American Journal of Biological, Chemical and Pharmaceutical Sciences
Vol. 1, No. 6, October 2013, PP: 35 - 41, ISSN: 2328 - 6814 (Online)
Available online at http://www.ajbcps.com/

Research article

Protein Secreted by *Bacillus subtilis* ATCC 21332 in the Presence of *Allium sativum*

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Abstract

Many studies have reported that the primary activity of most inhibitors of bacterial function is to modulate transcription processes at much lower concentrations than that required for antibiosis. Therefore, the bacteria might be produced and secreted more proteins in the mild stress surroundings (e.g. in the presence of low doses of antimicrobial agents) than in the normal environment. However, not much is known about unexpected ability of natural antimicrobial compounds at low concentration to become a signaling agent that capable to modulate biological functions in bacteria. Thus, this study aims to explore the potential of natural antimicrobial compound (*Allium sativum*) at sub-minimal inhibitory concentration (sub-MIC) in regulating proteins production by *Bacillus subtilis* ATCC 21332. The Minimum Inhibition Concentration (MIC) of *A. sativum* on *B. subtilis* resulting 14.29% was determined by microdilution assay. The bacteria cells were further exposed to *A. sativum* at sub-MIC (0.05 x MIC) in fermentation process. SDS-PAGE profile showed that two protein bands with approximate size of 51.36 kD and 9.74 kD were produced for the bacteria treated with *A. sativum*. LC-MS/MS analysis identified six possible proteins from the two bands expressed in mild stress condition. The proteins exhibited antimicrobial activity towards several Gram-positive and Gram-negative bacteria. Hence, *B. subtilis* ATCC 21332 in mild stress condition with the presence of 0.05 x MIC *A. sativum* could regulate bioactive proteins production.

Keywords: *Bacillus subtilis* ATCC 21332, *Allium sativum*, proteins, sub-MIC, antimicrobial agent, transcription

Introduction

Bacteria launch stress responses (chaperones) under hostile and challenging environmental conditions likes during depletion of nutrients, oxygen and changes in temperature due to improve their chances of adaptation and survival. In harsh condition, bacteria have developed special physiologic mechanisms including the production and secretion of specific proteins. Hence, the production of proteins was higher than the normal condition [1]. The stress responses are very specific and the mechanisms include metabolic alterations also the activation of chaperones and signal transduction cascade dedicated for sensing and responding to various stress [2].

Antimicrobials are one of the various stresses in the bacterial world. The antimicrobial agents or antibiotics have unexpected ability to modulate global transcription processes in target cells. This activity is detected at much lower concentrations than that required for inhibitory activity. The characteristic of possessing contrasting effects at low and high concentrations has been referred as hormesis [3]. *Allium sativum* (garlic) was popular as an antiviral and antibacterial from ancient days to modern times. A powerful antibiotic chemical in *A. sativum*, known as allicin is released when chewed or crushed [4]. Allicin can act as a weapon to inhibit or kill bacteria at high concentration and might be play as signaling molecules at low concentration due to hormetic property.

Bacillus sp. is a known producer of a wide arsenal of antimicrobial substances, including peptides and lipopeptides antibiotics, as well as bacteriocins. The bioactive substances have a great potential in biotechnological and biopharmaceutical applications. One of the most important species of genus *bacillus* is *B. subtilis* that is commonly recovered from water, soil and environment, and it can survive in extreme conditions of heat and desiccation because of the production of endospore. *B. subtilis* is considered a benign organism as it is non-pathogenic and non-toxigenic to humans, animals and plants and it does not possess any traits that cause disease [5]. Thus, scientists pay more attention to this organism with the aim to the commercial exploitation of *B. subtilis* as major "cell factories" for secreted heterologous proteins of interest.

It is well established that bacteria are exposed to and respond to many different extracellular signals in the environment. However, there are limited studies on the potential of natural antimicrobial compounds at low doses acting as signaling molecules to trigger biological functions in bacteria by activating or repressing the specific genes of interest via the signal given. Therefore, this study focuses on the role of antimicrobial compound (*A. sativum*) in inducing proteins production by *B. subtilis* ATCC 21332. Escalating incidents of life-threatening infections by antibiotic-resistant bacteria in recent years have provided strong impetus to discover new antibiotics. Hence, this study aims for future discovering of proteins that have a potential as a novel source of antimicrobial substances.

