EFFECT OF HIGH HYDROSTATIC PRESSURE ON
WHEY PROTEIN CONCENTRATE
FUNCTIONAL PROPERTIES

By

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To the Faculty of Washington State University:

The members of the Committee appointed to examine the Dissertation of XIAOMING LIU find it satisfactory and recommend that it be accepted.

Chair
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Abstract

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After high hydrostatic pressure (HHP) treatment at 600 MPa and 50°C for 30 min to whey protein concentrate (WPC), there were decreases in protein solubility at pH 4.6, and increases in aggregation and denaturation of whey proteins, especially at high WPC concentrations. During the come-up time of HHP treatment, dissociation of aggregates and formation of dimers were observed. With increasing HHP treatment time, monomers of β-lactoglobulin (β-LG), α-lactalbumin (α-LA), and bovine serum albumin (BSA) decreased and aggregates were formed.

An increase in tryptophan intrinsic fluorescence intensity and a 4 nm red-shift were observed after 30 min of treatment, which indicated changes in the polarity of tryptophan residues of whey proteins from a less polar to a more polar environment. HHP treatment for 30 min increased the number of binding sites of WPC for 1-anilino-naphthalene-8-sulfonate (ANS) from 0.16 to 1.10 per molecule of protein. HHP treatments did not show significant influences in the apparent dissociation constant of ANS except a 1.8-fold increase after 30 min HHP treatment. Increased binding affinities of cis-parinaric acid (CPA) were observed after come-up time or 10 min of HHP treatment.
HHP treatments increased the number of binding sites and the apparent dissociation constants of WPC for benzaldehyde. HHP treatment for 10 min increased the binding affinity of WPC for diacetyl, but no significant changes in the number of binding sites were observed after 10 min of HHP treatment. There were increases in the number of binding sites of WPC for heptanone and octanone after HHP treatment for the come-up time.

As observed by headspace analysis, HHP treatments did not result in significant changes in the retention for benzaldehyde in WPC solutions. Flavor retention of 100 ppm and 200 ppm heptanone and octanone in HHP treated (10 min) WPC was significantly lower than in untreated WPC and HHP treated WPC for come-up time or 30 min. Significant decreases were only observed at 100 ppm for flavor retention of nonanone in HHP treated (10 min) WPC solutions. Further research is required to evaluate the full potential of application of HHP to modify functional properties of WPC and its benefits to the food industry.
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CHAPTER ONE
INTRODUCTION

People are aware that high levels of saturated fat and cholesterol in the diet are linked to increased blood cholesterol levels and enhanced risk for heart disease. Thus, food manufacturers have tried to substitute fat with fat replacers to create products that meet the demands of health-conscious consumers (Miller and Groziak, 1996). In 1996-1997, thirty-eight percent of new product launches had lowfat claims (Goettsche, 2002). However the sales of lowfat and fat-free products has declined in recent years because of product quality issues. Fat has a unique functionality that enables it to react with flavor compounds and to have a specific pattern of flavor release in the mouth that no present fat replacers can provide (Li et al., 1997). Thus, the waning consumer interest in these products has prompted firms to scale back on reduced fat product production. The percentage of new lowfat products dropped to 11 percent of all new launches in 2001 (Goettsche, 2002).

The worldwide cheese manufacturing industry produces an estimated 190 billion pounds of whey annually, which contains an estimated 1.3 billion pounds of whey protein (Morr, 1984; Zall, 1984). The U. S. dairy industry produces 44 billion pounds of whey annually, which contains an estimated 360 million pounds of whey protein. Current statistical data indicate that annual U. S. whey protein concentrate (WPC) production is approaching 200 million pounds (Morr and Ha, 1993).

Whey products have been used successfully in the food industry for years. Cost efficiency and nutritional value are key drivers in using whey products. Whey products provide solubility and viscosity, form gels, emulsify, facilitate whipping and foaming, enhance color,
flavor and texture, and offer numerous nutritional advantages (U. S. Dairy Export Council, 1999).

Modification of whey proteins may enhance their functional properties, allowing for development of a variety of protein components for food products. The binding capacity of β-lactoglobulin (β-LG), a major whey protein, can be improved by heating at 70°C (Marin and Relkin, 1998; O’Neill and Kinsella, 1988). However, more severe thermal treatment will increase protein denaturation, accompanied by loss of solubility and functional properties (Kester and Richardson, 1984). High hydrostatic pressure (HHP) is an alternative technology to heat processing for food product modifications. It does not cause environmental pollution and eliminates the use of chemical additives in food products (Kadharmeston, 1998). Studies have been done to understand the effect of high pressure on some of the functional properties of whey proteins (Famelart et al., 1998; Galazka et al., 1995). However, little work has been done regarding the effects of high pressure on flavor-binding properties of whey protein concentrate. High pressure induces β-LG into a molten globule state, which may help improve the functional properties of flavor binding and release (Yang et al., 2001). Investigation of the effect of HHP treatment of WPC on its flavor-binding functional properties will assist in designing fat substitutes out of WPC that give similar flavor release profiles as the original food.

This dissertation is presented in six chapters. Chapter 2 provides a review of WPC composition and functionality, chemical and physical modification, high hydrostatic pressure mechanism and applications, and hypotheses. The following three chapters report the results of the research. Chapter 3 investigates the effects of HHP on protein solubility of WPC, and denaturation and aggregation of whey proteins. Chapter 4 evaluates the hydrophobic binding properties of WPC affected by HHP. Chapter 5 assesses the flavor-binding functionality of WPC affected by HHP. Chapter 6 summarizes and evaluates this research.
REFERENCES


CHAPTER TWO
LITERATURE REVIEW

Lowfat and nonfat product flavor problems

The flavor perception of a particular food product is a major factor determining consumer acceptance (Casimir, 1998). The quantity of flavor released into the oral cavity depends on the retention of flavor compounds in the food matrix and, therefore, on the nature of the ingredient-flavor interactions (Harrison and Miller, 1997). Among these factors, fat is very important because the majority of flavor components are dissolved to some extent in the lipid phases of food and are released slowly in the mouth, resulting in a pleasant aftertaste (Hatchwell, 1994).

Although fat is important for sensory qualities such as flavor, color, texture, and mouthfeel, manufacturers have made it a practice to substitute fat with fat replacers in order to create products that meet the demands of health-conscious consumers (Casimir, 1998). High fat intake is associated with increased risk for obesity, and saturated fat intake is associated with high blood cholesterol and coronary heart disease (American Heart Association, 1996; U.S. Department of Health & Human Services, 1988). As these substitutions are made, both the texture and flavor profile of the products may change. Fat has a unique functionality that enables it to react with flavor compounds and to have a specific pattern of flavor release in the mouth that no present fat replacers can provide (Li et al., 1997). As the concentration of the fat is significantly reduced, the flavor challenges are increased, and aroma chemicals may be perceived as harsh and unbalanced. For example, fat removal from vanilla ice cream results in drastic flavor profile change and loss in vanillin intensity during storage (Hatchwell, 1996).

Approved fat substitutes are mainly made out of carbohydrates and proteins. They may achieve quite satisfactory fatlike texture and mouthfeel. However, none of them can be
transformed to the same flavor derivatives as lipids during processing, nor will their interactions with the added flavors be comparable to fats (Leland, 1997).

**Whey protein concentrate production and application**

Whey comprises 80 to 90% of the volume of milk entering the cheesemaking process and contains about 50% of the solids present in the original whole milk, including 20% of the protein and most of the lactose, minerals, and water-soluble vitamins (Marshall, 1982). The U. S. dairy industry produces 44 billion pounds of whey annually, which contains an estimated 360 million pounds of whey protein (Morr and Ha, 1993). However, only about 55% of the whey produced is further processed in the United States (American Dairy Products Institute, 1999). Disposal of whey is difficult and costly because of its high biological oxygen demand (BOD) (Marshall, 1982). New high value-added products and technologies are crucial for the dairy industry to decrease the expenses of waste disposal (Yang and Silva, 1995).

Whey and whey products have been used successfully in the food industry for the past 30 years. Cost efficiency and quality improvement are key drivers in using whey products. The nutritional value of whey products is also an important reason why an increasing number of food manufacturers worldwide include whey products in their formulations. Whey products provide solubility and viscosity, form gels, emulsify, facilitate whipping and foaming, enhance color, flavor and texture, and offer numerous nutritional advantages (U. S. Dairy Export Council, 1999).

In the manufacture of cheese from milk, curds are formed by the action of rennet-type enzymes and/or acid. Whey is the liquid remaining after the recovery of the cheese curds (Marshall, 1989). In industrial practice, there are two major types of whey: sweet whey from the
manufacture of cheese from milk by the action of rennet-type enzymes with relatively little or no acidity development; and acid whey, where the milk is coagulated primarily with acid. Sweet whey has a minimum pH of 5.6 and acid whey a maximum pH of 5.1 (International Dairy Federation, 1978). Components of whey ranked in decreasing order of relative amount are: lactose, nitrogenous compounds (protein, peptides, and amino acids), ash, and lipids. For example, sweet whey is generally composed of 4.77% lactose, 0.82% protein, 0.53% ash, and 0.07% lipids (Bassette and Acosta, 1988). The principal whey proteins are β–lactoglobulin (β-LG) and α–lactalbumin (α-LA). These two proteins account for approximately 80% of the total whey protein. Other proteins include: bovine serum albumin (BSA), immune globulins (Ig), proteose peptones, and soluble caseins and a variety of minor proteins (enzymes, lactoferrin, etc.). The major mineral components of whey are calcium, phosphorous, sodium, and potassium (Morr and Ha, 1993).

Whey protein concentrate (WPC) is manufactured from sweet or acid whey that is processed to remove fat and some lactose (Morr and Ha, 1993). Producers try to remove as much lipid as possible from whey to improve WPC functionality. Lipid has long been recognized as being detrimental to the quality of whey protein concentrates with particular attention to the foaming and flavor qualities of the product (King, 1996). It is also of high priority to reduce lactose in WPC because lactose is a reducing sugar and can react with proteins via non-enzymatic browning to produce less nutritious and less functional products (King, 1996). Lactose can also crystallize in ice cream and result in texture defects such as sandy. Whey protein isolate (WPI) generally has higher levels of protein than WPC (WPI ≥ 90% protein). The WPI is accomplished either by ion exchange (IE) followed by concentration, or microfiltration followed by ultrafiltration (UF). Since WPI is more expensive, it is mainly used in nutraceutical beverages and bars (King, 1996).
Commercial-scale UF and diafiltration (DF) processes that utilize semipermeable membranes with molecular weight cut-off limits of 10 to 50 kDa for fractionating whey proteins from the low molecular weight components have become the processing methods of choice for manufacturing WPC (Morr and Ha, 1993). UF and DF are used to concentrate retentate and remove lactose, minerals, and other low molecular weight components (Morr, 1989).

The industry has expended a great amount of effort during the last 30 years to develop commercial-scale processes for manufacturing highly functional WPC suitable for use as a food and animal feed ingredient (Morr and Ha, 1993). These products contain 34 to 90% protein (Morr and Ha, 1993). WPC is a Generally Recognized As Safe (GRAS) ingredient. WPC can be used in any food product, unless restricted by specific standards of identity, providing the utilization is in accordance with Good Manufacturing Practices (Food and Drug Administration, 1981). WPC is not only a less expensive alternative to skim milk powder, but it also has many applications in foods (King, 1996). The functionality of commercial WPC products is generally related to the concentration of whey protein and the extent to which these proteins have been denatured intentionally or unintentionally during processing (Hugunin, 1987).

WPC that contains 34 to 55 % protein is used in animal feed manufacture (King, 1996). WPC that contain ≥ 70 % protein are used extensively as functional and nutritional ingredients in medical, pharmaceutical, and human food products, such as bakery, dairy, processed meats, confectionery, and beverages (King, 1996). For example, WPC with a 75% protein content can be used as a partial replacer of whole egg or egg whites in biscuits, sponges and icings (King, 1996). When WPC is used to supply milk solids and replace skim milk powder, the lower lactose concentration of WPC allows usage of higher levels of WPC than skim milk powder, contributing body and texture without developing sandiness in the ice cream (Young, 1999).
WPC can gel and increase viscosity of the products upon cooking. In nutriceutical bars and nutritious confections, the whipping abilities of WPC are used to produce a fluffy texture (King, 1996). Since WPC is stable in acidic conditions, WPC can be used to fortify juices and fruit drinks for the health and sports markets (King, 1996). Development of WPC with enhanced functional properties may meet the need of the industry for highly functional protein-rich ingredients.

**Basic properties and functional properties of whey proteins**

β-Lactoglobulin (β-LG) is a small, globular whey protein found in the milk of many mammals including cows, goats, camels, pigs, and dogs (Pervaiz and Brew, 1985). β-LG is the most abundant of the whey proteins and represents 56-60% of the total whey proteins (Morr and Ha, 1993). β-LG is a globular protein with a molecular weight (MW) of 18.3 kDa and consists of 162 amino acid residues that have been sequenced (Eigel et al., 1984). β-LG has five Cys/2 residues as two intermolecular disulfide bonds and one free SH group (Godovac-Zimmermann and Braunitzer, 1987). The structure of β-LG is dependent on the pH. β-LG exists as a dimer with a MW of 36.7 kDa in solutions above its isoelectric point of pH 5.2. Below pH 3.5 and above 5.2 the dimer polymerizes to a 147 kDa octomer (Swaisgood, 1982; Swaisgood, 1985). β–LG has about 15, 43, and 47% α-helix, β-sheet, and unordered structures, respectively, that are pH and temperature sensitive (Kinsella, 1984). While preserving more or less the same secondary structure, β-LG can adopt various tertiary structures that display different susceptibility to denaturing agents at different pH values (Frapin et al, 1993). β-LG undergoes time- and temperature-dependent denaturation above 65°C, which is accompanied by extensive conformational transitions that expose highly reactive thiol and amino groups (Kinsella, 1984).
β-LG binds fatty acids \textit{in vivo} (Perez et al., 1989) and a large variety of hydrophobic ligands \textit{in vitro} (Futterman and Heller, 1972), such as retinol, sodium dodecyl sulphate (Magdassi et al., 1996) and aroma compounds (Guichard and Langourieux, 2000). Although the protein-ligand interaction is mostly described in terms of flavor binding, it has been generally considered that β-LG is a possible carrier for flavor compounds and may be effective in protecting, delivering, or delaying release of flavor components. For instance, β-LG could be engineered to bind and protect a wide range of volatile and unstable flavors during food manufacturing or to release the flavors in a more or less controlled way by chemical modifications or heat treatment (Boundaud and Dumount, 1996).

α-LA, with a 14 kDa molecular weight, represents about 20% of the total whey proteins in milk (Eigel et al., 1984). This Ca$^{2+}$ binding protein consists of 123 amino acid residues (Ramboarina and Redfield, 2003). α-LA has eight Cys/2 residues and all of its sulfur amino acid residues are in the form of intramolecular disulfide bonds (Swaisgood, 1982). For many years, α-LA was considered to be the most heat stable of the whey proteins (de Wit, 1981). More recent evidence indicates that α-LA is quite susceptible to heat denaturation (Morr, 1976). Rüegg et al. (1977) showed that α–LA is denatured at 65°C and pH 6.7, and that at these conditions 80 to 90% of the denaturation is reversed upon cooling.

BSA consists of 582 amino acid residues, with a molecular weight of 66 kDa (Morr and Ha, 1993). This protein has 17 intramolecular disulfide bonds and one free sulfhydryl group (Eigel et al., 1984). BSA is a well-known transport protein for insoluble fatty acids in the blood circulatory system (de Wit, 1989).

Morr and Ha (1993) defined protein functional properties as those physicochemical properties that influence the structure, appearance, texture, viscosity, mouthfeel, or flavor
retention of the product. Most of the key protein functional properties may be classified into two main groups: hydration-related and surface–related properties (Morr and Ha, 1993). Hydration related functional properties include dispersibility, solubility, swelling, viscosity, and gelation. Surface-related properties include emulsification, foaming, and adsorption at air-water and oil-water interfaces. Other functional properties that do not fit either of these two categories include diffusion; molecular unfolding (denaturation); and protein-protein, protein-ion, and protein-ligand binding (Morr and Ha, 1993).

Flavor-binding property is an important functionality of dairy proteins, and interactions of flavor compounds with whey proteins in food systems have been investigated in the past 30 years (Li et al., 2000). Due to their complexity, flavor-protein interactions remain a major challenge for food scientists (McGorrin, 1996; Stevenson et al., 1996). The amount of flavors bound depends mainly on protein type, protein conformation, and type and position of functional groups of the flavors (McGorrin, 1996). The binding is also affected by temperature, pH, and the concentrations of the protein and the flavor compounds (McGorrin, 1996).

β-LG is known to interact with many flavor compounds, such as aldehydes, ketones (O’Neil and Kinsella, 1987), ionones (Dufour and Haertlé, 1990), and hydrocarbons (Wishnia and Pinder, 1966). Binding of the flavor compounds by proteins could be of different natures. For example, an increasing percentage of retention with increasing chain length for a series of alkanones and ethyl esters suggests hydrophobic binding, whereas benzaldehyde could be partially covalently bound (Sostmann et al., 1997). For series of ketones, aldehydes, alcohols and lactones, a good linear correlation was found between the logarithm of the binding constant measured by affinity chromatography and hydrophobicity of the ligands (Guichard and Langourieux, 2000). Thus, ketones, aldehydes, alcohols and lactones may bind into the
hydrophobic pocket of β-LG by hydrophobic interactions (Guichard and Langourieux, 2000). However, for terpene alcohols and phenolic compounds, such a linear relationship could not be obtained, showing that both hydrophobic and steric interactions of the molecule influence the binding with β-LG (Reiners et al., 2000).

