ABSTRACT

LILLARD, JOHN STEPHEN. Expanding the Utility of a Modified Whey Protein Ingredient via Carbohydrate Conjugation. (Under the direction of Dr. Christopher R. Daubert.)

Whey proteins are commonly used in foods for their thickening and emulsifying properties. As a one time by-product of the cheese making process, roughly 30% of whey protein still remains unused. Therefore, the need for ingredients with novel functionality provides the impetus to modify whey proteins for desired performance. Previously, an acidified, thermally treated whey protein concentrate (MWPC) was developed to produce a cold-set, thickening ingredient. For these studies, a MWPC was obtained from a whey manufacturer which contained around 7% lactose post processing. In an attempt to optimize the functionality, the Maillard reaction was employed to covalently couple, or conjugate, the MWPC with two separate dextrans (35kDa and 200kDa) under varying thermal conditions at pH 3.5. A commercially available whey protein concentrate (CWPC) was studied along with the modified ingredient for comparison purposes.

Maillard reactivity was confirmed through biochemical analyses including colorimetry, *o*-phthaldialdehyde assays (OPA), sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and ionization mass spectrometry. Colorimetry showed that the MWPC containing only lactose exhibited the greatest degree of color formation as a result of Maillard end products generated during heat treatment. These results were complemented by *o*-phthaldialdehyde assay data that showed a decrease in the level of free amino groups in all samples after thermal treatment, an indicator that covalent attachment of the saccharides had occurred. The SDS-PAGE banding profiles also displayed new and diffuse higher molecular weight bands formed after coupling as detected by protein (Coomassie) and glycoprotein staining methodologies. Ionization

mass spectrometry results revealed the lysine content in the MWPC to be 8.5% lactosylated as a result of the manufacturing process, itself.

Once verification of glyco-conjugation was established rheological analysis, as well as, emulsion stability, emulsifying capacity, and water holding capacity measurements were completed to assess possible functional benefits. Changes in both steady shear and small amplitude oscillatory rheology were dependant on the reducing sugar present. The MWPC samples containing dextran prior to heat treatment exhibited a 3.4 times greater apparent viscosity over the MWPC alone. After heat treatment, however, the apparent viscosity of the MWPC-dextran samples showed no increase over non-heated samples while the viscosity of the MWPC alone increased 3-fold measured at 50 s^{-1}. Excluded volume interactions due to the high proportions of polysaccharide present in the MWPC-dextran samples may have played a role in the rheological impact. The emulsion stability of MWPC-dextran fractions were 2 to 3 times greater than either MWPC alone or CWPC, based on the creaming index. The higher solids content and increased molecular size of the MWPC-dextran conjugate may have increased the steric stabilization of the system, and enhanced the stability of the emulsion. Emulsifying capacity measurements showed minimal differences between the samples with a slight improvement noted in MWPC-dextran (100 kDa to 200 kDa) samples. Although the water holding capacity of all samples decreased upon additional heat, the MWPC-dextran samples still held nearly six times their weight of water. Scanning electron microscopy was used to examine the microstructure of each sample and porous network formation was observed upon the addition of dextran which differed from the dense network observed in the MWPC sample, alone. The porous nature of the new network could have affected both rheological properties and the ability of MWPC-dextran samples to bind water.

It was concluded that modified whey ingredients can be further enhanced by conjugation with carbohydrates through non-toxic means such as the Maillard reaction. The specific application into which the ingredient will be introduced will dictate which carbohydrate is most appropriate to obtain the desired functionality.

EXPANDING THE UTILITY OF A MODIFED WHEY PROTEIN INGREDIENT VIA CARBOHYDRATE CONJUGATION

by
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DEDICATION

I dedicate this thesis to my beloved wife Marcy. You are my best friend, confidant, and the shoulder I look for to lean on. It's not only the words you choose but the actions you take that are a constant inspiration to me. Chicken Kitchen will always be the meal that sparked the fire and I am eternally grateful for you and that moment.

BIOGRAPHY

John Stephen Lillard was born on June 11, 1974 in Columbia, Missouri to Stephen and Katie Lillard. Soon after he was born John moved to St. Louis, Missouri and spent most of his grade school years there before moving to St. Charles, Missouri where he graduated from Duchesne High School in June of 1992. John then ventured on to begin his undergraduate work at Palm Beach Community College in West Palm Beach, Florida. After completion of his Associate of Arts Degree he went on to complete his Bachelor's Degree at the University of Florida in Gainesville, Florida. Gainesville provided John with the perfect microcosm for growth and a friend base he would carry to this day. John also worked closely with Dr. Bobbi Langkamp-Henken while at the University of Florida on a study she was conducting regarding arginine and immune function in the elderly. John would later take the skills he acquired while working with Dr. Langkamp-Henken and apply them towards work at Palm Beach Gardens Medical Center as Assistant Food Service Director.

John spent two years at Palm Beach Gardens Medical Center before trying his at hand at the business world as he forayed into telecom by becoming an Account Executive for BellSouth. His time at BellSouth helped to increase both his business acumen and his interpersonal communication skills. After five years John decided he missed the food industry and found a job that allowed him to utilize both his business skills, as well as, his nutritional background when he went to work for Balance for Life. While working at Balance for Life John began to realize the large gap in nutritional education and healthy food choices available to the majority of Americans. This realization along with some prodding from his supportive wife Marcy provided John with the impetus to return to school. After a meeting with Dr. Christopher R. Daubert, John

decided NC State University would be the perfect launching pad for a career in product development under Dr. Daubert's direction.

ACKNOWLEDGEMENTS

I would like to begin by thanking my advisor Dr. Daubert for making me feel immediately comfortable when we first met over 2 years ago. The moment I shook his hand after our first meeting I knew I had made the right choice in coming to NC State. Luckily, it appears Dr. Daubert thought I was a good fit for his rheology team and I will be forever indebted for his continual support, guidance, and dry sense of humor that was a nice complement to mine I might add. I also thoroughly enjoyed my time spent with his beloved pug Ulysses.

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To my uncle Pat and aunt Laurene for supporting all the crazy roads I travel.

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To Yifat, my Jewish connection, thanks for making the holidays special and getting me through FS 785.

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CHAPTER 1. LITERATURE REVIEW

1.1 Introduction

Proteins are a common food ingredient used by food technologists based on their emulsifying, stabilizing, and gelation properties. Modifications are often made to proteins to further enhance their functional benefits. Whey proteins in particular are often used because of their high nutritional value and their ability to provide clean labeling as a result of their classification as a dairy based ingredient. Previously, whey protein modification accomplished by thermal treatment, pH adjustments, and/or covalent attachment of carbohydrates lead to increased functionality over non-modified ingredients (Dickinson and Semenova, 1992; Dickinson and Izgi, 1996; Hudson *et al*., 2001; Mishra *et al*., 2001; Akhtar and Dickinson, 2003; Einhorn-Stoll *et al*., 2005). Therefore, the goal of the following literature review is to describe the components involved in protein modification as well as explain the methods used to assess increased functionality as a result of these modifications.

1.2 Proteins

Proteins are essential nutrients to human function and are comprised of amino acids linked by peptide bonds (Figure 1).

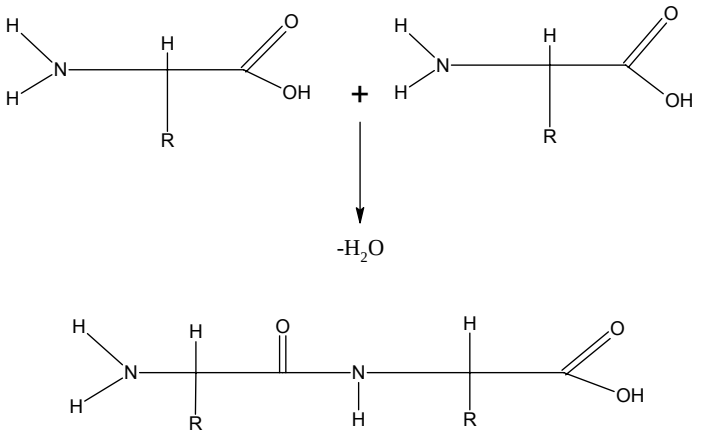

Figure 1. Formation of a peptide bond.

Each amino acid has a unique configuration comprised of a basic amino group and an acidic carboxyl group linked by a hydrocarbon chain. The primary structure of a protein is determined by the sequence of amino acids occurring along its backbone. Protein conformation, or three dimensional protein structure; contains secondary, tertiary, and quaternary components (when more than one component is present) (Figure 2).

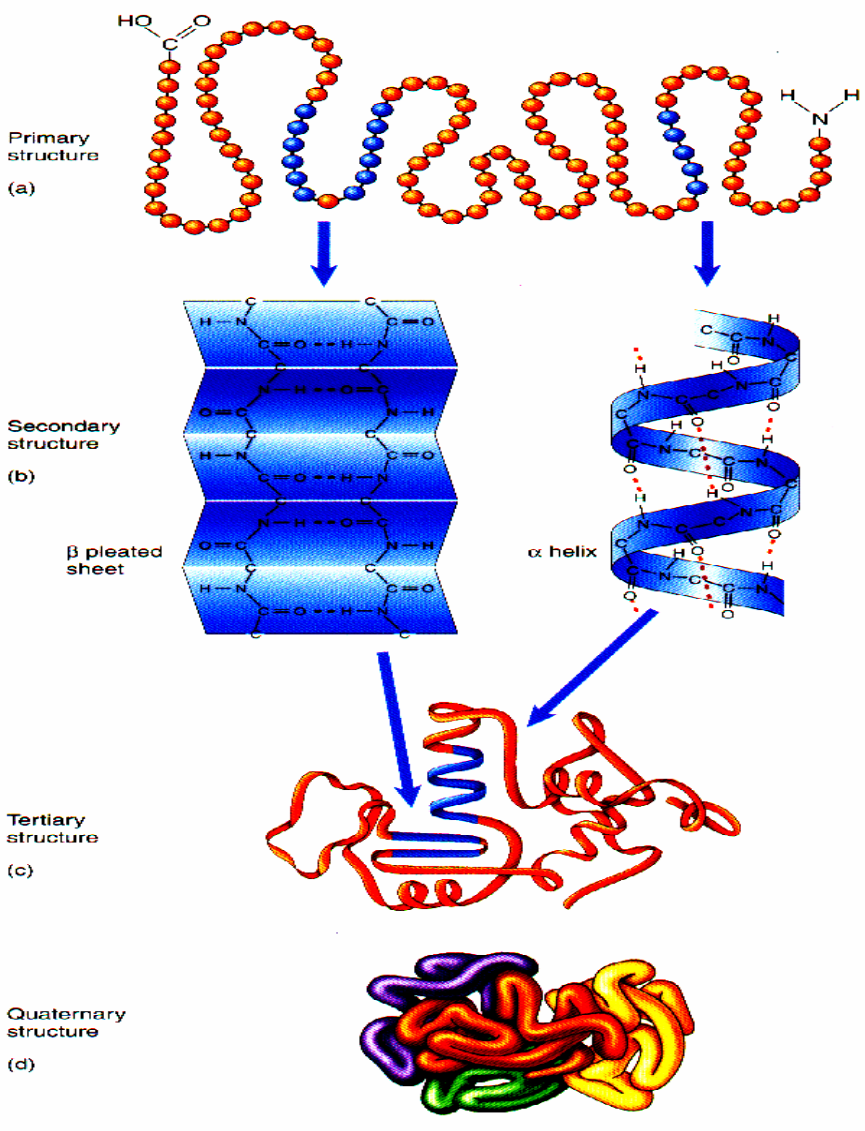

Figure 2. Schematic of primary, secondary, tertiary, and quaternary protein structure. academic.brooklyn.cuny.edu/.../page/3d_prot.htm

The linear sequence of amino acid residues may form alpha-helices or β-pleated sheets held together by hydrogen bonds, designated as secondary structure. The tertiary structure denotes an overall shape or folding pattern with linear polypeptide chains referred to as fibrous proteins, and tightly coiled spherical configurations termed globular proteins. Tertiary interactions within globular proteins, such as those found in whey, are stabilized by intramolecular hydrophobic forces, typically folded within the protein core, while the charged or hydrophilic residues on the protein surface are water-exposed. Disulfide bonds can form between cysteine residues also contributing to the protein tertiary structure. Whey proteins have an abundance of cysteine (SH) residues that promote intermolecular covalent bond formation, during high temperature processing, which play a significant role in heat-induced gelation or foam stabilization (Morr and Ha, 1993). The quaternary structure refers to the association of two or more polypeptide chains held together by non-covalent interactions, forming larger protein moieties and dictating function. Herein, the focus of these discussions will be directed towards whey protein and its combination with suitable carbohydrates leading to enhanced functionality.

1.2.1 WHEY PROTEINS

Bovine milk is comprised of two major protein types, whey and casein. When bovine milk is acidified to its isoelectric point of pH 4.6, at about 30°C, 80% of the protein precipitates and this is casein. The remaining 20% of the protein is soluble and defined as whey (Fox and McSweeney, 1998). Previous studies have helped highlight the nutritional and functional benefits whey proteins can impart both in food products and independently (Morr and Foegeding, 1990, Morr and Ha, 1993).

Presently, there are four main commercial methods to produce whey protein ingredients: 1) ultrafiltration/diafiltration of acid or rennet whey; 2) ion-exchange chromatography, 3) demineralization by electrodialysis and/or ion exchange, and 4) thermal denaturation. The first method, ultrafiltration/diafiltration of acid or rennet whey, removes lactose, leaving whey protein concentrates at 30-80% protein after spray drying (Figure 3). Ion-exchange chromatography of proteins occurs through absorption of the protein onto an ion-exchanger where the protein is then washed free of lactose and salts. The protein is then spray dried to produce whey protein isolates at about 95% purity. Demineralization and thermal denaturation steps are less common and produce lower functional yields (Fox and McSweeney, 1998).

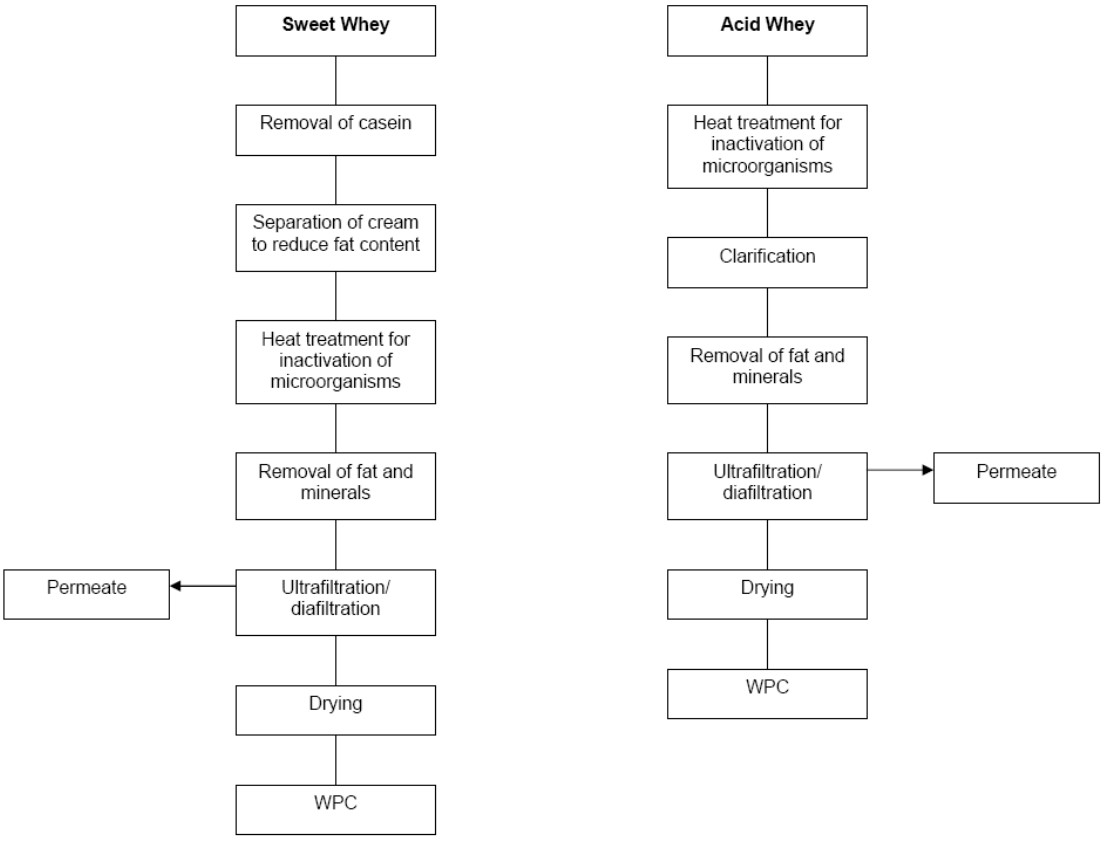

Figure 3. Separation process for manufacturing whey protein concentrate from acid and sweet whey (redrawn from http://www.apv.com).

Whey proteins can be obtained in powdered forms and vary in protein content. Whey protein concentrates (WPC) contain between 30-80% protein in addition to other milk components such as lactose and fat. In contrast, whey protein isolates (WPIs) generally contain as much as 90-96% protein and are often devoid of lactose and other small molecular weight entities (Brink, 2006). Both whey protein isolates and concentrates are comprised of four major proteins; β-lactoglobulin, α-lactalbumin, bovine serum albumin (BSA), and immunoglobulins (Broihier, 1999). The two proteins in highest proportion being β-lactoglobulin and α-lactalbumin are discussed below.

Beta-lactoglobulin (β-Lg) is the most abundant of the whey proteins comprising about 50% or greater of the total protein content (Morr and Ha, 1993). Beta-lactoglobulin consists of 162 amino acid residues, exhibiting a molecular weight (MW) of 18.3 kDa (Farrell *et al*., 2004). The isoelectric point, or point at which β-Lg carries no net charge, is about 5.2. At a pH between 5.2 and 7.5, β-Lg exists as a 36.7 kDa dimer, whereas below a pH of 3.5 or above 7.5, the dimer dissociates to a monomer. Between pH 3.5 and 5.2 β-Lg exists as an octomer (Swaisgood, 1982). The denaturation of β-Lg takes place above 65ºC thereby exposing sulfhydryl and ε-amino groups (Kinsella, 1984). Disulphide bonds can then be formed between the free sulfhydryl groups which in turn help to maintain structural integrity by lowering entropy and increasing the stability of the protein (Anfinsen, C.B. and Scheraga, H.A., 1975). Chevalier *et al*. (2001) studied the effects of attaching various sugars to β-Lg with respect to protein solubility, heat stability, emulsification, and foaming properties. Glycation of β-Lg with highly reactive pentose sugars, such as arabinose or ribose, improved emulsifying properties while foaming properties were improved when glycation occurred with hexose sugars, such as glucose or galactose. The amino acid lysine appears to be the preferred binding site for glycation

and in 1997 Leonil *et al*. found lysine residues (at positions 47 and 60) to be the reactive sites for conjugation to the carbohydrate lactose (Figure 4).

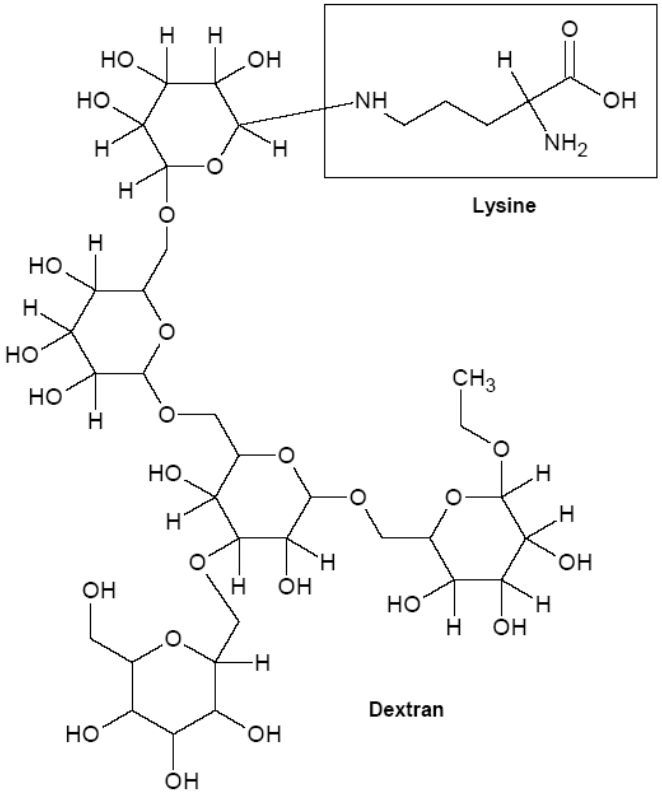

Figure 4. Schematic of lysine attached to dextran.

The whey protein component present in the second highest amount is α-lactalbumin (α-La) representing about 20% of total whey proteins (Morr and Ha, 1993). Alpha-lactalbumin is comprised of 123 amino acid residues and exhibits a MW of 14 kDa (Farrell *et al*., 2004). Alpha-lactalbumin is also known to be heat stable, attributed to the high potential for renaturation. A study by Chaplin and Lyster (1986) showed that heating α-La to 77ºC, and then immediately cooling it, gave rise to only 10% irreversible protein

denaturation. Although α-La may exhibit a high heat resistance, manufacturing techniques must be taken with great care or detrimental effects may occur, leading to low solubility and a non-functional product (Morr and Ha, 1993).

1.2.2 WHEY PROTEINS IN FOODS

Whey proteins serve as complete sources of essential amino acids, ideally suited for developing protein-enhanced foods (Hazen, 2003). The biological value or ability of the body to utilize a protein can be determined through measurement of nitrogen intake vs. nitrogen excretion of subjects on a test diet (Mitchell, 1923). Whey proteins have been shown to have the highest biological value over eggs, soy protein and wheat (Haines, 2005), attesting to the nutritive value of whey proteins. Furthermore, whey proteins are known to be among the richest source of branched chain amino acids (BCAAs), including leucine, isoleucine, and valine, which are essential amino acids.

Whey products can improve the overall functionality and nutrient delivery of foods which is a major benefit to food manufacturers. When introduced into infant formulas, whey can simulate human milk and stimulate the growth of beneficial bacteria in the intestinal tract (de Wit, 1998). When added to low-fat dairy products whey can be used to replace the fat content (McMahon *et al*., 1996).

Whey proteins are also frequently used as emulsifiers and stabilizers in dairy foods such as ice cream to prevent aggregation and coalescence (Euston *et al*., 2001). Whey stabilizes emulsions by forming a visco-elastic, adsorbed layer on oil droplets, resulting in a physical barrier to coalescence. Once adsorbed, the protein unfolds rearranging secondary and tertiary structures leading to the exposure of hydrophobic residues to the hydrophobic phase such as oil (Wilde *et al*., 2004).

1.2.3 Physicochemical Properties of Whey Proteins

The function of whey proteins in foods is governed through multiple chemical and physicochemical properties. Solubility may be the greatest property of native or non-denatured whey proteins occurring over a wide range of concentrations, pH, temperature, and ionic conditions (Morr and Ha, 1993). Processing treatments of native whey proteins to make WPCs, however, can lead to molecular unfolding and denaturation of the proteins which may diminish solubility. The reduced solubility is a causal effect of the proteins aggregating amongst themselves or other food particles (Kruif and Tuinier, 1999). Surface chemistry such as molecular chain flexibility, number and distribution of charged residues, reactive sulfhydryl residues and the number and distribution of hydrophobic and hydrophilic residues can impact viscosity and emulsifying capabilities (Morr and Ha, 1993). When a polysaccharide such as dextran is introduced into a system containing a whey protein component, phase separation can occur due to depletion interactions (Smith and Shaink, 1995). These interactions are a result of a depletion of polymer from a region of neighboring colloidal particles (Figure 5). This depleted region causes the osmotic pressure to be smaller than in the bulk leading to an attraction between the molecules (Tuinier *et al*., 2000). The mixing of proteins and polysaccharides can lead to incompatibility which is manifested in aggregative or segregative phase separation. Aggregative phase separation is where polymer-polymer interactions are favored over polymer-solvent interactions and segregative phase separation is when polymer-solvent interactions are favored over polymer-polymer interactions.

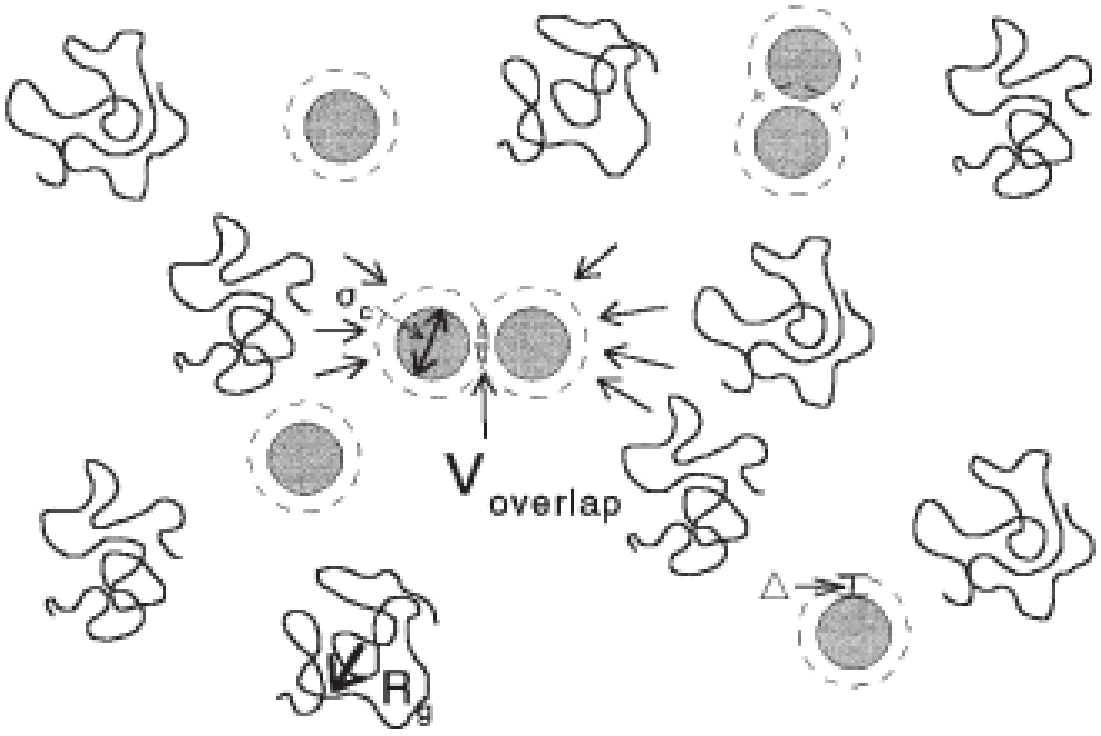

Figure 5. Schematic of depletion interaction induced by a polysaccharide of radius R_g interacting with a whey protein colloid of diameter σ_c. (Taken from de Kruif and Tuinier, 1999).

