Inactivation and morphological damage of *Vibrio parahaemolyticus* treated with high hydrostatic pressure

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**A B S T R A C T**

The effects of high hydrostatic pressure (HHP) treatments on *Vibrio parahaemolyticus* cells were investigated using viability counting, scanning and transmission electron microscopy, and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in order to determine optimal inactivation conditions and further understand the mechanisms of microorganism inactivation under HHP. The results showed that 300-MPa treatment for 10 min could extensively inactivate *V. parahaemolyticus*, with the number of viable bacteria decreasing from $10^9$ CFU/mL to no viable bacteria. Damage to the cell wall, cell membrane, and cytoplasmic components by HHP treatments can be observed on scanning as well as transmission electron microscopy images. SDS-PAGE results showed that the protein bands differed between HHP untreated and treated *V. parahaemolyticus*, whereas HHP decreased protein content and caused partial protein degradation. Therefore, our results indicate that HHP can be applied to inactivate *V. parahaemolyticus* by inducing morphological changes in internal and external structures in the cell, as well as by causing cell membrane damage, cell wall rupture, and membrane protein degradation.

**1. Introduction**

High hydrostatic pressure (HHP) is an emerging non-thermal technology that can achieve food safety via heat pasteurization while meeting consumer demand for fresher-tasting minimally processed foods. One of the principal advantages of the HHP process is an expanded shelf life and improved food safety due to the inactivation of pathogenic microbial populations. This technology has been approved by the Food and Drug Administration (FDA) and the US Department of Agriculture (USDA) and can be applied in food processing procedures. The National Advisory Committee on Microbiological Criteria for Foods (NACMCF) has therefore recommended that pasteurization be re-defined, and HPP is listed as a type of supplementary non-thermal pasteurization (Barbosa-Cánovas & Juliano, 2008). Several studies have shown that HHP treatment causes a number of changes in the cell morphology, cell wall, thermotropic phase in cell membrane lipids, ribosome dissociation, and biochemical reactions, and it results in the loss of genetic function in the responsible microorganisms. These observations are all proposed as possible reasons and mechanisms contributing to microbial inactivation due to HHP treatment (Rendueles et al., 2011). Apparently, no damage is caused to any cellular structure or function of the microorganisms; cell death occurs due to damage to multiple parts in the cell. When the accumulated damage exceeds the cell’s ability to repair, death occurs. Under several circumstances, the damaged cell can recover if post-treatment conditions are favorable (Follonier, Panke, & Zinn, 2012).

Electron microscopy offers the attractive possibility of identifying HHP-induced morphological and structural changes in microorganisms. In a transmission electron microscopy (TEM) study conducted by Yang et al. (2012), cell membrane damage, cell wall rupture, and chromosome DNA degradation was found in foodborne pathogens isolated from HHP-treated raw milk. HHP treatment resulted in morphological changes in internal and external structures of *Escherichia coli* and *Staphylococcus aureus*. Scanning electron microscopy (SEM) examination of *S. aureus* and *E. coli* O157:H7 cell, revealed that pressure treatment at 200 MPa and 400 MPa caused denaturation of membrane-bound proteins and induced phase transition of the membrane lipid bilayer (Pilavtepe-Çelik, Balaban, Alpas, & Yousef, 2008). In another study, *Saccharomyces cerevisiae* cell in apple juice were treated with HHP at 600 MPa for 7 min. Scanning electron microscopy
showed the degree of damage to the cell membrane-perforation and release of the cell wall; scars were also observed on the surface pressurized cells (Marx, Moody, & Bermúdez-Aguirre, 2011).

*Vibrio paraahaemolyticus* is an enteric pathogen that is widely distributed in marine and estuarine environments. This pathogen is frequently isolated from shellfish and can cause acute gastroenteritis characterized by diarrhea, headache, vomiting, nausea, and low fever after consumption of raw or partially cooked seafood (Calik, Morrissey, Reno, & An, 2002). Several recent outbreaks of *V. paraahaemolyticus* associated with oysters have heightened concerns about the safety of raw shellfish consumption in the USA, UK, and Australia (Kural, Shearer, Kingsley, & Chen, 2008). However, distinctive effects of HHP on *V. paraahaemolyticus* cell membrane integrity and ultrastructure have not been thoroughly characterized. The objectives of this study were to evaluate morphological damage and cellular death in HHP-treated *V. paraahaemolyticus* cells. We determined bacterial viable counts, morphology, and membrane protein changes by using different HHP treatments.

