J. Microbiol. Biotechnol. (2007), **17**(4), 616–623

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

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Effects of Inoculum Level and Pressure Pulse on the Inactivation of *Clostridium sporogenes* Spores by Pressure-Assisted Thermal Processing

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Received: September 21, 2006
Accepted: November 28, 2006

Abstract The effects of initial concentration and pulsed pressurization on the inactivation of *Clostridium sporogenes* spores suspended in deionized water were determined during thermal processing (TP; 105°C, 0.1 MPa) and pressure-assisted thermal processing (PATP; 105°C and 700 MPa) treatments for 40 min and 5 min holding times, respectively. Different inoculum levels (10^4, 10^6, and 10^8 CFU/ml) of *C. sporogenes* spores suspended in deionized water were treated at 105°C under 700 MPa with single, double, and triple pulses. Thermally treated samples served as control. No statistical significances ($p>0.05$) were observed among all different inoculum levels during the thermal treatment, whereas the inactivation rates (k_1 and k_2) were decreased with increasing the initial concentrations of *C. sporogenes* spores during the PATP treatments. Double- and triple-pulsed pressurization reduced more effectively the number of *C. sporogenes* spores than single-pulse pressurization. The study shows that the spore clumps formed during the PATP may lead to an increase in pressure-thermal resistance, and multiple-pulsed pressurization can be more effective in inactivating bacterial spores. The results provide an interesting insight on the spore inactivation mechanisms with regard to inoculum level and pulsed pressurization.

Keywords: *Clostridium sporogenes*, spore inactivation, spore clumps, inoculum level, pulsed pressurization, pressure-assisted thermal processing

Since food quality and safety have been in high priority over the last decade, their awareness and concern have led to continuous development in the new and emerging food preservation technologies from traditional thermal processing to ionizing radiation, ohmic heating, pulsed electric field processing, ultrasounds, and high pressure technology [11–13, 19, 26, 32]. The application of high-pressure processing to food preservation is receiving growing attention because of its microbial inactivation and shelf-life extension abilities. High-pressure processing can inactivate vegetative cells, but either high pressure or thermal treatment alone is not capable of inactivating bacterial spores [30]. The combination of high pressure and thermal treatments can be a more realistic approach to ensuring microbiological food safety [28, 29].

Bacterial spores under high pressure firstly induce germination and heat activation, and the germinated spores become more susceptible to high pressure and elevated heat [7, 27, 33]. Many researchers have reported that high pressure at elevated temperature increases the spore inactivation, leading to the leakage of intracellular substances such as metallic ions (Na^+, K^+, Mn^{2+}, Ca^{2+}, and Zn^{2+}), dipicolinic acid (DPA), and α/β small acid-soluble spore proteins (SASPs), the hydrolysis of core and cortex, and the decrease of intracellular pH [8, 20, 34]. Pressure-induced germination is more likely to occur at relatively lower pressure between 200 and 500 MPa [36]. However, the exact mechanisms of spore germination and inactivation have not been clearly understood during pressure-assisted thermal processing (PATP), specifically over 600 MPa.

Spore inactivation is more complicated at high pressure combined with high temperature and also linked to other complex metabolites. High pressure and temperature may cause different types of germination and inactivation, and an incomplete germination in the presence of superdormant spores and hydrophobic clumps, leading to a tailing phenomenon [37]. It is more difficult to differentiate between germinated spores and sublethally injured spores by PATP, some of which may not be inactivated by high pressure and temperature.

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Although it is obvious that PATP is effective in reducing bacterial spores close to a satisfactory sterilization, there still remains a question of whether the highly resistant bacterial spores can be completely inactivated by this processing technology. Therefore, understanding the inactivation behavior of bacterial spores by the PATP is essential for ensuring microbiological food safety. The objectives of this study were to 1) examine the spore inactivation mechanisms associated with inoculum level and pulsed pressurization and 2) investigate the effectiveness of PATP on the inactivation of *Clostridium sporogenes* spores at 105°C and 700 MPa. *Clostridium sporogenes* is a spore-forming anaerobic and Gram-positive spoilage microorganism and commonly used as a surrogate for validating thermal processing in lieu of *C. botulinum* [2].

