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Label free-based proteomic analysis of proteins in *Bacillus cereus* spores regulated by high pressure processing and slightly acidic electrolyzed water treatment

Liping Wang, Qiang Xia, Yunfei Li*

Department of Food Science and Technology, School of Agriculture and Biology, Shanghai Jiao Tong University, Shanghai 200240, PR China

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ABSTRACT

To investigate the mechanism of high pressure processing (HPP, 200 MPa/20 min + 500 MPa/20 min) and slightly acidic electrolyzed water (SAEW, 44 mg/L available chlorine concentration (ACC)) on *B. cereus* spore inactivation, a label-free quantitative proteomics approach based on high-resolution mass spectrometry (MS) data was used. Quantification of 2810 proteins was obtained with high confidence. A total of 93 and 83 proteins showed significant differential abundance after HPP-SAEW and HPP treatments, respectively, and 21 proteins changed significantly when both treatments were compared. The results indicated that the spore cortex-lytic enzyme was upregulated 3.15 fold after HPP treatment, and the germination was influenced by ATP generation. The metabolic, degradation, signaling, and biosynthesis pathways were involved in HPP-SAEW mediated spore inactivation. Amino acid-biosynthetic and quorum sensing were the main KEGG pathways mediated by SAEW under HPP conditions. Specifically, our results showed that the phosphorylation of the Spo0A transcription factor was mediated by the downregulation of the ABC transporter ATP-binding protein, which was associated with inactivation of spores.

1. Introduction

Bacillus cereus is a gram-positive, spore forming, motile, and rod-shaped facultative aerobic bacterium that may cause food poisoning (Wijnands, 2008). As a highly thermally resistant bacterium, *B. cereus* spores are among the most common food-borne bacterial pathogens, causing diarrhea and nausea (Evelyn & Silva, 2015; Wimalaratne, 2009). The number of outbreaks of zoonoses, zoonotic agents, and food-borne illness caused by *B. cereus* toxins was 287 (3073 cases, and 8% hospitalization) in European member states (MSs) in 2014, whereas 291 (3131 cases, and 3% hospitalization) were reported by nine MSs in 2015 (EFSA & ECDC, 2016; Osimani, Aquilanti, & Clementi, 2018). The dormant spores are extremely resistant to various environmental conditions that could potentially damage DNA, including heat, radiation, oxidizing agents, and desiccation (Setlow, 1995). Spores are in stress-resistant states, and their undesired germination affects food stability (Abhyankar, Stelder, Koning, Koster, & Brul, 2017). In contrast to the sporulation process, in which the spores are given the appropriate stimulus, these spores can return rapidly to vegetative state (Paidhungat et al., 2002), and germination only takes a few minutes (Wimalaratne, 2009).

The high pressure processing (HPP) technique inhibits a variety of bacteria, but its antibacterial effect on spores is limited. HPP can induce morphological changes in spore structures (Akasaka & Matsuki, 2005; Wang, Xia, & Li, 2017a), although individually HPP has a limited effect on the inactivation of spores. In addition, germination plays a key role in the inactivation of bacterial endospores by HPP treatment (Akasaka & Matsuki, 2005). Under 100–200 MPa pressure, spores germination depends on germinant receptors, then dipicolinic acid (DPA) is released, and cortex is hydrolyzed, whereas under 500–600 MPa pressure, spore germination is independent of germinant receptors, and the directly release of DPA activates cortex lytic enzyme, which results in cortex hydrolysis (Setlow, 2003). Studies of spores in various foods or food model systems indicated that HPP induced spores germination, but this effect was usually limited to a few log cycles (Luu-Thi, Grauwet,

* Corresponding author. Professor of School of Agriculture and Biology, Shanghai Jiao Tong University, No.800, Dongchuan road, Shanghai 200240, PR China.
E-mail addresses: wangliping8810@163.com (L. Wang), xqiang0713@hotmail.com (Q. Xia), yfli@sjtu.edu.cn (Y. Li).

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Vervoort, Hendrickx, & Michiels, 2014). DPA release was shown to be the first indicator of pressure-induced loss of heat resistance, and was the rate-limiting step for successful spore inactivation in a high-pressure thermal sterilization process (Reineke, Schlumbach, Baier, Mathys, & Knorr, 2013). Inactivation of *B. cereus* spores using the hurdle technology of HPP and slightly acidic electrolyzed water (SAEW) has been demonstrated to be effective, and conditions of 200 MPa HPP + SAEW with 44 mg/L ACC + 500 MPa HPP showed a potent inactivation effect against *B. cereus* spores (Wang, Xia, & Li, 2017b).