Increases in polysaccharide or protein can lead to an unstable mixture where separation into a polysaccharide rich phase and a protein rich phase can occur (de Kruif and Tuinier, 1999). Figure 6, shows the phase diagram associated with a stable mixture migrating to an unstable region as the concentrations of proteins and polysaccharide are increased. Since proteins and polysaccharides play a distinct role in both the texture and structure of foods, their separation in food systems can potentially lead to adverse affects on functionality (Tuinier *et al.*, 2000)

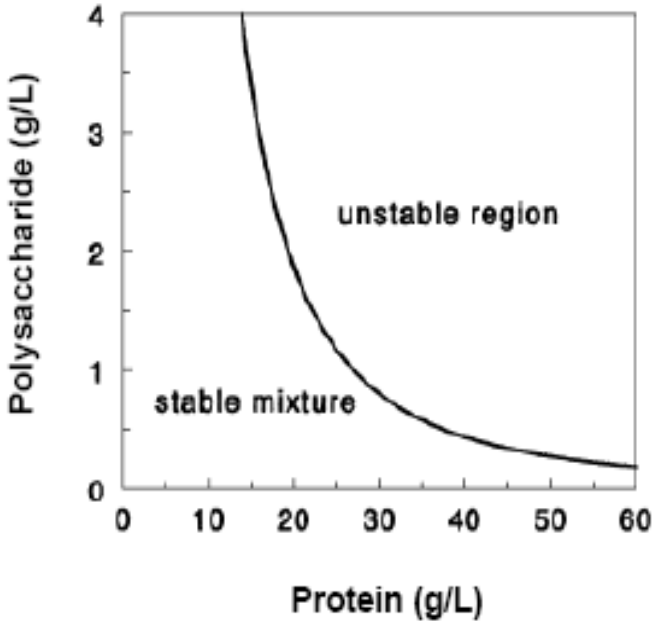

Figure 6. Phase diagram of a protein and polysaccharide mixture showing binodal separation of a stable and unstable region as concentrations of protein and polysaccharide are increased. (Redrawn from de Kruif and Tuinier, 1999).

1.2.4 Lysine

Lysine is an essential amino acid obtained from several foods including milk, meat, cheese, fish, and soybeans. Like the BCAAs, lysine is an essential amino acid because it is required for human health but cannot be manufactured by the body. Lysine is important for proper growth, playing an essential role in the production of carnitine, a nutrient responsible for converting fatty acids into energy and lowering cholesterol. Lysine helps the body absorb and conserve calcium, fulfilling an important role in the formation of collagen, a substance important for bones and connective tissues including skin, tendon, and cartilage (Civitelli *et al*., 1992).

When whey proteins are heated, lysine is known to be readily reduced by the Maillard reaction at pH 6.75 (Schaafsma, 1989). Multiple studies have shown that Maillard-type carbohydrate–protein conjugates are produced when the *ε*-amino group of lysine residues or *N*-terminal amino groups of proteins react with the carbonyl group of

carbohydrates under controlled conditions of temperature, pH, and relative humidity (Nakamura *et al*., 1992a; Nakamura *et al*., 1992b).

1.3 Carbohydrates

Carbohydrates are found in a wide variety of foods such as fruits, vegetables, and grains and provide the most readily utilized source of energy for the body. Carbohydrates can be placed into two categories; simple and complex. Monosaccharides, such as glucose and fructose, as well as disaccharides, such as sucrose are considered simple carbohydrates while oligosaccharides (i.e., maltodextrin) and polysaccharides (i.e., dextran) are considered complex carbohydrates. Simple sugars such as glucose contain six carbon atoms, twelve hydrogen atoms, and six oxygen atoms that form ring structures. The hydroxyl groups (-OH) associated with the ring structure are known to be the chemically reactive site and once the rings are opened the aldehyde or ketone groups present can be exposed producing a reducing sugar (Potter, 1986). The reducing sugar can then interact with other constituents such as protein components to form varying compounds.

1.3.1 Dextran

The complex carbohydrate dextran is comprised of varying numbers of glucose units connected by glycosidic linkages. Dextran can form straight chains consisting of alpha 1,6 glycosidic linkages, and branches consisting of alpha 1,3 glycosidic linkages (Figure 7). Dextran fractions are readily soluble in water and electrolytic solutions, a process that appears to be independent of pH. Dextran solutions exhibit Newtonian flow characteristics, that is, the flow rate is independent of shear stress (Oene and Cragg, 1962). Since dextran is a neutral polysaccharide, the viscosity is not significantly influenced by changes in pH or salt concentrations. Therefore dextran represents a

desirable carbohydrate with respect to its potential for conjugation with whey proteins. Application of a non-toxic chemical modification, such as the Maillard reaction, to form glycoproteins is of great interest to the food industry based on potential improvements in functional characteristics (Akhtar and Dickinson, 2003). The combined covalent linkage of a carbohydrate, such as dextran to a suitable site on a protein, such as β-Lg, is often referred to as a glycoconjugate and its benefits are briefly outlined in the following section (Akhtar and Dickinson, 2003; Chevalier *et al*., 2001).

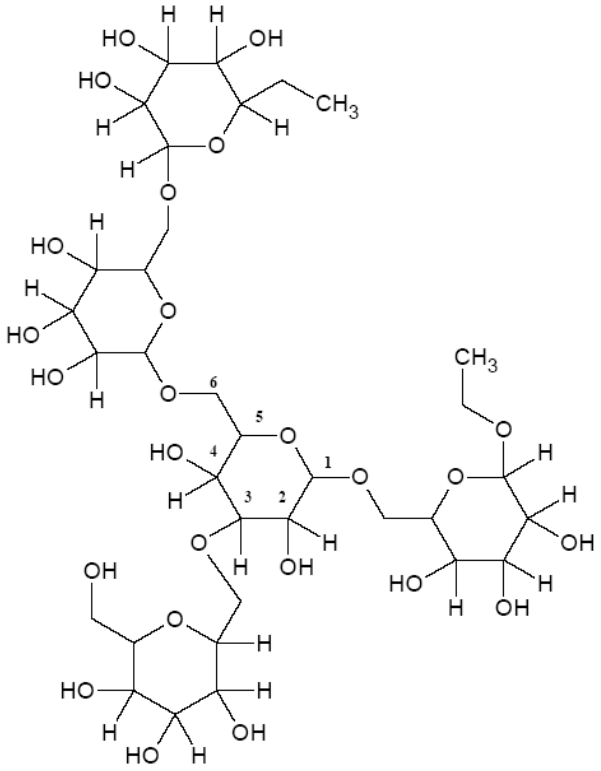

Figure 7. Schematic of dextran straight chains consisting of α1->6 glycosidic linkages, and branches consisting of α1->3 linkages

1.4 Glycoconjugates

Proteins are often used in foods as emulsifiers, while polysaccharides can be used as thickeners. Covalent linkage of the two by chemical means (Chen *et al*., 1989), or non-chemical means such as the Maillard reaction (Akhtar and Dickinson, 2003; Akhtar and Dickinson, 2007), produce high molecular weight biopolymers known as glycoconjugates (IUPAC, 1988). Creation of glycoconjugates is often achieved through conjugation or attachment of a suitable carbohydrate to a particular binding site on the protein. In a concentrated aqueous solution containing both a protein and a polysaccharide component with no association between the polymers, separation into two distinct phases will occur called 'simple coacervation' or thermodynamic incompatibility (Dickinson and Semanova, 1992). Multiple attempts have been made to improve the functionality of whey proteins through conjugation (Waniska and Kinsella, 1988; Bertrand-Harb *et al*., 1990; Dickinson, 1992; Akhtar and Dickinson 2003). Many of the positive benefits of producing Maillard generated conjugates include improvements in emulsifying activity, foaming properties, calcium complexing, solubility, and heat stability (Oliver *et al*., 2006). Recently, Akhtar and Dickinson (2007) found that glycoconjugates made with polysaccharides such as maltodextrin showed enhanced emulsifying properties as the molecular weight of the polysaccharide was decreased. This was attributed to the reduction in average droplet size obtainable with the lower molecular weight polysaccharides. Emulsion stability enhanced by the use of glycoconjugates can prevent aggregate formation which in turn prevents flocculation and coalescence, processes that can negatively lead to creaming (Dickinson, 1993).

1.5 Maillard Reaction

The Maillard reaction causes covalent attachment of a protein to a polysaccharide, and usually results when foods are processed at elevated temperatures or during prolonged periods of storage. The Maillard reaction can produce changes in color, flavor, and aroma, and the desirable effects may be found in such foods as beer, chocolate, and roast meats. The type of reducing sugar utilized in these reactions can greatly affect overall reactivity (Ames, 1990). Pentose sugars such as ribose react more readily than hexose sugars such as glucose. Both pentose and hexose sugars react more readily than disaccharides such as lactose.

The Maillard reaction is one of three reactions that can contribute to nonenzymatic browning along with caramelization and ascorbic acid oxidation. The Maillard process under acidic pH conditions, follows four main chemical sequences; A. sugar-amine condensation, B. Amadori rearrangement, C. sugar dehydration, and D. aldehyde-amine condensation (Figure 8).

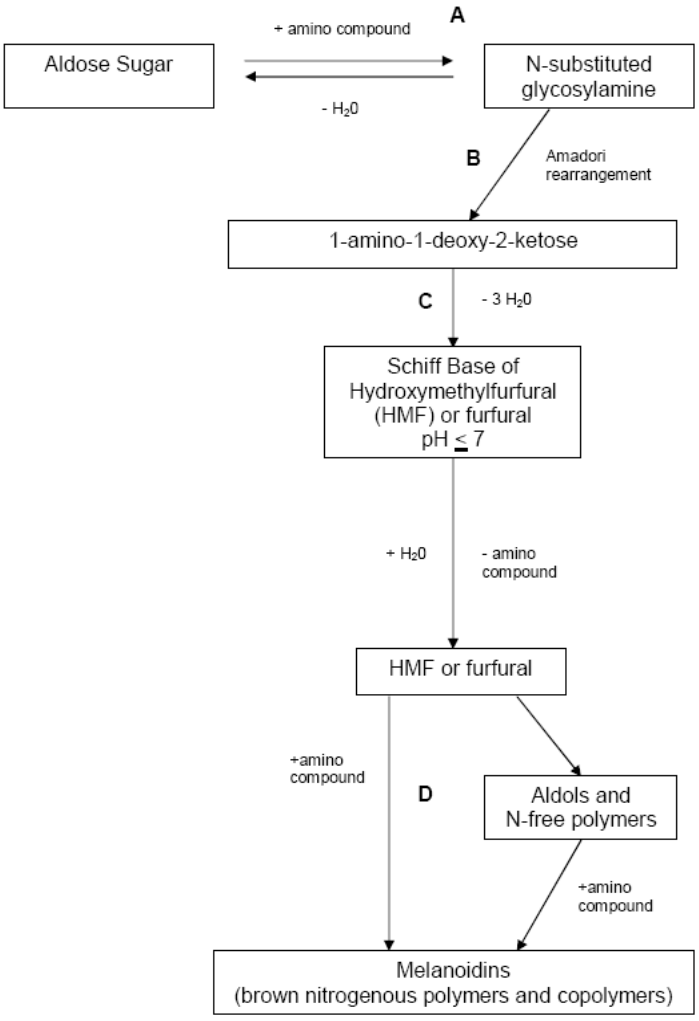

Figure 8. Reaction pathways for Maillard reaction at pH ≤ 7 (redrawn from Nursten 2005)

The first step involves sugar-amine condensation, a process initiated by the loss of a water molecule between the carbonyl group of an aldose sugar such as glucose and the free amino group of an amino acid, yielding an N-substituted aldosylamine. The electrophilic carbonyl group of the sugar is attacked by the nucleophilic NH_2 group of the reacting amino acid in which case, the product of this condensation step then goes through dehydration and is converted into a Schiff base (Nursten, 2005). At this stage, lysine complexes appear as ε-glycosylamine and remain nutritionally available (Mauron,

1981). Subsequently, Amadori rearrangements result in the formation of a ketosamine (Davies and Labuza, 2006), high amounts of water attributed to dehydration reactions are lost during this intermediate stage. Previously, Nursten (2005) suggested that browning was hindered in environments where the relative availability of water or water activity (a_w) was high due to a dilution of the reagents. Although the reactant concentration may increase at low a_w, mobility of the reactants may be lost leading to diminished browning.

Conjugation by a dry heating method is based on Amadori rearrangement steps in which terminal amines of the protein are linked to the reducing end of the polysaccharide. The Amadori rearrangement is considered to be the key step in the formation of major intermediates that cause browning effects, and overall the process is not reversible. Amadori products occur by one of three pathways depending on the pH conditions. At low pH, the reaction favors 1,2 enolisation via 3-deoxy-1,2-dicarbonyls while high pH values favor 2,3-enolisation via 1-deoxy-2,3-dicarbonyls (Nursten, 2005). Reactive lysine residues may become nutritionally unavailable as a result of the formation of ε-Amadori reaction products (Mauron, 1981).

Sugar dehydration follows Amadori rearrangement and dependent on the pH conditions, the reactants produce furfurals or reductones (Figure 9).

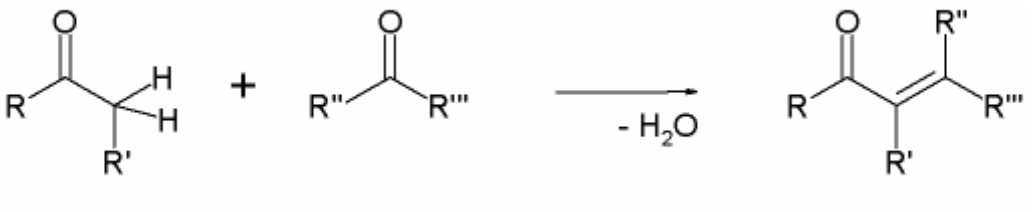

Figure 9. Sugar dehydration reaction

Under acidic conditions, D-xylose forms furfurals while D-glucose forms hydroxymethylfurfurals (HMF) due to the loss of three water molecules. The HMFs, themselves, are thought to exhibit low browning potential (Nursten, 2005). Subsequently the production of furfurals at this point can lead directly to the formation of melanoidins. Aldehydes, generated at this step, may react with each other via a condensation reaction, a process also leading to the production of melanoidins.

Aldehyde-amine condensation is the final step involved in the Maillard reaction at low pH (Nursten, 2005). In this step, α and/or β-unsaturated aldehydes react with an amine to produce high molecular mass, colored products, known as melanoidins. A study by Benzing-Purdie *et al*. (1985) showed that temperature was the major driving force for melanoidin production with the proportion of high molecular mass material increasing with temperature. The loss of products such as H_2O and CO_2 was shown to be 54% at 100ºC and 30% at 68ºC. Melanoidins are complex molecules and the nature of their properties is dictated by the conditions under which they were produced (Nursten, 2005). Since the conjugate is comprised of these Maillard end products, this complexity can greatly impact how the conjugate will perform in various food applications.

1.6 Rheological Analysis

Rheology is the study of how materials respond to applied stresses and strains. Therefore, an evaluation of rheological properties can help characterize and enable prediction of how a food will respond in both production and consumer settings. Viscosity, or resistance to flow, is an important characteristic of some foods that may

affect process design calculations and consumer acceptance. The apparent viscosity (η) of a food is determined by taking the shear stress (σ) or force applied parallel to the surface of the food and dividing it by the shear strain rate ($\dot{\gamma}$) or deformation occurring due to the applied force. The rheological data can then serve to assist with process engineering calculations, ingredient functionality, quality control, shelf life testing, and correlations with sensory data (Steffe, 1996).

1.6.1 Steady Shear

Steady shear viscosity is a property of all fluids and can be determined through methods such as rotational viscometry. Flow behavior is unique to a food system and can be either Newtonian in which case the fluid viscosity is independent of time or non-Newtonian where the fluid viscosity is time dependent (Cross, 1965). In the past, rheological analyses performed in a steady shear capacity have been used to probe changes in viscosity due to protein modifications (Resch and Daubert, 2002) as well as those stemming from protein-polysaccharide interactions in oil-in-water emulsions (Dickinson and Pawlowski, 1996; Ibanoglu, 2002).

The apparatus used for steady shear rheological experiments consists of a concentric cylinder attachment known as a bob with a radius of R_b, suspended inside a cylindrical cup with a radius of R_c, that contains the fluid being tested. The bob is typically connected to a torque (*M*) measuring device, and rheological properties are derived from a force balance around the bob:

$$\sigma_b = \frac{M}{2\pi hr^2} \qquad (2.0)$$

The radius (r) can be any location within the measured fluid such that $R_b \leq r \leq R_c$, with the height of the bob denoted by (*h*). Most viscometers in use today determine the apparent viscosity (η) of a test fluid as well as other important rheological properties as a function of shear rate ($\dot{\gamma}$) derived from the angular velocity of the bob (Ω) (Riande *et al.*, 2000). The viscometers may often utilize a simple shear model equation for determination of such results:

$$\dot{\gamma}_b = \frac{\Omega R_b}{R_c - R_b} \tag{3.0}$$

Use of model equations, such as the Power Law which involve the flow behavior (*n*) and the consistency coefficient (K), may be applied to demonstrate mathematical relationships between viscosity, shear stress, and shear rate:

$$\eta = K\dot{\gamma}_b^{\,n-1} = \frac{\sigma_b}{\dot{\gamma}_b} \tag{4.0}$$

Operational modes for steady shear analysis using a cup and bob geometry include the Couette configuration, where the cup rotates, or the Searle configuration where the bob rotates. End effects may also occur during measurements leading to erroneous data. These artifacts can be overcome by using a Mooney-Couette design where the bob has a bottom with a slight angle, which ensures the shear rate at the bottom is equivalent to the shear rate in the annulus or the area between the cup wall and sides of the bob. Application of serrated edges to the cup and bob can also help minimize errors due to slippage between the test material and the attachment.

1.6.2 Small Amplitude Oscillatory Shear

Most foods are considered viscoelastic, demonstrating both a viscous and elastic component. Steffe (1996) states that in order to analyze the behavior of food systems, both the viscous and elastic nature of a material must be taken into consideration. The following equation predicts shear stress (σ) as a function of the elastic nature ($G'\gamma$) and the viscous behavior ($\eta'\dot{\gamma}$) of the material:

$$\sigma = G'\gamma + \eta'\dot{\gamma} \tag{5.0}$$

Different methods can be employed to study these components, but oscillatory testing is well suited to study the chemical composition and physical structure of foods (Steffe, 1996). Multiple studies have successfully applied dynamic oscillatory measurements to study the rheology of mixed whey protein-carbohydrate systems (Aguilera and Rojas, 1997; Eleya and Turgeon, 2000; Braga and Cunha, 2004; Bertrand and Turgeon, 2006).

Instruments used to measure oscillatory shear, dynamically, can operate in a controlled strain environment where the strain is fixed and the stress is measured, or a controlled stress environment where the stress amplitude is fixed and the strain or deformation is measured (Steffe, 1996). The instrument used in these experiments operated in a controlled stress mode. Initially a stress sweep was performed to assess the linear viscoelastic region (LVR) of samples. For this type of testing, the amplitude of the frequency is held constant while the stress is increased. The LVR is identified as the maximum limit of stress where the complex modulus (G*) is independent of applied

stress. The storage modulus (G′), or energy stored within the food, and the loss modulus (G′′), or energy lost within the food, can be determined from the following equations:

$$G' = (G^*)\cos(\delta) \tag{6.0}$$

$$G'' = (G^*)\sin(\delta) \tag{7.0}$$

The phase angle (δ) is a measurement reflecting the degree of viscoelasticity ranging between 0° (purely elastic) and 90° (purely viscous). To perform the mechanical spectra analysis, a stress value is then chosen from the LVR while the frequency is increased over at least three orders of magnitude to establish a clear picture of the viscous and elastic behavior of the sample.

1.7 Emulsions

Emulsions are comprised of a mixture of two immiscible liquids and are categorized as oil-in-water (o/w) or water-in-oil (w/o) dispersions. The o/w emulsions are most common in foods and occur in milk, milk products, sauces, dressings and soups. On the other hand, w/o emulsions are found in food products such as margarine or butter. These systems involve two phases termed dispersed and continuous. In an o/w emulsion, the oil would be considered the dispersed phase whereas the water is considered the continuous phase. Figure 10 provides a graphical representation of emulsion formation.

Many components affect the surface activity between the two phases. Smaller droplet sizes, around 1μm in diameter, are known to provide better stabilization, but this stipulation may require that a great deal of energy be delivered into the system to decrease the droplet size (Genovese *et al*., 2007). The volume fraction of the dispersed

phase and the composition of the continuous phase can vary depending on the system. A volume fraction of the dispersed phase is normally between 0.01 and 0.4 for most foods; however, for mayonnaise the volume fraction may be as high as 0.8 (Fennema, 1996). Attractive and repulsive interactions, including van der Waals, electrostatic, steric, hydrophobic, and hydration forces, govern the stability of o/w emulsions stabilized by whey proteins (Keowmaneechai and McClements, 2002). The evaluation of how well an ingredient, such as whey proteins, may potentially stabilize an emulsion is a critical step when designing appropriate applications, and the degree of stability can be determined through multiple methods such as emulsifying capacity (EC) and emulsion stability (ES).

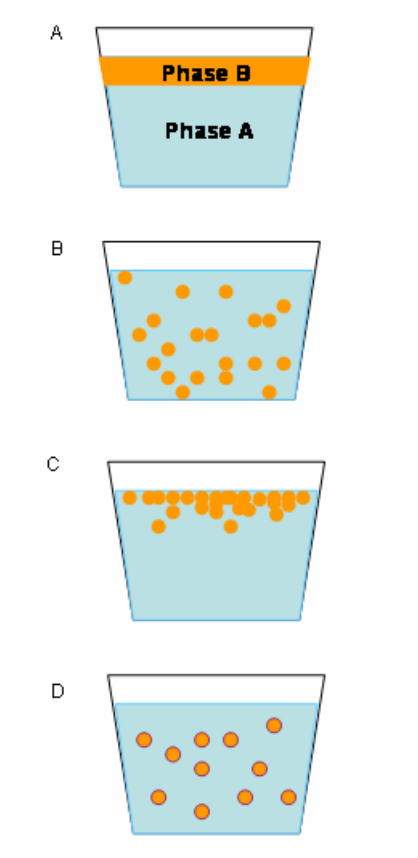

Figure 10. A. Two immisicible liquids, prior to emulsification; B. An emulsion of Phase B dispersed in Phase A; C. Separation of unstable emulsion; D. Conjugate emulsifier positions itself on the interfaces between Phase A and Phase B, stabilizing the emulsion.

1.7.1 Emulsifying Capacity

Swift *et al*. (1961) initially defined emulsifying capacity as the amount of oil required to reach the inversion point, when the emulsion collapses and the viscosity drops. This measurement was reported as the quantity of oil added per 100mg of protein. Webb *et al*. (1970), later found this method to be too subjective and devised a new procedure for measuring EC based on electrical resistance using meat and fish protein extracts. This protocol consisted of measuring resistance in an o/w emulsion where the protein-water phase was continuous. Since oils are nonconductors and protein-water mixtures are good conductors, a change in the continuous phase, due to aggregation of the oil droplets at the inversion point, would cause a spike in electrical resistance (Kato, 1985). The point at which this spike occurs was defined as the system's emulsifying capacity. Later studies by Marshall *et al*. (1975) and Wang and Kinsella (1976) added oil-soluble dyes to provide a visual means for measuring the inversion point. A diagram outlining the model created by Firebaugh (2004) for measuring EC is shown as Figure 11. This design was reconstructed for the purpose of measuring EC in this study.

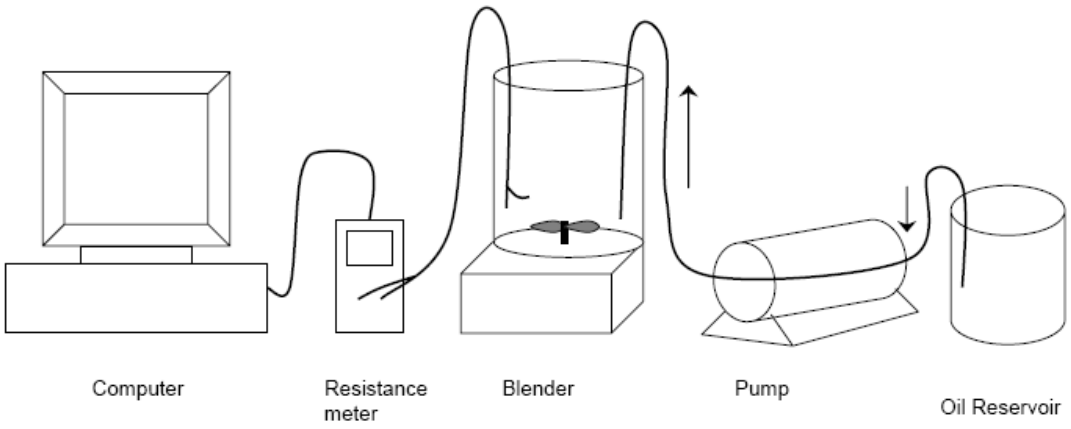

Figure 11. Schematic of emulsifying capacity measurement apparatus (Firebaugh, 2004).

1.7.2 Emulsion Stability

Emulsion stability is usually determined by measuring the percentage of fat separated into a top cream layer either by centrifugation or refrigerated storage (Morr and Ha, 1993). Demetriades and McClements (1999) evaluated emulsion stability by measuring the height of the interface between the optically opaque layer, termed droplet rich, and the less opaque layer, termed droplet depleted. The results were then reported as a "creaming index."

$$\text{Creaming Index} = 100 \times \left(\frac{HD}{HE} \right) \qquad (8.0)$$

The term HD was defined as the height of the droplet depleted layer and HE represented the total height of the emulsion. Other studies have used similar methods for measuring the emulsion stability of whey protein-carbohydrate conjugates based on separation of layers over time (Akhtar and Dickinson, 2003; Mishra *et al*., 2001).

