Inactivation Kinetics of Lactobacillus plantarum by High Pressure Carbon Dioxide

S.-I. Hong and Y.-R. Pyun

ABSTRACT
Inactivation kinetics of Lactobacillus plantarum by high pressure CO\(_2\) was investigated to understand the mechanism of microbial inactivation. The inactivation rates increased with pressure, temperature and exposure time, and with decreasing pH of media. Microbial inactivation was governed Essentially by penetration of CO\(_2\) into cells and its effectiveness could be improved by the enhanced transfer rate. Microbial reduction of 8 log cycles was observed within 120 minutes under CO\(_2\) pressure of 70 kg/cm\(^2\) at 30°C. We hypothesized that the cell death resulted from the lowered intracellular pH and damage to the cell membrane due to penetration of CO\(_2\). Pressurized CO\(_2\) treatment is a potential nonthermal technology for food preservation.

Key Words: high pressure, CO\(_2\), Lactobacillus plantarum, microbial inactivation, food preservation

INTRODUCTION
NON THERMAL METHODS FOR THE PRESERVATION OF FOODS are under intense research to evaluate their potential as an alternative or complementary process to traditional thermal methods (Mertens and Knorr, 1992; Gould, 1995; Barbosa-Cánovas et al., 1998). Some thermal preservation processes generally cause considerable reductions in nutritive values and drastic changes in sensory properties of foods. Nonthermal processes are expected to induce only minimum quality degradation of foods.

Research is increasing in the use of high pressure carbon dioxide for inactivation of microorganisms and some enzymes. Carbon dioxide under a pressure range of 10s to 100s atm has been reported to inactivate microorganisms and certain enzymes (Haas et al., 1989; Arreola et al., 1991; Chen et al., 1992; Lin et al., 1993, 1994; Ishikawa et al., 1995a, b, 1996; Ballestra et al., 1996; Hong, 1997; Hong et al., 1997a, b). This method conceptually differs from conventional heat sterilization in that the primary action of microbial inactivation is likely prompted by disturbance or damage to the balance of biological systems of the cell (Lin et al., 1993, 1994). Principally, pressurized CO\(_2\) not only inactivates effectively both airborne and exposed surface bacteria, but also penetrates easily into porous materials to reduce microbes inside the food. Published reports have demonstrated the feasibility of high pressure CO\(_2\) treatment as one of the alternative technologies for food sterilization. However, the mechanism for cell inactivation is not clear. With proper understanding of the mechanism of high pressure CO\(_2\) inactivation, some of limitations to practical use may potentially be overcome.

Lactic acid bacteria (LAB) are fermentative microorganisms capable of growth over a wide pH range in the presence of organic acids. Among LAB, most Lactobacillus species found on fermenting vegetables and dairy products can tolerate low pH and maintain a pH difference between the cytoplasm and the environment by their unique pH homeostasis (Padan et al., 1981; Kashket, 1987; McDonald et al., 1990; Hutkins and Nannen, 1993). Especially, homofermentative Lactobacillus plantarum strains which generally proliferate at the late stage of natural mixed fermentation of the vegetables reportedly are important in overacidification (Pederson and Albury, 1961, 1969; Mheen and Kwon, 1984). We chose L. plantarum as a typical acid tolerant bacterial cell to treat with CO\(_2\) under pressure, because further studies on representative microbial species were needed for a better understanding of its lethal effects. The microbial inactivation by high pressure CO\(_2\) was studied in terms of operating parameters and environmental conditions. Our objective was to investigate the mechanism for cell inactivation by high pressure CO\(_2\) and examine the possibility of its application to fermentative food preservation.

MATERIALS & METHODS

Cultivation of microorganism
Lactobacillus plantarum KFRI 815 isolated from the late stages of kimchi fermentation was obtained from the Dept. of Biotechnology, Korea Food Research Institute and used in the experiments. Stock culture was maintained on LBS agar (BBL Microbiol. Systems) slants at 0°C. The LAB was cultivated in 70 mL of MRS broth (DIFCO Lab.) in a 150 mL flask at 37°C for 12 h, and the final culture growth generally ranged from 2.4 × 10\(^8\) to 6.2 × 10\(^8\) CFU/mL. In some experiments, the cells incubated in MRS broth were centrifuged and suspended in a given buffer solution (0.1 M acetate, pH 4.5; 0.1 M phosphate, pH 7.0) or sterile distilled water (pH 6.0) of the same volume in an attempt to assess the effects of medium pH on inactivation. The experiments were also extended to cell cultures that were incubated at different temperatures (20, 30, 37°C) for various incubation times (12, 24, 50h) in order to evaluate the effects of environmental conditions of cell growth. The cultures were freshly prepared by the same procedures.

