Note

Effects of High-Pressure Carbonation on Intracellular ATP and NADH Levels and DNA Damage in *Escherichia coli* Cells

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In order to understand the microbial inactivation mechanism of high-pressure carbonation (HPC), we examined the changes in the activity of the respiratory chain and DNA damage in *Escherichia coli* cells. HPC was performed under 1–6 MPa at 30°C for 1 min. The increase in CO₂ pressure decreased the number of viable cells of *E. coli*, intracellular ATP, and intracellular NADH, and increased the number of apurinic/aprimidinic sites. These results indicate that HPC has a detrimental effect on the functioning of the respiratory chain in *E. coli* and induces DNA damage, which could result in the death of the bacterial cells.

Key words : Carbonation / Intracellular ATP / Intracellular NADH / *Escherichia coli*.

Temperature and pressure are the most important factors that affect the growth of microorganisms (Zhang et al., 2006). Currently, thermal processing is the most widely used food pasteurization method. However, an extra high temperature during the process may severely damage the organoleptic, nutritional, and physico-chemical properties of heat-sensitive food products (Garcia-Gonzalez et al., 2007). As a result, owing to the rise in consumer demand for good quality food, superior sensory perception of food, high safety standards, and extended shelf life, the efforts to reduce damage to these properties should be made. Thus, many alternative non-thermal treatments such as those involving high hydrostatic pressure and pulsed electrical fields have been extensively developed during the past 20 years (Garcia-Gonzalez et al., 2007; Jeyamkondan et al., 1999; Espachs-Barroso et al., 2003; Devlieghere et al., 2004).

Hydrostatic pressure from 100 to 1000 MPa is required to inactivate bacteria (Balasubramaniam et al., 2008). However, in the presence of CO₂, it is possible to lower the pressure requirement to 5–35 MPa (Garcia-Gonzalez et al., 2007). As a result, there is a growing interest in understanding the effects of high-

pressure carbonation (HPC) treatments on the inactivation of microbial activity in liquid food products such as orange juice, carrot juice, coconut water, tomato sauce, and egg mixtures (Arreola et al., 1991; Park et al., 2002; Damar and Balaban, 2005; Parton et al., 2003; Haas et al., 1989). A predictive model for HPC inactivation of microorganisms was also studied (Buzrul, 2009).

Hong and Pyun (2001) proposed that increasing the pressure would enable CO₂ diffusion through cellular membranes and its accumulation within the cell. Cytoplasmic CO₂ forms carbonic acid (H₂CO₃), which dissociates into bicarbonate (HCO₃[−]), carbonate (CO₃^{2−}), and hydrogen (H⁺) ionic species according to the following chemical equilibrium:

The resultant rise in cytoplasmic proton concentration could lead to the simultaneous entry of the CO₂ molecules into the cytoplasm. Eventually, the intracellular pH (pH_i) of the microbial cell would decrease to the value of the extracellular pH due to the enhanced dissociation of carbonic acid upon pressurization (Spilimbergo et al., 2005). Under such an unfavorable acidic cytoplasmic environment, microbial growth and metabolism

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are inhibited (Wu et al., 2007).

The mechanism of the effect of low-pressure carbonation of 1 MPa on the heat inactivation of *Escherichia coli* was previously investigated according to physiological damage analysis (Klangpetch et al., 2011). However, the correlation of elevating CO₂ pressure and the damage in *E. coli* cell remains unclear. We hypothesized that there exists a mechanism to inactivate bacteria subjected to even relatively mild pressure carbonation (1–6 MPa). In order to understand this mechanism, *E. coli* was used as a model organism. Most of the earlier studies have focused on physical factors such as disruption of cells, modification of the cell membrane, and extraction of cellular components from the HPC-treated cells (>7 MPa) (Damar and Balaban, 2006). However, this study focuses on the role of physiological factors within the respiratory chain, namely, the electron transport system and H⁺-ATPase system, in maintaining the cytoplasmic pH of a bacterium. Moreover, it has been proposed that hyperactivation of the electron transport system stimulates superoxide formation, which is extremely toxic (Kohanski et al., 2007; Wei and Lee, 2002) and readily damages cellular protein and DNA (Farr and Kogoma, 1991). Therefore, we evaluated the extent of the DNA damage after HPC treatment.