1.8 Summary

The addition of carbohydrates to previously modified whey proteins by non-toxic means such as the Maillard reaction shows promise for potential expansion of whey in food systems. For example, viscosity and emulsion stability could be greatly enhanced by such a modification. Further studies will be required to evaluate sensorial characteristics such as color, taste and texture of the protein-polysaccharide conjugate to establish its practical use in food applications. The newly formed ingredient may prove useful in systems such as whey-based beverages where stabilization of flavor oils is a required component.

1.9 References

Aguilera, J.M. and Rojas, G.V. 1997. Determination of kinetics of gelation of whey protein and cassava starch by oscillatory rheometry. Food Research International: 30, 349-357.

Akhtar, M. and Dickinson, E. 2003. Emulsifying properties of whey protein-dextran conjugates at low pH and different salt concentrations. Colloids and Surfaces B: Biointerfaces 31125-132.

Akhtar, M. and Dickinson, E. 2007. Whey protein-maltodextrin conjugates as emulsifying agents: an alternative to gum arabic. Food Hydrocolloids, 21: 607-616.

Anfinsen, C.B., Scheraga, H.A. 1975. Experimental and theoretical aspects of protein folding. Advanced Protein Chemistry. 29, 205.

Ames, J. M. 1990. Control of the Maillard reaction in food systems. Trends in Food Science and Technology, 1: 150-154.

Benzing-Purdie, L., Ripmeester, J. A., and Ratcliffe, C.I. 1985. Effects of temperature on Maillard reaction products. J. Agric. Food Chem. 33, 31-33.

Bertrand-Harb, C., Charrier, B., Dalgalarrondo, M., Chobert, J. M., and Haertle, T. 1990. Condensation of glycosidic and aromatic structures on amino groups of β-lactoglobulin via reductive alkylation. Solubility and emulsifying properties of the protein derivatives. Le Lait, 71, 205-215.

Bertrand, M., and Turgeon, S.L. 2006. Improved gelling properties whey protein isolate by addition of xanthan gum. Food Hydrocolloids: 21, 159-166.

Boratynski, J. and Roy, R. 1998. High temperature conjugation of proteins with carbohydrates. Glycoconjugate Journal 15: 131-138.

Braga, A.L.M., and Cunha, R.L. 2004. The effects of xanthan conformation and sucrose concentration on the rheological properties of acidified sodium caseinate–xanthan gels. Food Hydrocolloids: 18, 977-986.

Brink, W. 2006. The whey it is. http://www.bodybuildingforyou.com/protein/whey-protein-more-info-2.htm. Accessed May 2006.

Broihier, K. Milking the nutrition market. Food Process. 1999, 60, 41-44.

Chaplin, L.C., Lyster, R.L.J. 1986. Irreversible heat denaturation of bovine α-lactalbumin. J. Dairy Res. 53, 249.

Chen WS, Henry GA, Gaud SM, Miller MS, Kaiser JM, Balmaceda EA, Morgan RG, Baer CC, Borwankar RP, Hellgeth LC, Strandholm JJ, Hasenhuettl GL, Kerwin PJ, Chen CC, Kratochvil JF, Lloyd WL, inventors; Kraft Inc., assignee. April 28, 1989. Microfragmented ionic polysaccharide/protein complexes dispersions. U.S. patent 5,104,674.

Chevalier, F., Chobert, J., Popineau, Y., Nicolas, M.G.,Haertle, T. 2001. Improvement of functional properties of β-lactoglobulin glycated through the Maillard reaction is related to the nature of the sugar. International Dairy Journal 11, 145–152

Civitelli R, Villareal DT, Agnusdei D, Nardi P, Avioli LV, Gennari C. 1992. Dietary L-lysine and calcium metabolism in humans. *Nutrition*. 8(6):400-405.

Cross, M.M. 1965. Rheology of non-Newtonian fluids: A new flow equation for psuedoplastic fluids. Journal of Colloid Science 20, 417-437.

Davies, C.G.A and Labuza, T.P. The Maillard Reaction Application to Confectionery Products. http://faculty.che.umn.edu/fscn/Ted_Labuza/PDF_files/papers/maillard-confectionary.pdf. Accessed August 2006.

de Kruif, K. G., and Tuinier, R. 1999. Whey protein aggregates and exo-polysaccharides. International Journal of Food Science and Technology, 34: 487-492.

de Witt, J. N. 1998. Nutritional and functional characteristics of whey proteins in food products. J Dairy Sci 81:597–608

Demetriades, K., McClements, D.J. 1999. Flocculation of whey protein stabilized emulsions as influenced by dextran sulfate and electrolyte. Journal of Food Science, 64, 206-210.

Dickinson, E. 1992. Emulsifying properties of covalent protein-dextran hybrids. Colloids and Surfaces, 64, 299-310.

Dickinson, E., and Semenova, M. G. 1992. Emulsifying behaviour of protein in the presence of polysaccharide under conditions of thermodynamic incompatibility. J. Chem. Soc. 88(6), 849-854.

Dickinson, E. 1993. Trends Food Sci. Technol. Vol. 4, 330.

Dickinson, E., and Izgi, E. 1996. Foam stabilization by protein-polysaccharide complexes. Colloids and Surfaces. 113, 191-201.

Dickinson, E., and Pawlowski, K. 1996. Rheology as a probe of protein-polysaccharide interactions in oil-in-water emulsions. In: Phillips GO, Williams, PA, editors. Gums and stabilizers for the food industry. New York: IRL Press Ltd. P 181-191.

Einhorn-Stoll, U., Ulbrich, M., Sever, S., Kunzek, H. 2005. Formation of milk protein-pectin conjugates with improved emulsifying properties by controlled dry heating. Food Hydrocolloids: 19, 329-340.

Eleya, M.M., and Turgeon, S.L. 2000. Rheology of κ-carrageenan and β-lactoglobulin mixed gels. Food Hydrocolloids 14: 29-40.

Euston, S.R., Finnigan, S.R., Hirst, R.L. 2001. Aggregation kinetics of heated whey protein-stabilised emulsions: effect of low-molecular weight emulsifiers. Food Hydrocolloids:, 15, 253-262

Farrell, H. M., Jr., R. Jimenez-Flores, G. T. Bleck, E. M. Brown, J. E. Butler, L. K. Creamer, C. L. Hicks, C. M. Hollar, F. Ng-Kwai-Hang, and H. E. Swaisgood. 2004. Nomenclature of the proteinsof cows' milk—Sixth edition. J. Dairy Sci. 87:1641–1674.

Fennema, O.R. 1996. Food chemistry: third edition. Marcel Dekker, New York, NY. 133.

Firebaugh, J.D. 2004. Characterization and application of a derivatized whey ingredient. Master's Dissertation, NC State University, 2004.

Fox, P.F.; McSweeney, P.L.H. 1998. Dairy Chemistry and Biochemistry. pp. 149. Springer - Verlag.

Genovese, D.B., Lozano, J.E., Rao, M.A. 2007. The rheology of colloidal and noncolloidal dispersions. Journal of Food Science, Vol 00, Nr. 0, R1-R10.

Haines, B. 2005. Whey's protein star is rising. http://www.preparedfoods.com/CDA/Archives/def692deb5788010VgnVCM100000f932a8c0. Prepared Foods online. Accessed May 2006.

Hazen, C. 2003. Formulating function into beverages. Food Product Design. http://www.foodproductdesign.com/archive/2003/0103CS.html. Accessed March 2006. Cover Story. January.

Hudson, H.M., Daubert, C.R., and Foegeding, E.A. Thermal and pH stable protein thickening agent and method of making the same. 2001, US Patent No.6261624.

Ibanoglu, E. 2002. Rheological behaviour of whey protein stabilized emulsions in the presence of gum arabic. Journal of Food Engineering 52: 273–277.

IUPAC Compendium of Chemical Terminology, Electronic version, http://goldbook.iupac.org/G02651.html. PAC, 1988, 60, 1389. Accessed January, 2007.

Kato, A., Fujishige, T., Matsudomi, N., and Kobayashi, K. 1985. Determination of emulsifying properties of some proteins by conductivity measurements. *Journal of Food Science* 50:1, 56–58

Keowmaneechai, E. and McClements, D. J. 2002. Influence of EDTA and citrate on physicochemical properties of whey protein-stabilized oil-in-water emulsions containing CaCl2. J. Agric. Food Chem, 50: 7145–7153.

Kinsella, J.E. 1984. Milk proteins; physicochemical and functional properties. Food Science and Nutrition, 21, 197.

Leonil, J., Molle, D., Fauquant, J., Maubois, J. L., Pearce, R. J., and Bouhallab, S. Characterization by ionization mass spectrometry of lactosyl β-Lactoglobulin conjugates formed during heat treatment of milk and whey and identification of one lactose-binding site. Journal of Dairy Science. 80: 2270-2281.

Marshall, W.H.. Dutson, T.R., Carpenter, Z.L., and Smith, G.C. 1975. A simple method for emulsion end-point determinations. J. Food Sci. 40: 896.

Mauron, J. 1981. The Maillard reaction in food; a critical review from the nutritional viewpoint. Pergamon Press, Oxford 5:35.

McMahon, D.J., Alleyne, M.C., Fife, R.L., Oberg, C.J. 1996. Use of fat replacers in low fat mozzarella cheese. J Dairy Sci 79:1911-1921

Mishra, S., Mann. B., Joshi, V.K. 2001. Functional improvement of whey protein concentrate on interaction with pectin. Food Hydrocolloids. Vol. 15, 9-15.

Mitchell, H.H. 1923. A Method of determining the biological value of protein". Journal of Biol. Chem. 58 (3): 873.

Morr, C.V., Foegeding, E.A. 1990. Composition and functionality of commercial whey and milk protein concentrates and isolates: a status report. Food technol. Vol. 44, 100-112.

Morr, C.V. and Ha, E.Y.W. 1993. Whey protein concentrates and isolates: processing and functional properties. Critical reviews in Food Science and Nutrition. Vol. 33(6), 431-476.

Nakamura S, Kato A and Kobayashi K,. 1992a. Bifunctional lysozymegalactomannan conjugate having excellent emulsifying properties and bactericidal effect. *J Agric Food Chem* 40:735–739.

Nakamura S, Kato A and Kobayashi K,. 1992b. Enhanced antioxidative effect of ovalbumin due to covalent binding of polysaccharides. *J Agric Food Chem* 40:1734–1739.

Nursten, H. 2005. The Maillard reaction; chemistry, biochemistry, and implications. Royal Society of Chemistry. p. 7-8.

Oene, H.V., Cragg, L.H. 1962. Shear dependence in solutions of fractionated dextran: a variable-shear capillary viscometer for use with aqueous solutions. J. of Polymer Sci. 57, 175-185.

Oliver, C.M., Melton, L.D., Stanley, R.A. 2006. Creating proteins with novel functionality via the Maillard reaction: A review. Critical Reviews in Food Science and Nutrition, 46:337-350.

Potter, N. 1986. Food sciene: fourth edition. Van Nostrand Reinhold Company, Inc. New York, NY. p. 36-38.

Resch, J.J. and Daubert, C.R. 2002. Rheological and physicochemical properties of derivatized whey protein concentrate powders. International Journal of Food Properties 5(2), 419–434.

Riande, E., Diaz-Calleja, R., Prolongo, M., Masegosa, R., Salom, C. 2000. Polymer viscoelasticity: Stress and strain in practice. Marcel Dekker, New York, NY. 536.

Schaafsma, G. 1989. Effects of heat treatment on the nutritional value of milk. IDF Bull. Int. Dairy Fed., Brussels, Belgium. 238 p. 68.

Smith, J.A.M. & Schaink, H.M. 1995. Phase behaviour of protein-polysaccharide water systems and its role in food design. Industrial Proteins, 2: 8–9.

Steffe, J. F. 1996. Rheological methods in food process engineering: second edition. Freeman Press, MI. 1.

Swaisgood, H.E. 1982. Chemistry of milk proteins. *Developments in Dairy Chemistry, Vol 1 Proteins*. Fox, P.F., Ed., Applied Science, New York, 1.

Swift, C.E., Lockett, C. and Fryar. A.J. 1961. Comminuted meat emulsions: Capacity of meats for emulsifying fat. Food Technol. 15: 468.

Tuinier, R., Dhont, J. K. G., De Kruif, C. G. 2000. Depletion-induced phase separation of aggregated whey protein colloids by an exocellular polysaccharide. Langmuir, 16: 1497-1507.

Wang, J.C. and Kinsella, J.E. 1976. Functional properties of novel proteins: Alfalfa leaf protein. J. Food Sci. 41: 286.

Waniska, R. D., and Kinsella, J. E. 1988. Foaming and emulsifying properties of glycosylated β-lactoglobulin. Food Hydrocolloid, 2, 439-449.

Webb, N.B., Ivey. F.J., Jones, V.A., and Monroe. R.J. 1970. The measurement of emulsifying capacity by electrical resistance. J.Food Sci. 35: 501.

Wilde P, Mackie A, Husband F, Gunning P, Morris V. 2004. Proteins and emulsifiers at liquid interfaces. Adv Colloid Interface Sci. 20:63-71.

CHAPTER 2. MATERIALS AND METHODS

2.1 Introduction

Whey proteins are commonly added to foods such as dairy products, baked goods, and meat products due to their high nutritional value and ability to improve functionality (de Witt, 1998). These dairy proteins impart viscosity, bind water, coat a product, or improve emulsification or foaming properties (Morr, C.V., 1979; Morr, C.V., 1982; de Witt, 1984; de Witt, 1989; Kinsella *et al*., 1989; Mishra *et al*., 2001). The demand for whey, as well as the environmental and financial costs for whey disposal, has made it profitable for companies to convert a one-time waste stream into a marketable source of revenue. As of 2002, whey accounted for approximately 11% of the revenue from a modern cheese plant (Balagtas *et al*., 2003). In 2005, whey protein concentrate production for human consumption reached approximately 325 million pounds (USDA, 2006). At least 30% of the whey produced remains unused, leading developers to constantly work towards creating new ingredient opportunities (Balagtas *et al*., 2003).

Modifications to whey are often made to increase its function in foods. In 2001, Hudson *et al*. patented a method for modifying whey protein isolates that showed expanded functional characteristics over native whey, itself (Figure 1). This method involved lowering the pH of a whey protein solution to 3.35, followed by heating at 80ºC for 3 hours to induce gelation. The gel was then frozen, lyophilized, and ground to a fine powder. This modified whey protein displayed cold-set, thickening functionality without additional heat or salts. Further studies with whey protein isolates were focused on characterizing the emulsifying and foaming properties of the previously modified ingredient (Firebaugh, 2004).

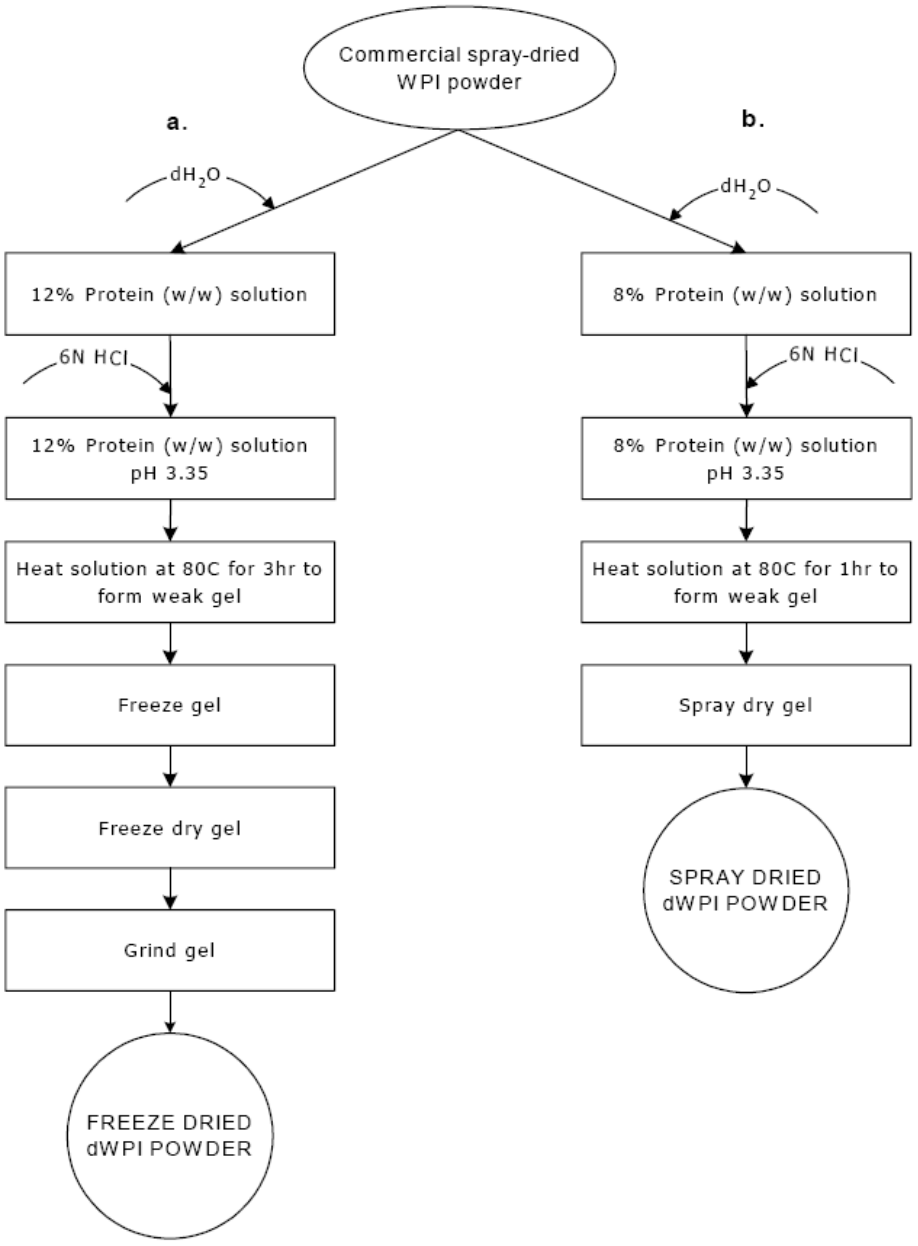

Figure 1. Diagram of A. whey protein modification and B. spray drying procedure (Firebaugh, 2004)

Improvements in whey functionality such as the stabilization of emulsions and foams, achieved through the covalent linkage of whey proteins to carbohydrates via non-

chemical means, such as the Maillard reaction, have been studied extensively (Dickinson and Semenova, 1992; Dickinson and Izgi, 1996; Akhtar and Dickinson, 2003; Mishra *et al*., 2001; Einhorn-Stoll *et al*., 2005). The Maillard reaction is a naturally occurring reaction, in contrast to other methods such as acetylation, deamidation, and succinylation (Oliver *et al*., 2006). These types of reactions may also result in significant changes to the functionality of the final product.

The objectives of this study were focused on conjugating the modified whey protein ingredient with test carbohydrates via the Maillard reaction in order to study changes in functionality. Characterization of hydration related properties such as viscosity, gelation, and water holding capacity as well as surface related properties including foaming and emulsification could then be assessed through multiple methodologies (Morr and Ha, 1993).

2.2 Materials and Methods

A commercial modified whey protein concentrate (MWPC) powder, manufactured according to the method of Resch and Daubert (2004), contained 70.4% (w/w) protein and was used for all experiments (Grande Custom Ingredients Group, Lomira, WI). A commercial whey protein concentrate (CWPC) containing 78.1% (w/w) protein was also acquired from Grande Custom Ingredients (Lomira, WI) for comparison purposes. Nitrogen content of the MWPC was analyzed by the Analytical Services Laboratory (Raleigh, NC) using a CHN Elemental Analyzer, Series II (Perkin Elmer Corporation, headquartered in Norwalk, CT). Protein content was calculated from the provided value using the equation (N x 6.38) (Onwulata *et al*., 2004; Table 1). Dextran (35 kDa – 45 kDa; DX 35) and (100 kDa – 200 kDa; DX 200) from leuconostoc mesenteroides were purchased from Sigma-Aldrich (St. Louis, MO). A Bicinchoninic Acid (BCA) protein assay

kit, *o*-phthaldialdehyde (OPA) assay reagent, and glycoprotein staining kit was obtained from Pierce (Rockford, IL), while precast Tricine SDS-Polyacrylamide Gradient Gels (10 - 20 %) and a Colloidal Blue staining kit were purchased from Invitrogen Life Technologies (Carlsbad, CA).

Table 1.
Analytical analyses of modified and commercial whey protein concentrates.

Sample	Protein[a] (%)	Moisture[b] (%)	Ash[b] (%)	Fat[b] (%)	Carbohydrate[b] (%)
MWPC	70.37	3.97	8.50	3.99	13.17
CWPC	78.09	4.56	2.70	4.81	9.84

[a] Determined by micro-Kjeldahl (N x 6.38) (Onwulata *et al*., 2004)

[b] Provided by Grande Custom Ingredients Group

2.3 Glycoconjugate Production

Initially, an appropriate ratio of protein to carbohydrate was determined by dissolving stock solutions of MWPC powder and DX 200 in deionized (DI) water at three ratios: protein to carbohydrate (1:0, 2:1, and 3:1), such that all dispersions contained 8 % solids (w/v). Sample 1, containing MWPC alone, was dispersed in DI water to form a 5.6 % protein (w/v) solution. Sample 2, was made at a 2:1 mixture of MWPC to DX 200 and dispersed in DI water to produce a 3.7 % protein (w/v) concentration, while sample 3 was prepared at a 3:1 ratio of MWPC to DX 200 and dispersed in DI water to produce a 4.2 % protein (w/v) concentration. The solutions were stirred at 150 rpm for 2 hours and the pH adjusted to 3.5 with 6N HCl. The solutions were then transferred to lyophilization vessels, attached to a 4.5 liter benchtop freeze dryer (Labconco, Kansas City, MO), and lyophilized for 48 hours. Once removed, the powder was ground to a fine particle size and placed in 400ml beakers for thermal treatment. The dried powder was then heated

in an Isotemp 630G convection oven (Fisher Scientific, USA) for 2 hours at 100ºC to form the glycoconjugate fraction. Measurement of the residual free amino groups by *o*-phthaldialdedhyde analysis showed that the 2:1 ratio, provided the greatest degree of conjugation based on normalized protein concentrations (Table 2). Subsequent whey-based protein solutions containing dextran (35 kDa – 45 kDa and 100 kDa – 200 kDa) were made in accordance with these findings.

Table 2.
Measurement of the free amino groups by *o*-phthaldialdehyde (OPA) Assay of MWPC, alone, and after dry heat treatment of MWPC with dextran (100 kDa – 200 kDa).

Sample	OD at 340 nm	µM / mg of protein
MWPC non-heated	0.378 [a]	597 [a]
2:1 ratio heated	0.101 [b]	328 [c]
3:1 ratio heated	0.101 [b]	383 [b]

[a-c] Letters next to readings represent significant differences ($P < 0.05$)

2.4 Bicinchoninic Acid (BCA) Assay

The bicinchoninic acid (BCA) assay is a colorimetric method for measuring protein concentration in a given sample. The first step is a Biuret reaction that reduces Cu^{+2} to Cu^{+1}, followed by the BCA reagent forming a complex with Cu^{+1} and producing a purple color detectable at 562 nm (Weichelmen *et al*., 1988). All samples were hydrated at 7 % (w/v) protein in DI water for $\geq$ 24 hours at 4 ºC prior to testing. A dilution of 1:30 (protein solution to DI water) of the 7 % (w/v) protein solutions was made to ensure all samples were within the range of the established standard curve (0 to 2 mg/mL). A standard curve was achieved by preparing 5 test tubes containing 2 mL of BCA reagent and adding increasing volumes (0-100 µL) of bovine serum albumin (BSA) standard (2.0 mg/mL). All test volumes were adjusted to 100 µL total with DI water. Then, 100 µL of

the appropriately diluted experimental samples were added to 2 mL of BCA reagent. All samples were incubated at 37 ºC for 30 minutes and the absorbance read at $A_{562\ nm}$ using a Gilford Instruments 2600 UV-Visible spectrophotometer. This analysis was performed in duplicate. Figure 2 shows a graphical representation of a BCA standard curve.

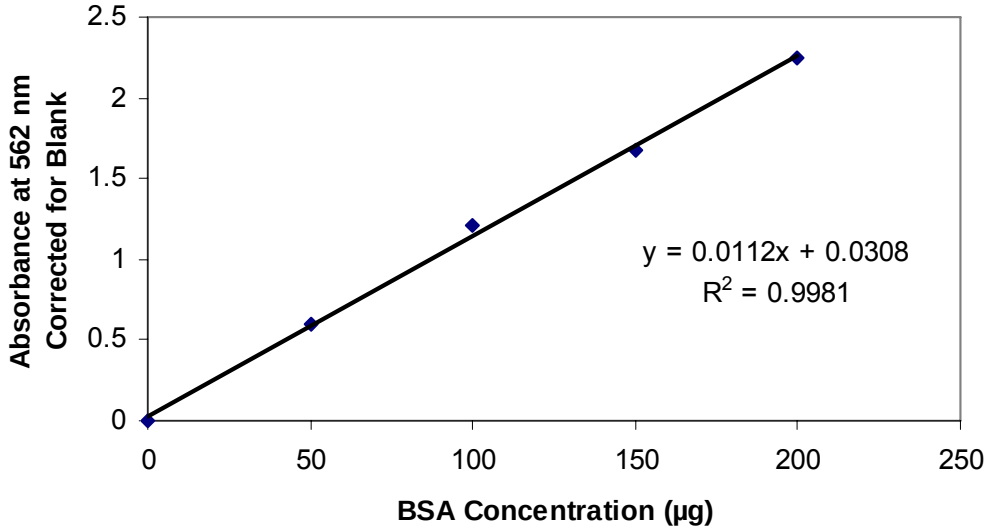

Figure 2. Representative standard curve using BCA reagents and known amounts of bovine serum albumin (BSA).

The absorbance readings were used to calculate the amount of protein remaining in each sample after treatment to ensure that equal amounts of protein were evaluated amongst all test samples. In this event, the results obtained can be compared based on the conjugation process, itself, since test MWPC solutions per se, contained equivalent amounts of protein. Previously, Smith *et al*. (1985) found that free sugars present in samples may cause erroneous results with respect to protein concentration measurements using bicinchoninic acid. However, in these experiments, an evaluation of the dextran used for this study showed no indication of such interference.