Apparatus
A cylindrical pressure vessel with internal volume of 140 mL (G7 Science Co., Korea) was used for CO\(_2\) pressurization. A chromel-alumel needle type thermocouple (Shane Corp., Korea) was placed in a water bath to agitate the samples, the cultures were freshly prepared by the same procedures.

Procedure
At the beginning of an experiment, 70 mL of L. plantarum culture in MRS broth or cell suspension in given buffers was placed in the pressure vessel. The vessel was tightly closed and immersed in a water bath at constant temperatures (20°C to 40°C), and then
cells were agitated by a magnetic stirrer at 250 rpm. When the temperature was equilibrated and all tubing connections were secured, commercially available CO₂ (N₂ in some experiments) was injected from gas cylinder into the vessel to reach desired pressures (50 to 80 kg/cm²). After the cells were exposed to CO₂ for a designated time, the pressure was released at the controlled rates. It took about 2 min for our system with the line filter to release the pressure of 70 kg/cm². The sudden pressure discharge did not have any notable influence in microbial reduction was previously reported (Arreola et al., 1991; Hong et al., 1997a). The sample was immediately removed from the vessel and, if necessary, diluted with 0.1% peptone (DIF-CO Lab.) solution. The viable cell counts were measured by plating 0.1 mL of the samples on MRS agar medium and incubating the plates at 37°C for 2 days. The microbial survival rate was expressed by the ratio of the viable cell counts after CO₂ treatment (N) to initial cell counts (N₀). Results are presented as averages and standard deviations of multiple samples (n=5). Statistical significance of mean values was examined by one-way analysis of variance at p<0.05.

Observation of cell structures

Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) observations were performed on L. plantarum cells treated at 30°C under 70 kg/cm² of CO₂ for 60 min. The untreated and treated cells were placed on an inorganic membrane (Anodisc 25, Whatman Lab.) and fixed with 0.2 M phosphate buffered saline containing 5% paraformaldehyde and 5% glutaraldehyde, dehydrated in ethanol and dried. Samples were observed with a SEM (JSM-5410LV, JEOL, Japan). The prefixed cells were fixed again with 0.2 M phosphate buffered saline containing 2% OsO₄. The fixed specimens were embedded in Epon after dehydration in ethanol. Thin sections were cut with a diamond knife and mounted to observe with a TEM (JEM-1010, JEOL, Japan).

RESULTS & DISCUSSION

Inactivation as influenced by operating parameters

The major operating parameters influencing microbial inactivation efficiency of the high pressure CO₂ treatment were pressure, temperature, and exposure time. These factors affected not only microbial inactivation by the characteristics of CO₂ mass transfer, but also biological activities of the microbial cells. The microbial inactivation by high pressure CO₂ was governed essentially by penetration of CO₂ into the cells, and its effectiveness could be improved by the enhanced transfer rate. In this respect, the inactivation was accelerated with increasing pressure of CO₂ ranging from 50 to 80 kg/cm² at 30°C (Fig. 2). It took about 150 min to reduce the cells by 5 log cycles at 50 kg/cm², while it took less than 50 min at 70 kg/cm². Principally, pressure controlled both the solubilization rate of CO₂ and its solubility in a suspending medium (Hong, 1997). Consequently, higher pressure enhanced CO₂ solubilization to facilitate its contact with the cells. When CO₂ penetrated into the cells and its concentration inside the cells reached a critical level, it would exert lethal effects on the cells to disturb or unbalance the biological systems.

The microbial inactivation under a constant pressure of CO₂ is dependent upon temperature. Not only pressure but also temperature were closely related to the characteristics of CO₂ mass transfer. Probably, higher temperatures stimulated the diffusivity of CO₂, and could also increase the fluidity of cell membrane to make its penetration easy. Inactivation of L. plantarum cells was affected by temperature (Fig. 3). Under a constant CO₂ pressure of 70 kg/cm², the inactivation rate increased with increasing temperature up to 30°C, but unexpectedly decreased at 40°C. Less effective inactivation at 40°C rather than 30°C might be attributed to the change of CO₂ solubility (or density) in a suspending medium by temperature variations occur in the region near the critical point of the solvent (Williams, 1981; Rizvi et al., 1986). As the temperature increases within this region, the density of the solvent decreases quite rapidly. Considering that CO₂ has the critical point of 31.1°C and 7.38 MPa, its solubility was expected to decline when temperature increased from 30°C to 40°C at a pressure of 70 kg/cm².

