Systematic and Applied Microbiology 34 (2011) 139–147

Contents lists available at ScienceDirect

Systematic and Applied Microbiology

journal homepage: www.elsevier.de/syapm

Changes in bacterial cells induced by high pressure at subzero temperature

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ARTICLE INFO

Article history:
Received 21 July 2010

Keywords:
High pressure and subzero temperature
Pressure inactivation of bacteria
Mechanisms of inactivation
ATPase activity
Integrity of cytoplasmic membrane
Nucleic acid stability

ABSTRACT

The aim of this study was to examine the effect of pressure treatment at 193 MPa and −20 °C on membrane damage, changes in activity of membrane-bound ATPases and degradation of nucleic acids. The experiments were carried out with three *Escherichia coli* strains, in the exponential and stationary phases of growth, and differing in sensitivity to pressure. All *E. coli* strains subjected to pressure in the exponential phase of growth were inactivated by 6 log cycles, independently of the strain, which was accompanied by a total loss of ability to plasmolyse, an increase in irreversible membrane permeability to PI, and a reduction of cellular ATP by more than 80%. After pressure treatment of stationary phase cells, the relationship between the inactivation level and the ability to plasmolyse was not as evident as in the case of exponential phase cells. Pressure treatment of two strains of *E. coli* K-12 and Ec160/59 in the stationary phase that decreased viability by no more than one log cycle led only to reversible permeabilization of bacterial membranes, while irreversible permeabilization was observed in the pressure sensitive *E. coli* IBA72 strain phase that was inactivated by 4.6 log cycles. The reduction of ATP and changes in ATPase activity after pressure treatment of tested *E. coli* strains in the stationary phase of growth depended on the stage of inactivation of the particular strain. Electrophoretic analysis showed degradation of RNA isolated after pressure treatment from cells of all *E. coli* strains tested in the exponential phase of growth. The changes of RNA induced by pressure were not visible in the case of cells in the stationary phase. The degradation of DNA isolated from pressure treated *E. coli* strains from the exponential as well as from the stationary phase of growth was not observed.

Introduction

High hydrostatic pressure is a useful technique that allows most microorganisms to be inactivated at much lower temperatures than those used in thermal pasteurization of food. However, in the wider practical application of this method for preservation of food products, especially with pH close to neutral, it is important to be certain that the parameters of the process ensure the death of the most pressure resistant vegetative cells of bacteria. Microorganisms, even strains of the same species, differ markedly in their sensitivity to pressure. Among the strains belonging to the same species of food-borne pathogens and spoilage bacteria there are those that survive treatment with very high pressure and those that are pressure-sensitive [2,24,28,33,34]. Therefore, it is necessary to understand the mechanisms of pressure inactivation and the reasons for differences in pressure sensitivity of microorganisms. Studies in this area have been presented in some research papers and reviews. As a result, it has been suggested that pressure-induced death of bacteria is caused by cell membrane damage, denaturation of proteins, changes in enzyme activity and ribosomal conformation [5,11,17,32,39]. Furthermore, although nucleic acids are much more resistant to pressure than proteins [4], changes of the nucleoid in pressure treated cells were shown to be visible with the electron microscope [22,27].

There are numerous data available on the lethal effect of high pressure related to the loss of membrane integrity of bacterial cells. High pressure induces changes in lipid composition and their transition phase, as well as tighter packaging of acyl chains within the phospholipid bilayer [10,38]. Disappearance of some outer membrane proteins has also been shown [35]. All these changes can lead to reversible or irreversible membrane permeabilization, including the outer membranes of Gram-negative bacteria [13,21,23,31,33,44]. The loss of plasmolysis response [33], leakage of ATP, protein and some other important components occur as a result of loss of the cytoplasmic membrane integrity [23,37]. Changes in activity of membrane-bound ATPases after pressure treatment have also been shown [45]. According to Ulmer et al. [44] the death of bacteria is preceded by sublethal injury of cells. The loss of ability to grow on selective media of some pressure treated species of bacteria has been reported [1,13,15,18,21,34,40].

The available data show that the loss of membrane integrity induced by high pressure is highly responsible for inactivation of exponential phase bacterial cells [3,7,33,44]. However, as Pagan and Mackey [33] and Klotz et al. [23] report, the mechanisms of bacte-

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0723-2020/$ – see front matter © 2011 Elsevier GmbH. All rights reserved.
doi:10.1016/j.syapm.2010.09.007

rial death in the stationary phase of growth are more complicated and unclear, and apart from the loss of membrane integrity, other changes induced by pressure can lead to the loss of cell viability. This shows that further studies are still necessary for completing the explanation of the mechanisms of microorganism death under high pressure conditions. Furthermore, a new possibility for processing and preservation of food could be created by using high pressure below 0 °C. Such high pressure and low temperature conditions allow microorganisms to be inactivated more effectively than pressure treatment in a specific temperature range above 0 °C [14,16]. However, the changes in cells that take place under such conditions and their contribution to the death of microorganisms are recognized in a limited range. According to Moussa et al. [31] mechanisms of bacterial inactivation differ at room and subzero temperature.

