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Inactivation Kinetics of *Listeria innocua* ATCC 33090 at Various Temperature Heating-up and Pressure Building-up Rates

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Abstract The effects of temperature heating-up rate and pressure building-up phase on the inactivation of *Listeria innocua* ATCC 33090 were evaluated in buffered peptone water. The number of *L. innocua* was reduced by 5.57 and 6.52 log CFU/mL during the nonisothermal treatment (the come-up time followed by isothermal process) and the isothermal treatment, respectively, at 60°C. When compared to the isothermal treatment ($0.76<D$ value<0.96), *L. innocua* exposed at the nonisothermal treatment ($1.58<D$ value<2.31) became more resistant to heat. The come-up time reductions in numbers of *L. innocua* significantly increased with increasing the heating rates ($p<0.05$). The pronounced reduction was observed by more than 5 log CFU/mL at 33.2°C/min of temperature heating-rate. The effect of the combined high pressure and thermal processing on the inactivation of *L. innocua* increased with increasing pressure and temperature. At all temperature levels from 40 to 60°C under 700 MPa, *L. innocua* was not detected by enrichment culture (>7 log reduction).

Keywords: high pressure, *Listeria innocua*, kinetics, nonisothermal, isothermal

Introduction

Microbial heat resistance in foods varies widely depending on species, growth conditions, food composition, and other environmental stresses (1, 2). In general, temperature above the optimum growth temperature exerts a stress and lethal effect. However, heating at a sublethal temperature may induce higher thermotolerant microorganisms known as an adaptive mechanism. Many studies have reported on the effect of heat shock on the thermal resistance of microorganisms such as *Escherichia coli*, *Salmonella typhimurium*, *Pseudomonas aeruginosa*, *Clostridium perfringens*, and *Listeria monocytogenes* (3-6). Nonisothermal heating-up phase can affect microbial heat resistance which increases as heated up slowly to a target temperature (5, 7). In traditional thermal processing, it is impossible to achieve desirable isothermal condition because of an unsteady-state heat transfer. Although high pressure processing, which currently has received much attention as an emerging technology for extending shelf-life and improving food safety, provides homogenous and immediate pressure distribution (8-10), the pressure build-up always causes an increase in temperature due to the adiabatic heating (11). Therefore, the come-up time required to reach the isothermal and/or isobaric conditions is an important variable in thermal processing as well as high pressure processing.

Thermal inactivation has been generally described by a first order kinetics under isothermal conditions, assuming that all bacterial cells in a population have the same sensitivity to thermal treatment (12). However, since survival curves do not always follow a first order kinetics, linear model is inadequate to predict nonlinear survival curves, leading to considerable misinterpretation on microbial resistance. Many researchers have used nonlinear models such as biphasic, n^{th} order kinetics, log-logistic, modified Gompertz, and Weibull distribution models, in order to describe microbial inactivation having a shoulder or a tail and both (13-21) but relatively few attempted to predict survival curves at the come-up time. Recently, the thermal inactivation kinetics has been extensively studied with regard to dynamic conditions in order to predict microbial inactivation in real time during processing (15, 22-25). Palou *et al.* (26) reported the effect of the come-up time on the inactivation of *Zygosaccharomyces balii*. Microbial reduction and resistance change during the come-up time may influence kinetic parameters describing heat and pressure resistance. Therefore, it is essential to take the significant effect of the come-up time into account for determining microbial resistance.

To our knowledge, no study has been done on the impact of the come-up time including nonisothermal and nonisobaric phase on the inactivation of microorganism. Nonpathogenic *L. innocua* used in this study is a useful surrogate for foodborne pathogen, *L. monocytogenes*, showing a similar heat resistance (27, 28). Listeriosis is an opportunistic disease, which varies and depends on an individual's health infection of *L. monocytogenes* generally appears as a mild enteric disease in healthy people, while it causes very serious disease to fetuses, newborns, infants, pregnant women, and immune deficient people (29). Foodborne outbreaks of listeriosis have been reported in pasteurized milk (1983), cheese (1985), chocolate milk (1994), and cooked hot dogs (1998) in the USA (30, 31). The Food and Drug Administration (FDA) and Food Safety Inspection Service (FSIS) have regulated the incidence of *L. monocytogenes* under a 'zero-tolerance' plan in precooked foods (32). In spite of the highly thermotolerance of *L. monocytogenes* (33, 34), little attention has been paid to its heat resistance during the come-up time. Therefore, the objective of this study was to

