AN ABSTRACT OF THE THESIS OF

Kalpesh K. Gandhi for the degree of Master of Science in Food Science and Technology presented on November 8, 2002
Title: *Bacillus subtilis* Endospore Coat Protein Solubilization Methods for Studying Effects of High Pressure Processing.

Abstract approved: _____

J. Antonio Torres

Spores of foodborne pathogens such as *Clostridium botulinum, Clostridium perfringens* and *Bacillus cereus* are widely distributed in nature. Presence of those spores in food products, particularly *C. botulinum* spores in vacuum packed, ready-to-eat low-acid products, is a great safety concern. The research here described is a first effort towards understanding the role of the spore coat proteins in the inactivation of bacterial spore using high pressure processing. This study proposes a coat protein solubilization methodology using non-ionic detergents minimizing protein damage and compatible with spectroscopy methods. The methodology developed here was compared with approaches proposed in the literature with respect to protein yield, protein fractions identified, amino acid composition and suitability with spectroscopy techniques for the further analysis of coat proteins.

Bacillus subtilis ATCC 6633 spore coat proteins were solubilized (n = 3) using octyl-β-D-glucopyranoside (OGP) at room temperature and urea/sodium dodecyl sulphate (UDS) at 37C and 70C. Analysis of variance (ANOVA) showed no significant (95% confidence) differences between the three repetitions of the three spore coat protein solubilization methods. Protein yield was significantly larger (95% confidence) when using UDS at 70C as compared to UDS at 37C. OGP gave the lowest protein yield but allowed

circular dichroism (CD) analysis of the spore coat protein solution with minimum blank signal. SDS-PAGE revealed that the UDS-70C coat protein solutions consisted of five major and six minor proteins ranging 6 to 65 kD while the OGP solution appear to consist of four major and nine minor bands in the same mw range. Amino acid analysis of the protein extracted by the OGP method was conducted using reverse phase HPLC (RP-HPLC) and compared with published information. The OGP spore coat protein solution showed a higher proportion of aspartate, glutamate, alanine and tyrosine.

Pressure, heat and time effects were studied on spore coat proteins obtained from untreated and pressure-treated *B. subtilis* ATCC 6633 spores. Pressure treatments of spores, and of extracted spore coat protein solutions, at 50 kpsi (345 mPa) and 85 kpsi (586 mPa) for 10 and 30 min at constant 85C along with appropriate heat- and pressure-only controls and untreated sample, were used to study the effect of pressure, heat and time on spore coat proteins. Both spore coat protein solubilization procedures showed a significant reduction in protein yield for pressure-only, heat-only and pressure/heat treated spores when compared with untreated spores. When OGP-solubilized proteins from untreated spores were pressure treated, SDS-PAGE profile showed an increasing overall band intensity with increasing pressure and time. In the case of protein solution obtained from pressure-treated spores the electrophoretic pattern showed the loss of higher molecular weight proteins.

The significance of this study is that for the first time we have observed extensive changes on spore coat proteins caused by pressure, as well as heat treatments. Future studies will examine what is the probable physiological role of the proteins damaged by these physical treatments. An advantage of the protein solubilization here developed will allow the application of spectroscopy techniques to characterize changes in spore coat proteins.

Bacillus subtilis Endospore Coat Protein Solubilization Methods for Studying

Effects of High Pressure Processing

by

Kalpesh K. Gandhi

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Master of Science

Presented November 8, 2002

Commencement June 2003

Master of Science thesis of <u>Kalpesh K. Gandhi</u> presented on <u>November 8, 2002.</u>
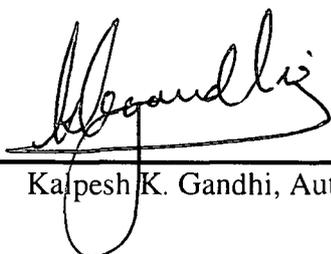
APPROVED:

Major Professor, representing Food Science and Technology

Head of the Department of Food Science and Technology

Dean of Graduate School

I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request

---

Kalpesh K. Gandhi, Author   11/15/'02

CONTRIBUTION OF AUTHORS

In Chapter 3, Dr. Anderson and Dr. Malencik contributed information on protein chemistry and analysis. Dr. Velázquez was involved with the writing and data interpretation of Chapter 4.

TABLE OF CONTENTS

TABLE OF CONTENTS (CONTINUED)

LIST OF FIGURES

Bacillus subtilis Endospore Coat Protein Solubilization Methods for Studying
Effects of High Pressure Processing

1. INTRODUCTION

Currently, the most effective and used method to inactivate enzymes in foodstuffs is the use of heat; however, there is an increasing interest in the protein denaturing ability of pressure. High hydrostatic pressure to process and preserve foods has been studied for over a century (Hoover et al., 1989). Thermal denaturation of proteins is caused by an enhanced vibration of molecules, leading to the breaking of intra- and intermolecular bonds. The mechanism of pressure denaturation, on the other hand, is governed by the principle of LeChatelier, which states that phenomena accompanied by a volume decrease are favored by pressure increase (Weemaes et al., 1996).

Recent advances in equipment and successful product development examples have lead to considerable commercial interest in High pressure processing (HPP) technology (Velazquez et al., 2002). Unlike thermal processing, HPP is an isostatic process, i.e., its effects are uniform and instantaneous throughout the food and do not follow a concentration gradient. Also, there is no waste generated and the process is environmentally friendly. The quality retention in HPP is related to the requirement for time-pressure treatments causing only minimum chemical changes. This capability becomes very attractive when processing natural and biologically active chemicals with disease-preventing and life-sustaining functions (Shukla, 1992).

The successful commercial introduction of avocado paste has set an HPP quality standard in the United States. Pressure-treated juices, currently sold elsewhere, are expected soon in the U.S. The developers of technologies for these juices and other products will need a thorough understanding of the mechanism for

the elimination of microbial pathogens and their toxins in food production, processing and storage while preserving the chemicals responsible for the claims on health promotion and life prolongation (Shukla, 1992). For example, HPP could be more widely applied if it were effective for bacterial spore inactivation. Products currently on the market rely on refrigeration, reduced water activity or low pH to prevent bacterial spore outgrowth.

Bacteria of the genera Bacillus and Clostridium form highly resistant endospores. Many of these bacteria are responsible for causing food poisoning. For example, *C. botulinum* is the causative organism of the potentially fatal disease botulism, because of the production of an extracellular toxin during spore germination. Strains of *B. cereus* and several Clostridia can also cause brief, but severe, bouts of diarrhea.

Many studies have shown the resistance of spores to pressure and other chemical and physical factors (Gould, 1983). Among the protective layers of a bacterial spore, the spore protein coats protect spores from mechanical damage and harmful chemicals, mainly by forming a permeability barrier to large molecules. Treatments, which break the coats, usually by disrupting disulphide bonds, allow access through the cortex of molecules such as multivalent cations, lysozyme and organic solvents.

The objective of this study were (1) to develop a coat protein solubilization methodology using non-ionic detergents and thus minimizing protein damage; (2) to compare the approach proposed with other available methods with respect to protein yield, protein composition and amino acid residue analysis; (3) to determine the compatibility of non-ionic detergents for structural analysis of coat protein using spectroscopic techniques such as circular dichroism (CD); and, (4) to study pressure, heat and time effects on spore coat proteins obtained from pre- and post-treated *Bacillus subtilis* endospores.

2. LITERATURE REVIEW

High pressure processing (HPP) is a non-thermal process that reduces spoilage microorganisms and bacterial pathogens in foods with minimum or no apparent changes in texture, color, and flavor (Cheftel 1995; Knorr 1993). Food preservation by HPP was first examined more than a century ago by Hite (1899). Although, his early research received little attention, the current demand for minimally processed, high quality and safe food products by consumers has renewed industry interest in HPP.

Research on HPP as a means for controlling or inactivating pathogens has shown that a 5-log reduction in many pathogens including *Salmonella typhimurium*, *Salmonella enteritidis*, *Listeria monocytogenes*, *Staphylococcus aureus* and *Vibrio parahaemolyticus* can be achieved by pressure treatments ranging from 275 to 700 mPa for 15 minutes or less (Mackey et al. 1994; Metrick et al. 1989; Patterson et al. 1995; Stewart et al. 1997; Styles et al. 1991).

2.1. Inactivation of spores by high pressure processing

Bacteria of the genera *Bacillus* and *Clostridium* form highly pressure resistant endospores. Many of these bacteria are responsible for food poisoning. For example, *C. botulinum* is the causative organism of the potentially fatal disease botulism caused by an extracellular toxin. Strains of *B. cereus* and several *Clostridia* can cause brief, but severe, bouts of diarrhea. The ability of bacterial spores to remain in a dormant state for a long period of time and resist physical and chemical agents is an important safety and quality problem to the food industry. Spores of foodborne pathogens such as *Clostridium botulinum*, *Clostridium perfringens* and *Bacillus cereus* are widely distributed in nature (Gould 1983 1989). Presence of those spores in food products, particularly *C. botulinum* spores in vacuum packed, ready-to-eat low-acid products, is a great safety concern.

Germination of these spores in temperature-abused products may lead to the production of toxins and cause foodborne illness if the product is consumed.

Bacterial spores, unlike vegetative cells, are much more resistant to pressure and may not be easily inactivated by HPP alone (Crawford et al. 1996; Mills et al. 1998; Reddy et al. 1999). The resistance varies depending on the type of microorganism, pressure level, processing temperatures, pH, water activity, and food composition (Cheftel and Culioli 1997; Raso et al. 1998). Nakayama et al. (1996) examined the pressure resistance of several types of *Bacillus* spores and found pressure treatments (981 mPa for 40 min or 588 mPa for 120 min) at 5 to 10 °C did not inactivate spores of *Bacillus stearothermophilus*, *Bacillus subtilis* or *Bacillus licheniformis*. Mills et al. (1998) reported no significant inactivation of *Clostridium sporogenes* spores treated at 600 mPa for 30 minutes at 20C. Recently, McClements et al. (2001) showed that the inactivation of *B. cereus* spores after 400 mPa for 25 minutes at 30C was only 0.45-log. These results indicate that meeting the Food and Drug Administration (FDA) requirement that low acid foods (pH above 4.5), held at room temperature in hermetically sealed containers, be free of *C. botulinum* remains a challenge for HPP. Fundamental studies on the mechanism of spore inactivation by pressure are needed to develop technologies meeting this FDA requirement in a safe and consistent manner. Currently, refrigeration, low pH or reduced water activity (aw) are used to prevent the outgrowth of spoilage and pathogenic bacterial spores in HPP products presently on the market.

Reddy et al. (1999) reported *C. botulinum* type E spores were reduced by approximately 5-log with a treatment of 827 mPa at 50-55 C for 5 min while no reduction of spores was observed at temperatures below 35C. Crawford et al. (1996) showed a 5-log reduction of *C. sporogenes* spores in chicken breast meat by a process of 689 mPa at 80°C for 20 min and at ambient temperature for 60 min. Inactivation of *C. sporogenes* spores, although less than 1-log decrease, was reported by a combined heat and pressure treatment (400 mPa at 60°C) for 30 min (Mills et al. 1998). Total inactivation of 10^{5-6} spores/mL *B. stearothermophilus* and

10^{6-7} spores/mL *B. licheniformis* was reached by 800 mPa for 3 min at 70 and 60C, respectively. *B. coagulans* spores were partially inactivated by 900 mPa for 5 min at 70C (Gola et al. 1996).

Although heated pressure and pressure cycling treatments have been reported to increase the effectiveness of HPP for spore inactivation, the efficacy seemed to depend on the types of spores, temperature, pH, pressure level, and processing time. Effects of these treatments on the resistance of *B. cereus*, *C. botulinum* and *C. sporogenes* spores to pressure have not been extensively studied. Further study is needed in order to develop an efficient HPP for their inactivation.

2.2. Spore Germination Induced by Pressure

Germination of a dormant spore into a vegetative cell results in the loss of its exceptional resistance to physicochemical agents. Inactivation of bacterial spores by HPP can occur in two steps: initial spore germination and subsequent inactivation of vegetative cells (Gould and Sale 1970; Clouston and Willis 1969).

Pressure processing can induce spore germination (Sale et al. 1970). Raso et al. (1998) reported that a pressurized treatment of 250 mPa for 15 minutes was optimal for inducing germination of spores for three *B. cereus* strains at 25C. Germination of *Bacillus* spores from various strains was reported to be more efficient at moderate (<500 mPa) than at higher pressure (>500 mPa) (Nakayama et al. 1996). This pressure-induced germination was most prominent at 40-50C and was virtually not observed below 10C. Increased germination of *Bacillus coagulans* spores was induced by pressures between 100 and 600 mPa at 45C with maximal inactivation of spores observed at 300 mPa (Gould and Sale 1970). Study on *B. subtilis* spores germination by low and high pressures showed that the same level of germination was observed at low (100 mPa) and high (500 to 600 mPa) pressures (Wuytack et al. 1998). However, spores germinated at 100 mPa were found more

sensitive than those germinated at 500 mPa to subsequent treatment at 600 mPa for 10 min. Small acid-soluble proteins (SASP) and dipicolinic acid (DPA) were released upon both low- and high-pressure germination, but SASP degradation, which normally accompanies nutrient-induced germination, occurred upon low-pressure germination but not upon high-pressure germination. The germination at 100 mPa resulted in rapid ATP generation, as in nutrient-induced germination, but no ATP was formed during germination at 600 mPa. These results suggest that spore germination can be initiated by low- and high-pressure treatments but is arrested at an early stage in the latter case.