Materials and Method

A. sativum, Bacterial Strains and Culture Conditions

A. sativum was purchased from local market outlets. The outer skins of garlic cloves were peeled and the cloves were crushed aseptically in sterile mortar and pestle. The fine garlic mash was transferred into microcentrifuge tube and centrifuged at 100 rpm for 10 min (Mini-Centrifuge, Eppendorf). The garlic extract was then pipette out into new microcentrifuge tube and ready to use for further analysis. *Bacillus subtilis* ATCC 21332 was obtained from American Type Culture Collection (ATCC) and grown in Mueller-Hinton Broth (MHB; Oxoid, USA). Test microorganisms used for antimicrobial screening were grown in MHB include:

(a) Gram-positive bacteria: *Bacillus cereus* ATCC 13061, *Bacillus subtilis* ATCC 11774 and *Enterobacter faecalis* ATCC 29212.

(b) Gram-negative bacteria: *Escherichia coli* ATCC 25922, *Klebsiella pneumonia* ATCC 13883 and *Salmonella thyphimurium* ATCC 13331.

Minimum Inhibition Concentration (MIC) Determination

The MIC of *A. sativum* towards *B. subtilis* ATCC 21332 was determined by microdilution assay [6]. Serial dilution of *A. sativum* extract was prepared and added to 10^7 cells/ml *B. subtilis* ATCC 21332 cultures in microtire plate. The plate was incubated for an overnight at 37°C. The bacterial viability was detected by 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT; Sigma Chemical Co., USA) solution. The lowest concentration of *A. sativum* could inhibit the growth of *B. subtilis* was considered as MIC value.

Microbial Proteins Production

A colony of *B. subtilis* ATCC 21332 was grown in 10 ml of MHB and incubated at 37°C with gentle agitation. *A. sativum* at sub-MIC (0.5 x MIC) was added to the bacteria culture during log (8 h of incubation) phase of cultivation period. The culture not treated with *A. sativum* served as a control. After 24 h of fermentation process, the microbial proteins were further isolated and extracted.

Microbial Proteins Extraction

Microbial proteins extraction was done according to Lash *et al*. (2005) [7]. The bacteria culture was centrifuged at 7000 x *g* for 6 min at 4°C. The supernatant was collected and filter sterilized by using 0.2 µm syringe filter (Millex®-GV, Millipore, Bedford, Massachusetts, USA) in order to produce sterile cell-free supernatant. About 60% (w/v) of ammonium sulphate (Sigma, St. Louis, Missouri, USA) was added and mixed-well with cell-free supernatant and left for 1 h at 4°C to precipitate the proteins. The crude proteins precipitate was then centrifuged for 20 min at 15,000 x *g* at 4°C. The resulting pellet was suspended into 200 µl phosphate-buffered saline (PBS, pH 6.8, Cambrex Bioscience, Verviers, Belgium) and centrifuged for another 10 min at 15,000 x *g* at 4°C. The pellet was then dissolved in deionised distilled water before further mixed with Laemmli sample buffer (Biorad, Singapore) and β-mercaptoethanol (Biorad, Singapore) in 1:1 ratio. The diluted proteins sample was then heated at 90-95°C for 5 min and ready for SDS-PAGE analysis.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Microbial Proteins Identification

Proteins were analyzed by electrophoresis on Any kD™ Mini-PROTEAN® TGX™ Precast Gel in Protean III electrophoresis system (Bio-Rad, Hercules, CA) with Precision Plus Protein™ Dual Xtra Standards (Biorad, USA). The protein bands were visualized by using Biosafe Coomassie blue staining (Bio-Rad, USA) and the bands of interest were identified by Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) based peptide sequencing. Spectra were analysed to identify proteins of interest using Mascot sequence matching software (Matrix Science) with Ludwig NR and SwissProt databases and taxonomy set to Bacteria.

Antimicrobial Activity Screening

Screening of antimicrobial activity *via* agar well diffusion method was based on Millette *et al*. (2007) [8]. Briefly, four equidistant wells of 6 mm in diameter were punched into the Mueller Hinton Agar (MHA; Oxoid, USA) by using a sterile cork borer and the plates were then stored for 2 h at 37°C to dry the humidity. Each bacterial culture with the concentration of 10^7 cell/ml was spread on MHA plates before further kept at 4°C for 12 h for pre-diffusion process. 50 µl of protein sample was loaded into respective well on agar plates. After 24 h of incubation at 37°C, the plates were examined for the presence of inhibition zones.

