Maillard-Induced Glycation of Whey Protein Using Matlodextrin and Effect on Solubility, Thermal Stability, and Emulsification Properties

A THESIS
SUBMITTED TO THE FACULTY OF
UNIVERSITY OF MINNESOTA
BY

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IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF MASTER OF SCIENCE

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June 2016

Acknowledgements

I would like to take the opportunity to thank my advisor, Dr. Baraem (Pam) Ismail, for everything she has done for me leading up to this point. Not only did Pam afford me the opportunity to work as a graduate student under her supervision, but she has also offered unwavering support, guidance, and encouragement throughout my journey towards a Master's degree. Thank you to my committee members, Dave Smith and Xiaoli Chen, for taking the time out of their busy schedules to serve on my thesis committee. I want to also thank Nancy Toedt and Dave Smith for helping me along in the process of joining the Food Science Master's program.

Thank you to everyone in the Protein and Phytochemical Lab at the University of Minnesota for your support in my journey towards a Master's degree. I especially want to thank Chathurada, Cati, Chelsey, Courtney, and Elizabeth for the training and assistance with my research.

The acknowledgements would not be complete if I did not extend a thank you to my family for everything they have done for me during my Master's research, and throughout my life. My mother, father, and sister have been the best friends, and support group anyone could ask for.

Dedication

This thesis is dedicated to Mary Beth and Kent Savre, who have given me their absolute best efforts as parents and as friends, which has been nothing short of amazing. I could not be prouder to call you my parents.

Abstract

With high nutritional value, and excellent physiological and functional properties, whey protein has a unique position in the protein market. Whey protein beverages, specifically, have high popularity among people looking for additional protein in their diet in an easy to consume and readily available form. Formulating beverages with whey protein, however, is not free of challenges. Despite the excellent solubility of whey protein over a wide range of pH, when whey protein beverages undergo thermal processing and prolonged storage, aggregation and a resulting loss of solubility can occur. Loss of solubility upon processing and during storage is especially prevalent near the whey protein isoelectric point (pI) (4.5). This hurdle makes production of acidic whey protein beverages (pH 3.8-4.5) with high protein content (>4.2%, to make a high protein claim) difficult. Current whey protein acidic beverages available on the market contain at most 4% protein and are formulated at pH $\leq$ 3.4, which makes them sour and astringent. In order to expand the market value, it would be ideal to develop shelf stable whey protein acidic beverages that can make the high protein label claim, and are produced at a slightly higher pH (3.8-4.5). Previous research attempted to achieve this goal through Maillard-induced glycation. But more work was needed to optimize this approach. It was hypothesized that the solubility and thermal stability of membrane filtered whey protein isolate will be enhanced upon glycation with food grade maltodextrin. Additionally, improvements in functionality will be recreated with a higher protein to carbohydrate ratio, with the assumption that this will eliminate the need for separation of unreacted carbohydrate, and generates a product with higher protein content.

Thus, the objectives of this study were twofold: (1) Optimize Maillard-induced glycation of membrane-filtered whey protein with food grade maltodextrin following an industry feasible approach. (2) Determine the effect of Maillard-induced glycation on solubility, thermal stability, and emulsification properties of whey protein.

Maillard glycation of whey protein was induced by incubating whey protein isolate with maltodextrin at 60°C, water activity (a_w) of 0.49, 0.63, or 0.74, and a 1:4 and 2:1 ratio of protein to maltodextrin over a period of 48-144 h. The extent of glycation was monitored via estimation of Amadori compound formation and browning, quantification

of free amino group and free lysine loss, and visualization of protein molecular weight distribution. Optimum conditions for the desired objectives were determined to be 96 h of incubation at 0.49 a_w, in a 2:1 ratio of whey protein to maltodextrin, due to a plateau in Amadori compound formation, limited browning, and minimal loss of free amino groups (11%) and lysine (0.45%). Unreacted maltodextrin was removed using hydrophobic interaction chromatography to produce a purified partially glycated whey protein (PGWP) constituting ~ 94% protein and ~4% carbohydrate.

The onset of denaturation of PGWP was monitored using differential scanning calorimetry (DSC), and solubility of PGWP were assessed at 5% protein concentration prior and post heat treatment at 80°C for 30 min. SDS-PAGE was used to visualize polymerization induced by heat treatment that would contribute to changes in solubility. Emulsification properties of PGWP were assessed as well, through emulsification capacity and stability measurements. Partial glycation of whey protein resulted in enhanced solubility and thermal stability of whey protein near the pI of WPI (pH 4.5) and under neutral conditions (pH 7). Around the pI of whey proteins, WPI lost ~60% of solubility, whereas PGWP remained almost entirely soluble (~8% loss). Under neutral conditions, the decrease in solubility of PGWP (~15% loss) upon heating was half as much as that of WPI (~32% loss). The enhanced solubility and thermal stability of PGWP was attributed to resistance to denaturation and reduced protein-protein interactions upon glycation. The emulsification capacity of WPI, on the other hand, was improved upon glycation by ~12%, while emulsification stability was reduced. The improvement in emulsification capacity was attributed to the conformational changes that whey protein underwent upon glycation.

Overall, this work showed for the first time that limited Maillard glycation can be induced using food grade maltodextrin to produce a partially glycated protein product with a protein content greater than 90%. Compared to WPI, this high protein product had enhanced solubility and thermal stability, even at the pI of whey protein, allowing for its application in both acidic and neutral beverages with an anticipated longer shelf life at protein concentration > 4.2%. Successful formulation at protein levels that allow a high protein claim, while maintaining longer shelf-life and overall quality, provides economic gain to producers and physiological benefits to consumers.

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1. Literature Review

1.1 Introduction and Objectives

After decades of being an unused byproduct of cheese production, whey has taken a significant role in the food industry. Whey is primarily water, along with some lactose, but there are valuable proteins in whey that can be concentrated for food use. Whey powders and protein ingredients had a total market value of $9.8 billion in 2013 and are projected to have a global market value of $11.7 billion in 2017 (ADPI, 2014a). Whey protein is a widely used ingredient due to its broad range of functionality, along with its exceptional nutritional and physiological properties. Whey protein is used in a number of food applications, including but not limited to protein supplements, baked goods, dairy, and meat products. Specifically, 75% of the protein supplements market is made up of bars and beverages, and for the latter, whey protein is ideal due to its excellent solubility (Schmidt et al., 2014).

The two types of whey protein beverages commercially available are neutral and acidic beverages. The neutral whey protein beverages generally have a pH around 6.8, and are opaque. Acidic whey protein beverages have a pH less than 3.5 and are relatively clear liquids. Acidic whey beverages require mild heat treatment, which leads to a more desirable sensory quality and longer shelf-life stability (Laclair & Etzel, 2009). One disadvantage of acidic whey protein beverages, however, is loss of protein solubility upon thermal treatment and during storage. Because of this limitation, current products contain a maximum of 4% protein. In order to be considered a "high protein beverages" a protein concentration of 4.2% is required by the FDA.

A variety of ways have been attempted to improve whey protein solubility through structural modification. Previous research has shown improved solubility of whey protein through limited enzymatic hydrolysis (Chobert et al., 1988). Even though hydrolysis may improve solubility, thermal stability remains a problem due to hydrophobic interactions and disulfide linkages that can result in polymerization and precipitation of proteins. An alternative way that has been researched to improve whey protein solubility is Maillard-induced glycation. The Maillard reaction involves condensation by an unprotonated

amino group of a protein and a carbonyl group of a reducing carbohydrate (Hodge, 1953). The glycation that occurs due to this reaction can be controlled to an extent, and has been shown to improve solubility and thermal stability of whey protein without reduction in digestibility or nutritional quality (Wang & Ismail, 2012).

Glycation of whey protein has also shown improvement in other functional properties, such as emulsification. Whey protein and carbohydrate conjugates produced through Maillard-induced glycation were shown to have a thick steric barrier, increased oil droplet surface hydrophilicity, and decreased oil droplet aggregation (Zhu et al., 2010). All of these factors contributed to improved emulsion stability and capacity of whey protein, when it was glycated.

The reported methods for glycation of whey protein are not entirely industry feasible. The glycation ratio (w/w) often used is 4:1 carbohydrate to protein mixture. This ratio created a need for separation of whey proteins and glycated whey proteins from unreacted carbohydrate. The separation and waste stream created may add a high cost to producing a glycated product. Furthermore using high carbohydrate to protein ratio resulted in a glycated product that only had 60% protein (Wang & Ismail, 2012). At such low protein concentration, the product is no longer an isolate. Also an ion-exchange whey protein isolate was used in the previously reported work (Chobert, 1988; Wang & Ismail, 2012; Martinez-Alvarenga et al., 2014). A considerable amount of whey protein ingredient supply comes from membrane separated whey protein isolate. The ion-exchange separated and membrane separated whey protein isolate differ in their protein profile, along with lactose and calcium content, and hence differ in their functionality. This warrants investigating the glycation effect on membrane separated whey protein isolate.