The major challenge for HPP to inactivate bacterial spores is that relative large numbers of spores remain as ungerminated dormant spores (Sale et al. 1970). Increasing germination during the HPP will result in greater inactivation of spores by the process. Although previous studies indicated that pressures up to 600 mPa were able to induce spore germination, knowledge of factors affecting pressure-induced germination is still limited. Further study is required to define optimal parameters for inducing spore germination by pressure treatments.

2.3. Research background supporting experimental procedures

Spore coat proteins play an important role in spore resistance to inactivation. Studies on the pressure effects on the spore coat proteins of *B. subtilis*, *C. botulinum*, *C. sporogenes*, *C. perfringens* and *B. cereus* are not yet available to the extent of our knowledge.

2.4. Key Morphological Structures in Spores

All bacterial spores share the basic morphological features of *Bacilli* spores which include the core, inner membrane, cortex, outer membrane, coat and, in many species, the exosporium. After germination, the spore core surrounding the inner membrane becomes the vegetative cell protoplast. The core has a

macromolecular composition similar to vegetative cells except for some spore-specific enzymes, proteins and the absence of some vegetative cell enzymes. The spore core is substantially dehydrated when compared with vegetative cells. X-ray microanalysis studies show that more than 95% of the Ca^{+2} present in *B. megaterium* spores is located in the spore core accounting for 2-4% of the spore dry weight. This concentration exceeds the mole sum of all other inorganic cations. The calcium is thought to be entirely chelated by pyridine-2,6-dipicolinic acid (DPA), a spore-specific compound which accounts for 5-15% of the dry weight of the spores in approximately a 1:1 molar ratio with calcium. The dehydrated state is probably maintained by the mechanical properties of the cortex and may be one mechanism by which a spore achieves its resistance properties.

The enzymes for the glycolytic pathway and other metabolic activity are present in the spore core, as demonstrated by their activity in broken spore extracts, but in the intact spore, no activity is detected. The spore core also has a large amount of several small molecules in addition to DPA and divalent metal ions. These molecules include 3-phosphoglyceric acid and glutamic acid, which form the basis for the spore energy usage during the early stages of germination. The spore core is surrounded by two membranes with opposing polarities. The inner membrane, which originates as the forespore cell membrane, comprises ~80% of the spore phospholipid and 66% of the spore neutral lipid, and appears to be complete. The outer membrane, which arises from the cell membrane of the mother cell does not appear to be complete, as it does not contain enough material to completely enclose the spore.

The spore cortex is a peptidoglycan polymer located between the spore inner membrane and outer membranes. In contrast with the core, the cortex contains a high level of mobile ions. The low degree of cross-linking of the spore cortex allows glycan chains to move more freely with respect to one another, permitting greater changes in response to changes in pH and ionic concentrations than in vegetative cell walls. Secondly, the cortex possesses a greater net negative

charge than the vegetative peptidoglycan. Because of these features, the cortex is expected to show a comparatively greater volume change in response to ionic changes. The main function of the cortex appears to be in the maintenance of dormancy, by keeping the core dehydrated. The anisotropically expanded cortex model states that the cortex expands during sporulation, due to cleavage of peptidoglycan chains and subsequent mutual repulsion of the negatively charged glycan chains in the absence of divalent cations. This radial expansion of the cortex causes an inward expansion compressing the spore core. During germination, limited cleavage of the cortex causes collapse of the cortex polymer, allowing uptake of water by the core and subsequent germination events.

2.5.   Spore coats

The spore coats consist mainly of protein and occupy ~50% of the spore volume, 30-60% of the spore dry weight and 40-80% of spore proteins. The coat proteins make up to 50-80% of the total protein in mature spores (Spudich & Kornberg 1968; Goldman & Tipper 1978). Analysis of coat proteins in mature spores by extraction with sodium dodecyl sulphate/dithioerythritol followed by electrophoresis shows that the coat contains four major proteins-36K, 20K, 12K and 11K and about ten others-65K, 59K, 33K, 30K, 26K, 24K, 15K, 10.5K, 9K and 8K.Quantitative densitometry of gels stained with Coomassie blue showed that the four major proteins bands accounted for about 50% of the total integrated density in the stained profile (Jenkinson et al. 1981). Mitani and Kadota (1976) reported that sodium dodecyl sulphate (SDS) and dithiotheritol solubilized 85% of the *B. subtilis* spore coat proteins and that the major protein component had a molecular weight of 14,000. Serine was the major amino terminal residue. The residual insoluble material (15% of the total spore protein) was rich in cysteine and was probably also derived from the spore coats (Goldman and Tipper 1978).

Aronson and Fitz-James (1976) reported that about 80% of the *B. subtilis* T spore coat was solubilized by dithioerythritol (DTT) plus SDS. Smaller quantities of complex carbohydrates and lipid and in some species phosphorus-containing material are present within the coats. The coat is highly elastic and flexible and thus difficult to disrupt physically. The coat composition varies considerably among species, containing between one and more than twelve polypeptide.

Hayakawa et al. (1998) in their investigation to understand the mechanism of inactivation of heat tolerant spores of *Bacillus stearothermophilus* IFO 12550 proposed that sterilization was due to the physical breakdown of spore coat, and was induced by its physical permeability of water at high pressure and temperature. Among the three devices tested for rapid decompression after compression to 200 mPa, the link-motion system was the fastest and decreased the D-value of the spores from 3000 min (100C, one atmosphere) to 6 min, 11 min, and 17 min at 95, 85 and 75C, respectively.

The coat physiological role is to protect spores from mechanical damage and harmful chemicals, mainly by forming a permeability barrier to large molecules. Treatments, which damage the coats, usually by disrupting disulphide bonds, allow access through the cortex, of molecules such as multivalent cations, lysozyme and organic solvents. An exosporium can be seen outside the coats of spores of most species and contains protein as a major component.

2.6. Determination of changes in protein structure

New analytical techniques have revolutionized protein science by providing the high-resolution structures needed to establish structure-function relations. However, these techniques have not been used to our knowledge to investigate changes in the secondary structure of spore coat proteins subjected to pressure (Kelly and Price 1997). Structural studies can be performed with techniques such as infrared (IR), circular dichroism (CD), nuclear magnetic resonance (NMR) and

X-ray techniques. The complete three-dimensional structure of proteins at high resolution can be determined by X-ray crystallography, but this technique requires the molecule to form a well-ordered crystal, which is not possible for every protein (Kurt 1996). NMR spectroscopy can be applied to proteins in solution to determine their secondary structure. Secondary structure refers to the local structure of specific segments of the polypeptide chain. However, obtaining secondary structure from NMR data requires more material and effort because sequence specific resonance assignments are required. In addition, the interpretation of NMR spectra for large proteins is very complex, so current applications are limited to small proteins (~15-25 kDa).

NMR limitations have led to the development of alternative optical methods such as circular dichroism (CD) and Fourier Transform Infrared Spectroscopy (FTIR) spectroscopy, which cannot generate protein structures with atomic resolution but can provide structural information, especially changes in secondary structure. The use of IR and CD to determine the structure of biological macromolecules has dramatically expanded (Kurt 1996). CD spectroscopy is a form of light absorption that measures the difference in absorbance of right- and left-circularly polarized light by a substance (Price 1995). CD is very sensitive to the changes in secondary structure of proteins and polypeptides resulting from heat, chemical, high pressure and other treatments and can quantify protein-ligand, protein-protein and protein-nucleic acid interactions.

Information about the secondary structure can be obtained by CD spectroscopy in the 190-250 nm region with the peptide bond as the chromophore. Structures like α-helix, ß-sheet and random coil each give rise to a characteristic CD spectra shape and magnitude. The approximate fraction of each secondary structure type present can thus be determined by analyzing its CD spectrum. The CD signal in the 250-350 nm spectral region is sensitive to disulfide bonds and responds to changes in tertiary structure. The chromophores in this region are the aromatic amino acids and the CD signals produced are sensitive to the overall

tertiary structure of the protein (Kurt 1996; Venyaminov and Kalnin 1990; Krimm and Bandekar 1986). CD becomes a limited technique when the solvent containing the protein is incompatible in terms of characteristic signals or higher blank absorbance. The complex aggregating nature of membrane proteins limits the number of detergents solubilizing proteins with minimum denaturation and spectroscopic interference. Preliminary experiments conducted in our laboratory demonstrated that the methods used by Mitani and Kadota (1976), Goldman and Tipper (1978), Aronson and Fitz-James (1976) were incompatible with CD because of the interference by the detergents used.

The infrared spectra of polypeptides exhibit a number of amide bands representing different vibrational modes of the peptide bond. The peptide group gives up to 9 characteristic bands named amide A, B, I, II ... VII. The amide I band, between 1600 and 1700 cm^{-1}, is mainly associated with the C=O stretching vibration and is directly related to conformation. These vibrational modes are sensitive to hydrogen bonding and coupling between transition dipole of adjacent peptide bonds and hence are sensitive to secondary structure. Amide II band is more complex than Amide I and is conformationally sensitive. It is found in the 1510 and 1580 cm^{-1} region and results from the N-H bending vibration and from the C-N stretching vibration (Krimm and Bandekar 1986). Water molecules have an intense IR band in the region of the amide I band and this requires that samples are measured in D_2O or that the solvent resonance is subtracted digitally. Several attempts have been made to extract quantitative information on protein secondary structure from analyses of the amide I bands (Byler and Susi 1986; Susi and Byler 1988; Fry et al. 1988; Surewicz et al. 1993). Both curve-fitting and pattern recognition techniques have been applied with varying success. Since the potential sources of error in CD and FTIR analyses of secondary structure content are largely independent, the two methods are highly complementary and could be used in conjunction to increase accuracies.

High pressure Raman experiments on lysozyme have shown that a pressure of 500 mPa induces an irreversible denaturation and that above this pressure not only intramolecular but also intermolecular changes are important (Heremans and Wong 1985; Wong and Heremans 1988). These authors obtained also IR spectra of the enzyme chymotrypsinogen at pressures reaching 3000 mPa. An irreversible denaturation was observed at 760 mPa with the contributions of the random coil and turn conformational substructures increasing dramatically at the expense of the contributions of the α-helix and ß-sheet substructures. Furthermore, these authors noted that the pressure at which denaturation started was higher when pressure was applied more slowly. This is important as it suggests that the dynamic aspects of pressure processing affect changes in protein conformation and thus may play an important role in spore inactivation. Therefore, pressure pulsing and the rate of pressure increase and release may be factors in designing effective HPP processes for spore inactivation.

Cheung et al. (1999) followed the chemical changes of *B. subtilis* spore components using attenuated total reflection FTIR. Under atmospheric conditions, protein modifications began immediately following the initiation of germination, and ~26% of the spore protein (1279 cm^{-1}) was hydrolyzed in the first 15 min. Within the same time, the chelation state of DPA to Ca^{+2} (1380 cm^{-1}) had changed by 11%. After one hour, the values were 34% and 17%, respectively. In conclusion, FTIR and CD provide a good combination to monitor conformational changes in spore coat proteins due to pressure.

2.7. Effect of high pressure on spore structures

HPP can cause changes in bacterial spore coats and create a disturbance in cortex permeability, which results in the inability to maintain spore dormancy and causes death of cells under high pressure environments (Clouston and Wills 1970; Hoover et al. 1989). However, no study has been conducted to determine effects of

pressures on spore coat proteins. A recent study on *B. stearothermophilus* spores treated with 200 mPa for 60 min at 75C followed by a rapid decompression (1.60 ~ 1.65 msec) resulted in destruction of spores that looked like brittle fragments of glass or ceramics (Hayakawa et al. 1998). The authors speculated that the destruction was caused by the physical breakdown of the spore coat.

Spore DNA is well protected against many different types of damage through the saturation of the spore chromosome with a group of DNA-binding proteins termed α/β-type small, acid-soluble spore proteins (SASP) (Setlow 1994, 1995a). *Bacillus subtilis* spores lacking SASP were much more sensitive to dry heat than wild-type spores (Setlow 1995b). SASP's and dipicolinic acid (DPA) are involved in spore resistance to UV light and hydrogen peroxide. Wuytack et al. (1998) studied the fate of these compounds during pressure-induced germination of *Bacillus subtilis* spores at moderate (100 mPa) and high (500-600 mPa) pressures. They found that DPA was released upon both low- and high-pressure germination, but SASP degradation, which normally accompanies nutrient-induced germination, occurred only upon low-pressure germination. Spores germinated at 100 mPa were more sensitive to pressure (>200 mPa), UV light, and hydrogen peroxide than were those germinated at 600 mPa. They concluded that the resistance to pressure inactivation of 600 mPa could be due to SASP's since mutants deficient in α/β-type SASP's were more sensitive to inactivation. Moreover germination at 100 mPa resulted in rapid ATP generation, as is the case in nutrient induced germination, but no ATP was formed during germination at 600 mPa. These results suggest that spore germination can be initiated by low- and high-pressure treatments but is arrested at an early stage in the latter case.

