論　文

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Thermal Properties of Water in *Bacillus cereus* Spores and Vegetative Cells*

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Bacillus cereus spores have an extremely high heat resistivity as compared to their vegetative form. The thermal behavior of the cell water was studied with special regard to these properties characteristic of the spores. The phase diagrams for both cell forms were obtained by single scannings with a differential scanning calorimeter. The intracellular water was divided into freezable and unfreezable fractions, the amount of which could be determined calorimetrically for both cell forms. The amount of freezable water was lower in the spores than in the vegetative cells. This fact seems to be important for the maintenance of the dormancy and heat resistivity of the spores. The unfreezable water content was slightly lower in the spores than in the vegetative forms.

1. INTRODUCTION

Some species of bacteria, including *Bacillus* and *Clostridium*, produce spores in their stationary phase of growth. Morphologically, spores are composed of three parts; coat, cortex and core. The spore coat is the outermost region of the cells and constitutes the major part of the spore. The core is the innermost region in which cell constituents important for metabolism are contained. The cortex makes the region in between the coat and the core. The spores have high dormancy and resistivity to heat and various chemicals. Various studies have been made by a number of investigators in order to explain these characteristic properties of the spores. Among these are the studies of the content and physical state of water in the spores. It has been reported that bacterial spores, particularly the core regions, have considerably low water contents, as estimated from isothermal adsorption curves for water vapor[1–3] and the refractive index obtained by use of the interference light microscope and the laser light scattering photometer[4–5]. Lewis *et al.* suggested that such a low water content of the core and the cortex itself arises through compressive contraction of the surrounding cortex[6]. However, Gould *et al.*[7–11] recently proposed the hypothesis that the enclosed core is in a dehydrated state caused by the osmotic pressure and expansion of the cortex matrix in which electronegative peptidoglycan and its counterions are included. In relation to the physical properties of water in bacterial spores, it was shown in terms of dielectric measurements in our previous paper[2] that the typical properties of spores as mentioned above might be due to the fact that mobility of water molecules in the spores are more restricted than in the vegetative forms.

This paper presents the thermal properties in subzero temperature regions of the cell water in the *Bacillus cereus* spores in comparison with the vegetative cells of the same strain by the use of a differential scanning calorimeter. The freezing point of intracellular water is depressed by the presence of solute materials, while that of water in the outerpart of the cells is less depressed for lack of the solute effect. Therefore, measurement of the amount of subzero freezing water makes it possible to distinguish between the intra- and extracellular water. On the other hand, part of the cell water is known to remain unfrozen down to considerably low temperatures. This unfreezable water seems to contain tightly bound water molecules and water molecules with restricted mobility. The sample frozen at low temperatures starts melting around at −20°C and this melting process

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completes around 0°C during the warming process of differential scanning calorimetry (DSC). During this warming process the sample consists of ice and liquid solution phase different in composition at each temperature, giving rise to the phase diagram obtained by plotting the melting temperatures against the water contents of the solution phase. The liquid water content at subzero temperatures is obtained by adding the water fraction melted by warming up to each temperature to the unfreezable portion which remained unfrozen during the previous freezing period. Both unfreezable and subzero freezable water fractions can be estimated by measuring the integral and differential heat of melting on DSC curves as described later.

2. EXPERIMENTAL

2.1 Microorganisms

Bacillus cereus IFO 1166 was used throughout the experiments. The spores of this strain were obtained from a 48h shaking culture at 30°C in a medium containing glucose, sodium-L-glutamate, yeast extract and salts[12]. The spores were purified by discontinuous density gradient centrifugation at 10,000rpm for 1h through the Urografin solution (20cm^3 of 'Urografin 60%' +6cm^3 of water)[13-14]. 'Urografin 60%' was purchased from Schering AG, Berlin. The vegetative cells of the same strain were obtained from a 6–8h shaking culture in the logarithmic phase of growth under the same conditions as with the spores. Both samples were washed several times with distilled water, stored at 4°C in suspension and washed again 2 or 3 times prior to use.

2.2 Preparation of the Coat and Soluble Fraction of the Spores

The spores prepared as mentioned above were disrupted by shaking 5 times for 1 min at intervals in the Braun cell disintegrator with glass beads of 0.1 mm in diameter. After removal of glass beads, the spore coat was precipitated and separated by centrifugation (6,000rpm for 10 min) from the solution mixture thus obtained, and washed several times with distilled water. The supernatant fluid was freeze-dried and used as a soluble fraction for the DSC measurement after the addition of an appropriate amount of water.

2.3 DSC Measurements and Construction of Phase Diagrams from DSC Curves

Phase diagrams were constructed basically according to the method for purity determination by the use of differential scanning calorimetry based on melting point depression of impure materials[15-16].