2.5 Colorimetric Assay

Reflectance measurements of dry powdered MWPC and MWPC-Dextran glycoconjugates were determined using a Konica Minolta CR-300 Chroma Meter (Tequipment, Long Branch, NJ) with diffuse illumination/0º. The readings obtained from the Chroma Meter were based on the Hunter L*, a*, b* scale (HunterLab, 1996) and analyzed in triplicate.

2.6 *o*-Phthaldialdehyde (OPA) Assay

The OPA spectrophotometric assay method used for these experiments is based on the method of Church *et al*. (1983). The assay is a rapid, convenient, and sensitive procedure for determination of available primary amines. Samples were microcentrifuged at 13,600 x g for 10 minutes, and the soluble protein remaining in the supernatant collected and diluted 1:10 with DI water. Afterwards, 50 µl of the diluted solution was added to 2.0 ml of OPA reagent and incubated for 5 min at 25 ºC. Absorbance readings were made at $A_{340\ nm}$ using a Gilford Instruments 2600 UV-Visible spectrophotometer with all values falling within a range of 0.1-1.0. The µM concentration of available amino groups was then calculated by dividing the absorbance readings at $A_{340\ nm}$ by the extinction coefficient (6000 m^{-1} cm^{-1}) (Church *et al*., 1983) and the length of the light path (1 cm). Samples were then normalized for protein concentration based on BCA results. All samples were analyzed in triplicate.

2.7 SDS-Polyacrylamide Gel Electrophoresis (SDS PAGE)

Tricine 10-20% gradient polyacrylamide gels were used to evaluate protein and glycoprotein banding patterns. Initially, BCA analysis was performed to ensure that equivalent amounts of protein (2 mg/ml) were loaded into each lane of the Tricine gel. Samples were mixed with 0.9 M Tricine sample buffer containing 8 % sodium dodecyl

sulfate (SDS) and 5.0 % β-mercaptoethanol, then heated at 100 ºC for 10 min prior to loading into the gel lane. Gels were electrophoresed using a Novex Power Ease 500 Power Supply (Invitrogen Inc., Carlsbad, CA) for 85 min at 125 V. After completion, samples were stained to visualize the protein bands using a colloidal Coomassie Blue staining reagent (Invitrogen Inc., Carlsbad, CA). Alternatively, glycoproteins were detected after development with a carbohydrate staining reagent [GelCode Glycoprotein Staining Kit (Pierce, Rockford, IL)].

2.8 Steady Shear Rheological Analysis

To evaluate the viscosity of newly formed glycoconjugates, samples were dispersed in DI water to a final concentration of 5.6 % and 7 % protein (w/v) solutions. These solutions were stirred at 150 rpm for 2 hours and the pH adjusted and maintained at 3.5. The samples were pre-sheared at 15 s^{-1} for 30 s and subsequent shear rate ramps (0.1-500 s^{-1}) were conducted at 25 ºC with a StressTech Controlled Stress Rheometer (ReoLogica Instruments AB, Lund, Sweden) for apparent viscosity (η) measurements.

2.9 Small Amplitude Oscillatory Shear

Viscoelasticity of the samples was determined by small amplitude oscillatory shear. Heated and non-heated samples were hydrated at a 2:1 ratio of protein to carbohydrate in DI water. A StressTech Controlled Stress Rheometer (ReoLogica Instruments AB, Lund, Sweden) was used to run both a stress sweep for LVR evaluation and a frequency sweep for G', G", and phase angle determination. Determination of the LVR was achieved by maintaining a frequency of 10 Hz while the stress was ramped from 0.01 Pa to 50 Pa. A frequency sweep was then performed

based on the LVR of 1.0 Pa found from the stress sweep data. The stress was held constant at 1.0 Pa while the frequency increased from 0.01 to 10 Hz allowing determination of G', G", and the phase angle associated with each sample.

2.10 Emulsion Stability

Since stabilization can occur in a system comprised of non-covalently linked protein and polysaccharide components (Dickinson, 2006), initial testing was performed to assess the degree of stabilization that occurred due to a simple mixing of these ingredients compared to the production of glycoconjugates found via the Maillard reaction (covalent bond formation). Stock solutions were prepared at 5 mg/ml protein with MWPC, MWPC-DX35, MWPC-DX200, and CWPC in DI water. All solutions were stirred for 2 hours at 150 rpm and hydrated overnight at 4 ºC. Sodium azide 0.02 % (w/v) was added to the solutions to prevent microbial growth. The pH of each solution was adjusted to 3.5, and blended at a 1:1 ratio with corn oil in a Waring blender for approximately 1 minute. The solutions were immediately homogenized in 2 passes using a Panda 2K NS1001L homogenizer (GEA Niro Inc., Columbia, MD) at a setting of 200 bar on stage 1 to form an emulsion. Particle size analysis was performed on each sample using a Shimadzu SA-CP4 centrifugal particle size analyzer (Columbia, MD), ensuring that the droplet size was in the same range and approached the ideal size of 1 μm (Genovese *et al*., 2007).

The stability of the emulsion was assessed based on an evaluation of the creaming index as described by Demetriades and McClements (1999). For these experiments, ten milliliters of each emulsion was placed in a 15mL centrifuge tube and stored at ambient temperature for a period of one week. During this time period, each sample separated into two layers with a droplet rich layer on top and droplet depleted

layer on the bottom. The total height of each emulsion (HE) and the height of the droplet depleted layer (HD) were measured in triplicate. The creaming index was reported as:

$$\text{Creaming Index (\%)} = 100 \times \left(\frac{HD}{HE} \right) \qquad (2.0)$$

2.11 Emulsifying Capacity

The emulsifying capacity (EC) was measured using a system described in Firebaugh (2004). The EC value represents the amount of oil each sample is capable of stabilizing. An inversion point, or the point at which the emulsion separated or converted to a water-in-oil emulsion, was obtained by measuring the resistance on an Omega HHM26 with a RS232 interface (Omega Engineering Inc., USA) connected to a Dell desktop computer. Test solutions were prepared at 0.1 % protein levels in DI water and the pH adjusted to 3.5 using 6N HCl. A Waring blender was employed to create suitable conditions approaching homogenization of the samples. Electrodes, connecting the Omega HHM26 resistance meter to the blender, were placed at an optimal height inside the blender to ensure accurate measurement of the inversion point. A 7 mm Tygon tube, used as the oil feed line, was placed at the same height directly across from the electrodes in the blender. A 200 ml fraction of each sample was loaded into the blender and mixed on high for one minute. Once equilibration was reached, oil was administered at a rate of 1ml per second with a peristaltic pump (Cole-Parmer Instrument Company, Vernon Hills, IL), with the blender running on high until the inversion point was reached. All test samples were analyzed in triplicate and the average volume of oil emulsified (ml) per 100 mg of protein was reported as the EC.

2.12 Scanning Electron Microscopy

All samples were placed in a 78 µm microporous capsule (Structure Probe Inc., West Chester, PA), and placed into 2 mL of cold 3 % glutaraldehyde, buffered with 0.1 M sodium acetate buffer, pH 3.5, for 24 hours. After this time, the capsule was transferred into a Petri dish containing the sodium acetate buffer. The liquid whey sample formed a solid mass enabling it to be cut into 2-3 mm^3 pieces. These pieces were then washed 3 times with buffer for 20 minutes at 4 °C. Dehydration steps were then performed using an ethanol series of 30 %, 50 %, 70 %, 95 % and 100 % for 20 minutes, each at 4 °C. A Samdri-795 critical point dryer (Tousimis, Rockville, MD) was used to dry the samples. The dried samples were sliced into 1 mm^3 pieces and coated with gold/palladium (25-30 nm) using an Anatech Hummer 6.2 sputter coater (Anatech Ltd, Denver, NC) for imaging.

2.13 Ionization Mass Spectrometry

A non-heated sample of MWPC and CWPC, as well as thermally processed MWPC samples were analyzed by the NC State University Metabolomics and Proteomics Laboratory using an ionization mass spectrometer to determine the amount of glycosylation that occurred during heating. Dried samples (50 µg) were reconstituted in 100 µL of 10 mM dithiothreitol prepared in 50 mM ammonium bicarbonate, pH 7.8, and heated for one hour at 56 ^{o}C. Upon cooling to room temperature, 100µl of 55mM iodoacetamide prepared in 50mM ammonium bicarbonate, pH 7.8, was added and held at room temperature for 45 minutes. Sequencing grade trypsin (Sigma Aldrich), 1µg, was then added, and the sample digested overnight at 37 ^{o}C.

Samples were analysed with a Thermo LTQ linear ion trap mass spectrometer with Surveyor HPLC and Autosampler (Thermo, San Jose CA). Briefly, a 5 μl sample was injected onto a 150 x 0.5 mm Jupiter Proteo C18 Phenomenex column (Torrance, CA), equilibrated in water: acetonitrile (97:3) containing 0.01 % trifluoroacetic acid (TFA) and 0.005 % heptafluorobutyric acid (HFBA). A linear gradient, 0-60 % acetonitrile, was applied over 70 minutes in which case, tryptic peptides were eluted. Data dependent tandem mass spectrometry (MS/MS) spectra were used to provide internal sequence identity of the eluting peptides, extracted ion chromatograms in MS-only mode were used for quantitative purposes. The MS/MS spectra were searched in Bioworks Browser 3.1 (Thermo), allowing for a dynamic modification of lysine residues (+342 amu) corresponding to lactosylation.

2.14 Water Holding Capacity

The water holding capacity (WHC) of the glycoconjugates was assessed by calculating the gram weight of water held per gram of powder. Dispersions of the modified ingredient and the glycoconjugates were made by preparing 15 g weight test samples. The samples were centrifuged in a Sorvall RC-5B Refrigerated Superspeed Centrifuge at a g force of 746 for 15 minutes at 25ºC (DuPont Instruments, Wilmington, DE). After centrifugation, the resulting pellet and supernatant were measured and the WHC was calculated from the grams of water in the pellet divided by the grams of protein in the 15 g of sample.

2.15 Statistical Analyses

Data were analyzed using SAS software (version 9.1; SAS Institute, Inc., Cary, NC). Significant effects of carbohydrate used and thermal treatment applied were evaluated by ANOVA with means separation (least squares means).

CHAPTER 3. RESULTS AND DISCUSSION

3.1 Introduction

Maillard reactivity occurring due to dry heat treatment of the modified whey ingredient in the presence of carbohydrates had varying benefits which were dependent upon carbohydrate selection. The following results highlight the degree of glycosylation that occurred in each sample, and the functional attributes that resulted.

3.2 Colorimetric Assay

Hunter L*, a*, b* measurements attributed to color development from the formation of Maillard reaction end products are shown in Table 3. The modified whey protein concentrate, prior to heat treatment, was used as a baseline for comparison. The degree of color formation going from darkest to lightest is represented in the following order based on L* values: MWPC heated (lactose present) > MWPC-DX200 > MWPC-DX35; as determined by the Chroma Meter. Hunter L* values have been successfully used to detect Maillard reactivity in food systems with the highest reactivity equating to L* values approaching zero (Reyes *et al.*, 1982; Nielson *et al.*, 1997). Presumably, increasing the amount of carbohydrate content would promote an increase in the number of reactive sites enabling a higher degree of conjugation. However, since these samples were prepared on a (w/v) basis, the lower molecular weight dextran (35 kDa – 45 kDa) actually reflected a higher molar concentration of reducing ends than the higher molecular weight dextran (100 kDa – 200 kDa). Since there was less color change observed with the MWPC-dextran (35 kDa – 45 kDa) the higher molar concentration may have lead to diffusion limitations and the blocking of potential reaction sites, thus preventing excessive color development (Kato, 2002). In a study by Clare et al. (2005) the lightness (L*) of heat treated milk samples decreased over time and with increased heat treatment, while redness, associated with a positive a value, increased. These

results were identified as Maillard reaction products and a similar observation was made in this work (Table 3).

Table 3.
Color Measurement of dry powdered MWPC and MWPC-glyconjugates.

Sample	L*	a*	b*
MWPC non-heated	97.92 [a]	-0.74 [c]	7.19 [d]
MWPC heated	88.72 [d]	0.51 [a]	18.59 [a]
MWPC-DX 35 heated	96.03 [b]	-0.55 [bc]	11.86 [c]
MWPC-DX 200 heated	93.70 [c]	-0.42 [b]	14.71 [b]

a +ve values: Red; a -ve values: Green
b +ve values: Yellow; b -ve values: Blue
L: white = 100, black = 0
[a-d] Letters next to readings represent significant differences ($P < 0.05$).

3.3 *o*-Phthaldialdehyde (OPA) Assay

Covalent coupling of MWPC to dextran was quantified via the OPA assay method. The Maillard reaction promotes covalent attachment of a reducing sugar, such as glucose, to an available amine group associated with lysine (Leonil *et al*, 1997). Increased coupling is evidenced by a reduction in the concentration of available amino groups. The degree of glycosylation may be dependent on the type of protein present and the number of accessible amino groups available for reactivity as well as the molar ratio of protein to polysaccharide (L. Jimenez-Castaro *et al*., 2007).

Initially, assessments to determine the appropriate ratio of protein to carbohydrate were performed using the higher molecular weight dextran (100 kDa – 200 kDa). The greatest loss of reactive amino groups was seen with the heated sample prepared at a ratio of 2:1 (protein to carbohydrate). This formulation was used for all subsequent experiments.

Heated and non-heated samples were tested in the presence and absence of dextran. The MWPC fraction, prior to heat treatment, was used to establish the baseline. The lowered absorbance readings at $A_{340\ nm}$ reflected formation of glycoconjugates. These results were in direct agreement with those obtained via the colorimetric assay with the greatest loss of free amino groups observed in the MWPC heated sample followed by the MWPC-DX200 and finally the MWPC-DX35 (Table 4). Significant differences were seen amongst all samples after normalization of the protein ($P < 0.05$).

Table 4.
Measurement of Free Amino Groups by *o*-phthaldialdehyde (OPA) Assay of thermally treated samples.

Sample	OD at 340 nm	µM / mg of protein
MWPC non-heated	0.905 [a]	298 [a]
MWPC-DX200 heated	0.491 [b]	206 [c]
MWPC-DX35 heated	0.458 [c]	272 [b]
MWPC heated	0.458 [c]	194 [d]

[a-d] Letters next to readings represent significant differences ($P < 0.05$).

Testing was also performed on mixtures of the modified ingredient and dextran with no application of heat treatment to verify that the presence of saccharides did not affect results (Table 5). No significant differences between samples were observed ($P > 0.05$).

Table 5.
Measurement of Free Amino Groups by *o*-phthaldialdehyde (OPA) Assay of samples prior to thermal treatment.

Sample	OD at 340 nm	µM / mg of protein
MWPC non-heated	0.191 [a]	463 [a]
MWPC-DX200 non-heated	0.197 [a]	487 [a]
MWPC-DX35 non-heated	0.196 [a]	475 [a]

[a] Letters next to readings represent significant differences ($P < 0.05$).

Differences in the readings between tests were observed (Table 4 and Table 5) due to a change in the dilution factor used.

3.4 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE results confirmed covalent coupling of MWPC to dextran under dry heating at 100ºC for 2 hours (Figure 3 and 4). Following protein and glyco-protein staining of the gels, characteristic whey protein bands, such as α-lactalbumin (MW: 14 kDa), and β-lactoglobulin (MW: 17 kDa) were observed (Figures 3 and 4). Smearing patterns were observed in the glycoprotein stains that did not appear in the protein stain and it has been found that glycoproteins block interactions with the Coomassie stain leading to reduced sensitivity of their appearance (conversation with Paul Haney a Research and Developer at Pierce Biotechnology on July 17, 2007).

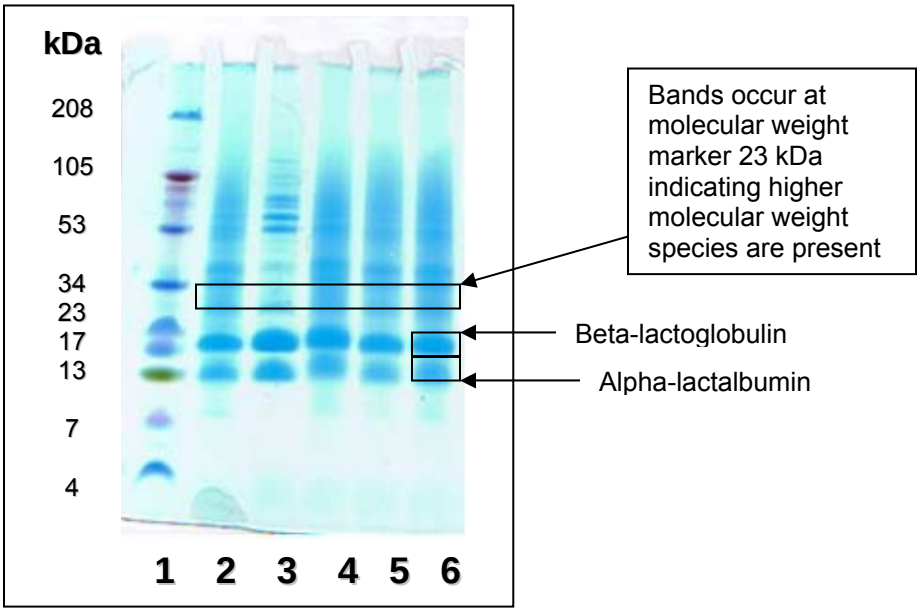

Figure 3. SDS-PAGE protein stain of Lane1, marker protein; Lane2, MWPC nonheated; Lane 3, CWPC; Lane 4, MWPC heated; Lane 5, MWPC-DX35, Lane 6, MWPC-DX200. Samples in lanes 4, 5, and 6 received a dry heat treatment of 100 ºC for 2 hrs. All samples are at pH 3.5 except for sample CWPC at pH 6.6.

Figure 4 depicts the SDS-PAGE glycoprotein banding pattern of MWPC and MWPC-Dextran.

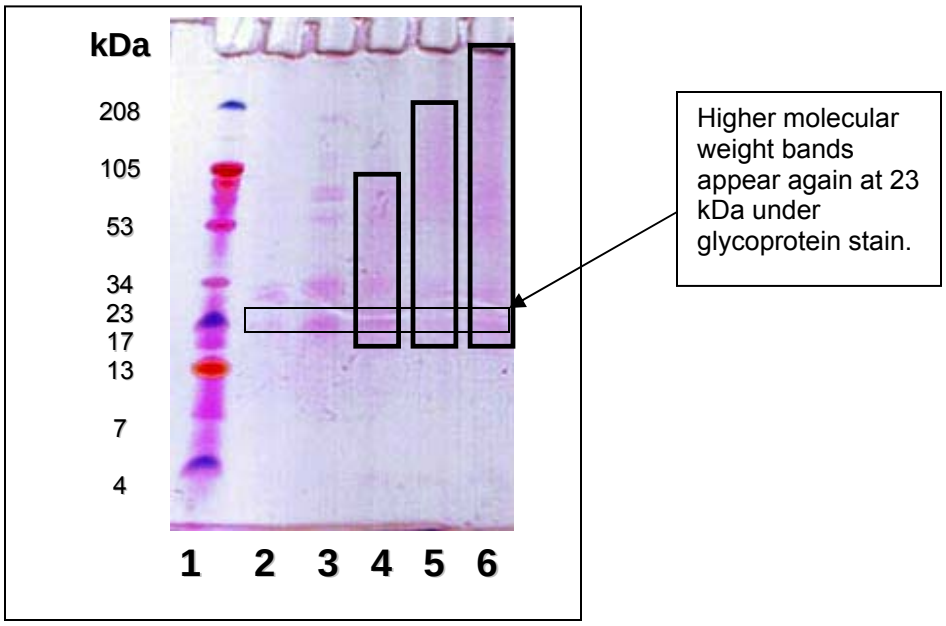

Figure 4. SDS-PAGE glycoprotein stain of Lane1, marker protein; Lane2, MWPC; Lane 3, CWPC; Lane 4, MWPC heated; Lane 5, MWPC-DX35, Lane 6, MWPC-DX200. Lanes 4, 5, and 6 received a dry heat treatment of 100 ºC for 2 hrs. Boxes indicate newly observed smearing pattern of conjugate. All samples are at pH 3.5 except for sample CWPC at pH 6.6.

A smeared carbohydrate staining band spanning the 53-200 kDa range as well as new bands appearing at 23 kDa evidenced of decreased mobility of the MWPC-dextran samples due to an increased molecular size of the conjugate. This is similar to results found by Akhtar and Dickinson (2003) that showed new distinct higher molecular weight bands were formed when whey protein isolate and dextran were heated under dry conditions. Also, Boratynski and Roy (1998), found a BSA-dextran thermal conjugate migrated as a high molecular weight diffuse band spanning a 75-200 kDa range. In their work the height of the band in each thermally treated sample coincided with the size of the dextran used for conjugation. Lane 3 in Figure 3, containing the commercial whey

protein concentrate, evidenced distinct bands at higher molecular weight positions. The appearance of these distinct bands suggests that glycosylation likely resulted from thermal processing and spray drying during manufacturing by the supplier. Also since the CWPC is produced close to neutral pH, 6-6.5, a more suitable condition for Maillard reactivity is encountered. Dry heated samples, at a pH of 3.5, including MWPC (Lane 4), MWPC-DX35 (Lane 5), and MWPC-DX200 (Lane 6), reveal more diffuse bands as the molecular size of the saccharide was increased which is also observed in previous work by Akhtar and Dickinson (2003, 2007) using whey protein and maltodextrin conjugates. To ensure that acid hydrolysis itself did not create false positives, a control gel containing dextran, alone, was electrophoresed and stained for carbohydrate (data not shown). At no point did dextran migrate into the gel suggesting that the diffuse bands observed were a direct result of covalent linkage between the protein and the polysaccharide.

3.5 Steady Shear Rheological Analysis

Rheological analysis of the conjugated and non-conjugated samples elucidated the effects that varying carbohydrates had on the viscosity of the system. It was originally hypothesized that covalent linkage of a large polysaccharide to a modified whey protein would increase the intrinsic viscosity or hydrodynamic radius of the particles, thereby, increasing the viscosity. The MWPC samples containing dextran at a 2:1 ratio of protein to carbohydrate, prior to thermal treatment, exhibited a 3.4 times greater apparent viscosity at a shear rate of 50 s^{-1} than the MWPC alone (Figure 5). The representative 50 s^{-1} shear rate was previously reported to be within the range of that observed during swallowing in humans (Shama and Sherman, 1973).

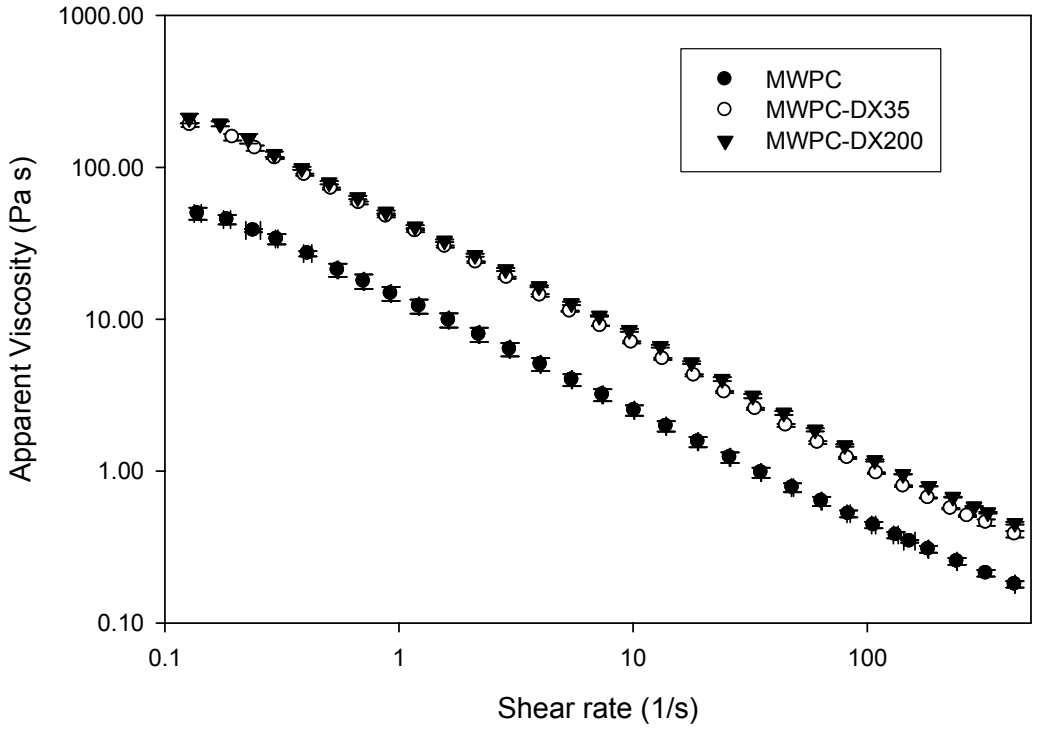

Figure 5. Apparent viscosity of non-heated 7% protein (w/v) MWPC (+/-) dextran prepared at a 2:1 ratio over the shear rate range of 0.1 to 500 s^{-1} at 25ºC. Error bars represent one standard deviation from the mean.

However, upon thermal treatment, the MWPC-dextran conjugate samples showed no further increase in apparent viscosity while the MWPC containing lactose exhibited a 3-fold increase in viscosity (Figure 6). A potential cause for the lack of increased viscosity in the dextran samples may be increased aggregation occurring upon heat treatment of the modified whey in the presence of dextran. The increased aggregation potentially leads to segregative phase separation causing a protein rich phase surrounded by a carbohydrate rich phase which in turn diminishes protein-protein interactions and entanglements (de Kruif and Tuinier, 1999). Lactose at high temperatures can convert into lactulose by the rearrangement of aldoses to ketoses and a study by Matsuda *et al*. (1991) found that heating β-Lactoglobulin in the presence of lactulose caused a high degree of polymerization of the protein. Increased

polymerization of the β-Lactoglobulin molecules without the obstruction of dextran may have lead to the increase in viscosity that was observed by just heat treatment of the MWPC in the presence of lactose.

