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Effect of Ionic Stress on the Stability of Bacterial Spores

Chong-Ouk, Rhee

Agricultural Products Utilization Division, Institute of Agricultural Engineering and Utilization, Suweon, 170, Korea

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세균 포자의 안정성에 미치는 이온 강도의 영향

李 鍾 旭

농공이용연구소, 농산물이용연구담당관실

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SUMMARY

High ionic strength is expected to enhance dissociation of Ca-DPA from spores and to contribute to a detrimental effect on spore stability or on spore heat resistance with a combined treatment of gamma-radiation. From this study, this hypothesis has become apparent as as follows; 1) Ca-DPA dissociation contributes to loss of stability of bacterial spores with respect to heat resistance, survival during storage, and 2) the cytoplasmic membrane plays a role in maintaining the stability of DPA-Ca-spore complex, apparently by serving as a permeability barrier.

INTRODUCTION

A hypothesis has been previously proposed that bacterial spore dormancy and resistance is due to a mineralized state of the cytoplasm[1], The mineralizing compound is thought to be Ca-DPA or Ca-DPA associated with a second ligand, i.e., some substance present in the spore, particularly in the innermost core, where Ca-DPA seems to be deposited[2].

If this hypothesis is correct then one would ex pect that conditions that are known to affect Ca-DPA dissociation would probably cause its release from the spore. Indeed such a correlation was found to be true at extremes of pH, where acid and basic pH contribute to Ca-DPA dissociation and enhance release of Ca-DPA from the spore; at the same time heat resistance of spores is considerably diminished[1]. However, the evidence from pH experiments so far cannot be interpreted unequivocally since extremes of pH reduce heat resistance of many cells including those having no Ca-CPA. Furthermore, extremes of pH affect also denaturation of many biopolymers such as enzymes, DNA, RNA etc. Therefore in order to validate our hypothesis, it is necessary to employ other systems which would contribute to dissociation of the hypothetical DPA-Ca-spore complex and to correlate dissociation of the DPA-Ca-spore complex with changes in spore stability.

The present paper explores the effect of high ionic strength achieved by NaCl concentrations up to 15%. This ionic strength is expectecd to enhance dissociation of Ca-DPA and if such dissociation occurs in the spore, one would anticipate a possible detrimental effect on either spore viability during storage, or on spore heat resis-

tance, or on both.

MATERIALS AND METHODS

Microorganisms. *Bacillus cereus* T from Dr. T. Hashimoto of Loyola University was selected for this experiment. For spore production, an inoculum of an active culture was grown by the active culture technique in Trypticase Soy Broth (TSB) from a single colony isolate by transfering stepwise a 10% inoculum until a total culture volume of 7.2 liters was obtained for inoculating into a Fermacell Industrial Fermentor(New Brunswick Sci.) containing 72 liter of modified G medium. Oxygen flow was maintained at 40 liter per min and pH kept at 7.0 by automatic titration with 300 ml 5N NaOH over the first 3.5 h. At this time the culture became increasingly turbid. The pH rose to 7.2 after 6 h and to its maximum of pH 8.0 by 18 h. 100% sporulation and 70% release of free spores were verified by phase contrast microscopy after 28 h. Spores were harvested after 30 h and isolated at 45,000 rpm in three Sharples S-16 steam turbine continuous centrifuges. Slpores were washed with a continuous flow of distilled deionized water, resuspended in 0.14 M NaCl and stored at 4°C.

The stock spore suspension contained no vegetative cells or cell debris. Spore content was determined by microscopic Petroff-Hausser count and by pour plate counts in Nutrient agar (Difco) incubated for 24 h at 30°C.

Effect of NaCl in the growth medium. In order to determine the NaCl sensitivity of irradiated spores, they were irradiated to 0.2 Mrad and then incubated for 3 days at 30 C in nutrient agar which contained 5% NaCl.

