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Growth Inhibition and Induction of Stress Protein, GroEL, of *Bacillus cereus* Exposed to Antibacterial Peptide Isolated from *Bacillus subtilis* SC-8

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Abstract This study was conducted to investigate the antibacterial effect of BSAP-254 on *Bacillus cereus* with the induced stress proteins. The BSAP-254 is an antimicrobial peptide isolated from soybean-fermenting bacteria, *Bacillus subtilis* SC-8. It had a narrow spectrum of activity against *B. cereus* group. The growth inhibitory effect of BSAP-254 (50 μg/mL) reduced the population of *B. cereus* from $>10^8$ to 10^4 colony-forming units per milliliter within 30 min. In *B. cereus* exposed to BSAP-254, 14 intracellular proteins were differentially expressed as determined by 2-DE coupled with MS. Of the differentially expressed proteins identified, the stress protein GroEL, which is heat shock protein, was induced in *B. cereus* exposed to antibacterial peptide.

Keywords Antibacterial effect · *Bacillus subtilis* · *Bacillus cereus* · Soybean-fermenting bacteria · Food-borne pathogenic bacteria · Heat shock protein GroEL

Introduction

Bacillus cereus is a potential food-borne pathogenic bacterium that easily contaminates naturally fermented foods. In the food industry, various methods such as physical [1–7] and chemical treatments [8, 9] have been evaluated for their ability to control food-borne pathogenic bacteria. Recently, antimicrobial peptides produced by fermenting Bacilli, which are generally recognized as safe (GRAS), have received a great deal of attention as biological control agents because they have low toxicity and biodegradability [10, 11].

The mechanisms by which antimicrobial peptides can destroy bacterial cells are membrane depolarization, damage to the intracellular processes, degradation of cell walls, modification of the lipid composition in the bilayer membrane, and formation of micelles [12]. However, bacteria have developed tolerance to antibacterial peptides via defense

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systems such as the production of protease and capsules [13, 14], modification of the cell wall [15], and the expression of stress response proteins [16].

In our lab, an antagonistic substance against *B. cereus* was isolated from *Bacillus subtilis* SC-8, which was obtained from the Korean traditional soybean-fermented food, *cheonggukjang*, and the properties of this substance were reported [17]. The substance showed specific antagonistic activity against *B. cereus* at low concentration on lawn cell plate. In addition, the substance was biodegradable and stable at pH ranging from 4 to 10 and temperatures as high as 60°C.

This study was to investigate antibacterial effect of BSAP-254 against food-borne bacteria *B. cereus* and stress proteins induced in *B. cereus* under the unfavorable condition exposed to antibacterial peptide.

Materials and Methods

Bacterial Strains and Culture Medium

The antagonistic strain *B. subtilis* SC-8, which was isolated from the traditionally soybean-fermented food, *cheonggukjang*, and *B. cereus* (KCTC 3624) were incubated in Luria–Bertani (LB) broth medium (BD Diagnostics, Sparks, MD, USA) composed of 10 g of tryptone, 5 g of yeast extract, and 10 g of sodium chloride per liter.

Preparation of Antibacterial Peptide

B. subtilis SC-8, an antagonistic strain against *B. cereus*, was incubated in 1 L of LB medium at 37°C with shaking at 250 rpm until the cell density reached 1.3 at A_{600}. The supernatant containing the antibacterial peptide, BSAP-254, was obtained according to a previously described method with minor modification [17]. HPLC was used to purify the antibacterial peptide. The sample was then filtered through a 0.45-μm polytetrafluoroethylene membrane (Schleicher & Schuell, Keene, NH, USA) and injected onto a C18 reversed-phase column [25 cm (length)×4.6 mm (internal diameter), 5 μm particle diameters; Shiseido, Tokyo, Japan]. The mobile phase consisted of 0.1% trifluoroacetic acid (TFA) in water (solvent A) and 0.1% TFA in acetonitrile (solvent B) using the following program: stage I, 60% A, 40% B, 0–4 min, a flow rate of 2 mL/min; stage II, 40–10% A, 60–90% B, 4–12 min, a flow rate of 0.7 mL/min; stage III, 100% B, 12–20 min, a flow rate of 1 mL/min; and stage IV, 0–60% A, 100–40% B, 20–25 min, a flow rate of 1 mL/min [18]. The sample injection volume was 20 μL, and the sample was monitored at 215 nm. The reserved fractions were collected for activity analysis and concentrated with a centrifugal concentration system (VS-802 Centra-vac, Vision Scientific, Bucheon, Korea).