Changes in spore structures caused by HPP remain unclear and the mechanism of a spore losing its dormancy due to pressure has not been fully investigated. This study investigates the effects of HPP on spore coat proteins to improve the understanding of the mechanism on spore injury and loss of dormancy caused by HPP. Such information will serve as the foundation for improvements in

HPP for effective inactivation of bacterial spores in food. To reach this goal, the objectives of this study were (1) to develop a coat protein solubilization methodology using non-ionic detergents; (2) to compare the approach proposed with other available methods with respect to protein yield, protein composition and amino acid residue analysis; (3) to determine the compatibility of non-ionic detergents for structural analysis of coat protein with spectroscopic techniques such as circular dichroism (CD); and, (4) to study pressure, heat and time effects on spore coat proteins obtained from pre- and post HPP-treated *Bacillus subtilis* endospores.

3. PROTEIN SOLUBILIZATION METHODS FOR STUDYING BACTERIAL ENDOSPORE COAT PROTEINS

Gandhi KK*, Anderson SR [†], Malencik DA [†] and Torres JA*
*Dept. of Food Science and Technology
[†]Dept. of Biophysics and Biochemistry
Oregon State University, Corvallis, OR 97331, USA

3.1. Abstract

Spore coat proteins from *Bacillus subtilis* ATTC 6633 spores were solubilized (n = 3) using octyl-β-D-glucopyranoside (OGP) at room temperature and urea/sodium dodecyl sulphate (UDS) at 37C and 70C. Protein concentration of the solutions obtained was estimated (n=3) by the modified Lowry method using bovine serum albumin (BSA) as standard. Amino acid analysis of the protein extracted by the OGP method was conducted using reverse phase HPLC (RP-HPLC). Analysis of variance showed no significant (95% confidence) differences between the three repetitions of the three spore coat protein solubilization methods. Protein yield was significantly larger (95% confidence) when using UDS at 70C as compared to 37C. OGP gave the lowest protein yield but allowed circular dichroism (CD) analysis of the spore coat protein solution with minimum background absorbance. SDS-PAGE revealed that the coat protein solutions consisted of three major and seven minor proteins ranging 5 to 66 kd. Amino acid analysis of the OGP spore coat protein solution showed a higher proportion of aspartate, glutamate, alanine and tyrosine.

3.2.   Introduction

Bacteria of the genera Bacillus and Clostridium form highly resistant endospores. Many of these bacteria are responsible for food poisoning. For example, *C. botulinum* is the causative organism of the potentially fatal disease botulism, caused by an extracellular toxin produced during spore germination. Strains of *B. cereus* and several Clostridia can also cause brief, but severe, bouts of diarrhea (USFDA 2000 & 2001; Roitt et al. 1998). Many studies have shown the resistance of spores to pressure and other physical and chemical factors (Sale et al. 1970; Gould 1983). Bacterial endospores are encased in a proteinaceous coat that provides resistance to lysozyme and harsh chemicals and influences the spore response to germinants (Henriques 2000). In conjunction with the exosporium, the coats protect the spore from a wide range of deleterious substances, particularly surfactants and enzymes (Warth 1978).

The coat proteins represent 50-80% of the total protein in mature spores (Spudich & Kornberg 1968; Goldman & Tipper 1978). Aronson & Fitz-James (1976) reported that about 80% of spore coat of *B. subtilis* T was solubilized by dithioerythritol (DTE) plus sodium dodecylsulfate (SDS). Hayakawa et al. (1998) in their investigation on the inactivation mechanism for heat tolerant spores of *B. stearothermophilus* IFO 12550 proposed that sterilization was caused by the physical breakdown of the spore coat and induced by the permeability to water at high pressure or temperature.

Detergents can solubilize membrane proteins by mimicking the lipid-bilayer environment. Micelles formed by detergents are analogous to the bilayers of the biological membranes. A layer of detergent molecules surrounds the hydrophobic regions of membrane proteins normally embedded in the membrane lipid bilayer and the hydrophilic portions are exposed to the aqueous medium. Complete detergent removal can cause aggregation due to the clustering of hydrophobic regions and, hence may precipitate membrane proteins (Calbiochem 2001).

Nonionic detergents, such as octyl-β-D-glucopyranoside (OGP), are far less denaturing than ionic detergents. OGP has uncharged hydrophilic head groups and is better suited for breaking lipid-lipid and lipid-protein interactions than protein-protein interactions. They are particularly useful for isolating functional protein complexes (Calbiochem 2001). OGP has a high critical micelle concentration (CMC) that will return to monomeric state upon detergent dilution, thus permitting rapid detergent removal by dialysis or ultrafiltration (Bollag & Edelstein 1991). This nonionic detergent was designed specifically for the extraction of membrane proteins in their active state (Schimerlik 2002; Anatrace 2002).

The spore coat protein is highly hydrophobic and difficult to solubilize with diluted aqueous salt solutions (Gould et al. 1970). In a study on the optimal conditions for solubilization of coat proteins, Pandey & Aronson (1979) determined the percent solubilization after extracting spores using urea/sodium dodecyl sulphate (UDS) buffer. A 30-min extraction with UDS resulted in a 27% solubilization and increased to 60% after 3 hr. Only 15% of the protein was solubilized in 30 min using the same buffer without urea. The proteins extracted were fractionated by electrophoresis on 15% acrylamide-SDS-urea slab gels showing six to seven major polypeptides in a reproducible manner. In addition to showing that urea was essential for complete extraction, this study demonstrated the need to examine extracts immediately (no dialysis, etc.) because of the ready aggregation of these proteins in UDS.

Circular dichroism (CD) analysis relies on the differential absorption of left and right circularly polarized radiation by chromophores possessing intrinsic chirality. Proteins possess a number of chromophores that can give rise to CD signals. In the far UV region (240-180 nm), which corresponds to peptide bond absorption, the CD spectrum can be analyzed to quantify regular secondary structural features such as α-helix and β-sheet (Kelly and Price 1997). CD becomes a limited technique when the solvent containing the protein is incompatible in terms of characteristic signals or high blank absorbance.

The complex aggregating nature of membrane proteins limits the number of detergents solubilizing proteins with minimum denaturation and spectroscopic interference. Therefore, the objectives of this study were to (1) develop a spore coat protein extraction methodology using non-ionic detergents and compare it with published methods using urea/sodium dodecyl sulphate (UDS) with respect to protein yield, protein and amino acid composition; and (2) confirm its suitability for structural analysis of coat proteins by CD.

3.3.   Materials and methods

The preparation of bacterial endospores and solubilization of spore coat proteins followed modifications of the procedures previously described (Aronson & Fitz-James 1976; Nakayama et al. 1977; Goldman & Tipper 1978; Pandey & Aronson 1979; Jenkinson et al. 1981). The protein extracts were analyzed by the modified Lowry method (Peterson, 1977) and SDS-PAGE. Amino acid composition by reverse phase HPLC (Malencik et al. 1990) and CD spectroscopy was used only for spore coat proteins extracted by OGP.

3.3.1. Preparation of bacterial endospores

Lyophilized *Bacillus subtilis* cultures purchased from the American Type Culture Collections (Manassas, VA, ATCC 6633), maintained at −20C until used, were grown at 37C for 5 days on AK agar #2 (Sporulation agar, Beckton Dickinson, NJ). Spore formation was confirmed by staining and microscopic observation. Counts were determined by standard plate counts at 37C for 24 hr using nutrient agar (Difco, Sparks, MD). For the strain used in this study, preliminary studies showed that the modified Schaeffer's medium recommended for sporulating Bacilli (Leighton & Doy 1971; Nicholson & Setlow 1990) gave unsatisfactory yield and small size spores.

Spores were harvested from 10 plates by adding twice 5ml of sterile distilled water and gently scrapping the agar surface with a sterile bent glass rod. Harvested cells were pooled together to make a uniform suspension and then washed thrice by centrifugation (Sorvall® refrigerated centrifuge, rotor F16/250, 10000 g or 8113 RPM, 15 min, 4C) using sterile distilled water. Washed spores were heated at 80C/10 min to destroy vegetative cells. Plate counts were taken pre- and post-heat treatment to estimate the actual spore count.

Harvested spore pellets were washed with KCl (1 M) and NaCl (0.5 M), and incubated for 60 min at 37C in Tris/HCl buffer (50 mM, pH 7.2) containing lysozyme at 200 µg/ml. After lyses of vegetative cells, spores were subjected to a series of washing with 1M NaCl, 0.14 M NaCl, 0.1% (wt/vol) SDS, 1 mM NaOH, and antibody buffer, followed by eight washings with sterile Milli-Q water. The antibody buffer consisted of 0.05 M sodium phosphate containing 0.05 M NaCl, 0.1% (wt/vol) sodium deoxycholate, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 0.1% Triton X-100, pH 7.5 (Pandey & Aronson 1979). Final preparations contained >98% phase bright spores.

3.3.2. Preparation of spore coat fractions

Clean spores were suspended to a density of 4-5 mg dry wt/ml in TEP buffer [Tris/ HCl (50 mM, pH. 7.2) containing EDTA (10 mM) and PMSF 2 mM], mixed with 0.10 mm diameter Zirconia/Silica beads (density 3.7 g/ml, Biospec Products Inc., Bartlesville, OK; 15 ml spore suspension plus 55g beads) and broken in a Beadbeater™ (Biospec Products Inc.) kept in an ice/water mixture for cooling. A total of 5 min, 2 min cooling after every 15s beating, was sufficient for total disruption of spores as revealed by subsequent spore staining. After beads settled out of suspension, the supernatant was removed by pipetting and beads were washed with 15-20 ml of TEP buffer. The washings and original supernatant were pooled and pelleted by centrifugation (Sorvall®, rotor F16/250, 15000 g, 15 min,

4C). The pellet was then resuspended in TEP buffer containing 200 µg/ml lysozyme and incubated for 60 min at 37C to hydrolyze the cortical and mother cell peptidoglycan. The suspension was subjected to a washing sequence with TEP buffer containing KCl (0.5M) and glycerol (1% w/v); NaCl (1M); SDS (0.1% w/v) in TEP buffer; TEP buffer; NaCl (1M); and eight times with sterile Milli-Q water containing Tween 80 (0.1% w/v), PMSF (2 mM), EDTA (5 mM) and iodoacetamide (0.5 mM). The residue was called 'Spore coat fraction'. As previously described (Aronson & Fitz-James, 1976), readily solubilized spore coat proteins are lost in this procedure.

3.3.3. Solubilization of spore coat protein

The spore coat fraction was suspended in freshly prepared 25ml UDS buffer [0.05 M dithioerythritol-1% SDS – 8 M urea in 0.005 M cyclohexyl amino methane sulfonic acid (CHES) and PMSF (2 mM)] at pH 9.8. After incubation with gentle stirring in a water bath for 3 hr at 37C (UDS-37C) or 1 hr at 70C (UDS-70C), the mixture was centrifuged (14000 g, 5 min) and the supernatant with the soluble coat proteins was retained. In a third solubilization alternative (OGP-RT), the protein was extracted at room temperature for 3 hr using 35 mM or 1% (w/vol) octyl-β-D-glucopyranoside (OGP) prepared in 10 mM phosphate buffer (pH 9.8).

3.3.4. Spore coat protein concentration

Protein concentration in the UDS-37C, UDS-70C and OGP-RT extracts was determined by the modified Lowry method using bovine serum albumin (BSA) as standard (Peterson 1977). Three aliquots of each triplicate of these three extracts obtained from the same spore suspension were analyzed.

3.3.5. SDS-polyacrylamide gel electrophoresis

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of the spore coat protein solution was carried out on 10-well, 50 µL, Tris- HCl 8-16% (4% stacking gels) (Biorad Cat. no 161-1225, Hercules, CA). Samples for electrophoresis were concentrated by adding 0.8 ml distilled water and 0.1 ml 72% chilled TCA to 0.2 ml samples. After incubation for 1 hr in ice, the mixture was centrifuged at 5000 g for 5 min and then the supernatant was carefully removed. The pellet was washed with ice-cold acetone and then air-dried.

The UDS-37C and UDS-70 solubilized protein pellet was dissolved in 100 µL UDS buffer and 10 µl Laemmli sample buffer (Laemmli & Favre 1973) and then heated for 5 min at 100C. The OGP-RT solubilized protein pellet was dissolved in 100 µl Laemmli buffer containing 5 µl mercaptoethanol and heated for 5 min at 100C. Electrophoresis was performed at 126 V using Broad Range Biorad® SDS-PAGE Gel standards (Cat. no 161-0317) as molecular weight markers. The gels were stained with Coomassie brilliant blue R250 (Biorad Cat. no 161-0400).