MATERIALS AND METHODS

Bacterium and Growth Culture Medium

A strain of *Clostridium sporogenes* (ATCC 7955) was purchased from the American Type Culture Collection (Manassas, VA, U.S.A.). *Clostridium sporogenes* was cultivated anaerobically in reinforced clostridial medium (RCM, Oxoid Inc., Ogdensburg, NY, U.S.A.) at 37°C for 24 h. After the second cultivation in RCM, the cultures were used to induce sporulation.

Spore Production

The fresh culture of *C. sporogenes* was spread-plated on reinforced clostridial agar (RCA; Oxoid Inc., Ogdensburg, NY, U.S.A.) containing 10 ppm of $MnSO_4$ (Fisher Scientific, Pittsburgh, PA, U.S.A.) in an anaerobic chamber (Type B, COY Laboratory Products Inc., Grass Lake, MI, U.S.A.) with auto airlock system (5% H_2/95% N_2 mix) and incubated anaerobically at 37°C until more than 95% bright spores were visible under phase-contrast microscopy. Spores were collected by flooding the surface with 9 ml of sterile distilled water and scraping the colonies with a sterile glass spreader. Each spore suspension was washed five times by differential centrifugation, ranging from 2,000 to 8,000 ×*g* for 20 min each at 4°C, sonicated for 10 min (SM275HT, peak power 270W, Crest Ultrasonic, ETL Testing Laboratories, INC., Cortland, NY, U.S.A.), and heated at 80°C for 10 min to destroy any remaining vegetative cells [28]. The spore pellet was resuspended in deionized water to approximately 10^9 CFU/ml and stored at 4°C prior to use.

Sample Preparation

The samples were inoculated at low, medium, and high inoculum levels in deionized water according to the counts of *C. sporogenes* spores as approximately 10^4, 10^6, and 10^8 CFU/ml, respectively. The suspensions were individually packaged in a sterile plastic pouch (5×2.5 cm) made from sterile filter bags (01-002-57; Fisher Scientific, Pittsburgh, PA, U.S.A.), and sealed using an impulse heat sealer (American International Electric, Whittier, CA, U.S.A.). All samples were preheated in a water bath at each initial temperature for different treatments immediately prior to thermal and pressure treatments. The sample suspensions were treated at 105°C thermal and 105°C under 700 MPa treatments for various time intervals.

Thermal Treatment

The samples suspended in deionized water were transferred to custom-fabricated aluminum tubes and heated in a 28-l oil bath at 105°C (Fisher Scientific) for 0, 1, 3, 5, 10, 20, and 40 min holding times. The sample temperature was monitored and recorded using a K-type thermocouple (Omega Engineering, Stamford, CT, U.S.A.) attached to a data logger (IOtech, Cleveland, OH, U.S.A.). The heating time (come-up time) was recorded when the sample temperature reached the target temperature (Table 1). The time required to reach the constant temperature of 105°C was 3.33 min.

Pressure Treatment

Pressure inactivation was carried out using custom-fabricated equipment (PT-1, Avure Technologies, Kent, WA, U.S.A.) available at the Food Safety Engineering Laboratory, Department of Food Science and Technology, Ohio State University. The 100% food grade of propylene glycol (57-55-6, Avatar Corp., University Park, IL, U.S.A.) was used as the pressure transmitting fluid. Depressurization occurred in less than 1 s. The sample temperature and chamber pressure were recorded every second during the entire treatment cycle using a K-type thermocouple sensor (Model KMQSS-04OU-7; Omega Engineering, Stamford, CT, U.S.A.) and pressure transducer (Model 3399 093 006,

Table 1. Typical pressure and temperature conditions used during various stages of thermal and pressure-assisted thermal processing[a].

Process pressure (MPa)	Bath temperature (°C)	Come-up time (min)	Preheating temperature (T_1, °C)	Initial sample temperature (T_2, °C)	Process temperature under pressure (T_3, °C)
0.1	105	3.29±0.06	-[b]	23.80±1.06	104.93±0.29
700	105	0.60±0.02	51.85±0.92	57.13±0.56	105.25±0.38

[a]Mean (n=3) values along with standard deviation.
[b]For thermal processing samples, no preheating was involved.