Viability of *B. cereus* Exposed to BSAP-254

B. cereus was incubated in 20 mL of LB medium at 37°C with shaking at 250 rpm until the cell density reached 1.2 at A_{600}. Cells from 1 mL of culture medium were then collected by centrifugation (14,000×*g*, 4°C, 15 min), after which the pellets were washed and suspended in 1 mL of dH_2O. Next, 50 μg of BSAP-254 was treated with 100 μL of cell suspension and reacted at 37°C for 10, 20, 30, and 60 min. *B. cereus* treated by BSAP-254 were incubated on LB agar plate at 37°C for 16 to 18 h, and the colonies of *B. cereus* were then counted.

Preparation of Whole-Cell Proteins for Proteome Analysis

B. cereus was incubated in 20 mL of LB medium (250 rpm, 37°C) until the cell density reached 1.2 at A_{600}, after which they were harvested by centrifugation (14,000×*g*, 4°C, 1 min). Pellets were suspended with 5 mL of fresh LB medium. Next, 200 μg of BSAP-254 was added to 5 mL of cell suspension and allowed to react at 37°C for 1 h. The reactant was then washed with dH_2O two times and resuspended in 5 mL dH_2O. Next, the suspended cells were sonicated four times for 30 s each. After centrifugation (14,000×*g*, 4°C, 5 min), the supernatant was dried with a centrifugal concentration system (Vision Scientific, Korea). The concentrate was then solubilized in 100 μL of lysis buffer containing 9 M urea, 65 mM dithiothritol (DTT), 65 mM 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), and 5% ampholytes (pH 3 to 10; Fluka, Buchs, Switzerland). Proteome analysis was conducted by the Korea Basic Science Institute.

Two-dimensional Electrophoresis

First-dimension isoelectric focusing (IEF) was conducted using an Ettan IPGphor 3 (GE Healthcare, Buckinghamshire, UK). Precast 18-cm, nonlinear gradient pH 3–10 immobilized pH gradient (IPG) strips were obtained from GE Healthcare. Whole-cell proteins (100 μg) obtained from the three independent experiments were resuspended with rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, trace of bromophenol blue, 20 mM DTT, and 0.5% corresponding IPG buffer) to a final volume of 150 μL. IEF was conducted at 101,000 Vh/h. After IEF, the proteins were reduced and alkylated using rehydration buffer (7 M urea, 2 M thiourea, 2% CHAPS, and 10 mg/mL DTT) and alkylation buffer (7 M urea, 2 M thiourea, 2% CHAPS, and 40 mg/mL iodoacetamide). The second-dimension electrophoresis was conducted using a standard 12.5% SDS–PAGE protocol. Gels were stained using a silver staining kit (Amersham Biosciences, AB, Sweden), after which the protein patterns were recorded as digitalized images using an Epson perfection V750PRO scanner (SEIKO EPSON, Nagano, Japan), and scanned images were analyzed using the Master 2D Platinum Software (version 7.0, GE Healthcare). Tryptic digestion was then performed as previously described [19].

