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LUMINOMETRIC AND DIFFERENTIAL SCANNING CALORIMETRY (DSC) STUDIES ON HEAT- AND RADIATION INACTIVATION OF *BACILLUS SUBTILIS luxAB* SPORES*

J. FARKAS, É. ANDRÁSSY, Z. FORMANEK AND L. MÉSZÁROS

Department of Refrigeration and Livestock Products' Technology,
Szent István University,
Ménesi út 45, H-1118 Budapest, Hungary

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A bioluminescent derivative of *Bacillus subtilis* containing a plasmid encoding a *luxAB* fusion under control of a vegetative promoter and gives bioluminescence upon addition of an exogenous long-chain aldehyde has been used as test organism. Its spore populations have been produced and their heat- and radiation survival curves established. Heat-sensitization effect of pre-irradiation of spores was proven not only by colony counting but also with differential scanning calorimetry. Under a linearly programmed temperature increase, the heat destruction of spores surviving 2.5 kGy gamma irradiation resulted in at a few centigrade lower temperature than that of untreated spores. Heat denaturation endotherms in the DSC-thermogram of irradiated spores were shifted to lower temperatures as well. Comparative turbidimetric, luminometric and phase-contrast microscopic studies of untreated, heat-treated and irradiated spore populations showed that the kinetics of germination and the light emission during germination of radiation-inactivated spores were the same as those of untreated spores, revealing that the pre-formed luciferase enzyme packaged into the spores during sporulation remained intact after an irradiation dose causing 90% decrease in number of colony forming spores. Therefore, in contrast to heat-treated spores, the initial bioluminescence reading upon germination of irradiated spores does not reflect the viable count of their population.

Keywords: *Bacillus subtilis*, *luxAB* spores, luciferase enzyme, gamma-irradiation, bioluminescence, differential scanning calorimetry

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Introduction

High resistance of bacterial spores against all antimicrobial treatments is of major importance for the efficacy of technologies aiming microbial safety and storage stability of foods. A recent development for monitoring test-organisms and studying effects of inimical processes to them involves luminescence, conferred by introduction of bioluminescence genes into model microbial strains. The genes encoding the proteins required for a bioluminescent phenotype in bacteria all reside on a single operon, the *lux* operon. Two of the substrates for the bioluminescent reaction – reduced flavin mononucleotide, and molecular oxygen – are both readily available; all that is required is to produce light from the flavin-mediated oxidation of a long-chain aliphatic aldehyde catalyzed by the bacterial luciferase enzyme. The luciferase genes alone (*luxA* and *luxB*) may be introduced into a bacterium either to be constitutively expressed or as a reporter of gene expression and bioluminescence is induced following the addition of exogenous aldehyde. Luminescence offers considerable advantages over other marker systems, as it quantifies the metabolic activity of the cell and can provide means of *in situ*, non-extractive detection.

Differential scanning calorimetry (DSC) is an established thermal analysis method, particularly suited as a tool for studying various heat related phenomena in foods and their components under dynamic temperature conditions [1]. DSC has been applied also to study thermal properties of dormant bacterial spores with reference to heat activation and inactivation [2–4].

Using a bioluminescent (*luxAB)* derivative of *B. subtilis*, the objectives of our studies were to investigate thermal transitions in bacterial spores by differential scanning microcalorimetry as compared to the loss of viability during heat treatment and its relation to the heat-sensitization of spores by gamma irradiation. Germination studies were performed to compare changes of *in vivo* bioluminescence of untreated, heat-treated and irradiated populations of the test organism in a growth medium

Materials and methods

A bioluminescent derivative of *Bacillus subtilis* which contains a plasmid *pSB340* encoding a *luxAB* fusion under control of vegetative promoter (P43) [5] was used as test-organism. The strain was obtained from the Department of Applied Biochemistry and Food Science of the University of Nottingham, U.K. Its mature, dormant spores are “dark”, because they have no detectable metabolism. However, germinating spores and vegetative cells give bioluminescence upon addition of an

exogenous long-chain aldehyde [6, 7]. The plasmids also confer erythromycin resistance on the spores.