Determination of heat resistance. To determine the heat resistance of the spores during storage in NaCl solution, 1 ml of each of the spore suspensions ($2x10^7$ spores/ml) were adjusted to 1.5% NaCl concentration by appropriate dilution. Heating of the spores was done in 10×150mm Pyrex test tubes in a water bath at 90 C. Tubes were removed from the bath according to a prearranged time schedule and cooled in a crushed ice water bath. Survivors were determined as described in the previous section. Thermal death was defined as failure to grow into visible colonies on nutrient agar plates.

Gamma irradiation. Samples were irradiated in Cesium-137 Gammator (Radiation Machinery Corp., Parsippany, N.J.) at a dose rate of 0.1 Mrad per h. To obtain oxygen free conditions, the spore suspension was bubbled prior to irradiation with purified nitrogen gas for 5min and the tubes were closed with parafilm. During irradiation, the spores were suspended consistently in 0.9% NaCl, and kept in crushed ice.

Release of ^{14}C-DPA. Lyophilized ^{14}C labeled spores were irradiated to 0.2 Mrad in N_2-atmosphere while the tubes were immersed in crushed ice. The spores were suspended in distilled water and appropriately diluted to give 5×10^8 spores per ml in 0.9%, 5% and 15% NaCl concentration with ca 4,000 cpm/ml of ^{14}C-DPA. The samples were stored at room temperature for 5 h, and tested for release of ^{14}C-DPA at prearranged time intervals. The spores for 1 ml samples were collected on 0.45 μm Millipore membrane filters (24 mm pore size, type HA) and washed with 4 ml of distilled water three times.

The filter discs with the spores were dried and placed into 10 ml to scintillation solution. The scintillation fluid contained 4 gm of omnifluor (98% PPO, 2% Bis-MSB, New England Nuclear) to one liter scintillation grade toluene for counting. ^{14}C radioactivity was determined in a Beckmann LS-200 Liquid Scintillation Counter.

Labelling of ^{14}C-DPA. ^{14}C-labelled pyruvate was selected as an appropriate precursor for the synthesis of ^{14}C-DPA. Sodium pyruvate-3-C^{14}(*C $H_3COCOONa$) was obtained from New England Nuclear as a crystalline soild (the most stable state). All of the crystalline soild was dissolved in 10 ml of sterile distilled water and then divided into five 2 ml samples and stored at -10°C until needed. The total activity per 2 ml sample was 10 uCi.

The heat-activated spores were inoculated into

50 ml of Modified G medium[3] and placed on the water-shaker bath until vegetative cells were observed. Then 10 ml of the inoculum was transferred into 300 ml of Modified G medium and returned to the water-shaker bath. Twenty-four hours later, when prespore cells were observed, 0.6 ml (3uCi) of sodium pyruvate-3-C^{14} was added to the medium and the culture was returned to the water bath until spores were released. The spores were then harvested and washed.

RESULTS

Loss of spore viability in NaCl solution. Our initial observation on loss of *Bacillus cereus* T spore viability was made on an extremely dilute sample, ca 100-200 spores/ml stored in the refrigerator, 2-4 C. As summarized in Fig. 1, spores at this concentration lost viability relatively rapidly in distilled water, but were protected by 0.9 % NaCl (physiological saline) and to a lower degree by 3.4% NaCl. A subsequent experiment(Fig. 2) showed that at a higher concentration(10^8 spores/ml) the spores were more stable in distilled water than those at the lower spore concentration. As further indicated in Fig. 3, 0.9% and 15% NaCl had essentially no effect on spore stability with $2x10^7$ spores per ml stored at 2-4°C up to 20 days. We found however, that 0.2 Mrad of gamma radiation induced sensitivity and rapid loss of viability of spores stored in 15% NaCl but not in 0.9% NaCl. As shown in Fig. 4, the radiation dose of 0.2 Mrad caused only a slight loss (ca 30%) of spore viability, though the spores became extremely sensitive to NaCl in the suspending growth medium, suggesting that 0.2 Mrad had damaged cytoplasmic membrane permeability,