Results and Discussion

Bacteria have inhabited the earth and colonized almost every conceivable habitat. It is well recognized that bacteria are confront to various stresses in the environment including exposure to antibiotic or antimicrobial agents. However, bacteria have physiologic mechanisms enabling them to survive in environments that preclude their growth. Bacteria under stress "switch on" a catalogue of genes, by which the protein promoters may be

affected. Any antimicrobial at sub-inhibitory concentration could act as an inducer or signaling agent by causing up- or down-expression of a large number of transcripts in different bacteria [9]. In this present study, *B. subtilis* ATCC 21332 was grown in the presence of *A. sativum* at various concentrations ranging from 1-100%. The effects of this antimicrobial on bacteria growth were identified in order to determine the MIC value for performing subsequent experiment. The bacteria viability was detected by MTT as an indicator. This colorant substance was added to the test solutions and incubated at 37ºC for 30 min. As shown in Figure 1, the blue colour showed bacteria growth due to the blue formazan formed, while the yellow colour indicated no bacteria growth. The MIC value was determined as the highest dilution or lowest concentration of *A. sativum* showed no bacterial growth [6]. *A. sativum* could inhibit the growth of *B. subtilis* ATCC 21332 at concentration 14.29%.

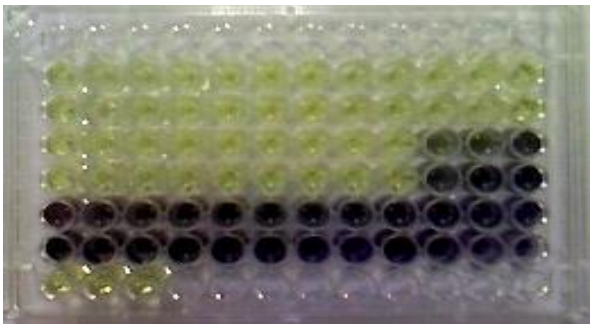

Figure 1: Determination of MIC value by microdilution assay using MTT as an indicator

Microbial secondary metabolites, including microbial proteins are usually not produced during the phase of rapid growth (log phase), but are synthesized during a subsequent production stage (stationary phase), which is when primary nutrient source is depleted [10]. A culture of *B. subtilis* cells that has exhausted one or more essential nutrients and experienced fluctuations in the surrounding will enter the stationary phase of growth. At this stage, regulons that function in sporulation, competence development, the production of extracellular proteins and enzymes as well as antibiotics are induced. These various phenomena reflect the complex response of the cells to stress and show that a rapid adjustment to environmental change is essential for survival. In this study, after bacteria cells were cultivated for 24 h in the presence of 0.05 x MIC *A. sativum*, two new proteins with approximate size of 51.36 kD and 9.74 kD were produced as shown in Figure 2. Hence, it demonstrated that *B. subtilis* ATCC 21332 cells were induced to introduce new proteins during stationary phase as an adaptation strategy in the sub-minimal stress condition caused by *A. sativum*.

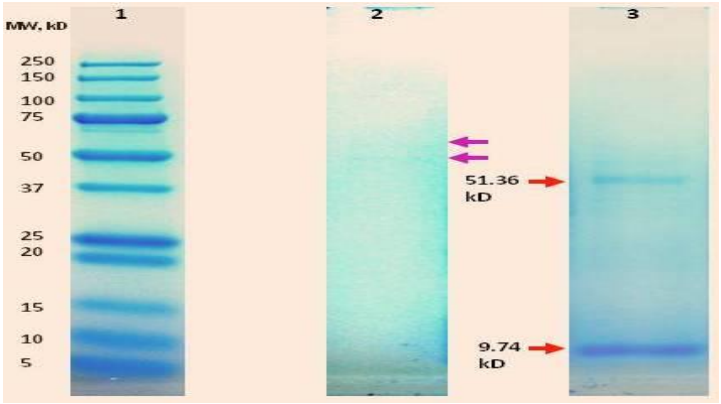

Figure 2: SDS-PAGE analysis on microbial proteins produced by *Bacillus subtilis* ATCC 21332: Lane (1) protein ladder (5-250 kD); Lane (2) in the absence of *A. sativum* (control); Lane (3) in the presence of *A. sativum*

The findings that *B. subtilis* ATCC 21332 tend to secrete new proteins after treating with *A. sativum* was accordance to the fact that *B. subtilis* lacks an outer membrane. Therefore, lots of proteins are secreted directly

into the growth medium and these proteins were considered as extracellular proteins [11]. Extracellular and surface-associated proteins play a most important role in many essential interactions and adaptations of *B. subtilis* to the environment. Heng *et al*. (2007) grouped almost all antimicrobial proteins or bacteriocins into four classes: Class I bacteriocins are modified lantibiotics which are less than 4 kD, while Class II bacteriocins are small, heat stable, non-modified peptides and less than 10 kD. Members of Class III are large, heat- labile proteins and larger than 30 kD. Lastly, Class IV bacteriocins comprises of cyclic peptides [12]. According to the classification of bacteriocins based on size, the two new proteins might be in the Class II bacteriocins (9.74 kD), which is heat stable peptides and Class III bacteriocins (51.36 kD), groups of heat-labile proteins.