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Table 1. Kinetic parameters of *L. innocua* ATCC 33090 during nonisothermal and isothermal treatments

Parameter	Nonisothermal treatment		Isothermal treatment
	[+Come-up time][1)]	[-Come-up time][2)]	[No Come-up time]
$D^{3)}$	2.26±0.09$^{c5)}$	1.58±0.10^{b}	0.76±0.04^{a}
R^2	0.946	0.936	0.914
$D^{4)}$	2.31±0.09^{c}	1.90±0.07^{b}	0.96±0.03^{a}
R^2	0.960	0.848	0.742
b	0.39±0.05^{a}	1.01±0.20^{b}	2.43±0.17^{c}
n	1.04±0.04^{b}	0.63±0.09^{a}	0.51±0.04^{a}
R^2	0.961	0.960	0.965

[1)]The kinetic parameters were calculated including the come-up time.
[2)]The kinetic parameters were calculated excluding the come-up time.
[3)]The decimal reduction time was calculated at the first linear potion of survival curve.
[4)]The decimal reduction time was calculated at entire processing time.
[5)]Means with different superscript letters (a-c) within a row are significantly different at $p<0.05$.

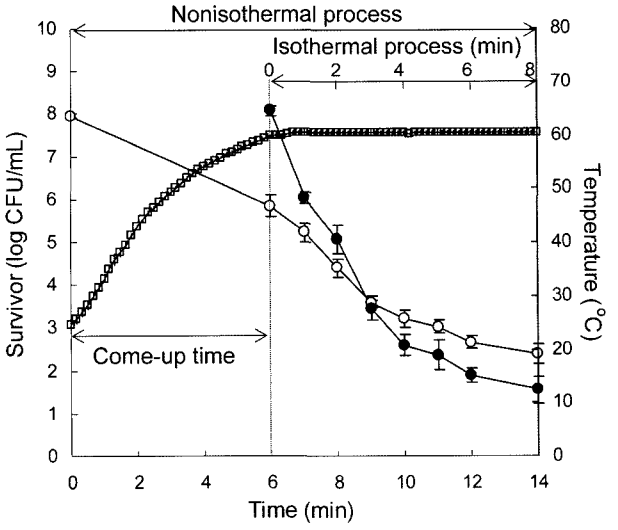

Fig. 1. Nonisothermal and isothermal inactivation curves of *L. innocua* ATCC 33090 suspended in buffered peptone water at 60 °C of target temperature. Nonisothermal ○, Isothermal ●, Temperature Profile □.

determine the effects of thermal heating rate and pressure building-up phase on the inactivation of *L. innocua* in terms of microbial carry-over resistance from nonisothermal phase through isothermal phase.

Materials and Methods

Bacterial culture and media The strain of *L. innocua* ATCC 33090, a non-pathogenic indicator, used *in lieu* of *L. monocytogenes* was purchased from the American Type Culture Collection (Manassas, VA, USA). *L. innocua* was cultivated in trypticase soy broth supplemented with 0.6% yeast extract (TSBYE; Difco, Becton Dickinson, Spark, MD, USA) at 30°C for 20 hr prior to use. After preculture, cells were harvested at 6,500×g for 10 min at 4°C. The cell pellet was resuspended to original volume with sterile buffered peptone water (BPW; peptone, 10.0 g/L; NaCl, 5.0 g/L; Na_2HPO_4, 9.0 g/L; NaH_2PO_4,1.5 g/L, pH 7.2). The harvested cells were diluted to approximately 10^8 CFU/mL in BPW for inoculation.