E. coli NBRC 3301 was obtained from the National Institute of Technology and Evaluation (Chiba, Japan). The cells were precultured in 7 mL of tryptic soy broth (TSB; Difco, Detroit, MI) at 30°C for 12 h, and 100 µL from the resulting culture was inoculated into 7 mL of freshly prepared TSB. The cells in this culture were allowed to reach the late logarithmic growth phase. Cells were harvested and washed three times by centrifugation at $2,000 \times g$ at 4°C for 10 min in 0.9% w/v sodium chloride solution, and the pellet was resuspended in the solution to yield a final cell concentration of 10^8 – 10^9 colony forming units (CFU/mL). This cell suspension was subjected to the inactivation treatment described below.

HPC was performed by dissolving CO₂ into the cell suspension at the pressure of 1–6 MPa. In the first step, the bacterial cell suspension was introduced into a CO₂-dissolving vessel, using sample pump I. CO₂ gas was then introduced into the vessel at a pressure of 1–6 MPa and dissolved, with stirring, into the cell suspension to the point of saturation at 4°C for 15 min. In the second step, the cell suspension was introduced into the residence column maintained at 30°C in a water-bath using sample pump II. The cell suspension was withdrawn via a pressure control valve after subjecting it to 1–6 MPa CO₂ pressure for 1 min (FIG. 1). The CO₂ concentration in the cell suspension was kept saturated at each treatment pressure. Saturation of CO₂ in the cell

FIG. 1. Schematic diagram of the apparatus used for HPC.

suspension was determined by the method described by Shimoda et al. (2002).

Appropriate serial dilutions of each sample were prepared with a 0.9% sodium chloride solution; 0.1 mL of the suspension was plated onto a tryptic soy agar (TSA; Difco) plate, and the number of viable cells was counted after incubation at 30°C for 24 h. The viable count (CFU/mL) was expressed as the mean $\pm$ standard deviation for 3 independent experiments.

The amount of ATP was measured using a CheckLite™ 250 Plus kit following the manufacturer's instructions (Kikkoman, Chiba, Japan). Briefly, 0.1 mL of the *E. coli* cell suspension was mixed with 0.1 mL of the ATP releasing agent. After 20 s, 0.1 mL of luciferin-luciferase reagent solution was added to the mixture, and luminescence was measured immediately with a Lumitester (C-110; Kikkoman). To eliminate extracellular ATP, a pretreatment was carried out with 1 mL of the sample solution that was mixed with 0.1 mL of the CheckLite™ ATP Eliminating Kit solution. Thirty minutes later, 0.1 mL of the aliquot was subjected to the bioluminescence assay described above using a CheckLite™ 250 Plus Kit. Relative light units (RLU), representing ATP, were used to calculate the ATP concentration by plotting the RLU value on the calibration setting of 10^{-12} – 10^{-7} M of the ATP concentration.

Yellow MTT; 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Nacalai Tesque, Kyoto, Japan) is reduced to purple formazan by a dehydrogenase (NADH→NAD⁺) in the respiratory system of living cells. Therefore, NADH_{in} consumption can be determined by measuring the amount of produced formazan (Mosmann, 1983).

To measure the NADH_{in} concentration, we used a modified version of the method described by Mosmann (1983). MTT stock solution was prepared by dissolving it in PBS buffer (pH 7.0) at a concentration of 5 mg/mL. Five hundred µL of this solution was added to 500

μL of the microbial cell suspension. The mixture was incubated at 37°C for 4 h. Dimethyl sulfoxide (Nacalai Tesque) was added to the mixture and mixed thoroughly to dissolve the dark blue crystals. After the mixture was left for a few minutes at room temperature to ensure complete dissolution, the absorbance was recorded at 490 nm using a spectrophotometer (UV-1700; Shimadzu, Kyoto, Japan). A standard curve was obtained by plotting the NADH concentrations (0.02–1.5 mM) versus the absorbances at 490 nm.

We estimated the extent of the DNA damage by measuring the number of AP (apurinic/apyrimidinic) sites of the previously extracted *E. coli* DNA by using the DNA Damage Quantification Kit (Dojindo Molecular Technologies) according to the manufacturer's protocol. The number of AP sites in the genomic DNA was determined using the calibration setting of 0–40 AP sites/100,000 bp of ARP (Aldehyde Reactive Probe)-DNA standard solution.

The effect of HPC on the log number of viable *E. coli* cells is shown in FIG. 2. Under the conditions of CO_2 saturation with the pressure ranging from 1 to 6 MPa at 30°C during 1 min of the treatment time, it appeared that the number of viable *E. coli* cells remarkably decreased with the increase in pressure. Therefore, it could be concluded that even at an appropriate growth temperature, the microbial inactivation increased with the increase in the pressure. The inactivation effect was attributed to the increasing concentration of the dissolved CO_2 , leading to a rise in CO_2 concentration in the cell upon pressurization (Shimoda et al., 2001; Wu et al., 2007). Hence, the HPC may indeed be the preferred method for a relatively mild pressure treatment, which is effective for microbial inactivation. However, our results were inconsistent with earlier find-

FIG. 2. Effect of HPC treatment (1–6 MPa) on the log number of viable cells of *E. coli*. HPC was carried out at 30°C for 1 min. Data are expressed as the mean $\pm$ standard deviation from 3 independent experiments.