The objective of this study was to investigate the relationship between sublethal injury of cells, membrane damage, changes in activity of membrane-bound ATPases, degradation of nucleic acids and viability loss of some bacteria after pressure treatment in the liquid state at subzero temperature. The loss of membrane integrity was assessed by measurement of uptake of propidium iodide (PI), the loss of ability to plasmolyse and ATP leaking from the cells. The experiments were carried out with bacteria, mainly *Escherichia coli* strains, from the exponential and stationary phase of growth with different sensitivity to pressure.

Materials and methods

Cultures and growth conditions

The following bacterial strains were used: *E. coli* K-12 PCM2560 (NCTC10538) from the Polish Collection of Microorganisms, Ludwik Hirszfeld Institute of Immunology and Experimental Therapy of the Polish Academy of Sciences, Wroclaw, Poland; *E. coli* IBA 72 (ATCC 11105) from the Research and Development Center for Biotechnology, Warsaw, Poland; *E. coli* CCUG 41424 and CCUG 11321 from the Culture Collection, University of Göteborg, Sweden; *E. coli* Ec160/59 and Ec 29/51 from the Czech National Collection of Type Cultures, Institute of Hygiene and Epidemiology, Prague, Czech Republic; *E. coli* MW (isolated from pork meat), *E. coli* M (isolated from milk), *E. coli* 7250 (isolated from cod meat) and *E. coli* 2140 (isolated from kefir), kindly provided by J.S.H. Laboratoria, Gdynia; *Pseudomonas fluorescens* WSRO 121 from the Collection of Dairy Cultures, Department of Microbiology, University of Warmia and Mazury, Olsztyn, Poland, and *P. fluorescens* L1 from the Department of Food Chemistry, Technology and Biotechnology, Gdansk University of Technology, Poland.

Inocula of strains were prepared by inoculating 100 mL of tryptone soy broth supplemented with 0.6% yeast extract (TSBYE) with 100 μL of liquid culture and incubating at 37 °C (*E. coli*) or 27 °C (*P. fluorescens*) for 24 h with shaking. Under these conditions the cells were in the stationary phase.

In order to obtain an appropriate growth phase of cells, 100 μL of the stationary phase culture were inoculated into 100 mL of fresh TSBYE and incubated with shaking at the optimal growth temperature. The bacterial growth was measured by determination of the optical density at 660 nm for 48 h. The middle, exponential and stationary phase were determined based on the growth curves obtained.

Preparation of cell suspensions

The cells in a particular phase of growth were centrifuged at 1300 × *g* for 20 min at 5 °C, and the pellets were resuspended in phosphate buffered saline (PBS) at pH 7.0 to give viable counts of approximately 10^8–10^9 CFU/mL.

Pressure treatment

The pressure was generated in an apparatus in similar way to that described by Hayakawa et al. [16], without using an oil pressure pump. The method is based on generating pressure as a result of the increasing volume of ice formed in a sealed vessel filled with water kept at subzero temperatures. Moreover, according to Bridgman [9], high pressure reduces the freezing point of water to –22 °C at 207.5 MPa. Therefore, above this temperature, the sample placed in a sealed vessel is affected by the pressure in an unfrozen state. The equipment used to generate pressure during the experiments was designed and constructed by Edward Dunajski at the Department of Food Chemistry, Technology and Biotechnology, Chemical Faculty, Gdansk University of Technology, Poland. The details of the procedure were previously described by Malinowska-Pańczyk et al. [29].

The samples were stored in an ice bath prior to determination of viable counts, and unpressurized samples were used as controls.

Enumeration of viable and injured cells

Pressure-treated samples and untreated control cell suspensions were serially diluted with buffered saline (pH 7.0). Dilutions were plated on the non-selective medium tryptone soy agar supplemented with 0.6% yeast extract (TSAYE), and the selective media TSAYE supplemented with 2.5% NaCl or MacConkey agar. The plates were incubated for 48 h at appropriate temperatures.

The data presented in the figures are average values of three replications with standard deviation. The differences between the treatments were evaluated statistically by analysis of variance (one-way procedure) using the program Statistica 8.0.

Measurement of osmotic response

Ability to plasmolyse in the stationary and exponential phase cells after pressure treatment was determined according to Pagan and Mackey [33] by measurements of optical density in PBS and PBS containing 0.75 M NaCl. The initial OD_{680} in PBS was approximately 0.2. The increase in OD_{680} is a reflection of the ability to plasmolyse and was calculated by subtracting the value obtained in PBS from that in PBS containing 0.75 M NaCl expressed as a percentage of the value obtained with PBS without NaCl.

Determination of the permeability of the cytoplasmic membrane with propidium iodide (PI)

Pressure-treated cells in the exponential or stationary phase, diluted in PBS to an OD_{600} of approximately 0.2, were stained with PI (Sigma–Aldrich), as described by Pagan and Mackey [33]. The fluorescence was measured using a spectrofluorimeter (Perkin–Elmer, model LS-55) at the excitation and emission wavelength of 495 and 615 nm, respectively. The slit width was 10 nm. PI was added before pressure treatment in order to also test for reversible permeabilization. The data were expressed as an increase in fluorescence related to the measurements obtained from unpressurized cells.

Measurement of intercellular ATP

Cell suspensions (1 mL) were centrifuged at 1300 × *g* for 20 min and the pellet was resuspended in 1 mL sterile distilled water. The amount of intracellular ATP in cells before and after pressure treatment was analyzed by using an ATP bioluminescent assay kit and ATP luminometer (HY-Lite, Merck, Germany). The ATP content was