Inactivation of Clostridium botulinum Type A Spores by High-Pressure Processing at Elevated Temperatures

N. R. REDDY,1*, H. M. SOLOMON,2 R. C. TETZLOFF,3 AND E. J. RHODEHAMEL3

1U.S. Food and Drug Administration and 2Illinois Institute of Technology, National Center for Food Safety and Technology, 6502 South Archer Road, Summit-Argo, Illinois 60501; 2Food and Drug Administration, Division of Microbiological Studies, 200 C Street S.W., Washington, D.C. 20204; and 3Cryovac, Sealed Air Corp., P.O. Box 464, Duncan, South Carolina 29334, USA

ABSTRACT

The effects of high-pressure treatments at various temperature-time combinations on the inactivation of spores of Clostridium botulinum type A strains 62-A and BS-A in phosphate buffer (0.067 M, pH 7.0) and in a crabmeat blend were investigated. The log unit reduction of strain 62-A spores increased significantly as the processing pressure increased from 417 to 827 MPa (from 60,000 to 120,000 lb/in²) at 75°C. The reduction of BS-A and 62-A spores in either medium increased as processing temperatures increased from 60 to 75°C and processing times increased from 5 to 20 min at a maximum pressure of 827 MPa. Approximately 2- and 3-log reductions of BS-A and 62-A spores, respectively, in phosphate buffer were obtained at the maximum pressure–maximum temperature combination of 827 MPa and 75°C for a processing time of 20 min. Processing for 15 min at the maximum pressure–maximum temperature combination resulted in maximum reductions of 3.2 and 2.7 log units for BS-A and 62-A spores, respectively, in the crabmeat blend. Results obtained in this study indicate that the crabmeat blend did not protect BS-A and 62-A spores against inactivation by high-pressure processing.

Recent interest in the use of high-pressure processing (HPP) technology for food preservation may be due to increased consumer demand for minimally processed, fresh-tasting, high-quality, additive-free, microbiologically safe foods with extended shelf lives (17, 21, 33). HPP is one of the nonthermal technologies that offer the potential to produce foods with natural flavor, color, and quality attributes while extending shelf life through the control of microorganisms and enzymes (4, 5). Several reviews and reports dealing specifically with the use of HPP in combination with low to moderate temperatures as a means of controlling or inactivating vegetative pathogens of importance in foods have been published (1, 2, 9, 10, 14, 16–19, 22, 40–42, 45, 46). The resistance of vegetative pathogens (namely, Aeromonas hydrophila, Yersinia enterocolitica, Salmonella Typhimurium, Salmonella Enteritidis, Listeria monocytogenes, Escherichia coli O157:H7, and Staphylococcus aureus) to HPP is variable. These pathogens can be inactivated at pressures ranging from 250 to 700 MPa at ambient temperature with various processing times (3, 11, 29, 31, 32, 47). Bacterial spores, however, are very resistant to high pressures and cannot be inactivated by high pressure alone (20, 25, 36). Bacterial spores survive pressures above 1,000 MPa unless pressurization is carried out at temperatures close to 100°C (5). The structure and thickness of the bacterial spore coat is believed to account for this strong resistance. There are several reports on the inactivation of spores of Bacillus spp. and Clostridium sporogenes by (i) combined heat and high-pressure treatment (6, 8, 12, 13, 15, 23, 25–27, 30, 36–38, 43), (ii) pulsed high pressure in conjunction with heat (8, 15, 23, 43), and (iii) high pressure with preservatives or bacteriocins and mild heat (39, 44). Nakayama et al. (26) reported that pressure treatments of 981 MPa for 40 min and at 588 MPa for 120 min did not inactivate the spores of Bacillus stearothermophilus IAM 12043, Bacillus subtilis IAM 12118, or Bacillus licheniformis IAM 13417 at 5 to 10°C. Crawford et al. (6) observed a 5-log reduction of C. sporogenes spores in chicken breast after the chicken breast had been processed at 680 MPa for up to 60 min at ambient temperature. Other researchers (20, 35, 36) have demonstrated that high-pressure (800 MPa) treatment in conjunction with high heat (80 to 108°C) can result in a 5-log reduction in C. sporogenes spore counts in a model food or buffer system.