Tecsis, Frankfurt, Germany). A data acquisition computer equipped with relevant hardware (Daq-Board/2000 16-Bit, 200 kHz PCI card, DBK 81 7-Channel thermocouple expansion card, DBK 203-expansion card; IOtech, Cleveland, OH, U.S.A.) and software (DasyLab 7.00.04; National Instruments Corp., Austin, TX, U.S.A.) was used to record the data. For the single-step pressurization, the samples were subjected to 105°C under 700 MPa for 0, 0.25, 0.5, 1, 2, 3, and 5 min holding times. The time needed to reach the treatment was 0.58 min, which is the come-up time starting at 57°C for initial temperatures, followed by holding at 105°C for final temperatures (Table 1). An external bath surrounding the pressure chamber was maintained at 105°C so that isothermal-process conditions could be maintained throughout the pressure holding time. For the pulsed pressurization, the samples were subjected to 105°C under 700 MPa for one, two, and three pulses at 0.25, 0.50, 0.75, and 1.00 min pressure holding times (Fig. 4). The initial temperature was 72°C and the bath temperature was maintained at 80°C. After high-pressure treatment, samples were immediately cooled in an ice-bath to avoid further inactivation.

Microbiological Analysis

Total inactivated spores were directly determined by pour plating and enrichment methods. Pressure-treated samples were heat-treated in a water bath at 80°C for 10 min to further inactivate germinated spores. The samples (1 ml) were serially (1:10) diluted with 0.1% peptone water and pour-plated in duplicate on TSA. The plates were anaerobically incubated at 32°C for 24 to 48 h. Enrichment method was used to confirm the samples (1:1) of less than 10 colonies. The samples (1 ml) were incubated in TSB (9 ml) for 24 to 48 h, followed by streaking on TSA, and then incubated for 24 to 48 h to examine for visible growth.

Inactivation Kinetics

The kinetic parameters for spore inactivation were analyzed using first-order reaction kinetics and the Weibull distribution model. *D* values were calculated at the initial linear portion of inactivation curves, assuming the logarithmic number of spores is a linear function of treatment time [3–5, 31]:

$$\log\frac{N}{N_0}=-\frac{t}{D} \tag{1}$$

where N and N_0 are the momentary and initial numbers of bacterial spores, respectively.

The Weibull distribution model assumes lethal events as the frequency function and inactivation curves as cumulative function for a continuous random variable [6, 9].

$$\log(N/N_0)=-bt^n \tag{2}$$

where b and n are pressure- and temperature-dependent coefficients, indicating a measure of resistance and a degree of curvilinear, respectively.

The data were also fitted using the Whiting-Buchanan model [35], which describes the biphasic inactivation kinetics of microorganisms:

$$\frac{N}{N_0}=\frac{f(1+e^{-k_1t_{lag}})}{1+e^{k_1(t-t_{lag})}}+\frac{(1-f)(1+e^{-k_2t_{lag}})}{1+e^{k_2(t-t_{lag})}} \tag{3}$$

where k_1 is the inactivation rate at the first fraction of the survival curve, k_2 is the inactivation rate at the second fraction of the survival curve, f is the initial resistant proportion at the first fraction, and t_{lag} is the lag time duration required for the initial inactivation of microorganisms.

Statistical Analysis

The spore inactivation curves were analyzed using Nonlinear Curve Fitting Function of Microcal Origin 7.5 (Microcal Software Inc., Northampton, MA, U.S.A.). Data were analyzed using the Statistical Analysis System software (SAS 8.0, SAS Institute Inc, Cary, NC, U.S.A.). The General Linear Model (GLM) and Fisher's least significant difference (LSD) procedures were used to determine significant differences among inoculum levels and pressure pulses at $p<0.05$. The paired *t*-test was used for the pulsed pressurization comparisons at the same pressure holding times.

RESULTS

The effect of inoculum levels on the inactivation of *C. sporogenes* spores was investigated at thermal alone (105°C, 0.1 MPa) and pressure-assisted thermal (105°C under 700 MPa) treatments as shown in Figs. 1 and 2.