2. Materials and methods

2.1. Bacterial culture preparation

*V. paraahaemolyticus* BCRC 10806 were obtained from Bioresource Collection and Research Center, Hsinchu, Taiwan. The strain was cultured in tryptic soy broth (Sigma Aldrich, France) supplemented with 2.5% NaCl at 37°C for 16 h until it reached stationary phase. The cell concentration in the final culture was generally approximately \(1 \times 10^8\) CFU/mL. The resulting cell suspensions were aseptically transferred to sterile polyethylene bags (internal length, 13 mm, and width, 6 mm; Yizuo Co., LTD., Taiwan) in 8-mL portions, vacuum packaged, and the bags were heat-sealed immediately. The samples were kept in an ice bath until HHP treatments.

2.2. HHP treatment

HHP treatments were performed using a laboratory high-pressure processing system with a 2-L vessel (HHP, L2-600/2; Tianjin Sai Mei Int. Trade Co., Ltd, Tianjin, China), that was capable of operating at up to 600 MPa. The pressure come-up rate was approximately 45 MPa/s, and the pressure release time was less than 10 s. The pressure transmission fluid used in this study was water, and pressurization times reported excluded the time for pressure increase and release time. Pressure levels and pressurization times were set manually. The bags containing bacteria were subjected to the pressure treatment in triplicate at 100, 150, 200, 250 or 300 Mpa, with a holding time of 10 min at room temperature (25°C). A control bag was held in an ice bath at atmospheric pressure (0.1 Mpa). After processing, samples were placed in an ice bath and immediately processed for cell viability counting, electron microscopic observation, and whole-cell protein pattern analyses.

2.3. Enumeration of surviving cells

After the pressure treatment, both treated and untreated bacterial suspensions were 10-fold serially diluted in 0.1% sterile peptone solution (Becton Dickinson, Cockeysville, Md.). Subsequently, 1 mL of these dilutions were assessed by the pour plate method by using trypticase soy agar (TSA) media at 37°C for 24 h. Plates containing 30–300 colonies were selected for counting. Results are expressed as CFU/mL, and the lethality rate was calculated as the percentage difference between colony counts of the untreated and treated samples divided by the untreated ones. All data are presented as averages ± standard deviation of 3 independent experiments.

2.4. SEM analysis

*V. paraahaemolyticus* cells were prepared from pressure-treated and untreated cell suspensions via centrifugation at 10,000× g for 10 min and then washed twice in 0.1 M phosphate buffer (pH 7.4). Cell pellets were then resuspended in 1 mL of 0.1 M phosphate buffer. Suspended bacterial cells were filtered (0.22-μm MF Millipore, GSWP; Millipore Corp., Billerica, MA, USA) and fixed on a membrane by using 10 mL of 1.0% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). Fixatives were left in contact with the cells overnight at 4°C. Membranes were subsequently transferred to glass vials and subjected to three 10-min washes in buffer, and then post-fixed for 1 h in 1% osmium tetroxide. Next, membranes were rinsed twice in buffer, for 10 min each time. Membranes were then dehydrated through a series of 10-mL ethanol solutions (10, 20, 30, 40, 50, 60, 70, 80, 90, 95, 100 and 100% ethanol), for 15 min in each solution. Samples were then soaked in isopentyl acetate before critical point drying in CO₂ medium with a critical point dryer (Hitachi HCP-2). Filters were then attached to large scanning electron microscope stubs by using double-sided sticky tape and then coated with gold–palladium. Sample observation was performed under a scanning electron microscope (Hitachi S4700) operating at 15 kV voltage, and photomicrographs were obtained.