The goal of this research was to produce a partially glycated whey protein product with enhanced solubility and thermal stability that can be feasibly produced on a large scale in the food industry. It is hypothesized that the solubility and thermal stability of membrane filtered whey protein isolate will be enhanced upon glycation with food grade maltodextrin. Additionally, improvements in functionality will be recreated with a higher protein to carbohydrate ratio, with the assumption that this will eliminate the need for

separation of unreacted carbohydrate, and generates a product with higher protein content. Therefore, the specific objectives of this research were:

1) Optimize Maillard-induced glycation of membrane-filtered whey protein with food grade maltodextrin following an industry feasible approach

2) Determine the effect of Maillard-induced glycation on solubility, thermal stability, and emulsification properties of whey protein.

1.2 Significance of Whey Protein

1.2.1 Economic Significance and Uses

Originally, whey was an unused waste stream from cheese production that caused difficulty in disposal, since its disposal into the bodies of water can harm the environment due to the pH of the waste stream, along with the nitrogen, mineral, and phosphorus content that result in high biological oxygen demand (Prazeres et al., 2012). To address the waste stream issue and to generate additional economic revenue, whey was utilized to develop value-added products such as whey protein ingredients (Bylund, 1995). With improvements in filtration and separation technology, manufacturers are able to concentrate and isolate whey protein, and hence generate a variety of uses for it. Due to its excellent nutritional qualities, whey protein has become widely used as a nutritional supplement in a variety of products. Whey protein also has outstanding functional properties, including gelation, emulsification, foaming, and solubility, allowing for its use in many food products.

In January 2015, 62 million pounds of dry whey products were produced in the US alone, 11 million pounds of which were whey protein isolates (USDA, 2015). Whey protein ingredients have come to hold a significant share in the food industry, reaching an estimated market value of $9.8 billion in 2013, and a projected value of $11.7 billion by 2017 (ADPI, 2014a). Trends toward healthy diets and consumer demand for high protein foods and supplements have contributed to this trend.

1.2.2 Nutritional Value and Physiological Properties

Whey protein is rich in all the essential amino acids, one of the main reasons why it is considered a high quality protein. Whey protein has a biological value 15% greater than that of egg protein, which is considered a standard protein for biological values (Smithers, 2008). This high biological value has led to its use in protein supplements and even baby formula.

Another major reason for being considered a high quality protein is that whey protein is the richest known source of naturally occurring branched chain amino acids. The branched chain amino acids (leucine, isoleucine, and valine) are taken up directly by skeletal muscles, bypassing metabolism in the liver unlike all other amino acids (Sowers, 2009). This quick uptake by skeletal muscles makes branched chain amino acids important for athletes after exercise. The branched chain amino acids in whey protein speed up recovery after an activity by aiding in the repair and rebuilding of muscle tissues at faster rates than other sources of amino acids (Ha & Zemel, 2003; Fujita et al., 2007).

Additionally, whey protein is known to have several other physiological benefits. Whey protein is an abundant source of methionine and cysteine, which both contain sulfur. These sulfur containing amino acids are involved in glutathione synthesis, which serves as an antioxidant that defends the body against free radicals and neutralizes toxins (Shoveller et al., 2005). Whey protein has been well documented to have antimicrobial activity, growth promotional activity and immunoreactivity (Smithers, 2008). These physiological properties are attributed to the naturally present proteins such as lactoferrin, growth factors, and immunoglobulins.

1.3 Whey Protein Composition

Whey protein constitutes 20% of the total protein in bovine milk (Swaisgood, 2008). The main components of whey protein are β-lactoglobulin, α-lactalbumin, bovine serum albumin (BSA), immunolobulins, and lacto-transferrin. β-lactoglobulin and α-lactalbumin make up the majority of whey protein, and these components represent around 50% and

20% of the total protein, respectively (Fox & McSweeney, 2006). The functionality of whey protein, as a whole, is largely based on the structure and composition of these two proteins.

1.3.1 β-lactoglobulin

β-lactoglobulin (β-lg) is composed of 162 amino acids and is globular in structure. There are two variants of β-lg, and they different in molecular weight with variant A being 18,362 Da and variant B being 18,276 Da. The difference in molecular weight comes from variant A having an aspartic acid where variant B has a glycine. The secondary structure of β-lg is 50% β-sheet, 15% α-helix, and 15-20% reverse turn (Wong et al., 1996).

Several key structural characteristics of β-lg influence its functionality. A single monomer of β-lg has nine strands of antiparallel β-sheets, and eight of which sheets wrap around to create a conical barrel (Fox & McSweeny, 2006). β-lg has two hydrophobic pockets, one lies within the conical barrel, while the other is on the surface of the structure. Having only one hydrophobic region on the surface of β-lg, makes this protein highly hydrophilic in native form. There is also a 3 turn α-helices on the surface of β-lg. Upon denaturation and exposure of the second hydrophobic pocket, interactions and binding to a wide variety of hydrophobic molecules occurs, including hydrophobic protein-protein interactions. Additionally, β-lg has five cysteine residues, four of which form disulfide bonds (Sava et al., 2005). The remaining free thiol group plays a significant role in thermally-induced protein polymerization when β-lg denatures at temperatures greater than 78°C (Havea et al., 2001).

Intermolecular association of β-lg is directly related to the pH of the solution it is in. Under acidic conditions (pH < 3.5), β-lg molecules exist as monomers due to a net positive charge that causes electrostatic repulsion. Between a pH of 3.5 and 5.2, which nears β-lg's isoelectric point of 5.4, β-lg aggregates into octamers due to the reduction in electrostatic repulsion. Between pH of 5.4-7.5 β-lg forms dimers, and above pH of 7.5 β-lg dissociates to form monomers again due to the net negative charges causing electrostatic repulsion (Swaisgood, 2008).

1.3.2 α-lactalbumin

α-lactalbumin (α-la) is a globular protein with two variants, just as β-lg is, but the variants are smaller in molecular weight with A being 14,147 Da and 14,175 Da for B. α-lactalbumin is composed of 123 amino acids, with Variant B having Arg in place of one of Variant A's Gln. α-lactalbumin is named as such due to the four α-helices that make up the secondary structure, along with one β-sheet (Fox & McSweeney, 2006). There are four disulfide linkages in the tertiary structure of α-la, which has a nearly spherical shape.

α-lactalbumin is the most susceptible to heat of the whey proteins, with a denaturation temperature of 62 °C, but its denaturation can be reversible (Chaplin & Lyster, 1986). The thermal stability of α-la is enhanced when bound to calcium. α-lacatalbumin has a high affinity to calcium and other metal ions (Wong, et al., 1996). Bound calcium is required for refolding and native disulfide bond formation in the denatured protein (Rao & Brew, 1989). This property enhances the gelation potential of α-la (Permyakov et al., 1981).

α-lactalbumin generally remains in its native structure between pH 5 and 7. However, below pH 4.2 unfolding occurs and the protein is considered to be in a molten globule state (Kim & Baum, 1998). In this molten globule state, protein-protein interactions increase which results in precipitation of the protein, and effectively, lowered solubility.

1.4 Whey Protein Ingredients

The composition of whey protein reflects the original processing source of the whey, along with the method used to concentrate the protein. Whey generated from the production of cheese using rennet is considered sweet whey. In addition to the whey protein components, sweet whey contains glycomacropeptide which is the hydrophilic fragment of κ-casein that is cleaved off by the rennet enzyme (Fox & McSweeny, 2006). Whey produced as a byproduct of acid casein production is called acid whey, which contains no glycomacropeptide (Neelima et al., 2013). In general sweet whey is used to produce different whey protein ingredients following either membrane filtration or chromatographic separation (Whey Protein Concentrate Handling, 2015). The

classification of the final product of whey protein powder is based on the percentage of total protein in the powder. The most common ingredients found on the market are whey protein concentrate, whey protein isolate, and whey protein hydrolysate.

1.4.1 Whey Protein Concentrate (WPC)

Whey protein concentrate (WPC), contains between 34-80% protein. The remaining constituents are lactose (10-55%), fat (1-10%), and minerals (<8%) (ADPI, 2014b). Whey protein concentrate is used in protein beverages and bars, along with confectionery and baked products. It also finds use in infant formula. Membrane filtration, along with precipitation and dialysis, can be used to produce and concentrate WPC. These techniques can also be used to further concentrate WPC to higher percentages of protein for the production of WPI (Schuck & Quest, 2013).

1.4.2 Whey Protein Isolate (WPI)

Whey Protein Isolate (WPI), which contains between 90-96% of protein, can be produced either by membrane filtration or by chromatography techniques. The membrane filtration techniques typically used include microfiltration, ultrafiltration, reverse osmosis, and nanofiltration (Adams, 2012). The chromatography techniques used include ion-exchange chromatography and radical flow chromatography (Pihlanto & Korhonen, 2003). The resulting WPI products from the two different concentration techniques differ in their constituents, and even in functionality.