Experimental design Three experiments were conducted in duplicate with three replicates. In experiment 1, the inactivation of *L. innocua* suspended in BPW (ca. 10^8 CFU/mL) was performed under nonisothermal and isothermal conditions. The nonisothermal treatment included the come-up time immediately followed by isothermal process (Fig. 1). The constant target temperature was maintained at 60°C throughout the whole isothermal treatment. In experiment 2, *L. innocua* suspensions (ca. 10^7 CFU/mL) were heated at 5 different heating-up rates from approximately 25 to 60°C (Table 2). In experiment 3, *L. innocua* suspensions (ca. 10^7 CFU/mL) were subjected to the pressure levels of 300, 500, and 700 MPa at 5 different target temperatures of 40, 45, 50, 55, and 60°C (Table 3)

Heating apparatus Heating rate was varied by adjusting temperature set of a 28-L circulating oil bath (Fisher Scientific, Pittsburgh, PA, USA). A K-type thermocouple (Omega Engineering, Stamford, CT, USA) was located at the geometric center of an uninoculated glass test tube (100 mm length, 13 mm i.d., and 1.0 mm wall thickness). The temperature was monitored with a data logger (IOtech, Cleveland, OH, USA). The thermal heating-up rates were estimated during the come-up time immediately after the temperature reached the target temperature.

Thermal inactivation For the isothermal treatment, a relatively small volume of *L. innocua* suspension (0.1 mL) was inoculated into a large volume of BPW (9.9 mL) preheated to 60.0±0.2°C. Each treatment in glass test tube

Table 2. Effect of temperature-heating rate on the come-up time reduction and decimal reduction of *L. innocua* ATCC 33090

Initial temperature (°C)	Final temperature (°C)	Temperature-heating rate (°C/min)	Come-up time reduction (log N_0/N)	D value (min)
24.7±0.9	60.0±0.5	5.72±0.08$^{a1)}$	2.63±0.22^{a}	2.30±0.19^{d}
25.7±0.4	60.1±0.1	11.47±0.14^{b}	4.11±0.12^{b}	0.73±0.01^{c}
24.6±0.1	60.3±0.4	17.83±0.25^{c}	4.83±0.05^{c}	0.42±0.01^{b}
24.9±0.8	60.0±0.1	23.40±0.85^{d}	5.37±0.16^{d}	0.37±0.01^{ab}
25.3±0.4	59.5±0.8	33.20±0.42^{e}	5.66±0.02^{d}	0.18±0.01^{a}

[1)]Means with different superscript letters (a-e) within a column are significantly different at $p<0.05$.

Table 3. Typical pressure and temperature conditions during the come-up time at different pressure and temperature levels

Process pressure (MPa)	Initial temperature (°C)	Final temperature (°C)	Temperature-heating rate (°C/min)	Compression heating factor (°C/100 MPa)
300	31.65±0.21	39.90±0.42	40.13±0.53	2.68±0.04
	34.24±0.12	44.00±0.81	47.15±1.77	3.14±0.12
	39.17±0.35	50.06±0.62	52.73±0.67	3.52±0.04
	43.10±0.41	54.53±0.81	54.98±1.03	3.67±0.07
	48.12±0.76	59.14±1.36	51.38±1.52	3.43±0.10
500	27.35±0.35	40.10±0.42	35.64±0.10	2.50±0.01
	30.65±0.34	44.85±0.35	39.86±0.01	2.79±0.01
	34.35±0.07	50.05±0.21	44.57±0.20	3.12±0.01
	37.40±0.57	54.95±0.49	49.07±0.10	3.44±0.01
	41.40±0.57	60.01±0.57	52.00±0.01	3.64±0.01
700	14.27±0.62	39.02±0.45	41.79±0.13	3.48±0.01
	19.81±0.42	44.78±0.39	43.17±1.20	3.60±0.10
	25.38±0.60	49.51±0.44	41.60±1.08	3.47±0.09
	27.60±0.57	54.80±0.42	46.89±1.09	3.91±0.09
	31.30±0.28	59.66±0.51	48.57±0.80	4.05±0.07

was held at 60°C for 0, 1, 2, 3, 4, 5, 6, and 8 min holding times as shown in Fig. 1. For the nonisothermal treatment, 10 mL of BPW inoculated with *L. innocua* was heated from 25 to 60°C at different thermal-heating rates (Table 2).