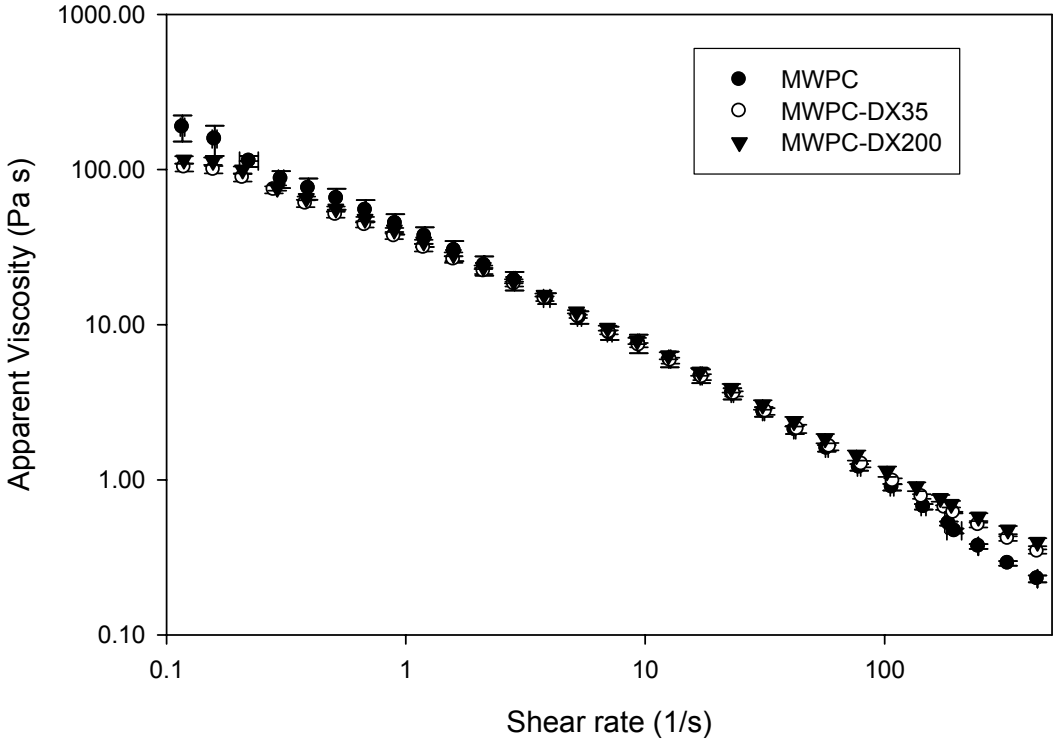

Figure 6. Apparent viscosity of 7% protein (w/v) MWPC (+/-) dextran prepared at a 2:1 ratio (after dry heat treatment at 100ºC for 2 hours) over the shear rate range of 0.1 to 500 s^{-1} at 25ºC. Error bars represent one standard deviation from the mean.

3.6 Small Amplitude Oscillatory Shear

Viscoelastic data of the newly formed glycoconjugates showed similar trends to data obtained through steady shear analysis. All samples displayed the formation of a viscous semisolid as indicated by G′ dominating G″ as the frequency was increased over three orders of magnitude. Initially the dextran-added samples prior to heat treatment

showed a greater degree of gel rigidity as indicated by the higher shear modulus (Figure 7).

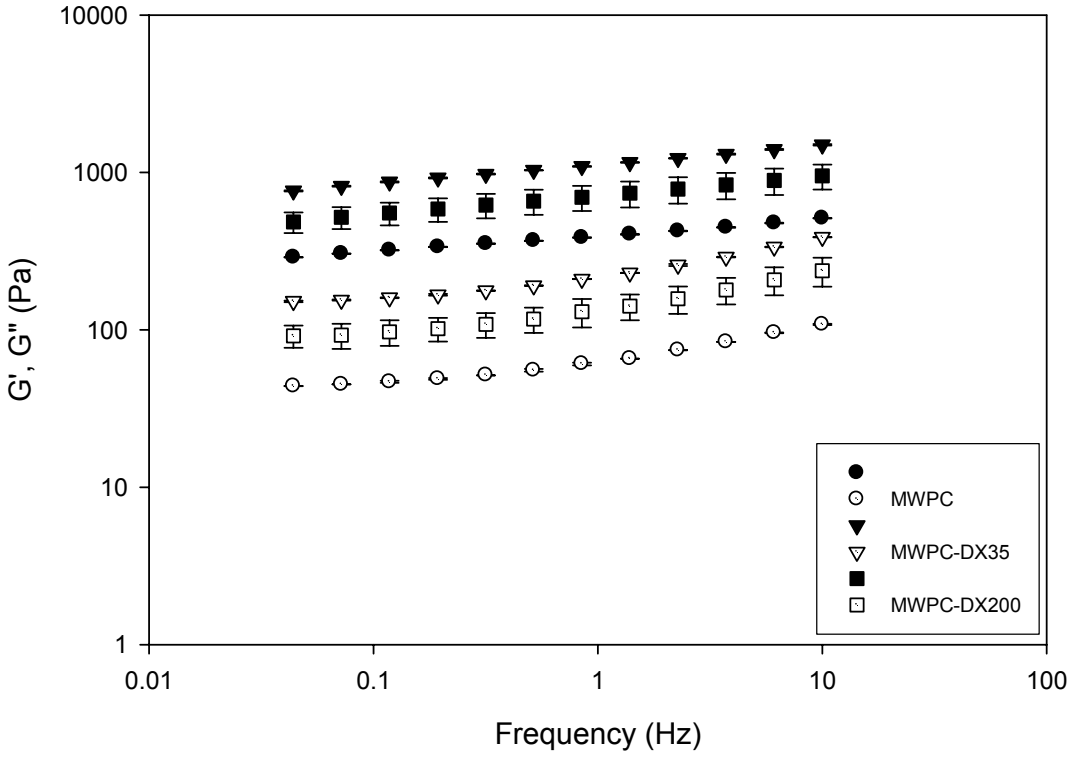

Figure 7. Small amplitude oscillatory shear data for non-heated MWPC (7% protein, w/v) (+/-) dextran prepared at a 2:1 ratio. Closed symbols indicate G′ values and open symbols indicate G′′ values. Error bars represent one standard deviation from the mean.

After heat treatment, the MWPC sample alone showed increased gel rigidity over the dextran-added samples (Figure 8). Rigidity is defined as the shear modulus (G), which is proportional to the complex modulus (G*), and is the force per unit area necessary to change the shape of a material (shear stress, σ) divided by the change in length per the height of the material (shear strain, γ) (Steffe, 1996). At a concentration of 7% protein (w/v) the MWPC-dextran, prepared at a 2:1 ratio, does not separate, producing a homogenous solution. Gel rigidity increased amongst all heated samples, although the highest degree of change was noted in MWPC containing only lactose,

where the highest color formation and loss of amino groups was previously observed (Table 3, Table 4).

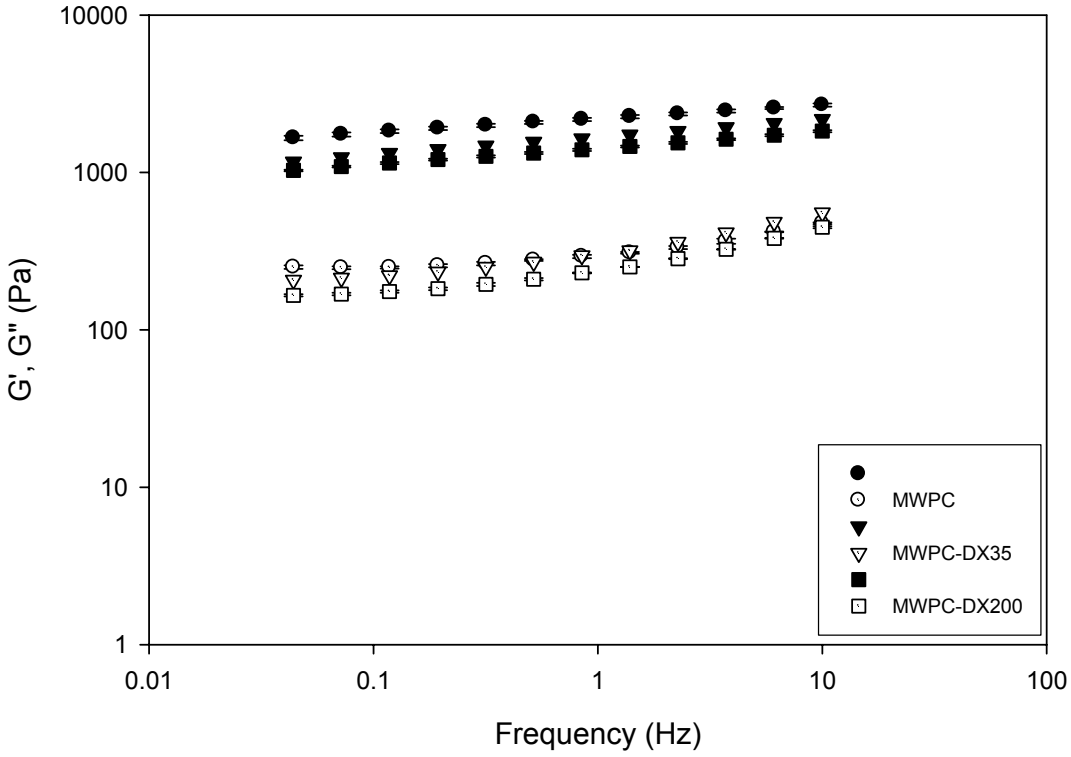

Figure 8. Small amplitude oscillatory shear data for 7% (w/v) MWPC (+/-) dextran prepared at a 2:1 ratio (after dry heat treatment at 100ºC for 2 hours). Closed symbols indicate G′ values and open symbols indicate G′′ values. Error bars represent one standard deviation from the mean.

3.7 Emulsion Stability

To stabilize emulsions, parameters such as viscosity of the continuous phase, as well as, repulsive and attractive forces can greatly influence the degree of creaming that occurs within a system. Creaming is a result of emulsion separation in which case, particles rise to the top while no interaction occurs between the particles. Proteins act as emulsifying agents where the aqueous environment is suitable for effective steric and electrostatic stabilization (Akhtar and Dickinson, 2003 and 2007). A conjugate which stabilizes an emulsion containing a solvent that promotes full solvation of the hydrophilic

chains into the medium is a necessary requirement for steric stabilization. In the case of the conjugate, the hydrophobic protein head is positioned firmly at the interface, while the hydrophilic tail of the polysaccharide, now covalently linked to the protein, repels itself restricting attractive forces (Figure 9). Attractive forces can be observed as flocculation, where emulsion particles clump together, or coalescence, where the surface of two particles ruptures upon contact, creating a larger particle. Formation of high molecular weight glycoprotein conjugates exploit both the ability of the protein to adsorb to the oil-water interface and the solvation capability of the polysaccharide in an aqueous phase medium (Dickinson and Galazka, 1991).

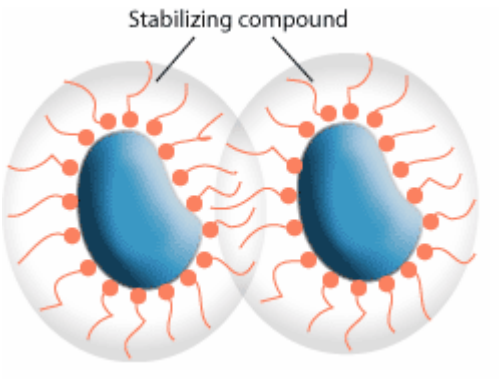

Figure 9. Diagram of steric stabilization. Hydrophobic head attached to dispersed phase interface with covalently linked polysaccharide hydrophilic tail protruding into aqueous phase medium. Taken from (http://www.specialchem4coatings.com/tc/dispersion/index.aspx?id=steric) accessed May 2007.

Creaming index calculations of the emulsified samples were made after storage for 1 week at ambient temperature (Figure 10). Particle size analysis of each sample determined the average modal diameter of the particles to be 3.8 μm, nearing the ideal size of 1 μm, necessary for stability of an emulsified system by hydrodynamic and interparticle forces as well as Brownian motion (Genovese *et al*., 2007). All samples were significantly different ($P < 0.05$), and exhibited a clear serum layer indicating phase

separation of the emulsion. Interestingly, the greatest stabilization occurred in the heated MWPC-dextran (35 kDa – 45 kDa) sample which also exhibited the least color change and greatest number of residual reactive amino groups remaining after thermal treatment. Increased stabilization was also exhibited by the MWPC-dextran (100 kDa – 200 kDa) sample, although to a lesser degree. Akhtar and Dickinson (2007) found similar results upon conjugation of whey protein isolate and maltodextrin. Samples containing a higher molecular weight maltodextrin (280 kDa) were less stable than those comprised of a lower molecular weight maltodextrin (8.7 kDa) based on the particle size obtainable with the lower molecular weight polysaccharide. It has been theorized that whey protein conjugates containing dextran at molecular weights higher than 100 kDa may prevent saturation of the protein around the oil droplet surface thereby limiting increased stability (Dunlap and Cote, 2005).

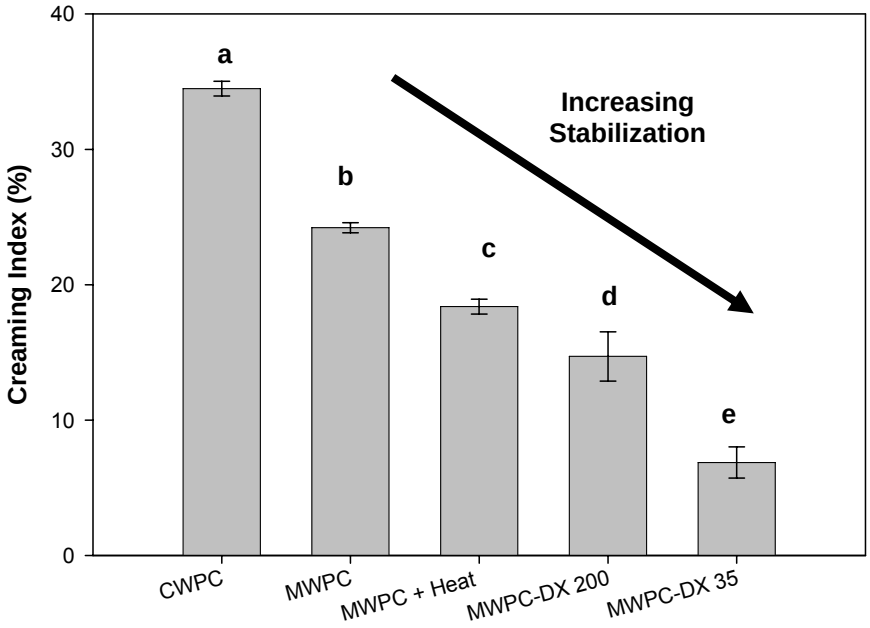

Figure 10. Plot of creaming index values for 5 mg/ml protein (w/v) solutions of CWPC, MWPC, heated MWPC, and heated MWPC-DX200 and MWPC-DX35 prepared at a 2:1 ratio measured after 1 week at ambient temperature. Error bars represent one standard deviation from the mean. All samples are at pH 3.5 except for sample CWPC at pH 6.6. $^{a-e}$ Letters next to readings represent significant differences ($P < 0.05$).

All emulsified samples containing MWPC showed greatly improved stability over CWPC with the MWPC-dextran samples exhibiting a creaming index percentage that was 2 to 3 times less. Samples were normalized based on protein content necessitating higher amounts of solids with respect to MWPC-dextran samples. The higher molar ratio of dextran in combination with the increased molecular size of the glycoconjugate may have led to enhanced steric stabilization amongst the particles (Akhtar and Dickinson, 2003 and 2007). Since stability can occur in protein systems containing a saccharide component that has not undergone prior heat treatment, MWPC-dextran (35 kDa – 45 kDa) samples were analyzed (+/-) dry heating at 100ºC for 2 hours (Figure 11).

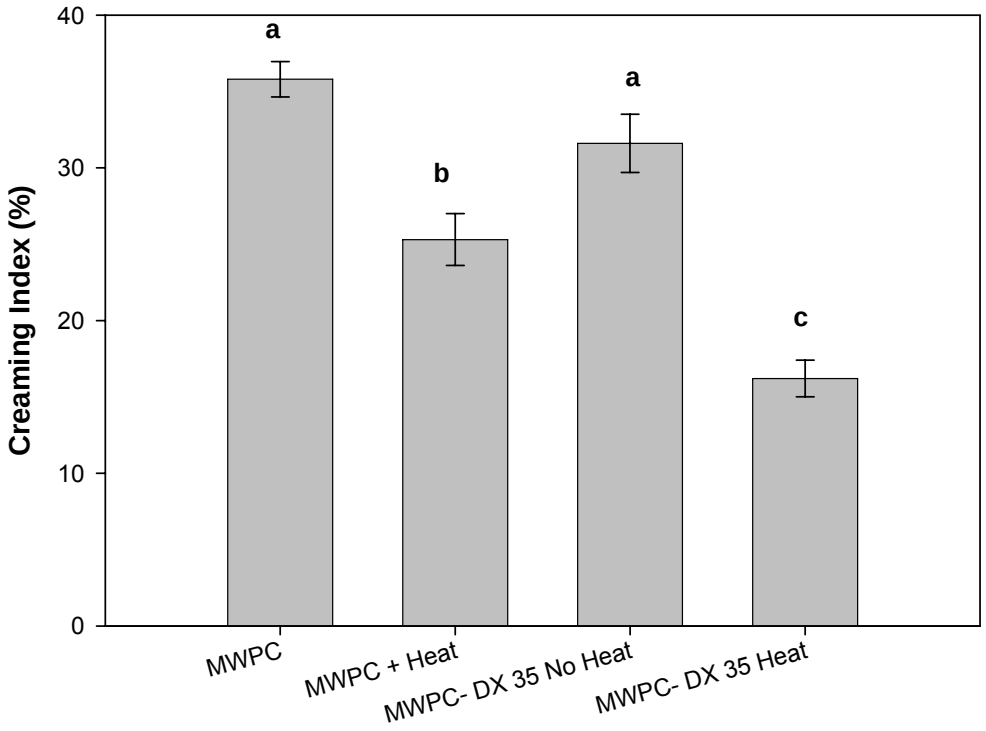

Figure 11. Plot of creaming index values for 5 mg/ml protein (w/v) solutions of MWPC and MWPC-DX35 mixtures (+/-) thermal treatment measured after 1 week at ambient temperature. [a-c] Letters next to readings represent significant differences ($P < 0.05$).

Significant differences were observed in the samples that had been thermally treated, with the dextran added sample showing the greatest stability ($P < 0.05$), while no significant differences occurred with the non-heated samples ($P > 0.05$). These results may have been due to simple coacervation or thermodynamic incompatibility occurring to a greater extent in the non-heated samples (Dickinson and Semanova, 1992).

3.8 Emulsifying Capacity

The volume of oil that the newly formed glycoconjugate could potentially stabilize was determined through measurement of the inversion point or the point at which the emulsion separated or converted to a water-in-oil emulsion. At the inversion point, a rapid increase in resistance was observed. A protein or protein-carbohydrate continuous phase serves as a good conductor, showing little to no resistance to the conduction of electricity, while oil acts as an insulator greatly restricting electrical conduction. When the system can no longer sustain the addition of oil, the emulsion breaks down, potentially inverting to a water-in-oil emulsion, a process associated with a high resistance. A representative depiction of this inversion point is shown in Figure 12.

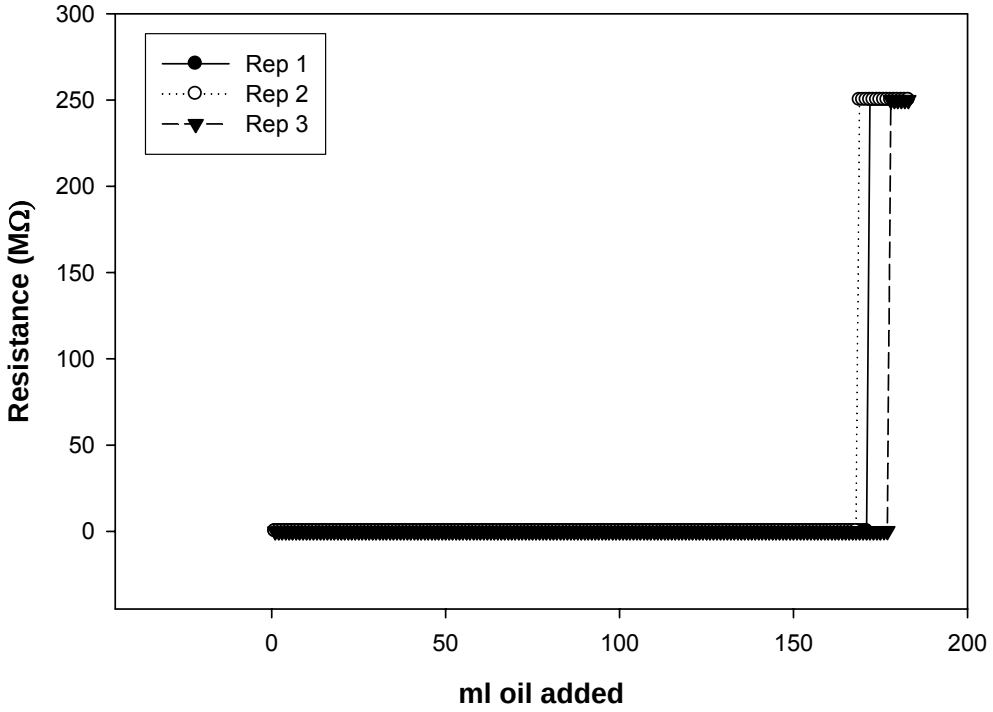

Figure 12. Resistance measurement showing the inversion point of a MWPC dispersion prepared at 1 mg/ml protein concentration, maintained at 25ºC, while corn oil was continuously added at the rate of 1 ml per second.

In this example, an oil level of 175 ml, spikes the resistance from a baseline value relatively close to zero to 250 MΩ. Significant differences were observed amongst the MWPC-dextran (100 kDa – 200 kDa) (both heated and non-heated), as well as, the heated MWPC-dextran (35 kDa – 45 kDa) and the non-heated MWPC ($P < 0.05$) (Figure 13). No major variations occurred between the heated MWPC and the non-heated MWPC-dextran (35 kDa – 45 kDa) ($P > 0.05$). The greatest capacity for oil stabilization appears to be in the MWPC-dextran (100 kDa – 200 kDa).

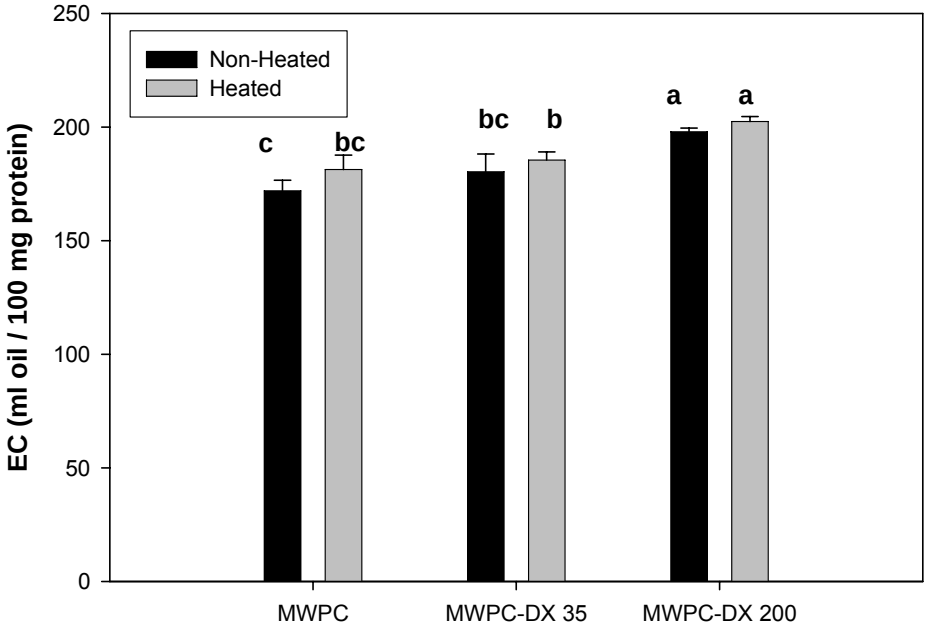

Figure 13. Emulsifying capacity of MWPC protein solutions (1 mg/ml) mixed with dextran at a 2:1 ratio of protein to carbohydrate (+/-) thermal treatment. Error bars represent one standard deviation from the mean. [a-c] Letters next to readings represent significant differences ($P < 0.05$).

3.9 Scanning Electron Microscopy

Scanning electron micrographs of MWPC and MWPC-DX200 samples prepared at a 2:1 ratio of protein to carbohydrate were taken at magnifications ranging from 1000X to 15,000X. These data illustrated variations in the microstructure as the higher molecular weight MWPC-DX200 conjugates were formed (Figure 14). Surface images exhibited a course, granular structure with small pockets (Image B in Figure 14) which were not apparent in the MWPC sample, alone (Image A in Figure 14). The number of pockets in the MWPC-DX200 sample begins to increase in direct correlation with increases in magnification, leading to the appearance of a highly porous structure (Image D in Figure 14) compared to the dense network of MWPC, alone (Image C in Figure 14). The glutaraldehyde and formaldehyde fixatives used to crosslink the whey proteins, enabled their imaging; however these reagents are unable to fix carbohydrates.

Therefore, these pockets or gaps may be indicative of the presence of carbohydrate in the protein network minimizing interactions between the protein structures (Dickinson and Chen, 1999).

