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Sporicidal interactions of ultraviolet irradiation and hydrogen peroxide related to aseptic technology

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Abstract

Synergistic UV–H_2O_2 spore killing is widely used in sterilization of packaging materials in aseptic processing and packaging, but the mechanisms for UV–H_2O_2 sporicidal activity are poorly defined. We found that UV–H_2O_2 spore killing was not highly temperature sensitive, in contrast to findings for moist heat killing, suggestive that synergistic killing does not involve cooperative reactions with high enthalpies of activation as does moist heat killing. Delayed synergistic UV–H_2O_2 killing of spores of *Bacillus cereus* var. *terminalis* initially exposed to H_2O_2 and then dried on glass surfaces for some hours before UV irradiation is described. Our overall findings were interpreted in terms of a view of synergistic killing involving concentrative uptake of H_2O_2 by dormant spores, formation of H_2O_2 adducts with spore core components and absorption of UV radiation by the adducts with resulting damage to multiple spore enzymes, including the glucose-phosphotransferase for sugar uptake, but not to spore DNA.

Keywords: Aseptic processing and packaging; Bacterial spores; Ultraviolet-peroxide killing; Enzyme inactivation; Hydrostatic pressure

1. Introduction

Hydrogen peroxide and ultraviolet (UV) irradiation are used extensively in a variety of industrial sterilization or disinfection processes. Their use is likely to grow because of expanding use of UV treatment, including pulsed UV, for drinking and process water and increased applications for aseptic processing and packaging (APP). APP is currently of major importance in the food industry for delivery of safe food products with greatly extended shelf life and reduced need for refrigeration. The technology is especially important in parts of the world where refrigeration is not prevalent as a major means to preserve foods. Moreover, the technology is being used increasingly for non-food products, such as pharmaceuticals, when there is a need to ensure safety under a variety of use conditions, including prolonged storage. Moreover, with current concerns about bioterrorism, aseptic packaging and processing has appeal for ensuring supplies of safe food and water.

The initial development of aseptic processing and packaging (APP) was closely tied to ultra-high-temperature (UHT) procedures for processing liquid foods, especially milk, to yield essentially sterilized product. UHT procedures take advantage of the low Z values for heat killing of spores and other organisms. The Z value is related to the familiar Q_{10} value. Z values can be estimated from plots of log D values versus temperature, where the D value is the time required for killing of 90% of the population of the organisms being tested. In essence, the Z value is the temperature interval over which there is a 10-fold change in D value. Spore killing by moist heat generally has a Q_{10} of about 10, that is, a 10-fold increase in the rate of killing is associated with a 10 °C increase in the killing temperature, and also a Z value of about 10. The high Q_{10} for heat killing can be compared with Q_{10} values of about 2 for many chemical reactions ($Z = 33.2$ °C) or approximately 1 for diffusion-limited reactions. Thus, an increase in process temperature can result in a 10-fold increase in the rate of killing but only a two-fold increase in reactions such as the loss of vitamins. Killing can be accomplished in one-tenth the time at a 10° higher temperature with substantially less loss of nutritional and other desirable properties of the food or other product. This same principle applies to continuous sterilization processes widely used for sterilizing media for industrial fermentation. APP of particulate foods involving

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use of ohmic heating has been growing, and again, offers advantages in terms of product quality and extended shelf life over conventional heat processing or use of food preservatives.

The prime example of an APP food is so-called UHT milk, which initially undergoes ultrahigh-temperature-short-time sterilization with subsequent delivery and rapid sealing into containers sterilized by use of hydrogen peroxide. H_2O_2 has major advantage for sterilization of packaging in not leaving toxic residues in the food. Concentrated solutions of H_2O_2 at high temperatures of some 60–70 °C are generally used to increase sporicidal activity. In general, bacterial spores are more resistant to H_2O_2 than are vegetative cells, and so, are generally the major targets in peroxide sterilization. However, the difference in resistance to H_2O_2 between bacterial endospores and vegetative cells or mould spores is not nearly as great as is the difference for moist heat. Moreover, it appears that H_2O_2 killing of spores is fundamentally different from killing of vegetative cells, in which DNA appears to be the major target for damage. Spore DNA is protected against peroxide and UV damage by small, acid-soluble, spore proteins (SASP) which bind to DNA and protect it [14,15].