β-LG possesses two distinct forms of binding: a strong affinity at a single, localized hydrophobic region for retinol; and a weaker affinity at one or several sites on the protein for fatty acids (Robillard and Wishnia, 1972a; Robillard and Wishnia, 1972b). Dimers or monomers of β-LG do not show effects on either the strong or the weak affinity (Robillard and Wishnia, 1972a). The binding site for retinol is well established and lies in the β-barrel of the protein (Cho et al., 1994). It is suggested that fatty acids bind at the external hydrophobic site between the sole α-helix and the β-barrel (Narayan and Berliner, 1998; Sawyer et al., 1998; Wu et al., 1999).

Factors affecting whey protein concentrate functionality

Composition

Numerous references in the literature document relationships between composition and functionality of WPC solutions (Schmidt et al., 1984; Liao and Mangino, 1987; Flingner and Mangino, 1991). Functionality of WPC is affected by a number of compositional factors, such as total and individual protein composition, pH, ionic strength, concentration of Ca$^{2+}$ and other individual ions, lipids, lactose, chemical emulsifiers, reducing and oxidizing chemicals (Flingner and Mangino, 1991).

There are wide differences in the gross composition of commercial WPC products manufactured worldwide (Morr, 1989; Morr and Foegeding, 1990; de Wit et al., 1986). Most of these compositional differences relate to the use of different processing conditions and
technologies for manufacturing whey and WPC (Morr, 1989; Schmidt et al., 1984). Most WPC products are manufactured by UF and DF technologies (Morr and Foegeding, 1990). Processing modifications with respect to whey manufacture, whey pretreatment, degree of fractionation by UF and DF, and spray-drying conditions are expected to alter the composition of WPC (Morr and Ha, 1993). Therefore, consistency in the composition of WPC is an important factor to predict WPC functionality and to manufacture WPC products with optimum functionality for each product application (Morr and Ha, 1993).

Hydrophobicity

The impact of hydrophobic interactions of food proteins on their functional properties has received major attention (Li-Chan and Nakai, 1989; Nakai and Li-Chan, 1989; Mangino, 1990). The hydrophobic interaction may arise from unfavorable interactions ($\Delta G > 0$) between water molecules and nonpolar residues on the protein molecule (Morr and Ha, 1993). These thermodynamically unfavorable interactions result in a change in water structure and a decrease in entropy (Morr and Ha, 1993). To minimize this reduction in entropy, nonpolar amino acid residues interact to form a hydrophobic core, thus reducing their area of contact with water (Morr and Ha, 1993).

Li-Chan and Nakai (1989) concluded that hydrophobic, electrical, and steric properties affect the functionality of proteins. Nakai (1983) demonstrated a close relationship between surface hydrophobicity and emulsion capacity of proteins. Increased fat binding capacity was associated with an increase in hydrophobicity of the protein (Voustsinas and Nakai, 1983). Harris et al. (1989) also reported a correlation between hydrophobicity and functionality of WPC.
Net charge on the protein molecule is one of the most important physicochemical properties for determining foaming properties, whereas foam capacity was most strongly correlated with the hydrophobicity and viscosity of the protein solution (Morr and Ha, 1993). Exposed hydrophobicity and SH group activity were important in determining thermal functional properties, such as coagulation, thickening, and gelation (Nakai, 1983).

**Protein Structure**

Knowledge about the relationship between the structural and functional properties of food proteins may help us to predict their functional properties. In the food industry, one of the most important applications of such studies is to modify the structure and texture, and hence the functional properties, of foods. Detailed knowledge about structure-functionality relationships will help food technologists to develop fabricated foods based on consideration of the underlying science rather than on trial-and-error manipulation of food ingredients (Hirose, 1993).

It seems difficult to predict precisely the functional property of a protein simply from its primary or tertiary structure (Gekko and Yamagami, 1991). For example, α-LA shows good emulsifying and foaming properties as compared to those of lysozyme, although there is a high similarity in the primary and tertiary structures of the two proteins. One of the reasons for such discrepancy may be because the dynamic structure of a protein is not taken into account in the prediction of the functional properties of proteins (Gekko and Hasegawa, 1986). X-ray analyses revealed that in some proteins there is void space that permits considerable internal motion in response to thermal or high hydrostatic pressure forces (Kundrot and Richards, 1987). An importance of the flexibility of food proteins has been pointed out from the correlation between the protease susceptibility and some properties such as foaming capacity (Nakai, 1983; Townsend and Nakai, 1983; Kato et al., 1985). Compressibility influences protein dynamics.
since it is directly linked to the possibility of volume changes in proteins (Cooper, 1976; Pain, 1987).

The partial specific volume ($\bar{\upsilon}$) of a protein in water consists of three contributions: (1) the constitutive volume estimated as the sum of the constitutive atomic or group volumes ($V_c$); (2) the volume of the cavity in the molecule due to imperfect atomic packing ($V_{cav}$); and (3) the volume change due to solvation or hydration ($\Delta V_{sol}$).

$$\bar{\upsilon} = V_c + V_{cav} + \Delta V_{sol}$$

Here, $V_{cav}$ involves not only the incompressible volume formed on the closest packing of atoms but also the compressible void space generated on the random close packing of atoms. $\Delta V_{sol}$ is ascribed to three types of hydration, electrostriction around the ionic groups, hydrogen-bonded hydration around the ionic groups, and hydrophobic affinity around the nonpolar groups. Each of them produces a negative volume change, and the resulting negative $\Delta V_{sol}$ cancels out the positive $V_{cav}$ almost completely. Since the constitutive atomic volumes may be assumed as incompressible, adiabatic compressibility of a protein is mainly due to the contributions of cavity and hydration (Gekko and Yamagami, 1991).

Although this type of fluctuation is thermodynamic and macroscopic, Gekko and Yamagami (1991) found that compressibility reflects the structural characteristics of globular proteins. They studied the adiabatic compressibility of 14 egg and milk proteins (including $\alpha$-LA, $\beta$-LG, and BSA), and found that the protease susceptibility, foaming capacity, and free energy of unfolding of proteins are positively correlated to the adiabatic compressibility. Their results indicate that the flexibility of the structure plays an essential role in the conformational stability and functional properties of food proteins (Gekko and Yamagami, 1991).

*Processing*
Modification of whey proteins may enhance or alter the combination of functional characteristics, allowing for development of a variety of protein components with a broad spectrum of functional properties (Dufour and Haertlé, 1991). These modified whey proteins may prove useful in the expanding area of fabricated foods (Dufour et al., 1992).

Modification can be accomplished by chemical or physical means (Dufour and Haertlé, 1991). The objective of chemical derivatization is to alter the noncovalent forces determining protein conformation in a manner that results in desired structural and functional changes (Dufour et al., 1992). But chemical derivatizations may have some effects upon amino acid bioavailability and have toxicologic consequences that make them less than ideal for food applications (Kester and Richardson, 1984).

Changes of protein functional performance through physical means can be achieved by thermal treatment (Cairoli et al., 1994). Hermansson (1979) discussed the effects of heating on the strength of protein gels, reporting that a balance between the rate of protein unfolding and aggregation is required for optimum gel strength. Mild heat treatment reportedly increased the overrun on whipping of WPC, but excessive heating inhibited foaming (Richert et al., 1974).

Marin and Relkin (1998) reported that the binding capacity of β-LG was improved by heating. The binding constant of β-LG to benzaldehyde increased ten-fold after the heat treatment in comparison with similar unheated solutions. The number of binding sites decreased by less than 10%, attributed to probable protein aggregation (Marin and Relkin, 1998). O’Neill and Kinsella (1988) reported that the flavor binding behavior of native β-LG was significantly altered by thermal or chemical modification. Upon heat treatment at 75°C for 10 and 20 min the binding affinity of β-LG for nonanone was reduced and the number of sites for binding was increased (O’Neill and Kinsella, 1988).
At or near 70°C, protein solubility, foaming activity, and emulsifying activity start to decline due to protein denaturation (Galazka et al., 1995). More severe thermal treatment will cause protein denaturation, accompanied by loss of aqueous solubility and overall functional behavior (Kester and Richardson, 1984). So partial denaturation, or combining partially denatured with native protein, has been suggested as a technique for intentional modification of functionality (Kester and Richardson, 1984).

High hydrostatic pressure technology and application in the food industry

Traditional food processing methods have relied on high temperatures as a way to ensure prolonged shelf life and food safety. However, the use of high temperatures is known to cause some detrimental changes in the processed products (Martin et al., 2002). Undesirable changes affect nutritional as well as organoleptic attributes. Several vitamins degrade under heat treatments as do color and flavor compounds (Martin et al., 2002).

High hydrostatic pressure (HHP) presents unique advantages over conventional thermal processing for food product modifications, including application at low temperatures, which permits the retention of food quality (Knorr, 1995a; Knorr, 1995b; Cheftel, 1992). It does not cause environmental pollution and eliminates the use of chemical additives in food products (Kadharmeston, 1998).

There has been considerable commercial HHP research and development activity in Japan and as a result a number of HHP processed products are available for retail sale, including low-sugar jams, fruit sauces, desserts, grapefruit juice and mandarin juice (Palou et al., 1999). In 1990, the first high-pressure product, a high-acid jam, was introduced to the Japanese retail market (Palou et al., 1999). The jams were vivid and natural in color and taste (Hayashi, 1995).
In 1991, yogurts, fruit jellies, salad dressings, and fruit sauces were introduced, and two fruit juice processors installed semi-continuous high pressure equipment for citrus juice bulk processing (William, 1994). A number of other products are at various stages of development; all of these products retain a remarkable degree of fresh flavor (Hayashi, 1995). In U. S. and Europe, developments are being made in fruit products, ready meals, dairy products, meats and fish (Palou et al., 1999). The guacamole from Avomex, Inc. is the first commercial HPP product in U.S., and consumer demand has exceeded expectations. Already a major user of HPP technology, Avomex, Inc. has installed a total of seven systems to process juices, avocado pulp, guacamole, salsa, guaca-salsa and avocado halves for food service and retail (Hoover, 1997).

The pressure range currently being investigated for use in food processing is roughly 100 MPa to 900 MPa, with pressures used in commercial systems between 400 and 700 MPa (Martin et al., 2002). Currently, the widest application of HHP processes within the food industry is mainly for extending the shelf life of food products, although as research progresses other uses are foreseen. These include solute diffusion, freezing-thawing, and modification of functional properties of proteins and other macromolecules (Martin et al., 2002).

Hayashi and Balny (1996a) reported that Sudachi juice could be sterilized by high pressure treatment and preserved for a long time retaining its natural flavor and quality. Combined treatment with high pressure and low temperature effectively inactivated *Saccharomyces cerevisiae* in strawberry jam with a pseudo-first order kinetics (Hayashi and Balny, 1996b).

The extent of microbial inactivation achieved at a particular pressure treatment depends on several factors, including type and number of microorganisms, magnitude and duration of HHP treatment, temperature, and composition of the suspension media or food (Palou et al.,
The patterns of HHP inactivation kinetics observed with different microorganisms are quite variable. Some investigators demonstrate first order kinetics for several bacteria and yeast (Hashizume et al., 1995; Smelt and Rijike, 1993). However, Cheftel (1995) observed a change in the slope of inactivation curve and a two-phase inactivation phenomenon: the first fraction of the population being quickly inactivated, whereas the second fraction appears to be much more resistant.

**Effects of high hydrostatic pressure on protein structure**

The basis of HHP is the *le Chatelier* principle, according to which any reaction, conformational change, or phase transition that is accompanied by a decrease in volume will be favored at high pressures, while reactions involving an increase in volume will be inhibited (Ledward, 1995; Cheftel, 1995). Pressure greater than 100-200 MPa often cause: (a) dissociation of oligomeric structures into their subunits, (b) partial unfolding and denaturation of monomeric structures, (c) protein aggregation, and (d) protein gelation if protein concentration and pressure are high enough (Cheftel, 1995).

The Gibbs energy determining the thermodynamic equilibrium among different conformers of a protein in solution is driven by pressure according to the following relation (Lassalle et al., 2003),

\[
\Delta V^0(p-p_0) - \frac{1}{2} \Delta \kappa (p-p_0)^2 + \Delta G^0 = \Delta G_p
\]

where \(p_0\) is the atmospheric pressure (1 bar), \(\Delta G^0\) and \(\Delta V^0\) are differences in the Gibbs energy and partial volume at 1 bar, respectively, and \(\Delta \kappa\) denotes the difference in compressibility. Pressure simply changes the conformational equilibrium by acting on volumetric properties, while denaturants such as urea directly perturb the interaction energy and entropy embedded in
ΔG° (Wu and Wang, 1999; AbouAiad et al. 1997). That is, pressure drives the equilibrium to increase the population of the lower volume conformer relative to the higher volume conformer (Weber and Drickamer 1983; Inoue et al. 2000).

A protein in solution is a dynamic entity, able to adopt a variety of conformations between the native (N) and the fully denatured (U) states. One of the conformations frequently observed in globular proteins under mildly denaturing conditions is a compact denatured state called the molten globule (MG), defined as a state with native-like secondary structure, but lacking fixed side-chain packing (Dolgikh et al., 1981; Ohgushi and Wada, 1983). The polypeptide chain in a MG is only loosely packed, typically showing a radius of gyration (Rg) of 10% larger than the corresponding Rg for the native state (Kataoka et al., 1997; Kamatari et al., 1999). The polypeptide chain must be hydrated, but the state of hydration is different from that of the fully unfolded state U, and spatial heterogeneity in hydration is also possible. As the polypeptide chain fold is loose, ample motions in proteins may lead to fluctuating packing density and volume fluctuations. Thus, the partial molar volume and compressibility are of general concern for the MG state (Kamatari et al., 1999).

Typical molten globules are found in vitro at low pH (Ohgushi and Wada, 1983; Ptitsyn, 1991; Ptitsyn and Uversky, 1994), in the presence of alcohol (Kamatari et al., 1998; Kamatari et al., 1999), after heat treatment (Chattopadhyay and Mazumdar, 2000), or after high pressure treatment (Zhang et al. 1995; Ruan et al. 1997; Jonas et al. 1998; Lassalle et al. 2000; Kitahara et al. 2002). In living cells, the MG state is likely to be present in equilibrium with the native state, and may be actively involved in various biologic processes such as targeting, transport, and aggregation (Bychkova and Ptitsyn, 1993).

Lassalle et al. (2003) showed that high pressure (from 30 to 2000 bar at 20°C) turned the α-LA MG into conformers with increasing disorder and hydration, which gives straightforward
evidence that the partial molar volume of the MG state is significantly larger than that of the fully denatured state. It is also important to note that the conformational changes were brought about reversibly with pressure under equilibrium conditions. This means that the MG state at 30 bar coexists with other conformers with partial unfolding at various degrees. The results verify that the MG state consists of a mixture of variously unfolded conformers from the mostly folded to the nearly totally unfolded that differ in stability and partial molar volume. The populations of the latter conformers are small compared to the main MG conformers found at 30 bar, but their fractions become significant at higher pressure because of their smaller partial molar volumes (Lassalle et al., 2003).

Pressure denaturation of protein is a complex phenomenon depending on the protein structure, pressure range, temperature, pH, and solvent composition (Palou et al., 1999). Oligomeric proteins are dissociated at relatively low pressures (200 MPa), while denaturation of monomeric proteins occurs at pressures greater than 300 MPa (Cheftel, 1995).

Unlike heat-denatured proteins, pressure unfolding of a protein does not correspond to the transfer of a nonpolar molecule from a nonpolar environment into aqueous solution (Hummer et al., 1998). The protein interior is largely composed of efficiently packed residues, more likely hydrophobic than those at the surface (Richards, 1974). Increasing hydrostatic pressure forces water molecules into the protein interior, gradually filling cavities, and eventually resulting in changes in the tertiary and quaternary structure of proteins (Hummer et al., 1998). The protein-water system may be packed more efficiently and have a lower total volume when water molecules are mixed into the structure (Sloan, 1990). Thus, pressure denaturation corresponds to the incorporation of water into the protein, whereas heat denaturation corresponds to the transfer of nonpolar groups into water.
Pressure may affect the secondary, tertiary, and quaternary structure of proteins (Palou et al., 1999). The fact that moderate pressure does not disrupt secondary structures is due to the little effect of pressure on hydrogen bonds that stabilize interaction of secondary structure (Masson and Cléry, 1996). On the other hand, disorganization of tertiary structure presumably results from pressure-induced disruption of hydrophobic interactions (Masson and Cléry, 1996). The main targets of pressure are the electrostatic and hydrophobic interactions in protein molecules (Palou et al., 1999). High pressure causes deprotonation of charged groups and disruption of salt bridges and hydrophobic interactions, thereby resulting in conformational and structural changes of proteins (Martin et al., 2002). Structural transitions are accompanied by large hydration changes (Masson, 1992). Hydration changes are the major source of volume decreases associated with dissociation and unfolding of proteins (Masson, 1992). Hydrophobic interactions in protein can be either disrupted or stabilized according to the magnitude of the applied pressure (Johnson et al., 1992).

High pressure affects the interaction of components by changing the distance between them. It has been hypothesized that HHP does not affect covalent bonds because that the length of covalent bonds is already limited by the Born repulsion that naturally exists among atoms that are close to one another (Barciszewski et al., 2002). Thus small molecules such as vitamins, color, and flavor compounds will remain unaffected after HHP treatment (Martin et al., 2002). This non-disruption of covalent bonds ensures the retention of nutrients and therefore leads to a more natural and “better” quality product compared to products obtained from thermal treatment (Tedford et al., 1998).

