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High hydrostatic pressure processing (HPP) is a nonthermal process in which foods are subjected to pressures in the range of 100-700 MPa at room temperatures or higher. HPP is effective in reducing microbial counts and enzyme activity. The ability of HPP to modify the functional properties of proteins has become the focus of research only recently. Covalent bonds within proteins are unaffected during HPP however; hydrogen bonds, covalent bonds and hydrophobic bonds are modified resulting in changes to protein structure and function.

The ability to control the protein denaturation process by applying HPP and low temperature has only recently been investigated as a method that minimizes the loss of native protein structure and reduces subsequent aggregation reactions. The objective of this thesis was to demonstrate that low temperature HPP treatment could be useful in controlled protein structural changes and thereby improving product functionality and quality.

The first part of this thesis investigated targeted denaturation of β-lactoglobulin through low temperature HPP treatment that optimizes the protein's ability to lower

surface tension at air-water interfaces, while minimizing protein aggregation. In addition, structure/function relationships of denatured β-lactoglobulin are investigated.

Samples treated at 510 MPa, for 10 minutes at 0.5 mg/ml, pH 7.5 at 8°C resulted in decreased aggregation as monitored by native PAGE and significantly higher surface pressure values at the air-water interface. Circular dichroism (CD) data indicated that secondary structural changes occurred. Intrinsic and extrinsic fluorescence and free thiol reactivity were also used to understand the degree of denaturation induced by HPP treatment.

The second part of the study was to apply the cold HPP treatment to yogurt to evaluate the quality benefits of this process in a dairy food system. The present study was conducted to investigate the effects of cold HPP treatment on the color, texture and microbiology of blackberry yogurt formulated with fruit and the shelf-stability of this product over time at refrigerated and room temperature storage conditions.

Low temperature HPP is an effective tool for targeted protein denaturation, which opens the potential for novel food products.

Effect of High Pressure at Low Temperature on the Molecular Structure and Functionality of Dairy Proteins

By

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Marcia K. Walker, Author

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CONTRIBUTION OF AUTHORS

Dr. Daniel Farkas was involved in the idea conception, design and data interpretation of this thesis.

Vicki Loveridge was involved in the research design and interpretation of Chapter 4.

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Introduction

Food product development has become an increasingly difficult task, as consumers are demanding more healthy, convenient and safe foods. This has offered a unique challenge to food scientists to develop ingredients or alter food properties that improve or enhance the functionality or quality of foods.

High hydrostatic pressure processing (HPP) of foods has made considerable advances in recent years by proving to be an effective treatment for microorganism inactivation, enzyme inactivation, and protein unfolding. The process minimizes the destruction of flavors, nutrients, and other quality attributes while delivering unique functional properties not observed with traditional processing methods. HPP treatment of proteins at room temperature has been investigated although undesireable aggregation results and the desired functional properties were not attained. HPP and low temperature were investigated in this thesis in model systems of pure protein and in a dairy food product.

Development of new and improved functional ingredients or food products has demanded an understanding of the importance of the ingredient's structural components and their relationship to functionality in the food system. Structural conformation and surface properties can determine the functional behavior of dairy proteins under a given set of environmental conditions. By using HPP and low temperature this thesis has sought to focus on understanding some of the factors that

determine and maintain desired structural conformations and that can result in increased functionality. Understanding the structural-functional relationship is necessary in order to understand the effects of new processing technologies and in creating value added ingredients or food products with superior functional capabilities.

Chapter 2

Structural-Functional Characteristics of β-Lactoglobulin

Marcia K. Walker

Introduction

Food product development has become an increasingly difficult task, as consumers are demanding more healthy, convenient and safe foods. This has offered a unique challenge to food scientists to develop ingredients that improve the functional properties of foods. Development of new and improved functional ingredients has demanded an understanding of the importance of the ingredient's structural components and their relationship to functionality in the food system.

Whey proteins once thought of as animal feed, are generated by the cheese-making process and are now being used for their high nutritional quality, versatile functionality and low price. The protein portion (.4-.8%) of the whey has gained considerable attention for the role it plays as a value-added food ingredient. Whey proteins have the added benefit of being generally recognized as safe ingredients (GRAS), making them safe to use and label-friendly to the consumer. Whey proteins are used in foods for functions such as mouth feel, gelation, foaming, emulsification and solubility.

Whey protein consists of four major proteins: β-lactoglobulin, α-lactalbumin, bovine serum albumin and a mixture of immunoglobulins. β-lactoglobulin accounts for approximately 60% of the total whey protein (Damodaran, 1997). Many of the functional properties and the thermal behavior of whey protein have been shown to be governed by the properties of β-lactoglobulin (de Wit, 1981). Therefore, structural and functional considerations will focus on β-lactoglobulin.

A major obstacle to increasing the use of or improving the functionality of whey proteins in food products is the understanding of structure function relationships. This paper examines the structural functional characteristics of β-lactoglobulin. As structure is dynamic and a function of the environment, this paper will also cover the effect of structure following certain external changes. It is beyond the scope of this paper to describe all of the functional properties of β-lactoglobulin, therefore, this paper is limited to two of the main functional properties: foaming and emulsions. Throughout the paper, analytical methods currently used to evaluate structural and functional properties of β-lactoglobulin were reviewed so that changes in structure or surface characteristics from a given environmental factor could be related to a change in functionality of the protein.

General Properties of Protein Structure

The functional behavior of food proteins is dependent on the four levels of protein structure: primary, secondary, tertiary and quaternary.

The primary structure is the linear sequence of amino acids and is different for each protein. This linear structure is made from the amino acid building blocks. The chain length and amino acid sequence ultimately helps determine the proteins three-dimensional structure. The amino acids are linked by peptide bonds. Because of the amino acid sequence and the peptide bond linkage, there appears to be a natural limitation to the flexibility so that the primary structure plays an important role in the functionality of the protein (Damodaran, 1997).

The secondary structure of proteins refers to the conformation of the polypeptide backbone. There are generally two types, helix and beta structures.

Predictions of allowed conformations of amino acids have been developed by Ramachandran et al. and known as a Ramachandran map (Ramachandran et al., 1968).

Of the secondary structures, the right-handed α-helix is the best known and easiest to recognize. The α-helix structure as proposed by Corey and Pauling consists of amino acid residues arranged in a repeating sequence of angles. There are 3.6 residues per turn of the α-helix (Kinsella et al., 1994). The α-helix can be viewed as a structure with interactions occurring between residues that are closely linked in the primary sequence. The detailed structure of the α-helix can vary depending on the environment so that the native protein structure will depend on the application of the protein.

The beta sheet structure is made up of beta strands that are lined up adjacent to each other such that hydrogen bonds form. The beta sheet can be described as a parallel, anti-parallel or mixed depending on the direction of the sheets. Generally, proteins with a predominance of beta type structures are more hydrophobic than alpha proteins. Proteins that contain higher amounts of beta sheets also appear to be more thermally stable (Creighton, 1997).

Reverse turns or loops are sometimes classified as a third type of secondary structure. The structure of most proteins is built-up of a mixture of both α-helices and β-sheets that are connected by loops or turns (reviewed by Branden et al., 1991).

In recent years, a category termed the supersecondary structure has become recognized as a level of protein structure (reviewed by Nakai, 1996). Regular associations of secondary structural elements give rise to the supersecondary structure. Examples of these include α-helix coiled coil, triple helix of collagen and other

specific combinations of secondary structural elements such as β–α–β (reviewed by Creighton, 1994). Most proteins appear to be made up of segments of secondary structure packed together and understanding the relationship between these segments is the focus of current research.

The tertiary structure of a protein generally refers to the overall structure formed by the folding of the secondary structural elements into a compact three-dimensional unit. Regions of a polypeptide chain can fold independently into a stable tertiary structure called a domain (Branden et al., 1991). It is the domains of a protein that interacts and gives proteins different tertiary structures. B-lactoglobulin for example is a small protein and only has one domain whereas larger proteins can contain more (Damodaran, 1997).

The quaternary structure of a protein describes the overall polypeptide chain folding patterns and how the tertiary structures are orientated in relation to each other (Creighton, 1993). The formation of tertiary and quaternary structures allow for amino acids that are far apart in the chain to be brought closer together. Several proteins are monomeric and lack a quaternary structure, while others have several polypeptide chains that fold to form the quaternary structure (Branden et al., 1991).

β-lactoglobulin

The molecular weight of β-lactoglobulin is 18.3 kDa and contains 162 amino acid residues (Green et al, 1979). Seven variants of β-lactoglobulin have been identified and are referred to as A, B, C, D, E, F and G. All variants contain the same number of amino acids, however, they vary by 1-3 amino acid residues (Manderson et

al., 1998). Variants A and B are the most abundant forms and differ by only two amino acid residues (Damodaran, 1997).

The primary structure of β-lactoglobulin has been well characterized for all the variants in milks from several different animals. The complete amino acid sequence has been reported (Green et al., 1979). It exists as a dimer between the pH ranges 3-7.5 and has a molecular weight of 36,000 kDa (Manderson et al., 1998).

The secondary structure of β-lactoglobulin has been determined through X-ray crystallography and NMR (Green et al., 1979; Papiz et al., 1986; Molinari et al., 1996). This data has been combined with investigations from circular dichroism and infrared spectroscopy so that secondary structure predictions have been established. β-lactoglobulin can be described as containing 10-15% α-helix , 50% β-sheet and 15-20% β-turns. Its compact structure resembles a flattened cone made of nine β-strands, an eleven-residue α-helix and three helical turns (Belloque et al., 1998; Manderson et al., 1998).

Disulfide bonds are formed between the Cysteine residues of β-lactoglobulin. Native β-lactoglobulin has two disulfide bonds (Cys-66-Cys 160) and (Cys 106-Cys 119) and a free thiol group (Cys 121) (Creighton, 1994). Proteins are capable of forming both intramolecular and intermolecular disulfide bonds. Intramolecular bonds are formed when a pair of cysteine residues is brought into close proximity by the folding of the protein molecule. Intermolecular disulfide bonding is formed when cysteine residues from neighboring molecules are brought together (Hoffmann et al., 1997)

The thiol group at Cys 121 is relatively unreactive at pH 2 however; as pH is increased and heat or high pressure is added the thiol group becomes more reactive. Thus, research has focused on agents that reduce the reactivity of the thiol group in an effort to stabilize the protein. (Moller et al., 1998).

Factors Influencing Structure of β-lactoglobulin

pH

The structure of β-lactoglobulin is significantly affected by environmental factors such as pH, temperature and pressure. The stability of the protein depends a great deal on the stabilizing forces of hydrogen bonding, hydrophobic interactions, disulfide bonding and electrostatic interactions. These stabilizing forces will be important to consider when researching the effects of pH, heat and pressure on whey protein.

The isoelectric point of β-lactoglobulin is approximately 5.2. Typically proteins are insoluble at their isoelectric point and precipitate out. β-lactoglobulin remains in solution near its PI (Kinsella et al., 1979). This unique characteristic suggests that forces other than electrostatic interactions are stronger. Thus, hydrophobic interactions may control protein structure at various pH values. At pH <2 or >7.5, β-lactoglobulin appears to exist as a monomer. At pH 5-7.5, it exists as a dimer and at pH 3-5 the dimers can form larger aggregates (Damodaran, 1997; Hoffmann et al., 1997). Specific structural changes have been characterized at different pH values. At alkaline pH, the protein structure is characterized by a highly flexible and more open structure whereas at acid pH, it takes on a tighter more

compact structure (Reddy et al., 1988). β-lactoglobulin is also more thermally stable at acid pH because of the associated structural changes.

Thermal Processing

The pasteurization of whey protein results in unfolding and aggregation. In simple terms, the process can be seen as an unfolding of the protein molecule into a disorganized structure. Early work in this area indicated that the proteins followed a simple two-step process with native protein unfolding and subsequent aggregation (de Wit, 1981). Current research would suggest that thermal unfolding of proteins follows a series of unfolding steps with several intermediate species (Qi et al., 1997; Cairoli et al., 1994; Iametti et al., 1996; Tani et al., 1995). The reactive amino acids are often located in the interior of the protein and this unfolding process exposes these hydrophobic amino acids and results in protein aggregation and ultimately insolubility (Bryant et al., 1998; DeWit, 1981). The pH, protein concentration and the presence of other ingredients will influence the conformation that β-lactoglobulin will adopt (Bryant, 1998; De Wit, 1981). The genetic variant of β-lactoglobulin also appears to influence thermal unfolding (Manderson, 1998). Although, it is unknown what accounts for the thermostability, they appear to follow the order of C>B>A (Kinsella, 1994). It has also been investigated recently, that the aggregation pathways for the genetic variants may be different and therefore studies are preferably done using single-variant systems (Manderson, 1998).