Packaging for APP can be sterilized by combinations of H_2O_2 treatment and ultraviolet irradiation taking advantage of the synergistic action of the combination initially described for bacterial spores [1] and then for vegetative cells [2]. In their original work, Bayliss and Waites [1,2] determined that bacteria had to be treated with the two agents together to achieve synergistic killing. It was difficult to define the nature of the synergistic interaction, although it appeared to occur within cells rather than outside. In other words, it was not due simply to radicalization of H_2O_2 in the environment and killing of cells by hydroxyl radicals ($OH^{\bullet}$). Possibly, such radicalization may occur within the cell cytoplasm. However, there is still no clear view of the basis for synergistic killing nor the main targets. Moreover, even though synergistic killing has been demonstrated for both vegetative cells and spores, it is well known that the mechanisms of peroxide killing and UV killing are different for the two cell types. H_2O_2 and UV are mutagenic for vegetative cells indicative of DNA damage and repair being important. However, they are generally not mutagenic for spores because of SASP binding to spore DNA to protect it against UV or peroxide damage. We have recently found that bacterial spores have very high capacities to take up H_2O_2 and to retain it during drying [11]. As a result, it was possible to separate H_2O_2 exposure and UV irradiation by as long as 24 h and demonstrate a type of delayed synergistic killing. This delayed action then allows for easier manipulation of the synergistic killing process in terms both of machine designs and experimental procedures.

In this paper, we present additional data on synergistic killing of bacterial spores and delayed synergy. In particular, we explore the effects of temperature on synergistic killing to estimate Z values for the process and relate the values to temperature effects on killing by peroxide alone or ultraviolet irradiation alone. We also present another example of enzyme damage in dormant spores caused by UV, H_2O_2 or the combined agents. Finally, based on the information available, we present a mechanistic view of UV–H_2O_2 killing of bacterial spores.

2. Materials and methods

2.1. Bacterial growth and spore preparation

Spores of *Bacillus megaterium* ATCC 19213 were prepared as described previously [6] from batch cultures grown aerobically in defined medium with limiting glucose. Spores of *B. cereus* var. *terminalis* were prepared similarly with use of the defined G medium described by Hashimoto et al. [5]. The spores were harvested by centrifugation after lysis of sporangia and purified by repeated centrifugation followed by removal of contaminating vegetative debris at the top of spore pellet. The final preparations consisted only of phase-bright spores. They were stored under USP 200 proof ethanol in the cold until used.

2.2. Killing assays

Spore killing was assessed by means of standard plate-count assays described previously [10] with at least 1:10 dilution of samples of treated spores in 1% (w/v) Difco peptone broth so that the H_2O_2 used would be neutralized by dilution and by association with peptides. Addition of catalase to the dilution medium did not improved recovery. UV irradiation with 254-nm radiation was by means of a Sterilaire Series unit (UVP Ultra-violet Products, Cambridge, UK). The unit has two 15-W mercury tubes producing mainly 254-nm UV light at an intensity of 1.6 mW/cm^2 at a distance of 30.5 cm. Output was measured with a UVX radiometer with a sensor calibrated for 254-nm radiation. For irradiation of aqueous suspensions, 5 ml of suspension was placed in a glass petri dish with area of 50 cm^2. 0.1-ml samples were withdrawn at intervals, diluted directly, and then samples from the dilution tubes were streak plated on trypticase-soy agar plates. The plates were incubated at 37 °C until colony formation was complete. For UV–H_2O_2 killing, the spores were first suspended in peroxide solution, usually 0.1% or 32.6 mM, and then immediately irradiated. The temperature of irradiation was controlled by keeping the petri dish on a solid, thermostated heat-block. Prior to the experiment, suspensions and solutions were brought to the desired temperature and then mixed. For irradiation of dried spores, aliquots of spore suspensions (0.04 ml of suspension containing 10^7–10^8 spores) were placed on standard glass coverslips (18 mm × 18 mm), placed on a heat block and dried at constant temperature. They were then irradiated directly. For counting of spores, the coverslips were transferred to dilution medium, the spores were removed from the coverslips by scraping, followed by homogenization and further dilution prior to plating.

2.3. Pressurization

H_2O_2 was added to spore suspensions in plastic syringes with attached Luer-Lok needles. The pointed ends of the needles were jabbed into rubber stoppers, and the syringes were placed in stainless steel chambers of the sort described previously [7]. The chambers were pressurized by use of an hydraulic pump with attached pressure gauge. At intervals, the chambers were rapidly decompressed, samples were taken, and the chambers