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In vitro and in situ antimicrobial action and mechanism of glycinin and its basic subunit

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ABSTRACT

Glycinin, basic subunit and β-conglycinin were isolated from soybean protein isolate and tested for their antimicrobial action against pathogenic and spoilage bacteria as compared to penicillin. The three fractions exhibited antibacterial activities equivalent to or higher than penicillin in the next order; basic subunit > glycinin > β-conglycinin with MIC of 50, 100 and 1000 μg/mL, respectively. The $IC_{50\%}$ values of the basic subunit, glycinin and β-conglycinin against *Listeria| monocytogenes* were 15, 16 and 695 μg/mL, against *Bacillus subtilis* were 17, 20, and 612 μg/mL, and against *S.* Enteritidis were 18, 21 and 526 μg/mL, respectively. Transmission electron microscopy images of *L. monocytogenes* and *S.* Enteritidis exhibited bigger sizes and separation of cell wall from cell membrane when treated with glycinin or basic subunit. Scanning electron microscopy of *B. subtilis* indicated signs of irregular wrinkled outer surface, fragmentation, adhesion and aggregation of damaged cells or cellular debris when treated with glycinin or the basic subunits but not with penicillin. All tested substances particularly the basic subunit showed increased concentration-dependent cell permeation assessed by crystal violet uptake. The antimicrobial action of glycinin and basic subunit was swifter than that of penicillin. The cell killing efficiency was in the following descending order; basic subunit > glycinin > penicillin > β-conglycinin and the susceptibility of the bacteria to the antimicrobial agents was in the next order: *L. monocytogenes* > *B. Subtilis* > *S.* Enteritidis. Adding glycinin and the basic subunit to pasteurized milk inoculated with the three bacteria; *L. monocytogenes, B. Subtilis* and *S.* Enteritidis (ca. 5 log CFU/mL) could inhibit their propagation after 16–20 days storage at 4 °C by 2.42–2.98, 4.25–4.77 and 2.57–3.01 log and by 3.22–3.78, 5.65–6.27 and 3.35–3.72 log CFU/mL, respectively.

1. Introduction

Plants produce a wide array of defense protein to control the attacks of microbial pathogens. As a result several classes of proteins with antibacterial and/or antifungal properties have been isolated, identified and recommended as antimicrobial agents (Kumarasamy et al., 2002). Plant antimicrobial cationic peptides or proteins (AMPs) which constitute a heterogeneous class of low molecular weight proteins, are important components of innate defense system directly interfering with the growth, multiplication and spread of microbial organisms (Garcia-Olmedo et al., 1998). The action of AMPs targets mainly the bacterial cell membranes (Zasloff, 2002) due to their positive net charge enabling the binding and permeation of negatively charged phospholipid membranes of bacteria (Shai, 2002).

The frequent and massive use of antibiotics gave rise to multidrug resistant bacteria (Charpentier and Courvalin, 1999). Hence, the quest for new antibacterial drugs and agents is a continuous mission. A highly basic small protein (finotin) was purified from seeds of *Clitoria ternatea* and found to have a broad and potent inhibitory effect on the growth of various important fungal pathogens (Kelemu et al., 2004). Small seed basic proteins isolated from *Robinia pseudoacacia* L. Rozynskiana (Leguminosae), manifested in vitro antibacterial activity against seven bacteria (Talas-Oğraş et al., 2005). Puroindoline A and puroindoline B from plant seeds as well as lactoferrin and lysozyme were proved as in-vivo antimicrobial agents against *Listeria monocytogenes* (Palumbo et al., 2010).

Antibacterial activities are not restricted to low molecular weight proteins but they can include high molecular weight ones e.g. achacin and aplysianin (56 and 320 KD, respectively) isolated from marine animals (Kamiya et al., 1986). Hydrophobic high molecular weight proteins (27 and 31 KD) isolated from fish were associated with strong pore-forming antibacterial activity (Ebran et al., 1999).

