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Proteomics study of silver nanoparticles toxicity on *Bacillus thuringiensis*

Fateme Mirzajani [a,b,*], Hossein Askari [a], Sara Hamzelou [a], Yvonne Schober [d], Andreas Römpp [d], Alireza Ghassempour [c,**], Bernhard Spengler [d]

[a] Department of Biotechnology, The Faculty of Renewable Energies & New Technologies Engineering (NTE), Shahid Beheshti University, G.C. Evin, Tehran, Iran
[b] Department of Nanobiotechnology, Protein Research Institute, Shahid Beheshti University, G.C. Evin, Tehran, Iran
[c] Department of Phytochemistry, Medicinal Plants and Drugs Research Institute, Shahid Beheshti University, G.C. Evin, Tehran, Iran
[d] Institute of Inorganic and Analytical Chemistry, Justus Liebig University Giessen, Schubertstrasse 60, 35392 Giessen, Germany

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ABSTRACT

Emerging technologies in functional genomics and proteomics provide a way of achieving high-throughput analyses, understanding effects on protein populations and sub-populations and follow up environmental stresses. To accomplish these, the action of homemade spherical Silver nanoparticles colloidal suspension (AgNPs) against *Bacillus thuringiensis* (isolate from *Oryza sativa* L. rhizosphere) was investigated by a proteomic approach (2-DE and NanoLC/FT-ICR MS identification). Thirty four responsive (up/down regulated) proteins were identified. Proteomic results revealed that an exposure of *B. thuringiensis* cells with different concentrations of AgNPs resulted in an accumulation of envelope protein precursors, indicative of the dissipation of a proton motive force. Identified proteins are involved in oxidative stress tolerance, metal detoxification, transcription and elongation processes, protein degradation, cytoskeleton remodeling and cell division. The expression pattern of these proteins and their possible involvement in the nontoxicity mechanisms were discussed.

1. Introduction

It has been discovered that silver and its related compounds are effective anti-microbial agents. They have a variety of applications including a prophylactic effect for human infections, the treatment of wounds and on plant and animal microbial diseases (Navarro et al., 2008). Silver nanoparticles (AgNPs) as a newborn form of silver represent a novel generation of anti-microbial, anti-fungal and anti-viral applications against a very broad range of micro-organisms (Kim et al., 2008; Rai et al., 2009). Environmental pollution caused by AgNPs, particularly contamination of soil and water resources, has been accelerated as a result of global industrialization and is considered a major risk for communities throughout the world (Navarro et al., 2008).

Soil ecosystems have been exposed to toxic compounds, among which are AgNPs (Filip, 2002). Some of the soil bacteria communities have an outstanding ability to adapt to a polluted environment according to employ various unique mechanisms (Wang et al., 2007). Consequently, polluted soil is faced with new kinds of micro flora; some have changed significantly, others are simply missing. Several negative effects on soil beneficial bacteria, suggesting that they have adverse reactions to pollution such as nanoparticles (Filip, 2002; Wang et al., 2007). *Bacillus thuringiensis* (Bt) is a Gram-positive soil-dwelling bacterium, commonly used as a biological pesticide. Its *cry* toxin is extracted and used as a pesticide. Many Bt strains produce crystal proteins (proteinaceous inclusions), called δ-endotoxins, that have insecticidal action. This compound has led to their use as an insecticide, and Bt genes are used to genetically modify crops recently (Höfte and Whiteley, 1989).

A bacterial cell can react simultaneously to a wide variety of stresses (Vollmer et al., 2008). Stress response mechanism of bacteria enables them to survive adverse and mutable conditions in their immediate environments. Various mechanisms have been suggested to bacterial responses following the different environmental fluctuations. The stress response in bacteria involves a number of systems that act against an external stimulus. A complex network of global regulatory systems in bacteria certifies that various stress response systems interact with each other and this leads to a coordinated and effective response.

Proteomics is a fastest developing field of research and it contributes substantially to our understanding of organisms at the cellular level (Lok et al., 2006; Navarro et al., 2008). In addition, this is the study of functions and regulation of biological

* Corresponding author at: Department of Biotechnology, The Faculty of Renewable Energies & New Technologies Engineering (NTE), Shahid Beheshti University, G.C. Evin, Tehran, Iran. Fax: +98 2122431598.
** Corresponding author. Fax: +98 2122431783
E-mail addresses: F_mirzajani@scientist.com (F. Mirzajani), aghassempour@scientist.com (A. Ghassempour).

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systems based on analysis of the protein expression profile. Furthermore, there is general agreement that soil bacterial proteomics may be a tool for its better management. Because of the ability of soil to stabilize extracellular proteins over various mechanisms, development of bacterial proteomics needs an assessment of the efficiency of protein extraction from various soil types. In the case of nanotechnology environment proteomics Li et. al. used the gel-base proteomics together with other biochemical studies to evaluate the AgNPs action on *Staphylococcus aureus* (Li et al., 2011). The aim of this study was to evaluate the action of homemade spherical colloidal AgNPs suspension against *Bacillus thuringiensis* (isolate from *Oryza sativa* L. rhizosphere) from proteomic point of view using the gel-base combined NanoLC/FT-ICR MSMS method.