Inactivation as influenced by environmental factors

The inactivation rates during high pressure CO₂ treatment were also affected by other factors including water content, pH, and constituents of suspending media, the culturing conditions and ages of cell growth, and the species of microorganisms. Among these factors, the pH and water content of the suspension in which the cells were treated with high pressure CO₂ were very important, because...
they could influence the cellular resistance to CO₂ penetration. Previous studies on the sterilization of dry foods indicated that water was essential for CO₂ to inactivate the microorganisms and that CO₂ under pressure would not be applicable to dry substances (Taniguchi et al., 1987; Haas et al., 1989). The reason why wet cells are more accessible to high pressure CO₂ treatment is probably a direct result of swollen cell walls and membranes due to the presence of water. Thus the cell walls and membranes were expanded to become more penetrable by CO₂ (Lin et al., 1993). Also, water is required for the hydration of CO₂ to form carbonic acid that could be attributed to the pH changes in suspension. With respect to the antimicrobial effects of CO₂, a lower pH inhibited microbial growth and diminished microbial resistance to inactivation (Daniels et al., 1985; Dixon and Kell, 1989; Hong and Pyun, 1997). When microorganisms are in acidic environments, acids enter the cells in the undissociated form and then dissociate. This causes acidification of the cytoplasm and reduction of the pH gradient across the membrane, resulting in inhibition of nutrient transport. In order to maintain pH homeostasis, the cells expel protons from the cytoplasm by H⁺-ATPase at the expense of ATP. Therefore, lowered pH may contribute to growth inhibition and diminishing resistance because of increased energy consumption to maintain pH homeostasis by the proton motive force (Hunter and Segel, 1973; Kashket, 1987; McDonald et al., 1990).

The inactivation rate was affected by initial environmental pH of the cell suspension during high pressure CO₂ treatment (Fig. 4). Since the acidic environmental conditions apparently facilitated the penetration of CO₂ into the cells, the microbial inactivation increased with lowering pH of the cell suspension. About 25 min in acetate buffer (pH 4.5), 35 min in sterile distilled water (pH 6.0), and 60 min in phosphate buffer (pH 7.0) were required to reduce the cells by 5 log cycles under CO₂ pressure of 70 kg/cm² at 30°C. Although the cell suspensions in MRS broth and 0.1 M acetate buffer had the same initial pH 4.5, the inactivation rate of L. plantarum cells in the acetate buffer solution was much higher than in MRS broth. The lipid and fat component contained in MRS broth possibly helped increase the resistance of the cells to high pressure CO₂ treatment. Lin et al. (1994) had observed that Listeria monocytogenes were more recalcitrant to CO₂ treatment when grown or suspended in a media that contained fat or oil. They proposed that the presence of fat in growth and suspending media probably resisted CO₂ penetration into cells by changing the surface structure of the cell walls. However, the inactivation was insignificant when environmental pH of the cell suspension was not lowered sufficiently by addition of 0.5 M sodium bicarbonate. The bicarbonate would react with carbonic acid made from the dissolved CO₂ and function as an intensive alkaline buffer (pH 8.0) in the medium, resulting in not lowering the pH of the cell suspension during CO₂ treatment. Thus the pH reduction due to dissolved CO₂ was important in the microbial inactivation by high pressure CO₂. However, a combination of the lowered pH that was produced by 0.1 M citrate buffer without CO₂ and high pressure N₂ treatment (70 kg/cm² at 30°C) was obviously not effective in microbial inactivation (data not shown). In that experiment, pressurization with N₂ was used as a pressure control to distinguish any unique effect of CO₂. Actually, the citrate buffer had the pH value of 3.5 even lower than the reduced pH values of 4.3 and 4.0 in nutrient broth due to the dissolved CO₂ under pressure of 70.3 and 210.9 kg/cm² at 35°C (Lin et al., 1993). Little reduction of viable cells possibly resulted from the low solubility of N₂ gas in water and low extraction power due to its extremely low critical temperature of -147°C (Lin et al., 1993; Nakamura et al., 1994). Thus, the fact that environmental acidification created by the buffer alone had no lethal effect on the cells exposed to high pressure N₂ indicated the importance of specific characteristics of the pressurized CO₂.