1.4.2.1 Membrane Filtered WPI

Membrane filtration is the separation and concentration of whey proteins on a size basis, but for good selective separation a two-fold difference in molecular weight is required. Membrane-produced WPI has variable amounts of glycomacropeptides (~22%) with molecular weights ranging from 2–10 kDa, and a total content of up to 17%. It also has a small amount (<6%) of peptides with molecular size < 2 kDa (Mercier et al. 2004;

Spellman et al., 2005). Membrane-produced WPI also has averages of 17% α-la and 44% β-lg, which are slightly higher and significantly lower, respectively, than the contents of ion-exchange produced WPI (Mercier et al., 2004). Since α-la and β-lg are the two major proteins in whey, differing compositions imply potential differences in functionality (Fox & McSweeny, 2006). Membrane-produced WPI has a higher calcium content than ion-exchange WPI as well, which can lead to better thermal stability of α-la at low pH as previously mentioned. The present calcium ions can play a significant role in WPI coagulation and gelation (Ju & Kilara, 1998). Gelation and self-assembly of α-la involves the formation of ionic bonds between calcium and carboxyl acids groups, especially upon enzymatic hydrolysis (Ipsen & Otte, 2007). With the increased calcium content, gelation and coagulation properties are improved, which means membrane filtered WPI is more applicable in products that involve gelation (Ju & Kilara, 1998). Although there are some difficulties to membrane filtration of WPI, such as membrane fouling, it is a more readily available product, due to lower cost than ion-exchange chromatography separated WPI (Fox & McSweeny, 2006).

1.4.2.2 Ion-Exchange WPI

Ion-exchange chromatography can be employed to more easily separate whey proteins by adjusting the pH of the proteins to provide an appropriate charge so that proteins can adsorb to ion-exchange media. After unwanted constituents are filtered through the column, the pH can be adjusted to desorb the desired proteins for concentration and spray-drying (Fox & McSweeny, 2006). The major whey proteins, β-lg, α-la, and BSA are all negatively charged at the pH of sweet whey (6.2 to 6.4), and the minor whey proteins lactoferrin and lactoperoxidase have a positive net charge at this pH. The difference in charge among components allows for separation of the major whey proteins, since the foundation of ion exchange separation is the reversible interaction between target proteins and the ion-exchange media, based on charge (Goodall et al., 2008). WPI prepared by ion exchange contains around 71% β-lg, and has less glycomacropeptide(~0.4%), immunoglobulins, lactoferrin, α-la (11%) than membrane-

filtered WPI (Mercier et al., 2004; Ounis et al., 2008; Fox & McSweeny, 2006). Accordingly, the functionality of this product is highly governed by β-lg.

Ion-exchange WPI also has less calcium and lactose content than membrane filtered WPI. Calcium content has implications in thermally processed beverages where gelation is not desired, hence ion-exchange WPI is preferred over membrane-filtered WPI in beverage applications. Lactose, on the other hand, may play a role in Maillard reaction. The Maillard reaction will result in browning, which is desirable in baked products, but is not desired in beverages (Bonnaillie & Tomasula, 2008).

Furthermore, the protein in ion-exchange WPI can be slightly more denatured than the protein in a membrane-produced WPI due to different pH states the protein is subjected to during processing (Bonnaillie & Tomasula, 2008; McSweeny & O'Mahony 2016). However, this difference in denaturation state has not contributed to major differences in functionality.

Limitations of ion-exchange separated WPI include the extra steps required to concentrate and purify the dilute protein eluate, and the increased risk of exposure to and growth of microbes (Lan et al., 2002; McSweeny and O'Mahony 2016). Also regeneration of ion-exchange resins creates added time and cost of productions (Lan et al., 2002). Although ion-exchange WPI has a more pure protein content with less lactose and calcium than membrane filtered WPI, the product may not be entirely desirable due to the increased cost of production. However, due to the lack of calcium, ion-exchange WPI has merit for use in whey protein beverages that undergo heat treatment leading to lower chances of gelation in the beverage.

1.4.3 Whey Protein Hydrolysate (WPH)

Whey protein hydrolysate (WPH) is a whey protein ingredient that has generally undergone enzymatic hydrolysis to cleave peptide bonds and generate peptides with lower molecular weight than the original protein (Nnanna & Wu, 2007). Hydrolysis of whey protein is typically achieved using a range of enzymes including microbial, plant, and digestive enzymes (Lahl & Braun, 1994; Nnanna & Wu, 2007; Morais et al., 2014).

The degree of hydrolysis (DH) and characteristics of the resulting peptides are dependent on many factors including the enzymes used and hydrolysis conditions.

Whey protein hydrolysate is produced to enhance functional and physiological benefits of whey protein. Whey protein hydrolysate generally has an improved solubility compared to WPI and WPC, due to smaller molecular weight and increased ionizable groups, namely carboxyl and amine groups (Perea et al., 1993; Herceg et al., 2005). Upon hydrolysis loss of tertiary and secondary structure also occurs resulting improved gelation, foaming, and emulsification, since WPH has potentially an even distribution of hydrophobic and hydrophilic regions (Perea et al., 1993). Whey protein hydrolysate may have health benefits attributed to the presence of bioactive peptides that may have antihypertensive, antioxidant, anti-cancerous, and antibacterial activities (Fields et al., 2009). On the other hand, the digestibility of WPH has been noted to be superior to WPC and WPI, as peptides in WPH are readily absorbed into the blood stream, and others are more readily digested than the intact counterpart (Webb, 1990; Boza et al., 2000). This improved digestibility in WPH has been shown to enhance the recovery process of athletes (Perea et al., 1993). While WPH seems to be a superior product in several facets, due to flavor challenges and added processing time and cost, WPI still dominates the whey protein market.

1.5 Whey Protein Functionality

Whey protein has a wide range of functionality food manufacturers seek. The functional aspects of whey protein that are most often targeted include gelling, water binding, viscosity, foaming, emulsification, and solubility (Beuschel et al., 1992). Whey protein forms heat-induced gels stabilized by hydrophobic interactions and di-sulfide linkages. The gelling properties of whey protein allow for use in products to improve water holding capacity, such as in meat products (Akoh, 1998). Whey protein also becomes a good foaming and emulsifying agent when denatured, due to the exposed hydrophobic and hydrophilic regions of the protein. The most important functionality of whey protein, however, is its solubility. Compared to other protein ingredients whey protein has superior solubility (Chobert et al., 1988). Yet, whey protein solubility can be

severely affected by thermal processing. Details on whey protein emulsification properties, solubility, and thermal stability are provided in the following subsections.

1.5.1 Emulsification

Whey proteins are able to stabilize emulsions by forming interfacial films between hydrophobic and hydrophilic phases. This property can be attributed to the composition of whey proteins, having hydrophobic and hydrophilic regions (Onwulata & Huth, 2009). Whey protein, upon denaturation, is able to produce thick films at the interface, which increase repulsive interactions among droplets in an emulsion, promoting stability (Barbana & Perez, 2011). Whey protein that is not adsorbed on the interface adds to the viscosity of the continuous phase, which will in turn help prevent droplets from coalescence (Onwulata & Huth, 2009). Whey proteins, however, are not highly amphiphilic, their hydrophobic and hydrophilic groups are distributed uniformly, and hence the proteins do not rearrange easily at the interface between oil and water. Casein, from bovine milk, has a more open structure than whey protein and provides a much more amphiphilic structure that is able to readily rearrange at the interface between oil and water (Wong et al., 1986; Schmidt et al., 1984). In order to improve the emulsification properties of whey protein, modification is necessary to expose the buried hydrophilic and hydrophobic groups, and enhance their surface activity.

1.5.2 Solubility

The degree of protein solubility can be defined as the retention of protein in the supernatant after centrifugation at a specified gravitational force for a given period of time. The solubility of proteins generally relates directly to surface hydrophobic and hydrophilic interactions. Hydrophobic interactions promote protein-protein interactions, whereas hydrophilic interactions promote protein-water interaction (Morrisey et al., 1987). Since whey protein has great surface hydrophilicity, it generally has excellent solubility over a wide range of pH (Wang & Ismail, 2012).

The major factor that contribute to solubility other than the hydrophilicity of a protein is the pH of the system. pH significantly affects the nature and distribution of charges on

a protein's surface, and therefor has a major effect on the solubility of that protein (Pelegrine & Gasparetto, 2005). Whey protein, however, has exceptional solubility over a wide range of pH, because it only has one hydrophobic region on the surface (Fox & McSweeney, 2006). This characteristic results in less protein-protein interaction. Even at the pI of whey protein, where the net charge on the molecular surface is zero, and repulsion between molecules is at a minimum, whey protein maintains excellent solubility as shown by Wang and Ismail (2012). This observation can be attributed to the hydrophilic interactions between whey protein and water being dominant over protein-protein interactions due to low percentage of hydrophobicity on the surface (Jimènez-Castaño et al., 2007). At pH values above and below the pI of whey protein, it will have more charged groups, which interact more with water (Pelegrine & Gasparetto, 2005). The excellent solubility of whey protein over a wide range of pH values can be greatly affected by the temperature at which a product is heated and at which a product is stored, which will be discussed in the following section.