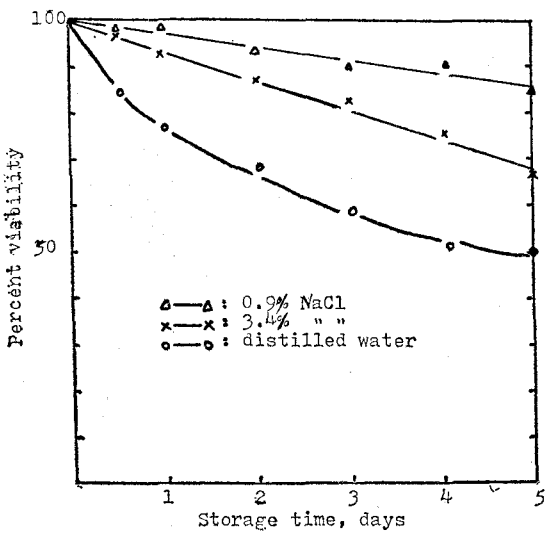

Fig. 1. Effect of NaCl on storage stability of spores of *B. cereus* T.
Spore concentration: 1×10^2 spores/ml
Temperature of storage: 2—4°C
Recovery medium: nutrient agar
Pour plating method by colony count

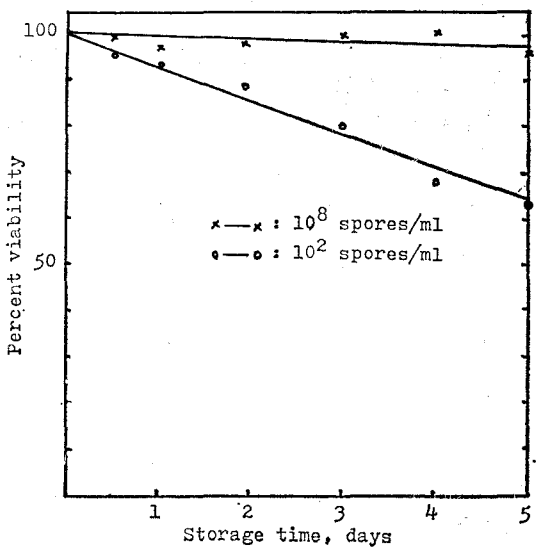

Fig. 2. Effect of spore concentration on storage stability of spores of B. cereus T in distilled water.
Temperature of storage: 2—4°C
Pour plating method by colony count

Effect of NaCl on spore heat resistance. Fig. 5 shows that during storage in 15% NaCl for 0, 6 and 12 days at 2-4°C, spores of *B. cereus* T progressively lost heat resistance when exposed to 90°C. As expected spores irradiated to 0.2 Mrad in order to damage their cytoplasmic membrane prior to storage, became more sensitive to heating than those not irradiated. However both unirradiated and irradiated spores consistently showed a greater loss of heat resistance during storage

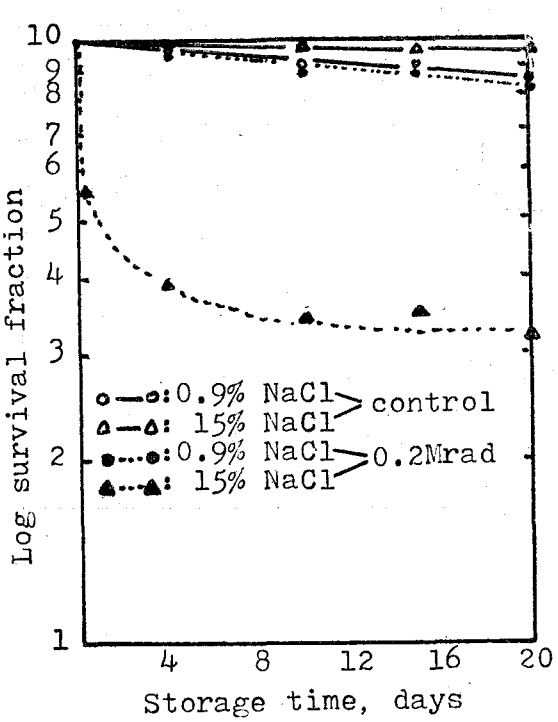

Fig. 3. Relation of NaCl conc. in suspending medium to viability of spores of *B. cereus* T during storage at 2—4°C. The spores were initially heat activated at 65°C for 30 min and irradiated up to 0.2 Mrad. After storage in 0.9% and 15% NaCl solution (2×10^7 spores/ml), the spores were appropriately diluted and plated on nutrient agar pour plates. Colonies were scored after incubation at 30°C for 24 h.