The functional properties of biological molecules are usually dependent on conformation and conformational changes. Any modification of the water shell around protein will alter the
spatial distribution of charges that could play a significant role in specific evolution of the protein conformation under high pressures (Dufour et al., 1994). The interactions between solvent and solute molecules and inter- and intramolecular interactions of the solute are influenced when subjected to pressure (Palou et al., 1999). Therefore, either beneficial or detrimental changes can be produced as a result of a high-pressure treatment (Johnson, 1995).

**Potential functionality improvement of high hydrostatic pressure on whey protein concentrate**

Studies have been done to understand the effect of HHP on some of the functional properties of whey proteins, such as gel formation (Famelart et al., 1998), emulsifying capacity (Galazka et al., 1995) and foamability (İbanoglu and Karatas, 2001). However, little work has been done regarding the effects of high pressure on WPC or whey protein-flavor binding. Changes in the surface hydrophobicity and aggregation effects have been observed with β-LG subsequent to treatments between 200 and 600 MPa (Nakamura et al., 1993; Dumay et al., 1994). Pressure-induced changes in protein molecules tend, in general, to increase the area accessible to the solvent and, as a consequence, alter surface properties (Nakamura et al., 1993; Dumay et al., 1994). Desirable functional characteristics of protein, such as high surface hydrophobicity, which facilitates the formation of stable foams, imply more binding of flavor components by hydrophobic interaction, compared to proteins of lower surface hydrophobicity (Fischer and Widder, 1997).

High pressure induces β-LG into the MG state (Yang et al., 2001). Semisotinov et al. (1991) reported that proteins in the MG state (bovine α-lactalbumin, bovine carbonic anhydrase and *Staphylococcus aureus* β-lactamase) exhibit high affinity for the hydrophobic probe 1-
anilino-naphthalene-8-sulfonate (ANS). Yang et al. (2001) reported a 3-fold increase in the ANS fluorescence intensity, indicating enhanced aromatic hydrophobic binding. The result suggests that HHP may help improve the functional properties of proteins, such as flavor binding and release. β-LG in the MG state induced by HHP exhibited a significant decrease in affinity for retinol and a significant increase in affinity for \textit{cis}-parinaric acid (CPA) and ANS compared to native β-LG (Yang et al., 2003).

The MG state of α-LA has become a paradigm for evaluating the properties of stable partially folded proteins (Kuwajima, 1996; Kuwajima et al., 1989; Ptitsyn, 1995). α-LA forms a MG state under a variety of conditions, including at low pH, at low salt concentrations in the absence of Ca\textsuperscript{2+} (neutral pH) (Dolgikh et al., 1981; Kronman et al., 1965; Kuwajima et al., 1976), or after pressure treatment (Tanaka et al., 1996; Chang et al., 2000; Lassalle et al., 2003). However, the stability of α-LA towards high pressure is greater than that of β-LG, probably due to the lack of free sulfhydryl groups in α-LA (Tanaka et al., 1996; Chang et al., 2000).

The secondary structure of BSA, the third major whey protein, is very stable under pressure as well. The stability of BSA was shown through specific rotation, fluorescence and electrophoresis (Hayakawa et al., 1992; Cheftel and Dumay, 1996). The resistance of BSA against high pressure may be due to the 17 intramolecular disulfide bonds of the molecule (Lopez-Fandino et al., 1996).

The presence of multiple proteins in WPC has significant influence on the behavior of whey proteins during high pressure and heat treatment. de Wit and Klarenbeek (1984) reported that although α-LA is the whey protein with the lowest denaturation temperature, and appears to be (at pH 6.0) most thermostable against protein aggregation because of its high capability of renaturation on cooling. This renaturation effect is not observed in WPC, which might be due to heat-induced interactions with β-LG and BSA. In the mixture of α-LA and β-LG, during HHP
treatment (1000 MPa, 30 min) β-LG promoted the oligomerization of α-LA (Jegouic et al., 1997). In this case, mixing and denaturation of β-LG with α-LA resulted in formation of a large heterogeneous population of oligomers including β-LG or α-LA/β-LG dimers (Jegouic et al., 1997).

Prediction of protein functionality on the basis of molecular structure for commercial WPC that contain a mixture of proteins is more difficult than prediction for well-defined, individual globular proteins (Patel and Fry, 1985). Each of the whey proteins, that is, α-LA, β-LG, BSA, Ig, and the minor whey proteins, as well as residual lactose and lipids, affect WPC functionality in different ways. Commercial WPC contains mixtures of proteins in varying ratios that have undergone varying degrees of heat denaturation and aggregation (Morr, 1982; Morr, 1989). It is extremely difficult to predict the functionality of such complex protein systems on the basis of simple solubility and functionality test results (Patel and Fry, 1985), and more studies are needed to understand the structure-functionality relationship of WPC.

**Objectives**

This research is based on the following hypotheses. High hydrostatic pressure (HHP) treatments cause conformational changes and aggregation of the major whey proteins, β-LG, α-LA, and BSA. After HHP treatments WPC exhibit greater hydrophobicity than the untreated WPC. Increased WPC hydrophobicity improves functional properties of whey proteins such as flavor-binding, with increases in the number of the binding sites and decreases in the apparent dissociation constants (increases in the binding affinity) for flavor compounds. HHP treated WPC can carry flavor compounds in formulated lowfat or nonfat food products and improve the
flavor profile during consumption. HHP treated WPC may be used as a food ingredient to improve the sensory quality of formulated reduced fat foods and promote the utilization of WPC.

The objectives of this research are to: (1) investigate structural properties of WPC affected by HHP, including protein solubility, protein denaturation and aggregation; (2) study hydrophobicity of WPC affected by HHP with selected aromatic and aliphatic hydrophobic probes; (3) study flavor binding functionality of WPC affected by HHP with selected aromatic and aliphatic flavor compounds.
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CHAPTER THREE

Effects of Heat and High Hydrostatic Pressure on Protein Solubility and Protein Composition of Whey Protein Concentrate

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ABSTRACT

After mild heat treatment (50°C, 30 min) or high hydrostatic pressure (HHP) treatment (600 MPa, 50°C, 30 min), whey protein concentrate (WPC) solutions in water and sodium phosphate buffer (0.01 M, pH 7.0) (0.02%, 0.2% or 2.0%) were studied with protein solubility assays (pH 4.6 and 7.0), size-exclusion chromatography, and polyacrylamide gel electrophoresis. No significant changes in protein solubility of WPC at pH 4.6 and 7.0 were observed after mild heat treatment. Mild heat treatment of WPC did not result in significant protein denaturation or aggregation. After HHP treatments, there were decreases in protein solubility at pH 4.6, and increases in aggregation and denaturation of whey proteins, especially at high WPC concentrations. During the come-up time of HHP treatment, dissociation of aggregates and formation of dimers of β-lactoglobulin (β-LG) were observed. With increasing HHP treatment time the amount of monomers of β-LG, α-lactalbumin (α-LA), and bovine serum albumin (BSA) decreased, and aggregates were formed. Overall, these results suggest that protein solubility of WPC, denaturation and aggregation of whey proteins are dependent on solution concentration and HHP treatment condition.

Keywords: whey protein concentrate; high hydrostatic pressure; solubility; protein composition; aggregation
INTRODUCTION

Whey and whey products have been used successfully in the food industry for years. Cost efficiency and quality improvement are key drivers in using whey products (Morr and Ha, 1993). The nutritional value of whey products is also an important reason why an increasing number of manufacturers worldwide include whey products in their formulations. Whey products provide solubility and viscosity, form gels, emulsify, facilitate whipping, foaming and aeration, enhance color, flavor and texture, and offer numerous nutritional advantages (U. S. Dairy Export Council, 1999).

One of the most important physicochemical and functional properties of whey proteins is their solubility over a wide range of protein concentration, pH, temperature, water activity, and ionic conditions (Morr, 1989). Native whey proteins remain soluble at their isoelectric point (pI), that is, throughout the pH range of 4 to 5; however, heat-induced denaturation renders whey proteins insoluble in this pH range (Morr and Ha, 1993). Thus, whey protein concentrate (WPC) protein solubility at pH 4.6 is useful for estimating protein denaturation (Morr and Foegeding, 1990).

Protein solubility depends on various endogenous physicochemical properties, including molecular weight, secondary and tertiary structure, hydrophobicity and hydrophilicity, and electrostatic charge (Morr and Ha, 1993). The solubility of WPC is markedly affected by solution conditions such as pH, temperature, and ion composition (Kinsella, 1984). Monovalent mineral ions and pH values away from pI enhance protein solubility by weakening intramolecular and intermolecular electrostatic interactions between the ionizing carboxyl and $\epsilon$-amino groups (Morr and Ha, 1993). Thus, pH and ionic composition of the solvent must be specified when reporting protein solubility (Morr and Ha, 1993).
Numerous references in the literature document relationships between composition and functionality of WPC solutions (Schmidt et al., 1984; Liao and Mangino, 1987). In practice, functionality is influenced by a number of compositional factors that affect the physicochemical properties of the proteins, that is, total and individual protein composition, pH, ionic strength, concentration of Ca\(^{+2}\) and other individual ions, lipids, lactose, chemical emulsifiers, and reducing and oxidizing chemicals (Morr and Ha, 1993).

There are wide differences in the gross composition of commercial WPC products manufactured worldwide (Morr, 1989; Morr and Foegeding, 1990; de Wit et al., 1986). Most of these compositional differences relate to the use of different processing conditions and technologies for manufacturing whey and WPC (Morr, 1989; Schmidt et al., 1984). Most WPC products are manufactured by ultrafiltration (UF) and diafiltration (DF) technologies (Morr and Foegeding, 1990). Processing modifications with respect to whey manufacture, whey pretreatment, degree of fractionation by UF and DF, and spray-drying conditions would be expected to alter the composition of WPC (Morr and Ha, 1993). Therefore, consistency in the composition of WPC is an important factor to predict WPC functionality and to manufacture WPC products with optimum functionality for each product application (Morr and Ha, 1993).

Traditional food processing methods rely on high temperatures as a way to ensure prolonged shelf life and food safety. However, the use of high temperatures results in some detrimental changes in nutritional and organoleptic attributes in the processed products (Martin et al., 2002). High hydrostatic pressure (HHP) presents unique advantages over conventional thermal processing for food product modifications, including application at low temperatures, which permits the retention of food quality attributes (Knorr, 1995a; Knorr, 1995b; Cheftel, 1992). The pressure range currently being investigated for use in food processing is roughly 100
MPa to 900 MPa, with pressures used in commercial systems between 400 and 700 MPa (Martin et al., 2002). The areas where high pressure offers potential are: the reduction of microbial numbers, the control of enzyme reactions, alteration to the conformation of biopolymers, and the control of phase transformations (Palou et al, 1999).

Pressure acts as a physicochemical parameter that alters the balance of intramolecular and solvent-protein interactions (Pitta et al., 1996). Pressure-induced protein unfolding is complex and can result in disruption of both internal hydrophobic interactions and salt bridges. Low protein concentrations and pressures (up to 200-300 MPa) usually result in reversible pressure-induced denaturation. Higher pressures have irreversible and extensive effects on proteins, including denaturation due to unfolding of monomers, aggregation and formation of gel structures (Pitta et al., 1996).

The formation of charged species in aqueous media is favored by high pressure because the electrostriction of water decreases the molar volume of total water (Kunugi, 1993). This means that an increase in pressure will weaken the electrostatic interactions between ions in pair, since formation of an interacting pair of charges will diminish the total net charge and liberate the hydrating water molecules back into the normal state in the bulk phase. On the other hand, hydrogen bond formation will be slightly strengthened by increases in pressure because the decreases in the interatomic distance lead to a smaller molecular size. Hydrophobic interactions have much more complicated characteristics. Interactions between aromatic compounds generally have negative reaction volumes and thus to be strengthened at higher pressure (Kunugi, 1993).

The major protein components of whey include β-LG (50%), α-lactalbumin (α-LA) (20%) and bovine serum albumin (BSA) (5%). Among the three major whey proteins, most
research has been focused on β–LG. Funtanberger et al. (1995) reported that high pressure processing (450 MPa, 25°C, 15 min, pH 7.0) induced partial unfolding and aggregation of β-LG isolate. Aggregation of β–LG was more extensive in pressure-resistant buffers than in phosphate buffer or in water (Funtanberger et al., 1995). Electrophoretic patterns also revealed the progressive formation of dimers to hexamers and of higher polymers of β–LG as a function of the type and molarity of buffer and of the pressure level (Funtanberger et al., 1995).

The stability of α-LA and BSA towards high pressure is greater than that of β-LG (Tanaka et al., 1996; Chang et al., 2000; Hayakawa et al., 1992). Baric oligomerization of α-LA alone is not observed in the absence of low molecular weight reducing thiols, even at high pressures applied for extended periods of time (Jegouic et al., 1996). This resistance to oligomerization is due to the fact that α-LA has no free sulphydryl groups capable of inducing sulphydryl disulfide exchange after unfolding of the protein by high pressure (Rao and Brew, 1989).

The secondary structure of BSA, the third major whey protein, is very stable under pressure as well. The stability of BSA was shown through specific rotation, fluorescence and electrophoresis (Hayakawa et al., 1992; Cheftel and Dumay, 1996). The resistance of BSA against high pressure may be due to the 17 intramolecular disulfide bonds of the molecule (Lopez-Fandino et al., 1996).

The presence of multiple proteins in WPC has significant influence on the behavior of whey proteins during high pressure and heat treatment. de Wit and Klarenbeek (1984) reported that although α-LA is the whey protein with the lowest denaturation temperature, it is most thermostable against protein aggregation (at pH 6.0) because of its high capability of renaturation on cooling. This renaturation effect is not observed in WPC, which might be due to heat-induced
interactions with β-LG and BSA. In the mixture of α-LA and β-LG, during HHP treatment (1000 MPa, 30 min) β-LG promoted the oligomerization of α-LA (Jegouic et al., 1997). In this case, mixing and denaturation of β-LG with α-LA resulted in formation of a large heterogeneous population of oligomers including β-LG or α-LA/β-LG dimers. The upper limit of molecular weights of these oligomers can be estimated as ~7 MDa. These oligomers are presumably composed of up to several hundreds of β-LG and α-LA molecules (Jegouic et al., 1997).

HHP induces β-LG into the molten globule state (Yang et al., 2001). Yang et al. (2003) reported a significant increase in the binding affinity of β-LG for 1-anilino-naphthalene-8-sulfonate (ANS) and cis-parinaric acid (CPA) after HHP treatment. However, commercial WPC contains mixtures of proteins in varying ratios, and each of the whey proteins affect WPC functionality in different ways. Given the information presented above, HHP treated WPC would be a good candidate for testing the practical utility of the application of HHP to modify the functional properties of WPC. Here, we investigate the effects of HHP on some structural and functional properties of WPC, including solubility, protein composition, hydrophobicity and flavor-binding properties.

MATERIALS & METHODS

Materials

RT-80 Grade A whey protein concentrate (WPC RT-80) was provided by Main Street Ingredients (La Crosse, WI). WPC RT-80 with the same lot number was used throughout the experiments. The product contained 84.9% protein, 3.9% fat, 3.4% ash, 3.5% lactose, and 3.7% moisture. The pH of a 2% solution at 20°C was 6.4. Standard proteins (β-LG, α-LA, BSA) were
obtained from Sigma Chemical Co. (St. Louis, MO) All of the chemicals used were of analytical grade obtained from Fisher Chemicals (Fairlawn, NJ) or unless otherwise specified.

**Heat treatment**

WPC solutions, at the protein concentration of 0.02%, 0.2% and 2.0% (w/v) in sodium phosphate buffer (0.01 M, pH 7.0) and water, were heated at 50°C for 30 min.

**High hydrostatic pressure (HHP) Treatment**

WPC solutions, at the protein concentrations of 0.02%, 0.2% and 2.0% (w/v) in sodium phosphate buffer (0.01 M, pH 7.0) and water, were treated with HHP of 600 MPa at 50°C for holding times of 0 (come-up time), 2.5, 5, 7.5, 10, 15, or 30 min. The come-up time (4.05 min) is the compression time required to reach a pressure of 600 MPa. After exposure to high pressure, WPC solutions were studied immediately or stored at 4°C for less than one month.

**Protein solubility at pH 4.6 and pH 7.0**

Protein solubility at pH 4.6 has been used as an index of the extent of whey protein denaturation. Solutions of WPC were adjusted to pH 4.6 with HCl and centrifuged at 1500 g for 15 min. The amount of proteins remaining in the total solutions and supernatant were determined by the Lowry method (Lowry et al., 1951). In this work, protein solubility at pH 4.6 is expressed as a percentage of the total protein content of the dispersion before centrifugation (Lee at al., 1992).

\[
PS = \left( \frac{\% \text{ protein, supernatant}}{\% \text{ protein, total}} \right) \times 100
\]

Protein solubility at pH 7.0 was also measured to give an indication of solubility of heated and HHP treated WPC for neutral pH food applications. The procedure was essentially similar to that described above for pH 4.6 solubility. Each analysis was performed in triplicate.
Size exclusion chromatography

The supernatants obtained from the untreated, heated, and HHP treated WPC solutions were filtered through a polyvinylidene difluoride (PVDF) membrane (pore size 0.45 µm) and fractionated by size exclusion chromatography (SEC) on a Protein-Pack SW 300 Glass column (8 × 300 mm, from Waters Corporation). The elution buffer was composed of 0.05 M sodium phosphate (pH 7.0). The flow rate was 0.5 ml/min, and the absorbance of the eluate was monitored by a Waters 440 UV/VIS detector at 280 nm.

