declare no conflicts of interest.

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