High pressure inactivation High pressure treatments were carried out using custom-fabricated equipment (PT-1; Avure Technologies, Kent, WA, USA). The 100% food grade of propylene glycol (Avatar Corp., University Park, IL, USA) was used as pressure transferring fluid. The decompression time was less than 1 sec. The pressure and temperature were recorded during the entire process.

Microbiological analysis The treated samples were serially (1:10) diluted with 0.1% sterile peptone water. The sample dilutions (0.1 mL) were plated on tryptic soy agar (TSA; Difco Laboratories, Detroit, MI, USA). The agar plates were incubated to determine the populations of *L. innocua* at 30°C for 48 hr. The samples less than 10 colonies were confirmed by enrichment culture in TSBYE for 24 to 48 hr.

Inactivation kinetics The kinetic parameters were estimated using log-linear and the Weibull distribution models. *D*-values were calculated as follows;

$$\log\left(\frac{N}{N_0'}\right)=-\frac{t}{D} \quad (1)$$

where, N_0 is the inoculum level, N_0' is the microbial count, measured immediately after the come-up time, and N is the count after exposure to the thermal or pressure-thermal treatment for a specific time (t).
The Weibull distribution model (17) is given by:

$$\log\left(\frac{N}{N_0'}\right)=-bt^n \quad (2)$$

where, *b* and *n* are the scale and shape factors, respectively.

Statistical analysis The microbial inactivation curves were analyzed using Nonlinear Curve Fitting Function of Microcal Origin® 7.5 (Microcal Software Inc., Northampton, MA, USA). Data were analyzed using the Statistical Analysis System software (SAS Institute, Inc., 1990). The general linear model (GLM) and least significant difference (LSD) procedures were used to compare means. Significant mean differences among treatments or storage times were calculated by Fisher's LSD at $p<0.05$.

Results and Discussion

In experiment 1, the effect of temperature and pressure come-up times on the inactivation *L. innocua* was investigated. The different inactivation patterns of *L. innocua* were observed at nonisothermal and isothermal treatments as shown in Fig. 1. During the thermal treatment, temperature increased from 25 to 60°C for 6 min of the come-up time (heating rate = 5.84°C/min). When compared to the initial population, the number of *L. innocua* was reduced by 2.12 log CFU/mL during the come-up time and further reduced by 3.45 log CFU/mL after the come-up time. After the come-up time, the survival curve followed a first order inactivation kinetics at the first 4 min and showed a slight tailing at the end of isothermal treatment. This observation suggests that there exist a heterogeneous distribution of *L. innocua* population and different resistant to heat (35). At the isothermal treatment directly exposed at the target temperature

without the come-up time, the initial number of *L. innocua* was more rapidly reduced by 6.53 log CFU/mL than that of *L. innocua* exposed to nonisothermal treatment including the come-up time. This observation indicates that *L. innocua* exposed to directly constant temperature (60°C) with negligible come-up time was more sensitive to heat than *L. innocua* reached to 60°C after certain come-up time. After approximately 4 min of isothermal treatment, the tailing was also observed. It suggests that *L. innocua* may be thermally adapted by constant-increasing temperature, leading to increasing heat resistance. Allan *et al.* (3) reported the heat shock response time of *P. aeruginosa* was as short as 1 min at 45°C. The heat resistance of *L. innocua* obtained from the come-up time resulted in different inactivation patterns during the isothermal treatment.

The survival curves of *L. innocua* fitted with linear and Weibull distribution model to determine its heat resistance. Table 1 summarizes the kinetic parameters obtained in different treatment conditions, 1) nonisothermal treatment including the come-up time, 2) nonisothermal treatment excluding the come-up time, and 3) isothermal treatment at the constant temperature (60°C). The Weibull distribution model (R^2>0.96) provided a better fit at all survival curves than linear model (0.74 < R^2 < 0.96). In Table 1, *L. innocua* was least sensitive to heat at the nonisothermal treatment including the come-up time, indicating highest *D* values, followed by the nonisothermal treatment excluding the come-up time and the isothermal treatment. Heat-adapted *L. innocua* cells during the come-up time are more likely to be adapted at additional isothermal treatment. This suggests that the come-up time during thermal processing could affect the interpretation of heat resistant of microorganisms. The *D* values were inversely correlated to the *b* values of the Weibull distribution model. The inactivation patterns varied with treatments, showing upward concavity (n<1) and downward concavity (n>1). The inactivation curve at isothermal treatment showed upward concavity (n=0.51) with a slight tail at the end of process, while that at nonisothermal treatment including the come-up time was close to straight (n=1.04). The fact that the different heat resistance of *L. innocua* varied with treatment conditions implies a potential carryover effect of resistance from the come-up time through isothermal condition. It suggests that microbial inactivation studies need to take the come-up time into consideration when looking into microbial resistance.