Individual whey proteins in the chromatogram were identified by means of a calibration curve with the logarithm of the molecular weight of standards as a function of the retention time. Different standards of lyophilized proteins were used: α-lactalbumin (α-LA, 14 kDa, L-5385), β-lactoglobulin (β-LG, dimers of variants A and B, 37.2 kDa, L-2506), bovine serum albumin (BSA, 66 kDa, A-2153) and bovine immunoglobulin G (IgG, 152 kDa, I-9640). Under the process conditions studied, linear separation with high resolution was possible for proteins with a molecular weight between 14 and 66 kDa. The relationship between the molecular weight ($M_w$, in Da) of the protein and the retention time ($t_R$, in min) within this Mw range was calculated as ($r^2 = 0.96$):

$$\log (M_w) = -0.159 \, t_R + 7.688$$

For β-LG and α-LA, quantitative measurements were obtained by SEC using regression lines in the concentration range 0-10 g/L for β-LG ($r^2 = 0.99$), and in the concentration range 0-5 g/L for α-LA ($r^2 = 0.99$). Each SEC analysis was performed in triplicate.

Polyacrylamide gel electrophoresis

The WPC samples were analyzed using the Mini-Protean Π Dual Slab Cell (Bio-Rad Laboratories, Hercules, CA). Polyacrylamide gel electrophoresis (4-20%) in the presence of
sodium dodecyl sulfate (SDS-PAGE), with or without β-mercaptoethanol (β-ME), was used according to instruction manual of Ready Gel Precast Gels (catalog number 161-0993, Bio-Rad Laboratories, Hercules, CA).

One milliliter of supernatant, obtained from the untreated, heated and pressurized WPC solutions, followed by centrifugation at 1500g for 15 min, was diluted with 3 ml of 62.5 mM Tris-HCl (pH 6.8), containing 25% glycerol, 0.01% bromphenol blue, 10% SDS, and/or 5% β-ME. Prior to analysis, solutions were heated for 5 min in a water bath at 100°C followed by cooling to room temperature with running tap water. Electrophoresis was run at ambient temperature for 35 min at 200 V. The gels were stained with a Coomassie blue solution containing 40% methanol, 10% acetic acid, and 0.1% Coomassie Blue R-250, destained with methanol/acetic acids solution, and preserved with a glycerol solution to prevent drying and deterioration. Each sample was analyzed in triplicate. Prestained SDS-PAGE standards (catalog number 1610318, Bio-Rad Laboratories, Hercules, CA) were used to calibrate the gels. The protein standards included myosin (203 kDa), β-galactosidase (120 kDa), bovine serum albumin (90.0 kDa), ovalbumin (51.7 kDa), carbonic anhydrase (34.1 kDa), soybean trypsin inhibitor (28.0 kDa), lysozyme (20.0 kDa), and aprotinin (6.4 kDa).

Statistical analysis

All experiments and analyses were done in triplicate. The analysis of variance test for significant effects of treatments and assay samples were determined using the General Linear Model procedure (PROC GLM) in SAS. Main effect differences were considered significant at the p < 0.05 level. Means separations were determined by Fisher’s Least Significant (LSD) for multiple comparisons (SAS Institute, Inc., 1993).
RESULTS AND DISCUSSION

Protein solubility at pH 4.6

Since whey proteins are generally recognized as being the nitrogenous fraction remaining soluble in the supernatant at pH 4.6 after precipitation of casein, the loss of solubility at this pH is commonly used to assess the extent of protein denaturation (Li-Chan, 1983; de Wit and Klarenbeek, 1984; Funtenberger et al., 1995). This criterion was used in the present work.

Table 1 shows that there were no significant changes in protein solubility of WPC at pH 4.6 after mild heat treatment. However, decreases in protein solubility of WPC at pH 4.6 were observed, especially at high WPC concentrations in sodium phosphate buffer. HHP treatments of 2.0% WPC solutions in sodium phosphate buffer resulted in complete loss of protein solubility of whey proteins at pH 4.6. The loss of protein solubility at pH 4.6 of WPC solutions after pressurization may be due to the formation of insoluble aggregates and denaturation of whey proteins.

Protein solubility at pH 7.0

The ability of a protein to remain soluble at pH 7.0 is important for the utilization in neutral pH food applications. There were no significant changes in protein solubility of WPC at pH 7.0 after mild heat treatment (Table 2). HHP treatment of 0.02% and 0.2% WPC solutions did not show significant effects on the protein solubility at pH 7.0, but decreases in protein solubility were observed after HHP treatment of 2% WPC solution in water and in buffer. WPC exhibited higher protein solubility in sodium phosphate buffer than in water because monovalent mineral ions enhance protein solubility by weakening intramolecular and intermolecular electrostatic interactions between the ionizing carboxyl and ε-amino groups (Morr and Ha, 1993). The higher solubility at pH 7.0 in comparison with pH 4.6 is due to a lower degree of
cross-links at pH 7.0 due to repulsion between negatively charged carboxyl groups which reduces protein aggregation (Cheftel et al., 1985; Camp et al., 1997).

**Size exclusion chromatography**

The SEC chromatograms of WPC samples were characterized by three protein peaks (Figures 1 through 8). On the basis of whey protein standards, peaks with retention times of $22.37 \pm 0.03$ and $19.56 \pm 0.02$ min were characterized as the $\alpha$-LA monomer with a molecular weight of 14 kDa, and as the $\beta$-LG dimer with a molecular weight of 36.5 kDa, respectively. Also, IgG and BSA were present in a protein fraction with retention time $16.98 \pm 0.01$ min, which corresponds to a molecular weight range 66.3-152 kDa.

Information on the pressure sensitivity of individual whey proteins may be gathered by the use of size exclusion chromatography. Based on Figures 1 through 8, mild heat treatments of WPC did not result in significant protein denaturation or aggregation at either pH 7.0 or pH 4.6, which is consistent with the results from the solubility assay. Increases in the amount of aggregates and decreases in the amounts of individual whey proteins were observed, especially at high WPC concentrations (Figures 1 through 8). $\beta$-LG is more sensitive to HHP treatments than $\alpha$-LA (Natamura et al., 1993), and a significant reduction in the amount of $\beta$-LG occurred after pressurization at pH 7.0. Thirty minutes of HHP treatment of 0.2% WPC solution in water resulted in 67% reduction in the amount of $\beta$-LG (pH 7.0), with no effects on $\alpha$-LA. At the same time, the amount of aggregates tripled. When WPC solution concentration increased to 2.0%, 30 min of HHP treatment resulted in the disappearance of $\beta$-LG peak (pH 7.0), 71% reduction in the amount of $\alpha$-LA, and a 4.5-fold increase in the amount of aggregates compared to the untreated WPC. HHP treatments of WPC solutions in sodium phosphate buffer showed similar patterns at pH 7.0.
When HHP treated WPC solutions (pH 7.0) were assayed at pH 4.6, fewer aggregates and fewer individual whey proteins were observed in the chromatographs than in chromatographs at pH 7.0. Thus, some of the aggregates formed during pressurization at pH 7.0 were insoluble at pH 4.6.

**Polyacrylamide gel electrophoresis**

The SDS gels exhibited four major bands for untreated WPC (Figure 9): monomers of α-LA and β-LG (Region I), BSA (Band III), and large aggregates (Region IV) (with ~200 kDa molecular weight). During the come-up time for HHP treatment, dissociation of aggregates and formation of dimers were observed. With increasing treatment time, the amount of monomeric β-LG, α-LA, and BSA decreased, and aggregates of various molecular weights formed. Region II, containing β-LG and α-LA dimers, was not well separated. Region IV contained proteins with apparent molecular weights larger than 60 kDa, corresponding with trimers and tetramers of whey proteins. The absence of single bands for dimers and other oligomers indicated a considerable extent of inter- and intramolecular disulphide bond diversity (Manderson et al., 1998).

Instead of large aggregates in untreated WPC solutions, we observed intermediate sized aggregates in HHP treated WPC (Region IV), with molecular weights from approximately 70 to 200 kDa. Due to the presence of free sulfhydryl groups in aggregates, aggregation is not limited to the formation of linear aggregates; branched aggregates can be formed (Schokker et al., 1999).

WPC samples were also analyzed in the presence of β-ME, which allowed evaluation of the contribution of disulfide-stabilized aggregates in the soluble protein fraction of both untreated and pressurized WPC solutions (Figure 10). Most aggregates observed in Figure 9 dissociated into the monomer form of α-LA, β-LG (Region I) and BSA (band III) in the presence
of β-ME, which indicated that these aggregates were stabilized by disulfide bonds. Dimers of β-LG disappeared in the presence of β-ME, while dimeric α-LA was still observed. Havea et al. (2000) also reported the presence of dimeric α-LA in the presence of β-ME, which may indicate the presence of a small quantity of nonreducible dimers of α-LA.

Although effects of high pressure on the aggregation and changes of whey protein concentrate have not been extensively studied, similar gel electrophoresis patterns of HHP treated WPC to heated WPC may suggest a similar mechanism. Heavea et al. (2001) observed that the 2D-PAGE patterns of a heated mixture of the three whey proteins (β-LG, α-LA and BSA) clearly demonstrated the presence of various disulphide homopolymers of each protein as well as various adducts of the three proteins. Initial aggregates are formed predominately by polymerization of BSA with itself while the aggregates involving β-LG and α-LA are generated at a later stage and appear to be in proportion to the quantity of monomeric protein present in the unheated sample. Because of the differences in thermal stability of the three proteins, during the initial stages of heating, BSA molecules will begin to unfold and aggregate (mainly via inter-molecular sulfhydryl-disulphide exchange, and to a lesser extent, non-covalent interactions) before β-LG. The exposed sulfhydryl groups of BSA molecules/aggregates could also react via sulfhydryl-disulphide interchange with one of the disulphide bonds of α-LA. Consequently, α-LA dimers and α-LA/BSA adducts could be generated. At later stages of heating, β-LG will unfold to expose a sulfhydryl group, which results in dimers, trimers and higher molecular weight polymers of β-LG or α-LA, as well as mixed aggregates of the two proteins.

Overall, HHP treatment of 0.02% and 0.2% WPC solutions did not show significant effects on the protein solubility at pH 7.0, but decreases in protein solubility were observed after HHP treatment of 2% WPC solution in water and in buffer. HHP treatments of WPC resulted in
decreases in protein solubility at pH 4.6 and increases in aggregation and denaturation of whey proteins, especially at high WPC concentrations. Changes that occur during HHP treatments may affect the functional properties of WPC, such as hydrophobicity and flavor binding.

CONCLUSIONS

Mild heat treatment did not result in significant changes in protein solubility at pH 4.6 and 7.0. HHP treatments of WPC resulted in decreases in protein solubility at pH 4.6 and increases in aggregation and denaturation of whey proteins, especially at high WPC concentrations. During the come-up time for the HHP treatment, dissociation of aggregates and formation of dimers were observed. With increasing treatment time, the amount of monomers of β-LG, α-LA, and BSA decreased, and aggregates of various molecular weights were formed. Overall, these results suggest that protein solubility, denaturation and aggregation of WPC are dependent on solution concentration and HHP treatment condition.

The changes occurring during the HHP treatments may affect the functional properties of WPC, such as hydrophobicity and flavor binding. Specific functional characteristics may be achieved by careful manipulation the processing conditions of WPC solutions, such as WPC concentration and HHP treatment conditions.

ABBREVIATIONS USED

β-LG, β-lactoglobulin; α-LA, α-lactalbumin; BSA, bovine serum albumin; HHP, high hydrostatic pressure; pI, isoelectric point; UF, ultrafiltration; DF, diafiltration; WPC, whey protein concentrate; WPI, whey protein isolate; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; β-mercaptoethanol, β-ME.
REFERENCES


Table 1. Effects of heat (50°C, 30 min) and HHP treatments on pH 4.6 protein solubility of WPC in water and 0.01 M (pH7.0) sodium phosphate buffer. Pressure processing was performed at 600 MPa and 50°C for various holding time: 5 min (HHP 5); 15min (HHP 15); 30 min (HHP 30).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Protein Solubility (%) in H$_2$O$^a$</th>
<th>Protein Solubility (%) in buffer$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.02 %</td>
<td>0.2%</td>
</tr>
<tr>
<td>WPC</td>
<td>82 ± 1</td>
<td>71 ± 2</td>
</tr>
<tr>
<td>Heated WPC</td>
<td>79 ± 1</td>
<td>68 ± 1</td>
</tr>
<tr>
<td>HHP 5 WPC</td>
<td>79 ± 0</td>
<td>62 ± 1</td>
</tr>
<tr>
<td>HHP 15 WPC</td>
<td>74 ± 1</td>
<td>51 ± 1</td>
</tr>
<tr>
<td>HHP 30 WPC</td>
<td>74 ± 1</td>
<td>48 ± 1</td>
</tr>
</tbody>
</table>

$^a$“±” values refer to 95% confidence limits

Table 2. Effects of heat (50°C, 30 min) and HHP treatments on pH 7.0 protein solubility of WPC in water and 0.01 M (pH7.0) sodium phosphate buffer. Pressure processing was performed at 600 MPa and 50°C for various holding time: 5 min (HHP 5); 15min (HHP 15); 30 min (HHP 30).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Protein Solubility (%) in H$_2$O$^a$</th>
<th>Protein Solubility (%) in buffer$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.02 %</td>
<td>0.2%</td>
</tr>
<tr>
<td>WPC</td>
<td>93 ± 1</td>
<td>83 ± 1</td>
</tr>
<tr>
<td>Heated WPC</td>
<td>93 ± 0</td>
<td>84 ± 1</td>
</tr>
<tr>
<td>HHP 5 WPC</td>
<td>89 ± 1</td>
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</tr>
<tr>
<td>HHP 15 WPC</td>
<td>88 ± 1</td>
<td>85 ± 1</td>
</tr>
<tr>
<td>HHP 30 WPC</td>
<td>85 ± 1</td>
<td>83 ± 2</td>
</tr>
</tbody>
</table>

$^a$“±” values refer to 95% confidence limits
Figure 1. SEC chromatogram of soluble proteins obtained from untreated, thermal treated, and HHP treated WPC solutions (0.2%, pH 7.0) in H₂O. From top to bottom: Untreated WPC, Thermal treated WPC (50°C, 30 min), HHP treated WPC (600 MPa, 50°C, 30 min). 1 = α-LA; 2 = β-LG; 3 = aggregates.
Figure 2. SEC chromatogram of soluble proteins obtained from untreated, thermal treated, and HHP treated WPC solutions (2.0%, pH 7.0) in H2O. From top to bottom: Untreated WPC, Thermal treated WPC (50°C, 30 min), HHP treated WPC (600 MPa, 50°C, 30 min). 1 = α-LA; 2 = β-LG; 3 = aggregates.
Figure 3. SEC chromatogram of soluble proteins obtained from untreated, thermal treated, and HHP treated WPC solutions (0.2%, pH 7.0) in 0.01 M sodium phosphate buffer. From top to bottom: Untreated WPC, Thermal treated WPC (50°C, 30 min), HHP treated WPC (600 MPa, 50°C, 30 min). 1 = α-LA; 2 = β-LG; 3 = aggregates.
Figure 4. SEC chromatogram of soluble proteins obtained from untreated, thermal treated, and HHP treated WPC solutions (2.0%, pH 7.0) in 0.01 M sodium phosphate buffer. From top to bottom: Untreated WPC, Thermal treated WPC (50°C, 30 min), HHP treated WPC (600 MPa, 50°C, 30 min). 1 = α-LA; 2 = β-LG; 3 = aggregates.
Figure 5. SEC chromatogram of soluble proteins obtained from untreated, thermal treated, and HHP treated solutions (0.2%, pH 4.6) in H$_2$O. From top to bottom: Untreated WPC, Thermal treated WPC (50°C, 30 min), HHP treated WPC (600 MPa, 50°C, 30 min). 1 = $\alpha$-LA; 2 = $\beta$-LG; 3 = aggregates.
Figure 6. SEC chromatogram of soluble proteins obtained from untreated, thermal treated, and HHP treated WPC solutions (2.0%, pH 4.6) in H₂O. From top to bottom: Untreated WPC, Thermal treated WPC (50°C, 30 min), HHP treated WPC (600 MPa, 50°C, 30 min). 1 = α-LA; 2 = β-LG; 3 = aggregates.
Figure 7. SEC chromatogram of soluble proteins obtained from untreated, thermal treated, and HHP treated WPC solutions (0.2%, pH 4.6) in 0.01 M sodium phosphate buffer. From top to bottom: Untreated WPC, Thermal treated WPC (50°C, 30 min), HHP treated WPC (600 MPa, 50°C, 30 min). 1 = α-LA; 2 = β-LG; 3 = aggregates.
Figure 8. SEC chromatogram of soluble proteins obtained from untreated, thermal treated, and HHP treated WPC solutions (2.0%, pH 4.6) in 0.01 M sodium phosphate buffer. From top to bottom: Untreated WPC, Thermal treated WPC (50°C, 30 min), HHP treated WPC (600 MPa, 50°C, 30 min). 1 = α-LA; 2 = β-LG; 3 = aggregates.
Figure 9. SDS-PAGE (without β-mercaptoethanol) patterns of untreated (N) and HHP treated (600 MPa, 50°C, for various holding time: 0, 2.5, 5, 7.5, 10, 15, 30 min) 0.2% WPC solutions. I: SDS-monomeric α-LA and β-LG; II: dimeric and trimeric α-LA and β-LG; III: SDS-monomeric BSA; IV: large aggregates.