2.5. TEM analysis

Bacterial samples were exposed to the same conditions as mentioned above for SEM analysis. Cell pellets were prepared from untreated and pressure treated cell suspensions by centrifugation at 3000× g for 10 min at 4°C and washed once with 50 mL of sterile distilled water. Cell pellets were transferred to sterile centrifuge tube (1.5 mL) and resuspended in 1 mL of 0.1 M phosphate buffer at pH 7.3. After the suspension was centrifuged, the pellet was embedded in 2% agarose. The agarose was cut into 1 mm³ pieces and postfixed for 1 h in 4% osmium tetroxide (OsO₄) in phosphate buffer, then the samples were rinsed in distilled water and stained for 1 h in 1% aqueous uranyl acetate. After dehydration through an ascending series of ethanol solutions (50, 70, 85, 90, 95, and 100%), cells in agar were transferred to propylene oxide, then were infiltrated and embedded in spurr’s resin, cut into sections with a diamond knife, and stained with uranyl acetate and lead citrate prior to examination under a transmission electron microscope (JEM-1400, Japan Electronics Co., Ltd.) at 60 kV.

2.6. Membrane protein electrophoretic analysis

Membrane protein electrophoretic analysis was used to assess the extent of protein injuries caused by HHP treatments. Proteins from each bacterial cell was extracted using B-PER protein extraction reagents (Thermo Fisher, Scientific Inc., IL, USA), according to the manufacturer’s instructions. Briefly, bacterial cells were pelleted by centrifugation at 5000× g for 10 min. Next, 4 mL of B-PER Reagent (containing 8 μ of lysozyme [50 mg/ml] and 8 μ of DNase I [2500 U/ml]) per gram of the cell pellet was added, and the suspension was repeatedly passed through a pipette until it was homogeneous. The mixture was then incubated for 15 min at room temperature. Protein extracts from the supernatant were obtained by centrifugation at 15,000× g for 5 min. Protein concentration were estimated using the dye-binding method of Bradford (1976) with Bio-Rad dye reagent concentrate (500-0006; Bio-Rad). Protein extracts were analyzed using 12% sodium dodecyl sulfate
polyacrylamide gel electrophoresis (SDS-PAGE), and the protein bands on the gel were visualized with Coomassie Brilliant Blue R-250 staining.

3. Results

3.1. HHP treatment and bacterial survival

Effects of HHP treatment on the viability of V. parahaemolyticus cells were determined using colony enumeration by the direct plating method. When the same volume and cell concentration of both the untreated and pressurized cell suspensions were spread on TSA plates and incubated at 37 °C for 24 h, the colonies from the untreated samples were larger in number as well as size than those from the pressure-treated sample. In addition, the viable colonies of cells treated at 250 MPa/10 min were smaller than that of untreated ones. Cell viability was measured directly after pressure treatment and followed similar patterns for 5 min and 10 min holding times (Fig. 1). V. parahaemolyticus was extensively inactivated by a pressure treatment at 300 MPa for 10 min, with the number of viable bacteria decreasing from 10⁸ CFU/ml to no viable cells. Cell viability was not significantly affected at 100 MPa treatments for 5 min or 10 min. However, the lethality significantly increased exponentially with increasing pressure. At 250 MPa treatment for 5 min, the bacterial population decreased by 3.9 log cycles, whereas at the same pressure treatment for 10 min, the same population decreased by 5.9 log cycles Fig. 2.

3.2. SEM and TEM after exposure to HHP

V. parahaemolyticus cells were collected before and after exposure to HHP treatments. SEM showed that envelope and intracellular damage of V. parahaemolyticus cells was apparent after exposure to 300 MPa compared to the untreated cells. The HHP-treated cells showed a progressive increase in the number of pimples and swellings with increased pressure treatment, and led to some cells being crushed and others shattered. No bacterial cell growth was observed in samples pressurized at 300 MPa for 10 min. TEM images are shown in Fig. 3; untreated cells exhibited intact cell walls, cell membranes, uniform cell cytoplasm, and electron-transparent regions of nucleoids that displayed a typical rod or comma shape. However, application of HHP leads to morphological changes in internal and external structures. The most distinct changes observed in the cells with the application of HHP were the enlargement of electron-transparent regions in the cytoplasm and the appearance of some clumps of aggregated cytoplasmic material. HHP-treatment at 300 MPa for 10 min resulted in cellular changes, which included an irregular shape, expanded nucleoid regions, breakdown of the peptidoglycan layer, rupture of the cell wall, and partial loss of the cell envelope.