Identification of Protein by MALDI TOF/TOF MS/MS

The proteins were identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI TOF/TOF MS/MS). Briefly, proteins were digested in gel with trypsin. The tryptic peptides were then dissolved in 0.5% (*v*/*v*) trifluoroacetic acid in 50% (*v*/*v*) acetonitrile, after which they were desalted using a ZipTip C_{18} pipette tip (Millipore, Bedford, MA). Next, the peptides were directly eluted onto MALDI plates using a-cyano-4-hydroxy-cinnamic acid (CHCA) matrix solution (10 mg/mL CHCA in 0.5% TFA/50% acetonitrile, 1:1, *v*/*v*). All mass spectra were acquired in reflection mode using an ultraflextreme TOF/TOF (Bruker Daltonics, Germany) equipped with a pulsed smartbeam. The spectra were then analyzed using Flex Analysis software (version 3.3, Bruker Daltonics, USA), and a search against the taxonomy of bacteria in the nonredundant NCBI (NCBInr) database was conducted using the MASCOT software (version 2.3).

Western Blot Analysis

Samples (25 μg) were run on 12% polyacrylamide gels and then transferred onto polyvinylidene fluoride membranes (Millipore, Bedford, MA, USA). The membranes were

Table 1 The viability of *B. cereus* exposed to BSAP-254

Time (min)	*B. cereus* (colony-forming units per milliliter)	
	None	Treatment (BSAP-254, 50 μg)
0	$2.17\times10^{8}\pm31.81$	
10	NA	$7.55\times10^{6}\pm13.43$
30	$1.76\times10^{8}\pm19.79$	$8.34\times10^{4}\pm1.15$
60	$1.59\times10^{8}\pm42.42$	$2.34\times10^{4}\pm1.52$

The data shown represent the means of three independent experiments

NA not assayed

blocked with phosphate-buffered saline (PBS) containing 3% BSA, pH 7.4, at room temperature for 1 h, after which they were incubated in 1:1,000 diluted anti-rabbit GroEL antibodies (Sigma-Aldrich Co. St. Louis, Mo. USA). After washing in 50% Tween-20 PBS, the membranes were incubated in a secondary anti-rabbit IgG conjugated with horseradish peroxidase (1:1,000 dilution by PBS) at room temperature for 1 h and then washed with 50% Tween-20 PBS. After washing, the bands were detected by diaminobenzidine (Koma Biotech., Seoul, Korea). The detected bands were quantified by the Molecular Imager® Gel Doc™ XR System (Bio-Rad Laboratories, Hercules, CA, USA).

Results and Discussion

Antibacterial Effect of BSAP-254 on *B. cereus*

The growth inhibitory effects of BSAP-254 on *B. cereus* are summarized in Table 1. Minimum inhibitory concentration of BSAP-254 on *B. cereus* in liquid culture media was 8 μg/mL and did not grow at all in the concentration of 30 μg/mL with 10^{5} cells (data not

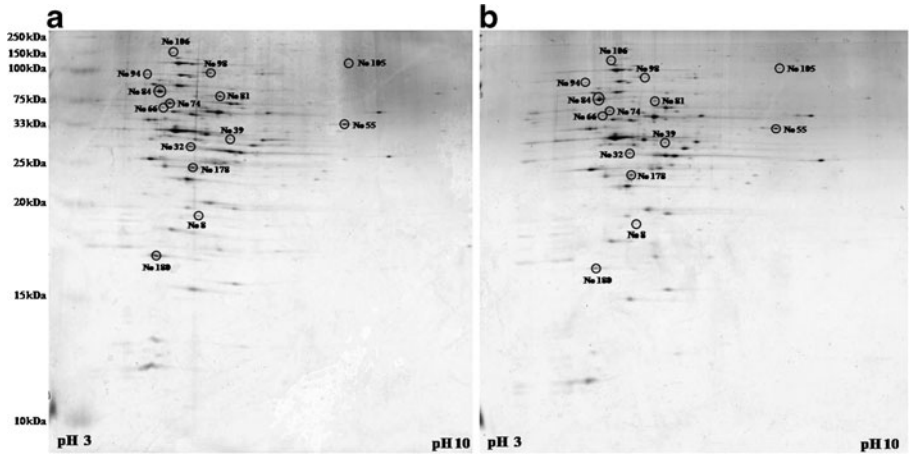

Fig. 1 2-DE proteome patterns of intracellular protein of *B. cereus*. **a** *B. cereus* (KCTC 3624), **b** *B. cereus* treated with 200 μg of BSAP-254. *B. cereus* was incubated in 5 mL LB medium at 37°C for 1 h