Figure 10. SDS-PAGE (with β-mercaptoethanol) patterns of untreated (N) and HHP treated (600 MPa, 50°C, for various holding time: 0, 2.5, 5, 7.5, 10, 15, 30 min) 0.2% WPC solutions. I: SDS-monomeric α-LA and β-LG; II: dimeric α-LA; III: SDS-monomeric BSA; IV: adducts.
CHAPTER FOUR

Effects of High Hydrostatic Pressure on Hydrophobicity of Whey Protein Concentrate

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ABSTRACT

The effects of high hydrostatic pressure (HHP) treatment (600 MPa, 50°C, 0 to 30 min) on intrinsic fluorescence of whey protein concentrate (WPC) and the binding properties of aromatic 1-anilino-naphthalene-8-sulfonate (ANS) and aliphatic cis-parinaric acid (CPA) probes were studied. HHP treatment of WPC resulted in an increase in intrinsic tryptophan fluorescence intensity and a 4 nm red shift after 30 min of treatment, which indicated changes in the polarity of tryptophan residues microenvironment of whey proteins from a less polar to a more polar environment. There was an increase in the number of binding sites of WPC for ANS from 0.16 to 1.10 per molecule of protein after HHP treatment for 30 min. No significant changes in the apparent dissociation constant of WPC for ANS were observed after HHP treatment, except for an increase from $1.8 \times 10^{-5}$ M to $3.3 \times 10^{-5}$ M after 30 min of HHP treatment. There were no significant changes in the number of binding sites of WPC for CPA. However, increased binding affinities of WPC for CPA were observed after the come-up time or 10 min of HHP treatment, with a decrease of apparent dissociation constant from $2.2 \times 10^{-7}$ M to $1.1 \times 10^{-7}$ M. The binding sites of WPC may become more accessible to the aliphatic hydrophobic probe CPA after the come-up time or 10 min of HHP treatment. These results indicate that HHP treatment of WPC resulted in increases in the number of binding sites for aromatic hydrophobic probe, while aliphatic hydrophobic binding affinity of WPC is enhanced after come-up time or 10 min of HHP treatment. HHP treatment shows potential for improving functionality of WPC and may provide opportunities for improvement of flavor in reduced fat products.

Keywords: whey protein concentrate; high hydrostatic pressure; hydrophobicity; fluorescence
INTRODUCTION

The impact of hydrophobic interactions of food proteins on their functional properties has received major attention (Burley & Petsko, 1985; Li-Chan & Nakai, 1989; Semisotnov et al., 1991). The functionality of protein molecules depends on hydrophobic, electrostatic, and steric parameters of the protein structure (Nakai, 1983). Nakai (1983) demonstrated an apparent close relationship between surface hydrophobicity, emulsion capacity and emulsion stability of proteins. Increased fat binding capacity was associated with an increase in hydrophobicity of the protein (Voutsinas & Nakai, 1983).

A number of methods have been developed for determining the hydrophobicity of proteins. These methods include: (1) hydrophobic probes employing 1-anilinonaphthalene-8-sulfone (ANS) and cis-parinaric acid (CPA) (Kato & Nakai, 1980; Matulis & Lovrien, 1998), (2) hydrocarbon binding capacity (Mangino et al., 1985), (3) hydrophobic chromatography (Ingraham et al., 1985), (4) hydrophobic partitioning, (5) triglyceride binding capacity (Voutsinas & Nakai, 1983), and (6) sodium docecyl sulfate (SDS) binding (Kato et al., 1984). The spectroscopic method using fluorescent probes is the most direct and efficient method to determine hydrophobicity (Kato & Nakai, 1980), because hydrophobic fluorescent probes ANS and CPA exhibit great quantum yields of fluorescence in nonpolar environments compared to aqueous solutions. The ANS and CPA probes are widely used to assay hydrophobicity of food proteins (Nakai, 1983; Li-Chan & Nakai, 1989). ANS is more sensitive to aromatic hydrophobicity, and CPA is more sensitive to aliphatic hydrophobicity (Nakai, 1983).

X-ray analyses revealed that there is some void space in globular proteins that permit considerable internal motion in response to thermal or high hydrostatic pressure forces (Kundrot & Richards, 1987). Flexibility and compressibility of globular proteins are linked to the
fluctuation of volume or internal cavities (Pain, 1987; Vihinen, 1987), and some functional properties such as foaming capacity (Nakai, 1983; Townsend & Nakai, 1983). Gekko & Yamagami (1991) found that compressibility reflects the structural characteristics of globular proteins. They studied the adiabatic compressibility of 14 egg and milk proteins, including α-lactalbumin (α-LA), β-lactoglobulin (β-LG), and bovine serum albumin (BSA), and found that the protease susceptibility, foaming capacity, and free energy of unfolded proteins are positively correlated to the adiabatic compressibility. Their results indicate that the flexibility of the structure plays an essential role in the conformational stability and functional properties of food proteins (Gekko & Yamagami, 1991).

Changes in the surface hydrophobicity and aggregation effects have been observed with β-LG subsequent to treatments between 200 and 600 MPa. HHP induced partial denaturation of the molecule, resulting in increased hydrophobicity and the formation of protein aggregates (Kunugi, 1993; Pittia et al., 1996; Hummer et al., 1998). Hayakawa et al. (1992) observed a remarkable reduction in α–helix content of β-LG as a consequence of treatment at 1000 MPa for 10 min. Fluorometry studies of ANS bound to β-LG at pH 7.0 showed an increase (40%) in the fluorescence intensity after pressurization (Galazkaa et al., 1996), which indicates a significant increase in protein surface hydrophobicity. HHP treatment of β-LG resulted in a reduction in the number of binding sites of retinol and CPA, respectively, indicating that structural changes of β-LG during HHP treatment alter the binding sites for retinol and CPA. The reductions in the number of binding sites and the affinity of β-LG for retinol result from conformational changes by HHP treatment in the calyx or adjacent to the calyx (Yang et al., 2003).

α-LA possesses four tryptophan residues, all of which are in the hydrophobic clusters (Tanaka et al., 1996; Redfield et al., 1999; Lassalle et al., 2003). The pressure-induced change in
the maximum intensity of the intrinsic fluorescence of holo-LA (Ca\(^{2+}\)-bound LA) was very small. The binding of ANS to holo-LA decreased from 0.1 to 100 MPa, but increased greater than 200 MPa (Masson & Cléry, 1996; Tanaka et al., 1996).

High pressure treatment of BSA up to 1000 MPa resulted in slight and gradual decrease in ANS fluorescence intensity and showed a decrease in the protein surface hydrophobicity (41%) after pressure processing at 800 MPa for 20 min (Galazkaa et al., 1996). The loss of surface hydrophobicity could be due to the lower number of hydrophobic groups binding to ANS because of intermolecular interactions (Hayakawa et al., 1992) or conformational changes occurred during pressurization (Galazkaa et al., 1996).

The presence of multiple proteins in WPC has significant influence on the behavior of whey proteins during high pressure and heat treatment (de Wit & Klarenbeek, 1984; Jegouic et al., 1997). Modification of WPC with HHP may enhance or alter the combination of functional characteristics, allowing for development of a variety of protein components with a broad spectrum of functional properties (Dufour & Haertlé, 1991). The objectives of this research were to investigate the effects of HHP on hydrophobicity of WPC.

**MATERIALS & METHODS**

**Materials**

RT-80 Grade A whey protein concentrate (WPC RT-80) was provided by Main Street Ingredients (La Crosse, WI). WPC RT-80 with the same lot number was used throughout the experiments. The product contained 84.9% protein, 3.9% fat, 3.4% ash, 3.5% lactose, and 3.7% moisture (measured by standard proximate analysis procedures). The pH of a 2% solution at 20°C was 6.4. Standard proteins (β-LG, α-LA, BSA) were obtained from Sigma Chemical Co.
(St. Louis, MO). All of the chemicals used were of analytical grade obtained from Fisher Chemicals (Fairlawn, NJ) or unless otherwise specified.

Concentrations of WPC solutions were determined spectrophotometrically by using the molecular absorption coefficients: \( \alpha\text{-LA} : \varepsilon_{278} = 28542, \beta\text{-LG} : \varepsilon_{278} = 17600, \) BSA: \( \varepsilon_{278} = 44488. \) The following molecular absorptions were used to calculate ligand concentration: ANS: \( \varepsilon_{350} = 5000, \) and CPA, \( \varepsilon_{304} = 71400. \)

**High Pressure Treatment**

WPC solutions, at concentrations of 0.2 % in sodium phosphate buffer (0.01 M, pH 7.0), were treated with HHP of 600 MPa at 50°C for holding times of 0 (come-up time), 2.5, 5, 7.5, 10, 15, or 30 min. The come-up time (4.5 min) is the compression time required to reach a pressure of 600 MPa. After exposure to high pressure, WPC solutions were studied immediately or stored at 4°C for less than one month.

**Intrinsic and extrinsic fluorescence spectra**

Conformational changes of WPC solutions were monitored by intrinsic tryptophan and extrinsic fluorescence spectra. Intrinsic fluorescence was assayed using an excitation wavelength of 295 nm (to avoid absorption by the tyrosine residues) and observing an emission wavelength of 350 nm. Extrinsic ANS fluorescence of WPC solutions was assayed using an excitation wavelength of 390 nm and observing emission at a wavelength of 470 nm. Extrinsic CPA fluorescence was assayed using an excitation wavelength of 325 nm and observing emission at a wavelength of 420 nm. For these assays, 36 \( \mu \)l of ANS (5.0 mM in 0.1 M phosphate buffer, pH 7.0) or 20 \( \mu \)l of CPA (2.5 mM in absolute ethanol containing equimolar butylated hydroxytoluene) solution were added to 3 ml of untreated or HHP treated WPC solutions (0.02%). Intrinsic and extrinsic fluorescence were collected with a FluoroMax-3
Spectrofluorometer (Jobin Yvon Inc., Edison, New Jersey), and fluorescence intensity was expressed as arbitrary units (a.u.).

**Fluorescent probe binding study**

Extrinsic aromatic hydrophobic ANS and aliphatic CPA fluorescence probes (Figure 1) are often selected to determine the hydrophobicity of proteins (Nakai, 1983). Due to aromatic structures, ANS probe was used to study aromatic hydrophobic binding. The CPA aliphatic probe was used to study aliphatic hydrophobic binding.

In the binding study, 4 µl of CPA (2.5 mM in absolute ethanol containing 2.5 mM butylated hydroxytoluene) was titrated to the untreated or HHP treated WPC solutions (1 µM) to reach a final concentration at 7 µM for CPA. The hydrophobicity of WPC was assayed as extrinsic CPA fluorescence using an excitation wavelength of 325 nm and observing emission at a wavelength of 420 nm.

CPA binding properties were evaluated with the Cogan method (Cogan et al., 1976). The number of accessible binding sites and apparent dissociation constants of CPA with WPC were calculated with the equation $P_0 \alpha = (1/n)(L_0 \alpha / (1-\alpha)(K'_d / n))$, where $P_0$ is protein concentration, $L_0$ is a given ligand concentration, $n$ is the number of binding sites per molecule of protein, $K'_d$ is the apparent dissociation constant, and $\alpha$ is the fraction of binding sites remaining free, assuming $\alpha = (F_{max} - F) / F_{max}$. $F_{max}$ is defined as the fluorescence intensity when protein molecules are saturated by the ligand.

Since β-LG exhibits only low affinity for ANS, the binding parameters for ANS can not be calculated from Cogan or Scatchard equations (Laligant et al., 1991). As suggested by Laligant et al. (1991), ANS binding parameters were evaluated according to the method of Wang & Edelman (1971). Two experiments were conducted for the ANS binding study (Yang et al.,
In experiment one, untreated or HHP treated WPC solutions (100 µM) were diluted with phosphate buffer (0.01 M, pH 7.0) to obtain 1 µM WPC solutions. Four microliters of ANS (5.0 mM in 0.1 phosphate buffer, pH 7.0) were titrated to the untreated or HHP treated WPC solutions to reach a final concentration at 55 µM for ANS. In experiment two, untreated and HHP treated WPC solutions (100 µM) were diluted with phosphate buffer (0.01 M, pH 7.0) to obtain WPC solutions with concentrations varying from 5 to 45 µM. Twenty microliters of ANS (100 µM in 0.1 phosphate buffer, pH 7.0) was added to 2 ml WPC solutions to obtain ANS concentrations of 1 µM. After mixing, extrinsic ANS fluorescence was determined with the spectrophotofluorometer using an excitation wavelength of 390 nm and observing emission at a wavelength of 470 nm. The apparent dissociation \(K'_d\) constants of ANS to WPC solutions were calculated with the equation 
\[
1/F = 1/F_{\text{max}} + \left[\frac{(K'_d/F_{\text{max}})}{L}\right]
\]
by varying ANS concentration, where \(F\) and \(F_{\text{max}}\) are the observed and final fluorescence intensities, respectively, and \(L\) is the free ligand concentration. In experiment one, \(L_0 >> P_0\) (total concentration of protein) and the total \((L_0)\) and free ligand concentration are rationally equal. Therefore, \(K'_d\) can be obtained by plotting \(1/F\) vs \(1/L\). The number of binding sites \((n)\) on WPC was calculated by varying WPC concentration with the equation 
\[
L_0/F = \left(\frac{1}{\varepsilon}\right) + \frac{K'_d}{\varepsilon(nP_0 - P_L)}
\]
where \(P_L\) is the concentration of the ligand-protein complex, and \(\varepsilon\) is a proportionality factor relating \(F\) to \(P_L\). In experiment two, since \(nP_0 >> L_0\) and \(nP_0 >> P_L\), the equation becomes 
\[
L_0/F = \left(\frac{1}{\varepsilon}\right) + \frac{K'_d}{\varepsilon P_0}
\]
\(L_0/F\) was plotted vs \(nP_0\) was calculated using the obtained value of \(K'_d\).

**Statistical analysis**

All experiments and analyses were done in triplicate. The analysis of variance test for significant effects of treatments and assay samples were determined using the General Linear
Model procedure (PROC GLM) in SAS. Main effect differences were considered significant at the p < 0.05 level. Means separations were determined by Fisher’s Least Significant (LSD) for multiple comparisons (SAS Institute Inc., 1993).

RESULTS AND DISCUSSION

Intrinsic tryptophan fluorescence

Fluorescence spectroscopy is a valuable tool in the investigation of structure, function and reactivity of proteins and other biological molecules (Chryssomallis et al., 1981). Fluorimetric method provides a relatively non-invasive and continuously controlled method for uninterrupted protein structure perturbation from which the relation of protein structure with their activity can be inferred (Dufour et al., 1994; Lakowicz, 1999). The intrinsic fluorescence of tryptophan residues is particularly responsive to microenvironments, so fluorescence is a very sensitive indicator of conformational changes (Dufour et al., 1994).

In most proteins, phenylalanine (Phe), tyrosine (Tyr), and tryptophan (Trp) residues can absorb ultraviolet (UV) radiation and can be raised to an excited state (Lakowicz, 1999; Eftink, 1991). The transfer back to the ground state can occur by (a) fluorescent or phosphorescent emission; (b) radiationless energy transfer to another absorbing center, e.g., from a Tyr to a Trp (Trp absorption band overlaps the Tyr emission band); and (c) quenching by a close group or molecule, with energy absorption without subsequent emission (Cowgill, 1967; Lakowicz, 1999). Examples of quenchers for Trp fluorescence in proteins include carboxyl groups of adjacent acidic amino acids, disulfide bonds (Hennecke et al., 1997), and dissolved oxygen (Chen & Barkley, 1998).
At atmospheric pressure (0.1 MPa), β-LG (in 50 mM Tris buffer, pH 7.0) and α-LA (in 10 mM Tris buffer, pH 7.0) display typical fluorescence emission spectra with a maximum at 332 nm (excitation: 295 nm) (Dufour et al., 1994; Tannaka et al., 1996). The recorded β-LG and α-LA fluorescence emission maximum (332 nm) is characteristic of tryptophan residues in a relatively hydrophobic environment, such as the interior of the globulin (Dufour et al., 1994). The hydrophobic character of the β-LG and α-LA tryptophan neighborhood is additionally suggested by the comparison with the fluorescence of free D,L-trptophan in aqueous solution (maximum near 355 nm) (Dufour et al., 1994; Lakowicz, 1999).

The intrinsic tryptophan fluorescence spectra of WPC in the present study were predominated by the spectra of β-LG and α-LA because of their high concentrations (~73% and ~23% of the major whey proteins, respectively) in the WPC (Figure 2). The fluorescence intensity increased and a red shift was observed after HHP treatment, which indicates changes in the polarity of tryptophan residues microenvironment of whey proteins from a less polar to a more polar environment. HHP treatment for come-up time resulted in a 2 nm red shift of the maximum emission wavelength from 335 nm to 337 nm. After HHP treatment of 2.5 min, there was a 1.1-fold increase in fluorescence intensity and the maximum emission wavelength shifted from 337 nm to 338 nm. Further HHP treatment up to 15 min did not cause changes in the maximum emission wavelength, and the increases in the fluorescence intensity showed smaller changes per unit of time, indicating major changes in tryptophan environment during pressurization occurred within 2.5 min. Thirty minutes of HHP treatment resulted in a further shift of maximum emission wavelength to 339 nm and a final 1.2-fold increase in intrinsic fluorescence.
The 4 nm red shift and the increases in the fluorescence intensity of WPC after HHP treatments are consistent with the results of HHP treatment of β-LG (Yang et al., 2001). The results indicated that the presence of multiple whey proteins did not significantly influence the behavior of Trp environments of individual whey proteins during pressurization. Both the results from the present study and Yang et al. (2001) report less than the 1.9-fold intensity increase and 13 nm red shift found for β-LG in 8 M urea (Yang et al., 2001). A reasonable explanation consistent with pressure studies on other proteins (Mohana-Borges et al., 1999) is that unfolding is more or less complete in 8 M urea but that partially folded, perhaps molten globular forms remain after the pressure treatment (Yang et al., 2001).