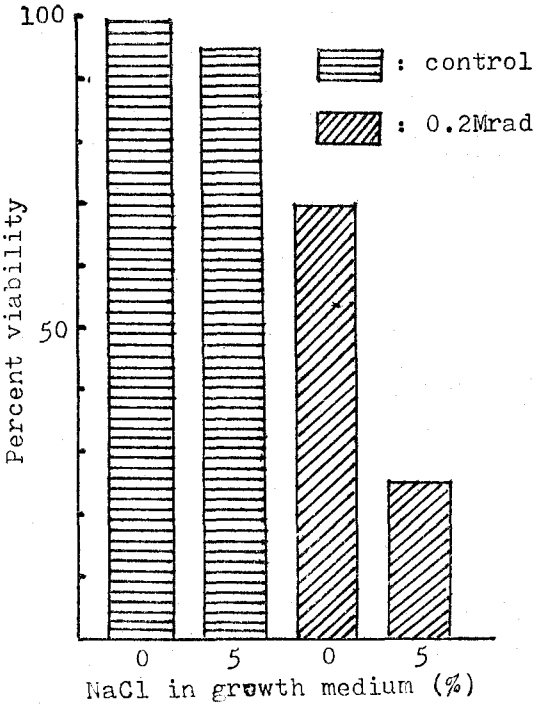

Fig. 4. Effect of 5% NaCl added to nutrient agar growth medium on the recovery of spores of *B. cereus* T as an indication of membrane permeability damage by 0.2 Mrad gamma-radiation.

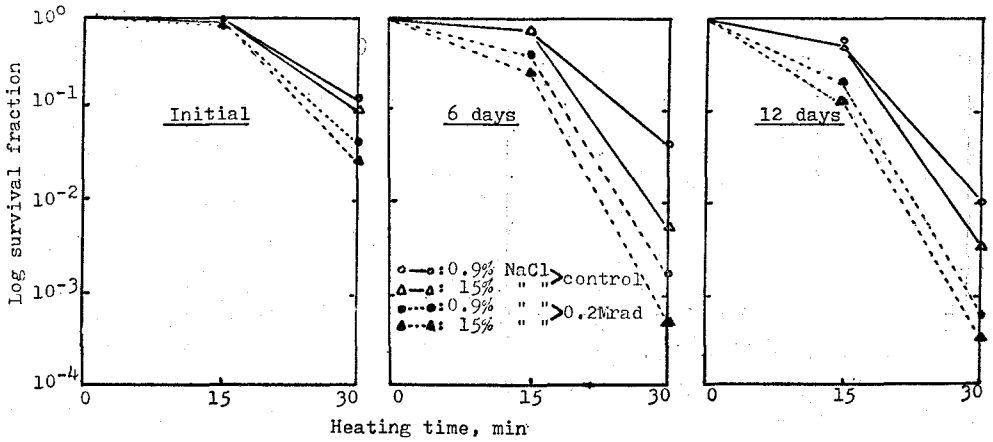

Fig. 5. Loss of heat resistance of spores of *B. cereus* T (2×10^8 spores/ml) during storage in 0.9% and 15% NaCl at 2—4°C for 0, 6 and 12 days. Prior to heating, the spores were diluted to give 2×10^7 spores/ml and 1.5% NaCl in the spore suspension during heating in all samples.

in 15% NaCl as compared with those stored in 0.9% NaCl.