At approximately 40°C β-lactoglobulin undergoes small but reversible conformational changes. Thermal unfolding of β-lactoglobulin follows a series of unfolding steps as the temperature is progressively increased from 60-80°C. At low

temperatures (<65°C) hydrophobic interactions dominate so that the folded state is favored (Bryant et al., 1998). Differential scanning calorimetry has shown the unfolding temperature of β-lactoglobulin to be 70°C. Experiments also show that up to this temperature the unfolding can be reversible. At temperatures near 130°C the protein unfolds and complete irreversible aggregation occurs. (DeWit, 1981; Laligant et al., 1991; Iametti et al., 1996). Recent research suggests that β-lactoglobulin unfolds to form a molten-globular structure between 65 and 130°C (Qi, et al., 1997, Tani et al., 1995). This molten globular structure appears to be neither fully folded or unfolded and is defined as having most of the secondary structural characteristics of the native state. Currently, there is disagreement at which temperature this state occurs. The most recent work suggests that most of the characteristics of the native secondary structure are lost by 70°C (Qi et al., 1997). However, this partial unfolding increases the exposure of inner hydrophobic groups and the thiol group. It has been observed that hydrophobic interactions and thiol-disulfide reactions can occur in this state leading to aggregation (Hoffmann et al., 1997). It is less clear at which point in the unfolding process these reactions occur. There is general agreement in the literature that thiol/disulfide reactions lead to the formation of intermolecular disulfide bonds that result in the heat induced aggregation (Cairoli et al., 1994, Iametti et al., 1996, Gezimati et al., 1997; Monahan et al., 1995; Laligant et al., 1991). Although the role of the thiol group in thermally induced aggregation has been studied extensively it is difficult to compare research findings. This is due to differences in experimental conditions and the fact that the mechanisms for aggregation of β-lactoglobulin vary significantly with temperature, pH and protein concentration.

Recent investigations have tried to elucidate the pathways involved in the aggregation of β-lactoglobulin and the role of pH (Hoffmann et al., 1997; De Wit et al., 1990; Belloque et al., 1998; Ju et al., 1999; Hoffmann et al., 1997; Manderson et al., 1998). At the iso-electric pH range near 5.2 the protein is the most heat sensitive (de Wit, 1981). Between pH range of 2.5 and 3.5 the protein appears to be the most heat stable and retains solubility (de Wit, 1981). There appears to be a critical range between pH 6 and 7. Heat treated β-lactoglobulin shows a great deal of protein aggregation at pH 6 with a marked decrease as the pH approaches 6.5. At pH above 6.8, experimental data indicates increased thermostability of the protein as illustrated by protein solubility.

Hoffmann et. al. (1997) recently concluded that the free thiol group of β-lactoglobulin plays a crucial role in the thermal aggregation by initiating the thiol/disulfide exchange reactions. Other studies indicate that at pH values close to and below the PI thiol reactions are limited, however above the PI they are increased (Monahan, 1995).

High Pressure Processing

It is well documented that HPP can result in the unfolding and gelatinization of proteins (Mozhaev et al., 1996; Balny et al., 1989; Hayakawa et al., 1996; Funtenberger et al., 1995; Kanno et al., 1998) though the mechanisms involved in these events have only recently been investigated and is not clearly understood (Hayakawa et al., 1996). The ability of high pressure to modify the functional properties of β-lactoglobulin has become the focus of research only in the last few years.

Pressure induced unfolding of β-lactoglobulin has been demonstrated through several basic studies at pressures of 100-1000 MPa for time periods up to 30 minutes (Tedford et al., 1999; Pittia et al., 1996). The degree of unfolding is complex and appears to be related not only to the level of pressure but also to temperature and pH. Following HPP treatments, researchers have observed changes in surface hydrophobicity and subsequent aggregation of β-lactoglobulin (Nakamura et al., 1993; Dumay et al., 1994). Generally, it is believed that HPP induced changes increase the area accessible to the solvent and consequently alters the surface properties of β-lactoglobulin (Cheftel et al., 1992). Currently, researchers conclude that structural changes upon HPP are different than thermal unfolding (Mozhaev et al., 1996). The pressure induced unfolding of β-lactoglobulin appears to allow for the destabilizing of the noncovalent interactions of the tertiary structure (Pittia et al., 1996, Tedford et al., 1999). Changes to the secondary structure are small and appear to resemble the molten globule state. Structurally, these proteins retain much of their secondary structure however, there is a small degree of unfolding and hydrophobic areas are exposed. It has been assumed that this is why protein aggregation occurs (Mozhaev et al., 1996; Tedford et al., 1999). Other researchers have investigated the pressure induced unfolding and the role of the SH and S-S groups. Generally, the unfolding of the protein during high-pressure treatment is followed by formation of hydrophobic and disulfide bonded aggregates after pressure release (Funtenberger et al., 1997). Future investigations are necessary to optimize the HPP treatment to minimize the aggregation of β-lactoglobulin and thereby increase its potential functionality.

Structural Analysis

Analytical Techniques

Many techniques have been used to characterize the structural changes in β-lactoglobulin during and following thermal and high pressure treatment (Haque et al., 1988; Damodaran et al., 1997; Qi et al., 1998; Belloque et al., 1998; Mozhaev et al., 1996). These techniques include UV, circular dichroism, Fourier transform infrared spectroscopy (FTIR), nuclear magnetic resonance (NMR) and fluorescence spectroscopy. Spectroscopic methods are widely used to determine protein stability and to follow structural changes such as unfolding. Consideration must be given to factors that will affect the choice of the method for a protein structure analysis. The amount of sample available, the resolution necessary, protein concentration, and whether secondary and tertiary information is desired are important considerations. Other important considerations are the availability of equipment and the expertise and the expense necessary to operate (Havel et al., 1996). In most cases, a single method may not provide all of the information needed and a combination of methods may compliment and support the data obtained. Because of their ability to determine both secondary and tertiary structure and the frequent use of these methods in both thermal and high pressure treatment of β-lactoglobulin this paper will be limited to the discussion of CD and NMR analysis.

Circular dichroism

Circular dichroism (CD) is the method of choice when considering expense, equipment operation and a technique that is sensitive to protein secondary and tertiary structure. The literature suggests that one of the most significant applications of CD is

to provide evidence that the structure of a protein has changed during a particular condition or treatment (Damodaran, 1996; Nakai et al., 1996). Because of the high precision of the equipment, CD analysis has become a standard technique for measuring protein structure (Creighton, 1997). The protein's optical activity results from its ability to rotate plane-polarized light. This plane rotates between two components right and left handed polarized light. Circular dichroism results when the two light components encounter a protein in solution and they are absorbed unequally (Damodaran, 1997). It produces highly reliable results when the CD is measured at 178 nm and compared to the x-ray structure of the proteins in database programs (Johnson, 1990). CD work is usually conducted in the far-UV (170-250 nm) and is dominated by the contribution of peptide bonds. Measurements taken in the far –UV are frequently used for secondary structure determination and to quantify secondary structural changes. It has been widely used for determining protein stability and to follow changes such as folding and refolding (Creighton, 1997). The α-helix displays a strong characteristic CD spectrum in this range. Other secondary structural components are less defined and determination is estimated from computer programs that compare protein structure to a base set of known proteins (Johnson, 1999). Several methods exist for the determination of secondary structural elements and reviews indicate that recent improvements to these programs make them a good analytical tool when established protocol is followed (Schmid et al., 1997, Johnson, 1999).

Measurements are also taken in the near-UV (240-320 nm) and it has been shown to be highly sensitive to conformational changes in protein and can be used

effectively to monitor tertiary structural changes (Nakai, 1996; Arai, 1998). Because the CD of the aromatic side chains is very low when the protein is unfolded, measurements can only be used to show differences between the folded and unfolded state of β-lactoglobulin and are not quantitative (Schmid et al., 1997; Havel et al., 1995).

In CD analysis, the current convention utilized for interpretation of the data is to express the difference spectrum in terms of the Beer-Lambert Law, where:

$\Delta\varepsilon=\varepsilon L - \varepsilon R= \Delta A/lc = A_L - A_R /lc.$

This equation is defined as follows:

$\Delta\varepsilon$ = molar absorbitivity coefficient

εL = left handed circularly polarized light

εR= right handed polarized light

l = pathlength in centimeters

ΔA = difference absorbance of left polarized light and right polarized light

c = concentration in moles per liter

The molor absorbitivity of a protein is different depending on the circularly polarized light and can be determined experimentally or calculated (Schmid et al., 1997; Johnson, 1990). Using data from the CD analysis, the protein can be compared through computer based programs to the CD of basis set proteins with known secondary structure that where determined by X-ray crystallography (Johnson, 1999). For determination of structures such as the molten globule, CD has been used successfully and showed good correlation with FTIR spectroscopy when used together

(Qi et al., 1997). Additionally, aggregation or gelling can diffract light and make the CD results inaccurate (Damodaran, 1996).

NMR Spectroscopy

Nuclear magnetic resonance (NMR) generally will give the most detailed structural information and has been correlated in quality with crystallography. NMR determinations are based on interproton distances and torsion angles (Havel et al., 1996). However, there are benefits and limitations to the use of NMR. If high resolution at an atomic level is necessary than the effort and expense of NMR is justified. This method is unfortunately expensive, time consuming, technically challenging, requires large samples, and is limited to analysis of proteins < 50kDa (Havel et al., 1996).

Surface Activity

Characteristics

Many processed foods exist as emulsions or foams because of the use of whey proteins and their unique surface activity. Emulsions and foams can be seen as a two-phase system in which an aqueous phases is dispersed in another phase (oil or air). The stability of this system is greatly influenced by the presence of surface-active agents without which phase separation would occur (Waniska et al., 1985). Surface-active agents or surfactants significantly reduce the surface tension between the two phases. In the food industry, whey protein has become very important as an effective tool in reducing the surface tension and aiding in the stability of emulsions and foams. Research in this area has indicated that not only does whey protein reduce interfacial

tension it can also form a film around oil or air droplets adding to its functionality (Dickenson, 1994).

The surface activity of β-lactoglobulin results from the fact that it is amphiphatic, it contains both hydrophobic and hydrophilic regions that can orientate at an interface. This property is determined by the protein's primary structure and the relative polar: nonpolar amino acid ratio or commonly called average hydrophobicity (Nakai et al., 1996). Solubility of proteins has often been related to the average hydrophobicity, the lower the hydrophobicity, the higher the solubility. This relationship failed to explain why β-lactoglobulin is soluble at its isoelectric point Thus, the average hydrophobicity does not appear to explain the large differences in surface characteristics among proteins. It is more likely that the surface hydrophobicity and hydrophilicity of β-lactoglobulin will play a more important role in surface activity characteristics (Damodaran, 1998).

Generally, diffusion to an interface has been described as an attachment or absorption of polypeptide segments at an interface with large portions of the protein suspended in the aqueous phase (Nakai et al., 1996). The next phase of adsorption is termed interfacial assembly and is characterized by a decrease in surface tension (increase in surface pressure) as increasingly more protein molecules interact at the interface (Kinsella et al., 1994). The ability of the protein to change conformation at the interface ultimately determines its ability as a surface active agent. The rearrangement and adsorption of β-lactoglobulin at the air-water interface at different pH values has been investigated. This property of β-lactoglobulin demonstrates the effect of environmental conditions on surface active properties. Researchers

concluded that the rate of adsorption of β-lactoglobulin at the interface was greatly influenced by pH, with the greatest values near the isoelectric point (Waniska et al., 1985). Interfacial protein adsorption increases near the isoelectric point because of the decreased electrostatic repulsion at the interface and the protein can pack to a greater extent into the interfacial film (Iametti et al., 1995). A high net charge appears to lower adsorption and protein-protein interaction. Therefore, β-lactoglobulin forms a strong viscous condensed film with maximum protein interactions between the pH ranges of 4.5-6.0 (Waniska et al., 1985). Increasing temperature and addition of salts, enhanced the rates of adsorption (Kinsella et al., 1994).

β-lactoglobulin has an internal free sulfhydryl group, which is not available for reaction however, as the protein adsorbs and partially unfolds at the interface, the sulfhydryl group becomes exposed and available for polymerization at the interface (Dickenson, 1994; Monahan et al., 1995; Gezimati et al., 1997). Thermal treatment and increased pH both lead to unfolding and increased exposure of the free sulfhydryl group resulting in increased protein interactions, intra and inter molecular thiol/disulfide interchange and thiol/thiol oxidation reactions (Monahan et al., 1995; Dickenson, 1994). Controlling the extent of these reactions can be an effective way of increasing the functionality of β-lactoglobulin. Once formed disulfide-linked proteins are less likely to unfold and have been used extensively to increase gel firmness and stability (Shimada et al., 1988). Disulfide linked proteins have been shown to decrease the formation of emulsions and foams because of their inability to unfold at the interface (Kim et al., 1987; Phillips et al., 1989). Once a foam or emulsion is formed, the stability has been shown to improve by the formation of

disulfide bonds (Dickenson, 1986, 1994). Thus, a great deal of research has been conducted on modifications that can control formation of disulfide bonds and result in an improvement in surface active properties (Hoffmann et al., 1997; Iametti et al, 1996; Waniska et al., 1985; Monahan et al., 1995).