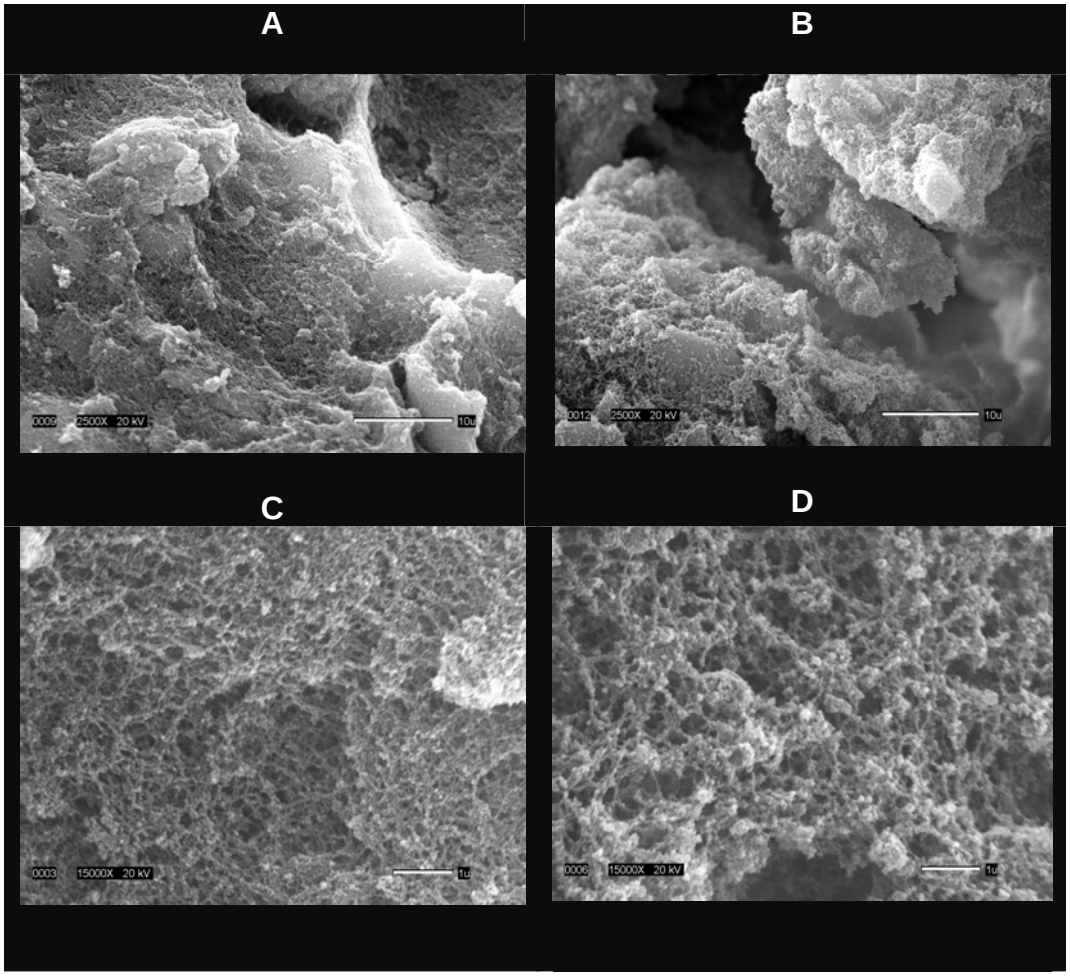

Figure 14. Scanning electron micrographs of MWPC 5.6% protein A) 2500X, C)15,000X and 2:1 ratio of MWPC to dextran (100 kDa – 200 kDa) B) 2500X, D) 15,000X showing surface variations and porous network formation (Image B and D).

3.10 Ionization Mass Spectrometry

Resulting chromatograms established that 8.5% and 21.4% of the total peptide (protein containing the appropriate residue) of the MWPC and CWPC respectively, existed in the lactosylated form as a result of processing (Figure 15). Based on previous work lysine residues at positions 47 and 60 were found to be the point of attachment

(Leonil *et al.*, 1997). Further treatment of MWPC under dry heating conditions increased lactosylation by 30%. The high incidence of lactosylation with respect to CWPC may be a result of its neutral pH promoting increased Maillard reactivity during pasteurization and spray drying. The results for MWPC were slightly less than those reported by Leonil *et al*. (1997), a finding which might be expected considering that their experiments were performed at a more favorable neutral pH in contrast to the acidic conditions utilized in the current work. An attempt was made to assess glycosylation in the MWPC-dextran conjugated samples but due to size restrictions of the carbohydrate, this method was not applicable.

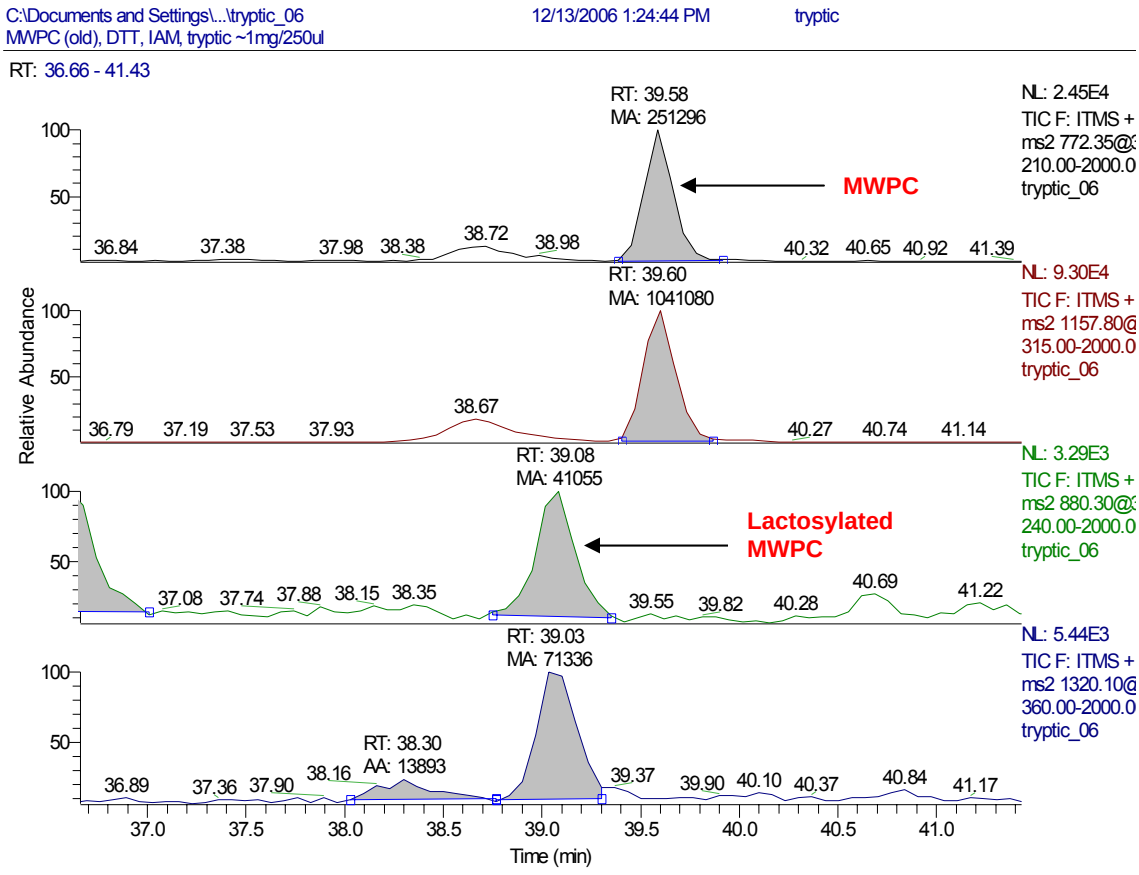

Figure 15. Ionization mass spectrometry chromatography data illustrating peaks of duplicate MWPC samples prior to heat treatment and lactosylation occurring after heat treatment.

3.11 Water Holding Capacity

Thermal processing of MWPC dispersions appeared to decrease their capacity to hold water (Table 6). All samples were significantly different and exhibited a pellet after centrifugation indicating their inability to retain water ($P < 0.05$). These results correlated with those data obtained with color and OPA measurements in which case the ability to bind water went in decreasing order as follows: non-heated MWPC > MWPC-DX200 > MWPC-DX35 > MWPC-lactose. Although the capacity to hold water was diminished after thermal treatment, the protein powders were still able to hold nearly six times their weight of water.

Table 6.
Water holding capacity of MWPC and MWPC-glycoconjugates at 25 ºC.

Sample	Water Holding Capacity[a] (gram water held/g protein)
MWPC non-heated	8.31 [a]
MWPC-DX200 heated	6.15 [b]
MWPC-DX35 heated	5.67 [c]
MWPC heated	5.21 [d]

[a-d] Letters next to readings represent significant differences ($P < 0.05$).

CHAPTER 4. CONCLUSIONS

4. Conclusions

Proteins are highly suited for modification purposes through thermal treatment and complexation with carbohydrates by non-chemical means such as the Maillard reaction. Normally the Maillard reaction is favored at a neutral pH (Nursten, 2005). The findings of this study suggest that the Maillard reaction can occur under acidic pH conditions in a dry environment as evidenced by a reduction in available amines of the protein and color formation occurring after thermal treatment. The specific carbohydrate selection was a key component that impacted the final functionality.

The effects of conjugation on viscosity appeared to be highly dependent on the saccharide present in the system. Increased viscosity was observed when the modified ingredient was heated in the presence of lactose; however, no increase occurred in the presence of higher molecular weight polysaccharides such as dextran. Gel rigidity was also enhanced upon heating to the greatest degree using MWPC test samples containing only lactose. This ability could potentially benefit whey protein concentrate manufacturers in regards to reducing added costs by utilization of lactose normally present in whey protein concentrates, as the carbohydrate complexing component.

The greatest benefit of conjugation with a higher molecular weight polysaccharide such as dextran appeared to be associated with its ability to stabilize emulsions. Both the total volume of oil stabilized and the storage time over which it remained stable were enhanced through conjugation. Emulsion stability was highest in the thermally treated dextran samples although the number of reactive lysine residues was higher and color formation was less compared to the MWPC, alone. This finding potentially affords a two-fold benefit for manufacturers in that functionality of the MWPC-dextran conjugate is increased while in a system where more bioactive lysine is available for consumers, as well as, the potential for off colors and flavors are reduced.

5. References

Akhtar, M. Dickinson, E. 2003. Emulsifying properties of whey protein-dextran conjugates at low pH and different salt concentrations. Colloids and Surfaces B: Biointerfaces 31125-132.

Akhtar, M. and Dickinson, E. 2007. Whey protein-maltodextrin conjugates as emulsifying agents: an alternative to gum arabic. Food Hydrocolloids, 21: 607-616.

Balagtas, J. V., Hutchinson, F.M., Krochta, J. M., Sumner, D. A. 2003. Anticipating market effects of new uses for whey and evaluating returns to research and development. J. Dairy Sci. 86: 1662-1672.

Boratynski, J. and Roy, R. 1998. High temperature conjugation of proteins with carbohydrates. Glycoconjugate Journal, 15: 131-138.

Church, F.C., Swaisgood, H.E., Porter, D.H. and Catignani, G.L. 1983. Spectrophotometric assay using *o*-phthaldialdehyde for determination of proteolysis in milk and isolated milk proteins. Journal of Dairy Science, 66: 1219-1227.

de Witt, J.N. 1984. Functional properties of whey proteins in food systems. Milk Dairy Journal, 38, 71.

de Witt, J.N. 1989. Functional properties of whey proteins. *Developments in Dairy Chemistry, Vol. 4* Elsevier Applied Science, New York, 285.

Demetriades, K., McClements, D.J. 1999. Flocculation of whey protein stabilized emulsions as influenced by dextran sulfate and electrolyte. Journal of Food Science, 64, 206-210.

Dickinson, E. 2006. Colloid science of mixed ingredients. Soft Matter, 2: 642-652.

Dickinson, E. and Chen, J. 1999. Heat-set whey protein emulsion gels : Role of active and inactive filler particles. J. Disp. Sci. and Tech., 20(1and2): 197-213.

Dickinson, E. and Galazka, V.B. 1991. Emulsion stabilization by ionic and covalent complexes. In A. M. Stephen (Ed). Food polysaccharides and their applications. New York: Marcel Dekker. 501-515.

Dickinson, E., and Izgi, E. 1996. Foam stabilization by protein-polysaccharide complexes. Colloids and Surfaces. 113, 191-201.

Dickinson, E., and Semenova, M. G. 1992. Emulsifying behaviour of protein in the presence of polysaccharide under conditions of thermodynamic incompatibility. J. Chem. Soc. 88(6), 849-854.

Dunlap, C.A., and Cote, G.L. 2005. β-Lactoglobulin-dextran conjugates: effect of polysaccharide size on emulsion stability. J. of Agric. and Food Chem.: 53, 419-423.

Einhorn-Stoll, U., Ulbrich, M., Sever, S., Kunzek, H. 2005. Formation of milk protein-pectin conjugates with improved emulsifying properties by controlled dry heating. Food Hydrocolloids: 19, 329-340.

Firebaugh, J.D. 2004. Characterization and application of a derivatized whey ingredient. Master's Dissertation, NC State University, 2004.

Genovese, D.B., Lozano, J.E., Rao, M.A. 2007. The rheology of colloidal and noncolloidal dispersions. Journal of Food Science, Vol 00, Nr. 0, R1-R10.

Hudson, H.M., Daubert, C.R., and Foegeding, E.A. Thermal and pH stable protein thickening agent and method of making the same. 2001, US Patent No.6261624.

HunterLab. 1996. Hunter lab color scale. Insight on color. Applications Note. Vol. 8, No. 9: 1-4.

Jimenez-Castano, L., Villameil, M., Lopez-Fandino, R. 2007. Glycosylation of individual whey proteins by Maillard reaction using dextran of different molecular mass. Food Hydrocolloids. 21: 433-443.

Kato, A. 2002. Industrial applications of Maillard-type protein-polysaccharide conjugates. Food Science and Technology Research, 8, 193-199.

Kinsella, J.E., Whitehead, D.M., Brady, J., Bringe, N.A. 1989. Milk proteins: possible relationships of structure and function. *Developments in Dairy Chemistry, Vol. 4* Elsevier Applied Science, New York, 55.

Leonil, J., Molle, D., Faubquant, J., Maubois, J.L., Pearce, R.J., Bouhallab, S. 1997. Characterization by ionization mass spectrometry of lactosyl β-lactoglobulin conjugates formed during heat treatment of milk and whey and identification of one lactose-binding site. Journal of Dairy Science. 80: 2270-2281.

Matsuda, T., Kato, Y., Nakamura, R. 1991. Lysine loss and polymerization of bovine β-Lactoglobulin by amino carbonyl reaction with lactulose. Journal of Agricultural and Food Chemistry, 39: 1201-1204.

Minton, A. 2005. Models for excluded volume interaction between an unfolded protein and rigid macromolecular cosolutes: macromolecular crowding and protein stability revisited. Biophysical Journal. 88: 971–985.

Mishra, S., Mann. B., Joshi, V.K. 2001. Functional improvement of whey protein concentrate on interaction with pectin. Food Hydrocolloids. Vol. 15, 9-15.

Morr, C.V. 1979. Review: functionality of whey protein products. Dairy Science Technology. 14, 185.

Morr, C.V. 1982. Functional properties of milk proteins and their use as food ingredients. *Developments in Dairy Chemistry, Vol 1 Proteins*. Fox, P.F., Ed., Applied Science, New York, 375.

Morr, C.V. and Ha, E.Y.W. 1993. Whey protein concentrates and isolates: processing and functional properties. Critical reviews in Food Science and Nutrition. Vol. 33(6), 431-476.

Nielson, B.R., Stapelfeldt, H., Skibsted, L. 1997. Early prediction of the shelf-life of medium-heat whole milk powders using stepwise multiple regression and principal component analysis. Int. Dairy Journal, 7: 341-348.

Nursten, H. 2005. The Maillard reaction; chemistry, biochemistry, and implications. Royal Society of Chemistry. p. 31-32.

Oliver, C. M., Melton, L. D., Stanley, R. A. 2006. Creating proteins with novel functionality via the Maillard reaction: A review, Critical Reviews in Food Science and Nutrition, 46: 337–350.

Onwulata, C.I., Konstance, R.P., and Tomasula, P. M. 2004. Minimizing variations in functionality of whey protein concentrates from different sources. J. Dairy Sci. 87:749-756.

Resch, J.J. and Daubert, C.R. 2002. Rheological and physiochemical properties of derivatized whey protein concentrate powders. International Journal of Food Properties, 5: 419-434.

Reyes, F.G.R., Poocharoen, B., Wrolstad, R.E. 1982. Maillard browning reaction of sugar-glycine model systems: changes in sugar concentration, color and appearance. Journal of Food Science, 47: 1376-1377.

Shama, F. and Sherman, P. 1973. Identification of stimuli controlling the sensory evaluation of viscosity. II: oral methods. J. Texture Stud. *4*, 111–118.

Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J., Klenk, D.C. 1985. Measurement of protein using bicinchoninic acid. Anal. Biochem. 150: 76-85.

Steffe, J. F. 1996. Rheological methods in food process engineering: second edition. Freeman Press, MI. 1.

USDA. 2006. http://www.ams.usda.gov/dairy/mncs/graphs/dy20060804MWPC_prod.pdf. Accessed January 2007.

Walstra, P. 2003. Physical Chemistry of Foods. New York: Marcel Dekker. 735.

Wiechelman K.J., Braun R.B., and Fitzpatrick J.D. 1988. Investigation of the bicinchoninic acid protein assay: Identification of the groups responsible for color formation. *Anal. Biochem.* 175: 231–237.

APPENDIX 1.

Calcium Effects on the Functionality of a Modified Whey Protein Ingredient

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ABSTRACT

The primary objective for this study addressed the effects of supplemental calcium on the functional properties of a modified whey protein concentrate (MWPC), prepared by acidification to pH 3.35, followed by extended heat treatment, gelation, and spray drying. In the presence of added calcium (MWPC-Ca^{++}), protein solutions showed increased thickening capacity, especially under refrigeration temperatures, compared to dispersions made with the MWPC, alone. A rheological assessment included the determination of (i) power law parameters, (ii) viscoelastic properties, and (iii) the effects of heating and cooling on these protein systems. The SDS-PAGE banding pattern revealed various disulfide linked molecular forms of β-lactoglobulin, bovine serum albumin, and immunoglobulin when electrophoresis was performed in the absence of β-mercaptoethanol compared to the pattern observed with commercial whey protein concentrate (CWPC) samples. An enhanced water holding capacity was observed in MWPC-Ca^{++} dispersions as measured by centrifugal methods. Differential scanning calorimetry established that addition of calcium salts caused a two-fold increase in the amount of bound, or unfreezeable water compared to MWPC controls. The physical appearance of the structural network varied significantly between glyceraldehyde fixed samples of MWPC-Ca^{++} versus MWPC dispersions when visualized with scanning electron microscopy. In the case of MWPC-Ca^{++} samples, the formation of large, rounded, spherical structures was noted and ascribed to an increased surface tension caused by the higher salt content. Ultimately, such attributes may afford distinct advantages for whey-based ingredients intended for application within food systems, especially under cold processing conditions.

A1.1 INTRODUCTION

Whey protein concentrates provide an excellent food resource because of the relatively high protein concentration, excellent nutritional quality, and exceptional functional characteristics. In previous work, Hudson *et al.* devised a process to modify a whey protein isolate (MWPI), forming a new dairy ingredient with improved water holding capacity and expanded gelation characteristics when compared to other commercial WPI products. This bioprocessing method involved a pH adjustment and thermal treatment (Hudson *et al*., 2000). After gelation, the materials were frozen, freeze dried, and ground into a powder. Later, Resch, Firebaugh, and Daubert extended these approaches using less costly whey protein concentrates (MWPC), and discovered that these alternative whey protein fractions also afforded instantaneous thickening capacity and cold-set gel functionality and stability (Resch and Daubert, 2002; Firebaugh and Daubert, 2005). Apparently, these expanded rheological characteristics can be directly attributed to the unique methodologies utilized during manufacture (Hudson *et al.*, 2001).

The added effects of monovalent or divalent salts on the physical/functional attributes of whey protein solutions have been well documented (Kuhn and Foegeding, 1991; Barbut and Foegeding, 1993; Sherwin and Foegeding, 1997; Vardhanabhuti *et al.*, 2001; Caussin *et al.*, 2003; Rattay and Jelen, 1995; Tang *et al.*, 1993; Ju and Kilara, 1998; Fujino *et al*., 1993; Fujino *et al.*, 1995). However, in most cases, these studies were performed using whey-based solutions prepared with a higher protein content and at a more neutral pH value than the dispersions used in this work. Although monovalent salts, such as sodium chloride, can alter viscoelastic parameters such as gelation times and/or temperatures, gel strength, viscosity etc., divalent cations, including barium and most notably calcium, appear to have a more significant impact on whey protein systems (Kuhn and Foegeding, 1991, Barbut and Foegeding, 1993, Sherwin and Foegeding, 1997; Caussin *et al.*, 2003; Rattay and Jelen, 1995; Ju and Kilara, 1998; Fujino *et al.*,

1993; Fujino *et al.*, 1995). Calcium induced aggregation presumably occurs as a result of three major events in which case: (1) electrostatic interactions are diminished as a result of charge neutralization, (2) ion-specific hydrophobic interactions are induced, and (3) Ca^{++}-protein bridges are formed, a process resulting in the crosslinking of adjacent anionic groups, such as glutamic and/or aspartic acid residues (Kinsella *et al.*, 1989).

Herein, the functional changes that occurred upon addition of supplemental calcium chloride to MWPC protein dispersions were systematically evaluated. Initially, an optimal ratio of [whey protein:salt] was defined in order to maintain a stable liquid suspension at room temperature. Afterwards, this formulation was analyzed using standard instrumental techniques to evaluate functional attributes including power law constants and viscoelastic properties. In earlier reports, Resch and Daubert (2002) identified the cold gelling characteristics associated with MWPC protein solutions; therefore, a storage study was performed with calcium supplemented MWPC dispersions, held at 4°C, in order to monitor changes in viscoelasticity over time. Also, the general effects of heating and cooling on this system were investigated.

In previous work, Resch and Daubert observed an ~8 fold enhancement in the water holding capacity of MWPC protein dispersions compared to those prepared with commercial WPC ingredients (2002). Since, the properties of water molecules can play such an important role in protein solution chemistry, we addressed the changes that occurred upon the addition of extra calcium ion and found that this particular functional parameter was increased even more. It was especially noteworthy that the amount of bound, or non-freezable, water was elevated in MWPC solutions containing supplemental calcium as compared to control MWPC samples based on differential scanning calorimetry measurements. In fact, the entire protein network structure was physically altered as viewed in scanning electron micrographs. Perhaps, hydrophobic associations were promoted as a result of the increased surface tensions caused by the

addition of divalent salts to these whey protein dispersions (Ohki and Zschornig, 1993). Ultimately, the results obtained from these studies may provide new insight for designing improved whey protein ingredients with expanded functionality for their use in novel applications within the food industry.

A1.2 Materials and Methods

Both the MWPC and CWPC used for these studies were prepared by Grande Cheese Incorporated (Lomira, Wiscosin). Heated whey protein solutions (5.6% protein), pH 6.5, were prepared by thermally processing at ~80°C for 90 minutes, a slight modification from the original method (Resch and Daubert, 2002). Calcium chloride was purchased from Fisher Scientific (Fair Lawn, New Jersey). All other reagent grade chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

A1.2.1 Sample Preparation

Protein solutions were hydrated with distilled water to a final concentration of 3%, 5.6%, or 7% (w/v) using CWPC or the MWPC ingredient for 1-2 h at room temperature prior to adding supplemental calcium chloride to a final concentration of 0, 25, 50, 75 or 100 mM. The pH was adjusted to ~ pH 3.5 with phosphoric acid, if necessary. In some experiments, the pH was raised to pH 6.5 using 1N sodium hydroxide. All hydrated test fractions were stirred, again, for a minimum of 3 hours at room temperature, then, stored overnight at 4°C prior to analysis unless otherwise noted.

A1.2.2 Rheological Analysis

A StressTech controlled stress rheometer (ATS Rheosystems, Bordentown, NJ) equipped with a 25mm serrated couette assembly was used for all tests. Prior to analysis, each sample was covered with a thin layer of mineral oil to minimize

dehydration. All dispersions were pre-sheared for 30 sec at 15 s^{-1} in order to establish a baseline shear history.

A1.2.2.1 Steady Shear

The apparent viscosity of MWPC and CWPC solutions was determined at 4°C and 25°C. Duplicate samples were subjected to a shear rate ranging from 0.1-500/s over 900 s. The apparent viscosity (η) data were modeled according to the power law equation which takes into account the flow behavior (n) of the material and the consistency coefficient (K) (Steffe and Daubert, 2006):

$$\eta = K\dot{\gamma}_b^{\,n-1} \qquad (4.0)$$

A1.2.2.2 Small Amplitude Oscillatory Shear

After determining the linear viscoelastic regions, MWPC dispersions and MWPC dispersions containing 100 mM calcium chloride (5.6% protein, pH 3.5) were analyzed at a stress setting of 0.7 Pa as the frequency was increased from 0.01 – 1 Hz. Under these experimental conditions, the storage (G') modulus and loss (G'') modulus were recorded at both 25°C and 4°C.

To simulate cold storage conditions, control MWPC (5.6% protein, pH 3.5) and calcium supplemented MWPC protein solutions (5.6% protein, 100 mM $CaCl_2$, pH 3.5) were hydrated for at least three hours at room temperature, loaded into the rheometer at a stress setting of 1 Pa with a frequency of 0.1 Hz, and maintained under refrigeration temperatures (4°C) for a period of 14h.

Heating and cooling ramps were also performed with MWPC dispersions prepared at 5.6% protein (w/v) in deionized water, in the presence and absence of 100

mM calcium chloride, and stirred for several hours at room temperature with extended hydration overnight at 4°C. All test solutions were analyzed at a stress level of 1 Pa and a frequency of 0.1 Hz. Heating and cooling cycles were accomplished according to the following regime: (1) heat treatment from 25°C to 80°C, (2) holding at 80°C for 5 minutes, (3) cooling to 4°C and holding for 30 min, then (4) reheating to 25°C. Heating and cooling rates were set at 1°C/min.

A1.2.3 SDS PAGE

All WPC and MWPC protein samples were appropriately diluted with distilled water, and mixed 1:1 (v/v) with 8% SDS, 0.9M Tris-Tricine sample buffer (InVitrogen Inc., Carlsbad, CA). In identified cases, β-mercaptoethanol was added to 5% in the sample buffer. All samples were then heated at 100°C for 5 min prior to loading onto 10-20% Tris-Tricine gradient polyacrylamide gels (InVitrogen, Inc). After electrophoresis, the samples were stained directly for visualization of protein bands using a colloidal Coomassie Blue staining reagent (InVitrogen, Inc.).

A1.2.4 Water Holding Capacity

The water holding capacity was determined according to the method described by Resch and Daubert (2002).

A1.2.5 Differential Scanning Calorimetry

Measurement of bound water was performed by using differential scanning calorimetry. The percentage of bound or unfreezeable water in MWPC test samples, pH 3.5, containing 8% solids (5.6% protein) and prepared (+/-) supplemental calcium chloride (100 mM), were measured with a DSC7 differential scanning calorimeter (Perkin

Elmer Instruments, LLC, Norwalk, Conn). The DSC was calibrated with indium (melting onset temp = 156.6 ºC; enthalpy = 28.45 J/g) and mercury (melting onset temp = -38.8 ºC). The reference, an empty stainless steel pan, weighed within 0.3 mg of the sample pan. Nitrogen, the purging gas, was maintained at a constant flow rate of 30 cc/min.