As soil microorganisms, *Bacillus* species secrete numerous enzymes which enabling them to degrade a variety of substrates and survive in a complex and continuously changing environment. *B. subtilis* secretes high levels of proteins under poor nutrient conditions. Further identification analysis on the isolated proteins secreted by *B. subtilis* ATCC 21332 after treatment with *A. sativum* was performed by LC-MS/MS based peptide sequencing. Identification of the purified proteins was essential in order to recognize the type of proteins or peptides secreted during mild stress condition. A configuration of peptide sequences to SwissProt and Ludwig NR databases revealed that different kind of proteins with various functional classes was produced by *B. subtilis* ATCC 21332 as shown in Table 1. Result showed that four possible proteins with different functions were identified for the first protein sample (BS 1, 51.36 kD). Meanwhile, the second protein sample (BS 2, 9.74 kD) indicated two possible proteins with different functions.

Table 1: Proteins identification by LC-MS/MS based peptide sequencing

Sample	Database Used	Proteins Significant Hits	Functional Classification
BS 1	SwissProt	Quinolinate synthase A	Nucleotide metabolism
		DNA ligase	Replication and repair
		Methane monooxygenase component C	Energy metabolism
		Silver-binding protein SilE	Bacterial silver resistance protein
BS 2	Ludwig NR	Murein lipoprotein	Cell envelope
		Tiorf107 protein	Conjugation process

In the antimicrobial study, six selected bacteria which are *B. cereus*, *B. subtilis* and *E. faecalis* classified as Gram-positive bacteria as well as *K. pneumonia*, *E. coli* and *S. thyphimurium* categorized as Gram-negative bacteria have been treated with microbial proteins. When a protein sample is dropped into a well on agar, the sample will diffuse from the well into the agar. If the microbial protein has the antimicrobial property, then there will be no growth of bacteria, forming a clear zone on the agar around the well as shown in Figure 3. As a control, microbial proteins without treating with antimicrobial were also tested for antimicrobial activity and labeled as PC. The protein produced by *B. subtilis* ATCC 21332 after treating with 0.05 x MIC *A. sativum* which is labeled as PS, showed high antimicrobial activity against *E. coli* and *K. pneumonia*, while indicated moderate antimicrobial activity towards *B. cereus*, *E. faecalis* and *S. thyphimurium*. Nevertheless, PS revealed no inhibitory effect towards *B. subtilis*. Meanwhile, the protein control (PC) as well as control sample (CS) did not show any antimicrobial activity against all the bacteria tested (Table 2).

Figure 3: Formation of clear zone represents antimicrobial activity

Table 2: Antimicrobial activity of proteins secreted by *B. subtilis* ATCC 21332

Samples	Test Bacteria					
	B. cereus	*B. subtilis*	*E. faecalis*	*E. coli*	*K. pneumonia*	*S. thyphimurium*
[a]PS	[e]+	[f]-	+	[d]++	++	+
[b]PC	-	-	-	-	-	-
[c]CS	-	-	-	-	-	-

[a]PS Protein sample: protein secreted by *B. subtilis* ATCC 21332 after treating with *A. sativum*; [b]PC Protein control: protein produced by *B. subtilis* ATCC 21332 without treating with *A. sativum*; [c]CS Control sample: *A. sativum* with broth only; [d]++ High antimicrobial activity: inhibition zone > 10 mm in diameter; [e]+ Moderate antimicrobial activity: inhibition zone ≤ 10 mm in diameter; [f]- No antimicrobial activity: no inhibition zone

Interestingly, the inhibitory substance from *B. subtilis* ATCC 21332 proteins was not able to inhibit the growth of *B. subtilis* ATCC 11774 which are generally recognized as beneficial bacteria or safe bacteria and have been used as probiotic strains. This finding led to the generalized assumption that such antimicrobial protein or bacteriocin can offer some advantages over existing antibiotics which kill both pathogenic and beneficial microorganisms. *B. subtilis* are being used as probiotics and competitive exclusion agents for both human and animal consumption, which can synthesize antimicrobial substances, likes bacteriocins to prevent the colonization of the gastrointestinal tract by pathogenic bacteria [13]. In this case, it is very important in the view of fact that this bacteriocin had no inhibition against other *B. subtilis* strains.

Conclusion

This study described that *B. subtilis* ATCC 21332 cells in mild stress condition with the presence of *A. sativum* at sub-MIC were able to induce proteins production by regulating the transcription process in bacteria. The microbial proteins displayed antimicrobial activity against Gram-positive as well as Gram-negative bacteria.

Acknowledgement

The authors acknowledge the Ministry of High Education Malaysia for the financial support through RACE Grant Scheme USIM/RACE/FST/STH/35/50412 and Universiti Sains Islam Malaysia.

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