Dufour et al. (1994) observed the changes in β-LG intrinsic fluorescence during the compression and decompression of HHP treatment. The compression of β-LG in Tris solution (pH 7.0) exhibited three distinct pressure regions (Dufour et al., 1994). Below 100 MPa, the maximum emission wavelength remained at 332 nm (Dufour et al., 1994). The increase of pressure from 100 MPa to 250 MPa was accompanied by a 12 nm red shift of the emission maximum, and a plateau value (344 nm) was reached at 250 MPa (Dufour et al., 1994). During decompression from 350 to 0.1 MPa, marked hysteresis was observed. In addition, the maximum emission wavelength (336 nm) observed after the release of pressure to 0.1 MPa did not return to the initial value (332 nm) (Dufour et al., 1994). These observations suggest that, after compression at 350 MPa, β-LG undergoes both reversible and irreversible folding changes at neutral pH.

Extrinsic ANS and CPA fluorescence spectra

In spite of relatively long-standing knowledge that β-LG tightly interacts in vitro with retinol (Futterman & Heller, 1972; Fugate & Song, 1980), the exact physiological role of β-LG is
unknown. However, it is reported that β-LG can also bind fatty acids and triacylglycerols (Brown, 1984; Diaz de Villegas et al., 1987), aromatic hydrocarbons (Farrell et al., 1987), ellipticine (Dodin et al., 1990), retinoic acid (Dufour & Haertlé, 1991), and CPA (Dufour et al., 1992). ANS and CPA fluorescence is very weak in aqueous solutions, but it is greatly enhanced when bound to β-LG (Dufour et al., 1994; Hamdan et al., 1996). Thus, ANS and CPA probes are widely used to assay hydrophobicity of food proteins (Nakai, 1983; Li-Chan & Nakai, 1989; Yang et al., 2001).

The ANS extrinsic fluorescence of HHP treated WPC exhibited three distinct regions (Figure 3). HHP treatment up to 2.5 min resulted in a 1.4-fold increase in the ANS extrinsic fluorescence intensity. A decrease in the fluorescence intensity to 1.1-fold was observed after further HHP treatment to 7.5 min. HHP treatment for 30 min resulted in a 2-fold increase in the ANS fluorescence intensity.

ANS fluorescence intensity of β-LG, α-LA, and BSA responded differently to HHP treatments (Yang et al., 2001; Tanaka et al., 1996; Galazka et al., 1996). Increases in the ANS fluorescence intensity were observed for β-LG and α-LA after HHP treatment above 200 MPa (Yang et al., 2001; Tanaka et al., 1996). However, HHP treatment of BSA up to 1000 MPa resulted in gradual decreases in ANS fluorescence intensity (Galazka et al., 1996). Thus, the ANS fluorescence spectrum of HHP treated WPC was the sum of the changes of individual whey proteins during pressurization. Within 2.5 min of HHP treatment, the increases in ANS fluorescence intensity from β-LG and α-LA overcame the decrease in the intensity of BSA, which resulted in an overall increase in the ANS fluorescence intensity for WPC. However, during further HHP treatments up to 7.5 min, the WPC ANS fluorescence spectra were dominated by the characteristic decrease in the ANS fluorescence intensity of BSA. When HHP
holding time increased above 10 min, the WPC ANS fluorescence spectra showed gradual increases in fluorescence intensity, which indicated the domination of the spectra by the increases in the ANS fluorescence intensity by β-LG and α-LA over the decrease in the intensity from BSA.

A feature of fluorescence studies is that conformational changes within a protein can affect the emission wavelength ($\lambda_{\text{max}}$) and the emission intensity at $\lambda_{\text{max}}$ ($I_{\text{Trp}}$) differently (Stapelfeldt et al., 1996). For example, Stapelfeldt et al. (1996) examined β-LG and reported that $\lambda_{\text{max}}$ increased at pressures up to 300 MPa, whereas $I_{\text{Trp}}$ attained a maximum value at a lower pressure of 200 MPa. Our study also showed that HHP treatment affected the ANS $\lambda_{\text{max}}$ and $I_{\text{Trp}}$ differently. In addition to the fluorescence intensity changes, a 6 nm blue shift was observed for the ANS maximum emission wavelength following 15 min of pressure treatment, and a 2 nm red shift appeared after further HHP treatment to 30 min. The blue shift during 15 min of HHP treatment indicates that HHP treated whey proteins bind ANS in a less polar environment compared to native whey proteins, which was also observed for β-LG after HHP treatment by Yang et al. (2001), although to a greater extent. Given that the ligand-free pocket of native β-LG is filled with water (Sawyer et al., 1999), such a polarity decrease is reasonable and consistent with pressure-induced MG formation. Further HHP treatment to 30 min resulted in a 2 nm red shift, indicating structural change with further pressure treatment. These results indicated that the presence of α-LA and BSA affected the binding of ANS to WPC, probably through the formation of aggregates as observed by the gel electrophoresis (Liu et al., 2004).

HHP treatment of WPC solutions resulted in decreases in fluorescence intensity of CPA and broadened peaks in CPA fluorescence spectra (Figure 4). After HHP treatment for 10 min, a 64% decrease in CPA fluorescence intensity was observed and a second peak appeared. The
large decrease of CPA fluorescence intensity and the appearance of a second emission peak suggest important conformational changes in CPA binding environment. Similar results were observed by Dufour et al. (1994) for β-LG retinol fluorescence during HHP treatment.

**Hydrophobic probe binding study**

One of the cardinal features of the native to molten globular transition is a loss of near UV Circular Dichroism (CD) intensity from the aromatic side chains, suggesting that the aromatic groups in particular become more mobile during the transition (Dolgikh et al., 1981). An increased mobility of the aromatic groups could lead to specific enhancement of their accessibility for binding with ANS (Yang et al., 2001). Besides differences in interaction energies between aromatic compounds as compared to those between aliphatic compounds, structural factors could also be important. Since the side chain packing of molten globules tends to be non-rigid, the clusters of hydrophobic side chains would be expected to assume more or less spherical form. Depending on their sizes, the spherical clusters could perhaps accommodate a more or less isometric structure like ANS, while not binding very well to a long, lean molecule such as CPA (Yang et al., 2001).

Titrations of WPC with ANS and CPA, presented in Figures 5 through 11, are plotted according to Cogan method (Cogan et al., 1976) and Wang & Edelman method (1971). WPC exhibited 0.16 binding sites per moluceld of protein for ANS with an apparent dissociation constant of $1.8 \times 10^{-5}$ M (Table 1), indicating one molecule of WPC bound with 0.16 molecules of ANS. Yang et al. (2003) reported that native β-LG exhibited 0.41 binding sites for ANS with a dissociation constant of $4.5 \times 10^{-5}$ M. The number of binding sites of WPC for ANS observed in this research is lower than the results Yang et al. (2003) reported for β-LG, which may indicate that the presence of aggregates in WPC decreased the accessibility of the binding sites.
for ANS. WPC exhibited 1.9 binding sites for CPA with an apparent dissociation constant of $2.2 \times 10^{-7}$ M (Table 2), indicating one molecule of WPC bound specifically with two molecules of CPA. Yang et al. (2003) reported that native β-LG exhibited 0.95 binding sites for CPA with a dissociation constant of $2.1 \times 10^{-7}$ M. The dissociation constant of WPC for CPA observed in the present research is similar to reports of Yang et al. (2003). The higher number of binding sites of WPC for CPA may indicate the generation of new binding sites by the aggregates present in WPC.

HHP treatments up to 15 min did not cause significant changes in the dissociation constants of WPC for ANS, but a 1.8-fold increase in the dissociation constant was observed after 30 min of HHP treatment (Table 1). Come-up time of HHP treatment resulted in an increase in the number of binding sites from 0.16 to 0.33, which may relate to the dissociation of the large aggregates present in untreated WPC and exposure of more binding sites for ANS. Further HHP treatments of WPC up to 30 min resulted in even higher number of binding sites for ANS (Table 1), indicating that structure modifications of WPC and formation of aggregates during HHP treatments generated new binding sites for ANS. Yang et al. (2003) reported that the number of binding sites for ANS interaction with β–LG did not change significantly during HHP treatment at 600 MPa and 50°C for 30 min. So the presence of α-LA and BSA in WPC may be responsible for the increases in the number of binding sites for ANS through the formation of aggregates during HHP treatments.

HHP treatments of WPC did not result in significant changes in the number of binding sites for CPA (Table 2), indicating formation of aggregates of WPC during HHP treatments did not significantly alter the binding sites of WPC for CPA. The apparent dissociation constant for CPA with WPC decreased to $1.1 \times 10^{-7}$ M after HHP treatment for come-up time, indicating an
increase in the binding affinity of WPC for CPA. This improvement may result from the
dissociation of aggregates (Creamer, 1995) present in untreated WPC. Further HHP treatment up
to 5 min resulted in decreases in the binding affinity, indicating some conformational changes
around the binding sites during HHP treatment. Changes in secondary structure (around the
loosely structured surface loops) may occur during HHP treatment (Creamer, 1995), which could
decrease the accessibility to the binding sites for CPA. HHP treatments of 7.5 or 10 min
decreased the apparent dissociation constant to $1.5 \times 10^{-7}$ M and $1.1 \times 10^{-7}$ M, respectively.
Further HHP treatment to 30 min resulted in a significant decrease in the binding affinity, with
the apparent dissociation constant increasing to $4.1 \times 10^{-7}$ M, which may be related to the
formation of aggregates.

Yang et al. (2003) reported that the surface hydrophobic site of HHP-induced β-LG
dimers were surrounded by hydrophobic amino acid residues, which resulted in an increase of
hydrophobic affinity of β-LG for CPA at the surface hydrophobic site (Yang et al., 2003). This is
consistent with the current finding, where increase in the binding affinity of WPC for CPA was
observed after 10 min of HHP treatment. However, the formation of β-LG dimers (Yang et al.,
2003) and aggregates from β-LG, α-LA and BSA monomers by disulfide bonds may decrease
accessibility of CPA to the surface hydrophobic binding site. Therefore, it follows that the
binding affinity of CPA to WPC decreased after further HHP treatment over 10 min to 30 min.

Wu et al. (199) reported that as the pH is raised from pH 6 to 7.5, changes in the
microenvironments of Glu-89 and Met-107 of β-LG resulted in increased accessibility of the
binding sites for palmitate. Narayan and Berliner (1998) observed that chemical modification of
Cys-121 of β-LG reduced the binding affinity of β-LG for palmitate by 10-fold as monitored by
intrinsic fluorescence. Further research regarding the effects of HHP treatments on the ligand binding sites will provide more information on the conformational changes of whey proteins.

CONCLUSIONS

The hydrophobic probe binding behavior of WPC is affected by the holding time of pressurization. HHP resulted in an increase in intrinsic tryptophan fluorescence intensity and a 4 nm red shift after 30 min of treatment, which indicate changes in the polarity of tryptophan residues microenvironment of whey proteins from a less polar to a more polar environment. HHP treatment for 30 min resulted in an increase in the number of binding sites for ANS from 0.16 to 1.10 per molecule of protein. No significant changes in the apparent dissociation constant of WPC for ANS were observed after HHP treatment, except for an increase from $1.8 \times 10^{-5}$ M to $3.3 \times 10^{-5}$ M after 30 min of HHP treatment. There were no significant changes in the number of binding sites of WPC for CPA. However, increased binding affinities of WPC for CPA were observed after the come-up time or 10 min HHP treatment, with a decrease of apparent dissociation constant from $2.2 \times 10^{-7}$ M to $1.1 \times 10^{-7}$ M.

These results indicate that during HHP treatments, conformational changes of whey proteins and aggregation affect the hydrophobicity of whey proteins. HHP treated WPC may display improved functionality and provide opportunities for increasing utilization of WPC in the food industry.

ABBREVIATIONS USED

β-LG, β-lactoglobulin; α-LA, α-lactalbumin; BSA, bovine serum albumin; Ig, immune globulin; HHP, high hydrostatic pressure; WPC, whey protein concentrate; ANS, 1-anilino-naphthalene-8-sulfonate; CPA, cis-parinaric acid; MG, molten globule; CD, circular dichroism.
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Yang J, Dunker AK, Powers JR, Clark S & Swanson BG 2003 Ligand and flavor binding functional properties of β–lactoglobulin in the molten globule state induced by high pressure. *Journal of Food Science* 68 444-452
Figure 1. Structure of fluorescent probes 1-anilino-naphthalene-8-sulfonate (ANS) and cis-parinaric acid (CPA).

Figure 2. Intrinsic tryptophan emission spectra of WPC solutions affected by HHP at 600 MPa and 50°C for various holding times from 0 to 30 min (H0-H30). Inset represents the maximum emission wavelength of the intrinsic tryptophan emission spectra of WPC solutions.
Figure 3. Extrinsic ANS emission spectra of WPC solutions affected by HHP at 600 MPa and 50°C for various holding times from 0 to 30 min (H0-H30). Inset represents the maximum emission wavelength of the extrinsic ANS emission spectra of WPC solutions.

Figure 4. Extrinsic CPA emission spectra of WPC solutions affected by HHP at 600 MPa and 50°C for various holding times from 0 to 30 min (H0-H30).
Table 1. Apparent dissociation constants ($K'_d$) and the number of ligand binding sites (n) of WPC for ANS after HHP treatment (600 MPa and 50ºC) for holding time of 0 to 30 min (H0-H30) calculated using the method by Wang and Edelman (1971) *.

<table>
<thead>
<tr>
<th>Ligands</th>
<th>WPC</th>
<th>n</th>
<th>$K'_d$ (M)$^2$</th>
</tr>
</thead>
<tbody>
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<td></td>
<td>Untreated</td>
<td>0.16$^a$</td>
<td>1.8×10$^{-5}$ M$^a$</td>
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<tr>
<td></td>
<td>H 0</td>
<td>0.33$^b$</td>
<td>1.7×10$^{-5}$ M$^a$</td>
</tr>
<tr>
<td></td>
<td>H 2.5</td>
<td>0.80$^c$</td>
<td>2.3×10$^{-5}$ M$^a$</td>
</tr>
<tr>
<td>ANS</td>
<td>H 5</td>
<td>0.72$^c$</td>
<td>2.1×10$^{-5}$ M$^a$</td>
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<td></td>
<td>H 10</td>
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<td></td>
<td>H 30</td>
<td>1.1$^d$</td>
<td>3.6×10$^{-5}$ M$^b$</td>
</tr>
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</table>

*: Data are means of three analyses calculated using method by Wang and Edelman (1971). 1, 2: means with different letters in the column are significantly different (p<0.05).

Table 2. Apparent dissociation constants ($K'_d$) and the number of ligand binding sites (n) of WPC for CPA after HHP treatment (600 MPa and 50ºC) for holding time of 0 to 30 min (H0-H30) calculated using the method by Cogan et al. (1976) *.

<table>
<thead>
<tr>
<th>Ligands</th>
<th>WPC</th>
<th>n</th>
<th>$K'_d$ (M)$^2$</th>
</tr>
</thead>
<tbody>
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<td>H 0</td>
<td>1.8$^a$</td>
<td>1.1×10$^{-7}$ M$^b$</td>
</tr>
<tr>
<td></td>
<td>H 2.5</td>
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<td>CPA</td>
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<td>H 7.5</td>
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<td></td>
<td>H 10</td>
<td>1.8$^a$</td>
<td>1.1×10$^{-7}$ M$^b$</td>
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<td>H 30</td>
<td>1.7$^a$</td>
<td>4.1×10$^{-7}$ M$^c$</td>
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</table>

*: Data are means of three analyses calculated using method by Cogan et al. (1976). 1, 2: means with different letters in the column are significantly different (p<0.05).
Figure 5. ANS binding to WPC plotted by Wang and Edelman (1971) method to calculate $K'_d$ (a) and $n$ (b) for untreated WPC and HHP treated (600 MPa and 50°C) WPC with 0 holding time (H0).
Figure 6. ANS binding to WPC plotted by Wang and Edelman (1971) method to calculate $K'_d$ (a) and $n$ (b) for HHP treated (600 MPa and 50ºC) WPC with 2.5 or 5 min holding time (H2.5 and H5).
Figure 7. ANS binding to WPC solutions plotted by Wang and Edelman (1971) method to calculate $K'_d$ (a) and $n$ (b) for HHP treated (600 MPa and 50°C) WPC with 7.5 or 10 min holding time (H7.5 and H10).
Figure 8. ANS binding to WPC solutions plotted by Wang and Edelman (1971) method to calculate $K'_d$ (a) and $n$ (b) for HHP treated (600 MPa and 50°C) WPC with 15 or 30 min holding time (H15 and H30).
Figure 9. CPA binding to WPC plotted by Cogan method (Cogan et al., 1976) to calculate $K'_d$ and n for Untreated WPC and HHP treated (600 MPa and 50°C) WPC with 0 or 2.5 min holding time (H0 and H2.5).
Figure 10. CPA binding to WPC plotted by Cogan method (Cogan et al., 1976) to calculate $K'_d$ and $n$ for HHP treated (600 MPa and 50°C) WPC with 5, 7.5 or 10 min holding time (H5, H7.5 and H10).
Figure 11. CPA binding to WPC plotted by Cogan method (Cogan et al., 1976) to calculate $K'_d$ and n for HHP treated (600 MPa and 50°C) WPC with 15 or 30 min holding time (H15 and H30).
CHAPTER FIVE

Effects of High Hydrostatic Pressure on Flavor-binding Properties of Whey Protein Concentrate

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ABSTRACT

The effects of high hydrostatic pressure (HHP) treatment on flavor-binding properties of whey protein concentrate (WPC) were determined with heptanone, nonanone, octanone, diacetyl, and benzaldehyde. After HHP treatment (600 MPa, 50°C, for 0, 10, and 30 min), flavor-binding properties of WPC were studied by intrinsic fluorescence titration and static headspace analysis. HHP treatments increased the number of binding sites and the apparent dissociation constants of WPC for benzaldehyde. HHP treatment for 10 min increased the binding affinity of WPC for diacetyl. HHP treatment of WPC for come-up time resulted in increases in the number of binding sites of WPC for heptanone and octanone. HHP treatments for 10 min resulted in an increase in the apparent dissociation constant of WPC from $2.5 \times 10^{-8}$ M to $3.9 \times 10^{-8}$ M for heptanone, from $2.2 \times 10^{-8}$ M to $3.1 \times 10^{-8}$ M for octanone, and from $1.9 \times 10^{-8}$ M to $2.7 \times 10^{-8}$ M for nonanone.