3.3. Effect of HHP on membrane protein

Effect of pressure on whole-cell protein of V. parahaemolyticus cells is shown in Fig. 4. The cells were harvested after hyperbaric exposure, and fractions of bacterial membranes were extracted and analyzed by SDS-PAGE; variations in the number and degree of brightness of the protein bands in the gel were observed. After HHP treatment, bacterial cell walls, cell membranes, some protein hydrogen bonds as well as covalent bonds were broken, and the intact protein chain was destroyed. Therefore, some protein fragments were lost after electrophoresis. Protein bands from high-pressure-treated V. parahaemolyticus cells were not as apparent as the untreated ones, even though the concentration of cells in both samples was similar. After pressure treatment at over 150 MPa, the bacterial cell population decreased, and associated membrane protein content was drastically modified at both 100 MPa and 300 MPa; treated cells presented different membrane protein patterns. Membrane extracts from cells treated at 200, 250 or 300 MPa showed a major protein band (40 kDa) appearing in a similar quantity as in the controls, and 2 proteins (130 kDa and 140 kDa) that were observed with the controls seemed to disappear with the pressure treatment. In addition, a portion of the protein extracted from cells treated with 200, 250 and 300 MPa appeared on the gel, but their concentrations were significantly lower than the controls and 100 MPa treated cells. The protein content in membrane fractions was drastically diminished and seemed to decrease with increased pressure treatment; however, there was no significant difference between controls and 100 MPa treated cells.

4. Discussion

The potential of high-pressure treatment for inactivation of food pathogens while preserving most of the sensory, nutritional, and functional properties of the treated products is of great interest in the food industry. High-pressure treatment has been proposed as an alternative to classic heat treatment to preserve foodstuffs. The development of HHP technology depends primarily on its capacity to eradicate microorganisms in foodstuffs. Research on HHP technology is still underway, and many concerning microbial inactivation must be fully studied and understood. Each microorganism has a different response to HHP treatments, depending on a number of factors. In order to ensure the microbial quality of the product, the correct processing conditions must be selected to completely inactivate all microbial cells without any possibility of recovery or compromising the physical (Mújica-Paz, Valdez-Fragoso, Samson, Welti-Chanes, & Torres, 2011). Several recent outbreaks of V. parahaemolyticus associated with shellfish have heightened concerns about the safety of raw seafood consumption. To control V. parahaemolyticus infections, the Interstate Shellfish Sanitation Conference (ISSC) proposed post-harvest treatment of shellfish by using interventions such as pasteurization. The standard set by the ISSC is a 5-log reduction of V. parahaemolyticus levels with an endpoint of non-detectable at the level of 10 CFU/g (Cook, 2003). High-pressure processing has been commercially used in the USA for several years to facilitate the shucking of raw oysters. The additional advantage of this technology is that it can inactivate V. parahaemolyticus and Vibrio vulnificus in oysters without compromising their sensory attributes (He, Adams,
Farkas, & Morrissey, 2002; Lopez-Caballero, Perez-Mateos, Montero, & Borderías, 2000). Kural et al. (2008) reported that to achieve a 5-log reduction of *V. parahaemolyticus* in live oysters, the pressure treatment must be at least 350 MPa for 2 min at temperatures between 1 °C and 35 °C, or 300 MPa for 2 min at 40 °C. Styles, Hoover, and Farkas (1991) reported that treatment at 170 MPa for 10 min and 30 min eliminated *V. parahaemolyticus* (10⁶ CFU/mL) in clam juice and phosphate buffer, respectively. Berlin, Herson, Hicks, and Hoover (1999) showed that treatment at 200 MPa for 10 min at 25 °C achieved greater than 6-log reductions of *V. parahaemolyticus* in homogenized raw oysters. Although numerous studies have reported inactivation of *V. parahaemolyticus* in oysters by HPP treatment under various conditions, no study has been conducted to identify the mechanisms for cellular damage of *V. parahaemolyticus* by HHP treatment.