1.5.3 Thermal Stability

Generally, an increase in temperature promotes protein-water interaction, and enhances solubility. Between 40 °C to 50 °C, whey protein solubility increases, but at higher temperatures and longer heating times, whey proteins become less soluble due to denaturation (Sava et al., 2005). Since β-lg and α-la are globular proteins, they are prone to denaturation when certain temperatures are reached (Anema, 2009). As temperature increases, globular proteins begin to unfold, which is initially a reversible unfolding. The reversible denaturation is a result of disrupted non-covalent interactions, such as hydrogen bonds, hydrophobic interactions and electrostatic interactions. With exposure to temperatures over 70 °C for prolonged time, the secondary and tertiary structure of whey protein can be disrupted to the point of irreversible denaturation. These disrupted interactions that stabilize the secondary and tertiary structures result in the exposure of hydrophobic groups and reactive sulfhydryl groups that were previously buried within the globular structure. The open structures are able to form intermolecular hydrophobic

interaction and disulfide linkages resulting in polymerization and precipitation (Thompson et al., 2009).

The denaturation of whey protein is also affected by pH. At neutral pH around 7, β-lg will start to denature at temperatures above 65 °C, but at pH of 2.5 the denaturation temperature increases to 75 °C. At lower pH values, the increased net negative surface charges create stabilization of the protein (Mulvihill & Donovan, 1987). At a pH of 4.5, the pI of whey protein, the protein denatures at even lower temperatures and exhibits lower solubility at temperatures as low as 60 °C (Laleye et al., 2008). Since whey protein has good thermal stability and solubility at acidic pH, it is applicable for use in acidic protein beverages, which will be discussed in the following section.

1.6 Whey Protein Beverages

1.6.1 Significance

Demand for protein-containing products is on the rise as health-conscious consumers become more aware of the benefits of protein in their diet. Protein products were originally a niche market for sports nutrition enthusiasts motivated by the benefits, but now the market is claiming a wider consumer audience (Fishel, 2011; Ha & Zemel, 2003). A major part of this rise is due to the benefits that protein has with weight management, and with faster recovery from exercise for athletes (Bowen et al., 2006). Additionally, high protein products promote satiety, which gives consumers a feeling of fullness for an extended period of time.

When it comes to protein products, whey protein is widely used because of its extensive health benefits and biological value as discussed earlier. Beverages, specifically, constitute a large portion of the protein-based products due to their convenience, and whey protein is ideal for this application since it has good solubility over a wide pH range (Fishel, 2011). There are many forms of whey protein beverages on the market with a large segment being neutral beverages (pH ~6.8). The neutral whey protein beverages are generally opaque and available in flavors such as chocolate, strawberry, and vanilla. Acidic whey protein beverages (pH < 3.5) are clear in

comparison to neutral whey protein beverages. The acidic whey protein beverages are preferred because they only require a mild heat treatment with the low pH values. The mild heat treatment is desirable because it leads to better sensory quality and shelf life stability (Etzel, 2004).

To maintain clarity over a reasonable shelf-life, current whey protein acidic beverages contain a maximum of 4% protein, which does not qualify the beverage as a "high protein beverage" according to the FDA (21 CFR 101.54 B). It would be ideal to develop whey acidic beverages with concentrations greater than 4.2%, in order to make a "high protein" claim, and raise the market value. This would help make better use of the nutritional and biological properties whey protein possesses. However, there are challenges when it comes to maintaining clarity in acidic beverages at high protein concentration (>4%) upon thermal processing and during storage.

1.6.2 Manufacturing Challenges

1.6.2.1 Solubility and Thermal Stability

When whey protein is in solution at higher concentrations, the occurrence of protein-protein association increases, thus driving down the protein's solubility (Golovanov et al., 2004). Thermal treatment of whey proteins lowers the solubility by promoting aggregation of the proteins through intermolecular hydrophobic interactions and disulfide linkages when the protein structures open up (Pelegrine & Gasparetto, 2005). In the study done by Pelegrine and Gasparetto (2005), it was shown that at the pI of whey protein, solubility decreased with thermal treatments as low as 40°C, and as temperature was increased up to 60 °C solubility dropped to 68%. The disulfide linkages that cause precipitation of proteins are irreversible, and pose problems with consumer acceptance. Some whey proteins during thermal treatment may not form disulfide linkages at the time, but presence of denatured protein leads to continued aggregation and precipitation during storage.

1.6.2.2 Sensory Quality and Shelf Life

There are some limitations when it comes to acidic protein beverages, chiefly customer acceptance due to sourness and astringency. Whey protein, like any other protein, is an excellent buffer, which means in solution there is a need for increased amounts of acid in order to reach the target pH (3.2-3.4) of acidic beverages. Beecher et al. (2006) made the claim that the astringency of whey protein acidic beverages could be attributed to the whey protein itself. However, Lee and Vickers (2008) attributed the astringency to the high amount of acid required to lower the pH of the beverages in the presence of protein. The perceived astringency requires large amounts of sugar to balance out the flavor (Beecher et al., 2006). This problem is exacerbated with higher protein concentrations.

Another problem with whey protein acidic beverages is that during storage turbidity can occur. Storage studies for up to six weeks at ambient temperature have shown that protein aggregation occurs resulting in precipitation and turbidity (Fox & McSweeney, 2006; LaClair & Etzel, 2009). Turbidity of beverages can cause a decrease in consumer acceptability, and forces producers to reduce shelf life claims, which is not economically desirable. Accordingly, these challenges need to be addressed in order to improve acceptability and extend the shelf life of acidic whey protein beverages.

1.7 Approaches to Enhance Whey Protein Solubility and Thermal Stability

Whey protein is known for having great solubility in comparison to other proteins, but achieving high protein concentrations in beverages is still a challenge. Since there is a large and increasing market for high protein supplements, especially beverages, the enhancement of whey protein solubility and thermal stability would have a great economic impact. Several approaches to improve whey protein solubility has been investigated, and the two major ones that will be discussed are enzymatic hydrolysis and Maillard-induced glycation.

1.7.1 Enzymatic Hydrolysis

Limited enzymatic hydrolysis using proteases has been shown to improve protein solubility. Solubility improvement of up to 20% at pH 3.5 in 0.1% protein solutions was shown by Perea et al. (1993), upon hydrolysis of whey protein (DH 16-20%) using Alcalase. The improvement in solubility was largely attributed to the reduced molecular weight of the proteins and an increase in free ionizable groups, mainly carboxyl and amine groups (Mutilangi et al., 1996; Jost & Monti, 1977). Enzymatic hydrolysis is an ideal process, due to the availability of a large number of enzymes that are recognized as being safe for use in the food industry.

Other research has shown, however, that hydrolysis with certain enzymes may lead to aggregation and precipitation upon thermal treatment and during storage due to the exposure of buried hydrophobic residues and reactive sulfhydryl groups. Chicón et al. (2009) reported that the thermal stability of WPH produced using pepsin, with complete proteolysis of α-la, determined by loss of α-la in SDS-PAGE gel, and limited proteolysis for β-lg, was slightly improved compared to WPI at pH 5. The same WPH was relatively unstable under other pH conditions. Whey protein hydrolysate usage in beverages may have hurdles that can hinder wide spread production. The thermal instability of WPH over a wide range of pH conditions can cause problems during processing, and may lead to aggregation and decreased solubility especially at relatively high concentration.

With DH greater than 10%, bitterness can be detected due to the accumulation of hydrophobic peptides that contain phenylalanine, tyrosine, and leucine (Cliffe & Law, 1990). Additionally, hydrolyzed proteins become better buffers due to the increased carboxyl and amine groups. Higher buffering capacity results in higher sourness and astringency in acidic beverages. With a potential lack of stability at pH conditions lower than 5, and possible undesirable flavors, WPH is not an ideal candidate for acidic protein beverages. Other modifications could offer more promise.

1.7.2 Maillard-induced Glycation

1.7.2.1 The Maillard Reaction

The Maillard reaction is a non-enzymatic browning reaction that occurs between a carbonyl group of a reducing sugar and an amine group of a peptide or protein. The reaction is most known for the aromas, taste, and coloring that occurs during cooking of breads and many other types of food (Davies & Labuza, 1997). This complex reaction involves multiple stages, and produces a vast number of reaction products. To simplify the reaction, the three main stages are the initial stage, intermediate stage, and final stage. In the initial stage, products are colorless, and a sugar-amine condensation occurs, followed by an Amadori rearrangement producing an Amadori product (glycated protein). In the intermediate stage, products range from colorless to yellow, and are produced as a result of sugar dehydration and fragmentation, along with amino acid degradation. Products from the intermediate stage may include carbon dioxide, flavor compounds, aminoketones, and aldehydes. In the final stage of the reaction, an aldol condensation occurs along with an aldehyde-amine condensation and polymerization of proteins (Hodge, 1953). In the final stage highly colored products, melanoidins, are formed. The initial stage of the reaction is of interest in protein modification, because the sugar-amine condensation and Amadori rearrangement result in a glycated protein that may have improved functional properties.