As observed by headspace analysis, HHP treatments did not result in significant changes in the flavor retention for benzaldehyde in WPC solutions. Flavor retention of 100 ppm and 200 ppm heptanone and octanone in HHP treated (10 min) WPC was significantly lower than in untreated WPC and HHP treated WPC for come-up time or 30 min. Significant decreases were only observed at 100 ppm for flavor retention of nonanone in HHP treated (10 min) WPC solutions. Further research is required to fully understand the effects of HHP treatment on flavor-binding and flavor release properties of WPC and to evaluate the full potential of this process in the food industry.

Keywords: whey protein concentrate; high hydrostatic pressure; flavor binding; fluorescence; gas chromatography
INTRODUCTION

The acceptability of a food depends mainly on its sensory qualities and in particular on its flavor (Casimir, 1998). To elicit a response, a flavor compound must achieve a sufficient concentration in the vapor phase (nasal) or aqueous phase (saliva) to stimulate the olfactory and taste receptors, respectively (Kinsella, 1990). Concentration of aroma compounds, and therefore aroma perception during eating, depends on the nature and concentration of the volatiles present in the food as well as on their availability to perception (Harrison, 1997). Availability is influenced in part by the process of eating, such as mastication, temperature and the effect of saliva, but mainly by interactions between aroma compounds and non-volatile food constituents, such as fats, proteins, and carbohydrates (Bakker et al., 1996). The types of interactions vary with the nature of the food component and the volatile compounds, such as entrapment, formation of covalent bonds, hydrogen bonds and physical adsorption via hydrophobic bonds (Kinsella, 1990). Thus the composition of a food product greatly influences the performance of a flavoring and therefore the sensory quality. Changes in a food matrix require changes or modifications of flavorings to optimize their performance (Harrison et al., 1997).

The fat content is an important variable in a food matrix. Although fat is important for sensory qualities such as flavor, color, texture, and mouthfeel, manufacturers have made it a practice to substitute fat with fat replacers in order to create products that meet the demands of health-conscious consumers (Casimir, 1998). High fat intake is associated with increased risk for obesity, and saturated fat intake is associated with high blood cholesterol and coronary heart disease (American Heart Association, 1996; U.S. Department of Health & Human Services, 1988). As fat substitutions are made, the flavor challenges are significantly increased, and aroma chemicals may be perceived as harsh and unbalanced (Hatchwell, 1994).
Indeed, fat cannot simply be removed, as it makes a significant contribution to the sensory properties of foods in several ways. One important point is that fat is a good solvent of flavor compounds and influences the vapor pressure of the volatiles, thereby affecting the perceivable aroma profile. Hence good fat based flavorings tend to become unbalanced or even off-flavored in aqueous or reduced fat systems (Hatchwell, 1994). Triacylglycerols also play an important part in the mouthfeel or texture of a food during eating. Removal or reduction of these lipids leads to an imbalanced flavor, often with a much higher intensity than the original full fat food. This is because the non-polar volatiles are no longer dissolved in the lipid phase and are released from the food as soon as eating begins. In vivo measurements of the release of flavor compounds from sweet biscuits and Frankfurter sausages have demonstrated that the lowfat versions release higher amounts of flavor compounds than do the same products containing higher fat concentrations (Ingham et al., 1996). The pattern of flavor release was also different between biscuits with different fat contents. Flavor release from the 16.5% fat biscuit reached a maximum at 5 s and then remained constant whereas flavor release from the 4.6% fat biscuit increased to a maximum at approximately 20 s and then decreased (Ingham et al., 1996).

In addition to fats, proteins belong to another important class of components in food systems capable of influencing flavor release. The market for functional protein-rich ingredients is expanding and is currently supplied by various proteins. Whey protein concentrate (WPC) represents a potentially significant source of functional protein ingredients for many traditional and novel food products. Its utilization as a flavor carrier, besides its other properties like emulsifying and gelation properties, could be very interesting for the food industry (Buhr et al., 1999).
\( \beta \)-lactoglobulin (\( \beta \)-LG), the major whey protein, is known to interact with many flavor compounds, such as aldehydes and ketones (O’Neil and Kinsella, 1987), ionones (Dufour and Haertlé, 1990), and hydrocarbons (Wishnia and Pinder, 1966). Although this observation is mostly described in terms of flavor binding, it has been generally considered that \( \beta \)-LG is a possible carrier for flavor compounds and may be effective in protecting, delivering, or delaying release of flavor components. For instance, \( \beta \)-LG could be engineered to bind and protect a wide range of volatile and unstable flavors during food manufacturing or to release them in more or less controlled way by chemical modifications or heat treatment (Boundaud and Dumont, 1996).

Modification can be accomplished by chemical or physical means (Dufour and Haertlé, 1992). Chemical derivatizations may reduce amino acid bioavailability and have toxicologic consequences (Kester and Richardson, 1984). The use of high temperature results in some detrimental changes on the processed products, which affect nutritional as well as organoleptic attributes (Martin et al., 2002). High hydrostatic pressure (HHP) presents unique advantages over both chemical and thermal processing for food product modifications, including application at low temperatures, which has little effect on food quality (Knorr, 1995a; Knorr, 1995b). HHP treatment does not cause environmental pollution and eliminates the use of chemical additives in food products (Knorr and Dornenburg, 1996).

Studies have been done to understand the effect of high pressure on some of the functional properties of whey proteins, such as gel formation (Famelart et al., 1998), emulsifying capacity (Galazka et al., 1995), and foamability (İbanoglu and Karatas, 2001). However, little work has been done on the effects of high pressure on whey protein-flavor binding. Changes in the surface hydrophobicity and aggregation have been observed with \( \beta \)-LG subsequent to HHP.
treatments between 200 and 600 MPa (Dumay et al., 1994; Nakamura et al., 1993). Pressure-
induced changes in protein molecules tend, in general, to increase the area accessible to the
solvent and, as a consequence, alter surface properties (Dumay et al., 1994; Nakamura et al.,
1993). High pressure induces β-LG into the molten globule (MG) state (Yang et al., 2001).
Proteins in the molten globule state usually retain the secondary structure of the native state and
exhibit a compact tertiary structure, but with increased mobility and looser packing of the protein
chain (Ptitsyn, 1995). Semisotinov et al. (1991) reported that proteins in the MG state (bovine α-
lactalbumin, bovine carbonic anhydrase and Staphylococcus aureus β-lactamase) exhibit high
affinity for the hydrophobic probe 1-anilino-naphthalene-8-sulfonate (ANS). Yang et al. (2001)
reported a 3-fold increase in the ANS fluorescence intensity after HHP treatment (600 MPa,
50°C, 32 min) in sodium phosphate buffer (0.01M, pH 7.0). β-LG in the MG state induced by
HHP exhibited a significant decrease in affinity for retinol and a significant increase in affinity
for cis-parinaric acid (CPA) and ANS compared to native β-LG (Yang et al., 2003).

Binding between flavor compounds and proteins has been studied by different authors
and with different techniques (Damodaran and Kinsella, 1980; O’Neill and Kinsella, 1988), such
as exclusion chromatography, equilibrium dialysis, static headspace, fluorimetry, dynamic
coupled column liquid chromatography, affinity chromatography, and sensory evaluation
(Guichard and Langourieux, 2000).

Fluorescence spectroscopy is a valuable tool in the investigation of structure, function
and reactivity of proteins and other biological molecules. Fluorimetric method provides a
relatively non-invasive and continuously controlled method for uninterrupted protein structure
perturbation from which the relation of protein structure with their activity can be inferred
(Dufour et al., 1994; Lakowicz, 1999). However, only the structure of the fluorescent probe and the immediate environment is reported.

Headspace gas analysis has been successfully applied to the food industry for over 20 years (Rouseff and Cadwallader, 2001). Analysis of volatiles in the gaseous headspace above foods is widely used to estimate the binding of flavors and to determine factors affecting their partitioning between the substrate and air (Damodaran and Kinsella, 1980; Guichard and Langourieux, 2000; O’Neill and Kinsella, 1988). However, headspace analysis of volatile flavors lacks sensitivity, and large volumes have to be used to obtain detectable concentrations, which frequently result in poor chromatography (Rouseff and Cadwallader, 2001). Therefore, a combination of fluorescence and headspace analysis may provide useful information on the effects of HHP on the interaction of WPC and flavor compounds.

The objectives of this research were to investigate the binding properties of selected flavor compounds with both untreated and HHP treated WPC. Intrinsic fluorescence titration and static headspace analysis were employed in the present research.

**MATERIALS & METHODS**

**Materials**

RT-80 Grade A whey protein concentrate (WPC RT-80) was provided by Main Street Ingredients (La Crosse, WI). WPC RT-80 with the same lot number was used throughout the experiments. The product contained 84.9% protein, 3.9% fat, 3.4% ash, 3.5% lactose, and 3.7% moisture. The pH of a 2% solution at 20°C was 6.4. All of the chemicals used were of analytical grade obtained from Fisher Chemicals (Fairlawn, NJ) or unless otherwise specified.
**High Pressure Treatment**

WPC solutions, at concentrations of 0.2% in sodium phosphate buffer (0.01 M, pH 7.0), were treated with HHP of 600 MPa at 50°C for holding times of 0 (come-up time), 10, or 30 min. The come-up time (4.5 min) is the compression time required to reach a pressure of 600 MPa. After exposure to high pressure, WPC solutions were studied immediately or stored at 4°C for less than one month.

**Flavor compound fluorescence binding study**

Benzaldehyde, diacetyl, heptanone, octanone, and nonanone were flavor compounds selected to bind with WPC (Figure 1). Benzaldehyde is the characteristic almond flavor. Diacetyl is the buttery flavor compound in many dairy products. Heptanone, octanone, and nonanone are typical flavors developed in yogurt.

The binding of flavor compounds with WPC was evaluated by following the quenching of intrinsic tryptophan fluorescence (Dufour and Haertlé, 1990; Marin and Relkin, 1998). In the binding study, 4 µl of benzaldehyde, heptanone, octanone, nonanone, or diacetyl (125 µM in absolute ethanol) was titrated to 2 ml of the untreated or HHP treated WPC solution (1 µM) to reach a final concentration of 2.5 µM. As the titration proceeded toward its end, larger amounts of flavor compounds were added from more concentrated solutions. Following each titration of flavor compound the system was thoroughly mixed and then allowed to equilibrate for 30 min prior to the recording of the fluorescence intensity. At the end of the various titrations the ethanol concentration did not exceed 3%. Intrinsic fluorescence was measured using an excitation wavelength of 295 nm and observing an emission wavelength of 350 nm. To eliminate the dilution of WPC solution by the added flavor solution and tryptophan fluorescence changes induced by alcohol, a blank containing WPC solutions titrated with ethanol was monitored as
described above. The fluorescence intensity changes of the blank were subtracted from fluorescence intensity measurements of the flavor/protein complexes for every considered titration point. In all cases, tryptophan fluorescence intensity at 350 nm was normalized to 1, and the fluorescence intensity was expressed as arbitrary units (a.u.).

Flavor-binding properties were evaluated with Cogan method (Cogan et al., 1976). The numbers of accessible binding sites and apparent dissociation constants of flavor compounds with WPC are calculated with the equation \[ P_0 \alpha = \frac{(1/n)(L_0 \alpha / (1-\alpha))(K'_d/n))}{}, \] where \( P_0 \) is protein concentration, \( L_0 \) is a given ligand concentration, \( n \) is the number of binding sites per molecule of protein, \( K'_d \) is the apparent dissociation constant, and \( \alpha \) is the fraction of binding sites remaining free, assuming \( \alpha = (F_{\text{max}} - F)/ F_{\text{max}} \). \( F_{\text{max}} \) is defined as the fluorescence intensity when protein molecules are saturated by flavor compounds.

**Headspace analysis**

Benzaldehyde, heptanone, octanone and nonanone were chosen to investigate the effects of HHP on flavor retention. Analyses were done in triplicate in amber flasks (40 ml) closed with mininert valves (Supelco, Bellefonte, PA). Two aroma concentrations (100 and 200 ppm) and one WPC concentration (0.2%, 5 ml) were tested. Analyzed solutions, with or without WPC, were stirred and equilibrated at 37°C for 30 min. Vapor phase samples (1 ml) were taken with a gastight syringe and injected onto a Carlo Erba 8000 gas chromatograph equipped with a DB-Wax column (J & W Sci., i.d. 0.32 mm, 30 m, film thickness = 0.5 µm). Temperature of injector and detector were respectively 250°C and 260°C. The \( \text{H}_2 \) carrier gas velocity was 1.9 ml/min.

**Statistical analysis**

All experiments and analyses were done in triplicate. The analysis of variance test for significant effects of treatments and assay samples were determined using the General Linear
Model procedure (PROC GLM) in SAS. Main effect differences were considered significant at the p < 0.05 level. Means separations were determined by Fisher’s Least Significant (LSD) for multiple comparisons (SAS Institute, Inc., 1993).

RESULTS AND DISCUSSION

Flavor compound binding fluorescence study

β-LG binds structurally different molecules such as fatty acids, retinol (Diaz de Villegas et al., 1987), and alkanone flavors (O’Neill and Kinsella, 1987). The affinity of β-LG for a flavor compound or a ligand is dependent on molecular structure of flavor compounds or ligand (Damodaran and Kinsella, 1980; Reiners et al., 2000). The fact that retinol, CPA, and ANS bind to β-LG indicates that aliphatic hydrophobic chains and aromatic hydrophobic rings are important structural components for molecules binding to β-LG. Palmitic acid affinity for β-LG is due to the aliphatic hydrophobic carbon chain. Reiners et al. (2000) observed that chain length contributed to the affinity of flavor compounds for β-LG by increasing hydrophobicity.

There are at least two distinct binding sites per monomer of β-LG for a variety of ligands (Sawyer et al., 1998; Wu et al., 1999). The primary hydrophobic binding site is located within the calyx formed by eight strands of antiparallel β–sheets, and a second hydrophobic binding site lies in a cleft between the helix and an edge of the barrel (Wu et al., 1999). The capacity of β-LG to bind chemically and structurally miscellaneous ligands suggests that β-LG, in addition to the presumable deep central pocket site, may potentially bind these ligands in the outer surface site framed by hydrophobic residues (Monaco et al., 1987).

The titration curves for benzaldehyde, diacetyl, heptanone, octanone and nonanone are presented in Figures 2 through 6. The fluorescence emission spectra of WPC solutions were
studied as a function of added compounds, and the observed tryptophan fluorescence quenching, due to changes of the polarity in the neighborhood of indoles (Lakowicz, 1999) is indicative of the formation of a complex. The addition of benzaldehyde, diacetyl, heptanone, octanone and nonanone to WPC solutions all produced fluorescence quenching, suggesting that these compounds bind to whey proteins or interfere with whey protein tryptophans.

β-LG is a relatively spherical protein having ca. 20 Å in radius; nearly 60% the mass of such a particle would be within 5 Å of the surface, and almost 90% within 10 Å (Meuresean et al., 2000). Real protein subunits are by no means spherical, so their mass is even closer to the surface. Although quenching studies using acrylamide and iodide as external quenchers indicated that the β-LG tryptophan residue(s) are buried, the “buried” tryptophan(s) must be very close to the protein surface (Meuresean et al., 2000). For untreated WPC, the maximum fluorescence quenching is obtained at a flavor-protein ratio of 1:5 to 1:3, which is lower than 1:1, results previously reported for a β-LG and flavor-binding study (Marin et al., 1998; Guichard and Langourieux, 2000). The decreases in the number of binding sites may indicate that some binding sites in β-LG are blocked, probably by the formation of aggregates, during the production of WPC, such as filtration.

Marin et al. (1998) studied the effect of heat treatment on the binding property of β-LG with benzaldehyde. Although the plateau value obtained from the intrinsic fluorescence study is reached for 1:1 molar ratio in both untreated and heat treated β-LG, the percentage of quenching is higher with previously heated protein solutions, indicating that the binding capacity of the protein is increased by heating (pH 6, 75°C, 10 min). O’Neill and Kinsella (1988) reported that exposure of β-LG (in 20 mM phosphate buffer, pH 7.6) to a heat treatment of 75°C for 10 or 20 min resulted in a decrease in binding affinity of β-LG for benzaldehyde with a concomitant
increase in the number of low affinity, non specific binding sites. In our study with benzaldehyde, 30 min of HHP treatment increased the number of binding sites from 0.20 to 0.36, and the apparent dissociation constant from $2.7 \times 10^{-8}$ M to $4.7 \times 10^{-8}$ M, which are consistent with the heat treatment results reported by O’Neill and Kinsella (1988).