Spore populations were produced on modified Schäffer's medium with a mineral supplement. The inoculated agar plates were incubated at 30 °C until the cultures had 99% phase-bright spores as examined by phase-contrast light microscopy. Spores were washed from agar plates with cold, sterile distilled water and centrifuged in a Beckman J2-21 centrifuge at 4000×g for 20 min at 5 ºC. The spore pellet was re-suspended and debris of sporangia was digested by lysozyme of 0.1 mg/ml (Reanal, Budapest, Hungary) at 5 ºC for 24 hours. After the lysozyme treatment the washing procedure was repeated 4 times. The heat survival at 100 ºC and gamma radiation resistance were established in 1/15 M Sörensen's phosphate buffer at pH 7.0.

Gamma irradiation was performed aerobically by a self-shielded ^{60}Co-radiation source (type RH-gamma-30) at the Central Food Research Institute, Budapest.

Isothermal heating in a boiling water bath was applied to determine heat-survival curve of spores of the test-organism.

Radiation- and heat-survivals were estimated on serial dilutions (diluent: 0.1% pepton and 0.9% NaCl in distilled water) of samples of spore suspensions taken during treatments by plating them into nutrient agar with or without erythromycin of 1 mg/l (Merck, Art. 3615), and incubated at 30 ºC for 96 hours.

DSC profiles of dense suspensions of untreated and irradiated spores were determined in a "SETARAM Micro-DSC III" microcalorimeter. Portions of 0.9 ml-s of buffered dense (approx. 10^{10} spores/ml) suspension of untreated or irradiated (2.5 kGy) spores were dispensed into hermetically closed stainless-steel sample holders of the microcalorimeter interfaced to a computer for data acquisition and data processing, then heated up from 20 to 120 ºC at a scanning rate of 0.5 ºC/min and using the same volume of cell-free buffer as reference sample. After heating, the sample holders were cooled down to their initial temperature and reheated at the same scanning rate as in the first run to investigate the reversibility of heat transitions observed at the first run.

Thermal death of unirradiated and irradiated spores parallel with the DSC-experiments was estimated by heating buffered spore suspensions of approx. 10^8 CFU/ml under a programmed heat treatment with a similar heating rate as in the MicroDSC calorimeter. During heating samples were removed at predetermined temperatures, cooled on ice and survivors were counted as described above.

Germination and outgrowth of untreated, heat-treated (100 ºC for 15 min) and irradiated (2.5 kGy) spore populations, respectively, were followed during incubation in an erythromycin-containing nutrient broth at 30 ºC, both by absorbance measurements in a Beckman DU-64 spectrophotometer at 520 nm and by measuring the light emission as described above. Changes in the cell-state distribution of the cultures during the

process of germination and growth were estimated periodically by phase-contrast microscopy, distinguishing visually the phase bright (refractile) spores, phase dark (germinated) spores, outgrowing cells, and vegetative cells.

Bioluminescence (luciferase) activities were measured by a "BioOrbit 1251" luminometer, integrating the arbitrary light output values (mVs) for the first 10 sec interval during light emission as an effect of addition of 50 µl of 1% nonanal (Merck) solution (in EtOH) to 0.3 ml aliquots of the suspension of the test organism.