Microbial inactivation varied with environmental conditions of cell growth and age of cells, even with the same species of microorganism. Generally, young cells are more susceptible to sterilization treatments than mature cells, and spores are much more resistant than vegetative cells. As shown (Fig. 5), L. plantarum cells of the late log phase (incubated at 37°C for 12 h) were more sensitive to high pressure CO₂ treatment than those of the stationary phase (incubated at 37°C for 24 h). Upon entry to the stationary phase of growth, bacteria synthesize new protein that protects cells against a variety of adverse conditions including high temperature, oxidative stress, high salt concentrations, and high pressure (Kolter, 1993; say what...).
Stationary phase cells of *L. plantarum* were also relatively protected against inactivation by high pressure CO\(_2\) but the mechanism for this effect was not clear. Additionally, *L. plantarum* cells were more or less resistant to inactivation when they were cultivated at 20°C or 30°C but not at 37°C (Fig. 6). Relative less inactivation of the cells grown at lower temperatures might be afforded by differences in the final pH of the culture broth. The microbial culture incubated at 37°C for 12 h had a final pH of 4.5, whereas pH 5.4 and pH 5.3 were observed at 20°C for 50 h and at 30°C for 24 h, respectively. As mentioned, microbial inactivation increased with lowering pH of the cell suspension. On the contrary, Lin et al. (1993) had observed that *L. monocytogenes* cells cultivated at 10°C were more sensitive to CO\(_2\) treatment than those at 27°C.

**Changes in cell structure**

Structural changes in cells exposed to high pressure CO\(_2\) have been reported in a few studies. Nakamura et al. (1994) observed morphological changes in baker’s yeast before and after CO\(_2\) treatment at 40 atm and 40°C for 5 h. Some deformation that was expressed as the appearance of “holes” and “wrinkles” was recognized in cell surfaces and some cells were found to have completely burst. Although the extent of cells encountering such morphological changes was not confirmed, they proposed that yeast cells, at least some, would be mechanically ruptured by the CO\(_2\) treatment. It was also demonstrated that some *E. coli* cells treated with 5 MPa of CO\(_2\) pressure at 35°C had deformed cell walls (Ballestra et al., 1996). However, more than 25% of the cells exhibited intact walls while the viability was only 1%. Based on the results, Ballestra et al. (1996) suggested that other mechanisms would be involved in the lethal actions of CO\(_2\) under pressure although the observed deformation of walls was the cause of some cell death. Contrary to previous reports, the SEM observation of *L. plantarum* cells treated with CO\(_2\) under pressure of 70 kg/cm\(^2\) at 30°C for 60 min showed intact cell walls without signs of deformation (Fig. 7). The morphological changes in cells exposed to high pressure CO\(_2\) could be variable according to
Inactivation of L. plantarum by High Pressure CO₂

Inactivation mechanism

Carbon dioxide has characteristically high solubility in water as well as lipid depending on pressure and temperature. Furthermore, CO₂ dissolves in aqueous solutions to form carbonic acid, which can contribute to pH changes in suspension media. Although an exact mechanism for the antimicrobial effects of CO₂ is not clear, lowered pH due to the dissolved CO₂ inhibits growth and metabolism of some microorganisms (Jones and Greenfield, 1982; Daniels et al., 1985; Dixon and Kell, 1989), and also appears to attenuate microbial resistance to inactivation (Lin et al., 1993, 1994). The pH changes in water and other aqueous solutions under high pressure CO₂ (Meyssami et al., 1992) were found to be sensitive to its pressures only in a limited range (< 7 MPa). For instance, the pH values in pure water and nutrient broth were lowered to 3.2 and 4.35 under CO₂ pressure of about 6.0 MPa at 32°C (Haas et al., 1989; Meyssami et al., 1992). Nonetheless, environmental acidification by pressurized CO₂ alone would be unlikely to provide a lethal effect on the microbes (Haas et al., 1989; Lin et al., 1993, 1994; Isenschmidt et al., 1995; Hong, 1997). Such effect of the acidified condition on microorganisms is particularly apparent at long exposure times. Some microorganisms, including especially LAB, are well known to have tolerance to acid environments through their pH homeostasis for very long periods (Padan et al., 1981; McDonald et al., 1990; Hutkins and Nannen, 1993). The cytoplasmic pH value is indeed higher than the environmental pH, when the microorganisms are rapidly producing acids or when they are in low pH conditions. In this respect, it is the internal pH rather than the external pH that ultimately has the greatest effect on cellular activity (Kashket, 1987).