At 0 time of storage only a slight perhaps insignificant difference was noted between spores stored in 15% and 0.9% NaCl. However after 6 days of storage considerable loss of heat resistance was noted with the same relative pattern i.e., greater loss in 15% NaCl vs 0.9% NaCl, and in irradiated vs unirradiated spores, respectively. Spores stored for 12 days showed essentially identical heat resistance pattern as those stored for 6 days.

Loss of ^{14}C-DPA. In this connection it was of interest to test for possible loss of DPA from spores during storage as a function of NaCl concentration. Fig. 6 summarizes our data which indicated that ^{14}C-DPA release from unirradiated spores was definitely enhanced by increasing concentration of NaCl.

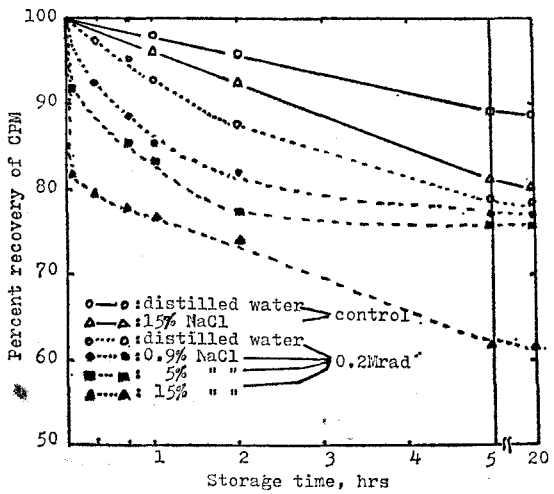

Fig. 6. Release of ^{14}C-DPA from spores of *B. cereus* T during storage as a function of NaCl solution. ^{14}C-DPA labelled spores (5×10^8 spores/ml) were stored at room temperature for the specific time periods.

Furthermore irradiation to 0.2 Mrad(to damage membrane) significantly enhanced loss of ^{14}C-DPA from the spores. The data show that a substantial amount of ^{14}C-DPA from irradiated spores was lost within the first 5 min of storage. Thereafter only slow additional loss was recorded in 0.9% and 5% NaCl up to some 2 h after which there was no further loss of ^{14}C-DPA; in 15% NaCl irradiated spores lost ^{14}C-DPA more abruptly and more extensively than in 0.9% and 5% NaCl during the first 5 min, but continued to lose additional ^{14}C-DPA at a slower rate up to 20 h (the length of this experiment). After 20 h, the fraction of initial ^{14}C-DPA lost was 50%, 20%, and 18% during storage in 15% NaCl, 5% NaCl and 0.9% NaCl, respectively.

DISCUSSION

Two important points have become apparent from this study, 1) Ca-DPA dissociation contributes to loss of stability of bacterial spores with respect to heat resistance, survival during storage etc., and 2) the cytoplasmic membrane plays a role in maintaining the stability of Ca-II-DPA association, apparently by serving as a permeability barrier. These two findings are potentially of great theoretical and practical significance. The role of Ca-II-DPA in maintenance of dormancy and stability of spores has been variously implied but no conclusive proof was available so far. Moreover, no systematic approach to the study of the stabilizing role of Ca-II-DPA was available. We consider the importance of this paper in that it specifically provides a hypothetical idea and conceptual strategy to the investigation of the role of Ca-II-DPA and DPA-Ca-Spore complexes together with the spore cytoplasmic membrane in maintenance of biological dormancy and resistance of bacterial spore.

From the practical point of view, the approach taken in this study shows a rational method for reducing heat resistance of bacterial spores and their viability during storage. The past literature contains much evidence that both heat resistance and viability during storage can be manipulated, although past studies did not provide a clear understanding of the underlying molecular events responsible for loss of spore stability. The relation of Ca-DPA to stability of bacterial spores has been summarized by Murrell[4]. In particular the time of synthesis of Ca-DPA during sporulation coin-

cides with the appearance of heat resistance of *B. cereus* and *C. botulinum*[5]. Furthermore, generally mutant spores containing no DPA[6] and spores which lost DPA as the result of germination[7] are also more sensitive to heat and radia tion. In addition it was shown more recently that the rate of Ca-DPA release during heating was more rapid in heat sensitive spores than in heat resistant spores of strains of *C. botulinum*[8] and strains of *B. stearothermophilus.*[9]