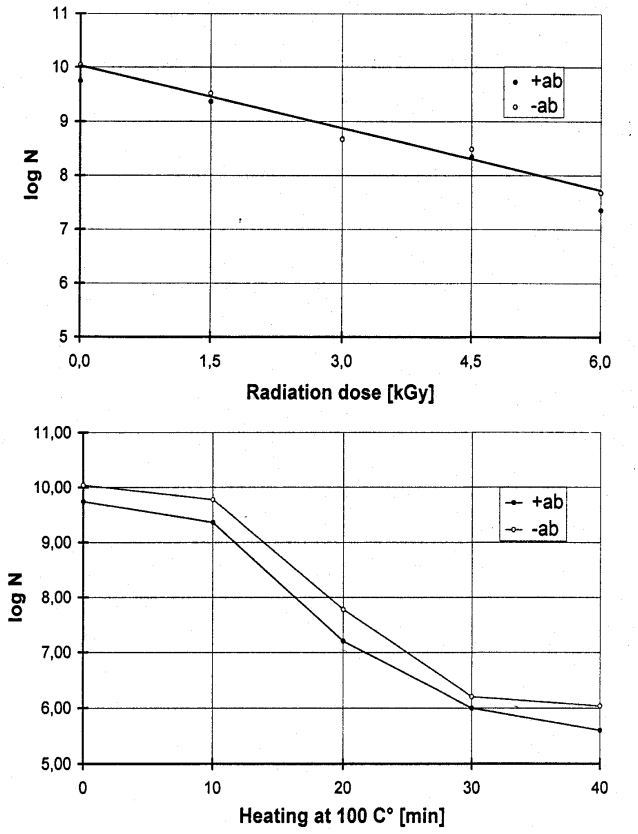

Fig. 1. Radiation- and heat survival curves of *Bacillus subtilis luxAB* spores

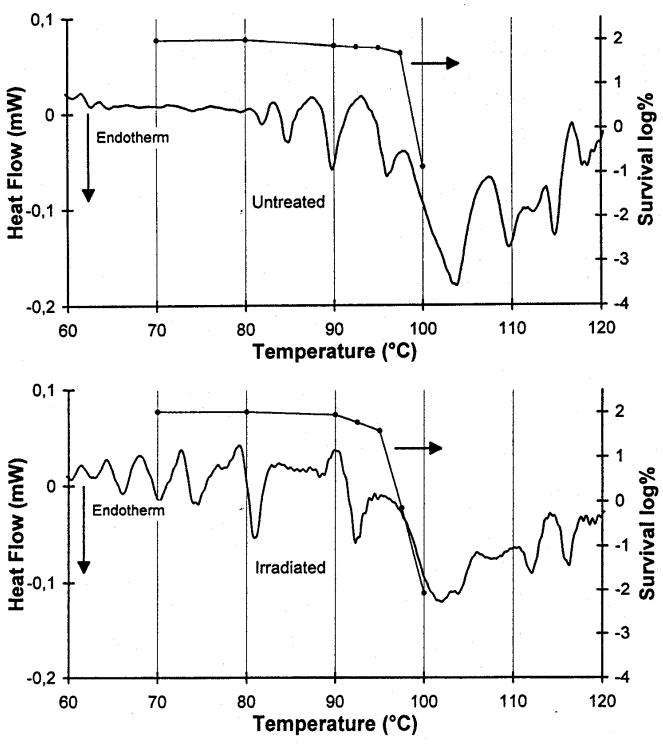

Fig. 2. Effect of pre-irradiation (2.5 kGy dose) on heat destruction (survival log %) and DSC-thermogram (heat flow, mW) of *Bacillus subtilis luxAB* spores

Results

Relation between bioluminescence activity and the viable cell count of vegetative cells of Bacillus subtilis lux AB *strain*

The logarithmic viable cell counts (CFU/ml) of serial dilutions of an overnight culture of the test organism and the logaritmic luminometric intensity (log mVs) of the samples showed good linear correlation expressed in the regression equation:

$$\log mVs = 0.89 \log cfu - 2.71$$
$$r = 0.990$$

in the range of 10^2–10^9 CFU/ml viable cell counts investigated.