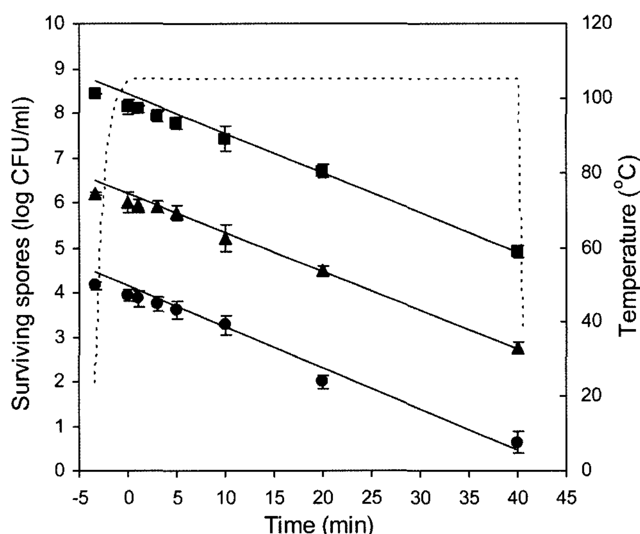

Fig. 1. Effect of thermal treatment (105°C and 0.1 MPa) on the inactivation of *C. sporogenes* spores suspended in deionized water. Temperature profile (·····); high inoculum (■, 2.63×10^8 CFU/ml); medium inoculum (▲, 1.58×10^6 CFU/ml); low inoculum (●, 1.45×10^4 CFU/ml). Thermal treatments had a come-up time of approximately 3.3 min at 105°C.

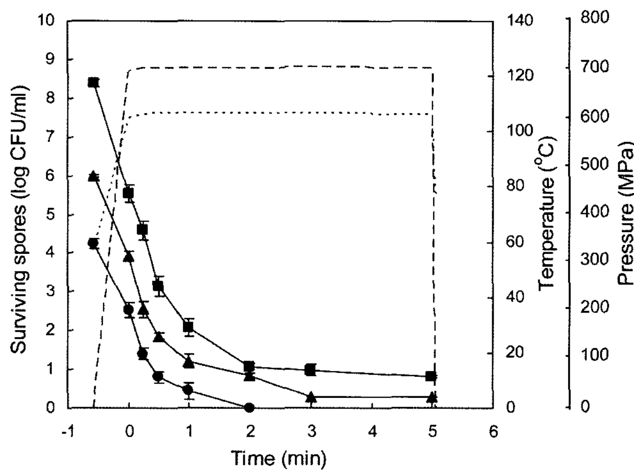

Fig. 2. Effect of pressure-assisted thermal treatment (105°C and 700 MPa) on the inactivation of *C. sporogenes* spores suspended in deionized water.

Temperature profile, (·····); pressure profile, (----); high inoculum (■, 2.40×10⁸ CFU/ml); medium inoculum (▲, 9.66×10⁵ CFU/ml); low inoculum (●, 1.78×10⁴ CFU/ml). PATP had a 0.58 min come-up time.

Compared with the initial populations, the numbers of *C. sporogenes* spores were not significantly reduced by 0.22, 0.21, and 0.28 log CFU/ml during the come-up time at low (4 log CFU/ml), medium (6 log CFU/ml), and high (8 log CFU/ml) inoculum levels, respectively (Fig. 1). *Clostridium sporogenes* spores still survived approximately 1.00, 2.75, and 4.93 log CFU/ml at low, medium, and high inoculum levels, respectively, after 40 min of thermal treatment. In Fig. 2, the numbers of *C. sporogenes* spores treated at 105°C under 700 MPa were significantly reduced by 1.72, 2.08, and 2.84 log CFU/ml at low, medium, and high inoculum levels, respectively, during the come-up time. Biphasic inactivation curves were observed at low, medium, and high inoculum levels during the pressure-assisted thermal treatment, which was described by a fast decline at the first phase and a slight tail at the second phase. The numbers of *C. sporogenes* spores within 1 min pressure holding time were reduced by 3.80, 4.78, and 6.31 log CFU/ml at low, medium, and high initial concentrations, while those after 1 min holding time were reduced by 0.30, 0.91, and 1.28 log CFU/ml at low medium, and high inoculum levels, respectively. Table 2 shows the parametric values estimated from the survival curves of *C. sporogenes* spores at thermal (105°C) and pressure-assisted thermal (105°C under 700 MPa) treatments. No significant differences in *D* value among low, medium, and high inoculum levels were observed at 105°C thermal treatment alone. However, there were significant differences in the scale factor (b) and inactivation rates (k_1 and k_2) among all inoculum levels. The b values were increased with increasing inoculum level. Low inoculum level had the highest inactivation rate (k_1=8.96) at the first portion of survivor curve, followed by 7.63 and 5.98 (1/min) for medium and high inoculum levels. The inactivation curves at thermal treatment alone showed linearity for all different inoculum levels. A similar inactivation pattern was observed for all inoculum levels at 105°C under 700 MPa treatment, showing a noticeable upward concavity (n<1). Regression coefficients (R^2) were larger than 0.92 for the survival curves of *C. sporogenes* spores at all treatments. In order to evaluate spore germination, the counts of inactivated spores after pressure-assisted thermal treatment and germinated spores after pressure-assisted thermal treatment followed by additional heat shock at 80°C for 10 min were observed in deionized water (Fig. 3). The numbers of surviving

Table 2. Kinetic parameters of the linear, the Weibull distribution, and the biphasic models for *C. sporogenes* spores suspended at different inoculum levels at 105°C and 105°C under 700 MPa treatments.