The heat-flux differential scanning calorimetry for the samples mentioned above was performed at a heating rate of 0.36 K/min after the preceding cooling down to −50°C at a rate of about 5 K/min using the low temperature type SSC 544 available from Daini-Seikosha Co. Ld. Thirty milligrams of the samples containing about 80% cell water were packed in tightly sealed sample vessels to eliminate the evaporation loss.

A typical example of DSC curve for the spores was given in Fig. 1 to illustrate the method for determination of the base line and real sample temperatures. The tentative base line AB drawn by extrapolating the lower temperature portion of the DSC curve did not intersect the DSC curve above 0°C. The difference BC between the tentative base line and the DSC curve is due to increase in the heat capacity caused by ice melting. Thus, a new base line was drawn by correcting for the heat capacity increase corresponding to the amount of water melted at each temperature. The final base line AQRC was obtained by repeating the correction procedure using a renewed base line in each step until successive steps converge. The sample temperature at a given point P on the DSC

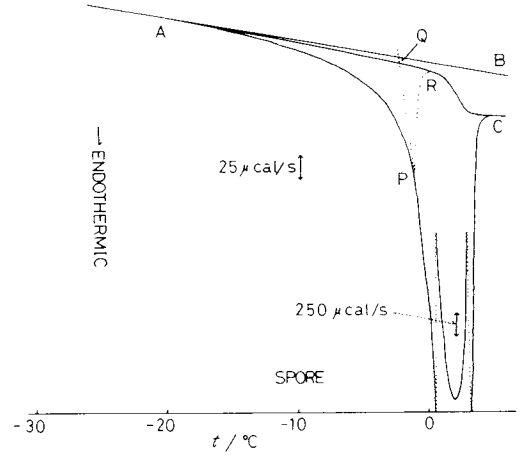

Fig. 1. Drawing of the base line and estimation of the sample temperature.

curve is given as the intersection of the line PQ and the base line AQC, since the sample temperature lags behind the temperature of the heat block due to the thermal resistance between the sample vessel and the heat block. The lag time can be estimated from the linearly rising portion of the DSC melting curve for pure water.

The amount of water melted below the point P is equal to the area bounded by the DSC curve AP, the exponential curve PR and the base line RQA. The exponential curve PR can be drawn using the time constant obtained from the exponentially falling portion which emerges on the DSC curve for pure water after completion of ice melting. The entire DSC curve was sectioned into small parts with an interval of 0.18 K (equivalent to the time interval of 30s), the temperature and the area corresponding to each point on the curve being determined in such a way as mentioned above. Thus, phase diagrams were constructed by the plot of temperatures against water contents obtained from each area. The calculation was performed using HITAC 8800/8700 computer system, the University of Tokyo.

The validity of this method was examined by the use of standard NaCl solutions at 2.4% and 4.8%. During warming process of the pre-frozen NaCl solution, a second small endothermic peak due to the melting of eutectic mixture is observed on DSC curves around at −22°C in addition to the main peak due to ice melting. The second peak was not taken into consideration as such in estimating water fraction from subdivided areas of the main peak obtained with those dilute standard solutions. The phase diagrams thus constructed for NaCl aqueous solutions were drawn in Fig. 2 which shows good agreement with the thermodynamic values cited from literature.

2.4 Water Vapor Sorption Isotherms

The samples of 100−200mg in weight were placed over the saturated solutions of several inorganic salts in desiccators at 30°C for 6 days until equilibrium was attained.

2.5 Determination of Water Content

The total water contents of the samples were determined as weight loss of the samples observed when dried at 105°C for 5h. Unfreezable water contents were obtained from the difference between the total amount of water determined as above and the amount of freezable water calculated from the total peak area of the DSC curve.

3. RESULTS AND DISCUSSION

Fig. 3 shows the phase diagrams constructed from the DSC curves for ice melting with the spores and vegetative cells of *B. cereus*. As seen from the figure, the cell water was divided into three parts on the curves. The higher water content regions above 40% (on wet basis) with the spores and 60% with the vegetative cells fail to cause any observable freezing point depression, because the cell water in this region is extracellular as to its

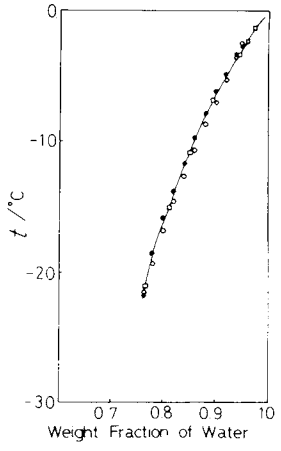

Fig. 2. Phase diagram for NaCl solutions.
●: 2.4%, ○: 4.8%,
⊣⊢: Values from literature