3.3.6. Amino acid analysis

Amino acid analysis of the OGP solubilized protein was conducted as described by Malencik et al. (1990).

3.3.7. CD spectroscopy

The OGP compatibility with CD analysis was evaluated at the OSU Dept. of Biochemistry & Biophysics Spectroscopy Laboratory. Cylindrical quartz cells with a 100 µm path length were used as a short path length cuts solvent absorbance by a factor of 200 over the commonly used 1 cm cell and extend the transparency of water to 176 nm (Johnson 1990). Also, cylindrical cells have lower birefringence

(baseline CD) and require slightly less sample. Protein solutions were scanned from 190-300 nm with a 2.0 nm bandwidth, sensitivity of 10 mdeg/FS, a time constant of 180 s and a scan speed of 20 nm/min. The baseline spectrum of the solvent/buffer was collected under the same conditions as the sample spectrum using the same cuvette and orientation with respect to the light beam. The baseline was subtracted from the sample spectrum to produce the final CD plot (Rodger 1997).

3.3.8. Statistical analysis

The reproducibility of the three spore coat protein solubilization methods was evaluated by analysis of variance (ANOVA) applied to triplicate protein concentration determinations for solutions obtained from three independent extractions of the same spore suspension. Tukey-HSD was used to determine the yield difference between solubilization methods. All statistical analyses were performed with STATISTICA (Version 5.1, StatSoft, Inc., Tulsa, OK).

3.4. Results and discussion

Spore coat proteins had poor solubilization at physiological pH (data not shown), an observation consistent with published research (Gould et al. 1970; Mitani & Kadota 1976). On the other hand, spore coat proteins were easily solubilized under the strong alkaline solutions widely used to solubilize spore coat proteins (e.g., Aronson 1976; Pandey, 1979). The absorbance spectrum of pure UDS buffer at pH 9.8 showed over 2 OD units in the 220-240 nm region making it unsuitable for protein spectral analysis. Zwitterionic detergents such as CHAPS (Bollag & Edelstein 1991; Anatrace 2002; Calbiochem 2001) are more efficient than a nonionic detergent such as OGP at overcoming protein-protein interactions but the absorbance of 1% CHAPS was significant in the 200-280 nm region (Fig. 1). On the other hand, 1% OGP in 10 mM phosphate buffer at pH 9.8 gave uniform and low blank readings (Fig. 1).

Figure 1: Absorption spectrum for two of the spore coat protein extraction buffers, CHAPS and octyl-β-D-glucopyranoside (OGP)
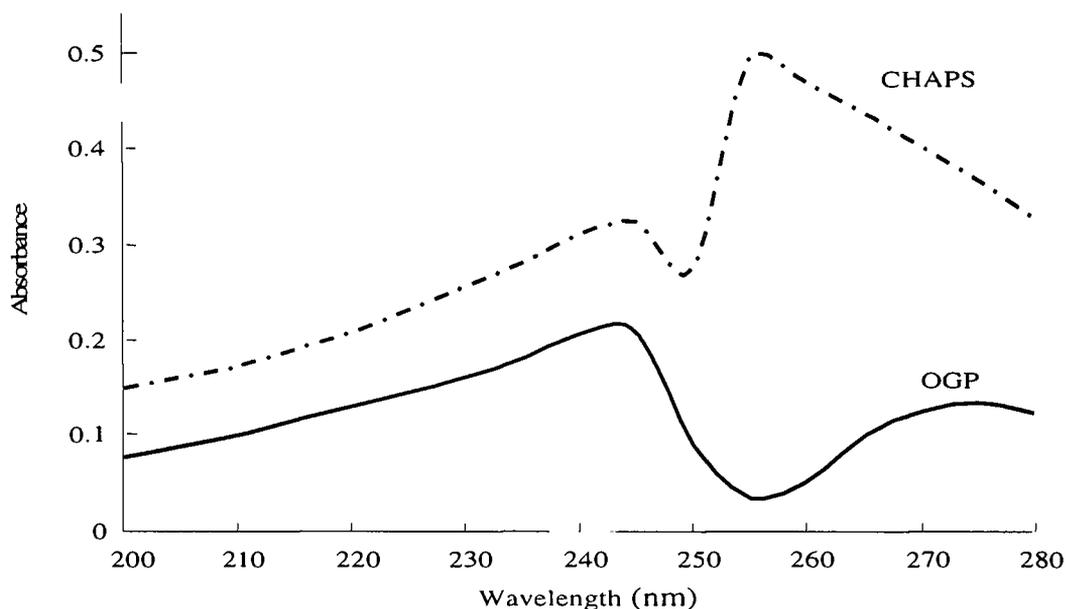
The selection of OGP at pH 9.8 for this study was based on the need to scan protein solutions in the 190-300 nm region for CD spectroscopy. Information about the secondary structure can be obtained by CD spectroscopy in the 190-250 nm region with the peptide bond as the chromophore. The characteristic CD spectrum obtained from coat protein solution obtained from OGP shows that the blank spectra is close to zero generating a corrected CD signal that is more reliable (Fig. 2). The obtained spectra have a response typical for a protein containing α-helix regions showing negative peak with separate maxima of similar magnitude at 222 nm and 208 nm (Rodger 1997). This indicates that α-helix is the dominant secondary structure in spore coat proteins (Fig. 2).

Figure 2: **Characteristic CD spectrum for coat proteins extracted from of** *Bacillus subtilis* **ATCC 6633 spores by octyl-β-D-glucopyranoside (OGP)**
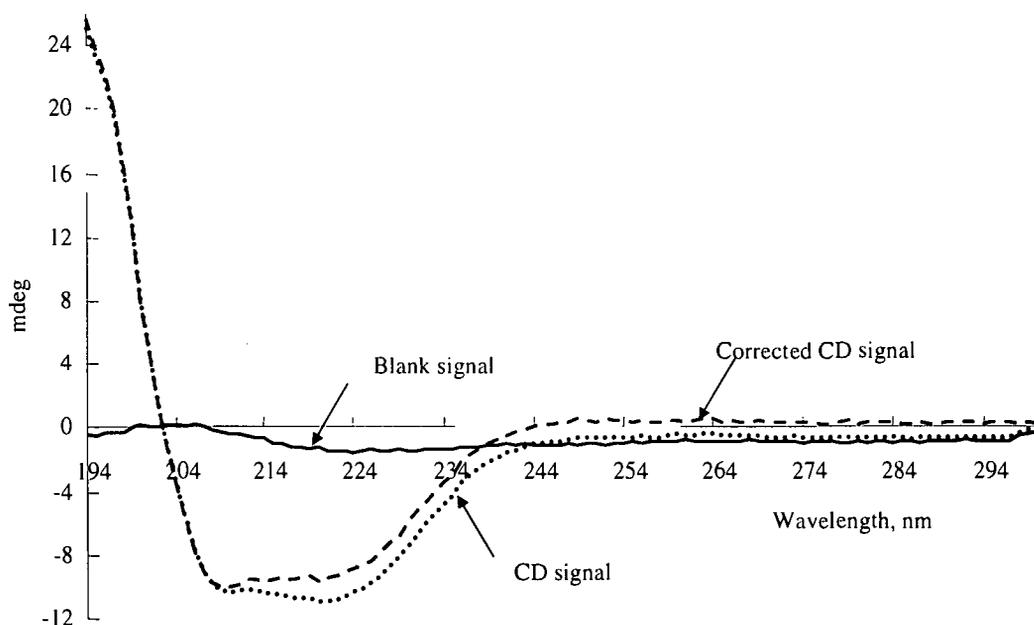
3.4.1. Protein yield

The statistical analysis of the means of protein concentration showed no significant difference (95% confidence) between the independent extractions of the same spore suspension and hence average values are shown in Fig. 3.

On the other hand, the Tukey HSD test showed that the three methods have significantly different yield. The larger yield for UDS at 70C as compared to 37C could be due to the higher extraction temperature that opens protein structure thereby facilitating detergent interaction with the protein. In addition to temperature, the difference between UDS and OGP at ambient temperature (~23C) is the UDS content of 1% SDS and 8 M urea; both are strong denaturing agents.

Figure 3: Coat protein yield by extraction of *Bacillus subtilis* ATCC 6633 spores with octyl-β-D-glucopyranoside at room temperature (OGP-RT) and urea/sodium dodecyl sulphate (UDS) at 37C and 70C at pH 9.8
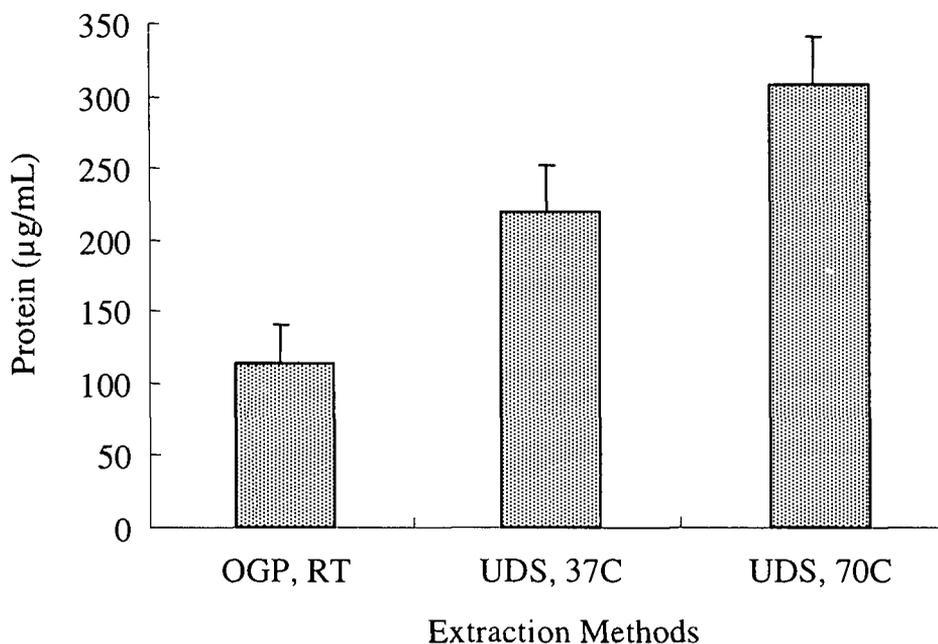
3.4.2. SDS-PAGE

SDS-PAGE showed the separation of the spore coat proteins solubilized by using UDS at 37C with fainter bands associated with the lower extraction yield (data not shown) and at 70C with easier to identify bands (Fig. 4). As an aid to identification, and not as an accurate molecular weight determination, each protein band is labeled using its apparent molecular weight. The major protein bands observed, 9, 12, 33 and 50 kD, in addition to minor protein bands at 20, 22, 24, 26, 59 and 65 kD were repeatedly observed (Fig. 4a). These findings are consistent with the results obtained by other researchers (Aronson 1976; Mitani 1976; Goldman 1978; Munoz 1978; Pandey 1979) and particularly those observed by Jenkinson et al. (1981) who found bands in the 8-65 kD range (Fig. 4b) using a similar extraction procedure but a different spore strain and sporulation medium. In the case of our work we saw also the presence of higher molecular weight

fraction suggesting the probable protein aggregates that are not fully solubilized by the spore coat protein extraction procedure.

The SDS-PAGE for the spore coat proteins solubilized by OGP at room temperature showed a total of nine distinct bands ranging from ~5 to ~66 kD (Fig. 5). Two major protein bands, 66.2 and 26.5 kD, dominate the coat fraction. An additional seven protein bands at 49, 22.5, 18, 16, 12, 7 and 5 kD were consistently observed.