As the protein equivalent of genomics, proteomics is the study of proteins and their interactions in a cell, and it provides the foundation for constructing and extracting useful knowledge for biomedical research (Cho, 2007). The rapid growth of proteomics technologies, combined with increased genome sequence information, has led to proteomics analysis becoming one of the most efficient methods to explore protein expression levels, protein modifications, and protein-protein interactions in diverse biological systems (Liu et al., 2016). Traditional proteomic quantification (dyes, fluorophores, and radioactivity) requires high-resolution protein separation, and is incapable of revealing the identity of the underlying protein (Bantscheff, Schirle, Sweetman, Rick, & Kuster, 2007). Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) overcomes these problems. Mass spectrometry (MS) can identify and quantify thousands of proteins in complex samples, and most quantitative MS methods reply on differential labeling of protein samples with stable isotopes (Luber et al., 2010). However, the stable isotope labels used as internal standards are expensive, whereas label-free quantification uses direct MS signal intensities of peptides or the number of acquired spectra matching a peptide/protein as indicators of the respective amounts in a sample (Bantscheff et al., 2007). The advantage of this approach is that it can be applied to any proteomic sample directly without isotopes for quantification (Liu et al., 2016; Luber et al., 2010). In principle, there is no limit to the number of experiments that can be compared, and this technique can thus provide a higher dynamic range of quantification than stable isotope labeling (Bantscheff et al., 2007). Considering the overall experimental process, however, the label-free approach is the least accurate among the MS quantification techniques, and it is difficult to match thousands of peptides across samples. Additionally, there is variability in LC-MS resulting in retention time shifts, and introduction of errors in the sample fractionation steps (Tao et al., 2013). Hence, the number of experimental steps should be kept to a minimum and each step should be reproducible (Bantscheff et al., 2007). Proteomics technology has been used to characterize the spore proteins of *Bacillus subtilis* and *Clostridium difficile* 630 spores (Kuwana et al., 2002; Lawley et al., 2009). The bactericidal mechanism of sodium new houttuyfonate (SNH) against *Streptococcus pneumoniae* based on iTRAQ proteomics revealed that numerous proteins related to the production of reactive oxygen species were upregulated by SNH (Yang et al., 2016).

There are few reports on the mechanism by which HPP-SAEW inactivates spores. In this study, we utilized a label-free quantitative proteomic analysis technique to determine the changes in protein levels after *B. cereus* spores treated with HPP-SAEW. The mechanism of HPP-SAEW was investigated using the Gene Ontology (GO) annotation and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways.

2. Material and methods

2.1. Bacterial strains and preparation of spores

B. cereus (ATCC 11778) was obtained from Shanghai North Connaught Biotechnology Co. Ltd. (Shanghai, China), and spores were prepared as previously described (Wang et al., 2017a).

2.2. HPP and SAEW treatments

The spores were inoculated in SAEW, and 0.1 M 2-(N-morpholino) ethanesulfonic acid (MES) buffer (pH 6.1) for multi-cycle HPP treatment. The SAEW was generated using a flow-type electrolysis apparatus (Prosperity Prosperous Sanitary Products Co., Ltd, Shanghai, China), and the ACC, pH and oxidation reduction potential of SAEW were 44 mg/L, 6.08, and 980 mV, respectively. The multi-cycle HPP treatment was carried out with a HHP-750 instrument (BaoTou KeFa Co., Ltd., Inner Mongolia, China) with a 5-L cylinder (90 mm internal diameter × 320 mm internal height). The multi-cycle HPP process was as follows: the first cycle of HPP was 200 MPa for 20 min, and the second cycle of HPP was 500 MPa for 20 min. Spores inoculated in MES buffer without multi-cycle HPP treatment were used as controls, and the multi-cycle HPP combined with SAEW (200 MPa + SAEW with 44 mg/L ACC + 500 MPa) or MES (200 MPa + MES + 500 MPa) was referred to as 25S and 25M, respectively.

2.3. Extraction and quantification of proteins

After germination, all samples were extracted as previously described (Su, Xiong, Chen, Mu, & Liu, 2015; Kuwana et al., 2002). Sample pellets obtained was centrifuged at 4000 × *g* for 15 min at 4 °C, 400 μL of SDT lysis buffer (4% SDS, 100 mM Tris-HCl, 100 mM dithiotreitol (DTT), and pH 7.6) was added, and the cells were transferred to 2-mL grinding tube with silica sand for MP FasterPrep-24 (MP Biomedical, CA, USA) homogenization (24 × 2, 6.0 M/S, 60 s, two times). The samples were then subjected to ultrasonic (ultrasound JY92-II, Scientz, Zhejiang, China) treatment (power: 80 W, frequency: 20 KHz, 10 s operation and 15 s break for 10 cycles), and incubated in a boiling water bath for 15 min. Finally, after centrifugation at 14 000 × *g* for 30 min, the supernatant was filtered through a 0.22-μm membrane. The proteins collected at the filter were quantified using the bicinchoninic acid (BCA) protein assay kit (Beyotime Biotech Inc, Shanghai, China) according to manufacturer's instruction. The absorbance was recorded at 562 nm in a Multiskcan FC spectrophotometer (Thermo Scientific).

2.4. SDS-PAGE

Samples containing 20 μg of proteins were incubated with 5 × loading buffer (10% SDS, 0.5% bromophenol blue, 50% glycerol, 500 mM DTT, 250 mM Tris-HCl, pH 6.8) at a ratio of 5: 1 (v:v) in a boiling water for 5 min, then centrifuged at 14 000 × *g* for 10 min. The supernatant was subjected to 12.5% SDS-PAGE at a constant current of 15 mA for 60 min (electrophoresis SE260, GE Healthcare Life Science, Boston, USA), following by coomassie brilliant blue staining.

2.5. FASP enzymolysis of proteins

Filter aided sample preparation (FASP) was prepared according to the reference (Su et al., 2015) with a little modification. Samples comprising 200 μg of proteins were mixed with DTT to a final concentration of 100 mM and boiled for 15 min. After cooling, the samples were mixed with 200 μL of UA buffer (8 M Urea, 150 mM Tris-HCl pH 8.0), transferred to a 30 kDa Microsep, and centrifuged at 14 000 × *g* for 15 min. Next, 200 μL of UA buffer was added, and the samples were centrifuged at 14000 × *g* for 15 min. After the filtrate was discarded, 100 μL of IAA (iodoacetanide, 500 mM IAA in UA) was mixed with the samples at 600 rpm for 1 min, incubated in