Correlation between Dipicolinic Acid (DPA) Release and Heat Resistance of *C. botulinum* Type A and *C. sporogenes* Spores during Thermal Processing

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Abstract

Introduction: Dipicolinic acid (DPA) is a major constituent of bacterial spores and may provide protection against spore inactivation by various processes. Thermal processing induces DPA release from spores, followed by loss of heat resistance and inactivation of DPA-free spores. This suggests that the resistance of spores to thermal processes may rely on their ability to retain DPA.

Purpose: To determine if any correlation exists between DPA release and thermal inactivation of *C. botulinum* and *C. sporogenes* spores, and whether DPA release may be a quantifiable indicator of heat resistance.

Methods: Spores of *C. botulinum* (Giorgio-A) and *C. sporogenes* (PA3679) were diluted in ACES buffer (0.05 M, pH 7.0) to 7 log spores/mL, aliquoted into NMR tubes, and heat-sealed. Spores were thermally treated at 101°C, 105°C, and 108°C for predetermined treatment times in an oil bath. Surviving spores were enumerated by 5-tube MPN with 10-12 weeks incubation. Spore suspensions were filtered through a 0.22 µm membrane, heat-treated (85°C, 10 minutes) to denature any toxin, and analyzed for DPA by UPLC-MS/MS. Experiments were repeated in triplicate. Correlation between MPN log reductions versus % DPA release was performed using Pearson's correlation coefficient.

Results: Released DPA content from spores and log reductions increased with increase of treatment time and process temperature from 101 to 108°C for strains Giorgio-A and PA3679. DPA released from Giorgio-A spores at 105°C after 15 minutes was 56.20±7.71% of the total DPA content with 3.89±0.39-log reduction. DPA content in unprocessed controls was below limit of quantification. A strong, positive correlation was observed between % DPA release and log reduction of spores from Giorgio-A and PA3679 at all thermal treatments (r = 0.8033-0.9120).

Significance: These results suggest that a correlation exists between DPA release and heat resistance of spores of Giorgio-A and PA3679. Further studies are underway with other *C. botulinum* strains.

Introduction

DPA is complexed with calcium ions in the endospore core and accounts for 5-15% of a bacterial spore's dry weight (Powell and Strange, 1953; Church and Halvorson, 1959). Biosynthesis of DPA occurs during sporogenesis and results in the appearance of heat resistance in spores (Perry and Foster, 1955; Church and Halvorson, 1959). Spore inactivation by heat begins with DPA release from spores, loss of heat resistance and inactivation of DPA-free spores (Margosch et al. 2004; Doona et al. 2017). This suggests resistance of spores to various processing treatments may depend on their ability to retain DPA.

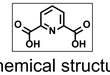

Figure 1. Chemical structure of DPA

The present study investigates retention of DPA in spores after subjecting to various temperature/time combinations and aims to identify if any correlation exists between DPA retention and heat resistance of *C. botulinum* and *C. sporogenes* spores.

Materials and Methods

Preparation of Spores

Preliminary screening for DPA was performed on *C. botulinum* strains (Table 1):

Table 1. Total kill DPA release from *C. botulinum* spores*

C. botulinum strain	DPA (average µg/mL)
69-A	1.13
Giorgio-A	3.56
17-B	0.60
2-B	0.60
Ham-B	0.59
QC-B	3.28
17844-B	0.90
610-F	1.05
202-F	0.94

*Total kill samples at 10^6-10^7 spores/mL

- Proteolytic strain ***C. botulinum* Giorgio-A** and thermal surrogate ***C. sporogenes* PA3679** were selected for the current study

Spores were prepared using biphasic media method (Reddy et al. 2013) and spore toxin serotype was confirmed by Endopep-MS assay. Spores of Giorgio-A and PA367 were individually diluted into ACES (*N*-(2-acetamido)-2-aminoetahnesulfonic acid) buffer to a concentration of 10^7 spores/mL. Aliquots of 1.7 mL were dispensed into nuclear magnetic resonance (NMR) tubes and heat-sealed in duplicate.

Thermal Inactivation

NMR tubes were submerged in Duratherm S oil in a calibrated oil bath (high precision bath 7321, Fluke Corp.) and heat treated (± 0.01°C) at different temperature/time combinations:

- Giorgio-A: 101°C – 10, 20, 30, 40 min
  105°C – 3, 7, 11, 15 min
  108°C – 3, 5, 7, 11 min
- PA3679: 101°C – 60, 120, 180, 240 min
  105°C – 30, 60, 90, 120 min
  108°C – 10, 20, 30, 40 min
- Total kill for both strains was obtained at 121°C for 30 min

NMR tubes were quickly removed from the oil bath and placed in an ice bath to stop the thermal treatment. Spore suspensions were removed from NMR tubes using sterile blunt needle syringe.

Enumeration and DPA Analysis

MPN

1.0 mL was removed for enumeration of surviving spores by 5-tube MPN method using Trypticase-Peptone-Glucose-Yeast (TPGY) extract broth.