Soybean storage proteins lie within the class of high molecular weight proteins. Globulins represent the majority of seed soybean proteins and can be subdivided into two main types according to their sedimentation coefficients: glycinin (11S) and β-conglycinin (7S). Glycinin has a molecular mass of 360 KD and is composed of 6 constituent subunits ($A_{1a}B_2$, A_2B_{1a}, AB, $A_5A_4B_3$, A_3B_4 and $A_{1b}B_2$),

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each of which consists of an acidic and a basic polypeptide, linked together by a disulfide bond (Kitamura and Shibasaki, 1975 and Nielsen, et al., 1989). The relative molecular masses of basic and acidic subunits are 20 and 34 KD, respectively (Kitamura et al., 1976). The subunit β-conglycinin is a trimeric glycosylated protein with a molecular mass of 150–200 KD (Ladin et al., 1987; Utsumi, 1992). The antimicrobial activities of these two main soybean protein subunits have never been investigated before due to the lack of direct traits of AMPs such as the cationic nature, low molecular weight and hydrophobicity. However, the basic subunit of glycinin is both hydrophobic and cationic and may be able to react with the bacterial cell wall and membrane in spite of its attachment to the acidic subunits. Based on previous reports that basic proteins or peptides can have antimicrobial activity (Dhatwalia et al., 2009), the objective of the current work was to specify the potential antimicrobial action of soybean subunits, particularly glycinin which has half of its subunits as basic polypeptides or the separated basic subunit against bacteria. These fractions were tested against *L. monocytogenes* and *Salmonell*a *enterica* subsp *enterica* serovar Enteritidis and one spoilage bacterium (*Bacillus subtilis*) as compared to penicillin. *L. monocytogenes* causes epidemic listeriosis (Schuchat et al., 1991; Denny and McLauchlin, 2008). *S.* Enteritidis is the cause of salmonellosis, another foodborne disease (Scallan et al., 2011) and some strains of *B. subtilis* may occasionally cause food poisoning (Pavic et al., 2005).

2. Materials and methods

2.1. Materials

Soybean (*Glycine max* L.) seeds were purchased from local market, Zagazig city, Sharkia, Egypt. *L. monocytogenes* Scott A and *S. enterica* subsp. *enterica* serovar Enteritidis PT4 strains used in this study were kindly obtained from Prof. George Nychas, Laboratory of Food Microbiology and Biotechnology, Department of Food Science and Technology, Agricultural University of Athens, Athens, Greece. *B. subtilis* was isolated from soil and identified according to Bergy's Manual systematic of bacteriology. For experimental use *L. monocytogenes* Scott A, *S.* Enteritidis and *B. subtilis* cultures were maintained on nutrient agar slopes at 4 °C and subcultured every 4 weeks.

2.2. Isolation of soybean protein subunits

Soybean seeds were ground to pass through a 1 mm^2 sieve and the resulting powder was defatted using a mixed solvent of chloroform: methanol (3:1 v/v) for 8 h. Soybean protein isolate was separated using the procedure of Johnson and Brekke (1983). The total nitrogen was determined in soybean protein isolate according to AACC (2000) and multiplied by the conversion factor 6.25 to get the total protein content. Soybean protein isolate was used for the isolation of glycinin and β-conglycinin according to Nagano et al. (1992).

Basic subunits were separated from the glycinin according to methods described by Damodaran and Kinsella (1982) with some modifications. Glycinin was dissolved in 30 mM Tris buffer (pH 8.0) containing 15 mM β-mercaptoethanol (at 0.5% w/v). The protein solution was heated to 90 °C for 30 min and then centrifuged at 10000 x *g* at 4 °C for 20 min. The precipitate (basic subunit) was washed twice with 30 mM Tris buffer (pH 8.0), suspended in distilled water, and freeze-dried.

