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Combined effects of ultra-high hydrostatic pressure and mild heat on the inactivation of *Bacillus subtilis*

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ABSTRACT

Effects of combined ultra-high hydrostatic pressure and mild heat (HPMH) treatments on the food-borne microorganism *Bacillus subtilis* were evaluated in this study. The *B. subtilis* culture (counted spores less than 2 log [CFU/mL]) was subjected to 100–500 MPa at 40 °C–60 °C for 15 min at pH 7.0. Treatment with HPMH increased membrane permeability by 10%–89% as determined by the uptake of propidium iodide. Changes in membrane lipids, proteins and DNA were detected in regions 3000 to 2800 cm^{-1} and 1300 to 900 cm^{-1} by Fourier transform–infrared spectroscopy (FT-IR). The membrane phospholipid molecules changed from a liquid crystalline state to a gel state, with a decrease in membrane fluidity. HPMH decreased the α-helix content (about 8–22%) while increased the random coil content (about 5–19%) of the cellular protein, which resulted in protein denaturation. Flow cytometry results indicated that HPMH treatment at 60 °C caused 63% damage to the esterase activity of the cells more than HPMH treatment at 40 °C. All of these results revealed the mechanism of HPMH, which is essential for the successful application of high hydrostatic pressure in neutral-pH food processing.

1. Introduction

Thermal processing has been commonly applied to inactivate pathogenic and spoilage microorganisms to maintain food safety and increase shelf life, respectively. However, thermal processes can damage texture and alter the flavour and colour of food. Nonthermal methods can eliminate or minimize this degradation of food quality.

Ultra-high hydrostatic pressure (UHHP) is a nonthermal technique that can maintain the natural qualities of food (Nguyen et al., 2010) as a result of its limited effects on covalent bonds (Yang, Jiang, Wang, Zhao, & Sun, 2009). However, in a neutral-pH environment, more than 600 MPa of pressure is required to eliminate pressure-resistant bacteria (Chen, 2007a). Thus, hurdle technologies that make use of mild heat (Akhtar, Paredes-Sabja, Torres, & Sarker, 2009; Zimmermann, Schaffner, & Aragão, 2013), high-intensity ultrasound (Lee, Heinz, & Knorr, 2003) or carbon dioxide (Watanabe et al., 2005) have been used in combination with UHHP to inactivate the pathogens and spores in foods with a neutral pH.

Bacteria are sensitive to temperature under pressurization. Studies have shown that some bacterial cells are resistant to UHHP at temperatures of 20 °C–35 °C but become sensitive to pressurisation at higher temperatures (Alpas, Kalchayanand, Bozoglu, & Ray, 2000). Previous studies reported that the optimum process parameters for a 6-log-cycle reduction of *Bacillus subtilis* were 479 MPa at 46 °C for 14 min (Gao & Jiang, 2005). However, under such process parameters the efficiencies of *B. subtilis* reduction in milk buffer and food matrix displayed some differences. Soybean protein, sucrose and pH significantly affected the reduction of *B. subtilis* (Gao, Ju, Qiu, & Jiang, 2007). In addition, the inactivation of bacterial cells (Alpas et al., 2000) and spores (Paredes-Sabja, Gonzalez, Sarker, & Torres, 2007) increased as the pH decreased under pressurisation. The composition of nutrients in the food system may affect the inactivation of microbial cells; for example, milk may exert a greater protective effect against inactivation by pressure than does water (Aouadhi et al., 2013).

San Martín, Barbosa-Cánovas & Swanson (2002) showed that even very high pressure levels (1000 MPa) at ambient temperature do not effectively inactivate bacterial spores. However, for some bacteria, effective inactivation can be achieved by combining UHHP

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with temperatures greater than 40 °C (Ju, Gao, Yao, & Qian, 2008; Kalchayanand, Dunne, Sikes, & Ray, 2004). Previous studies have concluded that a combination of pressure and moderate heat is always required to effectively inactivate spores of *Bacillus* species such as *Bacillus anthracis* (Cléry-Barraud, Gauberg, Masson, & Vidal, 2004) and *B. subtilis* (Gao, Ju, & Jiang, 2006; Nguyen Thi Minh, Dantigny, Perrier-Cornet, & Gervais, 2010).

B. subtilis is one of the most pressure-resistant *Bacilli* (Gao et al., 2006). Recently, the contamination of food products with *B. subtilis* has been shown to underlie food-borne diseases in humans (From, Pukall, Schumann, Hormazábal, & Granumh, 2005). Although the primary target for UHHP in bacterial cells is believed to be the cytoplasmic membrane (Klotz, Mañas, & Mackey, 2010), the underlying mechanism by which microorganisms are inactivated by UHHP and mild heat is still not fully understood.

The objective of this study was to investigate the effects of ultra-high hydrostatic pressure and mild heat (HPMH) in environments with a neutral pH by testing its effect on the food-borne microorganism *B. subtilis* in terms of membrane damage, the denaturation of protein and nucleic acid and metabolic activity. A full understanding of this combined mechanism is essential for the successful application of UHHP in the food-processing field.