A considerable amount of evidence is available from many experiments in the past that various chemical stresses that cause dissociation of Ca-II-DPA also reduce stability of bacterial spores with respect to survival during storage, heat resistance etc. Thus extremes of pH contribute to loss of heat resistance of spores. Coordination chemistry considerations show that there exists a close correlation between loss of heat resistance and dissociation of Ca-DPA as a function of pH[1]. Several lines of evidence also indicate that presence of chelating substances in the heating menstruum cause reduction in heat resistance of bacterial spores.

The present study provides another line of evidence indicating the role of Ca-DPA in storage stability and heat resistance of bacterial spores. It is evident from Figures 6.7 that Ca-DPA dissociation occurs relatively rapidly and is lost from spores if the membrane is made permeable by 0.2 Mrad irradiation. The membrane seems to present a permeability barrier to DPA loss as well as to pH since unirradiated spores lost DPA much slower and in the final analysis much less (after 20 h of storage) than irradiated spores. However, ^{14}C-DPA loss even from unirradiated spores was strongly affected by NaCl concentration in the suspending medium. Since increasing NaCl concentration caused more rapid and more exhaustive loss of ^{14}C-DPA from spores (Fig. 6), it may be concluded that ^{14}C-DPA loss was due to dissociation of ^{14}C-DPA form under the increased ionic stress exerted by the NaCl as would be expected from the hypothetic model of DPA-Ca-spore complex.

In this sense the present study supports our original hypothesis[10] of a "spore cement" or a mineralized spore cytoplasm similar to bone or dentin structure. Furthermore, the results of the present study suggest new and novel directions for further investigation of the hypothetical DPA-Ca-spore complex.

In summary it is clear that loss of DPA(Fig.6) as well as loss of spore viability as the result of ionic stress (Fig.1,3) occurs very rapidly. Furthermore the data also clearly demonstrate that spore permeability is a factor and should be taken into account in future experiments and industrial sterilization practices.

At this point it is attractive to speculate as to the actual state of Ca and DPA within the spore. The β-attenuation studies of Leanz and Gilvarg[2] show that DPA is located in the spore core and not in the cortex as was previously assumed. Two possibilities may be envisioned here with respect to the organization of Ca-DPA in the spore: the first possibility is that Ca-DPA in the form of a trihydrate may be crystallized in the cytoplasm. Since Ca-DPA forms long needle-like crystals it is conceivable that it would compactly pack in the cytoplasm, physically displace free cytoplasmic water and actively entrap other cytoplasmic components within its matrix. A second possibility is that proposed by Grecz et al[1] that hypothetical DPA-Ca-spore complexes of spore structure or spore cytoplasmic pool material may exist in the core. From thermodynamic considerations such a mixed chelate would offer an explanation for the rapid rates of changes during germination, especially if one considers the substantial Coulombic forces set up by the second ligand attachment. The loss of DPA from irradiated(permeable) spores may support the idea of a Ca-DPA trihydrate which would not be as expected to be strongly affected by NaCl concentration in the medium, but would depend primarily on membrane permeability.

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요 약

세균포자의 안정성과 관련하여 DPA-Ca-spore complex의 해리에 영향을 주는 여러 요인중 15% 까지의 NaCl 농도에 의해서 이루어 지는 높은 이온 강도의 효과에 대하여 실험하고 다음과 같이 결론을 얻었다.

1) NaCl에 의한 포자 현탁액중의 이온강도의 변화는 포자로 부터 Ca-DPA의 해리를 촉진시켜 주며 이런 현상은 포자의 열 저항성을 감소시키고 따라서 보관중 포자의 안정성이 상당히 저하 되었다.

2) 세포질의 membrane은 포자내에서 Ca-DPA의 안정성을 유지하는데 permeability barrier로써 중요한 역할을 한다.

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