Spores of Clostridium botulinum types A, B, E, and F are widely distributed in nature and have been associated with human botulism outbreaks. Nonproteolytic type E spores have low heat resistance and cause problems primarily in pasteurized or unheated foods, including seafood products. Proteolytic C. botulinum strains such as type A strains produce heat-resistant spores that are of major concern in the processing of low-acid, shelf-stable foods. Our previous study (34) evaluated the effects of high pressure (827 MPa) in combination with moderate heat (35 to 55°C) on spores of C. botulinum type E strains (Alaska and Beluga) in a phosphate buffer system. Approximately 5-log reductions of spores of Alaska and Beluga were obtained at 50 and 55°C, respectively, after processing at pressures of 827 MPa for 5 min when 2% sodium benzoate solution was used as a pressurizing medium. The objective of this study was to investigate the effects of high pressure and elevated temperatures on spores of C. botulinum type A.

* Author for correspondence. Tel: 708-728-4135; Fax: 708-728-4177; E-mail: rukma.reddy@cfsan.fda.gov.
strains BS-A and 62-A in phosphate buffer and in a crabmeat blend.

**MATERIALS AND METHODS**

*C. botulinum* type A spore preparations. The *C. botulinum* type A strains (BS-A [isolated from beef stew] and 62-A [isolated from cow liver]) used in this study had previously been isolated from food products implicated in cases of foodborne botulism outbreaks. These strains were maintained by the U.S. Food and Drug Administration (Washington, D.C.). Spores of these individual strains were produced in Trypticase-peptone-glucose-yeast extract (TPGY) medium at 35°C for 10 days as described in the *Bacteriological Analytical Manual* (7). Spores of each strain were harvested by centrifugation (10,000 × g), washed three times with sterile distilled water, and resuspended in sterile distilled water. The number of spores per milliliter in each strain suspension was determined by the three-tube most-probable-number (MPN) method with TPGY broth as the culture medium as described in *Bacteriological Analytical Manual* (7). The spore mixture was stored at 4°C until it was used.

**Preparation of crabmeat blend.** Cans of pasteurized crabmeat (Phillips Seafood [MASABATE, Inc.], Bacolod City, Philippines) weighing 16 oz were purchased from a local retailer. These cans contained handpicked backfin meat and were marketed in the United States by Phillips Foods, Inc. (Baltimore, Md.). Portions (ca. 100 g each) of pasteurized crabmeat were sterilized by autoclaving for 40 min at 121°C in shallow covered pans and refrigerated until use. The crabmeat blend was prepared by mixing 100 g of sterile crabmeat with 120 ml of sterile distilled water in a sterile jar and blending the mixture for 1 min at high speed in a Waring Commercial Laboratory Blender. The fresh crabmeat blend was prepared on the day of processing.

**Packaging of *C. botulinum* spores and HPP.** High-barrier pouches (4.0 by 10.0 cm; CN-530 series, Cryovac, Sealed Air Corporation, Duncan, S.C.) for the packaging of spores were sterilized by gamma irradiation with cobalt-60 as a source at a dosage level of 8.0 to 10.0 kGy (Isomedix Operations, Inc., Morton Grove, Ill.). About 9.0 ml of cold Sorensen phosphate buffer or crabmeat blend and 1.0 ml of a diluted mixture containing either 62-A or BS-A spores (1.0 × 10^6 spores per ml) were placed in a sterile high-barrier pouch and sealed with a heat sealer (Metric Model HS-C sealer, Doboy Packaging Machinery, Inc., New Richmond, Wis.) with the exclusion of air. Sealed pouches containing spores and either phosphate buffer or crabmeat blend were placed individually into slightly larger high-barrier pouches (6.0 by 14.0 cm; P640B series, Cryovac) and heat sealed. Two of the resulting double-bagged pouches were placed into a larger high-barrier bag (10.0 by 20.0 cm; P640B series, Cryovac) and heat sealed again. The final concentration of spores in each of the sample pouches was 1.0 × 10^5 spores per ml. The sample pouches were subjected to a combination of elevated temperatures (60, 65, 70, and 75°C) and pressure levels (414 to 827 MPa, i.e., 60,000 to 120,000 lb/in^2) for up to 30 min with a Quintus Model QFP-6 High Pressure Food Processor (Flow Autoclave Systems, Inc., Columbus, Ohio).