Figure 4: **Comparison of SDS-PAGE patterns (stained with Coomassie blue) of spore coat proteins extracted from** *Bacillus subtilis* **ATTCC 6630 spores with urea/sodium dodecyl sulphate (UDS) at 70C (a) with the work published by Jenkinson et al. (1981) (b). Solid and empty arrows indicate major and minor protein bands, respectively.**

(a)   (b)
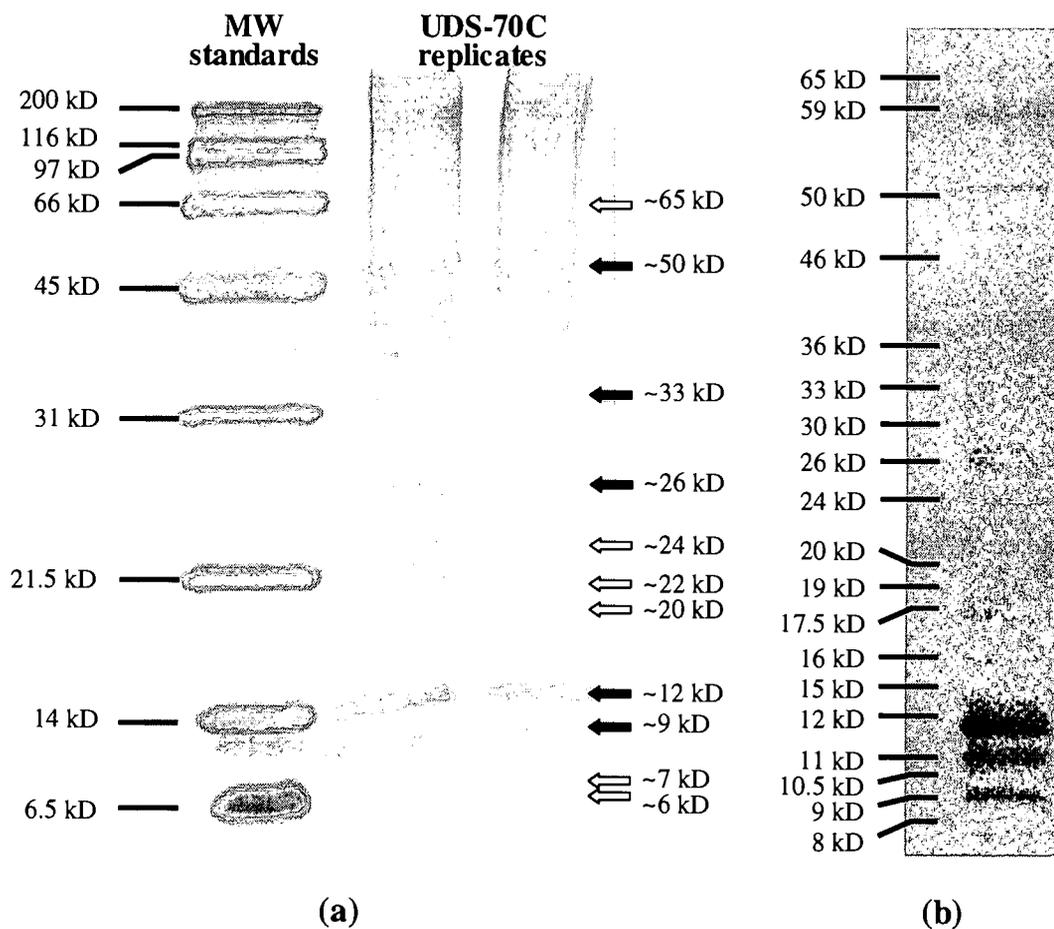
Figure 5: SDS-PAGE patterns (stained with Coomassie blue) of spore coat proteins extracted from *Bacillus subtilis* ATCC 6633 spores with octyl-β-D-glucopyranoside at room temperature (OGP-RT). Solid and empty arrows indicate major and minor protein bands, respectively.
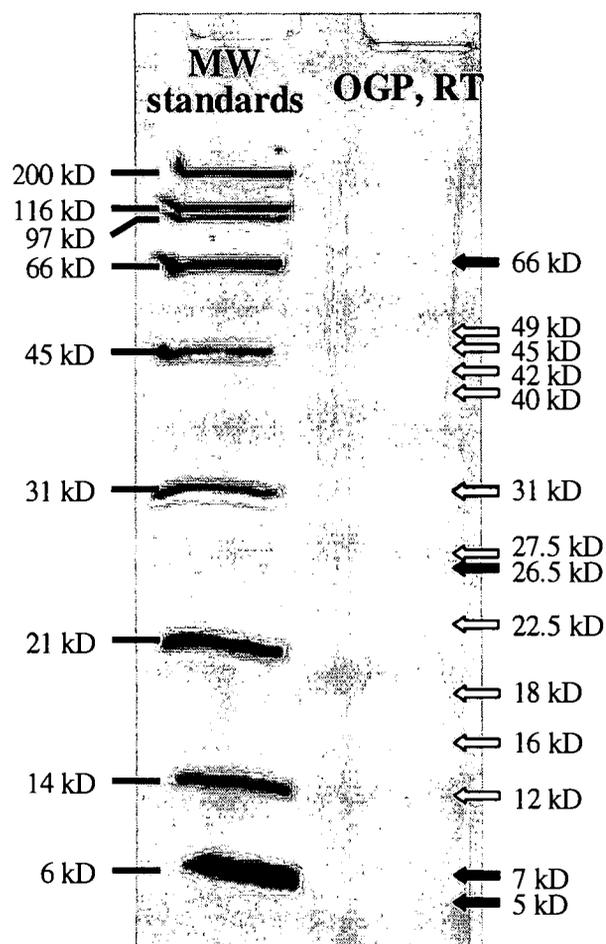
3.4.3. Amino acid analysis

The amino acid composition of the coat proteins obtained by the OGP extraction method was compared with published data. The differences between the values obtained in this study with those summarized in Table 1 may arise from differences in bacteria strain, methods employed for washing and solubilization of proteins and subsequent handling. For example, Mitani and Kadota (1976) used *B. subtilis* ATCC 6051 and distilled water for all washings and the solubilization

buffer consisted of 1% SDS, 0.1M dithiothreitol (DTT) in 0.1M sodium borate. Goldman and Tipper (1978) used *B. subtilis* 168 and washing was conducted with salt solutions. Coat protein solubilization buffer was 0.05M sodium carbonate-biocarbonate with 50mM DTT. Pandey and Aronson (1979) used *B. subtilis* JH642 and the protein solubilization was CHES buffer containing 8M urea in addition to 1% SDS and 0.5M DTT.

Table. Amino acid composition of soluble spore coat fraction

Amino acid	This study [b]	Published data				
		(c)	(d)	(e)	(f)	(g)
Aspartic acid	12.2	8.5	12.2	6.9	4.9	8.12
Glutamate	7.9	8	6.8	6	4.4	5.96
Serine	3.8	3.4	7.3	7.6	7.1	8.38
Threonine	3.4	3.5	5	4.6	4.8	6.11
Glycine	6.7	12.4	14.3	21.1	20.3	7.09
Alanine	7.4	7.8	8.7	7	7.7	13.55
Proline	4.6	5.3	3.6	2.8	16.5	4.78
Valine	8.9	6.9	5.2	4.4	3.6	8.3
Arginine	1.6	3.7	4.9	4	2.6	2.9
Methionine	1.1	0.6	1.7	_	0.91	0.8
IsoLeucine	5.8	5.7	4.3	3.2	2.7	0.72
Leucine	5.1	8	5.6	3.6	3.1	12.92
Phenylalanine	5.8	7.5	4.6	6.2	7.5	4.8
Lysine	6.8	7.4	6.1	6.6	2	4.8
Histidine	1.9	3.9	2	3	2.2	6.25
Tyrosine	7.0	4.5	7.1	11.2	8.9	2.3
Cysteic acid		2.8			0.74	

[a] Values of amino acids are expressed as mole percent, [b] *Bacillus subtilis* ATCC 6633 spore coat proteins solubilized by OGP at room temperature, [c] Pandey & Aronson (1979), [d] Mitani & Kadota (1976), [e] Spudich & Kornberg (1969), [f] Goldman & Tipper (1978), [g] Munoz & Doi (1978)

In this study, hydrophobic amino acids such as glycine, alanine, valine and tyrosine were in higher proportion which is consistent with published values. The hydrophobic nature of the coat proteins explains the coat insolubility in water or phosphate buffer. Finally, the high concentration of aspartic and glutamic acid suggests that the spore coat protein is acidic.

3.5. Conclusions

Temperature and pH of the extraction buffer had a significant effect on the yield of the spore coat proteins obtained by UDS. The same pH effect was noted in the case of OGP (data not shown). A solubilizing agent is required to maintain spore coat proteins in solution because of their tendency to aggregate. Among the two agents tested, UDS and OGP, the latter exhibited the lowest blank absorbance. The extracted spore coat proteins were in yields suitable for analysis by spectral analysis such as FTIR and CD to obtain further information on the effect of physical and chemical treatments on spore coat proteins. The quantification of the effect of chemical and physical processes to inactivate spores is more reliable for anionic detergents than solubilization methods using urea and SDS. The proteins obtained are more likely to suffer less damage during the extraction process.

4. EFFECTS OF HIGH PRESSURE PROCESSING ON BACTERIAL ENDOSPORE COAT PROTEINS

Gandhi KK*, Anderson SR[†], Malencik DA[†],Velázquez, G.* and Torres, JA*

*Dept. of Food Science and Technology
[†]Dept. of Biophysics and Biochemistry
Oregon State University, Corvallis, OR 97331, USA

4.1.   Abstract

Spore coat proteins from *Bacillus subtilis* ATTC 6633 were solubilized by octyl-β-D-glucopyranoside (OGP) at room temperature and urea/sodium dodecyl sulphate (UDS) at 70C. Pressure treatments of spores and of extracted spore coat protein solutions, at 50 kpsi (345 mPa) and 85 kpsi (586 mPa) for 10 and 30 min at constant 85C along with appropriate heat- and pressure-only controls and untreated samples, were used to study the effect of pressure, heat and time on spore coat proteins. Both spore coat protein solubilization procedures showed a significant reduction in protein yield for pressure-only, heat-only and pressure/heat treated spores when compared with untreated spores When OGP-solubilized proteins from untreated spores were pressure treated, SDS-PAGE profile showed an overall increasing band intensity with increasing pressure and time. In the case of the coat protein solution obtained from pressure-treated spores, the electrophoretic pattern showed the loss of higher molecular weight proteins.

4.2. Introduction

High pressure processing (HPP) is a non-thermal or minimum heat processing technology to inactivate spoilage microorganisms and bacterial pathogens with minimum or no apparent change in food texture, color and flavor (Cheftel 1995; Knorr 1993). Unlike thermal processing, HPP effects are uniform and instantaneous throughout a food and do not follow a concentration gradient. HPP can be used to produce safe, extended shelf-life foods with minimum or no effects on the chemical nature of the product.

Bacteria of the genera Bacillus and Clostridium form highly resistant endospore and many are frequently associated with food poisoning. The ability of bacterial spores to remain in a dormant state for a long period of time and resist physical and chemical agents is an important problem to the food industry. For example, *C. botulinum* the organism associated with the potentially fatal disease botulism, produces an extracellular toxin during spore germination. Strains of *B. cereus* and several Clostridia can also cause brief, but severe, bouts of diarrhea (USFDA 2000 & 2001; Roitt et al. 1998). Certain strains of the bacterium *B. cereus* are capable of producing a heat-labile diarrhea-causing enterotoxin, a heat-stable emetic enterotoxin and other toxins (Kramer & Gilbert 1989).

In his early work, Bridgman (1914) studied protein denaturation by pressure without heat. High pressure can induce protein denaturation by altering the equilibrium between the amino acid group interactions that stabilize the folded confirmation of native proteins. Pressure denaturation of proteins is a complex phenomenon depending on the protein structure, the pressure range and external parameters such as temperature, pH and solvent composition (Masson 1992). In general, and as proposed by Wong & Heremans (1988), secondary structure changes occur at very high pressure and lead to irreversible denaturation. It is not yet possible to predict the modifications of secondary structure caused by pressure. For example, irreversible denaturation of chymotrypsinogen induced at 760 mPa is

characterized by a dramatic decrease of α- helix and β- sheet contributions. In the case of lysozyme there is a decrease of α-helix and an increase of β-sheet structures at pressures beyond 360 mPa (Heremans & Wong 1985). Pressures of 400 to 600 mPa at room temperature can readily denature proteins such as ovalbumin and β-lactoglobulin.

Most food microorganisms are readily killed at 600 mPa for 15 min at 20-30C (Hayakawa 1991; Hayakawa et al. 1992a, b). However, the application of high pressure to low acid foods is problematic because bacterial spores must be inactivated (Gola et al. 1996). Bacterial spores have proven to be pressure-resistant and combined pressure and heat treatments are needed to inactivate them (Gould & Sale 1970; Sale et al. 1970). Hayakawa et al. (1994) found out that spore inactivation increases with the number of pressure cycles. These authors suggested that spore sterilization could be explained by the adiabatic expansion velocity of pressurized water or by a weakening of the physical strength of the spore coat under high pressure.

Spore DNA is extremely well protected against different types of damage through the saturation of the spore chromosome with DNA-binding proteins termed α/β-type small, acid-soluble spore proteins (SASP) (Setlow 1994, 1995a). *Bacillus subtilis* spores lacking SASP are much more sensitive to dry heat then wild-type spores (Setlow 1995b). The resistance to pressure inactivation of 600 mPa appears to be due to SASP's since mutants deficient in α/β-type SASP's are more sensitive to inactivation. SASP's and dipicolinic acid (DPA) are involved in spore resistance to UV light and hydrogen peroxide. Wuytack and others (1998) studied the fate of these compounds during pressure-induced germination of *Bacillus subtilis* spores at moderate (100 mPa) and high (500-600 mPa) pressures. They found that DPA was released upon both low- and high-pressure germination, but SASP degradation, which normally accompanies nutrient-induced germination, occurred upon low-pressure germination but not upon high-pressure germination. Spores germinated at 100 mPa were more sensitive to pressure (>200 mPa), UV light, and hydrogen

peroxide than were those germinated at 600 mPa. Germination at 100 mPa resulted in rapid ATP generation, as is the case in nutrient induced germination, but no ATP was formed during germination at 600 mPa. These results suggest that spore germination can be initiated by low- and high-pressure treatments but is arrested at an early stage in the latter case.