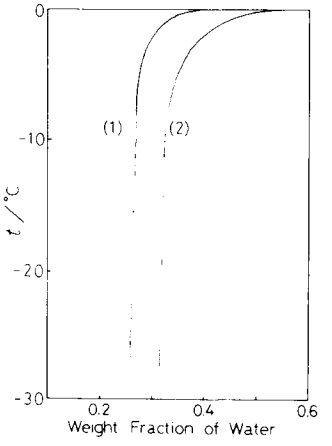

Fig. 3. Phase diagrams for the spores and vegetative cells of *B. cereus*.
(1): Spore, (2): Vegetative cell

location. The freezing point depression was also undetected in the lower water content regions below 26% with the spores and 32% with the vegetative cells, because water in this region is unfreezable in the observed temperature range. The intermediate regions in which the temperature vs. water content curves deviate from 0°C give rise to detectable freezing point depressions due to the solution effect of intracellular materials. The cell water in those intermediate region is to be frozen at some subzero temperature. When expressed on dry basis, the contents of such freezable water were 32% in the spores and 103% in the vegetative cells, while the unfreezable water fractions were estimated as 35% in the spores and 47% in the vegetative cells.

Fig. 4 shows the phase diagrams for the spore coat and soluble fraction prepared from the disrupted spores as mentioned in EXPERIMENTAL. The spore coat gave a curve similar in shape to that of the intact spores shown in Fig. 3, while the soluble fraction resulted in freezing point depression as expected over wider ranges of water content (concentration). These facts show that the phase behavior of the intact spores reflects that of the spore coat rather than the soluble fraction which has been contained in the core and cortex parts of the spores before disruption. It seems that the soluble fraction does not appreciably contribute to the thermal behavior of the intact spores in the subzero temperature range, since it is in a highly dehydrated state in the spores as proposed by a number of investigators.

The water vapor sorption isotherms of the spores and the vegetative cells are given in Fig. 5, indicating that the unfreezable water in both samples was in equilibrium with vapor pressures below 95% in relative humidity at the temperature employed.

The intracellular freezable water is regarded as making a solvent for various cell constituents and playing a significant role for cell metabolism. The results that the spores are appreciably lower in their freezable water content as compared with the vegetative forms (Fig. 3) and that such freezable water is mainly contained in the spore coat region as mentioned above (Fig. 4) may be related to the characteristic dormancy and heat resistivity of the spores.

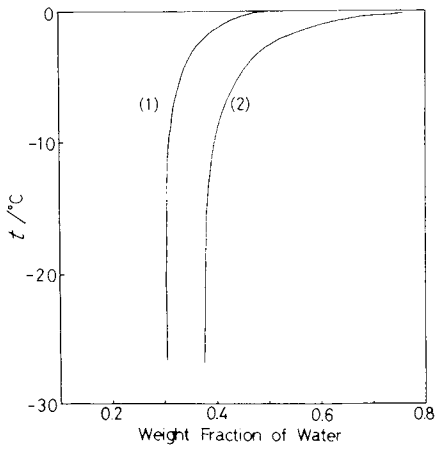

Fig. 4. Phase diagrams for the spore coat and soluble fraction.
(1): Spore coat, (2): Soluble fraction

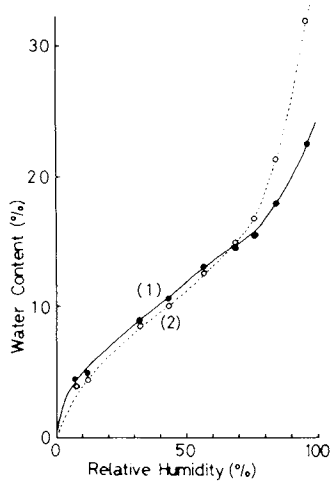

Fig. 5. Sorption isotherms for the spores and vegetative cells of *B. cereus*.
(1): Spore, (2): Vegetative cell

More recently, we reported that the 56°C peak on the DSC curve for the wet spore coat agrees with that for the wet spores and that the heat activation of bacterial spores may be attributed to the thermal denaturation of the spore coat at that temperature[17-18]. Since it was observed that the denaturation temperature of spore coat shifted to lower ranges with increase in water contents (data to be published elsewhere), the freezable water located in the spore coat region seems to facilitate the heat activation of the spores.

The amount of unfreezable water was found as mentioned above smaller in the spores than in the

vegetative cells. It seems that the presence of unfreezable water in smaller quantity may also favor the maintenance of dormancy and resistivity of spores. The unfreezable water has considerably high vapor pressures as shown in Fig. 5 and it has been known that the physiological activity, *e.g.* the respiration rate of partially dried yeast cells, develops in those regions of higher vapor pressure[19].

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〖会員の頁〗

★生物カロリメトリー国際シンポジウム

(International Symposium on Bio-Calorimetry)

1981年9月20日～26日，ソ連邦グルジア共和国のツビリッシで開催される。参加者数は約100名に制限されている。

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★第8回熱物性ヨーロッパ会議

(8-th European Conference on the Thermophysical Properties)

1982年9月27日～10月1日，西ドイツのBaden-Badenで開催。会議のChairmanおよび連絡先は下記のとおりである。

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なお期間中に装置の展示も行われる。

（以上　徂徠道夫）