This processor was equipped with a thermocouple (Flow Autoclave Systems, Inc., Vasteras, Sweden) that continuously monitored the temperature of the pressurization medium inside the cylinder during compression. Come-up times ranged from 122 to 145 s for pressures ranging from 414 to 827 MPa (i.e., 60,000 to 120,000 lb/in^2). Depressurization took <2 s. For all experiments in this study, the processing time did not include the pressure come-up time or the depressurization time. A preliminary curve developed for temperature versus pressure in water was used as a basis for the adjustment of the initial temperature of the pressurization medium inside the cylinder. A 50.0% water glycol fluid (Houghto-Safe 620-TY, Houghton International Inc., Valley Forge, Pa.) was used as a pressurizing medium. Compression heating initially increased the temperature of the pressurization medium during the processing cycle to 6 to 18°C above the processing temperatures, depending on the processing time at the start of the cycle (see Table 1). The processed bags were held under crushed ice and water at 4°C until they were opened for the enumeration of surviving spores. The experiments were repeated at least once.

**Enumeration of surviving spores and toxin determination.** Spores surviving in the processed pouches were enumerated by the five-tube MPN method with TPGY broth (7). Each pouch was aseptically cut open, and its contents were serially diluted in TPGY broth and inoculated into MPN tubes. An unprocessed control pouch containing phosphate buffer plus spores or crabmeat blend plus spores was included for each experiment to obtain the actual initial spore count. The inoculated MPN tubes were incubated aerobically at 35°C for 2 weeks. All tubes for which no growth was observed were sealed with a layer of sterile vaspar (4 ml) and mineral oil (0.5 ml) to maintain anaerobiosis and further incubated at room temperature for 4 months to allow pressure- and heat-injured spores to recover, germinate, and grow. If no growth was observed after 4 months, the tubes were considered sterile. For a number of tubes for each of the samples, outgrowth was observed from 2 weeks to 4 months of incubation. The presence of type A toxin was confirmed for tubes showing positive growth after incubation by the standard mouse bioassay (7). Reported spore counts are averages (±0.6 log units [standard deviation]) for duplicate or triplicate experiments. The spore count reduction was expressed as the ratio of the log count for a sample after treatment to that for the sample before treatment (control).

**Statistical analysis.** The effect of pressure, temperature, time, and suspending medium on the reduction of spores was analyzed by analysis of variance (SPSS Inc., Chicago, Ill.).

**RESULTS AND DISCUSSION**

Studies on the effects of high pressures ranging from 414 to 827 MPa (i.e., 60,000 to 120,000 lb/in^2) in combination with a temperature of 75°C on spores (1.0 × 10^5 spores per ml) in phosphate buffer were conducted for spores of strain 62-A only. Log unit reductions of spores of strain 62-A increased significantly (P < 0.05) as processing time increased from 5 to 20 min and pressure increased from 414 to 827 MPa (Fig. 1). At lower pressures (<689 MPa), the 62-A spore count was reduced by <2.0 log units with a processing time of 20 min. Increasing the pressure from 689 to 827 MPa further increased the reduction of 62-A spores to 3.0 log units with a processing time of 20 min. Maggi et al. (20) and Gola et al. (12) reported similar findings for spores of *C. sporogenes* PA3679. Results of the study by Maggi et al. (20) indicated that an
increase in pressure from 600 to 1,000 MPa at 80°C resulted in a >4.8-log reduction of spores in phosphate buffer (pH 7.0) with a processing time of 5 min. Initial spore counts for these samples ranged from 4.8 to 5.5 log units. No surviving spores were detected in phosphate buffer (pH 7.0), carrot broth, or meat broth (pH 7.0) following treatments at pressures of ≥800 MPa at 80°C for 5 min. Further, Gola et al. (12) also found that an increase in pressure from 600 to 900 MPa at a pressure of 827 MPa at temperatures ranging from 35 to 50°C significantly (P < 0.05) increased reductions of spores of C. botulinum type E Alaska in phosphate buffer (pH 7.0) by up to 5 log units with a treatment time of 5 min. Since a pressure of 827 MPa at 75°C brought about the maximum reduction of 62-A spores in phosphate buffer (pH 7.0), this pressure was used in the subsequent experiments with various combinations of processing temperatures and times.