Parameter	Treatment	Inoculum level		
		Low[a]	Medium[b]	High[c]
Linear				
D	105°C	11.86±0.92[x]	12.64±1.22[x]	12.78±0.22[x]
	105°C+700 MPa[d]	0.27±0.01[x]	0.23±0.02[y]	0.26±0.01[xy]
Weibull[e]				
b	105°C+700 MPa	2.04±0.08[z]	2.47±0.15[y]	3.01±0.03[x]
n	105°C+700 MPa	0.35±0.02[xy]	0.29±0.02[y]	0.35±0.01[x]
Biphasic[e]				
f	105°C+700 MPa	0.82±0.02[z]	0.93±0.01[y]	0.98±0.01[x]
k_1	105°C+700 MPa	8.96±0.17[z]	7.63±0.39[y]	5.98±0.21[x]
k_2	105°C+700 MPa	0.64±0.18[z]	0.32±0.06[y]	0.27±0.04[x]

[a]Low inoculums were 1.45×10⁴ and 1.78×10⁴ CFU/ml at 105°C and 105°C under 700 MPa, respectively.
[b]Medium inoculums were 1.58×10⁶ and 9.66×10⁵ CFU/ml at 105°C and 105°C under 700 MPa, respectively.
[c]High inoculums were 2.63×10⁸ and 2.40×10⁸ CFU/ml at 105°C and 105°C under 700 MPa, respectively.
[d]The *D* values were calculated from the linear portion of survival curves.
[e]Data from thermal inactivation were not calculated.
[x–z]Means with different superscripts within a row are significantly different at $p<0.05$.

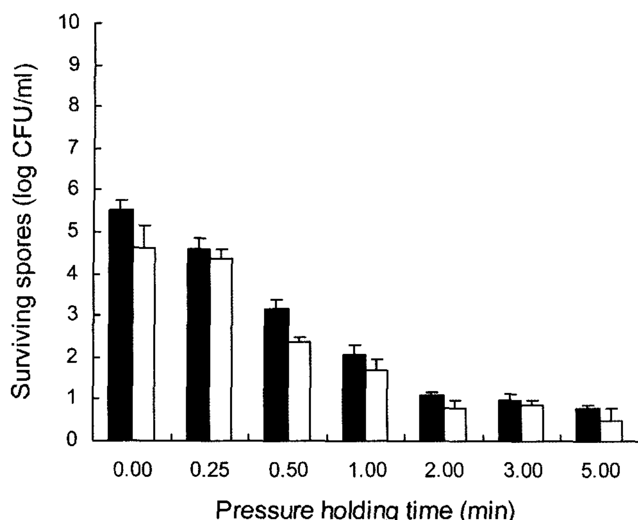

Fig. 3. Effects of pressure-assisted thermal treatment (105°C and 700 MPa) on the inactivation and germination of *C. sporogenes* spores suspended in deionized water.

The number of initial spores was 2.45×10^{8} CFU/ml. Surviving spores after pressure-thermal treatment (■, inactivation); surviving spores after pressure-thermal followed by heat shock (□, germination).

spores after pressure-assisted thermal treatment were not significantly different from those after pressure-assisted thermal treatment followed by additional heat shock in terms of spore inactivation and germination.