Analysis of Surface Activity

β-lactoglobulin surface properties have been investigated by measuring surface tension (Waniska et al., 1985; Suttiprasit et al., 1992). The absorption and interfacial orientation can be monitored by surface or interfacial tension and surface concentration measurements (Waniska et al., 1985). The surface tension of protein solutions reaches equilibrium only after several hours. Therefore, the practical methods available are limited. One of the most convenient methods for determining surface tension is Du Nouy ring tensiometry. This ring method is based on the force required to pull the ring from a liquids surface. The force measurement given by the scale reading is an apparent surface tension, that must be multiplied by a correction factor to get a true surface tension value (Suttiprasit et al., 1992).

Surface tension values over time can be used to determine the area cleared per molecule during the adsorption and rearrangement phases (Waniska et al., 1985). During protein adsorption at the interface, protein molecules will continually be adsorbing and rearranging at the interface. During this process, the protein molecules at the interface must be compressed to make way for new protein molecules. This value of compression can be calculated from surface tension and compared over time with the surface tension before protein adsorption. From the calculations, an estimation of the size of the protein at the interface and the average area cleared per

protein at different surface tensions can be determined. Additionally, calculation of the apparent number of amino acids at the interface is possible from these relationships. These relationships have been used to investigate the properties of protein films which are critical for the understanding of the foaming properties of proteins in foods (Waniska et al., 1985, Kinsella et al., 1994).

Analysis of Surface Characteristics

Hydrophobic characteristics of β-lactoglobulin have been studied and related to foaming and emulsification properties. Research indicates a strong correlation between the surface hydrophobicity and emulsification capacity (Voutsinas et al., 1983). Therefore, quantification of surface hydrophobicity has become an important step in prediction of protein functionality. β-lactoglobulin surface hydrophobicity has been determined by hydrophobic interaction chromatography and fluorescence probe method (Nakai, 1983).

Studies have frequently, utilized the fluorescent probe method since analysis is relatively easy (Laligant et al., 1991). This method has also been used to investigate the thermal and high pressure changes on β-lactoglobulin (Pittia et al., 1996). The surface hydrophobicity can be determined by binding with the hydrophobic fluorescent marker, 1,8 anilinonaphthalenesulfonate (ANS) or cis-parinaric acid (CPA) (Pittia et al., 1996; Iametti et al., 1995; Monohan et al., 1995; Nakai, 1983; Cairoli et al., 1994; Pagliarini et al., 1990). The method is based on the affinity of the fluorescent markers to bind with the hydrophobic patches on the protein surface. The relative fluorescence can be measured using fluorometric techniques and converted into a quantification of binding and thereby an index of surface hydrophobicity

(Laligant et al., 1991). ANS binding is the most common ligand used in these techniques, however it's validity has been questioned (Laligant et al., 1990; Lee et al., 1992). These researchers concluded that the binding of ANS by β-lactoglobulin was much lower than binding of retinol. It has also been suggested that fluorescent probes only bind to well defined hydrophobic patches on the surface of proteins and will give results that are low. Despite critisism, this method has shown to be useful in monitoring the unfolding of β-lactoglobulin, showing a increase in binding as the protein unfolds and a subsequent decrease as the protein aggregates (Nakai, 1996). Correlations have been made between surface hydrophobicity determined fluorometrically and surface tension and emulsification activity (Voutsinas et al., 1983).

As discussed, the internal thiol group plays an important role in surface characteristics when β-lactoglobulin is unfolded due to heat or HPP. Monitoring the presence or absence of these reactive groups is important in determining the proteins end functionality. Current testing of the reactivity of the free thiol of β-lactoglobulin is based on its reactivity with Ellman's reagent, 5,5'-dithiobis(2-nitrobenzoic acid) or (DNTB) (Moller et al., 1998; Hoffman et al., 1997; Lee et al., 1992; Pittia et al., 1996). DTNB reacts with the thiol components of β-lactoglobulin to produce p-nitrothiolphenol, which is yellow in color. In native β-lactoglobulin the thiol group is buried internally and remains very unreactive, however as it unfolds it will show an increased reactivity with the DNTB. Absorbance readings are determined spectrophotometrically and concentration of thiol groups can be computed. Close control of pH is essential with this method because of increased reactivity of the thiol

with increased pH (Moller et al., 1998). This method has also been employed for following protein refolding for several days following thermal or HPP treatment (Moller et al., 1998).

Functional Properties

General Characteristics

Several definitions for functional properties of foods exist. Kinsella et al. (1976) defined functional properties of food proteins as "those physical and chemical properties, which affect the behavior of proteins in food systems during processing, storage, preparation, and consumption. Different applications require different functional properties and thus prediction of the structural–functional relationship becomes complex. The protein's functional properties are interrelated with its physical, chemical, and structural properties and include their size, shape, amino acid composition and sequence, net charge, and hydrophobicity/hydrophilicity ratio. Also important considerations are the secondary, tertiary and quaternary structure, molecular flexibility and rigidity in response to external environmental conditions and interaction with other food components (Nakai et al., 1996). In a broad view, the functional properties of food proteins may be seen as a combination of two molecular aspects of proteins: conformational and surface-related properties. The conformational properties relate to size, shape, and molecular flexibility. Whereas, the surface related properties are controlled by the hydrophobic/hydrophilic characteristics of the protein surface.

The relationship between the conformational properties, surface properties and functional properties is poorly understood. Most of the research available in this area

is based on model aqueous solutions and not complex food systems. Additionally, there is a lack of standardized methods for analysis of protein functional properties. Comparison of literature results is difficult because of the variations in methods and procedures.

Foams

Foams are a two-phase system in which an air phase is surrounded by a continuous liquid phase (Phillips et al., 1990). Foaming properties are usually represented by two parameters, foamability and foam stability. Adsorption and rearrangement rates determine foamability and flexible, cohesive film formation determines foam stability (Ibanoglu et al., 1999). Phillips et al. (1995) proposed that foaming properties are dependent upon the protein's surface hydrophobicity and flexibility. Their research sought to determine the structural changes of β-lactoglobulin during foam formation and this research showed a strong correlation between the foaming properties and the secondary structure as determined by CD measurements. Other factors such as pH, heat and pressure will alter structural and surface properties that will influence foam formation. Ultimately, foaming formation will be dependent upon any change that alters the surface hydrophobicity and molecular flexibility (Phillips et al., 1995).

Research indicates that proteins in general foam best at pH levels where the molecules are most flexible and less compact (Kinsella et al., 1994). Foam overrun, a frequently used measure of foam formation had the lowest values at pH between 3-4, which corresponds to the pH range where β-lactoglobulin is rigid and difficult to unfold (Phillips et al., 1990).

Research indicates that mild heat treatment increases the surface hydrophobicity of β-lactoglobulin leading to increased foamability (Nakai, 1996). However, as the thermal treatment becomes more severe, poorer foaming characteristics of β-lactoglobulin resulted due to undesirable aggregation. Few studies are available that determine the effect of HPP on foam characteristics. However, available research suggests that HPP negatively effected foam behavior due to pressure induced undesirable aggregation (Pittia et al., 1996).

Methods of Evaluating Foam Properties

In 1990, researchers from nine laboratories collaborated over four years to evaluate a reliable, standard measurement for evaluating foaming properties of proteins (Phillips et al., 1990). Prior to this study, several methods were used to measure foaming and included whipping, bubbling or sparging and shaking (Phillips et al., 1987). The collaborative study determined that the whipping method was the method that related best to industrial processes. In the whipping method, the protein solution is whipped in a blender. Foam overrun or density, is measured after whipping for set time intervals and foam stability is determined from rates of drainage. This research suggested that even when using this method, previous results were difficult to compare due to variability in equipment and procedures. This research effort set to standardize procedures that with proper training and using a standard household blender could reliably detect differences in foaming properties of food proteins.

Foam formation was followed by measuring overrun. This was calculated by the following equation:

$$\%\text{overrun} = \frac{(\text{wt 100 ml protein}) - (\text{wt 100 ml foam})}{(\text{wt 100 ml foam})} \times 100$$

In determining a standardized method for overrun, the collaborators examined possible sources of variabilities. The main source of variability was the type of mixer used. These factors included the number of beaters, the bowl uniformity, and mixer speed. Each of these parameters was subsequently standardized among collaborating labs. Protein hydration, dispersion and quantity in solution were other sources of variability that needed standardization.

Drainage or foam collapse has been used previously as a reliable index of foam stability (Phillips et al., 1987; Coke et al., 1990). The collaborative study of Phillips et al. (1990) used a stainless steel bowl modified by drilling a hole in the bottom. At ambient temperature, immediately after whipping, the sample was weighed and the hole in the bowl was unplugged allowing the foam to drain into a beaker. The drained liquid was weighed and when 50% drainage was attained the time was used as the index of foam stability. In the process of standardizing this procedure the researchers determined that the major source of variation was the time following whipping that they initiated drainage. When drainage time was initiated immediately when whipping stopped, they obtained the most reproducible results. Additionally, blockage of the drainage hole was checked to ensure proper drainage.

Differences in whip time to achieve maximum overrun will vary depending on the protein and mixer used (Phillips et al., 1987). This point of maximum overrun is often the point of minimum foam stability for many proteins because the maximum amount of air has been incorporated. Thus, the collaborative study avoided using this point as the only criterion for performance of a foam. Instead, comparison of foam stability and overrun measurements at several whip times was more reproducible and

offered a better comparison of results between different proteins. The final standardized method to evolve from this study has been widely used to detect significant differences among foams produced by different proteins (Kinsella et al., 1996).

Conductivity has recently been used to measure foaming properties of HPP treated β-lactoglobulin (Pittia et al., 1996). The method is based on the ability of electrical current to travel around bubbles to pass through the foam. The bubbles carry no electrical current but contribute to the foam density. Additionally, because size of the bubble can influence foam stability this method has had limited acceptance as a tool for foam stability (Wilde et al., 1996). Critics of this method believe that it does not relate the bubble size distribution to the foam conductivity (Wilde et al., 1996). Recently, Wilde et al. (1996) demonstrated that by using single orifice sparging they could control the bubble size distribution and eliminate this as a variable. The foaming properties were monitored by using a microconductivity meter with a built in electrode and connected to a recorder. Initial foam conductivity (C_0)of the bulk solution is measured then nitrogen gas is sparged into the solution to create a foam and the conductivity after sparging (C_s) is recorded. Foamability was expressed :

$$\text{Foamability (\%)} = \frac{C_o}{C_s} \text{ X } 100$$

After sparging the sample is continuously monitored for conductivity for a set time, usually five minutes, and the (C_t) is recorded. Foam stability is thus defined by the following relationship:

$$\text{Foam Stability (\%)} = \frac{C_t}{C_o} \text{ X } 100$$

Rate of foam formation was also calculated by recording the time taken to generate the foam. Additionally, relative foam density and relative foam conductivity were calculated by the following relationships:

$$\text{Relative Foam Density (\%)} = \frac{\text{initial sample volume} - \text{volume liquid beneath foam}}{\text{Foam volume}}$$

$$\text{Relative Foam Conductivity (\%)} = \frac{\text{Foam Conductivity}}{\text{Sample Conductivity}}$$

Bubble size was measured using a microscope. This researcher ran samples of β-lactoglobulin and concluded that the microconductivity technique was a sensitive tool for responding to subtle changes in interfacial properties and composition of foams.

Emulsions

β-lactoglobulin, because of it's amphiphilic structure can concentrate at the interface between phases. Food emulsions are generally of two types: oil-in-water, in which the oil is dispersed in an aqueous continuous phase, and water-in-oil, in which the water is dispersed in a continuous oil phase. These dispersions are thermodynamically unfavored and they tend to immediately separate into two phases unless an emulsifier is present at the interface. Even in the presence of an emulsifier, these dispersions will separate over time. Therefore, not only is the proteins ability to form the emulsion important but also the ability to stabilize the emulsion over time is a critical functional property. Emulsifying properties and stability are affected by solubility, surface hydrophobicity, processing conditions, competing food ingredients and conformation (Damodaran, 1997).

Measurement of emulsifying properties

Solubility has been considered important in the ability of a protein to emulsify and stabilize an emulsion (Nakai, 1983; Voutsinas et al., 1983). Disagreement and contradictory results exist over the importance of this index in emulsification capabilities (Voutsinas et al., 1983; Nakai, 1983; Iametti et al., 1995). Solubility is generally correlated with superior functional properties and some researchers propose that increased solubility is closely related to surface activity of the protein (Kinsella et al., 1979). Others have demonstrated that high solubility was not the only factor responsible for good emulsification properties and suggest that the surface hydrophobicity measurements in combination with solubility measurements provide a more accurate prediction. Additionally, solubility can be affected dramatically by processing, manufacturing and storage. The method of Morr et al (1985) is standard for determining food protein solubility and is simple, rapid and inexpensive. This was a collaborative study that sought to standardize a procedure that could be used to characterize large numbers of protein products. Their approach was to modify and standardize the widely accepted nitrogen solubility index procedure. This measurement can be used to support surface hydrophobicity measurements and also in the determination of processing induced aggregation.