Inoculated MPN tubes were incubated aerobically at 37°C for 5-6 days. Following incubation, all remaining tubes with no growth were sealed with a layer of sterile vaspar (3 mL) and mineral oil (1 mL) to maintain anaerobiosis for 10-12 weeks incubation at room temperature to allow recovery of heat-injured spores. The log reduction of the spore count was calculated as $\log(N/N_0)$.

LC-MS/MS

The remaining 0.7 mL was filtered through a 0.22 µm membrane filter. A secondary heat treatment (85°C, 10 minutes) was performed to denature any remaining toxin in the filtrate. Samples were then ready for analysis of DPA release using the UPLC-MS/MS method described by Redan et al. 2022.

Correlation between MPN log reductions versus % DPA release of total kill (µg/mL) was determined using Pearson's correlation coefficient.

Results

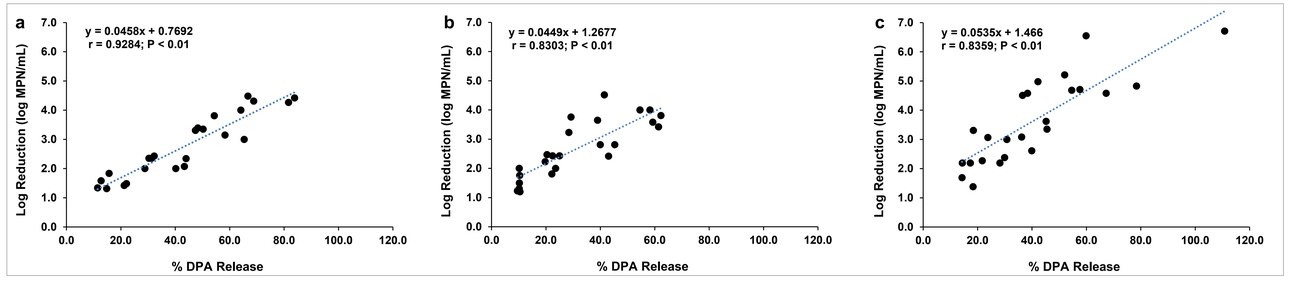

Figure 2. Linear correlation between log reduction of *C. botulinum* Giorgio-A spores and DPA release during thermal processing at 101°C **(a)**, 105° C **(b)**, and 108° C **(c)**. Significant (P < 0.01) correlation was determined using Pearson's correlation coefficient.

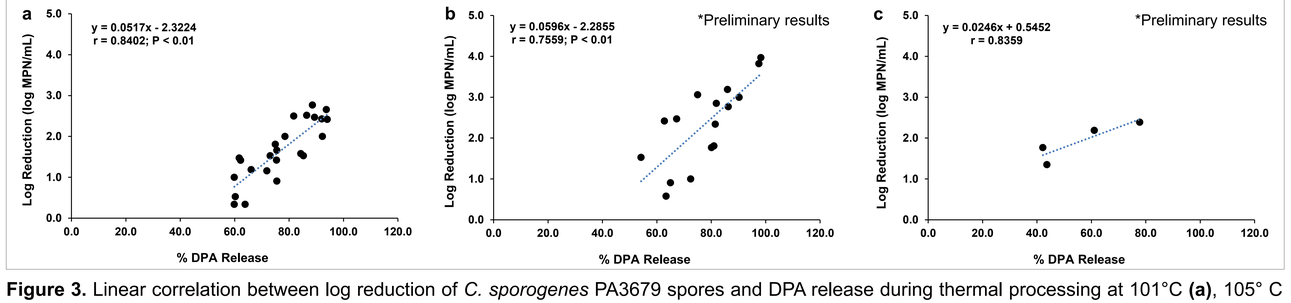

Figure 3. Linear correlation between log reduction of *C. sporogenes* PA3679 spores and DPA release during thermal processing at 101°C **(a)**, 105° C **(b)**, and 108°C **(c)**. Significant (P < 0.01) correlation was determined using Pearson's correlation coefficient at 101°C and 105°C. **C. sporogenes* PA3679 thermal studies are ongoing at 105°C and 108°C, current preliminary results.

Discussion

- The amount of DPA released from Giorgio-A and PA3679 increased with an increase in the treatment time for each process temperature.
- A strong, positive correlation (P < 0.01) between spore DPA release and log reduction was observed from *C. botulinum* Giorgio-A with r = 0.9284, 0.8303, and 0.8359 at 101°C, 105°C, and 108°C, respectively.
- At 101°C, *C. sporogenes* PA3679 demonstrated a strong, positive correlation (r = 0.8402, P < 0.01) between DPA release and log reduction. Preliminary results suggest the continuation of this correlation, further confirmation will be needed.
- DPA content in all unprocessed controls was below the limit of quantification (< 0.2 µg/mL) for Giorgio-A and PA3679.

Conclusion

These results suggest that DPA release may be correlated with spore reduction under thermal treatment. Further experiments will explore these findings in additional *C. botulinum* spore strains.

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