2. Materials and methods

2.1. Bacterial strain and growth conditions

B. subtilis ATCC 6633 was preserved in our laboratory and cultured in Oxoid's trypticase soy broth. The cultures were examined for the presence of spores by heating the cell suspension at 80 °C for 10 min. The cell suspensions were diluted in ten-fold series in phosphate-buffered saline solution (PBS; pH 7.0). Selected dilutions of cell suspensions were plated on tryptic soy agar, following incubation at 37 °C for 3 days, the number of the colonies were counted. The experiments were performed at least in triplicates. The concentration of the spores in all cultures used in the experiments was less than 10^2 CFU/ml (Shen, Urrutia Benet, Brul, & Knorr, 2005). The cells were harvested by centrifugation at 6,000 *g* at 4 °C for 15 min, and the pellets were resuspended in 0.05 mol/L of phosphate-buffered saline solution (PBS; pH 7.0). The final concentration was 10^8–10^9 CFU/mL. The bacterial suspension was then stored at 4 °C until the HPMH treatment within 1 h.

2.2. HPMH processing

Approximately 3 mL of bacterial suspension was placed in Corning tubes (Corning Incorporated, Corning, NY) and sealed after the removal of air bubbles. The samples were pressurised with a high-pressure unit (FPG5740, Stansted Fluid Power Co, UK) with 1,2-propanediol as a pressure-transmitting medium. The pressure level, time and temperature were computer-controlled. The temperature of the medium in the pressure vessel was measured by K-type thermocouples during pressurisation. The vessel water jacket temperature was also controlled to obtain the desired temperature at the end of the process. After depressurisation, the samples were cooled in an ice bath and stored at 4 °C for up to 6 h before enumeration.

The cell cultures were pressurised at 100, 200, 300, 400 or 500 MPa for 15 min at 40 °C, 50 °C or 60 °C. The time was fixed at 15 min because times exceeding this value have no industrial feasibility (Serment-Moreno, Fuentes, Barbosa-Cánovas, Torres, & Welti-Chanes, 2015). All HPMH experiments were performed in triplicate on separate days.

2.3. Determination of viable counts

Immediately after the HPMH treatment, the surviving bacteria were serially diluted in PBS and plated on tryptic soy agar to determine the viable cell counts. After aerobic incubation at 37 °C for 48 h, the number of surviving cells was enumerated. Each count was calculated as the mean of three dishes for each dilution. The inactivation effect was expressed as Log (N_0/N), where N_0 was the initial count of the untreated sample and N was the corresponding viable number of cells after HPMH treatment.

2.4. Determination of substances that absorb ultraviolet light

Absorption of ultraviolet light (UV) was carried out as described by Hong and Pyun (2001). After exposure to HPMH, the cells were centrifuged at 10,000 *g* for 10 min. The upper supernatant was removed, and the UV absorption was measured at a wavelength of 260 nm with a spectrophotometer (model UV-2450, Shimadzu, Japan). The measurements were made in triplicate and were corrected to account for absorbance by the medium used for HPMH treatment.

2.5. Determination of propidium iodide uptake by cells

The uptake of propidium iodide (PI) by cells was performed according to the method of Klotz et al. (2010). To test whether the HPMH treatment caused membrane damage, the cells were stained with 3 μmol/L of a DNA-binding PI probe before treatment. Stock solutions of PI (Sigma) were prepared in sterile distilled water to a final concentration of 0.3 mmol/L. After exposure to HPMH as described above, the cells were incubated in the dark at 37 °C for 10 min. The samples were then centrifuged and washed twice in 0.1 mol/L of PBS at pH 7.2. Fluorescence was measured by a spectrofluorophotometer (model F-7000, Hitachi, Japan) using an excitation wavelength of 495 nm and an emission wavelength of 615 nm. The fluorescence values obtained for the untreated cells were subtracted from the experimental values. This procedure was performed in triplicate.

The fluorescence of cells stained with PI after heating at 90 °C for 10 min was set as 100%, and the HPMH-treated cell membrane permeabilisation was expressed as the percentage of heat-treated cells.

2.6. Fourier transform–infrared spectral measurements

The Fourier transform–infrared (FT-IR) measurement method of Al-Qadiri, Al-Alami, Al-Holy, and Rasco (2008) was used in this study with slight modifications. Untreated cells and HPMH-treated cells were washed twice and resuspended in sterile water. Suspension aliquots (200-μL) were transferred onto a ZnSe plate and dried at room temperature to produce a transparent film. The films were then directly analysed by FT-IR spectroscopy. All of the spectra were collected with a Nicolet Nexus 470 FT-IR spectrometer (Thermo Electron Corp, Waltham, MA). Attenuated total reflection (ATR) spectra were recorded from 4000 to 500 cm^{-1} at a resolution of 4 cm^{-1}. Sixty-four interferograms were averaged for each spectrum. Omnic software (Thermo Electron Inc, San Jose, CA) was used to analyse the ATR spectra.

The region of 1700 to 1600 cm^{-1} in the FT-IR spectra mainly represented amide I, which indicated the secondary structure of proteins. A quantitative estimation of the secondary structure was made through second derivative calculation and Fourier self-deconvolution curve-fitting analysis obtained with the use of Omnic software (Omnic 8.2, Thermo Electron Corp, Waltham, MA). Curve-fitting was conducted with Peakfit software(Peakfit 4.12,