Further studies on the pressure effect on spores are needed in order to develop an efficient HPP treatment for their inactivation. The effects of pressure on spore coat proteins have not been studied and might provide an opportunity to identify additives with synergistic pressure effects. The objectives of this study were to (1) use two different solubilization methods for spore coat proteins and measure the protein yield obtained as an indication of the effects of pressure-only, heat-only combined heat and pressure treatments, and (2) determine the effect of pressure on spore coat proteins as revealed by SDS-PAGE electrophoresis.

4.3.   Materials and Methods

The preparation of bacterial endospore and the solubilization of the spore coat proteins by OGP at room temperature and by urea/sodium dodecyl sulphate UDS at 70C were described by Gandhi & Torres (2002). Protein concentration in the UDS and OGP extracts was determined by the modified Lowry method using bovine serum albumin (BSA) as standard (Peterson 1977). Three aliquots of each triplicate of the OGP and UDS extracts obtained from the same spore suspension were analyzed. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of the solubilized spore coat proteins was carried out as described by Gandhi & Torres (2002). The gels were stained with Coomassie brilliant blue R250 (Biorad Cat. no 161-0400) and subsequently silver stained (Biorad Cat. no 161-0449).

4.3.1. Pressure Treatments

The high pressure processing facility at Avure Technologies Inc. (Kent, WA) was used for all experiments. These were grouped into treatments of spores prior to solubilization and into treatments of solubilized coat proteins obtained from untreated spores (Fig. 6). Spore (15 mL) and coat protein solutions (0.5 mL) were packed in heat sealed pouches with the least amount of air possible.

Figure 6: **Experimental design showing untreated samples (U), heat-only (H), pressure-only (P) and pressure/heat (P/H) treated spore and coat protein solutions from** *Bacillus subtilis* **ATCC 6633**
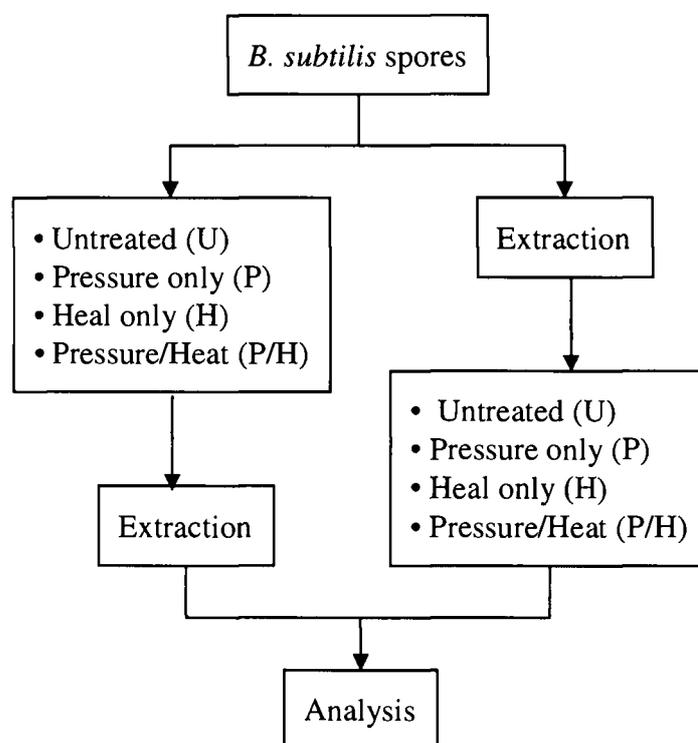
All combined pressure/heat (P/H) treatments were conducted at a constant temperature of 85C at 50 and 85 kpsi for 10 and 30 min. Heat-only (H) controls were treated at 85C for 10 and 30min while pressure-only (P) controls were treated at 50 kpsi and 85 kpsi for 30min.

All experimental treatments, including untreated (U) samples, H, P and P/H treatments were conducted in one experimental run using triplicate samples. The come-up time of the pressure vessel was 8s and the hydrostatic fluid was water.

4.3.2. Statistical Analysis

The protein yield reproducibility obtained for untreated samples and H, P, P/H treatments was evaluated by ANOVA with 95% confidence and Tukey- HSD test performed with STATISTICA (Version 5.1, StatSoft, Inc., Tulsa, OK).

4.4.   Results and Discussion

The results obtained are expressed in terms of yield of spores and differences in the patterns obtained by SDS-PAGE electrophoresis. Results obtained from the yield replicates for untreated samples and pressure and heat treatments showed that both coat protein solubilization procedures were statistically reproducible.

4.4.1. Spore Coat Proteins Yield

As shown previously by Gandhi & Torres (2002), the yield of spore coat proteins by OGP was significantly lower (130 µg/mL) than for UDS at 70C (~255 µg/mL). The yield of proteins solubilized by UDS at 70C (Fig. 7) and OGP at room temperature (Fig. 8) for H, P, and P/H treated spores showed a significant reduction when compared with untreated samples (U). The reduction in the yield for all treatments was about 80% and 40% for coat proteins solubilized by OGP at room temperature (Fig. 8) and UDS at 70C (Fig. 7), respectively. The difference in the extent of the reduction reflects the change in the temperature and nature of the spore coat protein extraction solution. Urea and SDS are strong detergents and more effective in separating protein aggregates than anionic OGP.

In addition, the solubilization by urea/SDS is at 70C and thus the action of the detergent on the spore coat proteins will be increased. Room temperature is used in the OGP solubilization procedure to minimize changes caused on proteins while obtaining yield at an acceptable value.

The decrease in yield as a result of pressure treatments, alone or in combination with heat, could be due to increase in the aggregation or packing of coat physically resulting from the negative volume change caused by pressure. This would limit detergent accessibility to the spore coat proteins. Zipp & Kauzmann (1973) stated that the volume change for protein folding is positive at atmospheric pressure but becomes negative at higher pressures. Protein undergoes a standard volume change for unfolding (ΔV°) and thus the partial molar volume of the denatured protein is smaller than that of the native protein (Prehoda et al. 1998).

Dityrosine has been shown to cross-link structural proteins (Anderson 1964; Aeschbach 1976; Gracia-Castineiras 1978) and consequently pressure and heat could increase dityrosine cross-linking and decrease the amount of protein that can be solubilized by the detergents.

Figure 7: **Yield (μg/ mL) of spore coat proteins solubilized by urea/sodium dodecyl sulphate (UDS), pH 9.8 and 70C from untreated and pressure-treated** *Bacillus subtilis* **ATCC 6633 spores**
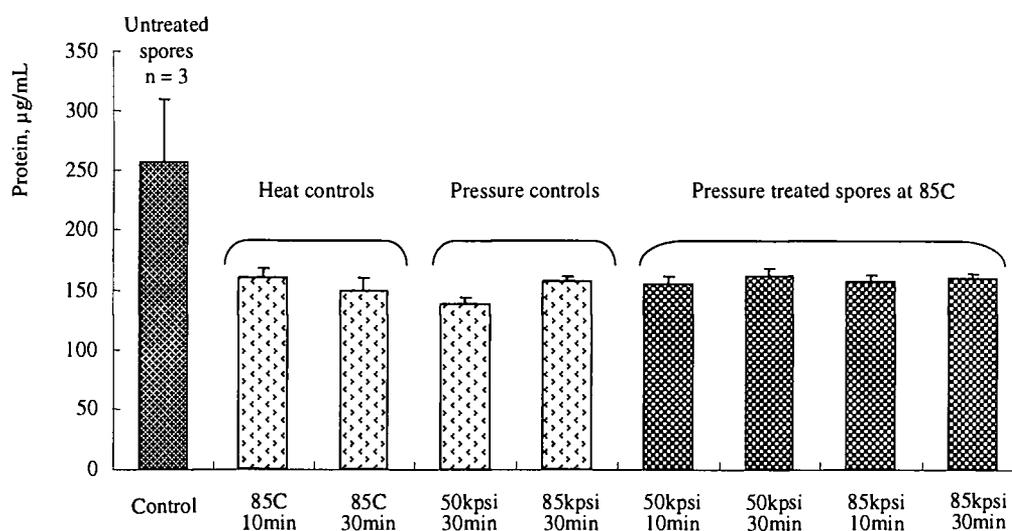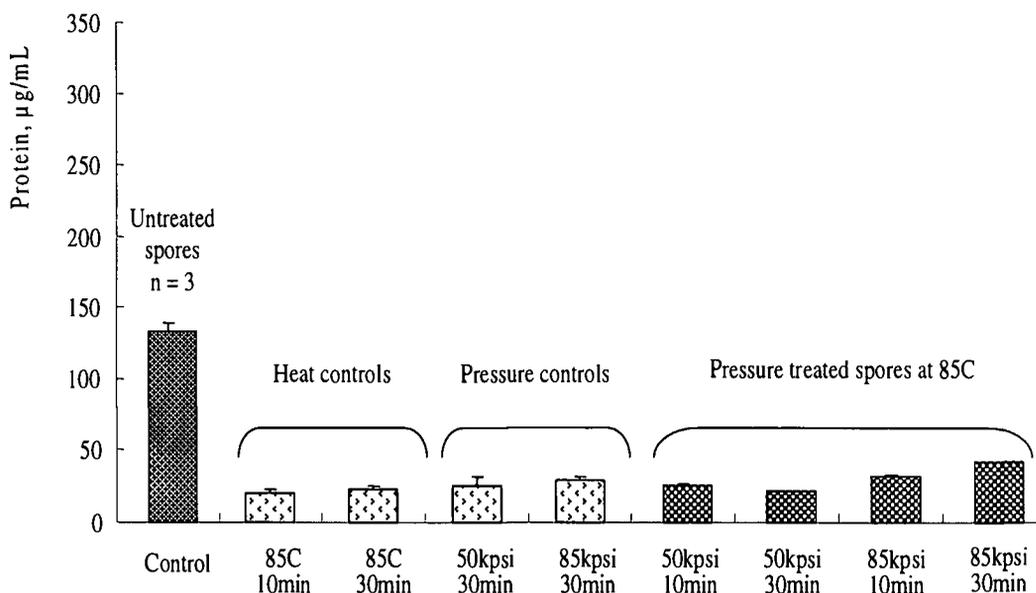
Figure 8: **Yield (µg/ mL) of spore coat proteins solubilized by octyl-β-D-glucopyranoside (OGP) at pH 9.8 and room temperature from untreated and pressure-treated** *Bacillus subtilis* **ATCC 6633 spores**


4.4.2. SDS- PAGE Electrophoresis

Proteins obtained from pressure treated spores were loaded using equal volumes. Therefore, the overall difference between the band intensities among pressure treated solutions (Fig. 9, lanes 3-6) and pressure treated spores (Fig. 9, lanes 7-10) reflects the decrease in the yield of proteins obtained from pressurized spores (Figs. 7 & 8).

Spore coat proteins pressurized in the form of solution responded to pressure differently than proteins present in intact spores. For example, the SDS-PAGE profile of pressure-treated coat protein solutions extracted from untreated spores using OGP showed an overall intensity of bands increasing with pressure and time (Fig. 9, lanes 2-6). Moreover there could be an increase in the unfolding of proteins that exposes hidden residues to bind with SDS thereby increasing the overall intensity of bands. Also, aggregated proteins can be seen in larger amounts at the bottom of the wells for the more severe treatments of the same stock of coat

protein (Fig. 9, lanes 2-6). On the other hand, the spore protein profile from pressure-treated spores showed a loss of high molecular weight fractions (Fig. 9, lanes 7-10) reflecting a likely cross-linking of these spore coat protein fractions in the intact spore. This would prevent their solubilization by OGP.