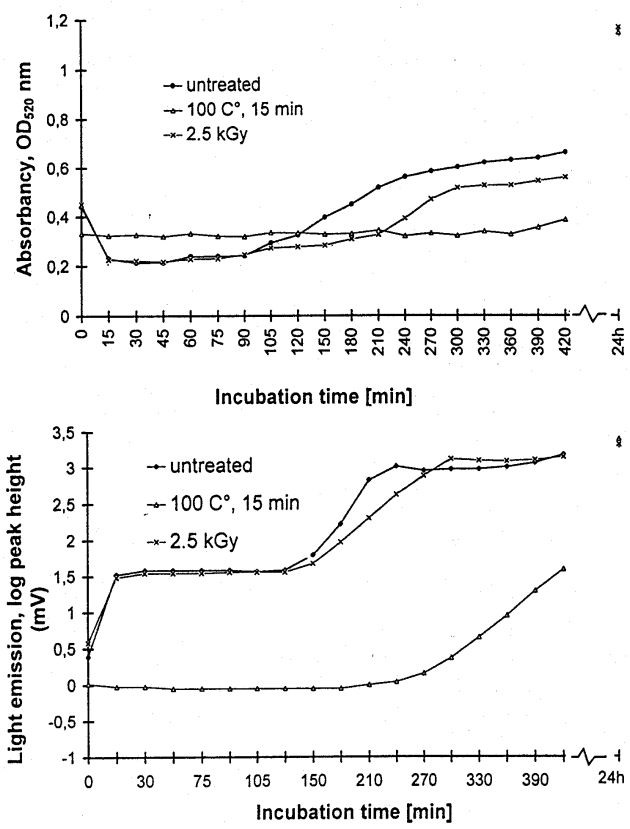

Fig. 3. Turbidimetric (top graph) and luminometric (bottom graph) investigations of spore germination, outgrowth and vegetative growth of *Bacillus subtilis luxAB* as affected by a heat treatment (100 °C, 15 min) or irradiation (2.5 kGy) of spores

Radiation- and heat-resistance of spores of the test organism

Radiation- and heat-survival curves are compared in Figure 1. Radiation inactivation showed a semilogarithmically straight-line survival kinetics with a D_{10}-value of approx. 2.5 kGy, whereas the heat-survival curve had a sigmoidal character. The presence (+ab) or absence (–ab) of the antibioticum erythromycin in the recovery medium did not influence the spore survival.

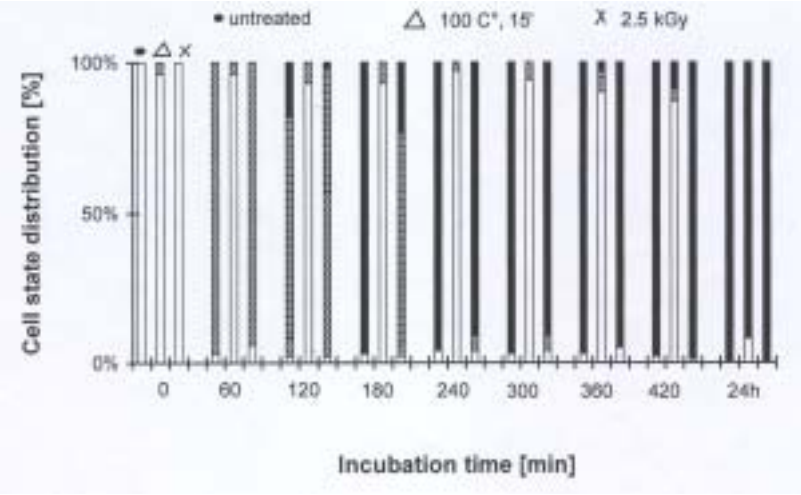

Fig. 4. Changes in cell-state distribution during incubation of *Bacillus subtilis luxAB* in nutrient broth at 30 °C.
Refractive spores; dark (germinated) spores; outgrowing cells; vegetative cells

Characteristics of DSC-thermograms of dense suspentions of unirradiated and irradiated B. subtilis luxAB *spores*

The initial-scan profile showed several endothermic transitions whereas no transitions were noted in the re-scan profile of the spores, indicating that all of the initial scan transitions were irreversible. Difference DSC-thermograms obtained by substracting the re-scan from the initial scan for untreated spores or that irradiated with 2.5 kGy, a dose killing 90% of the spore population, respectively, are shown in Fig. 2, together with the loss of viability of the spores under a heat treatment with the same heating rate as that of the microcalorimetry. The onset of the major endothermic transition with a peak of 104 ºC for the untreated spores and 102 ºC for the irradiated ones coincided with the initial temperatures of the loss of viability, resulting in 90% inactivation of colony forming units when reaching 99 ºC in case of untreated spores, and 97 ºC in case of the radiation survivors. As an effect of pre-irradiation, endotherms preceeding the "DSC-peak of heat killing" were shifted even more to lower temperatures.