Particularly, CO₂ can diffuse through the cellular membrane that consists of lipid bilayer and accumulate within the cells. Under pressure, it is possible that a large number of CO₂ molecules passed through the membrane and then lowered the internal pH enough to exceed the buffer capacity of the cytoplasmic pool and to collapse the pH gradient or the proton motive force across the membrane. The lowering of pH inside the cells may cause inhibition or inactivation of key enzymes involved in essential metabolic and regulatory processes such as glycolysis, amino acids and peptide transport, active transport of ions, and proton translocation (Hutkins and Nannen, 1993). In addition, highly pressurized CO₂ could reportedly extract the vital constituents including phospholipids and hydrophobic compounds from cell walls and membranes, which would lethally disturb or alter balance of the biological systems to cause microbial inactivation without rupture of cell walls (Kamihira et al., 1989; Lin et al., 1993, 1994). However, the extractability of lipid substances by CO₂ seems to be stronger in the supercritical regions.

CONCLUSION

MICROBIAL INACTIVATION BY HIGH PRESSURE CO₂ WAS GOVERNED PRINCIPALLY BY PENETRATION OF CO₂ INTO THE CELLS, AND ITS EFFECTIVENESS COULD BE IMPROVED BY ENHANCED TRANSFER RATES. THE INACTIVATION RATES INCREASED WITH INCREASING EXPOSURE TIME, PRESSURE AND TEMPERATURE, AND WITH DECREASING INITIAL ENVIRONMENTAL pH OF THE CELL SUSPENSIONS. THE INACTIVATION DURING HIGH PRESSURE CO₂ TREATMENT WAS AFFECTED BY OTHER FACTORS INCLUDING CONSTITUENTS OF SUSPENDING MEDIA, CULTURING CONDITIONS, AND AGES OF CELL GROWTH. SIGNIFICANT MODIFICATION IN THE CELL MEMBRANES WITH EXCEPTION OF THE WALLS WAS OBSERVED BY SEM AND TEM. WE PROPOSE THAT CELL DEATH RESULTED FROM THE LOWERED INTRACELLULAR pH AND DAMAGE TO THE CELL MEMBRANE DUE TO PENETRATION OF CO₂ RATHER THAN THE PHYSICAL RUPTURE OF CELL WALLS. RESULTS SHOWED THAT THE HIGH PRESSURE CO₂ TREATMENT COULD BE USED AS AN EFFECTIVE NONTHERMAL TREATMENT FOR FOOD PRESERVATION. FURTHER RESEARCH IS NEEDED TO INVESTIGATE THE EXACT MECHANISM OF BIOLOGICAL INACTIVATION AND TO PROVIDE METHODS FOR IMPROVEMENT AND APPLICATION TO SPECIFIC FOOD SYSTEMS.

REFERENCES


the species of microorganisms with different cell structures of walls and membranes. Regardless of damage to membranes, cells with rigid structure of walls, for example, would maintain apparently their external shape after CO₂ treatment. In fact, the microstructural observation by a TEM of L. plantarum cells treated with CO₂ showed modifications in the cell membrane with possible leakage of cytoplasm (Fig. 8). Some features found in the treated cells included the enlarged periplasmic space between the walls and the cytoplasmic membranes, fractures in membranes, and the empty aspect of the cytoplasm (Fig. 8). Some features found in the treated cells included the enlarged periplasmic space between the walls and the cytoplasmic membranes, fractures in membranes, and the empty aspect of the cytoplasm in which dense blocks were reduced and tended to be located at the cell periphery.

Fig. 8—Transmission electron micrographs of L. plantarum cells untreated (a) and treated (b, c) with 70 kg/cm² of CO₂ pressure at 30°C for 1 h. (×60,000)


Ms received 9/17/98; revised 3/14/99; accepted 3/21/99.

We thank Ms. Kim, K.W. in Electron Microscopy Lab. of SNU for experimental assistance in SEM and TEM observation.