2.3. Agar well-diffusion assay

Soybean protein fractions were tested for antimicrobial activity by conventional well-diffusions methods against *L. monocytogenes*, *S.* Enteritidis and *B. subtilis* (Nanda and Saravanan, 2009; Zgoda and Porter, 2001) with some modifications. The pure cultures of bacterial strains were subcultured on MHB (Mueller Hinton broth) and incubated on a rotary shaker at 200 rpm at 37 °C (*L. monocytogenes* and *S.* Enteritidis) or 28 °C (*B. subtilis*) for 24 h. An aliquot (0.1 mL) of the last culture was transferred into 10 mL MHB and incubated at 37 °C (*L. monocytogenes* and *S.* Enteritidis) or 28 °C (*B. subtilis*) for 24 h to reach a count of 1.05×10^9 CFU/mL. Each strain was spread uniformly onto individual plates using sterile cotton swabs. Wells of 6-mm diameter were made on Mueller Hinton Agar (MHA) plates using gel puncture. Aliquots (40 μL) of different protein concentrations (0, 25, 50, 100, 250, 500 and 1000 μg/mL) were transferred onto each well of all plates. After incubation at 37 °C (*L. monocytogenes* and *S.* Enteritidis) or 28 °C (*B. subtilis*) for 24 h, the different levels of zones of inhibition were measured using a transparent ruler and the diameter was recorded in mm to conclude the minimum inhibitory concentration (MIC). Penicillin was used as a positive control with the concentrations of 0, 25, 50, 100, 250 and 500 μg/mL.

2.4. Minimum inhibition concentration (MIC)

Soybean protein isolate or its fractions (glycinin, β-conglycinin and the basic subunit) was tested for antimicrobial activity by conventional broth dilution assay against *L. monocytogenes, S.* Enteritidis and *B. subtilis* (Murray et al., 1995). Minimum inhibitory concentration (MIC) was evaluated using standard inoculums of 1×10^5 CFU/mL (Rex et al., 2001). Serial dilutions of the test compounds, previously dissolved in sterilized distilled water, were prepared to final concentrations of 0, 25, 50, 100, 250, 500 and 1000 μg/mL of MHB. To each tube, 100 μL of the inoculum was added and incubated for 18–24 h at 37 °C (*L. monocytogenes* and *S.* Enteritidis) or at 28 °C (*B. subtilis*). At the end of incubation time, MIC was visually identified as the lowest concentration of the test compound which inhibits the visible growth and confirmed by measuring the OD_{600} of all treatments. Tests using sterilized distilled water as negative control and penicillin as positive control were carried out in parallel. All tests were performed in triplicate.

2.5. Rate of cell kill assay

The rate of cell kill of the studied bacteria strains upon treatment with protein fractions and penicillin was determined as described by Culafic et al. (2005) with some modifications. Overnight growth cultures of the three studied bacteria were grown in MHB up to 2×10^9 CFU/mL. An aliquot (100 μL) of the last suspension was inoculated into fresh media and grown up to 10^5 CFU/mL (Rex et al., 2001). All cultures were treated with 100, 200 and 300 μg/mL of the antimicrobial agent (equivalent to 1×, 2× and 3× MIC). Control tubes were similarly prepared but without adding the antimicrobial protein. All treatments were incubated either at 37 °C (*L. monocyotgenes* and *S.* Enteritidis) or 28 °C (*B. Subtilis*) for 24 h and OD_{600} was measured using JENWAY 6405 UV/visible spectrophotometer (UK) at the end of incubation time. All the determinations were done in triplicates.

2.6. Crystal violet assay

Alteration in membrane permeability was determined by crystal violet assay (Vaara and vaara, 1981). After overnight growth in MHB at 37 °C (*L. monocyotgenes* and *S.* Enteritidis) or 28 °C (*B. Subtilis*), the stationary phase cultures of the strains were adjusted to a concentration of 1×10^9 CFU/mL. An aliquot (1.5 mL) of the cell culture was centrifuged at $4500\times g$ for 15 min at 4 °C and the pellet was separated and washed three times before finally suspending in 1.5 mL peptone buffer solution (PBS) (0.1% w/v, pH 7.4). All antimicrobial agents were added to the cell suspension at 0, 50 and 100 μg/mL and incubated at 37 °C (*L. monocyotgenes* and *S.* Enteritidis) or 28 °C (*B. Subtilis*) for 30 min. To follow the kinetics of the permeation, all antimicrobial agents as well as EDTA were added to the cell suspension at 100 μg/mL and incubated at 37 °C (*L. monocyotgenes*