1.7.2.2 Early Stage Maillard-induced Glycation

The early stage Maillard reaction involves a carbohydrate-amine condensation followed by an Amadori rearrangement as previously mentioned, which is considered glycation. Glycation is a covalent bonding of a protein or lipid to a carbohydrate molecule without assistance from an enzyme. This early stage glycation has been used as an avenue for modification of protein ingredients, including WPI. In a study by Wang and Ismail (2012), early stage Maillard glycation was used to successfully improve solubility and thermal stability of whey protein, without any loss in nutritional value or digestibility. For example, at a pH of 4.5 and protein concentration of 5%, glycated whey protein showed ~85% increase in solubility compared to non-modified WPI when both were heated at 80 °C for 30 minutes. In the mentioned study, the Maillard reaction was controlled to limit progression to the intermediate and final stages.

The Maillard reaction is greatly influenced by the temperature and relative humidity under which a product is stored or incubated. The reaction may not occur at room temperature, but with increased temperature it moves along at a faster pace (Davies & Labuza, 1997). The Maillard reaction occurs under relative humidities (RH) ranging from 20 to 100%, with a maximum rate between 60-80% RH (Wolfrom & Rooney, 1953). In order to avoid advancing the Maillard reaction past initial stage, researchers have generally carried out the reaction between 30-50% RH and temperatures below 65 °C (Chobert et al., 2006; Wang and Ismail 2012; Martinez-Alvarenga et al., 2014). There are other important factors that affect the rate and extent of the Maillard reaction, which will be discussed further in the following sections.

1.7.2.2.1 Effect of Carbohydrate Types and Sizes on the Rate of the Maillard Reaction

The common reducing sugars in the Maillard reaction are xylose, ribose, glucose, fructose, and lactose (Laroque et al., 2008). Other sources of reducing compounds include corn starches and maltodextrins (Davies & Labuza, 1997). The rate of the Maillard reaction is influenced by the different types of sugars, as the ring structures of sugars open at different rates. In order to participate in the Maillard reaction, sugars need to be open-chained in form, which is the reducing form (Davies & Labuza, 1997). The molecular weight of reducing sugars also plays a major role in the reaction rate. Glycation between polysaccharides and proteins occurs at a much slower rate than it does with monosaccharides, disaccharides, or oligosaccharides. This is attributed to the steric hindrance of the bulky carbohydrate chain of larger molecular weight sugars, and the lower amount of reducing carbonyls (Davies & Labuza, 1997).

In a study done by Chevalier et al. (2001a), β-lg was glycated with ribose, arabinose, galactose, glucose, rhamnose, and lactose. The number of amino groups modified in β-lactoglobulin varied based on the size of the sugar used, with the smallest sugar used, ribose, producing the most modified amino groups and the largest sugar used, lactose, having the least amount of modified amino groups. When whey protein was glycated with 11 kDa dextran under aqueous conditions (10% protein – 30% carbohydrate)(wt/wt

basis) at 60°C for 24 h, the extent of glycation was determined to be limited due to remaining pronounced β-lg and α-la bands in SDS-PAGE gels (Zhu et al., 2008). This limited glycation was also seen with whey protein glycated with 10 kDa dextran under dry conditions (a_w = 0.49) at 60°C for 96 h, as shown by limited Amadori compound formation and furosine production, a product formed upon degradation of glycated lysine (Wang & Ismail, 2012). In order to have a limited propagation of the Maillard reaction larger polysaccharides can be used in order to minimize the amount of amino groups modified.

1.7.2.2.2 Effect of the Ratio of Protein to Carbohydrate on the Rate of the Maillard Reaction

The initial stage of the Maillard reaction is dependent on the concentration of both carbonyl and amine groups. The effects of increasing the molar ratio of glucose to glycine in solution was studied by Baisier and Labuza (1992), and as the ratio of glucose to glycine increased, the browning rate increased drastically. Although browning is an indication of advanced stage Maillard reaction products, this is still a good measure of how the ratio between sugar and amino acid greatly affects the reaction rate. In a study by Schnickels et al. (1976) the effect of glucose to lysine ratio on Maillard browning rate was measured as well. As the ratio of glucose to available lysine was increased, available lysine loss was increased. With more reducing sugars available to participate in the Maillard reaction with available lysine, the reaction rate and extent were increased. In order to limit the Maillard reaction, a lower ratio of reducing carbonyl groups available to the amount of amine groups available can be employed.

1.7.2.3 Physicochemical and Structural Modifications of Whey Protein Upon Maillard-induced Glycation

The glycation of whey protein changes its physicochemical characteristics and structure, and hence influences its function. A change in the pI was noted by Wang and Ismail (2012) in a study on glycation of whey protein. The pI of glycated whey protein

shifted from 4.86 to 4.2, and this was attributed to the blockage of some amino groups that would usually be protonated at pH values lower than the pKa values. In effect, the net negative charge was increased upon glycation. There was also an observed decrease in surface hydrophobicity of the glycated whey protein, which was attributed to blockage of surface hydrophobic regions by glycated amino groups, and attachment of a hydrophilic component. These modifications to the physicochemical characteristics of whey protein resulted in improvements of solubility and thermal stability (Wang & Ismail, 2012).

The structural changes of glycated whey protein were investigated by Wang et al. (2013), using Raman spectroscopy. Significant structural variations were noted in disulfide and hydrophobic regions. Also there was an increase in β-sheet configuration, and ionization of carboxyl groups at all pH values that were tested (3.4, 4.5, 5.5, and 7). A resistance to denaturation upon thermal treatment of the glycated whey protein was attributed to the molecular rigidity of the whey proteins due to structural modifications. Through the noted physicochemical and structural modifications of whey protein when it is glycated, the process of glycation has shown good promise for improvements in functionality of whey protein that undergoes thermal processing.

1.7.2.4 Effect of Glycation on the Solubility and Thermal Stability of Whey Protein

As mentioned earlier, two of the main reasons for modifications to whey protein are improvement in solubility and thermal stability. Nacka et al. (1998) were able to produce glycated β-lg and α-la that exhibited higher solubility compared to pure β-lg and α-la over a pH range of 3-9. An improvement in heat stability of β-lg was observed under neutral pH at concentrations of 2 mg/mL, when the protein was glycated with arabinose and ribose (Chevalier et al., 2001a). In the research done by Wang and Ismail (2012), the lowered pI value of glycated whey protein in comparison to whey protein showed major promise in increasing solubility at lower pH values, with the goal of application in acidic protein beverages. The increase in net negative charge due to the shift in pI upon blocking of ionizable amine groups resulted in repulsion between molecules and increased solubility. The repulsion was due to same charges as the bulky polysaccharides

attached, which created steric hindrance that reduced the chances of protein-protein interactions. The decrease in surface hydrophobicity improved solubility over a wide range of pH values, due to the decrease in intermolecular hydrophobic interactions between proteins. The decrease in hydrophobic interactions also played a role in an improved thermal stability of glycated whey protein. When heating at 75 °C, significant increase in whey protein hydrophobicity was observed after 10 minutes of heating, while for glycated whey protein significant increase in hydrophobicity was only noted after 40 minutes of heating (Wang & Ismail, 2012). Glycation resulted in protein structure rigidity resisting unfolding and exposure of the hydrophobic interior (Wang et al. 2013). The improvement of solubility and thermal stability of whey proteins through glycation is well documented, yet the practice of glycation for improvement of these functionalities has not been recreated in the food industry, according to public knowledge, due to a variety of reasons that will be elaborated on in the sections that follow.

1.7.2.5 Effect of Glycation on the Emulsification Properties of Whey Protein

Whey proteins are also known for having good emulsification properties, but they can diminish through thermal processing due to aggregation and precipitation of the proteins. However in previous research, emulsification stability was improved through glycation. When whey protein was glycated with maltodextrin in a 1:1 protein-carbohydrate ratio at a water activity of 0.79 for 2 h, emulsification stability was improved based on the observed decrease in average droplet size of whey protein-maltodextrin conjugates (Akhtar & Dickinson, 2007). The emulsification stability improvement could be a result of the already good emulsification properties of whey protein, and the steric stabilizing effect of the attached carbohydrates (Yang et al., 2015). However, there is not much information on glycation of whey proteins and improvement on emulsion capacity, which is the amount of oil that can be emulsified per gram of the emulsifying agent. The glycation employed to improve the functionality of whey proteins is beneficial in that aspect, but if it were to affect nutritional quality, that would be undesirable, which will be discussed in the following section.