The stainless steel calorimeter pans were filled with MWPC test fractions (~60 mg), sealed with a press, and loaded into the DSC. The dispersions were then cooled to -50ºC at 10ºC/min, held for 5 min at -50ºC, and heated to 30ºC at 5ºC/min. Deionized water (~60 mg) was also analyzed in this manner. The heat of fusion for water was calculated using the peak analysis function of the Pyris software program (version 5.0, Perkin Elmer Instruments, LLC, Norwalk, Conn.) and found to be within 1% of the known value (ΔH=335 J/g).

The phase transition of water in the MWPC sample during heating was recorded as the endothermic peak. The fraction of the freezable and unfreezable water in the sample was determined using the following equation (Wang and Gunasekaran, 2006), which assumes that the heat of fusion of free water in the MWPC sample was the same as that for the distilled water/ice.

$$X_{BW} = X_{TW} - \left(\frac{Q_{endo}}{Q_f} \right) \tag{9.0}$$

For these calculations, X_{TW} represents the total water fraction in MWPC, X_{BW} - the bound water, Q_{endo} - the heat of fusion for freezable water as obtained from the DSC thermogram (J/g), and Q_f - the heat of fusion measured for distilled water. X_{TW} was estimated by assuming that the total amount of water in each dispersion was 92% of the sample weight [100%-8% solids].

A1.2.6 Scanning Electron Microscopy

Experimental MWPC dispersions were prepared at 5.6% protein (w/v) in the presence and absence of 100mM calcium chloride, pH 3.5. In all cases, ~ 0.5 mL of each sample was placed in a 78 µm microporous capsule (Structure Probe Inc., West Chester, PA). The capsule was left in 0.1M sodium acetate buffer at pH 3.5 containing 2 ml of cold 3% glutaraldehyde for 24 hours. After 24 hours, the capsule was transferred to a Petri dish containing 0.1M sodium acetate buffer. The whey sample formed a solid mass that was cut into 2-3mm^3 pieces, washed 3 times with 0.1M sodium acetate buffer for 20 minutes at 4°C, and dehydrated using an ethanol series of 30%, 50%, 70%, 95% and 100% (20 minutes each) at room temperature. The final dehydration step was accomplished in 100% ethanol for 20 minutes at 4°C. Critical point drying was achieved with a Samdri-795 dryer (Tousimis, Rockville, MD). Afterwards, a 25-30nm coating of gold/palladium was applied onto each test sample using an Anatech Hummer 6.2 sputter coater (Anatech Ltd, Denver, NC).

A1.3 RESULTS AND DISCUSSION

A1.3.1 Effect of pH Adjustment and Heat Treatment on the Viscoelastic Properties of CWPC vs. MWPC Dispersions in the Absence of Supplemental Calcium

In order to compare the viscoelastic properties of CWPC, pH 6.5, versus MWPC dispersions, pH 3.5, the consistency coefficient, *K*, and flow behavior index, n, were determined. Instrumental readings for *K* and n power law parameters were calculated as the slope and intercept, respectively, obtained from a logarithmic plot of shear stress versus shear rate (Steffe and Daubert, 2006). Other solution parameters, such as viscosity, for example, simply report instrumental readings collected at a single shearing speed and may not be representative of the total changes that occur.

The flow characteristics of MWPC *vs.* commercial WPC protein dispersions differed significantly, even in the absence of supplemental calcium. In MWPC samples, the *K* value was amplified with increasing protein concentration while the flow behavior indexes (n) were lowered (Table 1).

Table 1.
Viscoelastic properties of CWPC, pH 6.5, *vs*. MWPC, pH 3.5, dispersions

Sample 25°C	**0m $CaCl_2$**	**25 mM $CaCl_2$**	**50 mM $CaCl_2$**	**75 mM $CaCl_2$**	**100mM$CaCl_2$**
3% CWPC **pH 6.5**	*K* = 0.0023 n = 0.98 r^2 = 0.920	*K* = 0.0023 n = 0.98 r^2 = 0.920	*K* = 0.0023 n = 0.98 r^2 = 0.920	*K* = 0.0023 n = 0.98 r^2 = 0.920	*K* = 0.0023 n = 0.98 r^2 = 0.920
5.6% CWPC **pH 6.5**	*K* = 0.0051 n = .86 r^2 = .996	*K* = .0044 n = 0.88 r^2 = 0.995	*K* = 0.0041 n = 0.89 r^2 = 0.997	*K* = 0.0042 n = 0.89 r^2 = 0.997	*K* = 0.0044 n = 0.87 r^2 = 0.996
7.0% CWPC **pH 6.5**	*K* = 0.0051 n = 0.87 r^2 = 0.987	*K* = 0.0043 n = 0.88 r^2 = 0.985	*K* = 0.0042 n = 0.90 r^2 = 0.988	*K* = 0.0039 n = .92 r^2 = 0.990	*K* = 0.0052 n = 0.86 r^2 = 0.985
3% MWPC – **pH 3.5**	*K* = 0.0167 n = 0.86 r^2 = 0.999	*K* = 0.0183 n = 0.86 r^2 = 0.999	*K* = 0.0117 n = 0.88 r^2 = 0.998	*K* = 0.0054 n = 0.87 r^2 = 0.986	*K* = 0.0045 n = 0.86 r^2 = 0.978
5.6% MWPC **pH 3.5**	*K* = 0.1799 n = 0.75 r^2 = 0.999	*K* = 1.1174 n = 0.47 r^2 = 0.993	*K* = 6.004 n = 0.26 r^2 = 0.991	*K* = 14.584 n = 0.17 r^2 = 0.954	*K* = 10.545 n = 0.14 r^2 = 0.873
7% MWPC **pH 3.5**	*K* = 28.508 n = 0.24 r^2 = 0.968	*K* = 55.743 n = 0.07 r^2 = 0.578	*K* = 57.22 n = 0.05 r^2 = 0.392	*K* = 13.608 n = 0.20 r^2 = 0.359	*K* = 22.699 n = 0.09 r^2 = .237

In contrast, these parameters were mostly not affected using WPC protein dispersions *per se*. Solanki and Rizvi (2001) reported similar trends while studying such variables using skim milk retentates obtained after microfiltration. Notably, the *K* readings for MWPC dispersions, pH 3.5, containing 5.6% protein, were considerably higher than equivalent WPC protein solutions, pH 6.5, likely caused, at least in part, by the additional heating steps used in the manufacture of the MWPC ingredient, itself.

The effects of heating, alone, were examined when WPC dispersions (5.6% protein), pH 6.5, were thermally treated at ~80°C for 90 minutes, a slight modification from the original method (Resch and Daubert, 2002). Upon cooling to ambient temperatures, the consistency coefficient exhibited a significant fold enhancement, compared to non-heated controls prepared at equivalent pH values (Table 2). These trends differed from the ones seen by other investigators in which case concentrated milk fractions were used versus whey protein materials (Vélez-Ruiz and Barbosa-Cánovas, 1998). In this case, the consistency coefficient decreased with increasing temperature; however, the experimental conditions between the two test systems differed significantly in many respects including the materials used, pH settings, and actual thermal treatments of test protein dispersions.

The effect of simply dropping the pH of commercial WPC protein solutions (5.6 %) from pH 6.5 to 3.5 caused an ~20 fold increase in the consistency coefficient (Table 2). Also, the apparent viscosity of 5.6% MWPC protein solutions, pH 3.5, measured ~69 mPas compared to readings of 2.8 mPas at 50 s^{-1} for control WPC samples, pH 6.5 (Figure 1). Jelen *et al*. and Rattay and Jelen (1992, 1995) made a similar observation when they demonstrated that viscosity measurements of WPC dispersions, prepared at pH values less than 4.0, were significantly higher than those prepared at pH 6.8. In addition, MWPC dispersions exhibited shear thinning behavior evidenced by decreasing viscosity with increasing shear rate, a pattern not seen in equivalent, non-heated, whey protein dispersions prepared with analogous commercial WPC products (Figure 1).

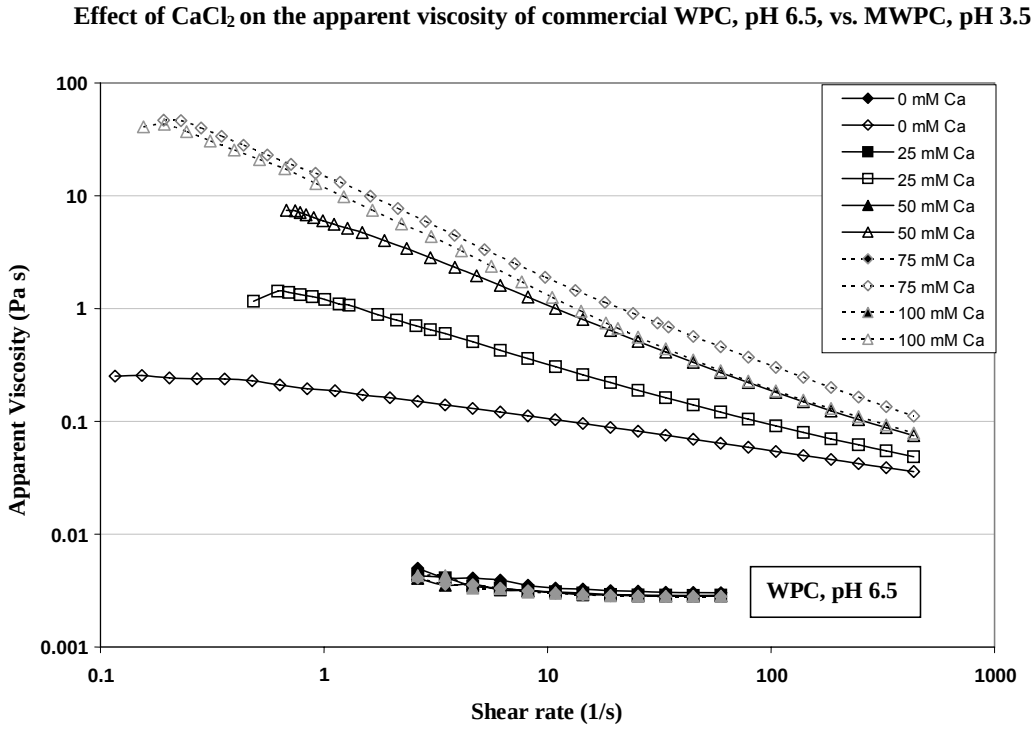


Figure 1: Apparent viscosity changes elicited by the addition of calcium chloride. A CWPC protein solution [closed circles, 5.6% protein (w/v), pH 6.5] and a modified whey protein concentrate dispersion [open circles, MWPC, 5.6% (w/v), pH 3.5] were prepared at 25°C (+/-) supplemental calcium.

A1.3.2 Effect of Salt Addition on CWPC vs. MWPC Dispersions

In earlier work, Hudson *et al.* examined the effect of sodium chloride on several functional parameters of modified whey protein isolates (MWPI); however, in their experiments, the protein concentrations were significantly higher, ~10%, while the sodium chloride content ranged from 0-50 mM (2000). Under these reaction conditions, a fine stranded gel was formed, exhibiting increased strain and decreased stress at fracture, which correlated with lower amounts of the monovalent salt.

The effect of divalent cations on heated whey protein suspensions has also been well documented and reportedly causes even more dramatic changes with respect to certain functional attributes (Kuhn and Foegeding, 1991, Barbut and Foegeding, 1993,

Sherwin and Foegeding, 1997; Caussin *et al*., 2003; Rattay and Jelen, 1995; Ju and Kilara, 1998; Fujino *et al*, 1993; Fujino *et al.*, 1995). In one example, Kuhn and Foegeding (1991) described a generalized effect of divalent cations with respect to increased shear stresses and shear strains at failure using WPI gels containing supplemental calcium chloride, magnesium chloride, or barium chloride compared to those changes brought about by the inclusion of monovalent salts such as Na, Li, K, Rb, and Cs. Their work clearly demonstrated that addition of divalent salts to heated whey protein solutions promoted formation of molecular crosslinks, resulting in an increased firmness of the gel.

Hence, the first series of experiments were designed to characterize the rheological changes elicited by the addition of calcium salts over a protein concentration ranging from 3% - 7% (w/v) while supplemental calcium chloride concentrations varied between 0-100 mM. A summary of these results is presented in Table 1 and Figure 1.

Calcium effects were mostly negligible in all WPC dispersions, prepared at pH 6.5, in which case, thermal exposure was limited to the pasteurization process, itself (Figure 1). Furthermore, additional heating (~80°C, 90 min) produced a ~320 fold rise in the consistency coefficient of these dispersions (pH 6.5), compared to non-heated control solutions. The added impact of 25 mM supplemental calcium increased this *K* value only 2X more and remained relatively constant over the entire range of salt tested (0mM – 100 mM; Table 2). By comparison, the *K* readings for equivalent MWPC samples (5.6% protein), pH 3.5, were ~35 fold higher than comparable WPC solutions, pH 6.5; however, the impact of calcium addition was much more dramatic over the entire concentration range (0 mM–100 mM). In fact, 5.6% MWPC solutions containing added 75 mM calcium exhibited an ~3500 fold increase in the *K* reading compared to analogous whey protein samples, pH 6.5. Moreover, an ~80-fold rise was observed in MWPC dispersions (5.6% protein), pH 3.5, that were supplemented with 75mM calcium

salt compared to the control. Generally speaking, the viscosity of MWPC dispersions, pH 3.5, was higher than equivalently prepared WPC solutions, pH 6.5, and the additional effects of supplemental calcium even still more evident (Figure 1).

Table 2.
Effect of heat and pH on the viscoelastic properties of MWPC dispersions containing 5.6% protein and $CaCl_2$

Test Sample	0m $CaCl_2$	25 mM $CaCl_2$	50 mM $CaCl_2$	75 mM $CaCl_2$	100 mM $CaCl_2$
5.6% WPC **pH 6.5**	*K* = 0.0051 n = .86 r^2 = .996	*K* = .0044 n = 0.88 r^2 = 0.995	*K* = 0.0041 n = 0.89 r^2 = 0.997	*K* = 0.0042 n = 0.89 r^2 = 0.997	*K* = 0.0044 n = 0.87 r^2 =0.993
5.6% WPC, ~80°C-1.5 h, **pH 6.5** -Effect of heat	*K* = 1.623 n = 0.37 r^2 = 0.993	*K* = 3.465 n = 0.28 r^2 = 0.954	*K* = 3.296 n = 0.34 r^2 = 0.995	*K* = 3.30 n = 0.34 r^2 = 0.997	*K* = 2.696 n = 0.36 r^2 = 0.993
5.6% WPC **pH 3.5** -Effect of pH	*K* = 0.0967 n = 0.75 r^2 = 0.999	*K* = 0.0986 n = 0.74 r^2 = 0.999	*K* = 0.0989 n = 0.74 r^2 = 0.999	*K* = 0.0963 n = 0.75 r^2 = 0.999	*K* = 0.0998 n = 0.74 r^2 = 0.999
5.6% MWPC **pH 3.5**	*K* = 0.1799 n = 0.75 r^2 = 0.999	*K* = 1.1174 n = 0.47 r^2 = 0.993	*K* = 6.004 n = 0.26 r^2 = 0.991	*K* = 14.584 n = 0.17 r^2 = 0.954	*K* = 10.545 n = 0.14 r^2 = 0.873

Previously, Sherwin and Foegeding (1997) noted that the impact of calcium on the aggregation of whey protein isolates occurred when the ratio of $CaCl_2$ (mM) to protein (%, w/v) fell between 3.3 and 23.3. In a similar manner, the values obtained during these experiments ranged from ~ 4-18 using MWPC dispersions containing 5.6% protein and 25 mM-100 mM $CaCl_2$. Previously, Ju and Kilara (1998) examined turbidity changes upon varying the amount of calcium chloride added to whey protein solutions (w/w) and also observed that the highest aggregational state was achieved at optimal ratios. Likewise, these same investigators reported that specific proportions of protein/calcium impacted gel formation and gel hardness using WPI solutions (Ju and Kilara, 1998). Taken together, these findings may be explained, at least in part, on the basis of thermal denaturation of the whey protein constituents, a process that may cause

increased exposure of partially buried aspartic/glutamic acid residues promoting an enhanced calcium binding capacity, especially with respect to β-lactoglobulin (Pappas and Rothwell, 1991).

Not surprisingly, MWPC dispersions manifested the classical rheological characteristics of a thickened fluid in that data curves revealed a zero shear plateau at low shear rates and exhibited shear thinning behavior at moderate speeds (Figure 1). This pattern was observed in all MWPC test samples containing 5.6% protein, (+/-) supplemental calcium. At lower protein concentrations (3%), a phase separation occurred in dispersions prepared with either the CWPC or MWPC ingredient, attributed to the fact that the amount of protein was below the critical concentration required for network formation. At higher amounts, such as 7% protein, the r^2 values (MWPC) did not evidence a linear instrumental response to increasing shear rates, likely caused by the dramatic thickening of the solution, itself. In fact, upon the addition of 25 mM calcium salts, there was a significant deviation from linearity, $r^2 = 0.578$, a finding that could be attributed to increased electrostatic repulsion caused by a higher protein content, itself. In contrast, test dispersions prepared with CWPC powders to 7% protein, pH 6.5, were extremely fluid even in the presence of calcium.

A1.3.3 Effect of Calcium Addition on Rheological Attributes

During the first set of experiments, it was observed that 5.6% protein solutions, containing a minimum of 75 mM calcium chloride, afforded maximal viscosity with optimal solution stability. In fact, inclusion of 100 mM calcium ion resulted in a highly thickened dispersion that was very resistant to flow even at ambient temperatures (Table 1, Figure 1). When these dispersions were refrigerated overnight at ~4°C, a significant hardening of the gel was noted; therefore, MWPC dispersions, prepared at 5.6% protein,

with 100 mM added calcium chloride were selected for further study since a primary goal for this work was focused on developing a cold set gelling agent.

A series of small strain oscillatory experiments were performed to evaluate the response of this test system to varying harmonic oscillations caused by mechanically imparted stress. The data was then used to evaluate the elasticity and/or viscosity of MWPC test dispersions over a range of different frequencies. As illustrated in Figure 2, protein dispersions, devoid of added salt, exhibited more “liquid-like” characteristics evidenced by the fact that G" (loss modulus) dominated G' (storage modulus) at both 4°C and 25°C. Furthermore, [$G'_{4°C}$, $G''_{4°C}$] moduli data were slightly elevated compared to those values observed at 25°C, indicative of increased thickening at colder temperature settings even in the absence of supplemental calcium. However, upon inclusion of 100 mM $CaCl_2$ into the system, a predominant “gel-like” characteristic of the MWPC protein solution was observed in which case, G'>G" at both 25°C and 4°C. Although MWPC dispersions containing 100 mM calcium chloride were essentially “gelled” at room temperature, the gel strength was significantly higher (~10X) for chilled samples. Furthermore, there was a very dramatic increase in the storage modulus (G') of MWPC-Ca^{++} dispersions maintained under refrigeration temperatures compared to control solutions lacking additional calcium, especially at low shear rates (Figure 2). Apparent viscosity measurements were performed at 4°C as well, in which case, shear thinning characteristics were also observed in MWPC dispersions (5.6% protein, pH 3.5) in the presence and absence of supplemental calcium (data not shown).

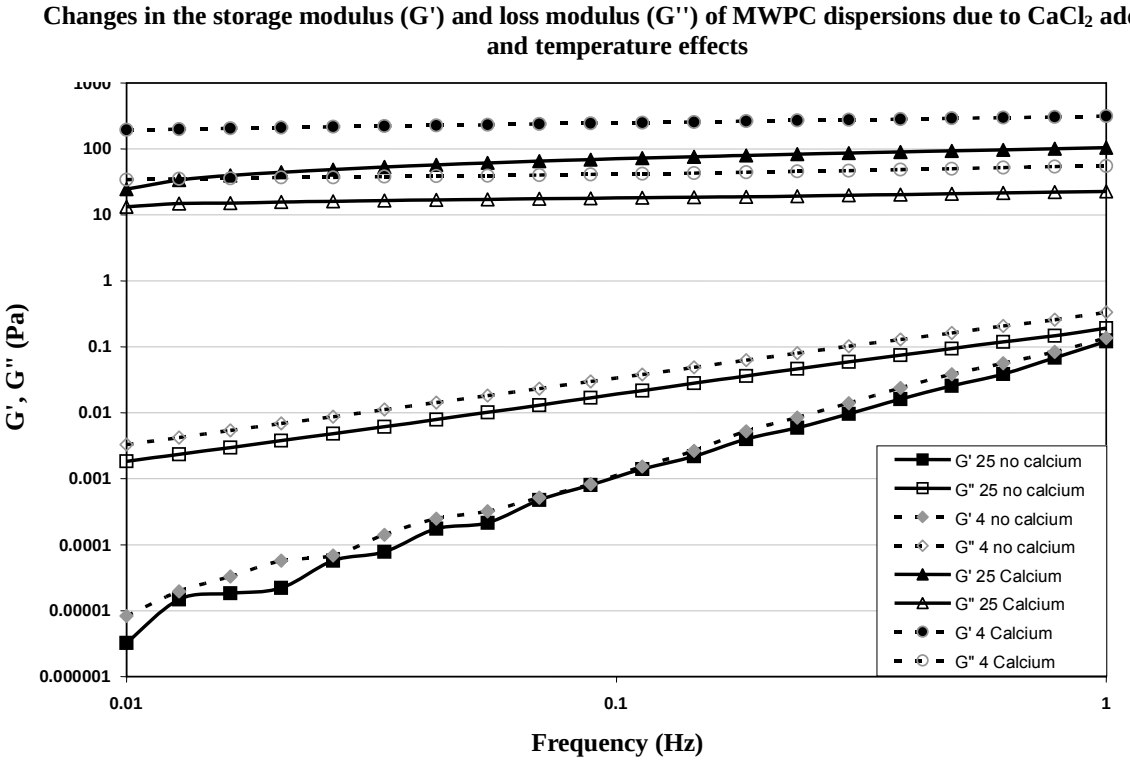


Figure 2. The effect of supplemental calcium chloride (100 mM $CaCl_2$) on the storage modulus (G') and loss (G'') of modified whey protein dispersions (5.6%, *w/v*), pH 3.5, at 4°C and 25°C.

A1.3.4 Effect of Supplemental Calcium on Apparent Viscosity During Long Term Storage at 4ºC

One of the more striking results revealed that the gel strength, reflected by G' readings of calcium supplemented MWPC dispersions, pH 3.5, was dramatically higher than that of equivalent control solutions, especially at 4°C (Figure 3). Likely, this finding can be at least partially attributed to the exposure of protein constituents to an acidic solution environment coupled with thermal unfolding of globular whey proteins, such as β-lactoglobulin, which occurred during processing (Resch and Daubert, 2002). At these pH values, the addition of calcium may reduce electrostatic repulsion as a result of charge neutralization effects between the salt and α-carboxylic acid groups in which the average pKa value is ~2.2 (Anonymous, 2007). Concomitantly, hydrophobic forces could

be enhanced as a result of thermal exposure resulting in protein aggregation and gelation. Ultimately, it is this combination of multiple molecular interactions that result in network formation creating a more “solid-like” protein matrix especially under refrigeration temperatures.

Because the increased thickness of the calcium supplemented MWPC sample was so pronounced at lower temperatures, these changes were also investigated during long term cold storage of the dispersion (~4°C). Maintenance of the protein solution under refrigeration temperatures showed a steady climb in the viscosity over a 14 h interval time (Figure 3). Apparently, the strength of the gelling network continued to progress, albeit at a slow rate, upon holding at 4°C, suggesting that the elastic rigidity of the protein/salt suspension was enhanced during cold storage.

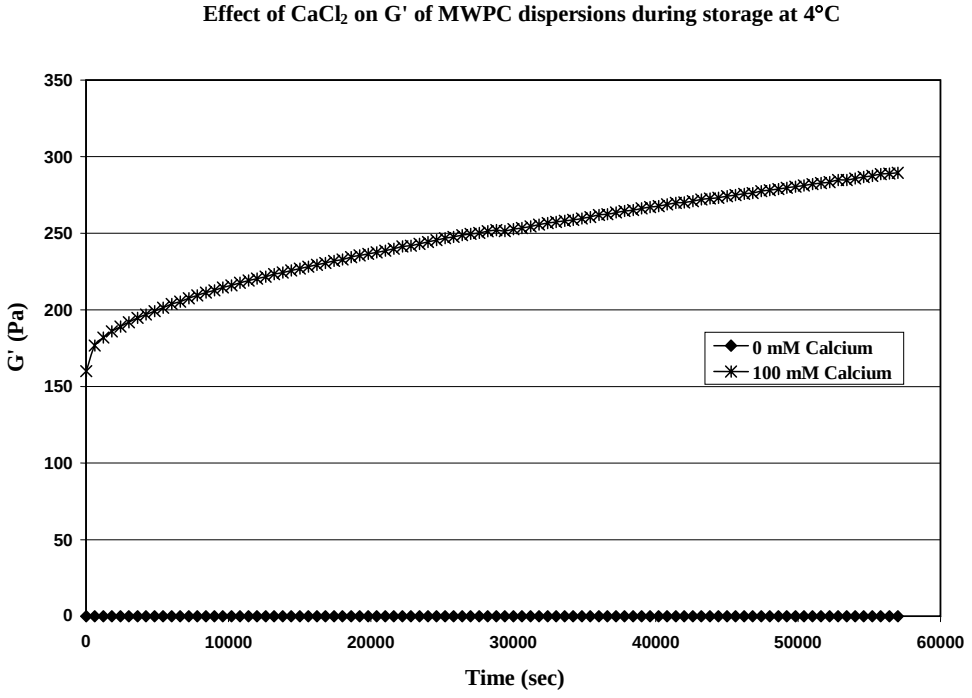


Figure 3. Gel strengthening at 4°C during storage of a MWPC protein dispersion [5.6% protein (*w/v*), pH 3.5] containing supplemental calcium chloride (100 mM) as measured by an increased storage modulus (G′).

A1.3.5 Effects of Heating and Cooling on the Rheological Attributes of MWPC Protein Dispersions

The effect of heating and cooling on these whey protein systems was examined, and the data revealed that the complex viscosity slightly increased in control MWPC samples (5.6% protein), lacking supplemental Ca^{++}, upon heating from ~47°C to 61°C (Figure 4). Furthermore, these values continued to climb, albeit at a slower rate, for the remainder of the cycle. This rise might be attributed to additional unfolding and further denaturation of the MWPC starting material resulting in a larger hydrodynamic radius for various protein components. In addition, hydrophobic associations may be promoted under these experimental conditions.