Figure 9: **Coomassie (R- 250) stained SDS-PAGE electrophoresis patterns of spore coat proteins solubilized by octyl-β-D-glucopyranoside (OGP) at pH 9.8 and room temperature from untreated and pressure-treated** *Bacillus subtilis* **ATCC 6633 spores. RP, Reference proteins; U, Untreated sample**
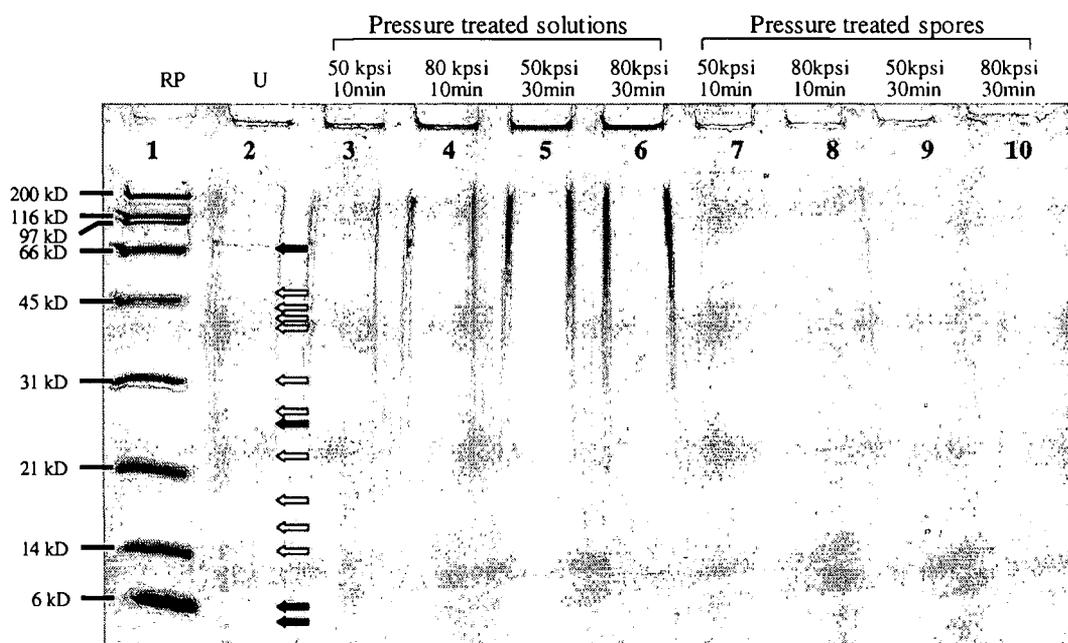
Silver staining revealed a change in the electrophoretic mobility of low molecular weight proteins (bands B & C, Fig. 10). This is not a gel artifact because the band at ~26kD (band A, Fig. 10) is parallel to the two lowest bands (band D, Fig. 10) for all gel lanes. The apparent increase in the molecular weight of these proteins needs further studies.

Figure 10: Coomassie (R- 250) + Silver stained SDS-PAGE electrophoresis patterns of spore coat proteins solubilized by octyl-β-D-glucopyranoside (OGP) at pH 9.8 and room temperature from untreated and pressure-treated *Bacillus subtilis* ATCC 6633 spores. RP, Reference proteins; U, Untreated sample.
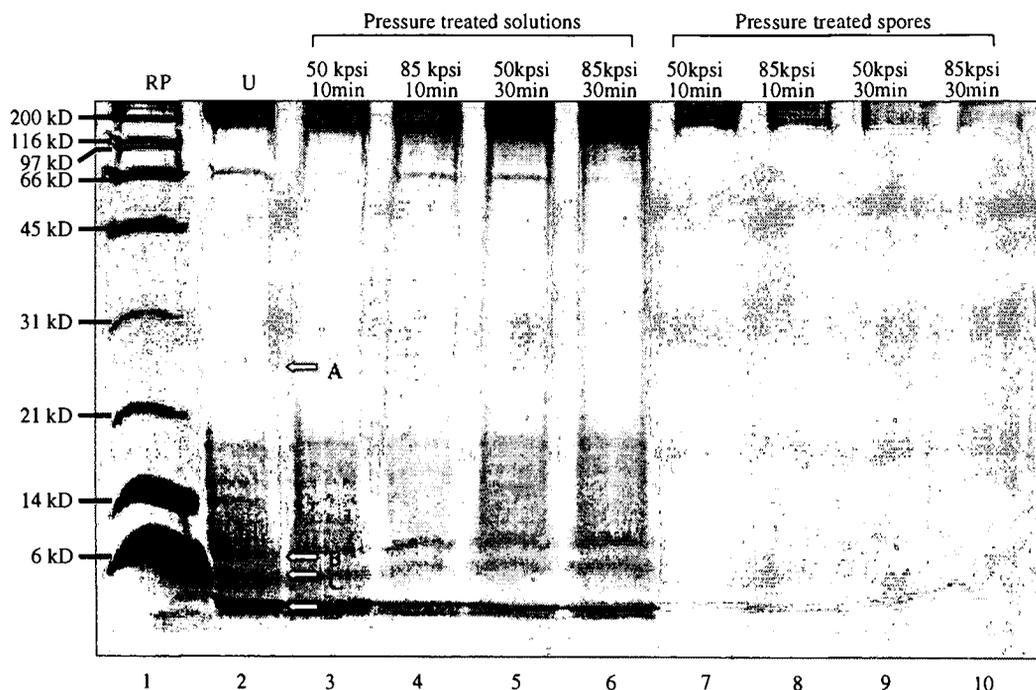
4.5. Conclusions

This study provides the first evidence of the extensive coat protein damage by heat, pressure or combined heat and pressure. The large reduction in the extraction yield by UDS and OGP are clear evidence of extensive changes in the spore coat protein, a key structure to the spore resistance to chemical and physical factors. Further changes observed by PAGE include increase in the amount of extracted proteins not entering the gel, increase in the aggregation of high mw fractions, and band intensity increase in the lower mw fractions require further study.

4.6.   Acknowledgments

5. GENERAL CONCLUSIONS AND FUTURE STUDIES

A most challenging goal for the present day food industry towards offering safe products is to make it free from heat resistant and toxin producing bacterial spores. Significant research has been done on the bacterial spore structure and the role of its different constituents with respect to the spore resistance to physical and chemical inactivation. Protective barriers include the spore coat that is difficult to disrupt with food preservation techniques and protects the DNA and core from harmful environmental conditions. High pressure is emerging as a promising food preservation technology and has the same challenge faced by other techniques, i.e., to inactivate heat-resistant spoilage and pathogenic bacterial spores.

This research is the first piece of information showing changes in spore coat proteins due to pressure. The key issue of isolating the proteins in a condition suitable for future studies has been addressed in this project. The methodology here proposed will be useful for implementing ideas outlined in this discussion. The decrease in the extracted spore coat protein yield and the changes observed in SDS-PAGE gels as a result of heat and pressure indicates that the treatment under considerations has effects on the proteins. The information obtained may not be comprehensive to fully explain the complex mechanism underlying the pressure and heat effects but will serve as basic preliminary information for conducting future in depth research targeted in this area leading to eventual practical applications, e.g., to help identify additives and pressure processing conditions increasing spore inactivation by pressure.

An example of how the procedures developed in this thesis could be used in future studies is to compare pressure-only and heat-only effects on spore coat proteins. Upon comparing the heat only and pressure only controls, there is a distinct difference. Bands A observed for heat treatments at 85C for 10 and 30min (lanes 3 and 4, Fig. 11) are not observed for pressure treatments (lanes 5 & 6, Fig. 11).

Figure 11: Coomassie (R- 250) stained SDS-PAGE electrophoresis patterns of spore coat proteins solubilized by octyl-β-D-glucopyranoside (OGP) at pH 9.8 and room temperature from heat-only (85C for 10 and 30min) and pressure-only (50 and 80kpsi for 30min) *Bacillus subtilis* ATCC 6633 spores. RP, Reference proteins; U, Untreated sample
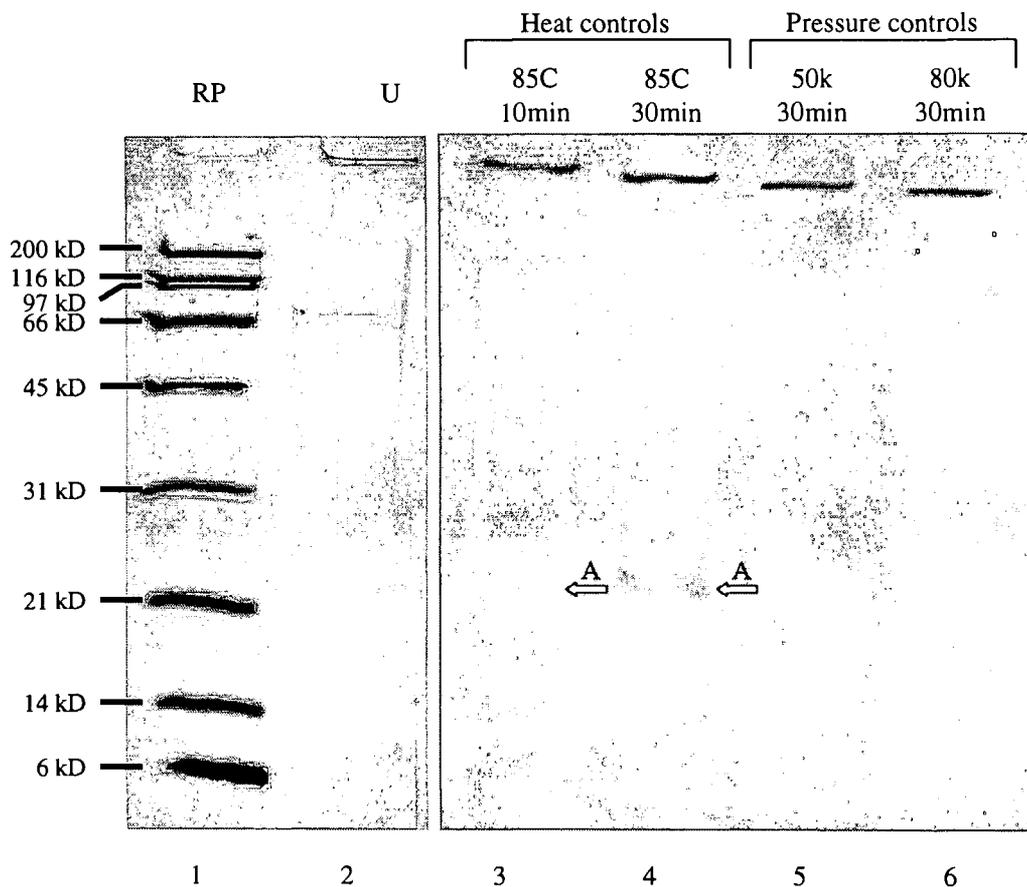
Another difference observed is that there is an absence of protein bands in the low mw range for spores heated at 85C for 10 minutes (lane 3, Fig. 11) but they are seen in spores heated for 30 min (lane 4, Fig. 11). Thus, it can be concluded that changes by heat and pressure on spore coat proteins are different. Native gel electrophoresis was not performed, as the detergent is necessary for the spore coat proteins to remain in solution.

This project focused on *B. subtilis* spores to allow comparisons with published literature, however future research will need to consider disease causing Bacillus or Clostridium spores, particularly *B. cereus*, *C. perfringens* and *C. botulinum*. In addition to studying other sporeformers, there are several continuation study possibilities to get a better understanding of the effects of pressure on spore coat proteins. The extract obtained from the proposed extraction methodology consists of a mixture of proteins with different molecular weights and properties. The challenge would be to separate this mixture and subject them individually to pressure and heat treatments.

The extraction protocol by OGP facilitates circular dichroism (CD) spectroscopy and could be used to obtain information on the changes in spore coat protein structure. CD connected in line with a high-pressure vessel could facilitate real time measurements to determine the duration and type of pressure treatments necessary for a protein structure change. These real time measurements could also be used to provide information on the coat protein structure changes during compression and decompression.

BIBLIOGRAPHY

Aeschbach R, Amado R, Newkom H. 1976. Formation of Dityrosine cross links in proteins by oxidation of tyrosine residues. Biochim Biophys Acta 439:292-301.

Anatrace 2002. Technical Bulletin No.101. Those Polyoxyethylene Detergents.

Anderson SO. 1964. The cross links in resilin identified as dityrosine and trityrosine. Biochim Biophys Acta 93:213-215.

Aronson AI, Fitz-James PC. 1976. Structure and morphogenesis of the bacterial spore coat. Bacteriol Rev 40:360-401.

Bollag DM, Edelstein SJ. 1991. Chapter 1: Preparation for protein Isolation. In Protein Methods, Wiley-Liss Division of John Wiley and Sons, Inc. p 1-25.

Bridgman PW. 1914. The coagulation of albumen by pressure. J Biol Chem 19:511-512.

Byler DM, Susi H. 1986. Examination of the secondary structure of proteins by deconvolved FTIR spectra. Biopolymers 25:469-487.

Calbiochem. 2001. Detergents: A guide to the properties and used of detergents in biological system. Bhairi SM, editor.

Cheftel JC. 1995. Review: High pressure, microbial inactivation and food preservation. Food Sci & Tech Int. 1, 75-90.

Cheftel JC, Culioli J. 1997. Effect of high pressure on meat: A review. Meat Science 46(3): 211-236.

Cheung HY, Cui J, Sun S. 1999. Real time monitoring of Bacillus subtilis endospore components by attenuated total reflection Fourier-transform infrared spectroscopy during germination. Microbiol. 145:1043 1048.

Clouston JG, Wills PA. 1969. Initiation of germination and inactivation of Bacillus pumillus spores by hydrostatic pressure. J Bacteriol 97, 684-690.

Clouston JG, Wills PA. 1970. Kinetics of Initiation of germination and inactivation of Bacillus pumillus spores by hydrostatic pressure. J Bacteriol 103, 140-143.

Crawford YJ, Murano EA, Olson DG, Shenoy K. 1996. Use of high hydrostatic pressure and irradiation to eliminate Clostridium sporogenes spores in chicken breast. J Food Prot 59(7):711-715.