A widely used method of evaluating emulsifiers has been to directly measure the area of contact between the two phases (Pearce et al., 1978). The turbidity of a suspension of spherical particles is related to its interfacial area based on the Mie theory of light scattering. Pearce et. al. (1978) used this relationship to derive an emulsifying activity index (EAI). The EAI formula incorporates turbidity, volume of

dispersed phase and weight of protein and is related to the interfacial area of the emulsion. They sought to develop a standardized method for measuring stability of protein emulsions that was simple, rapid and reliable. This method measures the turbidity at a single wavelength (500nm) to assess droplet size and by implication, the interfacial area of the emulsion (Lee et al., 1992). This method has been widely used in the research of β-lactoglobulin as an emulsifier (Lee et al., 1992; Cameron et al., 1991; Voutsinas et al., 1983). In 1991, Cameron et al. disputed the units used in the calculation of EAI . They compared results using a Malvern particle sizer with EAI results. The Malvern particle sizer uses laser diffraction and the interfacial area can be computed from the droplet size distribution. This instrument is highly accurate especially for measurements over time, however is expensive and not available in most laboratories. The researchers were able to develop a corrected EAI from its original methodology to a point where good correlation with the particle sizer was obtained. This provides a useful test for comparison of emulsifiers using standard laboratory equipment.

Emulsifying properties have also been evaluated using conductivity techniques (Kato et al., 1985; Suttiprasit et al., 1993). The conductivity of emulsions can be measured using a special conductivity electrode connected to a conductivity meter. The emulsion activity (EA) is the difference between conductivity of the original protein solution and the conductivity of the emulsion (Suttiprasit et al., 1993). This research concluded that the EA of β-lactoglobulin increased with protein concentration. Suttiprasit et al. also concluded that emulsion stability estimation is

improved by utilizing the entire conductivity curve. Previous research considered only the first slope of the curve (Kato et al., 1985).

Conclusion

Extensive research has been directed toward the major whey protein, β-lactoglobulin and understanding the relationship between structural conformation and functionality. Structural conformation and surface properties can determine the functional behavior of β-lactoglobulin under a given set of environmental conditions. There are many conditions that can influence the structure or surface properties of β-lactoglobulin, this paper has sought to focus on understanding the factors that determine and maintain desired structural conformations that can result in increased functionality. Understanding the structural-functional relationship is necessary in order to understand the effects of new processing technologies and in creating value added ingredients with superior functional capabilities.

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Chapter 3

Effect of High Pressure Processing at Low Temperature on the Molecular Structure of β-Lactoglobulin and the Impact on Surface Properties

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Abstract

Development of new and improved functional ingredients has demanded an understanding of the importance of the ingredient's structural components and their relationship to functionality in the food system. The primary protein in whey, β-lactoglobulin, is significantly affected by environmental factors such as pH, temperature and pressure. The ability of high pressure processing (HPP) to modify the functional properties of β-lactoglobulin has become the focus of research only in the last few years.

The research objective was to optimize the HPP treatment to minimize the undesirable aggregation of β-lactoglobulin and thereby increase its potential functionality.

The degree of structural unfolding is complex and appears to be related not only to the level of pressure but also to temperature, concentration and pH. β-lactoglobulin was treated with HPP at 420, 510, and 600 MPa for 10 minutes at 25°C . At each pressure, four pH values, 5.2, 6, 7 and 7.5 and four concentrations 0.2, 0.5, 1.0 and 1.5 mg/ml were included.

Surface functional changes following HPP treatments were determined using a Du Nouy ring tensiometer to measure changes in surface tension.

Native gel electrophoresis was used to monitor aggregation.

HPP treatments at higher pressures 510 and 600 MPa, pH 7.5 and concentration of 0.5 mg/ml resulted in the lowest surface tension values. These values were selected as optimum for pressure, pH and concentration. Further HPP samples were run using these optimized parameters at 8°C and 25°C. Surface tension and circular dichroism (CD) were used to observe changes in surface activity and secondary structure.

Samples treated at 600 MPa, for 10 minutes at 0.5 mg/ml, pH 7.5 at 8°C resulted in decreased aggregation and low surface tension values. CD data indicated that desirable secondary structural changes occurred with HPP treatment at 8°C and not at 25°C. There are many conditions that can influence the structure or surface properties of β-lactoglobulin, this research has sought to focus on understanding the HPP treatment conditions that result in structural changes that increase functionality. Understanding the structural-functional relationship is necessary in order to understand the effects of new processing technologies and in creating value added ingredients with superior functional capabilities.

Introduction

A major obstacle to increasing and improving the functionality of whey proteins in food products is understanding how functionality is related to structure. To maximize the utilization of whey proteins it is essential to understand the changes to protein structure during unfolding as related to functionality.

Bovine β-lactoglobulin, the 162 amino acid whey protein with a molecular weight of 18.3 kDa, has been the subject of extensive research (Green, D. et al.,1979; Bell, K., 1967; Dong, A, 1996; Manderson, G. A. et al.1999; Qi, X., et al., 1997). A wide variety of spectroscopic and analytical tools have been utilized to determine it's molecular structure and it's sensitivity to chemical, thermal and high pressure processing (HPP) unfolding. A relationship has been established between its molecular structure and functionality (Perez, et al.,1995; Sawyer et al., 2000; Jameson et al., 2002; Gross, et al., 1994). As a result, functionality can be improved through targeted unfolding. (Iametti et al., 1998; Manderson et al., 1995; Prabakaran et al., 1997; Cornec

et al., 2001). The structure and ability to form dimers are pH dependent and predominate at pH 5.5-7.5. Circular dichroism measurements indicate that the secondary structure of β-lactoglobulin can be described as containing 10-15% α-helix, 50% β-sheet and 15-20% β-turns (Manderson et al., 1999).

Protein Denaturation

Structural Effects. The classic approach in studying protein unfolding was based on a two-state theory of the globular protein unfolding transition, there being only two protein structures: native and highly unfolded. (Privlov et al., 1988). This theory explained protein unfolding as a cooperative process without involving any stable intermediates. It has now been clearly established that stable intermediates do exist and have been observed following mild treatments of heat, pressure, change of pH and addition of chemicals. (Manderson et al., 1999; Pittia, 1996; Yang et al, 2001; Yang, et al, 2002; Semisotvov, et al., 1991; Balny et al., 2002; Heremans et al., 1998).

Ohgushi and Wada (1983) first described the molten globule as having common structural component with a native-like secondary structure and an unfolded tertiary structure. There is now considerable evidence to support this molten globule theory from a range of kinetic studies using nuclear magnetic resonance (NMR), circular dichroism (CD) and hydrophobic probe techniques binding (Pittsyn, et al., 1995; Yang et al, 2001; Semisotnov, et al., 1991.

Native β-lactoglobulin can convert to the molten globule state when heated or exposed to chemical unfolding agents such as urea (Kuwajima, K. et al., 1996; Qi, et al., 1997; Cornec, et al., 2001). Yang et al. (2001) determined that HHP at 600 MPa and 50°C induced β-lactoglobulin into the molten globule state that was stabilized by the

formation of disulfide bonds. They concluded that the HHP induced molten globule state of β-lactoglobulin showed an increased ANS fluorescence compared with native β-lactoglobulin (Yang et al., 2002).

β-lactoglobulin has an internal free sulfhydryl group which is not available for reaction however, as the protein partially unfolds and adsorbs at the interface, the sulfhydryl group becomes exposed and available for polymerization at the interface (Dickenson, 1994; Monahan et al., 1995; Gezimati et al., 1997). Thermal treatment and increased pH both lead to unfolding and increased exposure of the free sulfhydryl group resulting in increased protein interactions, intra and inter molecular thiol/disulfide interchange and thiol/thiol oxidation reactions (Monahan et al., 1995; Dickenson, 1994). Controlling the extent of these reactions can effectively increase the functionality of β-lactoglobulin.

High Pressure Effects on Proteins

Pressure induced effects on proteins is largely attributed an initial penetration of water into the protein structure resulting in unfolding (Boonyaratanakornkit et al., 2002; Heremans et al.,1998; Balny et al., 2002; Smeller , 2002). In contrast, heat induced unfolding appears to take place prior to water penetration. This difference may explain why pressure induced unfolding is less likely to lead to protein aggregation than heat induced unfolding (Balny et al., 2002). Water penetration under pressure has been found to induce the protein to adopt the more functional conformation of a molten globule (Olivera, 1994).

HPP And Temperatures $\geq$ *25°C.* Bridgman first reported the combination effects of pressure and temperature on egg proteins in 1912. Later work on the effects of pressure

and temperature on protein unfolding were on ovalbumin and hemoglobin with the data indicating that the denaturation was both pressure and temperature dependent (Suzuki, 1960). At higher pressures and room temperature (>500 MPa, 73, 000 psi) protein aggregation and absence of intermediary structures was observed as a result of hydration (Wroblowski et al., 1996). β-lactoglobulin unfolding and loss of native structure by HPP processing at 50-1000 MPa and ≥ 25°C has been previously investigated by using methods such as fluorescence (Dufour et al., 1994; Stapelfeldt et al., 1996, 1999; Yang et al., 2001, 2002) differential scanning calorimetry (DSC), (Dumay et al., 1994), circular dichroism (CD) (Iametti et al., 1996; Pitta et al., 1996, Tedford et al., 1999), Fourier transform infra red (FTIR) spectroscopy and NMR (Heremans et al., 1997). Pittia et al. (1996) used pressure (300-900 MPa) in conjunction with temperature (25°C) and holding time to investigate the structural changes of β-lactoglobulin and the effect of HPP on functionality. They concluded that pressure modified β-lactoglobulin displayed reduced emulsifying capacity and foamability compared to native β-lactoglobulin due to an increased potential for undesirable aggregate formation. Previous research has also suggested that unfolding at pressures of 150-450 MPa and 25°C favor aggregation (Dumay et al., 1994). Studies revealed at high protein concentrations and room temperature treatment there was extensive protein aggregation (Heremans et al., 1998).

HPP And Low Temperature. Kolakowski et al, (2001) concluded that lowering the temperature under pressure enhanced the exposure of the hydrophobic areas of B-LG to water. Using relatively low pressures (300 MPa) they concluded that low temperature pressurization likely minimized the loss of native structure (determined by DSC) and

reduced undesirable aggregation (determined by gel permeation chromatography). They hypothesized that these changes were due to a more hydrated protein structure than when pressure treating at ambient temperature. Their research strongly suggests that low temperature pressurization may be useful to minimize protein aggregation and therefore losses in protein functionality. The pressure induced penetration of water into the protein at low temperatures and high pressures has also been interpreted as the first step in the cold denaturation process according to the theory of Wu (1931). Valente-Mesquita et al. (1998) also investigated the effects of hydrostatic pressure at low temperature on the dimeric structure of β-lactoglobulin. Application of pressures up to 350 MPa and 3°C caused an increase in intrinsic fluorescence emission. They concluded that the dimer unfolds during compression and upon decompression partial but incomplete refolding into the native conformation occurred. The research focused on the HPP unfolding mechanisms of β-lactoglobulin and the effect of concentration. There were no correlations made to functional properties.

Previous high pressure and low temperature studies utilized relatively low pressure. With the current commercial trend towards using higher pressures for shorter times there is a need for investigating the effects of higher pressures on the unfolding of β-lactoglobulin. The objective of this study is to induce targeted unfolding of β-lactoglobulin that optimizes the protein's ability to lower surface tension at air-water interfaces, while minimizing undesirable protein aggregation. In addition, structure/function relationships of unfolded β-lactoglobulin are investigated.

Materials and Methods

Proteins and Chemicals

Chromatographically purified, lyophilized β-lactoglobulin (no. L3908), ANS fluorescent probe (no. A-1028) and Ellman's reagent (D-8130), (5, 5'- Dithio-bis(2-nitrobenzoic acid), DTNB) were purchased from Sigma Chemical Co. (St. Louis, MO).

Treatment Optimization

Freshly prepared sodium phosphate buffer (10 mM) was used to prepare β-lactoglobulin solutions at four concentrations (0.2 mg/ml, 0.5 mg/ml, 1.0 mg/ml, 1.5 mg/ml) and four pH values (5.2, 6.0, 7.0, 7.5). The final concentrations were spectrophotometrically confirmed using a Shimadzu Bio Spec 1601 (Kyoto, Japan) at 280 nm. pH was verified using a Fisher Scientific Accumet AR25 pH meter. 60-mls of sample were packaged into polyethylene pouches (KAPAK, Minneapolis, MN) and heat-sealed. Samples were HPP treated at three pressures (420, 510, 600 MPa) for 10 minutes using a 7-in diameter by 36-inch long Engineered Pressure Systems (Haverhill, MA), 22L isostatic press. The compression time to achieve pressure was approximately 6-7 minutes and decompression time was less than one minute. Samples were run in triplicate at ambient temperature (22-27°C).

Low Temperature Processing. Samples at 0.5mg/ml, pH 7.5 were HPP treated at 8°C for 10 minutes at pressures 420, 510 and 600 MPa. Compression heating was controlled by cooling of the vessel by filling the unit with ice water and allowing to equilibrate for 2 hours. The samples where also equilibrated at 4°C and packed in 4°C ice water for loading. Samples were monitored for temperature before and after processing.

Freeze Drying. In order to create a shelf-stable ingredient, some samples were freeze dried immediately following pressure treatment. Samples were slab frozen in a blast

freezer at –40°C for 24 hours and then freeze-dried with the product temperature remaining < 25°C during the drying cycle. The final moisture content ranged from 4.38-5.6 %. Freeze dried samples were transferred into double lined polypropylene bags.