The effect of pulsed pressurization in the inactivation of *C. sporogenes* spores was determined at 105°C under 700 MPa. The pressure and temperature profiles were recorded for single-, double-, triple-pulsed pressurizations with 0.25, 0.50, 0.75, and 1.00 min holding times (Fig. 4). Fig. 5 describes the variables of pulse pressurization including process temperature, pressure, multiple pulses (single-, double-, and triple-pulsed pressurizations), and time periods (come-up and pressure holding times). The reduction of *C. sporogenes* spores increased with increasing holding time during the single-pulsed pressurization (Table 3). At the single-pulsed pressurization, *C. sporogenes* spores were reduced by less than 3 log CFU/ml within the 1 min holding time and by more than 3 log CFU/ml after the 1 min holding time. Most reductions occurred at the first come-up and holding times (log S_1/S_3). The reduction ratio of *C. sporogenes* spores gradually decreased from about 2 log to less than 1 log throughout the entire process. During

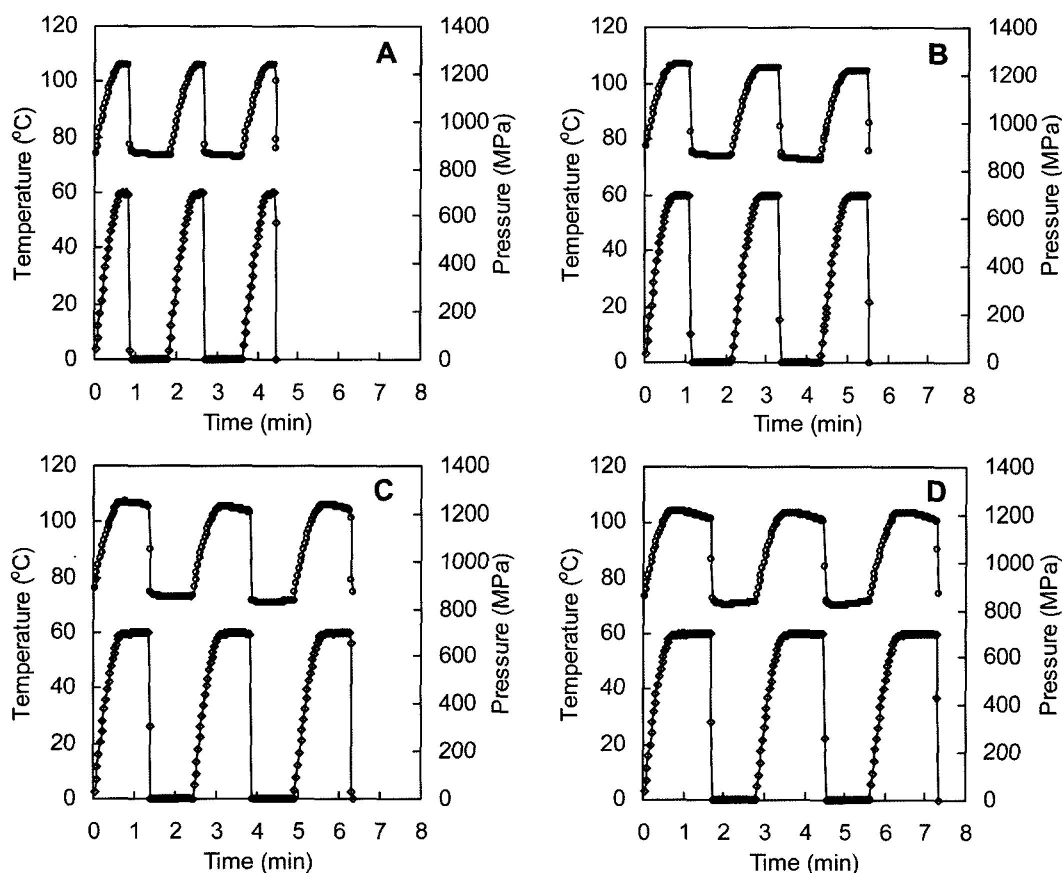

Fig. 4. Pressure and temperature profiles during pulsed pressurization at 0.25 (**A**), 0.50 (**B**), 0.75 (**C**), and 1.00 (**D**) min holding times.

Temperature profile (◇); pressure profile (□).

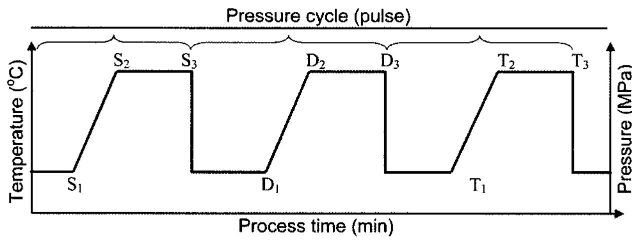

Fig. 5. Variables of pulsed pressurization.
Single-pulse (first come-up, S_1–S_2; 1st hold, S_2–S_3), double-pulse (second come-up, D_1–D_2; 2nd hold, D_2–D_3), and triple-pulse (third come-up, T_1–T_2; 3rd hold, T_2–T_3).