Fry DC, Byler DM, Susi H. 1988. Solution structure of the 45 residue MgATP-binding peptide of adenylate kinase as examined by 2-D NMR, FTIR, and CD spectroscopy. Biochem 27:3588-98.

Gandhi KK, Torres JA. 2002. Chapter 1. Protein solubilization methods for studying bacterial endospore coat proteins. In Bacillus subtilis endospore coat protein solubilization methods for studying effects of high pressure processing. Masters thesis submitted to Oregon State University.

Gola S, Forman C, Carpi G, Maggi A, Cassara A, Rover P. 1996. Inactivation of Bacterial spores in phosphate buffer and in vegetative cream treated with high pressure. In Hayashi R, Balny C, editors. High pressure bioscience and biotechnology: Proceedings of the International Conference on High Pressure Bioscience and Biotechnology, Kyoto, Japan, November 5- 9, 1995. New York: Elsevier Science Publication. p 253-259.

Goldman RC, Tipper DJ. 1978. Bacillus subtilis spore coats: complexity and purification of a unique polypeptide component. J Bacteriol 135:1091-1106

Gould GW, Sale JH. 1970. Initiation of germination of bacterial spores by hydrostatic pressure. J Gen Microbiol 60:335-346.

Gould GW, Stubb JM, King WL. 1970. Structure and Composition of Resistant Layers in Bacterial Spore Coats. J Gen Microbiol 60:347-355.

Gould GW. 1983. Mechanisms of resistance and dormancy. In: Gould GW, Hurst A, editors. The Bacterial Spore 2. London: Academic Press. p 173-209.

Gould GW. 1989. Mechanisms of action of food preservation procedures. London, New York: Elsevier Applied Science. 441

Gracia-Castineiras S, Dillon J, Spector A. 1978. Detection of bityrosine in cataractous human lens protein. Science 199:897-899.

Hayakawa I. 1991. Life multiplication engineering-Life engineering series V. In: Yamaguchi H editor. High Pressure Effects on Life Science. Tokyo: Shokabo Publication Co. Ltd. p 29-57.

Hayakawa I, Oda M, Kajawara J. 1992a. Food processing by ultra high pressure & twin screw extrusion. In: Hayakawa I, editors. Study of Pressure Bearibility of Baker's Yeast under Buffer Conditions. Lancaster, PA: Technomic Publishing Company. p 47-56.

Hayakawa I, Kajihara J, Morikawa K, Oda M, Fujio Y. 1992b. Denaturation of bovine serum albumin (BSA) and ovalbumin by high pressure, heat and chemicals. J Food Sci 58:288-292

Hayakawa I, Kanno T, Yoshiyama K, Fujio Y. 1994. Oscillatory compared with continuous High Pressure Sterilization on Bacillus stearothermophilus Spores. J Food Sci 59(1):164-167.

Hayakawa I, Furukawa S, Midzunaga A, Horiuchi H, Nakashima T, Fujio Y, Yano Y, Ishikura T, Sasaki K. 1998. Mechanism of inactivation of heat-tolerant spores of Bacillus stearothermophilus IFO 12550 by rapid decompression. J Food Sci 63(3):371-374.

Henriques AO, Moran CP. 2000. Structure and Assembly of the Bacterial Endospore Coat. Methods: A Companion to Methods in Enzymology 20(1): 95-110

Heremans K, Wong PTT. 1985. Pressure effects on the Raman spectra of proteins: Pressure induced changes in the conformation of lysozyme in aqueous solutions. Chem Phys Letters 118:101-104.

Hoover DG, Metrick C, Papineau AM, Farkas DF, Knorr D. 1989. Biological effects of high hydrostatic pressure on food microorganisms. Food Technol. 43(3):99-107.

Jenkinson WD, Sawyer WD, Mandelstam J. 1981. Synthesis and Order of Assembly of Spore Coat Proteins in Bacillus subtilis. J Gen Microbiol. 123:1-16.

Johnson CW. 1990. Protein Secondary Structure and Circular Dichroism: A Practical Guide Structures. PROTEINS: Structure, Function, and Genetics. 35:307-312.

Kelly SM, Price NC. 1997. Applications of circular Dichroism to studies of protein folding and unfolding. Biochim Biophys Acta 1338:161-185.

Knorr D. 1993. Effects of High-Hydrostatic-Pressure Processes on Food Safety and Quality. Food Tech 47(6): 156-161.

Kramer JM, Gilbert RJ. 1989. Bacillus cereus and other Bacillus species. In Foodborne Bacterial Pathogens. Doyle MP, editor. New York: Marcel Dekker. p 21-70.

Krimm S, Bandekar J. 1986. Vibrational spectroscopy and conformation of peptides, polypeptides, and proteins. Adv Protein Chem 38:181.

Kurt DB. 1996. Protein Secondary Structure. Course of Karolinska Institute, Stockholm, Sweden.

Laemmli UK, Favre M. 1973. Maturation of the head of bacteriophage T4.I.DNA packaging events. J Mol Bio 80:575-599

Leighton TJ, Doi RH. 1971. The Stability of Messenger Ribonucleic Acid during Sporulation in Bacillus subtilis. J Biol Chem 246:3189-3195.

Mackey BM, Forestiere K, Isaacs NS, Stenning R, Brooker B. 1994. The effect of high hydrostatic pressure on Salmonella thompson and Listeria monocytogenes examined by electron microscopy. Lett Appl Microbiol 19(6):429-432.

Malencik DA, Zhao Z, Anderson SR. 1990. Determination of Dityrosine, Phosphotyrosine, Phosphothreonine, and Phosphoserine by High-Performance Liquid Chromatography. Anal Biochem 184:353-359.

Masson P. 1992. Pressure denaturation of proteins. In Balny C, Hayashi R, Heremans K, Masson P, editors. High Pressure and Biotechnology. 224. Colloque INSERM/ John Libbey Eurotext Ltd. p 89-99.

McClements JMJ, Patterson MF, Linton M. 2001. The effect of growth stage and growth temperature on high hydrostatic pressure inactivation of some psychrotrophic bacteria in milk. J Food Prot 64(4):514-522.

Metrick C, Hoover DG, Farkas DF. 1989. Effect of high hydrostatic pressure on heat-resistant and heat-sensitive strains of Salmonella. J Food Sci 54(6):1547-1549 and 1564.

Mills G, Earnshaw R, Patterson MF. 1998. Effects of high hydrostatic pressure on Clostridium sporogenes spores. Lett Appl Microbiol 26:227-230.

Mitani T, Kadota H. 1976. Chemical features of spore coat of Bacillus subtilis. J Gen Appl Microbiol 22: 51-63.

Munoz LE, Nakayama T, Doi RH. 1978. Expression of spore coat gene, an 'early sporulation gene', and is relationship to RNA polymerase modification. In: Chambliss G, Vary JC editors. Spores VII. Washington, DC: American Society for Microbiology. p 213-219

Nakayama T, Munoz LE, Doi RH. 1977. A procedure to Remove Protease Activities from Bacillus subtilis Sporulating Cells and Their Crude Extracts. Anal Biochem 78:165-170.

Nakayama A, Yano Y, Kobayashi S, Ishikawa M, Sakai K. 1996. Comparison of pressure resistances of spores of six Bacillus strains with their heat resistances. Appl Environ Microbiol 62(10):3897-3900.

Nicholson WL, Setlow P. 1990. Sporulation, Germination and Outgrowth. In Harwood and Cutting editors. Molecular Biological Methods for Bacillus. New Jersey: John Wiley and Sons. p 391-449.

Olsen SJ, MacKinon LC, Goulding JS, Bean NH, Slutsker L. 2000. Surveillance for Foodborne Disease Outbreaks United States, 1993-1997. Division of Bacterial and Mycotic Diseases. National Center for Infectious Diseases. 3:49(SS01).1-51.

Pandey NK, Aronson AI. 1979. Properties of the Bacillus subtilis spore coats. J Bacteriol.137:1208-1218.

Patterson MF, Quinn M, Simpson R, Gilmour A. 1995. Sensitivity of vegetative pathogens to high hydrostatic pressure treatment in phosphate buffered saline and foods. J Food Prot 58(5):524-529 .

Peterson GL. 1977. A simplification of the Protein Assay Method of Lowry et al. Which is more Generally Applicable. Anal Biochem 83:346-356

Prehoda KE, Mooberry S, Markley JL. 1998. Pressure Denaturation of Proteins: Evaluation of Compressibility Effects. Biochem 37:5785-5790.

Price NC. 1995. Protein analysis by circular dichroism. In Meyers RA editors. The Encyclopedia of Molecular Biology and Molecular Medicine vol 1. Weinheim: VCH Press. p 384 396.

Raso J, Barbosa-Canovas G, Swanson BG. 1998. Sporulation temperature affects initiation of germination and inactivation by high hydrostatic pressure of Bacillus cereus. J Appl Microbiol 85:17-24.

Reddy NR, Solomon HM, Fingerhut GA. 1999. Inactivation of Clostridium botulinum type E spores by high pressure processing. J Food Safety. 19(4):277-288.

Rodger A, Norden B. 1997. Circular Dichroism and Linear Dichroism. New York: Oxford University Press. p 1-32.

Roitt I, Wakelin D, Williams R, Mims CA, Playfair JN. 1998. Chapter 20. Gastrointestinal Tract Infections. In Mims editor. Medical Microbiology. 2nd edition. St. Louis: Mosby. p 253-286.

Sale AJH, Gould GH, Hamilton WA. 1970. Inactivation of bacterial spores by hydrostatic pressure. J Gen Microbiol 60:323-334.

Schimerlik MI. 2002. Personal communication. Dept. of Biochemistry and Biophysics, Oregon State University, Corvallis, OR

Setlow P. 1994. Mechanisms which contribute to the long- term survival os spores of Bacillus species. J Appl Bacteriol 176 (Symp. Suppl.):49S-60S.

Setlow P. 1995a. Mechanisms for the preventation of damage to the DNA in spores of Bacillus species. Annu Rev Microbiol 49:29-54.

Setlow B, Setlow P. 1995b. Small, Acid-Soluble Proteins Bound to DNA Protect Bacillus subtilis Spores from Killing by Dry Heat. Appl Environ Microbiol 61(7):2787-2790.

Shukla TP. 1992. Nutraceutical foods. Cereal Foods World 37:665-666.

Spudich JA, Kornberg A. 1968. Biochemical studies of bacterial sporulation and germination. VI. Origin of spore core and coat protein. J Biol Chem. 243:4588-4599.

Stewart CM, Jewett Jr FF, Dunne CP, Hoover DG. 1997. Effect of concurrent high hydrostatic pressure, acidity and heat on the injury and destruction of Listeria monocytogenes. J Food Safety 17: 23-36.

Styles MF, Hoover DG, Farkas DF. 1991. Response of Listeria monocytogenes and Vibrio parahaemolyticus to high hydrostatic pressure. J Food Sci 56(5) 1404-1407.

Surewicz WK, Mantsch HH, Chapman D. 1993. Determination of protein secondary structure by Fourier transform infrared spectroscopy: a critical assessment. Biochem 32(1):389-94

Susi H, Byler DM. 1988. Fourier Deconvolution of the Amide I Raman of Proteins as Related to Conformation. Appl. Spectroscopy 42(5):819-825.

U. S. Food and Drug Administration Center for Food Safety and Applied Nutrition June 2, 2000. Kinetics of Microbial Inactivation for Alternative Food Processing Technologies Overarching Principles: Kinetics and Pathogens of Concern for All Technologies, http://vm.cfsan.fda.gov/~comm/ift-over.html

U. S. Food and Drug Administration Center for Food Safety and Applied Nutrition June 20, 2001. Foodborne Pathogenic Microorganisms and Natural Toxins Handbook. In Bad Bug Book. http://vm.cfsan.fda.gov/~mow/chap12.html

Velázquez, G., Gandhi K, Torres JA. 2002. Hydrostatic pressure processing: A review. Biotam 12(2): 71-78.

Venyaminov SY, Kalnin NN. 1990. Quantitative IR spectrophotometry of peptide compounds in water (H2O) solutions. I. Spectral parameters of amino acid residue absorption bands. Biopolymers 30(13):1243 1257

Warth AD. 1978. Molecular Structure of the bacterial spore. Advances in Microbial Physiology 17, 1-45.

Weemaes C, DeCordt S, Goossens K, Ludikhuyze L, Hendricks K, Hermans K, Tobback P. 1996. High Pressure, Thermal, and combined Pressure-Temperature Stabilities of α-amylases from Bacillus Species. Biotechnol Bioeng 40:396-402.

Wong PTT, Hermans K. 1988. Pressure effects on protein secondary structure and hydrogen deuterium exchange in chymotrypsinogen: a Fourier transform infrared spectroscopic study. Biochem Biophys Acta 956:1-9.

Wuytack EY, Boven S, Michiels CW. 1998. Comparative study of pressure induced germination of Bacillus subtilis spores at low and high pressures. Appl Environ Microbiol 64(9): 3220-24.

Zipp A, Kauzmann W. 1973. Pressure denaturation of metmyoglobin. Biochemistry. 12, 4217-4228.