Effects of HHP on microorganism morphology have been documented for a long time (Hoover, Metrick, Papineau, Farkas, & Knorr, 1989). The electron micrographs showed various levels of resistance to pressure, and some changes in the cell morphology or intracellular enzymes activity after pressure treatments were dependent upon the genera (Mackey, Forestiere, Isaacs, Stenning, & Brooker, 1994; Simpson & Gilmour, 1997). In the present study, electron microscopy revealed that pressure treatment induced morphological changes in of *V. parahaemolyticus* cells. These observations suggest that cellular structures could be targets of HHP treatment. In addition, SEM and TEM examination showed that 300 MPa treatments could induce some cell disruptions. Similar results have been obtained with *Salmonella* spp., *E. coli*, *Shigella* and *S. aureus* in other studies (Yang et al., 2012). TEM indicates that high pressure induces cell envelope and intracellular damage compared to the control. In a previous study involving SEM, similar effects of high pressure on *Listeria monocytogenes* membranes were observed (Ritz, Tholozan, Federighi, & Pilet, 2001). Results from another SEM study revealed that pressure treatment caused bud scars on the

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**Fig. 2.** SEM micrographs of *V. parahaemolyticus* cells. (a), (b) And (c), untreated cells; (d), (e) And (f), cells treated with 300 MPa of pressure at 25 °C for 10 min.

**Fig. 3.** TEM micrographs of *V. parahaemolyticus* cells. (a), (b) And (c), untreated cells; (d), (e) And (f), cells treated with 300 MPa of pressure at 25 °C for 10 min.
surfaces of cells and that membrane integrity was lost in most of the cell population. These observations suggest that the cellular wall or membrane could be a target of high-pressure treatment, but intracellular damage should not be overlooked. Intracellular regions of low density observed in V. parahaemolyticus (Fig. 3) are similar to those observed by Garcia-Gonzalez et al. (2010) in pressure-treated L. monocytogenes and E. coli. These authors suggest that these low-density regions are caused by transient membrane invaginations under pressure and are subsequently reversed upon pressure release, leaving the low-density regions adjacent to the cell membrane. Interestingly, these low-density regions were not observed in high-pressure-treated Salmonella enterica serovar Thompson (Mackey et al., 1994), suggesting that membrane damage and its relation to cell inactivation may vary according to the species and phase of bacterial growth (Pagán & Mackey, 2000).

Previous studies have also demonstrated that HHP treatment disrupts not only the cell morphology but also membrane proteins, resulting in changes in morphology and modification of the physical characteristics of the microbial cell (Hauben et al., 1997; Hoover et al., 1989). Previous studies have shown disorder of protein composition of the bacterial membrane after HHP treatment in Salmonella typhimurium (Ritz, Freulet, Orange, & Federighi, 2000). In this present study, we confirmed the role of HHP in the inactivation of cell structures and we have characterized a physical target of this treatment. Our results show that hyperbaric exposure drastically alters cell morphology and membrane proteins, leading to inactivation of V. parahaemolyticus. There seemed to be a good relation between the onset of membrane damage and loss of viability in the bacterial strain. Cell viability of V. parahaemolyticus was extensively decreased at 300 MPa, because this treatment caused the cells to rupture. This finding is in contrast with the results of Bertoloni, Bertucco, De Cian, and Parton (2006) and Spilimbergo, Mantooan, Quaranta, and Della Mea (2009), who observed that the initial cellular envelope damage was not lethal for the cells. In these studies, membrane on the basis of release of green fluorescent protein in E. coli cells following high-pressure carbon dioxide treatment at 40 °C and 8.5 MPa. Other authors have claimed that bacteria are not mechanically destroyed by pressure treatments. Our results demonstrated increasing changes in cell morphology with increasing pressure treatment. However, membrane disruption was not observed in experiments of Butz et al. (1986), even at the highest pressure treatments. Notable changes in cell aspects after a pressure treatment, as demonstrated by SEM probably result in important changes in cell volumes measured at the highest pressure. The cell membrane generally appears to be one of the targets of high-pressure treatment and results in some cell permeabilization (Macdonald, 1984). When the membrane is extensively permeabilized, it may cause cellular damage at varying levels.