2. Material and methods

2.1. Silver nanoparticles (AgNPs): synthesis and characterization

The homemade silver nanoparticle colloidal suspension (AgNPs) was prepared and characterized according to the previously reported method (Mirzajani et. al, 2011). Briefly, colloidal AgNPs with spherical morphology and distinct diffraction peaks of crystalline plans of cubic including (111), (200), (220) and (311), have a maximum absorbance (λ_{max}) at 426 nm. Their size was 18.34 nm (X99) with the homogeneity of their size within the range of 0.1–1000 nm.

2.2. Bacterial identification, cultivation and AgNPs treatment

Rice (*O. sativa* L.) rhizosphere (the soil near the surface of the root) bacteria were s collected from a paddy farm at Sari, Mazandaran Province, Iran, at April, 2010. The *B. thurigiensis* SBURR1 (Bt) was isolated and characterized according to the previously reported method (Mirzajani et al., 2013). In brief, the soil samples were dried and suspended in sterile saline solution (0.85% NaCl). The bacteria were originally isolated by plating the100 mL of soil suspension (in the range of 10^{-1}–10^{-5}) on nutrient agar and were then incubated at 36 ± 0.5 °C for 22 h. The developed colonies which varied in shape and color were picked up and purified by streaking on nutrient agar. Among the purified and identified strains (Mirzajani et al., 2013) based on microscopic examination, Gram staining, biochemical assay and 16S rRNA gene sequencing the Bt were chosen for the proteomics study (Fig. 1a). To identify *Bacillus* isolates, the gene, encoding 16S rRNA, was amplified by PCR using universal primers (27F:50-AGAGTTTGATCCTGGCTCAG-30; 1492R:50-TACCTTGTTACGACTT-30), subsequently the amplified nucleotide product was sequenced and similar sequences were identified using online BLAST in NCBI nucleotide database [http://blast.ncbi.nlm.nih.gov/Blast.cgi]. A multiple alignment and a phylogenetic tree were obtained using the CLUSTALX 2.0 and MEGA4 software packages (Larkin et al., 2007), respectively (Mirzajani et al., 2013). Additionally, for the detection of *B. thuringiensis*, the presence of cry genes coding for the insecticidal toxins was studied. Briefly, in order to detect the subgroups of cry genes, universal primers as follow: cry1 (forward 5_-CATGATTCATGCGGCAGA-TAAAC-3_; reverse 5_-TTGTGACACTTCTGCTTCCCATT-3_), cry 2 (forward 5_-GTTATTCT-TAATGCAGATGAATGGG-3_; reverse 5_-CGGATAAAATAATCTGGGAAATAGT-3_), cry3 (forward5_-CGTTATCGCAGAGAGATGACATTAAC-3_; reverse 5_-CATCTGTTGTTTCTG-GAGGCAAT-3_) and cry4 (forward5_-GCATATGATGTA GCGAAACAAGCC-3_; reverse 5_-GCGTGACATACCCATTTCCAGGTCC-3_) were used according to the reported protocol (Ben Dov et al., 1977; Kumar et al., 2008).

Since there is no specific antibacterial guideline for nanoparticles, the antibacterial effect of homemade AgNPs (the method of synthesis and characterization was previously reported) was evaluated by macro-broth dilution methods according to the standard methods recommended by CLSI (12) with some modifications (Mirzajani et al., 2011). The minimum concentrations of AgNPs required for inhibition (MIC) or killing (MBC) of the tested bacteria were 1 and 5 μg/mL, respectively. Furthermore, our previous results (Mirzajani et al., 2011, 2013) confirmed the pit formation and leakage through the bacterial membrane (Fig. 1c). Growth curves of Bt including the exposed ones (at MIC and MBC level) and unexposed ones to AgNPs were determined based on the absorbing value of 600 nm in the MHB media at 36 ± 0.5 °C while stirring at 120 rpm, during 24 h for each 20 min (Mirzajani et al., 2013). The appropriate sampling time for proteome extraction was chosen based on our previous report (Fig. 1b). Briefly, the exponentially growing curve of the treated Bt cultured in MHB medium was studied during 700 min at 600 nm (OD600) (Mirzajani et al., 2013). Tests of four different AgNPs treatments of 0, MIC=1, MBC=5 and between MIC and MBC=2.5 μg/mL at the exponential growth phase (OD600=0.1−0.2) were monitored. However, in an absence of AgNPs, Bt continued at the exponential phase for 520 min, and in the presence of AgNPs (1 μg/mL) slower growth continued for more than 200 min. Exposed to 2.5 μg/mL of AgNPs, the Bt growth lagged to 430 min, followed by the exponential growth for 90 min. When the concentration of AgNPs was 5 μg/mL, the delay was more evident and no growth of bacteria could be detected within a time frame of 370 min. Based on these results, the bacterial mass of 0, 1, 2.5 and 5 μg/mL of AgNPs treatment were collected (22,000 g at −4 °C) at 450 and 550 min of culturing, respectively.