HHP treatment of 10 min increased the binding affinity of WPC for diacetyl, with a decrease of apparent dissociation constant from $2.7 \times 10^{-8}$ M to $1.5 \times 10^{-8}$ M and no significant influence on number of binding sites. The effects of 10 min of HHP treatment on the number of binding sites and apparent dissociation constant of WPC for diacetyl and CPA were similar. This indicates that CPA and diacetyl may bind to the same binding sites. Yang et al. (2003) reported that the conformational changes and formation of dimers during HHP treatment of β-LG resulted in an increase of affinity for CPA at the surface hydrophobic site (Yang et al., 2003). The increases in the binding affinity of WPC for diacetyl after 10 min of HHP treatment may be related to the conformational changes occurred during pressurization. Further HHP treatment for 30 min resulted in a decrease in the binding affinity of WPC for diacetyl compared to that of HHP treated (10 min) WPC, which may be due to the formation of larger aggregates among the whey proteins.

Three aliphatic methyl ketones (2-heptanone, 2-octanone, and 2-nonanone) were used to evaluate the flavor-binding properties of WPC. Their apparent dissociation constants for WPC were $2.5 \times 10^{-8}$ M, $2.2 \times 10^{-8}$ M, and $1.9 \times 10^{-8}$ M, respectively. The interactions between β-LG and methyl ketones are hydrophobic (O’Neill and Kinsella, 1987) since the affinity constants increase with increasing hydrophobic chain length (Sostmann and Guichard, 1998).

HHP treatment of come-up time resulted in an increase in the number of binding sites of WPC from 0.23 to 0.39 per molecule of protein for heptanone, and from 0.21 to 0.40 for
octanone. SDS-PAGE results showed that during the come-up time of HHP treatment, dissociation of aggregates occurred (Liu et al., 2004), which may expose more binding sites for heptanone and octanone. However, no changes in the number of binding sites of WPC for nonanone occurred during HHP treatments. Frapin et al. (1993) studied the interactions of fatty acids with porcine and bovine β–LG. One β–LG fatty acid binding pocket can accommodate best an aliphatic fatty acid chain constituted by 16 carbon atoms. Apparently, the new binding site of WPC for methyl ketones can accommodate aliphatic chain constituted by no more than 8 carbon atoms.

HHP treatment of 10 min resulted in an increase in the apparent dissociation constants of WPC from $2.5 \times 10^{-8}$ M to $3.9 \times 10^{-8}$ M for heptanone, from $2.2 \times 10^{-8}$ M to $3.1 \times 10^{-8}$ M for octanone, and from $1.9 \times 10^{-8}$ M to $2.7 \times 10^{-8}$ M for nonanone. The increases in the apparent dissociation constants of WPC for the methyl ketones may be due to the formation of aggregates in WPC, which affects the accessibility of the binding sites. However, HHP treatments of 30 min resulted in decreases in the apparent dissociation constants of WPC for heptanone, octanone, and nonanone. Dissociation constants returned close to the values of the WPC for these three methyl ketones. It seems that additional conformational changes occurred during 30 min of HHP treatment, which compensated for the effects of aggregates on the binding affinity.

Tanaka et al. (1996) reported the presence of ‘hard binding sites’ and ‘soft binding sites’ of a protein. When a hydrophobic ligand binds to incompressible ‘hard binding sites’ of a protein, the binding is destabilized under high pressure (Torgerson et al., 1979). At elevated pressure, an apolar ligand bound at a hard binding site is replaced with incompressible water because the total compressibility of the system is reduced when the compressible ligand is not in a hard cage. On the other hand, pressure-stabilized binding is the characteristic of ‘soft binding
sites’. In this case, incompressible water in the soft cage is replaced with the compressible ligand to reduce the dimensions under high pressure (Tanaka et al., 1996). Therefore, the changes in the binding affinity and the number of binding sites we observed during pressurization may relate to changes that happened around these two types of binding sites. HHP may create and modify the ‘soft binding sites’ and increase the binding affinity and the number of the binding sites of WPC to flavor compounds. On the other hand, HHP may decrease the accessibility of ligands to the ‘hard binding sites’ in WPC.

**Headspace analysis**

The effect of high pressure on the retention of flavors by WPC was studied by static headspace analysis. Static headspace analysis measures the volatiles contained in the air above a food, usually in a sealed system at equilibrium. The composition of the headspace depends on the partitioning of volatiles between the air phase and the different phases present in the food (such as oil and water). Other factors that affect partitioning of the compounds between the air and the food, such as surface area and temperature, also influence the headspace composition (Taylor and Linforth, 1996).

The volatility of the flavor compounds decreased in the presence of WPC (Figures 12 through 19), mainly due to hydrophobic interactions between the flavors and the proteins (Guichard, 2000). Many researchers observed increases in flavor retention in the presence of whey proteins (Guichard and Langourieux, 2000). Androit et al. (1999) reported that addition of β–LG reduced the perceived aroma intensity of methyl ketones in aqueous solutions and increased the retention of methyl ketones. Marin et al. (1999) observed an increase in the retention of benzaldehyde in β–LG solution.
The retentions of flavors by WPC in decreasing order are: nonanone > octanone > heptanone > benzaldehyde for flavor concentrations of both 100 and 200 ppm. The percentage of retention of 200 ppm benzaldehyde, heptanone, octanone, and nonanone were 18.5%, 27.9%, 38.5%, and 40.5%, respectively. The retention of 100 ppm benzaldehyde, heptanone, octanone, and nonanone were higher, at 19.3%, 32.2%, 38.6%, and 50.7%, respectively. The percentage of retention of benzaldehyde and the methyl ketones is consistent with previously reported results for the retention of flavors in β–LG solution (Roozen and Legger, 1997; Jouenne and Crouzet, 1996; Charles et al., 1996; Espinoza and Voilley, 1996).

For benzaldehyde at both 100 ppm and 200 ppm, HHP treatments did not result in significant changes in the flavor retention in WPC solutions (Figures 12 through 13), although the results from the fluorescence titration showed decreases in the dissociation constants of WPC for benzaldehyde (Table 1). The amount of volatiles released to the gaseous phase is influenced by many factors of the flavor compounds, such as vapor pressure, solubility, concentration, partitioning of volatile among air and water phase, and interactions with other food constituents (Landy et al., 1996; Kinsella, 1990). The discrepancy may also arise from the fact that headspace experiments of flavor and WPC solutions were equilibrated at 37°C instead of room temperature, which was used for the fluorescence titration experiments.

Flavor retention of 200 ppm heptanone, octanone, and nonanone in HHP treated WPC solutions for 10 min was significantly lower than in untreated WPC and HHP treated WPC for come-up time and 30 min (Figure 14, Figure 16, and Figure 18). The decreases in retention of the methyl ketones in WPC solutions after 10 min of HHP treatment are consistent with the results from the fluorescence titration (Table 1). Significant decreases in flavor retention at 200 ppm were only observed for heptanone and octanone in WPC solutions after HHP treatment for 10
min, while no significant differences in flavor retention of nonanone at 100 ppm were observed among the untreated WPC and HHP treated WPC (Figure 15, Figure 17, and Figure 19). The decreases in retention of the methyl ketones in HHP treated (10 min) WPC solutions may be caused by conformational changes of whey proteins or formation of aggregates which decrease the binding affinity of the methyl ketones for WPC.

Static headspace analysis utilizes sealed system and allows equilibrium to be attained. This approach simplifies the analytical procedure, but it is doubtful whether equilibrium is actually achieved when food is eaten (Taylor and Linforth, 1996). Time-intensity assessment of flavor release may provide useful information regarding flavor perception (Bakker et al., 1996), and more research is needed to explore the potential of application of HHP treatment to improve the flavor-binding properties of WPC in food applications.

CONCLUSIONS

HHP treatments increased the number of binding sites and the apparent dissociation constants of WPC for benzaldehyde as observed by intrinsic fluorescence titration. HHP treatment for 10 min increased the binding affinity of WPC for diacetyl, but no significant changes in the number of binding sites were observed after 10 min of HHP treatment. HHP treatment for come-up time resulted in increases in the number of binding sites of WPC for heptanone and octanone. HHP treatments of 10 min resulted in an increase in the apparent dissociation constant of WPC from $2.5 \times 10^{-8}$ M to $3.9 \times 10^{-8}$ M for heptanone, from $2.2 \times 10^{-8}$ M to $3.1 \times 10^{-8}$ M for octanone, and from $1.9 \times 10^{-8}$ M to $2.7 \times 10^{-8}$ M for nonanone. No changes in the number of binding sites of WPC for nonanone were observed after HHP treatment.
As observed by headspace analysis, HHP treatments did not result in significant changes in the retention for benzaldehyde in WPC solutions. Retention of 100 ppm and 200 ppm heptanone and octanone in HHP treated (10 min) WPC solutions was significantly smaller than in untreated WPC and HHP treated WPC for come-up time and 30 min. However, for retention of nonanone in HHP treated (10 min) WPC solutions, significant decreases were only observed at 100 ppm. These results suggest that HHP treatments of 0.2% WPC can modify the flavor-binding properties of WPC; the effects depend on flavors and HHP treatment time.

In both fluorescence titration and static headspace analysis, equilibrium was attained. While eating, flavor profile and sensors respond to the pattern of flavor release with time (Taylor and Linforth, 1996). Further research is required to fully understand the effects of HHP treatment on flavor-binding and flavor release properties of WPC and to evaluate the full potential of this process in the food industry.

**ABBREVIATIONS USED**

β-LG, β-lactoglobulin; HHP, high hydrostatic pressure; WPC, whey protein concentrate; ANS, 1-anilino-naphthalene-8-sulfonate; CPA, *cis*-parinaric acid; MG, molten globule.
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Figure 1. Structures of flavor compounds used in fluorescence binding studies.
Table 1. Apparent dissociation constants ($K'_d$) and the number of flavor binding sites ($n$) of WPC and WPC after HHP treatment (600 MPa and 50°C) for holding time of 0, 10 or 30 min (H0, H10 and H30) calculated using the method by Cogan et al. (1976)*.

<table>
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<tr>
<th>Ligands</th>
<th>WPC</th>
<th>$n^1$</th>
<th>$K'_d$ (M)$^2$</th>
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<td></td>
<td>H 0</td>
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<td>H 10</td>
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<td>H 30</td>
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<td>4.7×10$^{-8}$ M$^b$</td>
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<tr>
<td></td>
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<td>Nonanone</td>
<td>Untreated</td>
<td>0.20</td>
<td>1.9×10$^{-8}$ M$^a$</td>
</tr>
<tr>
<td></td>
<td>H 0</td>
<td>0.19</td>
<td>2.1×10$^{-8}$ M$^a$</td>
</tr>
<tr>
<td></td>
<td>H 10</td>
<td>0.22</td>
<td>2.7×10$^{-8}$ M$^b$</td>
</tr>
<tr>
<td></td>
<td>H 30</td>
<td>0.23</td>
<td>1.8×10$^{-8}$ M$^a$</td>
</tr>
</tbody>
</table>

*: Data are means of three analyses calculated using method by Cogan et al. (1976).

$^1$, $^2$: means with different letters in the column are significantly different (p<0.05).
Figure 2. Fluorescence titration curves for WPC and HHP treated (600 MPa and 50°C) WPC with 0, 10 or 30 min holding time (H0, H10, and H30) with benzaldehyde.

Figure 3. Fluorescence titration curves for WPC and HHP treated (600 MPa and 50°C) WPC with 0, 10 or 30 min holding time (H0, H10, and H30) with diacetyl.

Figure 4. Fluorescence titration curves for WPC and HHP treated (600 MPa and 50°C) WPC with 0, 10 or 30 min holding time (H0, H10, and H30) with heptanone.
Figure 5. Fluorescence titration curves for WPC and HHP treated (600 MPa and 50°C) WPC with 0, 10 or 30 min holding time (H0, H10, and H30) with octanone.

Figure 6. Fluorescence titration curves for WPC and HHP treated (600 MPa and 50°C) WPC with 0, 10 or 30 min holding time (H0, H10, and H30) with nonanone.
Figure 7. Benzaldehyde binding to WPC or HHP treated WPC (600 MPa and 50°C) with 0, 10 or 30 min holding time (H0, H10, and H30) plotted by Cogan method (Cogan et al., 1976) to calculate $K'_d$ and n.
Figure 8. Diacetyl binding to WPC or HHP treated WPC (600 MPa and 50ºC) with 0, 10 or 30 min holding time (H0, H10, and H30) plotted by Cogan method (Cogan et al., 1976) to calculate $K'_d$ and n.
Figure 9. Heptanone binding to WPC or HHP treated WPC (600 MPa and 50°C) with 0, 10 or 30 min holding time (H0, H10, and H30) plotted by Cogan method (Cogan et al., 1976) to calculate $K'_d$ and $n$. 
Figure 10. Octanone binding to WPC or HHP treated WPC (600 MPa and 50°C) with 0, 10 or 30 min holding time (H0, H10, and H30) plotted by Cogan method (Cogan et al., 1976) to calculate $K'_d$ and n.
Figure 11. Nonanone binding to WPC or HHP treated WPC (600 MPa and 50°C) with 0, 10 or 30 min holding time (H0, H10, and H30) plotted by Cogan method (Cogan et al., 1976) to calculate $K'_o$ and n.
Figure 12. Static headspace analysis of benzaldehyde (200 ppm) in WPC or HHP treated WPC (600 MPa and 50°C) with 0, 10 or 30 min holding time (H0, H10, and H30).

Figure 13. Static headspace analysis of benzaldehyde (100 ppm) in WPC or HHP treated WPC (600 MPa and 50°C) with 0, 10 or 30 min holding time (H0, H10, and H30).
Figure 14. Static headspace analysis of heptanone (200 ppm) in WPC or HHP treated WPC (600 MPa and 50°C) with 0, 10 or 30 min holding time (H0, H10, and H30).

Figure 15. Static headspace analysis of heptanone (100 ppm) in WPC or HHP treated WPC (600 MPa and 50°C) with 0, 10 or 30 min holding time (H0, H10, and H30).
Figure 16. Static headspace analysis of octanone (200 ppm) in WPC or HHP treated WPC (600 MPa and 50°C) with 0, 10 or 30 min holding time (H0, H10, and H30).

Figure 17. Static headspace analysis of octanone (100 ppm) in WPC or HHP treated WPC (600 MPa and 50°C) with 0, 10 or 30 min holding time (H0, H10, and H30).
Figure 18. Static headspace analysis of nonanone (200 ppm) in WPC or HHP treated WPC (600 MPa and 50°C) with 0, 10 or 30 min holding time (H0, H10, and H30).

Figure 19. Static headspace analysis of nonanone (100 ppm) in WPC or HHP treated WPC (600 MPa and 50°C) with 0, 10 or 30 min holding time (H0, H10, and H30).
CHAPTER SIX
CONCLUSIONS

High hydrostatic pressure (HHP) treatment at 600 MPa and 50°C for 30 min of whey protein concentrate (WPC) resulted in a decrease in protein solubility at pH 4.6 an increase in aggregation and denaturation of whey proteins, especially at high WPC concentrations. During the come-up time of the HHP treatment, dissociation of aggregates and formation of β-lactoglobulin (β-LG) dimers were observed. With increasing HHP treatment time, monomers of β-LG, α-lactalbumin (α-LA), and bovine serum albumin (BSA) decreased, and aggregates were formed. Overall, these results suggest that protein solubility, denaturation and aggregation of HHP treated WPC are dependent on solution concentration and HHP treatment condition.

HHP treatments resulted in an increase in tryptophan intrinsic fluorescence intensity and a 4 nm red shift after 30 min of treatment, which indicate changes in the polarity of tryptophan residues microenvironment of whey proteins from a less polar to a more polar environment. HHP treatment of WPC for 30 min resulted in an increase in the number of binding sites for 1-anilino-naphthalene-8-sulfonate (ANS) from 0.16 to 1.10 per molecule of protein. No significant changes in the apparent dissociation constant of WPC for ANS were observed after HHP treatments except for an increase from $1.8 \times 10^{-5}$ M to $3.3 \times 10^{-5}$ M after 30 min. There were no significant changes in the number of binding sites of WPC for cis-parinaric acid (CPA) after HHP treatments. However, increased binding affinities of WPC for CPA were observed after the come-up time or 10 min HHP treatment, with a decrease of apparent dissociation constant from $2.2 \times 10^{-7}$ M to $1.1 \times 10^{-7}$ M. The binding sites of WPC may become more accessible to the aliphatic hydrophobic probe CPA after come-up time or 10 min HHP treatment. These results
indicate that HHP affects the hydrophobicity of whey proteins. Aliphatic hydrophobicity is enhanced after the come-up time and 10 min HHP treatment.

HHP treatments increased the number of binding sites and the apparent dissociation constants of WPC for benzaldehyde. HHP treatment for 10 min increased the binding affinity of WPC for diacetyl, but no significant changes in the number of binding sites were observed after 10 min of HHP treatment. There were increases in the number of binding sites of WPC for heptanone and octanone after HHP treatment for the come-up time. HHP treatments of 10 min resulted in an increase in the apparent dissociation constant of WPC from $2.5 \times 10^{-8}$ M to $3.9 \times 10^{-8}$ M for heptanone, from $2.2 \times 10^{-8}$ M to $3.1 \times 10^{-8}$ M for octanone, and from $1.9 \times 10^{-8}$ M to $2.7 \times 10^{-8}$ M for nonanone.

As observed by headspace analysis, HHP treatments did not result in significant changes in the retention for benzaldehyde in WPC solutions. Flavor retention of 100 ppm and 200 ppm heptanone and octanone in HHP treated (10 min) WPC was significantly lower than in untreated WPC and HHP treated WPC for come-up time or 30 min. Significant decreases were only observed at 100 ppm for flavor retention of nonanone in HHP treated (10 min) WPC solutions. These results suggest that HHP treatments of 0.2% WPC can modify the flavor-binding properties of WPC; the effects depend on flavors and HHP treatment time. Further research is required to evaluate the full potential of application of HHP to modify functional properties of WPC and its benefits to the food industry.