Table 2 The protein identified by 2-DE gel electrophoresis of whole-cell protein extracts of *B. cereus* exposed to BSAP-254

Spot no.[a]	Protein identity	Mowse score[b]	MW (Da)/pI[c]	Peptide sequence	No of peptide matched	Average ratio[d]	Accession no. (Mascot database)
8	Hypothetical protein PAU_01189 [*Photorhabdus asymbiotica*]	51	42,028/5.20	K.YGCVVEPTAGSDATDGSSGGLGGGGR.D	1	−1.56±0.31	gi|253988670
55	Arginine deiminase [*Bacillus cereus* ATCC 14579]	52	47,022/6.11	K.EHDYFAQTLR.N	1	−1.50±0.20	gi|30018614
84	Hsp60 [*Bacillus thuringiensis*]	168	42,081/4.76	K.VASIVAEGDEATGINIVLR.A K.SSIAQVAAISAADEEVGQLIAEAMER.V	2	1.63±0.01	gi|15706393
94	Molecular chaperone DnaK [*Bacillus* cereus ATCC 14579]	92	65,783/4.66	K.AVITVPAYFNDAER.Q	1	−1.37±0.48	gi|30022393
98	Pyruvate kinase [*Bacillus cereus* ATCC 14579]	70	62,343/5.02	R.GDMGVEIPPEEVPLVQKR.L	1	−3.07±0.01	gi|30022674
180	Alkyl hydroperoxide reductase C22 [*Bacillus* cereus ATCC 14579]	146	20,864/4.79	R.TITTNFNVLMEEEGLAAR.G R.GTFIIDPDGVIQSMEINADGIGR.D	2	−2.06±0.11	gi|30018585

[a] Spot number was defined according to its position in the 2-DE gels

[b] Individual ion scores >49 indicate identity or extensive homology ($p<0.05$)

[c] Theoretical MW (daltons)/pI are based on the amino acid sequence of the identified protein

[d] Average ratio between *B. cereus* cultured with BSAP-254 and without BSAP-254. These values were calculated as the average from three replicates. Spot intensity was increased (+) or decreased (−) in *B. cereus* cultured with BSAP-254

shown). Based on these results, 50 μg/mL of BSAP-254 as the concentration of enough damage was used for antibacterial effect on 10^8 cells of *B. cereus*. After treatment of BSAP-254, the population of *B. cereus* decreased from $>10^8$ to 10^6 colony-forming units per milliliter in 10 min and 10^4 colony-forming units per milliliter in 30 min. These results demonstrate that BSAP-254 is effective against *B. cereus* in 10 min and that a 50% reduction in the population of *B. cereus* occurred within 30 min.

Generally, antibiotic lipopeptides produced from *B. subtilis* consist of a linear or cyclic peptide with C8 to C18 fatty acid chains and are classified as cationic or anionic peptide [12, 20, 21]. Disruption of the membrane integrity of antimicrobial lipopeptides often leads to rapid death of bacteria. Cationic peptides such as gramicidin S and polymyxins inhibit many Gram-negative and a few Gram-positive bacteria via cytoplasmic membrane depolarization [22]. Anionic lipopeptides such as surfactin and daptomycin exert detergent-like action and depolarization on biological membranes [12, 20].

The BSAP-254 is the putative lipopeptide-like antagonist (molecular weight, 3,400 Da; 36 amino acids; lipid moieties) and degraded by protease and lipase [17]. Therefore, it could not be placed in the category of the major surfactin, fengycin, and iturin-like compounds (m/z 1,016 to 1,515; four to nine amino acids) [20, 23].