Adiabatic Compression Heating. Adiabatic compression heating was monitored by inserting thermal couples into protein samples and pressure transmitting fluid inside an Engineered Pressure Systems, (Haverhill, MA) 2-liter 100,000 psi isostatic press. 50 ml sample pouches were run at 510 MPa, for 10 minutes. Pressure come up time was approximately 4 minutes and pressure release was <1 minute. The chamber was packed with ice and allowed to cool for 1 hour. Samples were prepared and equilibrated in a water bath at 8°C. Thermocouples were inserted into the sample and pressure transmitting fluid. The sample was put into an outer bag filled with ice water and heat sealed.

Surface Tension Measurements

Immediately following HPP treatment samples were analyzed for surface tension using a Du Noüy Ring Tensiometer (model 70535, CSC Scientific Co., Inc, Fairfax, VA). A volume of 20mls of room temperature solution was poured into 60 X 15 mm tissue culture dish (#3002, Becton Dickson Labware, Lincoln Park, NJ). A 6-cm Platinum-Iridium Ring (70537, CSC Scientific Co.) was used for measurements. The ring was cleaned between each measurement by rinsing with deionized water followed by flame treatment. The ring was hung from the vertical arm and lowered about 5mm below the surface of the solution. The assembly plate was then lowered until the ring was just below the surface of the liquid and centered with respect to the dish. The ring was pulled from the surface and apparent surface tension (ρ) was recorded (Suttiprasit et al.,

1992). Apparent surface tension is the force required to pull the ring from the surface. This force is equal to the weight of the ring plus the downward pull of the solution due to surface tension (CSC manual). The scale reading of the tensiometer must be converted to true surface tension by multiplying by a correction factor (F) to account for the force needed to support the weight of the liquid clinging to the ring at the breakpoint. The equation $\gamma = \rho \times F$ was used.

Samples were monitored over a period of time up to three hours. It was determined that samples remained constant after one hour and subsequently surface pressure results were recorded after the sample equilibrated for one hour.

Native Polyacrylamide Gel Electrophoresis

Equipment, sample buffers, molecular weight standards (161-0362) 10-250 kDa, running buffer, 10-20% Tris HCL-30µl load size ready gels, staining and destaining solutions were obtained from BIORAD (Bio-Rad Labs, Hercules, CA). Samples were diluted 1:2 in BIORAD native sample buffer (161-0738) and 25µl was loaded into the wells.

Protein Structure Characterization

Circular Dichroism (CD). Far UV CD spectra were measured from 184 to 260 nm and near UV was measured from 260-320 nm using a Jasco Spectropolarimeter (J720, Japan Spectroscopic Co., Inc. Tokyo, Japan). Far UV measurements were performed using a 0.1cm square quartz cell and near UV measurements were performed using a 1 cm cylindrical quartz cell at ambient temperature. The spectral data were collected at 1 nm intervals using a scan rate of 20nm/min. Triplicate spectra were run on all samples

and averaged by the instrument software. Base line samples of buffer were run and all samples were baseline corrected prior to calculation. Instrument software was used to smooth all spectra. The instrument was calibrated using an aqueous solution of (+)-10-camphorsulfonic acid (CSA) at a concentration of 1 mg ml^{-1} in a 1mm cell. CSA has a ΔA of 1.02 X 10^{-3} or an ellipticity of 32 mdegrees at it's CD maximum of 290.5nm. (Johnson, 1999)

Samples were diluted 3:1, with buffer, to assure signal within linear range and final concentration was calculated using Beer's Law.

CD Data Analysis of Secondary Structure. CD spectrum were converted using the methods of J. P. Hennessey, Jr., W.C. Johnson, Jr. (1981, 1990) and P. Manavalan and W. C. Johnson, Jr. (1987). Data is reported as percent alpha helix, 3_{10}-helix, β-sheet, β-turn, polyproline-like 3/1-helix and other.

Intrinsic Fluorescence. Measurements were taken using a Perkin Elmer Luminescence Spectrometer, L550 at room temperature according to a modified method of Cairoli et al. (1992). Spectra were monitored using an excitation wavelength of 290nm and an emission wavelength of 450nm.

Surface Hydrophobicity - Extrinsic Fluorescence. Determination of surface hydrophobicity was carried out using ANS as a fluorescent probe according to modified methods of Creamer et al. (1997) and Yang et al. (2001). The fluorescence intensity was measured at room temperature using a Perkin Elmer Luminescence Spectrometer, L550. ANS fluorescence was assayed using an excitation of 390nm and an emission of 500nm.

Reactivity of Exposed Thiol Groups – A modified method by Shimada et al. (1989) based on Ellman's reagent was employed using a Shimadzu Bio Spec 1601 (Kyoto, Japan) spectrophotometer for absorbance measurements at 412 nm. To a 2.55 ml aliquot of the protein model solution 0.45 mls of DTNB solution was added (40mg/ml DTNB/10 ml of standard buffer). After the solutions were mixed, the absorbance was recorded after 15 minutes of equilibration at 25°C against a blank containing only DTNB and buffer. An extinction coefficient of 13,600$m^{-1}cm^{-1}$ was used to calculate the concentration of sulfhydryl groups reacting.

Results and Discussion

Treatment Optimization

Effects of Pressure, Concentration ,and pH.

β-lactoglobulin concentration during HPP treatment was optimized (data not shown) to determine the highest protein concentration that would give maximum surface pressure in protein/buffer model solutions while avoiding aggregation (as determined by Native PAGE). This was obtained at a β-lactoglobulin concentration of 0.5 mg/ml. Buffer pH was optimized based on the same parameters and 7.5 was selected. Samples at pH 6 resulted in high surface pressure values as well but β-lactoglobulin did not remain soluble during storage at this pH. Recently, it has been documented that HPP compression of foods may shift the pH of solution or food during treatment (Kolakowski et al., 2001; Stippel et al., 2002). Changes in pH would ideally be monitored will under pressure. Additional information obtained from Native PAGE (not shown) supported that the pressure treatment at 510 MPa resulted in the least amount of aggregation as determined by formation of higher molecular weight

compounds. For the efficiency of further experiments, the conditions chosen of 510 MPa, pH 7.5 and 0.5 mg/ml gave the maximum pressure and protein concentration without losing functionality due to aggregation.

Low Temperature High Pressure Effects

Effects of Low Temperature and Pressure on Surface Activity. Following treatment optimization at room temperature, low temperature was added as a variable to investigate the effect of temperature during pressurization and the resulting effect on surface activity of β-lactoglobulin solutions. Solutions treated at 510 MPa, 8°C and pH 7.5 showed optimum surface pressure results (Table 1). Based on this data, it was concluded that less denaturation was occurring at the lower pressure used (420 MPa) and denaturation with aggregation was occurring at higher pressures (600MPa). Native PAGE gel results (Figure 1) confirmed the presence of aggregation at 600 MPa. Surface pressure (Π) for model solutions containing β-lactoglobulin pressure treated at 8°C was significantly higher than for solutions containing β-lactoglobulin pressure treated at 25°C (Table 1). Beyond the initial diffusion controlled stage, changes in Π arise not only from new molecules arriving at the interface but also from conformational changes in those already absorbed. The Π of a protein solution increases with time as the surface tension decreases with time. Therefore, an increased value of Π also indicates a decreased amount of free energy at the interface (Waniska et al., 1985). These results imply that unfolding β-lactoglobulin by HPP and low temperature results in a conformation that gives it better functionality at the air water interface.

Table 1. Effect of HPP Treatment Temperature on Surface Pressure of β-lactoglobulin model solutions treated at 510 MPa, 10 minutes, pH 7.5, 0.5 mg/ml.

	Control	25°C	8°C
Π , Surface Pressure (mN/m)	6.14[a] (.108)	12.58[b] (0.107)	15.05[c] (0.174)

Mean of triplicate measurements ($p \leq .05$), standard deviations (in parentheses).
[a,b,c] For each treatment, means with different superscript are significantly different from each other.

Effects of Temperature and HPP on Aggregation of β-lactoglobulin determined by Native PAGE. Results are shown in Figure 4 of the Native PAGE comparing the formation of higher molecular weight molecules following HPP at 8°C and 25°C. Pressure treated samples showed a trend to contain higher molecular weight compounds than the control samples. However, samples treated at 510 MPa 8°C showed lighter bands at higher molecular weights and therefore it was concluded that there was unfolding but less aggregation with this treatment than the higher pressure (600MPa) treatment. Samples treated at 420 MPa and 8°C (not shown) showed no formation of higher molecular weight compounds and were similar to the control. At 420 MPa and 25°C higher molecular weight compounds were formed and it was concluded that aggregation had resulted from this treatment. These results are consistent with Kunugi et al. (2002) who concluded that β-lactoglobulin aggregates were detected by gel permeation chromatography when pressurized at 25°C but not at lower temperatures.

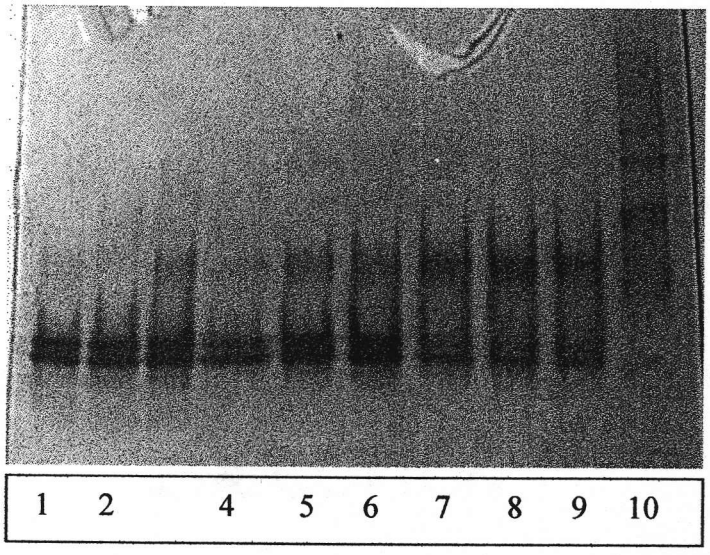

Figure 1. Native PAGE of HPP Treatments

1&2 – Control Native- 0.5 mg/ml, pH 7.5

4,5 HPP 0.5 mg/ml, pH 7.5, 510 MPa , 10 min, 8°C
6,7 HPP 0.5 mg/ml, pH 7.5, 600 MPa, 10 min., 8°C
8 HPP 0.5 mg/ml, pH 7.5, 600 MPa 10 min, 25°C
9 HPP 0.5 mg/ml, pH 7.5, 510 MPa, 10 min, 25°C
10 Standard

Adiabatic Compression Heating

Because temperature was an integral part of this research, it was necessary to document the changes during a treatment cycle. Temperature change during HPP is shown in Figure 2.

A thermocouple in the center of the sample package and the thermocouple placed near the top of the pressure unit in the pressure transmitting fluid were almost identical indicating good temperature distribution within the chamber. Even though the sample entered the cycle at 8°C, reached a high temperature of 28.6°C and upon decompression returned to a temperature of 11.8°C this treatment appears mild enough in temperature to unfold β-lactoglobulin without resulting in temperature induced aggregation.

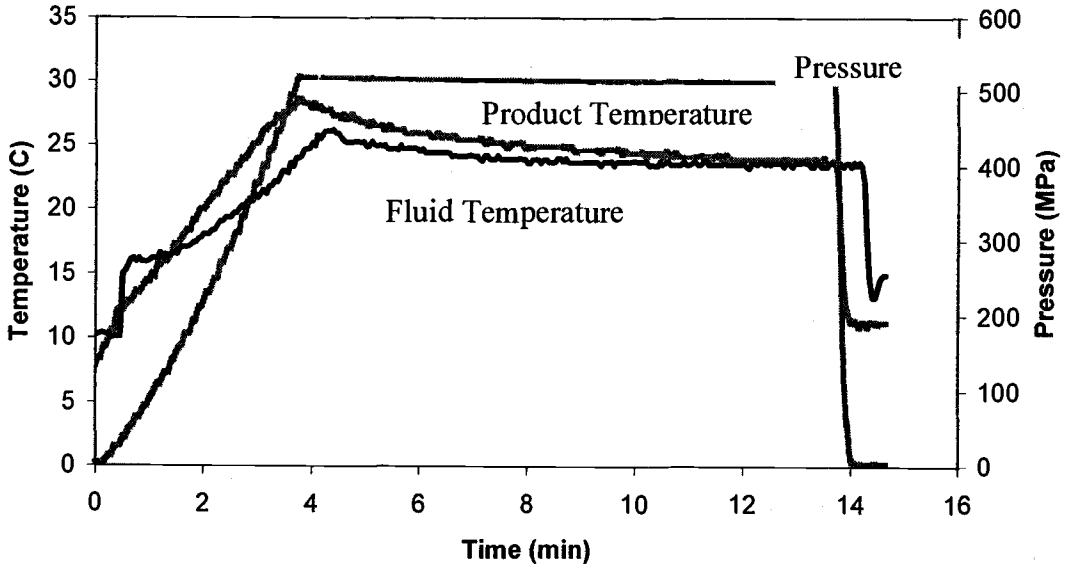

Figure 2. Adiabatic Compression Heating of HPP Treatment 510 MPa, 8°C, 10 minutes

Changes in β-lactoglobulin Secondary and Tertiary Structure Following HPP

Far UV. The CD for the alpha-helix component is dominated in the region of 200-220 nm with an intense negative band at about 222nm and another at approximately 208nm. Extending the CD to 180nm extends the far CD information due to distinct bands between 200-178. The random coil component shows an intense negative band at 198nm additionally, the β-sheet has a positive band in this region (Johnson, 1990). The changes in the far UV CD spectra of HPP samples treated are shown in Figure 3.