In experiment 2, the come-up time reductions in the number of *L. innocua* were examined at different temperature-heating rates as shown in Table 2. The initial numbers of *L. innocua* significantly decreased during the come-up time with increasing temperature-heating rates (p <0.05). As temperature-heating rate increased from 5.72 to 33.20°C /min, the come-up time reductions increased from 2.63 to 5.66 log CFU/mL and the *D* values decreased from 2.30 to 0.18 min. The observation suggests that increasing temperature-heating rate increases heat sensitivity of *L. innocua*. This is good agreement with the report that the slower heating rate induces the higher thermotolerant microorganisms (5). Our results could not clearly explain the relationship between the heat resistance developed during the come-up time and the inactivation during the isothermal treatment. However, the development of thermotolerance obtained from the come-up time may increase heat resistant to successive constant thermal treatment.

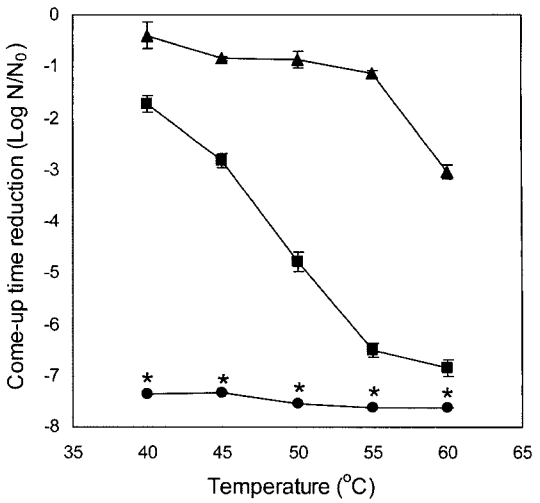

Fig. 2. Inactivation of *L. innocua* ATCC 33090 suspended during the come-up time at temperatures ranged from 40 to 60°C and different pressure levels. The mark (*) indicates that no survival colony was observed after enrichment culture. 300 MPa ▲, 500 MPa ■, 700 MPa ●.

In experiment 3, the combined effectiveness of pressure and temperature on the come-up time reduction of *L. innocua* was investigated at 300 to 700 MPa and 40 to 60°C as shown in Fig. 2. In general, the come-up time reductions increased with the increase in pressure and temperature. At 300 MPa, the reductions were approximately 1 log CFU/mL up to 55°C (p>0.05), while the number of *L. innocua* was significantly reduced by more than 3 log CFU/mL at 60°C (p<0.05). This observation indicates that *L. innocua* was more sensitive to temperature change after 55°C at constant 300 MPa. At 500 MPa, the reductions steadily increased up to 6.84 log CFU/mL at 60°C. No *L. innocua* survivor was detected in the range of 40 to 60°C at 700 MPa. The results suggest that the combinations of high pressure and temperature could provide a significant improvement in reducing foodborne pathogens over the traditional thermal processing. The come-up time will be considered to design and optimize thermal and high pressure processing. Further systematic study is underway to determine the effect of the temperature and pressure come-up rates on the inactivation of microorganisms during high pressure processing.

In conclusion, the heat resistance obtained from the come-up time caused an increase in thermotolerance during the isothermal treatment, showing the characteristic tailing. With increasing temperature heating-up rate, *L. innocua* was more sensitive to heat during the come-up time. The combined pressure-thermal treatment resulted in significant reduction of *L. innocua* when compared to thermal treatment alone. Therefore, these results highlight the importance of considering the come-up time for study of microbial thermal inactivation, and high pressure

processing could be more potential to inactivate foodborne pathogens as an emerging technology ensuring high quality and safety of food products.

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