2.3. Protein extraction

Total protein was isolated from cells by TRIzol reagent and available guidelines (Molecular Research Center, Inc., Cincinnati, OH, USA). Briefly, the bacteria were ground using mortar in liquid nitrogen and homogenized by TRIzol reagent. It was then incubated with the chloroform and centrifuged for 15 min at 12,000 g at 4 °C. It was mixed with absolute ethanol for 15 s, incubated for 3 min and finally centrifuged at 2000 g for 5 min at 4 °C. The proteins in the phenol/ethanol supernatant were precipitated by the addition of absolute acetone and dispersed in the washing agent (0.3 M guanidine hydrochloride in 95% ethanol and 2.5% glycerol). The latter was repeated 3 times and followed by washing in ethanol containing 2.5% glycerol. The precipitated protein was dissolved in lysis buffer (consisted of 9 M Urea, 4% CHAPS, 1% DTT, 1% pH 3–10 ampholytes, 35 mM Tris base) and stored at −80 °C. Protein concentration was determined according to the Bradford assay kit (BioRad Co., Hercules, CA, USA) in the comparison with the BSA as the standard.

2.4. 2 dimension gel electrophoresis (2-DE)

2-DE was carried out based on the previous report (National Committee of Clinical Laboratory Standards, 2005). IPG strips, 18 cm, pH 4–7, linear (BioRad Co., CA, USA) were loaded with the proteins during the rehydration process for 16 h at room temperature in reswelling tray (Amersham Pharmacia Biotech, Sweden). For analytical and preparative gels, 30/70 μg and 0.8 mg of proteins were loaded, respectively. The IPG strips were covered with silicon oil and all the IEF separations were performed horizontally at an optimized temperature of 20 °C using a Multiphore II system (Amersham Pharmacia Biotech) for a total of 70,000 Vh. Second-dimension SDS-PAGE separations were performed on the gels (245 × 180 × 0.5 mm; 12.5% polyacrylamide) using a PROTEAN II Multi Cell (BioRad Co., CA, USA). Separations were performed at 4 °C. The protein spots in analytical and preparative gels were visualized by silver nitrate and colloidal CBB G-250, respectively.

2.5. Image and data analysis

The analytical gels were immediately scanned using GS-800 calibrated densitometer (BioRad Co., CA, USA)at 600 dpi resolution. The spots of triplicate gels (experimental replicates) of control and experimental group were detected and matched using Melanie 3 software (GeneBio Co., Switzerland). The molecular mass (kDa) and (isoelectric point) p*I* of the spots were calculated by standard protein markers (Amersham Pharmacia Biotech) and interpolation of missing values on IPGs, respectively. Quantitative comparison of protein spots was based on their percent volumes. The one-way analysis of variance (ANOVA) and comparison of treatment means were carried out by SAS programs. Only those statistically significant spots ($P \leq 0.05$) were accepted and they had to be consistently present in all the replications. The accepted spots were filtered based on the average expression level of two-fold or consistent and significant changes in at least three nanoparticle levels.

2.6. Protein digestion and peptide extraction

The spots, showing comparable differences were cut out manually from a preparatory gel. The spots were washed with water and destained using 10 mM ammonium hydrogen carbonate in 50% (v/v) MeCN. They were fully dehydrated in 100% (v/v) MeCN and dried using a SpeedVac system (Thermo Fisher Scientific Co., MA, USA) at 20 °C, 14,000 g for 15 min. Subsequently, the dried gel pieces were rehydrated with ~1–4 μL trypsin (Promega, Madison, WI, USA) solution for 30 min (0.1 mg/mL in 30 mM NH_4HCO_3 buffer) tryptic digestion was carried out at 37 °C for 20 h. The peptides were extracted from the gel pieces in three steps, using a MeCN containing 5% formic acid under sonication for 10–15 min. The OMIX C18 10 μL tip (Varian, Inc., Palo Alto, CA, USA) was used to cleanup and to per-concentrate the extracted solution.

2.7. Mass spectrometry measurement

Measurements of the peptides were accomplished on an Ultimate binary nanohigh-performance liquid chromatography (nano-HPLC pump/autosampler) system for HPLC analysis (LCPackings/Dionex Co., Idstein, Germany). Volumes of 5 μL of the sample were pre-focused on a trap column (Dionex Co., C18 PepMap, i.d. 300 mm, length 5 mm) and separated on a fused-silica C18 PepMap100 capillary column (Dionex Co., 3 mm, 100 Å; i.d. 75 mm; length 150 mm). The flow rate was 0.2 μL/min. Solvent A consists of water containing 2% MeCN (v/v) and 0.1% formic acid (FA) (v/v). Solvent B was the MeCN containing 20% water (v/v) and 0.08% FA (v/v). Separation was performed as follows: first B was increased from 0% to 25% in