1.7.2.6 Effect of Glycation on the Nutritional Quality of Whey Protein

Even though functional improvements have been identified with the glycation of whey protein, the product would not be of value if there was significant loss in nutritional quality. The identified glycation sites that occur due to the Maillard reaction have been narrowed down to mainly the ε-amino group of the lysine residues, and to a lesser extent the guanidine group of arginine residues, the imidazole group of histidine, and the indole group of tryptophan (Ames, 1992). Lysine is an essential amino acid, so excessive glycation of lysine would result in a loss in nutritional quality. In a study done by Jiménez-Castaño et al. (2007) lysine blockage of β-lg incubated with 10 kDa dextran at 60 °C at a water activity of 0.44, was reported to be 1.65% (on a molar basis) after 72 hours of incubation. However, in the study done by Wang and Ismail (2012) the reported lysine blockage of the whey protein was 0.6% (on a molar basis). Other studies have shown much greater loss of lysine with up to a 13.4% loss shown in commercial whey proteins glycated with maltodextrin at temperatures between 100 to 120°C for 0 to 20 min (Rufián-Henares et al., 2006). It is desirable to limit lysine blockage while still improving the functionality of whey protein through partial glycation.

A decrease in digestibility of glycated whey protein is also of concern, since a decrease in digestion will cause a decrease in the nutritional value of the whey proteins as a whole. It is believed that the addition of bulky sugars to proteins can shield peptide bonds that are targeted by digestive proteases. However, contradictory findings were reported. In a study done by Chevalier et al. (2001a) where 20% of available amino groups were blocked due to glycation at 60 °C for 72 h, a lower tryptic digestibility of glycated whey proteins was observed by the presence of fewer peptides after digestion of glycated β-lg in comparison to native β-lg. However, in the research done by Wang and Ismail (2012), there was an increase in digestibility (8.9%) of glycated whey protein in comparison to whey protein. This observation was attributed to partial unfolding of the protein and the limited extent of glycation (1.4% loss of available amino groups). The contradictory results in different studies on the digestibility of glycated whey protein could be attributed to differences in the extent of glycation per protein molecule, size of

the carbohydrate, and the conformational change of the protein (Cheveliar et al., 2001; Hiller & Lorenzen, 2010).

1.8 Feasibility of Production

1.8.1 Dialysis of WPI

One problem encountered with the use of membrane filtered WPI is the residual lactose of about 2%. The residual lactose has potential to participate in the Maillard reaction, and with its small size may take precedence over other carbohydrates for glycation sites (Chevalier et al., 2001b). Additionally, its high reducing power compared to a bulky carbohydrate will result in fast propagation of the Maillard reaction to advanced stages during the incubation period. In order to limit the amount of residual lactose in membrane filtered WPI, dialysis can be employed with a molecular weight cutoff that retains all the proteins of WPI. Dialysis uses a semipermeable membrane in order to allow molecules smaller than the membrane pore size to diffuse through to a dialysate solution. In order to perform this removal on an industrial scale, nanofiltration has potential for use. Nanofiltration is a pressure driven membrane filtration that is already used in the dairy industry to produce lactose-free milk (Atra et al., 2005).

1.8.2 Incubation Conditions

In a research lab setting, in order to induce Maillard glycation, incubation is performed under controlled RH and temperature using desiccators containing saturated salt solutions placed in an incubator at controlled temperatures. In order to carry out Maillard-induced glycation on a larger scale in the food industry, controlled climate chambers can be used, in which temperature and RH can be easily controlled for incubation. Controlled climate chambers use steam generators, or atomizers to control the amount of moisture within the chamber. These chambers allow for specified control of the conditions under which glycation is performed, and would not be difficult to do on a larger scale.

1.8.3 Use of Chromatography for Separation of Unreacted Carbohydrates

Unreacted carbohydrate from the glycation of whey proteins needs to be removed for a variety of reasons. The unreacted, or free, carbohydrate has potential to interfere with the protein's functionality, such as increasing the viscosity of beverages. Free carbohydrates that remain in a glycated whey protein mixture would also be available to participate in the Maillard reaction, allowing for progression to intermediate and final stages during storage, which would be undesirable for glycated whey protein as an ingredient or in a product. Additionally, the unreacted carbohydrates are undesired in healthy products containing high protein, due to the increase in calories. Finally, having excess carbohydrate in the final product dilutes the final protein content.

Since the molecular weight of the carbohydrates often used are close to the molecular weights of the protein, membrane filtration is not a viable option and chromatography separation would be needed. Hydrophobic interaction chromatography (HIC) uses a hydrophobic solid phase media along with a salt solution to promote hydrophobic interactions between proteins and the hydrophobic solid phase media in a column. This process allows for the separation of whey protein from unreacted carbohydrate after incubation (Wang & Ismail, 2012). On a smaller scale this can be performed with relative ease, but when scaling up to produce large amounts of glycated whey protein, this can cause a variety of problems. This step creates a waste stream of salt solution and unreacted carbohydrate, although the carbohydrate could be reused. The salt solution used in HIC requires an added removal step, typically dialysis, since the salt affects the nature of the protein, and is an unwanted additive. The removal of salt adds additional time and cost to processing, along with additional steps in which microbes could be introduced to the whey protein.

The added HIC step could be considered time consuming, and would possibly not be cost effective in producing a WPI product with improved functionality. Ideally, unreacted carbohydrate after incubation would be minimized in order to avoid the need for separation. This could be done by lowering the starting amount of carbohydrate used for glycation of whey proteins.

1.8.4 Waste Streams

The potential waste streams created by the glycation of whey protein may limit the industry value of the product. Added waste streams increase cost of production, due to added time and money involved with disposal. Ideally, waste streams can be used in other products, which is how whey protein initially found a place as an ingredient in the food industry.

The waste that would be created from nanofiltration of WPI to remove lactose would be minimal. There is not much lactose in membrane filtered WPI in the first place, so further removal would require the same disposal process that is already used when concentrating whey protein isolate. This step in the glycation process will most likely only add minimal costs to the product. The major concern for added waste steams is the unreacted carbohydrate from the incubation of whey protein and carbohydrate. Using HIC for separation enables the removal of excess carbohydrates. The HIC process however, generates a waste stream rich in carbohydrate, thus adding to the cost of production.

Generally, research that has been conducted in the past, such as the study done by Wang and Ismail (2012), used a high ratio of sugar to protein, in order to maximize available sugars for glycation of the whey proteins. A major component of the current study is finding an alternative to the HIC separation step. In order to do that, various parameters of the glycation conditions of whey protein will be manipulated. By increasing the protein to sugar there is a possibility of minimizing unreacted sugars. Other parameters that will need to be manipulated in order to drive the initial stage of the Maillard reaction to completion, without advancing to the intermediate stage, include an increase in the relative humidity under which samples are incubated. A RH between 60-80% may increase the rate of the Maillard reaction, so carrying out the incubation under that range has potential to drive glycation to completion in a protein favored ratio sample. However, the incubation time needs to be reduced to avoid propagation to advanced stages. Finding the best balance between initiating the reaction and preventing propagation is key. Increasing temperature can also enhance the rate of the reaction, however an increase above 60 °C is not desired, because of the susceptibility of whey

protein to denaturation. Combinations of protein to carbohydrate ratio and RH conditions will be attempted in order to avoid the separation step.

1.9 Conclusion

Whey protein is an excellent protein source with great physiological benefits, excellent functionality, and numerous uses in the food industry. Whey protein isolate, in particular, has been used in many products for this reason, especially in functional food products like protein beverages. However, there are some limitations in the production of whey protein beverages related to protein solubility and stability upon thermal processing and during storage. The precipitation of whey proteins also limits other functionality, such as emulsification ability. Limited and controlled Maillard-induced glycation is a well-investigated approach for improving whey protein solubility and thermal stability, but previous research has limitations for reproducibility in the food industry. In this research, the attempt is to expand upon previous findings by optimizing incubation conditions to eliminate additional processing steps and to produce glycated product that is still considered an isolate (>90% protein) with superior solubility and thermal stability over a wide range of pH for the ultimate benefit to producers, processors, and end users.

2. Materials and Methods

2.1 Materials

Whey protein isolate (WPI) (Prolibra® 190, 90% protein) (Appendix A) was generously provided by Glanbia Nutritionals, Inc. (Fitchburg, WI, USA). Maltodextrin (MD 01956), molecular weight range 1,000-10,000 kDa (dextrose equivalence of 7.4), was provided by Cargill, Inc. (Hammond, IN, USA). WPI (BiPRO®, 92.7% protein) (Appendix A**)** was kindly provided by Davisco Foods International, Inc. (Eden Prairie, MN, USA). Snakeskin™ dialysis tubing (3.5kDa MWCO) (38035), bovine serum albumin liquid standard (23210), and Pierce™ bicinchonic acid (BCA) protein assay kit (23227) were purchased from Thermo Scientific (Waltham, MA, USA). UV-specific (Costar® 3635) 96-well polystyrene microplates were purchased from Corning, Inc. (Corning, NY, USA). High performance phenyl sepharose media (17-1082-01) and XK-50/20 column were purchased from GE® Healthcare (Little Chalfont, UK).O-phthaldialdehyde (OPA) (79760), L-lysine standard (56871), and Sudan Red 7B (6368-72-5) were purchased from Sigma Aldrich (St. Louis, MO, USA). Criterion™ TGX Precast 18% Tris-Hcl gels (567-1094), Laemmli sample buffer (161-0737), prestained SDS-PAGE standards, broad range (345-0024), and concentrated tris-tricine-sodium dodecyl sulfate running buffer (161-0744) were purchased from Bio-Rad Laboratories, Inc. (Hercules, CA, USA). Furosine standards (SC494) were purchased from the PolyPeptide Group (San Diego, CA, USA). All other reagent grade chemicals were purchased from Fisher Scientific (Waltham, MA, USA) or Sigma-Aldrich.