In the next series of experiments, the effects of phosphate buffer (0.067 M, pH 7.0) and a model food system, namely, a crabmeat blend, on the inactivation of BS-A and 62-A spores (1.0 × 10^5 spores per ml) were studied. Log unit reductions of BS-A and 62-A spores in phosphate buffer at 827 MPa increased significantly (P < 0.05) with an increase in processing temperature from 60 to 75°C and an increase in treatment time from 5 to 20 min (Figs. 2 and 3). Approximately 1.3- and 0.7-log reductions of BS-A and 62-A spores, respectively, occurred in phosphate buffer at 60°C with treatment at 827 MPa for 20 min. Larger reductions of BS-A and 62-A spores in phosphate buffer were obtained at temperatures of >70°C under similar conditions. Treatment with a pressure of 827 MPa at 75°C for up to 20 min resulted in ca. 2- and 3-log reductions of BS-A and 62-A spores, respectively, in phosphate buffer (Figs. 2 and 3). Increasing the processing time from 20 to 30 min at the maximum pressure (827 MPa) and temperature (75°C) further increased the reduction of BS-A spores from 2 to 3 log units in phosphate buffer (data not shown).

Freshly prepared crabmeat blends had pHs of 7.20 to

---

**TABLE 1. Mean temperatures of pressure fluid during compression at a pressure of 827 MPa for 5, 10, 15, and 20 min at various processing temperatures**

<table>
<thead>
<tr>
<th>Processing time (min)</th>
<th>Processing temp (°C)</th>
<th>Initial pressurization medium temp (°C)</th>
<th>Maximum pressurization medium temp (°C)</th>
<th>Final pressurization medium temp (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>60</td>
<td>32.0 ± 1.6</td>
<td>68.4 ± 1.3</td>
<td>62.5 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>65</td>
<td>35.1 ± 2.3</td>
<td>71.2 ± 1.4</td>
<td>65.3 ± 1.4</td>
</tr>
<tr>
<td></td>
<td>70</td>
<td>39.5 ± 3.2</td>
<td>75.7 ± 0.9</td>
<td>68.8 ± 1.5</td>
</tr>
<tr>
<td></td>
<td>75</td>
<td>45.7 ± 3.4</td>
<td>81.0 ± 1.7</td>
<td>74.2 ± 1.2</td>
</tr>
<tr>
<td>10</td>
<td>60</td>
<td>33.8 ± 7.0</td>
<td>69.9 ± 3.7</td>
<td>61.5 ± 2.5</td>
</tr>
<tr>
<td></td>
<td>65</td>
<td>37.6 ± 5.3</td>
<td>72.7 ± 3.4</td>
<td>65.0 ± 1.4</td>
</tr>
<tr>
<td></td>
<td>70</td>
<td>46.9 ± 2.9</td>
<td>80.0 ± 1.5</td>
<td>70.2 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>75</td>
<td>52.4 ± 1.5</td>
<td>85.9 ± 1.5</td>
<td>75.5 ± 1.6</td>
</tr>
<tr>
<td>15</td>
<td>60</td>
<td>40.5 ± 6.5</td>
<td>72.7 ± 4.4</td>
<td>59.1 ± 1.7</td>
</tr>
<tr>
<td></td>
<td>65</td>
<td>47.3 ± 6.3</td>
<td>79.0 ± 5.5</td>
<td>64.6 ± 2.1</td>
</tr>
<tr>
<td></td>
<td>70</td>
<td>55.0 ± 3.1</td>
<td>87.1 ± 3.6</td>
<td>70.2 ± 1.3</td>
</tr>
<tr>
<td></td>
<td>75</td>
<td>62.0 ± 3.2</td>
<td>95.6 ± 4.4</td>
<td>76.1 ± 1.0</td>
</tr>
<tr>
<td>20</td>
<td>60</td>
<td>43.4 ± 5.7</td>
<td>75.8 ± 3.1</td>
<td>60.4 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>65</td>
<td>47.3 ± 5.6</td>
<td>79.3 ± 5.6</td>
<td>64.6 ± 1.4</td>
</tr>
<tr>
<td></td>
<td>70</td>
<td>56.1 ± 1.1</td>
<td>87.7 ± 2.7</td>
<td>70.0 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>75</td>
<td>60.3 ± 1.1</td>
<td>92.4 ± 2.3</td>
<td>74.3 ± 0.5</td>
</tr>
</tbody>
</table>

*a* Values presented are averages for 5 to 12 replicates ± standard deviations.

*b* Initial pressurization medium temperature at the start of pressurization cycle.

*c* Maximum pressurization medium temperature at the start of the processing time at the processing pressure (827 MPa). The temperature gradually decreases and is close to processing temperature as the processing time ends.