Protein denaturation has been suggested as the main mechanism for bacterial inactivation by HHP treatment (Hayakawa, Kanno, Tomita, & Fujio, 1994; Hoover et al., 1989). The mechanism of bacterial inactivation by HHP may differ, depending on the pressure applied. HHP causes inhibition of protein synthesis and reversible protein denaturation at 50 MPa – 100 MPa, membrane damage at 200 MPa, and irreversible protein denaturation at ≥300 MPa (Hauben, Wuytack, Soontjens, & Michiels, 1996, 1997; Hoover et al., 1989). The irreversible denaturation of protein at pressure ≥300 MPa corresponds to the range of pressure sufficient to inactivate most vegetative bacterial cells (Lado & Yousef, 2002). Pressure application leads to the disruption of weak bonds, causing unfolding of proteins. Upon release of pressure, proteins refold in different configurations (Ray, Kalchayanand, Dunn, & Sikes, 2001). HHP also leads to the inactivation of bacterial enzymes through protein denaturation (Simpson & Gilmour, 1997). A study on the effects of high-pressure treatment on inactivation kinetics and proton efflux showed that high-pressure treatment caused increased membrane permeability, partial inactivation of FoF1 ATPase, and impairment of acid efflux mechanisms (Wouters et al., 1998). High-pressure treatment decreases the metabolic activity and membrane potential of L. monocytogenes (Ritz et al., 2001). Whereas in S. enterica serovar Typhimurium, high-pressure treatment damaged the outer membrane more than the cytoplasmic membrane, and some of the membrane proteins disappeared (Ritz et al., 2001). It was reported that differences in susceptibility of E. coli to pressure-induced membrane damage may be due to the different protein components of the membrane. The cell membrane is often considered as the first site of damage in pressure-injured bacteria, and sub-lethal damage or membrane damage accompanied by changes in membrane fluidity as a result of HHP treatment could facilitate the access of NaCl to the cytoplasm of bacteria. At the same time, the cytoplasm and other intracellular substances may outflow, suggesting that the injured survivors became sensitive to NaCl after pressurization, even for the NaCl-tolerant S. aureus. The TEM images also showed that the membrane and cell wall were damaged after HHP treatment. HHP treatment can cause alterations of membrane-bound protein and enzyme functionality (Kato & Hayashi, 1999); these changes can occur after treatment at 350 MPa or 400 MPa as well. Outer and internal membrane protein contents of S. Typhimurium were drastically modified, and some proteins were modified almost entirely. Previous studies on mechanisms of inactivation of microorganisms by HHP focused on perturbation of the cell membrane, damaged cell wall and cell membrane function, inhibition or inactivation of essential enzyme systems, ribosome destruction, and changes in cell morphology. Our results are in agreement with those of some previous studies, and they further confirm that the cell membrane and cell wall of the E. coli and S. aureus were totally damaged following different pressure treatments. This study is the first one using SDS-PAGE to analyze protein alteration before and after V. parahaemolyticus was subjected to pressure-treated membrane. In our survey, SDS-PAGE results showed a difference in the pattern of protein bands between untreated and HHP-treated V. parahaemolyticus cells, as well as differences in protein concentration; these results indicate that the bacterial protein was damaged during pressure treatment. This could be one of the most important potential mechanisms of inactivation by HHP.

**Fig. 4.** SDS-PAGE analysis of membrane protein from V. parahaemolyticus cell after pressurization (100–300 MPa).
In conclusion, this study made it possible to investigate various cellular targets for high-pressure treatment of bacterial cells. It has shown that pressurization significantly elicited modifications of morphological characteristics of cells by using electron micrographs. In addition, some physical damage was inflicted, as reflected by the occurrence of buds on the cell surface, and membrane integrity was lost in the latter part of the cellular population. Measurement of a fall in membrane protein potential showed that the morphological damage resulted in partial loss of the cell envelope or cell wall. The effects of high-pressure treatment on cell components such as nucleic acids or ribosomes have been studied, but our data represents the first observations of the effects of high-pressure treatment on bacterial cell wall protein. However, the mechanisms for inactivation of V. parahaemolyticus possibly include cell membrane damage, cell wall rupture and membrane protein degradation. HHP treatments have induced different degrees of damage to different cells types with particular characteristics in each one, but all of them have disrupted the cell wall, releasing cytoplasmic content and altering cell membrane permeability, in addition to causing other biochemical changes that might occur at the same time, therefore, we believe that the results of our study provide valuable information for future studies.

Acknowledgements

This research work was supported by the Ministry of Economic Affairs, 101-EC-17-A-03-04-0719, Taiwan, Republic of China.

References