2.2 Experimental Plan

In order to optimize incubation conditions to eliminate additional processing steps and to produce glycated product that is still considered an isolate (>90% protein) with superior solubility and thermal stability over a wide range of pH, whey protein was glycated under varying conditions and ratios. Following glycation, whey proteins were analyzed for extent of glycation through monitoring Amadori compound formation,

browning, and free amino group loss. Once an optimum glycation condition was selected, the whey protein was separated from unreacted carbohydrate using hydrophobic interaction chromatography (HIC) for functionality testing.

2.3 Maillard-Induced Glycation

2.3.1 Preparation of Partially Glycated Whey Protein (PGWP)

2.3.1.1 Initial Phosphate Buffer Preparation, Lyophilization, and Incubation of Samples and Controls

Prolibra® membrane-filtered WPI and maltodextrin were mixed at either 1:4 (w/w) ratio or 2:1 (w/w) ratio, dissolved in 0.001 M phosphate buffer, pH 7, and lyophilized. The dried mixture was then pulverized to a powder consistency, and incubated in triplicate in sealed desiccators under various water activities (a_w = 0.49, 0.63, and 0.74) at 60°C. WPI that had previously been dissolved in 0.001 M phosphate buffer and lyophilized served as a control. The water activity of the desiccators was maintained using saturated salt solution slurries of sodium bromide (a_w = 0.49), potassium iodide (a_w=0.63), and sodium chloride (a_w=0.74). Samples were removed from the desiccators at pre-determined time-points, 48, 72, 96, 120, and 144 hours, to examine the effects of time on the extent of glycation, and all samples were then stored at -20 °C until analysis. Upon initial UV-Vis spectroscopy readings at 304 nm it was determined that the considerable amount of lactose (~2%) in WPI was causing Amadori compound formation in the control samples. Therefore, a dialysis step for WPI was deemed necessary. The samples incubated with a_w = 0.49 were chosen for further investigation, as the a_w = 0.63 samples showed significant visible browning, an indication of undesired propagation to advanced stages of the Maillard reaction, and a_w = 0.74 samples exhibited caking and browning (Appendix B).

2.3.1.2 Dialysis of Components

Whey protein isolate was dissolved in distilled, deionized water (DDW) to produce a 10% (w/v) solution, and dialyzed against DDW in dialysis tubing (3.5 kDa molecular weight cut-off) to remove residual lactose. Maltodextrin was dissolved in DDW to produce a 10% (w/v) solution, and dialyzed against DDW in dialysis tubing (3.5 kDa molecular weight cut-off) to remove lower molecular weight matlodextrins. Lower molecular weight carbohydrates with higher reducing power, more readily participate in Mailliard-induced glycation as previously mentioned, so lower molecular weight maltodextrins were removed for more controlled glycation. The dextrose equivalent of the maltodextrin was 7.4 before dialysis, and 4.6 after. Dialyzed whey protein and maltodextrin were shell frozen, lyophilized, and stored at -20 °C until future testing.

2.4 Monitoring Extent of Maillard-Induced Glycation

2.4.1 UV-Vis Spectroscopy for Monitoring Amadori Compound Formation and Browning

Estimation of Amadori compound formation and browning of WPI incubated with maltodextrin over time was estimated following UV-Visible difference spectroscopy as outlined by Jiménez-Castaño et al. (2007), Zhu et al. (2008), Wang and Ismail (2012), with minor modifications. WPI incubated with maltodextrin, WPI incubated without maltodextrin, and non-incubated controls were analyzed in triplicate. For each, a 0.2% protein solution (w/v) was prepared in DDW, and centrifuged at 13,800 x g for 10 min. The absorbance of the supernatant was measured at 280 nm to correct for protein. Absorbance at 304 nm was recorded to estimate the extent of Amadori compound formation, and absorbance at 420 nm was recorded to estimate the extent of browning. The change in Amadori compound formation and browning over time was determined by comparing WPI incubated with or without maltodextrin to the non-incubated controls.

2.4.2 Loss of Free Amino Groups Determined Using OPA Procedure

Percent loss in free amino group content of WPI incubated with maltodextrin over time was monitored using the o-phthalaldehyde (OPA) method as outlined by Goodno et al. (1981) and Rao et al. (2012), with modifications. WPI incubated with maltodextrin, WPI incubated without maltodextrin, and non-incubated controls were analyzed in triplicate. For each, a 0.2% protein solution (w/v) was prepared in 1% sodium dodecyl sulfate solution (SDS) (w/v), and centrifuged at 15,682 x g for 10 min. The supernatant was then removed for analysis. Standards of L-lysine ranging from 0-200 μg/mL were prepared in 1% SDS (w/v) for generation of a standard curve. OPA reagent was prepared by dissolving 3.810 g of disodium tetraborate decahydrate and 0.5 g SDS in DDW. Two mL of ethanol was used to dissolve 80 mg o-phthalaldehyde, which was then added to the reagent solution along with 200 μL β-mercaptoethanol, and the final volume was brought to 100 mL using DDW. The OPA reagent was shielded from light and used within 30 min of preparation. A 50 μL aliquot of each standard and sample was loaded into polystyrene microplates specific for UV-readings followed by 200 μL of OPA reagent. Plates were incubated at room temperature for 2 min before reading the absorbance at 340 nm with a microplate reader (Biotek, Winooski, VT, USA). Data were corrected for protein content in supernatants using the BCA assay kit, following the manufacturer's instructions. The concentration of free amino groups in each sample was adjusted for the protein content to obtain the % free amino groups in each sample. The % loss in free amino group content over time was determined using **Equation 1**. Sample calculations can be found in **Appendix C.**

Equation 1:

$$\%\ \text{Remaining Free Amino Groups} = 100 - \frac{100 * (\%\ \text{free amino groups}_0 - \%\ \text{free amino groups}_{incubated})}{\%\ \text{free amino groups}_0}$$

$\%\ \text{free amino groups}_0 = \%$ of free amino groups in initial WPI sample

$\%\ \text{free amino groups}_{incubated} = \%$ of free amino groups after incubation

2.4.3 Determination of Lysine Blockage

Lysine blockage of the 96 h incubated glycated whey protein sample before HIC separation was determined by measuring 2-furoyl-methyl-Lys (furosine), the Amadori compound formed upon the reaction of a lysine residue with a reducing sugar (Guerra-Hernandez et al., 2002), following the hydrolysis procedure outlined by Jiménez-Castaño et al. (2007) and high performance liquid chromatography analysis reported by Krause et al. (2003), with some modifications. Glycated whey protein and WPI were analyzed in triplicate. For each, 3.75 mL of 7.95 N HCl was added to 0.02 g protein in a 13 x 100 mm vial. The vials were flushed with nitrogen, sealed, and heated at 43.3°C in an Equatherm TempBlok® heating block (Lab-Line Instruments, Thrippunithura, India) for 24 h. After digestion, samples were cooled to room temperature, supernatants were removed and centrifuged at 10,000 x g for 1 min to settle any debris from the acid digestion. Supernatants were then filtered through a 0.45 µm PVDF low binding filter. Each filtrate (0.5 mL) was applied to a previously activated Sep-Pack C_{18} light cartridge at a rate of 0.5 mL min^{-1}. Furosine was eluted with 2 mL of 3 N HCl and neutralized with 0.33 mL of 18 N NaOH. To quantify furosine content, an HPLC system (Shimadzu Scientific Instruments, Columbia, MD, USA) equipped with SIL-10AF auto injector, SPD-M20A photo diode array detector and a CTO-20A column oven was used. A YMC pack ODS AM-12S05-2546WT C-18 reverse phase column (250 mm x 4.6 mm, 5 µm) and a guard column (20 mm x 4 mm) of the same material were used. Column temperature and flow rate were maintained at 35°C and 1.2 mL min^{-1}, respectively. Solvent A was 5 mM sodium heptanesulphonate and 0.2% formic acid in DDW, and solvent B was 80% acetonitrile (ACN) containing 5mM sodium heptanesulphonate and 0.2% acetic acid. Following the injection of 20 µL of a sample, elution was performed at 15% ACN (81.3% Solvent A: 18.7% Solvent B). A standard curve was prepared using known furosine standard concentrations (from 0.5 to 20 ppm). UV detection was measured at 280 nm. Furosine was quantified based upon its peak area at the retention time of approximately 6.8 min and transferred into amount of blocked lysine per mole of protein using a series of calculations and conversions. A sample calculation can be found in **Appendix D.**