*d* Final pressurization medium temperature at the end of the processing time at the processing pressure (827 MPa).

---

**FIGURE 1. Reduction of C. botulinum type A strain 62-A spores in phosphate buffer with high pressure at 75°C. Each datum point value is an average ± 0.6 log units (standard deviation).**
7.40. An increase in the processing temperature from 60 to 75°C and an increase in the treatment time from 5 to 20 min at a pressure of 827 MPa resulted in significant ($P < 0.05$) reductions of BS-A and 62-A spores in the crabmeat blend (Figs. 4 and 5). Reductions of BS-A and 62-A spores in the crabmeat blend amounted to $<1$ log units with combinations of high pressure and lower temperatures (60 and 65°C) with a treatment time of 20 min. Maximum reductions of BS-A spores (3.2 log units) and 62-A spores (2.7 log units) in the crabmeat blend occurred at a temperature of 75°C in combination with high pressure and a processing time of 15 min. However, an increase in processing time from 15 to 20 min with a combination of high temperature and high pressure resulted in lower reductions of BS-A spores (2.5 log units) and 62-A spores (2.3 log units) in the crabmeat blend (Figs. 4 and 5). Crawford et al. (6) reported that the use of a constant high pressure (689 MPa) and temperature (80°C) for 20 min was most effective in reducing $C.\ sporogenes$ ATCC 7955 spores in chicken breast meat. These investigators obtained a reduction of about 2 log units and concluded that a combination of heat and pressure was more effective in destroying spores than was pressure alone. Several other investigators (12, 20, 24) reached similar conclusions. Maggi et al. (20) observed no effect of treatment with a combination of high pressure (1,500 MPa) and low temperature (20°C) for 5 min on spores of $C.\ sporogenes$ in phosphate buffer (pH 7.0), carrot broth (pH 7.0), or meat broth (pH 7.0); however, these investigators did observe $>5$-log reductions of spores of $C.\ sporogenes$ in phosphate buffer (pH 7.0) with various combinations of high pressure and high temperature (1,500 MPa and 60°C, 1,000 MPa and 80°C, and 800 MPa and 90°C) after 5 min of processing. Miglioli et al. (24) also reported that treatments involving combinations of high pressure and high temperature were necessary to destroy $C.\ sporogenes$ PA3679 spores in meat broth (pH 7.0). These investigators found that treatments consisting of 1,400 MPa at 60°C, 1,000 MPa at 80°C, and 800 MPa at 90°C for 5 min completely destroyed $3 \times 10^5$ spores per ml. It appears that spores of $C.\ botulinum$ type A strains BS-A and 62-A are pressure resistant and can be partially inactivated in either medium with the use combinations of high pressure and high temperature. The use of the crabmeat blend as a model food suspension medium had no significant ($P < 0.05$) effect on the inactivation of 62-A spores. However, after treatment with 827 MPa at 75°C for 15 min, the reduction of BS-A spores in the crabmeat blend (3.2 log units) was significantly ($P < 0.05$) larger than reduction of BS-A spores in phosphate buffer (1.7 log units). The increased reduction of BS-A spores in the crabmeat blend may be due in part to proteins and other components of the crabmeat blend.

It can be concluded that treatment with a combination of high pressure (827 MPa) and high temperature (75°C) resulted in the partial inactivation (i.e., $\leq 3$-log reductions)
of spores of *C. botulinum* type A strains BS-A and 62-A in model buffer or food systems such as phosphate buffer and a crabmeat blend. Since the pressures used in this study were slightly higher than those likely to be used commercially, it is evident that in order for HPP to be effective in destroying *C. botulinum* spores in low-acid, shelf-stable food products (i.e., commercially sterile products), processing temperatures will need to be increased. Further studies to investigate the effects of high temperatures (up to 120°C) and high pressures (up to 689 MPa) on type A spores in model buffer or food systems are under way.

**ACKNOWLEDGMENTS**

We acknowledge the cooperation and assistance of the Cryovac division of Sealed Air Corp. and Flow International Corp. This publication was partially supported by cooperative agreement no. FD-000431 from the U.S. Food and Drug Administration and the National Center for Food Safety and Technology. Its contents are solely the opinions of the authors and do not necessarily represent the official views of the U.S. Food and Drug Administration.

**REFERENCES**