the double-pulsed and the triple-pulsed pressurizations, *C. sporogenes* spores were reduced by less than 1 log CFU/ml at each come-up and hold period with the exception of the second come-up time (log D_1/D_2) by 1.03 log reduction. *Clostridium sporogenes* spores were not detected in enrichment cultures after double-pulsed pressurization with 0.75 and 1.00 min holding time, whereas single-pulsed pressurization showed positive in enrichment cultures after a 5 min holding time. All pressure-pulsed treatments rapidly reduced the numbers of *C. sporogenes* spores in the first 1 min of pressure holding time. The numbers of *C. sporogenes* spores inactivated by double and triple pulses were significantly higher than those by single-pulsed pressurization (Table 4). Compared with the single-pulsed pressurization, the double-pulsed pressurization with 0.50, 1.00, and 1.50 min holding times effectively reduced the numbers of *C. sporogenes* spores by 6.93, 7.28, and 7.67 log, respectively. At 0.75 min holding time, the number of *C. sporogenes* spores was reduced by 6.50 log at single-pulsed pressurization, whereas the corresponding number was 7.53 at triple-pulsed pressurization. The single-pulsed pressurization showed the extended tailing until the 5 min holding time. However, the double- and triple-pulsed pressurizations showed negative in enrichment method at 2.00 and 1.50 min holding times, respectively.

Table 4. Comparisons of pulsed pressurization on the inactivation of *C. sporogenes* spores at 105°C and 700 MPa.

Holding time (min)	Pulsed pressurization		
	Single [log S_1/S_3]	Double [log S_1/D_3]	Triple [log S_1/T_3]
0.50	6.12±0.21	6.93±0.09[a]	-[c]
0.75	6.50±0.09	-	7.53±0.19[a]
1.00	6.56±0.12	7.28±0.18[b]	-
1.50	6.90±0.17	7.67±0.13[b]	ND[d]
2.00	7.21±0.21	ND	-
2.25	7.42±0.05	-	ND
3.00	7.38±0.21	-	ND

[a]Means within a row are significantly different at $P<0.01$.
[b]Means within a row are significantly different at $P<0.05$.
[c]Pulsed-pressurization was not performed.
[d]No survival colony was detected.

DISCUSSION

This study demonstrated the effects of inoculum level and pulsed pressurization on the inactivation of *C. sporogenes* spores suspended in deionized water. Within the ranges of experimental study, the thermal inactivation curves at 105°C followed a first-order reaction kinetics, whereas the pressure-assisted thermal treatment showed biphasic inactivation curves. The inactivation rates of *C. sporogenes* spores at thermal treatment alone were not influenced by different inoculum levels. Compared with the first linear phase, the second linear phase showed a considerable decrease in inactivation rate during the pressure-assisted thermal treatment. These results indicate that high pressure-assisted thermal treatment is mainly responsible for the rapid lethal effect on the inactivation of *C. sporogenes* spores at the initial process time rather than a cumulative effect throughout the entire process [38]. The order on the inactivation rates of *C. sporogenes* spores at the pressure-assisted thermal treatment was low (4 log CFU/ml)>medium (6 log CFU/

Table 3. Effect of pulsed pressurization on the come-up and hold time reduction of *C. sporogenes* spores ($S_1=1.82\times10^8$ and log $S_1/S_2=3.74$).

Hold-time (min)	Log S_2/S_3	Log S_3/D_1	Log D_1/D_2	Log D_2/D_3	Log D_3/T_1	Log T_1/T_2	Log T_2/T_3
0.25	0.79±0.12	0.91±0.26	1.03±0.03	0.45±0.05	0.35±0.16	0.23±0.13	0.02±0.04
0.50	2.38±0.43	0.55±0.13	0.32±0.23	0.29±0.02	0.26±0.23	0.02±0.04	0.73±0.05
0.75	2.75±0.38	0.40±0.04	0.60±0.05	0.21±0.20	0.59±0.36	ND[a]	ND
1.00	2.82±0.34	0.87±0.38	0.40±0.53	0.57±0.23	ND	ND	ND
1.50	3.15±0.21	0.62±0.12	NP[b]	NP	NP	NP	NP
2.00	3.46±0.44	0.59±0.15	NP	NP	NP	NP	NP
2.25	3.68±0.35	0.48±0.13	NP	NP	NP	NP	NP
3.00	3.64±0.10	0.88±0.35	NP	NP	NP	NP	NP
5.00	3.77±0.32	0.75±0.18	NP	NP	NP	NP	NP