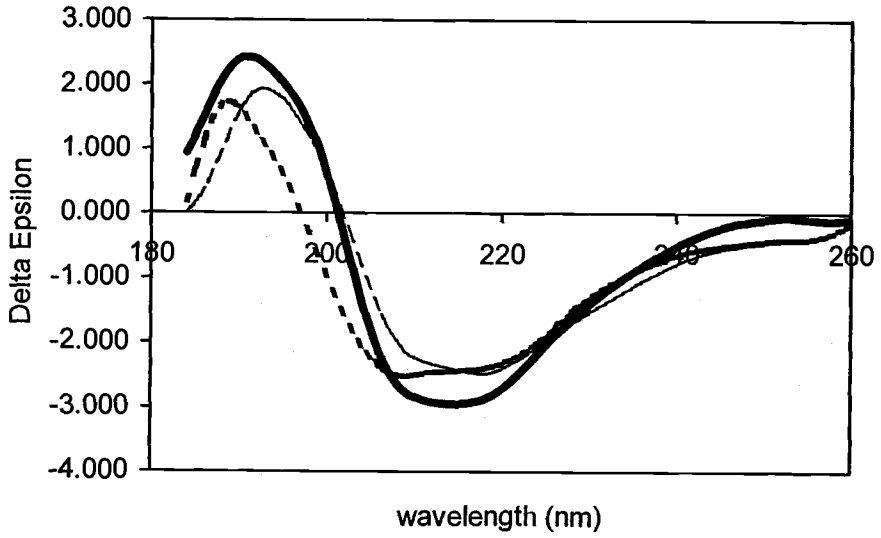

Figure 3. – Far UV CD of HPP Samples at Time Zero pH 7.5, 0.5 mg/ml, 510MPa, 10 min. Treatment: — Control (native); ---- 25°C; — - — 8°C.

An algorithm to transform CD data to secondary structure components was utilized (Johnson, 1990; Hennessey et al., 1981; Manavalan, 1987). Comparisons between the

secondary structure of native and HPP treated samples immediately following pressure treatment and after 24 hours held at 4.4° C are presented in Table 2. The native sample was stable in solution for a 24 hour period. HPP treatment at 8°C resulted in a decrease in α-helix and a slight increase in the β-sheet component. After 24 hours, this sample showed evidence of renaturation and resembled the secondary structural composition of the native sample. Samples HPP treated at 25°C had significant changes to the α-helix immediately following HPP and these changes remained after the 24 hour storage period at 4.4°C. This indicates that the sample lost secondary structure and had unfolded and formed a non-native structural conformation. The unfolding was not reversible following the 24 hour storage period.

Table 2. Secondary Structure of Native and HPP treated β-Lactoglobulin at pH 7.5 as determined by CD

Sample	Temp HPP	Time after HPP (Hours)	α – Helix	3_{10}-helix	β-sheet	β-turn	Polyproline-like 3/1-helix	Other
Native		0	.16	.04	.24	.14	.06	.37
Native		24	.17	.04	.24	.13	.07	.36
510 MPa	8°C	0	.11	.04	.26	.14	.07	.38
510 MPa	8°C	24	.18	.04	.20	.14	.07	.37
510 MPa	25°C	0	.08	.05	.26	.13	.07	.41
510 MPa	25°C	24	.07	.05	.24	.14	.07	.42

Near UV. CD bands in the near UV region of a folded protein are observed (250-320 nm) from the presence of aromatic amino acids. The signs, magnitudes and

wavelengths of the aromatic CD bands cannot be quantified, however the near UV spectrum represents a highly specific criterion for identifying the native folded state of a protein. It is therefore commonly used as a sensitive tool for comparing changes in conformation.

The near-UV CD spectra of HPP samples were compared with native β-lactoglobulin (Figure 4) immediately following pressure treatment. This spectrum indicates large changes in the tertiary structure of β-lactoglobulin HPP treated at 25°C. HPP treatment at 8°C resulted in a spectrum more closely resembling that of the native protein. The bands at 285-295 nm can be attributed to tryptophan and to tyrosine at 265-277 nm. The decrease in the tyrptophan bands at 285 and 295 indicates a loss of tertiary structure (Matsuura et al., 1994). Pressure clearly disrupts the tertiary structure of β-lactoglobulin with the extent of disruption increasing with treatment temperature. Beginning the pressure cycle at a temperature of 8°C was sufficient to reduce significantly the pressure unfolding of β-lactoglobulin.

Changes in the near UV CD spectra compare with previous reported results that the tertiary structure of β-lactoglobulin decreased with increased time of high pressure treatment. Tedford et al. (1999) observed that increased temperature was the most influential processing variable resulting in the most significant changes in tertiary structure. Yang et al. (2001) reported complete disappearance of tertiary structure following 8min. HPP treatment at 600MPa and 50°C.

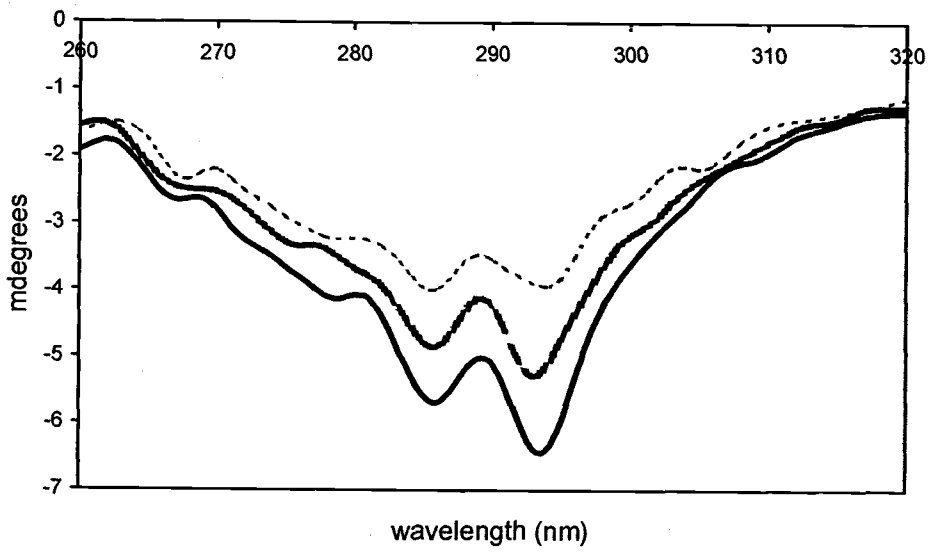

Figure 4. Near UV CD of HPP Samples at Time Zero, pH 7.5, 0.5 mg/ml, 510 MPa, 10 min. Treatment: — Control (native); ---- 25°C; — - — 8°C.

Effects of Freezing and Freeze Drying of HPP Samples. Changes in the secondary structure of β-lactoglobulin frozen or freeze dried and stored are listed in Table 3. Processing alone (freezing or freeze-drying) did not have a significant effect on the native secondary structure of β-lactoglobulin. However, samples that were HPP treated at both 8 and 25° C were not stable and changes in the α-helix component occurred when held frozen up to 12 days. Dumay et al. (1994) and Kolakowski et al. (2001) report similar reversibility when treating at lower pressures (450MPa) and 25°C. Freeze dried samples also underwent conformational changes during processing. Samples treated at 8°C retained the most native like structural conformations and optimum functional properties as measured by surface pressure (Table 4). This suggests that the samples treated at low temperature (8°C) were able to retain much of

their native secondary structure but with a degree of unfolding that gave optimum surface characteristics.

Table 3. Secondary Structure of Native and HPP treated β-Lactoglobulin at pH 7.5 followed by freezing as determined by CD

Sample	Time after HPP (Days)	α - Helix	3_{10}-helix	β-sheet	β-turn	Polyproline-like 3/1-helix	Other
Native							
Frozen	1	.18	.04	.21	.13	.07	.37
Frozen	12	.18	.02	.26	.10	.08	.35
Freeze-Dried	7	.17	.02	.24	.10	.09	.38
510 MPa/8°C							
Frozen	1	.17	.04	.23	.13	.06	.37
Frozen	12	.10	.03	.28	.12	.08	.39
Freeze Dried	7	.18	.03	.22	.12	.09	.36
510 MPa/25°C							
Frozen	1	.10	.05	.23	.13	.08	.40
Frozen	12	.17	.05	.18	.13	.08	.39
Freeze-Dried	7	.04	.02	.29	.11	.09	.43

Table 4. Effect of Freezing and Freeze Drying on Surface Pressure

Sample	Time after HPP (Days)	Surface Pressure (mN/m)
Control		
Frozen	1	8.98
Frozen	12	7.45
Freeze-Dried	7	8.86
510 MPa/8°C		
Frozen	1	14.81
Frozen	12	14.09
Freeze dried	7	14.3
510 MPa/25°C		
Frozen	1	11.86
Frozen	12	10.62
Freeze-Dried	7	11.46

Intrinsic Fluorescence Spectra. β-lactoglobulin exhibits an increase in intrinsic tryptophan fluorescence and a red shift upon unfolding. Manderson et al. (1999) used heat denaturation to consider fluorescence changes in Trp^{19} and Trp^{61} and suggested that both residues become separated from strong fluorescent quenchers upon denaturation. Yang et al. (2001) observed similar increases in intensity of intrinsic tryptophan concluding that HPP treatment also causes movement away from fluorescent quenchers. Kunugi et al. (2002) observed conformational changes in pure β-lactoglobulin and concluded that using low temperature and high pressure resulted in

tyrosine and tryptophan becoming more exposed to an aqueous environment. They concluded that different conformations exist under various P/T conditions.

The tryptophan fluorescence spectra for the treatments selected are shown in Figure 5. An increase in fluorescence was observed, indicating unfolding occurs in both HPP treatments and thus changes to the tertiary structure of the protein. However, HPP 25 °C treatment resulted in a larger change than samples HPP 8°C. This is in agreement with the tertiary changes as determined by near UV CD of HPP samples (Figure 4).

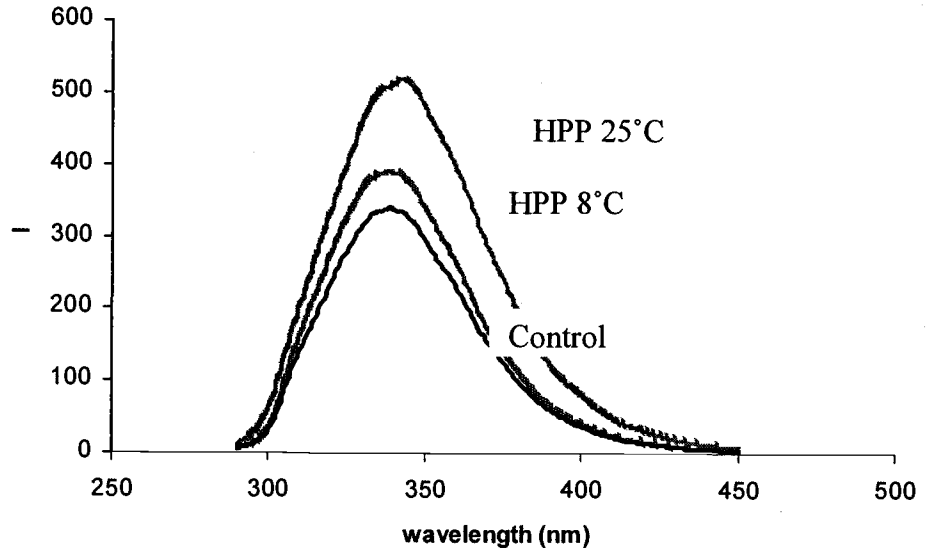

Figure 5. β-Lactoglobulin Fluorescence Time Zero pH 7.5, 0.5 mg/ml, 510 MPa, 10 min. Treatment.

Hydrophobic Probe Binding/Extrinsic Fluorescence. The native structure of β-lactoglobulin contains an internal hydrophobic binding site located within the β–barrel and an external hydrophobic site located between the β-barrel and the α-helix (Yang et al., 2002; Collini et al., 2000). 1-Anilinonaphthalene-8-sulfonate (ANS) is a hydrophobic probe that is only weakly fluorescent in aqueous solution but increases when it binds to the hydrophobic sites on β-lactoglobulin. Usually the hydrophobic core of β-lactoglobulin is protected from solvent by the rigid tertiary structure giving it a low affinity to hydrophobic binding probes like ANS. By disrupting the tertiary structure, the affinity of β-lactoglobulin to ANS increases (Semisotnov et al., 1991). Increased ANS binding was observed as an increase in extrinsic fluorescence when samples were HPP treated at 25°C compared to the untreated control or 8°C HPP samples (Figure 6). These results further support that HPP induces a degree of structural change in β-lactoglobulin that is both temperature and pressure dependent. An increase in ANS binding suggests that the protein is unfolded to different degrees depending on the treatment used (8 or 25°C) exposing buried internal hydrophobic groups for the solutions treated at 25°C.