Retained germinability and luciferase activity of irradiated spores as compared to those of untreated or heat-treated spore populations

Germination of luxAB spores runs parallel with their luxAB expression because electron transport starts in a very early stage of germination. Comparative turbidimetric, luminometric and phase-contrast microscopic studies (Figs 3 and 4, respectively) of untreated, heat-treated and irradiated spore populations showed that kinetics of germination and the light emission during germination of radiation-inactivated spores were the same as those of untreated spores, revealing that the pre-formed luciferase packaged into the spores during sporulation remained intact after a radiation dose causing 90% decrease in number of colony forming spores.

Discussion

The light output of untreated populations of the bioluminescent recombinant strain studied correlates well with their viable cell counts in a wide range of population densities and detecting luminescence of the bioluminescent test-organisms is a much more sensitive technique than measuring the population density by turbidimetry.

The comparative turbidimetric, luminometric and phase-contrast microscopic studies of untreated, heat-treated and irradiated spore populations (both the latter two are causing 90 % decrease in number of colony-forming spores) showed that unlike heat-inactivated spores, the radiation-inactivated spores produced the same light emission during germination as the untreated spores. This means that the pre-formed luciferase “packaged” into the spores during sporulation remained intact after irradiation. Therefore, unlike in case of heat-treated spores or those exposed to ethylene oxide [7], the bioluminescence of irradiated spores does not reflect the viable count of their population and the bioluminescence of the test organism cannot be used as biological indicator of a radiation decontamination treatment. It can be seen from Fig. 3 that *in vivo* bioluminescence which appears to be associated with the various stages of transition of spores to vegetative cells reflects more sensitively the metabolic activity during outgrowth and vegetative development stages than the traditional techniques. The production of germination-dependent bioluminescence from lux containing spores has been shown to be a sensitive real-time monitor of the germination and outgrowth process.

The DSC scans show larger numbers of distinct thermal transitions than those reported earlier for *B. megaterium* [4] and *Bacillus cereus* T [8]. Under a linearly programmed temperature increase, the heat destruction of spores surviving 2.5 kGy gamma irradiation was a few ºC lower than that of untreated spores. Heat denaturation

endotherms in the DSC-thermogram of irradiated spores were shifted to lower temperatures as well. Thus, a heat-sensitization of bacterial spores by pre-irradiation, known for many years from viability studies, could be noted also from the thermograms of the spore biomasses. Endothermic transitions in the DSC profiles of H_2O_2-treated spores of *B. megaterium* occurred also at lower temperatures than those for untreated spores [9]. Since the lethal action of both H_2O_2 and ionising radiation involves formation of hydroxyl radicals, which damage nucleic acid, proteins and lipids, the mechanism of heat sensitization might be also similar. The heat sensitization effect of irradiation seems to be related to a partial re-hydration and perhaps a certain mobilization of minerals of the spore protoplast as a consequence of radiation damage of the spore cortex [8], a characteristic structural component of spores, which has a major role in the maintenance of "dehydrated" state of spore-protoplast and thereby the heat resistance of spores [10].

The thermal characteristics observed at lower temperatures than the commencement of thermal inactivation may be attributed to heat activation [2–4].

The DSC thermogram of vegetative bacterial cells has a reversible endothermic peak after immediate re-scan [11–14]. Such reversible transitions could not be observed in the re-scanned thermograms of spores. A reason for this could be that calcium-DPA intercalated with the DNA in spores [15], or binding the spore DNA with small, acid-soluble spore proteins [16] are changing its thermal properties.

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