[a]Spores were not detected in enrichment cultures.
[b]No pulsed pressurization was conducted over the 1 min holding time.

ml)>high inoculum (8 log CFU/ml) levels at the first and second linear phases. Furukawa *et al.* [17] reported that spore clumps present in the initial suspensions decreased the inactivation rates of *B. subtilis* and *B. licheniformis* spores during PATP treatment. Since the *C. sporogenes* spores used in this study were collected by differential centrifugation, sonication, and microscopic examination to obtain a uniform dispersion with consistent spore size, the different inoculum levels may not contribute to the inactivation rates of *C. sporogenes* spores at the first straight phase of pressure-assisted thermal treatment. The inactivation ratio of *C. sporogenes* spore suspensions increased with the inoculum level decreased at the second linear phase, which is in good agreement with a previous report in which the *D* values calculated from the second straight portion of *B. subtilis* spores were increased from 13 min for the initial concentration of 10^4 CFU/ml to 65 min for 10^9 CFU/ml. These results suggest that no spore clumps were present in the initial spore suspensions and the spore clumps were formed through the pressure-assisted thermal treatment, but not in thermal treatment alone. The fact that spore inactivation increases with decreasing inoculum level is evidence that pressurization causes hydrophobic clumps resulting in the spore tailing phenomenon [16]. Hydrophobic surface properties cause spore aggregation and increase resistantce to high pressure and temperature [15, 22]. Recently, the use of surface active agent and filtration has been proposed to prevent the formation of spore clumps under pressurization [15–17]. According to the *b* values of the Weibull distribution, the resistance of *C. sporogenes* spores at the pressure-assisted thermal treatment was the highest at low, followed by medium and high inoculum levels. The resistance may be resulted from the first linear phase associated with inoculum levels and initial log reductions. The observed low *n* values suggest that the spores are inactivated immediately at the first phase of the survivor curve, and the remaining spores are more likely to be adapted to heat and pressure at the second phase leading to tailing [1, 10, 23]. Therefore, in interpreting the pressure and heat resistance of bacterial spores, kinetic models have been paid more attention, along with other factors such as inoculum level and come-up time reduction.

The magnitude of the inactivation of *C. sporogenes* spores used in this study was more pronounced with increasing pressure pulse and holding time. Compared with the equivalent single-pulsed pressurization, the double- and triple-pulsed pressurizations were more effective in inactivating *C. sporogenes* spore. These results suggest that the pulsed pressure treatments can enhance spore lethality and also avoid extended tailing during the pressure-assisted thermal processing. Furukawa *et al.* [14] suggested that pulsed pressure increases the susceptibility of bacterial spores when compared with continuous pressurization [18, 21, 25]. *Bacillus stearothermophilus* spores were reduced by approximately 6 log at the pulsed pressurization of 600 MPa and 70°C, whereas no comparable reduction was observed at continuous pressurization under 800 MPa and 70°C for 60 min [21]. The inactivation of bacterial spore is influenced by temperature, pressure, process time, and the number of pulses [25]. No significant differences between the numbers of *C. sporogenes* spores inactivated and germinated by single-pulsed pressurization were observed at 105°C and 700 MPa for a 5 min holding time, suggesting that germinated sensitive spores may be mostly initiated by double- and triple-pulsed pressurizations. The effect of pulsed pressurization on spore inactivation is due to pressure-induced germination, which helps to achieve complete inactivation of bacterial spores [24, 25]. The pulsed pressurization may trigger bacterial spores to activate, germinate, increase their sensitivity through complex processes, and then inactivate most germinated spores. Multiple-pulsed pressurization can be a better method in terms of complete inactivation of bacterial spores. By the viewpoint on microbiological food safety, the application of pulsed pressurization to the food industry may be desirable. However, further study is needed to evaluate the effectiveness of pulsed pressurization on spore inactivation, considering processing variables such as pressure come-up, pressure holding, and depressurizing, and also cost-effective ways to implement food safety and quality.

Acknowledgment

The authors gratefully acknowledge the financial support of the Center for Advanced Processing and Packaging Studies (CAPPS).

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