DNA and K^+ were released in *B. cereus* exposed to antibacterial peptide BSAP-254. DNA release was slightly increased from 20 to 70 ng/μL for 30 min. The K^+ efflux, however, showed a rapid increment, from 30 to 50 ng/mg for 10 min (data not shown). Therefore, BSAP-254 was found to inhibit *B. cereus* (10^8 colony-forming units per milliliter) in 10 min and decreased the cell population to 10^4 colony-forming units per milliliter in 30 min (Table 1). The maximum number of *B. cereus* in food has international distinction, with less than 10^4 or 10^6 colony-forming units per milliliter generally being allowed. BSAP-254 was isolated from food microorganisms (GRAS) and has properties of biodegradation and nonhemolysis [17]. Therefore, it could be effectively used as a biological control agent for the exclusion of *B. cereus* in food.

Differential Expression of Intracellular Protein in *B. cereus* Treated with BSAP-254

Generally, when bacteria are exposed to stress conditions, their stress proteins are induced for survival. To analyze differential expressed proteins in *B. cereus* exposed to BSAP-254, cells of *B. cereus* were used four times more than those for viability studies. We analyzed stress proteins induced in *B. cereus*, which was exposed to fourfold increased antibacterial peptide (200 μg/mL), using two-dimensional electrophoresis (2-DE) coupled with MS.

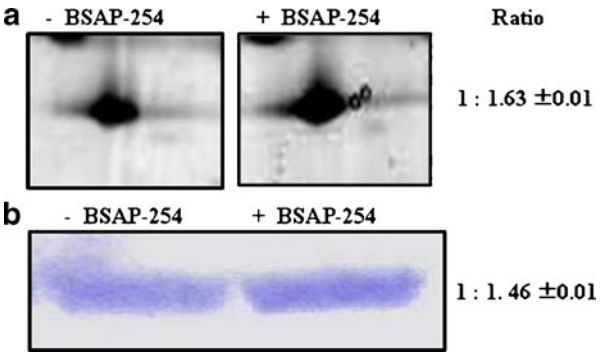

Fig. 2 Western bolt analysis of the upregulated HSP60 (GroEL) in *B. cereus* cultured in medium containing BSAP-254. **a** 2-DE gel spot (no. 84), **b** western blot analysis by GroEL antibody

The lysate of *B. cereus* cultured with BSAP-254 was analyzed by 2-DE using a nonlinear pH gradient of 3–10. Fourteen spots were consistently up- or downregulated (Fig. 1). Of these, one protein was upregulated and the rest were downregulated. Six of the 14 spots were identified by MALDI TOF/TOF MS/MS sequence analysis (Table 2). The upregulated protein was identified as heat shock protein (HSP60) (spot 84), while the other five spots were downregulated proteins that were identified as hypothetical protein PAU_01189 (spot 8), arginine deiminase (spot 55), molecular chaperone Dnak (spot 94), pyruvate kinase (spot 98), and alkyl hydroperoxide reductase C22 (spot 180). To confirm the expression level of the upregulated protein HSP60, western blot analysis was conducted using GroEL antibody (Sigma-Aldrich, St. Louis, MO, USA). The expression level of protein HSP60 analyzed by western blot corresponded to the results obtained by 2-DE (Fig. 2).

Upregulated heat shock protein HSP60, which matched chaperonin GroEL of *B. cereus*, is a specific modulator of the Controlling Inverted Repeat of Chaperone Expression regulon [24]. Classical heat shock proteins (HSPs) such as DnaK, GroEL, and ClpP play roles in protein folding, assembly, and repair, as well as the prevention of aggregation under stress and nonstress conditions. In HSPs of *B. cereus*, these proteins are induced by heat and ethanol. Specifically, GroEL, DnaK, DnaJ, ClpC, ClpP, ClxP, and FtsH are increased after exposure to ethanol [25]. The induction of these proteins by heat or chemical exposure is required for survival of *B. cereus*. For these reasons, the increment of GroEL in *B. cereus* treated with BSAP-254 will also be influenced by stress response.

In conclusion, the cells of *B. cereus* exposed to BSAP-254 isolated from *B. subtilis* SC-8 were decreased more than 50%, which was reduced from 10^8 to 10^4 within 30 min. The heat shock protein HSP60, which is generally expressed after a sudden increase in the ambient temperature, was increased in *B. cereus* exposed to BSAP-254.

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