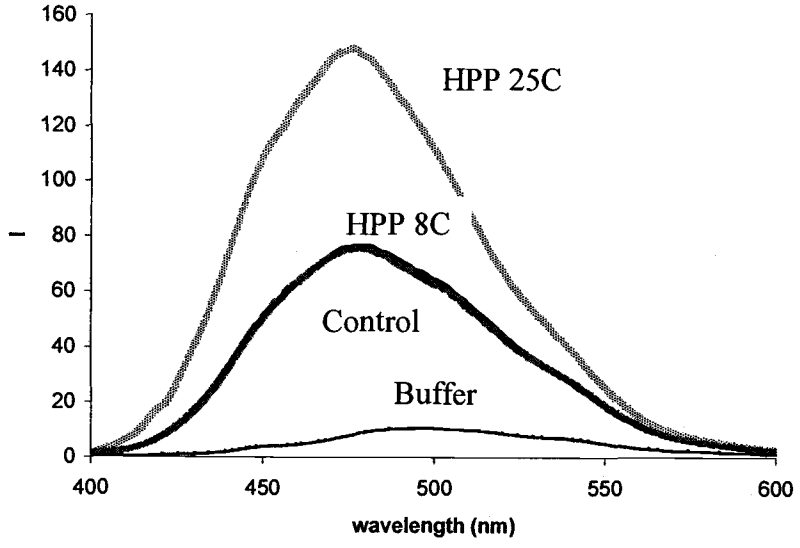

Figure 6. β-Lactoglobulin + ANS Fluorescence Time Zero for samples HPP treated at pH 7.5, 0.5 mg/ml, 510 MPa, 10 min.

Reactivity of Exposed Thiol Groups . The internal thiol group plays an important role in surface characteristics when β-lactoglobulin is unfolded due to heat or HHP. Monitoring the presence or absence of these reactive groups is important in determining the proteins functionality. Current testing of the reactivity of the free thiol of β-lactoglobulin is based on its reactivity with Ellman's reagent, 5,5'-dithiobis(2-nitrobenzoic acid) or (DNTB) (Moller et al., 1998; Hoffman et al., 1997; Lee et al, 1992; Pittia et al., 1996). DTNB reacts with the thiol components of β-lactoglobulin to produce p-nitrothiolphenol, which is yellow in color. In native β-lactoglobulin, of the five cysteines, four form intramolecular disulfide bonds and only Cys-121 is present as a free thiol group. This free thiol group is buried internally and remains very unreactive, however as it unfolds it will show an increased reactivity with the DNTB. Partial unfolding by HPP increases the exposure of inner hydrophobic groups and the

thiol group. It has been observed that hydrophobic interactions and thiol-disulfide reactions can occur in this state leading to aggregation (Hoffmann et al., 1997). It is less clear at which point in the unfolding process these reactions occur. There is general agreement in the literature that thiol/disulfide reactions lead to the formation of intermolecular disulfide bonds that result in the heat induced aggregation (Cairoli et al., 1994, Iametti et al., 1996, Gezimati et al., 1997; Monahan et al., 1995; Laligant et al., 1991). Although the role of the thiol group in thermally induced aggregation has been studied extensively it is difficult to compare research findings. This is due to differences in experimental conditions and the fact that the mechanisms for aggregation of β-lactoglobulin vary significantly with temperature, pH and protein concentration. By studying the reaction of DTNB with the thiol group of β-lactoglobulin we sought to gain additional information on the pressure induced unfolding and accessibility of the internal free thiol.

The UV absorbance of β-lactoglobulin in the presence of DTNB at 412nm significantly increased when HPP treated at 8°C and 25°C compared to the control. (Table 5). The higher reactivity of the free thiol in the HPP treated samples suggest that unfolding to different degrees has occurred in order to expose the internal thiol group for reaction.

Table 5. Effect of HPP Treatment Temperature on Free Thiol reactivity of β-lactoglobulin with DTNB (Ellman's Reagent) in model solutions treated at 510 MPa, 10 minutes, pH 7.5, 0.5 mg/ml.

	Control	25°C	8°C
ABS at 412 nm	0.164^{a}	0.195^{b}	0.182^{c}
	(.008)	(0.002)	(0.003)
Free Thiol Concentration (m)	1.2×10^{-4}	1.4×10^{-4}	1.34×10^{-4}

Mean of triplicate measurements ($p \leq .05$), standard deviations (in parentheses)

[a,b,c] For each treatment, means with different superscript are significantly different from each other.

Structure-Function Relationship

Upon adsorption at the air-water interface, there is a change in protein conformation from that of the native state (Cornec et al., 1999). The molecular rearrangements that occur as β-lactoglobulin moves towards and into the liquid interface lead to the partial unfolding of the molecule and a loss of tertiary structure occurs (Ptitsyn, 1995; Dickenson et al., 1994). Additionally, the conformation of the protein molecule in solution will greatly influence the rate and extent of the conformational change upon adsorption. Specifically, it appears that increased molecular flexibility facilitates the protein's rearrangement at interfaces (Cornec et al., 2001; Cornec et al., 1999; Wang et al., 1997). This research investigated improving the surface activity of β-lactoglobulin based on these unfolding theories (Jameson et al., 2002; Cornec, et al 2001; Tedford, 1996; Pittia et al., 1996). The surface pressure of β-lactoglobulin model solutions were increased when subjected to high pressure. Pressure treatment at room temperature caused significant and permanent changes to secondary and tertiary protein structure.

However, the treatment failed to lead to optimum surface activity in model solutions because of protein aggregation. In contrast, pressure treatment at low temperature caused changes in tertiary structure while secondary structure changes were minimal and substantial renaturation occurred during storage. In spite of the renaturation the gain in surface activity of the model solutions was high. This was most likely due to the fact that protein aggregation minimal. It seems likely that β-lactoglobulin in the partially denatured state (510 MPa , 8C and pH 7.5) has a more flexible, hydrophobic surface and therefore readily adsorbs at the interface whereas the control and lower pressure (410 MPa) treated proteins have a more tightly folded conformation and therefore have a lower probability of collision at the interface.

This suggests that the adsorbed protein at the interface has an average structure lying between the native folded and the completely unfolded state and fits the definition of the molten globule state. Research indicates that β-lactoglobulin in the molten globule state exhibits an increased affinity for ANS compared to the native state. (Yang et al., 2001; Yang et al., 2002; Semisotnov, 1991). In this research, the pressure induced denaturation of β-lactoglobulin at 8°C and storage at 4.4°C for 24 hours resulted in a structure that has the conformation as defined as being the molten globule with native-like secondary structure and disrupted tertiary structure and increased ANS binding.. These results support a favorable effect of unfolding on surface functionality when aggregation is avoided. Due to the unique effects of HPP and low temperature on the structural and functional properties of β-lactoglobulin further investigations should be explored in further optimizing this process. The optimization of the HPP treatment by

using low temperatures could lead to the development of novel food products and ingredients.

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Chapter 4

Effect of High Hydrostatic Pressure Treatment on Yogurt Quality and Shelf-life

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Abstract

High hydrostatic pressure processing (HPP) and low temperature was used to create a high quality extended shelf-life yogurt with fruit pieces that can be stored at ambient temperature. The quality and shelf-life was monitored by testing samples for viscosity, color pH and microbiological changes over a 60 day period. Samples were stored at 4.4 and 25°C in foil containing laminated pouches. An untreated control was included and stored at 4.4°C.

Samples HPP treated at 5°C for 10 minutes at 550 MPa showed significantly higher viscosity than the untreated control. Color changes resulted in a decrease in L* and an increase in chroma that was greatest in the HPP samples stored at 25°C. HPP at low temperature was effective in creating a microbiologically stable yogurt for storage at both 4.4 and 25 °C. Control samples steadily decreased in pH and microbiologically deteriorated after 40 days.

Introduction

The development of space foods has changed considerably over the past 30 years. There are still many unresolved problems related to space foods such as nutritional requirements, variety of foods, and methods of preservation for long duration space flights. Despite recent increases in the variety of foods, nutrient intake for Shuttle astronauts has not been adequate (Bourland et al., 2000).

Demand for high quality shelf-stable foods has increased with the developments of the International Space Station. Extended stay missions at the space station require a more palatable diet that must meet rigid nutritional requirements (Bourland et al.,

1993). Foods are designed with a minimum shelf-life of nine months and have conventionally been preserved through irradiation, dehydration, freeze drying and thermal processing. These processes often cause undesirable quality changes in the foods.

High hydrostatic pressure processing (HPP) has emerged over the past several years as an alternative to thermal processing to eliminate microorganisms (Smelt, 1998; Farkas et al., 2000; Velazquez, G. et al., 2001). The advantage of HPP processing in preserving foods has been the retention of color, flavor, and nutrient content of foods (Aleman et al., 1994, 1996, 1997). HPP has also been investigated as a means of developing new or improved functional properties and for enzyme inactivation (Cheftel et al 1992; Shook et al., 2001; Nienaber et al., 2001). The quality of heat processed yogurt is very poor and previous investigations by Walker et al. (1996; 2000) demonstrated that HPP processing at a temperature below 20°C produce a high quality shelf-stable yogurt with fruit that possessed superior sensory characteristics. Texture is an important factor in consumer acceptance of yogurt (Skriver et al., 1999; Vélez-Ruiz J et al., 1997). Traditional yogurt rheology has been extensively studied, however the effect of HPP treatment on yogurt rheology has not been investigated (Tunick et al., 2000; O'Neil et al., 1979).

With refrigeration and freezer space at a minimum on space flights, HPP foods offer a solution to the design of food systems for extended stay missions by increasing the shelf-life, improving the quality, reducing the requirements for costly refrigeration and by adding more variety and nutrition to meal selection.

The present study was conducted to investigate the effects of HPP on the color, texture and microbiology of blackberry yogurt formulated with fruit and with shelf-stability at both refrigerated and room temperature storage conditions.

Materials and Methods

Fresh nonfat vanilla yogurt was obtained from a regional dairy processor. The yogurt was manufactured from grade A nonfat milk and contained the active cultures *Lactobacillus bulgaricus, Streptococcus thermophilius, Lactobacillus acidophilus* and *Bifidobacterium bifidum.*

The yogurt was further formulated to contain the following

Blackberries, canned, drained	9.1%
Natural Blackberry Flavor	0.25%
Elderberry juice concentrate	0.01%
Nonfat vanilla yogurt	90.6%

Ingredients were mixed and subsequently degassed for 10 minutes at 25 inches vacuum. Six ounces of product was filled into foil laminate pouches and hermetically heat-sealed after displacing head space air. The pouches were held at 5°C until HPP processing.

Pouches were HPP treated at 4°C for 10 minutes at 550 MPa using a 7-inch diameter by 36-inch long Engineered Pressure Systems, Inc. 22-liter isostatic press (Haverhill, MA). Cooling of the HPP vessel was accomplished by packing the chamber with ice water and allowed it to equilibrate for two hours. Samples were monitored for

temperature before and after processing. The compression time to reach pressure was one minute and decompression was < 1 minute.

Following pressure treatment samples were put into temperature controlled rooms at 4.4°C and 25°C. An untreated control was placed into 4.4°C storage.

Adiabatic Compression Heating

Adiabatic compression heating was determined by inserting thermal couples into a sample and the pressure transmitting fluid inside an Engineered Pressure Systems (Haverhill, MA) 2-liter isostatic press.

Microbiological Analysis

Samples were tested at 0, 10, 40, and 60 days for aerobic plate count, yeast and mold, and lactic acid bacteria. 3M Aerobic Plate Count Petrifilms (product #6400) (St. Paul, MN) and Yeast and Mold Petrifilms (product #6407) were plated in triplicate for enumeration of these microorganisms. Difco Lactobacilli MRS Agar (Becton, Dickenson and Company, Sparks, MD) was used to enumerate remaining viable cells of the active cultures. Inoculated plates were run in triplicate and anaerobically incubated for 48 hours at 38°C.

Quality Analysis

pH was verified at each testing period using a Fisher Scientific Accumet AR25 pH meter.

Texture Analysis

A Brookfield DV-ll+ calculating Digital Viscometer (Middleboro, MA) was used for monitoring changes in viscosity during storage. Fruit pieces were removed carefully

and the yogurt was placed into a 250ml Griffin low form beaker. Triplicate samples were allowed to equilibrate covered at 5°C overnight. A modified method used by Skriver et al. (1999) was used for testing viscosity. Timed measurements were taken for 1 minute using spindle 4, 1 RPM and at a product temperature of 5°C. The mean value in centipoises was reported.