FIG. 3. Effect of HPC treatment (1–6 MPa) on ATP_{in} and NADH_{in} in *E. coli* cells. HPC was carried out at 30°C for 1 min. Data are expressed as the percentage per viable cell and log (percentage per viable cell). Data are expressed as the mean $\pm$ standard deviation from 3 independent experiments.

ings that have suggested that the *E. coli* cells are not inactivated below 4.5 MPa pressure (Oule et al., 2006); this may be attributed to the inadequate solubility of CO_2 in their study.

Generally, an excess amount of protons in a cell are excreted by proton pumps like the H^+ -ATPase system and the electron transport system (Garcia-Gonzalez et al., 2007). Therefore, both systems are typically influenced when there is a proton excess inside the cell due to HPC. The relative amount of ATP_{in} (calculated as percentage per viable cell) in *E. coli* cells after HPC treatment (1–6 MPa at 30°C for 1 min) is shown in FIG. 3. Here, freshly prepared *E. coli* cells contained a high level of ATP_{in} (10^{-8} M/viable cell), and this ATP concentration was set to 100%. Similar to the inactivation curve illustrated in FIG. 2, log (ATP_{in} percentage per viable cell) also decreased linearly with the increasing CO_2 pressure. Therefore, the inactivation of *E. coli* cells could be at least partly due to the consumption of ATP_{in} that was spent for pumping out the excess protons during HPC.

Loss of the metabolic activity in HPC-treated cells, an indicator of the activity of the electron transport system, was estimated by determining the concentration of NADH_{in} with tetrazolium chloride. Similar to the ATP_{in} measurement, the freshly prepared *E. coli* cells contained a high level of NADH_{in} (1.3×10^{-12} M/viable cell), and this value was set to 100%. The result shows that log NADH_{in} decreased linearly with the increasing CO_2 pressure. Thus, it could be concluded that both ATP_{in} and NADH_{in} decreased with the increase in CO_2 pressure.

FIG. 3 also suggested that HPC could enhance the oxidation of NADH to NAD^+ . This oxidation acts as a

FIG. 4. Effect of HPC (1–6 MPa) on the number of AP sites/100,000 bp of *E. coli* DNA. HPC was carried out at 30°C for 1 min. Data are expressed as the mean $\pm$ standard deviation from 3 independent experiments.

trigger for the formation of the hydroxyl radical, an extremely toxic reactive oxygen species (ROS) responsible for the oxidative damage of DNA (Farr and Kogoma, 1991). Formation of AP sites is one of the major types of DNA damage caused by ROS. These sites are usually created during the course of base excision repair of oxidized, deaminated, or alkylated bases. AP sites are repaired by AP endonucleases during the process of base excision repair (Sun et al., 2001). However, an imbalance in this DNA repair system may cause mutations as well as cell death (Boiteux and Guillet, 2004). Thus, the level of AP sites detected in cells is a good indicator of DNA damage.

As shown in FIG. 4, the AP site number increased with the increasing CO₂ pressure to 28/100,000 bp at 6 MPa. The AP site number of *E. coli* upon HPC at 1 MPa was approximately 6/100,000 bp. Therefore, this result suggests that HPC caused DNA damage in *E. coli* cells.

This study indicates that HPC at relatively mild conditions (1–6 MPa, 30°C, 1 min) has a remarkable inactivating effect on *E. coli* cells. Simultaneously, we showed the dramatic decrease of ATP_{in} and NADH_{in}, and the increase in the number of AP sites in the HPC-treated cells. The damage level was increased with increasing CO₂ pressure. From the results of this study, we propose that carbonation treatment has a detrimental effect on the functioning of the respiratory chain in *E. coli*, i.e., the exhaustion of ATP_{in} and NADH_{in} resulted from excreting the excess H⁺ during HPC and also induces DNA damage, which could lead to the death of the bacterial cells (FIG. 5). As there is no thermal process required, this process might reduce the detrimental effects of antimicrobial treatments on the sensory assessment of freshness, and on the nutritional, and physical properties of food. This treatment also

FIG. 5. Schematic diagram of a possible HPC inactivation mechanism against bacterial cells.

appears promising in various applications in the food industry.

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