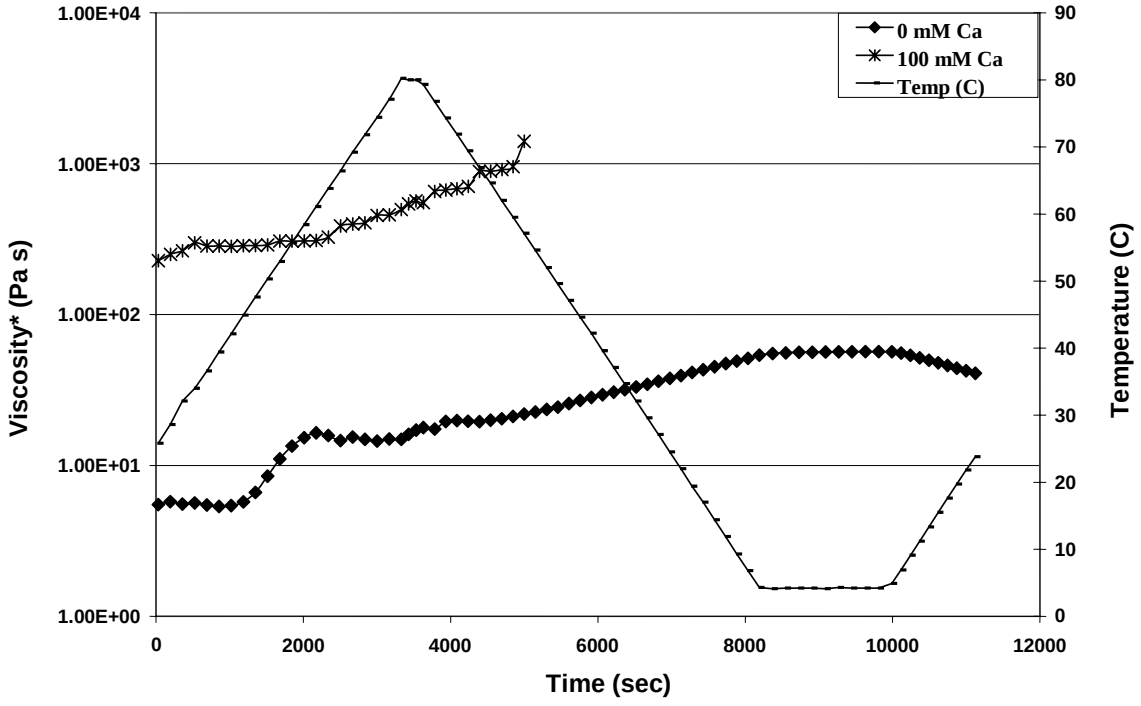


Figure 4. The effect of heating and cooling on the complex viscosity of MWPC protein dispersions (5.6%, *w/v*), pH 3.5, prepared in the presence and absence of 100 mM calcium chloride.

A1.3.6 The Added Effects of Calcium Salt on Heating and Cooling Determined by the Complex Viscosity Profile

Addition of calcium into this system had a significant effect, especially during the heating phase (step 1, Figure 4). Initially, the complex viscosity was much higher in calcium supplemented MWPC samples as noted before. Also, the viscosity rose during the heating cycle although the temperature at which this rise occurred was significantly higher than in those dispersions lacking additional calcium (~72°C *vs.* 47°C). With continued thermal exposure to ~75°- 80°C, followed by subsequent cooling, the viscosity continued to increase; however, upon removal of the sample from the cup, a water layer appeared to have formed on the top of the whey protein dispersion, attributed to a phase separation of the test sample. Ultimately, such changes prevented a collection of reliable rheological instrumental data during cycles 3 and 4 (data not shown).

A1.3.7 Protein Banding Profile of CWPC vs. MWPC

Since the rheological profile of MWPC dispersions differed so significantly from commercial CWPC protein solutions, the SDS-PAGE banding patterns of the two were investigated (Figure 5). The manufacturing process used for preparing either of the dried powders involved HTST pasteurization of raw milk, followed by a second HTST pasteurization of the whey, itself (Grande cheese, personal communication). However, MWPC was further heated after a pH adjustment to ~3.35 (Hudson *et al.*, 2000; Hudson *et al.*, 2001). Based on the electropherograms, obtained after electrophoresis under reducing and non-reducing conditions, the results revealed that extensive disulfide bond formation had occurred in the MWPC ingredient end product. Evidently, the extra heating step promoted crosslinking reactions, especially notable with respect to β-lactoglobulin (β-Lg), bovine serum albumin (BSA), and the immunoglobulin (Ig) fraction.

In the absence of βME, the staining intensity of monomeric β-Lg was somewhat diminished in MWPC samples compared to those prepared with commercial WPC powders, while BSA and Ig bands were essentially eliminated in MWPC test fractions. Instead, these banding patterns evidenced a broad "smearing configuration", likely indicative of the formation of higher molecular weight, disulfide linked aggregates, such as β-Lg polymers, BSA aggregates, and/or hybrids: β-Lg-BSA, BSA-Ig, β-Lg-Ig and so forth, all of which migrate with increased molecular size.

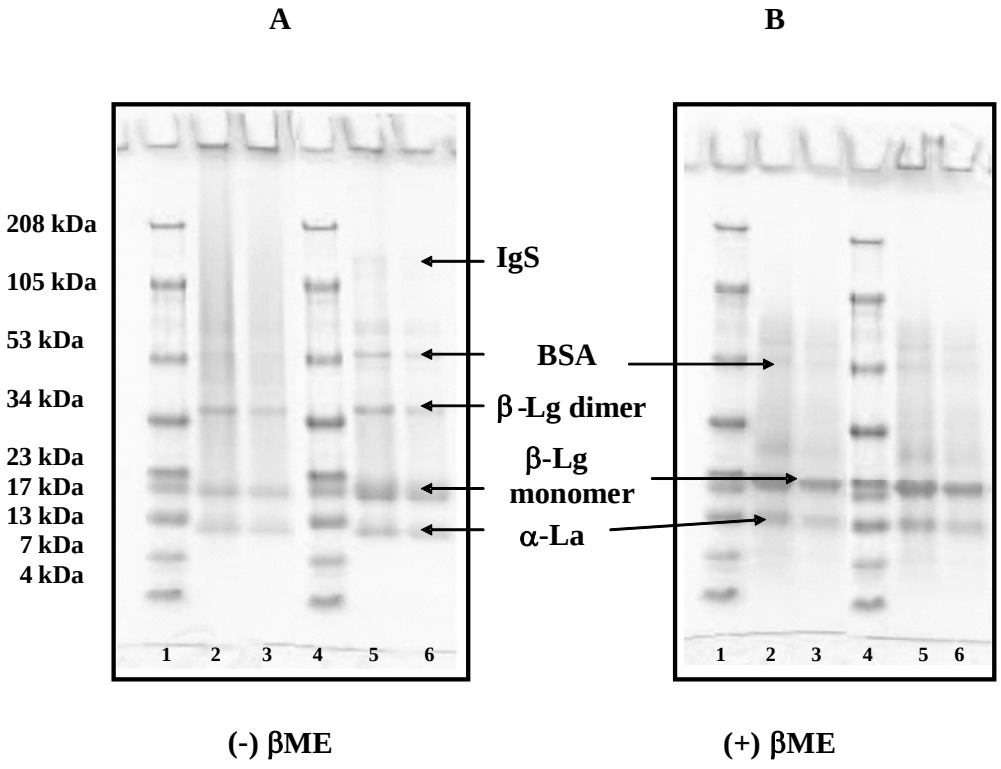


Figure 5. SDS-PAGE banding profile of CWPC, pH 6.5, and MWPC protein dispersions, pH 3.5, prepared at 5.6% protein (*w/v*) in the absence (A) and presence (B) of β-mercaptoethanol. Panel A: Lane [1]: Marker, [2]: MWPC, 40 μg, [3] MWPC, 30 μg, [4] Marker, [5] CWPC, 40 μg, [6] CWPC, 30 μg. The samples seen in panel B were loaded in an identical manner.

A1.3.8 Water Holding Capacity

Whey proteins can undergo irreversible denaturation upon heating, and oftentimes, gel formation occurs. If the gel is later dried, the protein displays increased water holding capacity (WHC) attributable to enhanced capillary action within the insoluble protein network (Fennema, 1985). In previous work, the WHC of the MWPC ingredient was determined by Resch and Daubert using centrifugal methods (2002). These investigators reported that 1 gram of a MWPC powder held ~8 grams of water. Herein, a similar finding was made although the water holding capacity of MWPC dispersions containing added calcium was even yet slightly higher, especially at 4°C (Figure 6). In a similar manner, Barbut and Foegeding (1993) demonstrated that salt induced WPI gels exhibited an improved water holding capacity, while Hongsprabhas and Barbut (1997) also reported an increased WHC, especially at lower temperatures, using whey protein–calcium test systems.

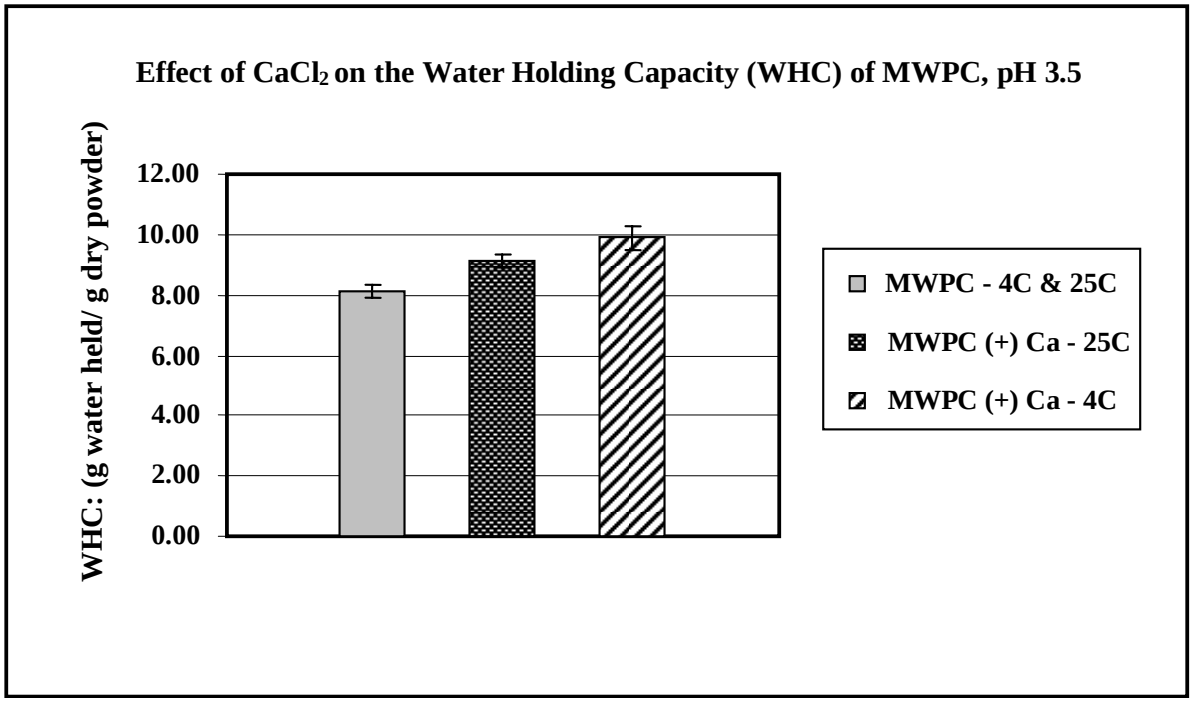


Figure 6. Changes in the water holding capacity of MWPC dispersions (5.6%, *w/v*), pH 3.5, upon the addition of 100 mM calcium chloride at 25°C and 4°C.

A1.3.9 Differential Scanning Calorimetry: Bound or Non-Freezable Water

Bound water, defined as non-freezable water at -40°C, exhibits different properties from that of "bulk" water molecules (Fennama, 1985). It is often detected by differential scanning calorimetry (Duckworth, 1971) in which case the fusion of ice in the sample is measured as an endothermic peak, and the area under the curve assumed to be proportional to the amount of freezable water. Others have reported that such measurements are independent of the initial moisture content if calculated from enthalpy values (Roos, 1986). Consequently, in these experiments, the amount of non-freezable water was calculated as a percentage of the total water in the sample.

Herein, we observed that the amount of bound water in whey protein solutions supplemented with calcium (MWPC-Ca^{++}) was at least two times higher than the amount detected in MWPC dispersions (Figure 7). Presumably, calcium ions hold water more tightly resulting in a decreased mobility of bound water molecules, an attribute that could be due to the strength of the electric field that was generated (Fennama, 1985).

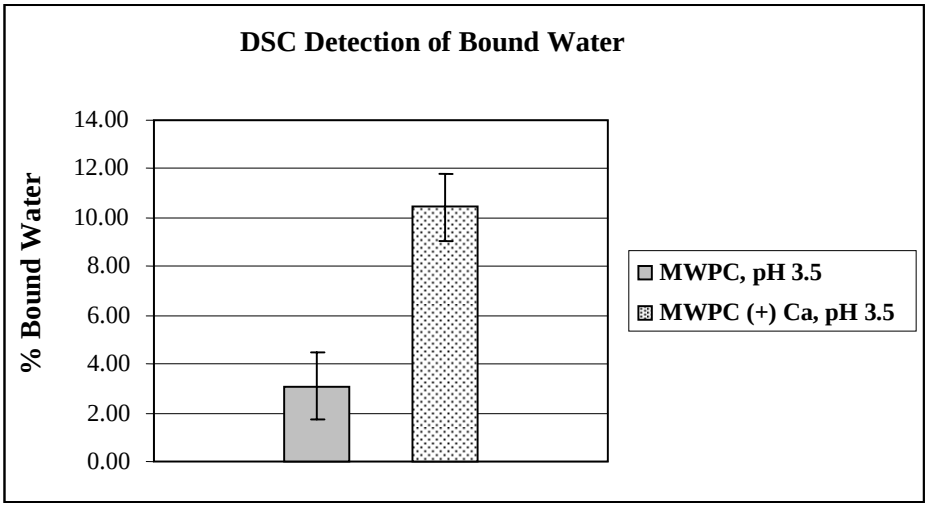


Figure 7. Differential scanning calorimetry analysis of non-freezeable (bound) water in MWPC protein dispersions (5.6%, *w/v*), pH 3.5, prepared in the presence and absence of 100 mM calcium chloride.

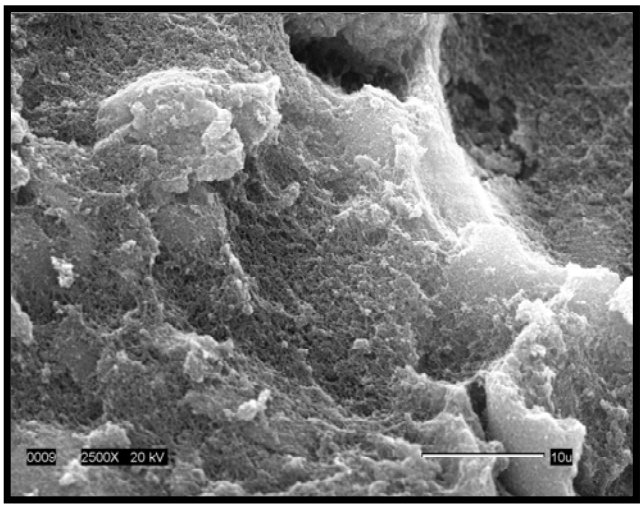
A1.3.10 Scanning Electron Microscopy

Since calcium ions bind water more tightly, these effects can increase hydrogen bonding between surrounding water molecules. In turn, this type of solution configuration may represent an unfavorable thermodynamic state attributable to decreased entropy within the system. Therefore, in order to minimize such unstable molecular interactions, hydrophobic groups quite often associate. Harwalker and Kalab found that whey protein solutions prepared at low pH and high ionic strength lowered the ionic forces and enhanced hydrophobic interactions (Harwalker and Kalab, 1985). Also, Shimizu *et al.* noted that the relative hydrophobicity of β-lactoglobulin, a major constituent of whey protein fractions, was increased ~ 200 fold simply by adjusting the pH from 7.0 to 3.0 (Shimizu *et al.*, 1985).

As shown in figure 8, scanning electron micrographs clearly depicted whey protein aggregates, which appeared as large, rounded, “ball-like” structures in MWPC solutions containing supplemental calcium. Perhaps, this particular configuration was created as a result of hydrophobic effects which minimized surface contact between neighboring water molecules with protein hydrophobic side chains leading to a more thermodynamically stable state. Also, the increased surface tension caused by the higher salt content may play a contributing role in the formation of these structures.

Previous investigators showed that the size of the aggregate formed during calcium induced gel formation of whey protein isolates was larger than those of non-calcium supplemented samples and such findings were ascribed to hydration effects caused by the increased salt content (Ju and Kilara, 1998). In a separate article, Britten and Giroux (2001) suggested that spherical polymers prepared with whey protein solutions containing 4 mM calcium, pH 6.5, appeared to be extensively aggregated and formed softer gels.

A. MWPC, pH 3.5



B. MWPC (+) 100 mM $CaCl_2$, pH 3.5

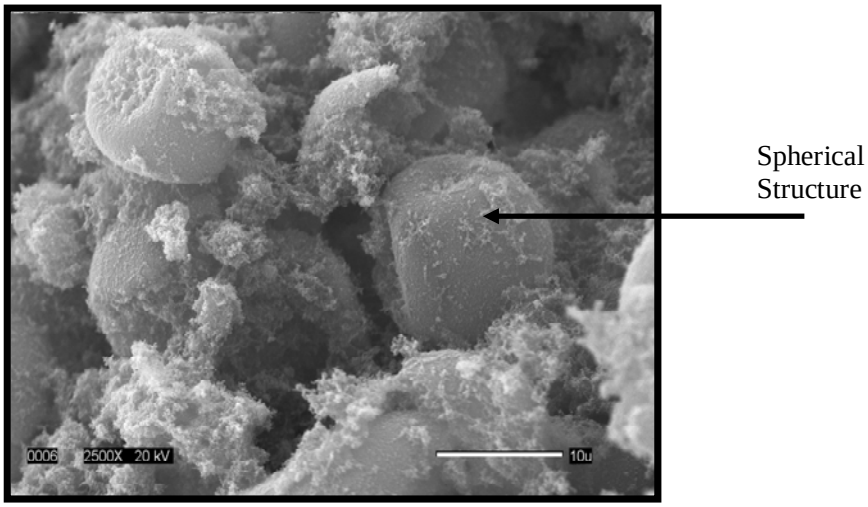


Figure 8. Scanning electron micrographs of MWPC protein dispersions (5.6%, *w/v*), pH 3.5, prepared in the absence (A) and presence (B) of 100 mM calcium chloride. The magnification is 2500X.

A1.4 Conclusions

A process was developed for creating whey protein formulations that exhibited gel-like characteristics at both ambient and refrigeration temperatures. The amount of protein required to form such a network was somewhat less in the presence of 75 mM supplemental calcium, i.e., 5.6% MWPC protein, compared to the report of Tang *et al.*, (1993) who suggested that a minimum of 6% protein was required to form a gelling matrix using CWPC powders.

Ultimately, formulations can be manipulated to yield whey protein ingredients that deliver specific functional attributes under well defined processing conditions. Therefore, in order to design whey based ingredients that deliver consistent characteristics for specific food applications, the details regarding the raw materials, “pre-processing” and storage conditions, and numerous experimental variables, such as ionic strength, temperature, and pH must be identified.

Taken together these results established that the inclusion of calcium into protein solutions prepared with MWPC powders enhanced the functionality of the final whey ingredient, delivering superior performance characteristics when compared with other commercial whey protein-based ingredients currently available on the market. Ultimately, these approaches may yield unique whey protein formulations that deliver specific functional attributes for development in a wide variety of food applications.

A1.5 References

Anonymous. 2007. www.sci.sdsu.edu/TFrey/Chem365/ThermoWaterAA/AminoAcidsCh365.html. Accessed June, 2007.

Barbut, S. and Foegeding, E.A. 1993. Calcium-induced gelation of pre-heated whey protein isolate. *Journal of Food Science*, *58*, 867-871.

Britten, M. and Giroux, H. 2001. Acid-induced gelation of whey protein polymers: effects of pH and calcium concentration during polymerization. *Food Hydrocolloids*, *15*, 609-617.

Caussin, F., Famelart, M., Maubois, J., and Bouhallab, S. 2003. Mineral modulation of thermal aggregation and gelation of whey proteins: from β-lactoglobulin model system to whey protein isolate. *Lait*, *83*, 1-12.

Duckworth, R.B. 1971. Differential thermal analysis of frozen food systems. I. The determination of unfreezable water. *J. Food Technology*, *6*, 317-327.

Fennema, O.R. 1985. Amino acids, Peptides, and Proteins. In *Food Chemistry*, Second Edition, Revised and Expanded. Tannenbaum, S.R. and Walstra, P., Editors; Marcel Dekker Inc., New York, New York and Basel, Switzerland, p. 37-286.

Firebaugh, J.D. and Daubert, C.R. 2005. Emulsifying and foaming properties of a derivatized whey protein ingredient. *International Journal of Food Properties*, *8*, 243-253.

Fujino, H., Muguruma, M., ITO, T., Ohashi, T. 1993. Effects of pH, sodium chloride and calcium chloride on heat aggregation of whey protein concentrate and whey protein isolate. *Journal of the Japanese Society for Food Science and Technology-Nippon Shokuhin Kagaku Kogaku Kaishi* , *40*, 309-315.

Fujino, H., Muguruma, M., Oniki, H., Ito, T., and Ohashi, T. 1995. Role of calcium on heat aggregation of whey-protein isolate – (Studies on available utilization of whey proteins). *Journal of the Japanese Society for Food Science and Technology- Nippon Shokuhin Kagaku KogaKu Kaishi*, *42*, 769-775.

Harwalker, V. and Kalab, M. 1985. Thermal denaturation and aggregation of β-lactoglobulin at pH 2.5. Effect of ionic strength and protein concentration. *Milchwissenchaft*, *40*, 31-34.

Hongsprabhas, P. and Barbut, P. 1997. Effect of Temperature on Ca^{2+}-Induced Gelation of Whey Protein Isolate. *Lebensmittel-Wissenschaft und-Technologie*, *30,* 45-49.

Hudson, H.M., Daubert, C.R., and Foegeding, E.A. 2000. Rheological and physical properties of derivitized whey protein isolate powders. *J. Agric. Food Chemistry*, *48*, 3112-3119.

Hudson, H.M., Daubert, C.R., and Foegeding, E.A. 2001. Thermal and pH stable protein thickening agent and method of making the same. US Patent No.6261624.

Jelen, P., Hostin, S., Jeschke, J., and Kerr, G. 1992. Effects of low temperature on viscosity and gelling properties of concentrated whey protein solutions. *Proceedings International Dairy Federation*, IDF Seminar, Munich.

Ju, Z.Y. and Kilara, A. 1998. Aggregation induced by calcium chloride and subsequent thermal gelation of whey protein isolate. *J. Dairy Science*, *81*, 925-931.

Ju, Z.Y. and Kilara, A. 1998. Gelation of pH-Aggregated whey protein isolate solution induced by heat, protease, calcium salt, and acidulant. *J. Agric. Food Chem.*, *46*, 1830-1835.

Kinsella, J.E., Whitehead, D.M., Brady, J., and Bringe, N.A. 1989. Milk proteins: possible relationships of structure and function. Pages 53-93 in Development in Dairy Chemistry – 4. Functional Milk Proteins. P.F. Fox, ed. Appl. Sci. Publ., London, United Kingdom.

Kuhn, P.R. and Foegeding, E.A. 1991. Mineral salt effects of whey protein gelation. *J. Agric. Food Chem*. *39*, 1013-1016.

Ohki, Z. and Zschornig, O. 1993. Ion-induced fusion of phosphatidic-acid vesicles and correlation between surface hydrophobicity and membrane-fusion. *Chemistry and Physics of Lipids*, *65*, 193-204.

Pappas, C.P. and Rothwell, J. 1991. The effect of heating, alone or in the presence of calcium or lactose on calcium binding to milk proteins. *Food Chemistry*, 42: 183-201.

Rattay, W. and Jelen, P. 1995. Viscous behavior of whey protein concentrate dispersions. *International Dairy Journal*, *5*, 673-684.

Resch, J.J. and Daubert, C.R. 2002. Rheological and physiochemical properties of derivatized whey protein concentrate powders. *International Journal of Food Properties*, *5*, 419-434.

Roos, Y.H. 1986. Phase Transitions and Unfreezable Water Content of Carrots, Reindeer Meat and White Bread Studied using Differential Scanning Calorimetry, *J. of Food Science*, *51*, 684-687.

Sherwin, C.P. and Foegeding, E.A. 1997. The effects of calcium chloride on aggregation of whey proteins. *Milchwissenschaft-Milk Science International*, *52*, 93-96.

Shimizu, M., Saito, M., and Yamauchi, K. 1985. Emulsifying and Structural Properties of Beta-Lactoglobulin at Different pHs. Agric. Biol. Chem. Tokyo 49, p 189-194.

Solanki, G. and Rizvi, S.S.H. 2001. Physio-chemical properties of skim milk retentates from microfiltration. *J. Dairy Science*, *84*, 2381-2391.

Steffe, J.F. and Daubert, C.R. 2006. Rheological properties of biological fluids. Page 5 in Bioprocessing Pipelines: Rheology and Analysis. Freeman Press, East Lansing, MI.

Tang, Q.N., Munro, P.A., and McCarthy, O.J. 1993. Rheology of whey-protein concentrate solutions as a function of concentration, temperature, pH, and salt concentration. *Journal of Dairy Research*, *60*, 349-361.

Tang, Q., McCarthy, O.J., Munro, P.A. 1993. Oscillatory rheological study of the gelation mechanism of whey protein concentrate solutions: effects of physiochemical variables on gel formation. *J. Dairy Research*, *60*, 543-555.

Vardhanabhuti, B., Foegeding, E.A., McGuffey, M.K., Daubert, C.R., and Swaisgood, H.E. 2001. Gelation properties of dispersions containing polymerized and native whey protein isolate. *Food Hydrocolloids*, *15*, 165-175.

Vélez-Ruiz, J.F., Barbosa-Cánovas, G.V. 1998. Rheological properties of concentrated milk as a function of concentration, temperature, and storage time. *J. Food Engineering*, *35,* 177-190.

Wang, T., and Gunasekaran S. 2006. State of water in chitosan-PVA hydrogel. *Journal of Applied Polymer Science*, *101*, 3227-3232.