Color Analysis

A Hunter Lab ColorQUEST 45/0 attached to a PC with Universal Software version 3.0 (Reston, VA) was standardized and used to monitor color during storage. Triplicate pouches were emptied and stirred to achieve uniform color. The yogurt with berries was put into an optically flat glass dish for measurements. An average of three measurements was reported as L, a*, b*. These numbers were further used to calculate chroma.

Results and Discussion

Adiabatic Compression Heating

Because temperature was an integral part of this project, it was necessary to document the changes during a treatment cycle. Temperature change during HPP is shown in Figure 1.

A thermocouple in the center of the sample package and the thermocouple placed near the top of the pressure unit in the pressure transmitting fluid indicated that the yogurt maintained a colder temperature than the pressure transmitting fluid within the chamber. This could indicate that the adiabatic compression heating varies depending on the food product. Products with a high water content would show a different

degree of heating than complex foods with multiple ingredients. This also reinforces the importance of monitoring temperature during HPP treatment especially when used to inactivate microorganisms. Even though the sample entered the cycle at 4°C, reached a high temperature of 16.6°C and upon decompression returned to a temperature of 3°C this treatment appears mild enough in temperature to retain high quality characteristics in the yogurt.

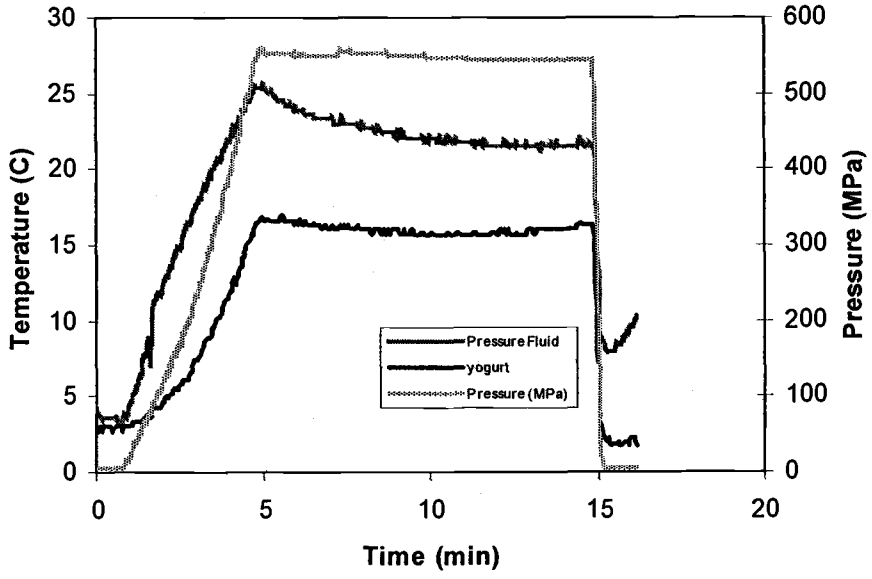

Figure 1. Adiabatic Compression Heating for HPP Yogurt Samples Treated at 550MPa 10 minutes, 4 °C initial temperature.

Rheological measurements

The viscosity of the blackberry yogurt increased significantly following low temperature HPP treatment (Figure 2). HPP treated yogurt had a visibly thicker and smoother appearance than the untreated control. The HPP samples continued to

maintain a consistently higher viscosity at both 4.4 and 25°C storage over the 60 day testing period. The samples stored at 25°C had a significantly higher viscosity than either the HPP samples stored at 4.4°C or the untreated control stored at 4.4°C. The untreated control stored at 4.4°C maintained a consistently lower viscosity than the HPP samples until it microbiologically deteriorated at 40 days storage. These results are consistent with previous work by Walker et al. (1996) that showed that HPP treatment at <20°C resulted in a product with improved sensory characteristics.

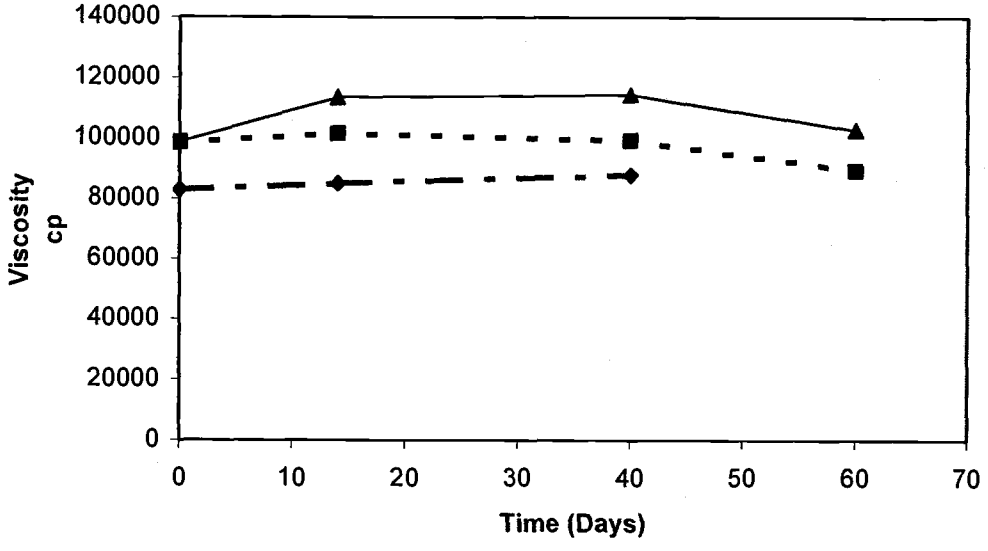

Figure 2 -Texture of HPP yogurt during storage ▲ HPP 25°C ; ■ HPP 4.4°C; ♦ Control

Color Measurements

HPP treatment had no significant effect on the color of the yogurt immediately following treatment (Table 1). Variations in color were observed throughout the 60

day storage period with L* decreasing and chroma increasing. This change indicates a trend toward a darker, deeper colored product. The yogurt took on a deeper purple color, which may have resulted from color from the fruit pieces migrating into the yogurt during storage. This trend was the greatest for HPP samples stored at room temperature (25°C). Browning, or a negative change in a* and b* was not observed in any of the stored samples.

Table 1 – L, a*, b * values for HPP Blackberry Yogurt stored at 4.4°C and 25°C for 60 days.

Sample	Storage Time (Days)	L*	a*	b*	Chroma
Control	0	72.36	10.45	1.09	10.5
HPP	0	76.36	8.98	2.41	9.3
Control	10	67.0	14.25	1.04	14.28
HPP 4.4°C	10	75.57	11.34	1.04	11.4
HPP 25°C	10	75.82	9.84	3.46	10.4
Control	40	72.05	12.51	1.98	12.65
HPP 4.4°C	40	71.34	12.06	.74	12.08
HPP 25°C	40	65.32	12.62	2.41	12.84
HPP 4.4°C	60	71.63	11.69	1.99	11.85
HPP 25°C	60	63.31	11.90	2.75	12.21

Microbiological and pH Analysis

pH results for the yogurt throughout the 60 day storage period are reported in Table 2 for all samples. Control samples steadily decreased in pH due to microbiological deterioration by 40 days of storage. Samples HPP treated and stored at both 4.4 and 25 °C decreased slightly by 14 days of storage but remained unchanged for the remainder of the project.

Table 2 – pH measurements in Blackberry Yogurt During Storage

Sample & Storage Condition	Storage Time (Days)	pH
Control	0	4.32
HPP 4.4°C	0	4.35
HPP 25°C	0	4.35
Control	14	4.11
HPP 4.4°C	14	4.25
HPP 25°C	14	4.27
Control	40	4.15
HPP 4.4°C	40	4.23
HPP 25°C	40	4.24
Control	60	spoiled
HPP 4.4°C	60	4.25
HPP 25°C	60	4.24

HPP treatment of acid foods have previously shown an inactivation of 10^6 CFU/g of *E.coli* 0157:H7, *Listeria* spp., *Salmonella* spp. and *Staphylococcus* spp.in products such as salsa and apple juice using pressures of 580 MPA and 15 minutes at 25°C (Farkas et al., 2000; Reilly et al., 2000). Yeast and mold are typically pressure sensitive and inactivated at pressures of 344 MPa for 15 minutes at 25°C (Aleman et al., 1994; Rasco et al., 1998). The yogurt raw material had low APC and yeast and mold counts (Table 3) and these were maintained throughout the 60 day storage period for samples that were HPP treated and stored at both 4.4 and 25 °C. Lactic acid bacteria have been reported to be pressure resistant (Smelt, 1998). Results in this study, indicate that immediately following HPP treatment a small number of lactic acid bacteria survived (Table 3) when monitored at 0 and 14 days storage. By 40 days and at both storage temperatures, there were <10 CFU/g. The untreated control stored

at 4.4°C steadily decreased in pH and increased in lactic acid bacteria growth causing the product to be microbiologically deteriorated at 40 days of storage.

Table 3 – Microbiological Results for Blackberry Yogurt During Storage

Sample	Storage Time (Days)	Aerobic Plate Count (CFU/g)	Yeast & Mold (CFU/g)	Lactic Acid Bacteria (CFU/g)
Control	0	<10	<10	25×10^{8}
HPP 4.4°C	0	20	<10	42×10^{1}
HPP 25°C	0	20	<10	42×10^{1}
Control	14	<10	<10	9.5×10^{7}
HPP 4.4°C	14	35	<10	33×10^{1}
HPP 25°C	14	40	<10	40×10^{1}
Control	40	<10	45	TNTC
HPP 4.4°C	40	21	<10	<10
HPP 25°C	40	25	<10	<10
Control	60	spoiled	spoiled	spoiled
HPP 4.4°C	60	23	<10	<10
HPP 25°C	60	15	<10	<10

Typically, HPP in conjunction with heat has been investigated as an effective microbial inactivation process. This research suggests that HPP and low temperature (5°C) treatment of an acid food product is effective in creating a microbiologically shelf-stable high quality food product for storage at 4.4 or 25°C in extended storage applications. The benefits of using low temperature and HPP to modify quality characteristics such as viscosity through protein denaturation could have a great deal of potential in creating new or improved dairy food products.

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Chapter 5

Conclusion

The surface pressure of β-lactoglobulin model solutions were increased when subjected to high pressure. Pressure treatment at room temperature caused significant and permanent changes to secondary and tertiary protein structure. However, the treatment failed to lead to optimum surface activity in model solutions because of protein aggregation. In contrast, pressure treatment at low temperature caused changes in tertiary structure while secondary structure changes were minimal and substantial renaturation occurred during storage. In spite of the renaturation the gain in surface activity of the model solutions was high. This was most likely due to the fact that protein aggregation minimal. It seems likely that β-lactoglobulin in the partially denatured state (510 MPa , 8C and pH 7.5) has a more flexible, hydrophobic surface and therefore readily adsorbs at the interface whereas the control and lower pressure (410 MPa) treated proteins have a more tightly folded conformation and therefore have a lower probability of collision at the interface.

This suggests that the adsorbed protein at the interface has an average structure lying between the native folded and the completely unfolded state and fits the definition of the molten globule state. Research indicates that β-lactoglobulin in the molten globule state exhibits an increased affinity for ANS compared to the native state. (Yang et al., 2001; Yang et al., 2002; Semisotnov, 1991). In this research, the pressure induced denaturation of β-lactoglobulin at 8°C and storage at 4.4°C for 24 hours resulted in a structure that has the conformation as defined as being the molten globule with native-like secondary structure and disrupted tertiary structure and increased ANS binding..

These results support a favorable effect of unfolding on surface functionality when aggregation is avoided. Due to the unique effects of HPP and low temperature on the structural and functional properties of β-lactoglobulin further investigations should be explored in further optimizing this process. The optimization of the HPP treatment by using low temperatures could lead to the development of novel food products and ingredients.

Chapter 4 on yogurt showed that the viscosity of the blackberry yogurt increased significantly following low temperature HPP treatment. HPP treated yogurt had a visibly thicker and smoother appearance than the untreated control. The HPP samples continued to maintain a consistently higher viscosity at both 4.4 and 25°C storage over the 60 day testing period.

HPP treatment had no significant effect on the color of the yogurt immediately following treatment. Variations in color were observed throughout the 60 day storage period with L* decreasing and chroma increasing. This change indicates a trend toward a darker, deeper colored product. The yogurt took on a deeper purple color, which may have resulted from color from the fruit pieces migrating into the yogurt during storage. This trend was the greatest for HPP samples stored at room temperature (25°C). Browning, or a negative change in a* and b* was not observed in any of the stored samples.

Control samples steadily decreased in pH due to microbiological deterioration by 40 days of storage. The yogurt raw material had low APC and yeast and mold counts and these were maintained throughout the 60 day storage period for samples that were HPP treated and stored at both 4.4 and 25 °C. Lactic acid bacteria have been reported

to be pressure resistant (Smelt, 1998). Results in this study, indicate that immediately following HPP treatment a small number of lactic acid bacteria survived when monitored at 0 and 14 days storage. By 40 days and at both storage temperatures, there were <10 CFU/g. The untreated control stored at 4.4°C steadily decreased in pH and increased in lactic acid bacteria growth causing the product to be microbiologically deteriorated at 40 days of storage.

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