2.5 Purification of Partially Glycated Whey Protein (PGWP) from Unreacted Maltodextrin

Separation of unreacted maltodextrin from the protein was performed using hydrophobic interaction chromatography (HIC). A Shimadzu Chromatograph system, equipped with a UV detector (Shimadzu Corp., Kyoto, Japan), was used. An HIC column, 11 cm x 50 mm, was packed with phenyl sepharose high performance media and equilibrated with 1.25 M ammonium sulfate. Incubated protein/maltodextrin mixture was dissolved in DDW (3% protein, w/v). An aliquot (10 mL) of the solution was injected into the column and the flow rate was set at 10 mL min^{-1}. Free maltodextrin was washed with 450 mL of 1.25M ammonium sulfate followed by 170 mL of DDW. Non-glycated and glycated whey protein, collectively termed partially glycated whey protein (PGWP), was eluted with 200 mL DDW. Elution volumes were experimentally determined to ensure separation of free maltodextrin and recovery of proteins. Elution of the protein was monitored at 280 nm. Collected PGWP was diluted to 300 mL with DDW and then neutralized to pH 7. To remove any remaining ammonium sulfate, the collected PGWP was divided into ~80 mL aliquots in 3.5 kDa cut-off dialysis tube, and placed with stir bars in separate buckets containing 7.5 L DDW each. The buckets were placed in a walk in cooler room (7^{o}C) and DDW was changed every 4 h for 24 h. After dialysis, the aliquots were combined in a flask, neutralized to pH 7, and lyophilized. Non-glycated WPI was run and collected through the same process in order to serve as a control during functionality testing. Additionally, a non-incubated mixture of maltodextrin and WPI were run through the column for the mass balance studies and confirmation of the separation of the maltodextrin from whey protein.

2.5.1 Total Protein Quantification of Samples

To determine the protein content of WPI and PGWP before and after HIC separation, the Dumas nitrogen combustion method (AOAC 990.03, AOAC International, 1998) was followed using a Nitrogen Analyzer (LECO® TruSpecN™, St. Joseph, MI, USA). A nitrogen conversion factor of 6.38 was used.

2.5.2 Total Carbohydrate Analysis of Samples

Total carbohydrate content of the ammonium sulfate wash, DDW wash, and protein elution fractions, and of the HIC separated WPI and final PGWP products was determined experimentally using the AOAC phenol-sulfuric acid method (Official Method 988.12, AOAC International, 1988), with modifications. Collected fractions and lyophilized protein samples were diluted with DDW to reach approximately 50 μg/mL carbohydrate. An aliquot (1 mL) of diluted sample was taken, and 25 μL of 80% (v/v) phenol and 2.5 mL concentrated sulfuric acid were added. All samples were vortexed for 5 s and allowed to stand for 10 min to cool to room temperature. Glucose standards ranging from 0-100 μg/mL glucose were prepared in a similar fashion to construct a standard curve. The absorbance of each standard and sample was measured at 490 nm using a spectrophotometer.

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

The change in the protein molecular size distribution of PGWP compared to HIC WPI, membrane-filtered WPI, and ion-exchange WPI was monitored by running SDS-PAGE as outlined by Laemmli (1970), and Schägger and von Jagow (1987), with modifications. For each sample, a 0.01% protein solution (w/v) was prepared in DDW before being diluted 1:1 (v/v) with Laemmli buffer for non-reducing conditions and with addition of β-mercaptoethanol in the Laemmli buffer (5 μL β-mercaptoethanol in 95 μL Laemmli buffer) for reducing conditions. All samples were then heated in boiling water for 5 min and allowed to cool to room temperature. An aliquot (5 μL containing 10.6 mg of protein) of each sample was loaded onto a precast 18% tris-HCl peptide gel, along with a 10 μL aliquot of a prestained molecular weight ladder. The gel was electrophoresed with tris-tricine-SDS buffer at 200 V for 1 h. Gels were then stained using Coomassie blue stain. Coomassie blue staining was carried out by first immersing gels in Coomassie blue stain solution (45% methanol (v/v), 10% glacial acetic acid (v/v), and 0.3% Brilliant Blue R250 (w/v) for 1 h while shaking. Gels were destained by four rinses with a destaining solution (85% DDW (v/v), 10% glacial acetic acid (v/v), 5%

methanol (v/v)). Imaging of the gel was captured using a Gel Doc XR Camera (Bio-Rad Laboratories, Inc.) with the gel positioned over a fluorescent back-lit platform for optimal resolution.

2.7 Thermal denaturation

Differential Scanning Calorimetery (Model 2920 Modulated DSC, TA Instruments, New Castle, DE, USA) analysis was carried out to determine the temperature of denaturation and enthalpy of WPI, HIC WPI, and PGWP following the method outlined by Wang and Ismail (2012). Solutions of WPI and PGWP (20% protein, w/v) were prepared and hermetically sealed in aluminum pans. Samples were allowed to equilibrate for 2 h at 23 °C prior to taking measurements. Isothermal conditions prior to analysis were set at 20 °C for 10 min. Temperature scans were conducted from 20 °C to 95 °C at a rate of 5°C min^{-1}. Areas under the curves (of endothermic peaks) were integrated and corrected based on protein content, to obtain the corresponding enthalpy (ΔH).

2.8 Solubility and Thermal Stability

Solutions (1 mL in DDW) of membrane-filtered WPI, ion exchange WPI, HIC WPI, and PGWP were prepared in 1.5 mL microcentrifuge tubes, in triplicate, at 5% protein concentration (w/v). The solutions were adjusted to pH 3.4, 4.5, or 7 using aliquots of 1M HCl or 1M NaOH and an Orion™ ROSS Ultra™ pH Electrode (Thermo Scientific, Waltham, MA, USA). Solutions at each pH were either left at room temperature or subjected to heating at 80ºC for 30 min in a water bath. The protein content of each solution, before heating and pH adjustment, was determined by the DUMAS method using LECO® TruSpecN™ nitrogen analyzer as described in section 2.5.1. All samples were centrifuged (15,682 x g for 10 min at 23ºC), and protein content in the supernatant of heated and non-heated samples was determined. Solubility was expressed as the percentage of protein content in the supernatant to the total protein content of the initial solution.

2.8.1. Determination of Protein and Carbohydrate Content of Heated pH 4.5 PGWP and HIC WPI Pellet and Supernatant

Upon initial viewing, the pellet size of the pH 4.5 heat treated PGWP and HIC WPI samples for solubility seemed too large based on the solubility percentage that was found (Fig 13, Appendix G). A Mass balance was carried out in order to confirm solubility percentages. The supernatant from the heat treated samples was removed and analyzed for protein content and carbohydrate content as outlined in sections 2.5.1 and 2.5.2 respectively. The pellet was then reconstituted in 975 µL of 1% SDS and 25 µL of β-mercaptoethanol followed by analysis for protein content and carbohydrate content.

2.8.2 SDS-PAGE of Heated Samples

Solutions of membrane-filtered WPI, ion exchange WPI, HIC WPI, and PGWP were prepared in the same way as outlined in section 2.7.1 under which each pH treatment underwent the specified heat treatment of 80ºC for 30 min in a water bath. The solutions were then vortexed and diluted appropriately to achieve the desired 0.01% protein solution (w/v) and an SDS-PAGE gel was run based on the method outlined in section 2.6.

2.9 Emulsification

2.9.1 Emulsification Capacity

Emulsification capacity was measured following the method outlined by Rickert et al. (2004) with some modifications. Solutions (10 mL in DDW) of membrane-filtered WPI, PGWP, and HIC WPI were prepared in triplicate at 1% protein concentration (w/v). The solutions were adjusted to pH 7.0 and 5 mL of protein solution was transferred into a 150 mL beaker. Sudan red 7B (2.4 mg) was added to 600 mL of Mazola corn oil. Corn oil containing sudan red was titrated at a steady flow rate of 4 mL min^{-1} into each protein solution while blended using a homogenizer (IKA® RW 20 Digital, IKA Works, Inc.,

NC, US) with the blade (IKA, R 1342, 4 blade, 50 mm diameter) rotating at 860-870 rpm. Once phase inversion was observed, the volume of oil titrated was recorded and converted to mass using the oil density (0.868 g/mL). The emulsification capacity was expressed as grams of oil per g of protein.

2.9.2 Emulsification Stability

The emulsification stability was measured according to the method outlined by Tang et al. (2003). Aliqouts (6 mL) of protein solutions (0.1% protein w/v) were prepared in DDW in triplicate. Two mL of soybean oil was added to each sample and then homogenized using a homogenizer with a 20 mm shaft and 20 mm open slotted coarse generator (Scilogex D500 Scilogex, LLC, Rocky Hill, CT, US) set to a rotational speed of 10,000 rpm for 1 min. Immediately following homogenization, 50 µL of the emulsion was diluted in 5 mL of 0.1% SDS, vortexed for 5 sec, and absorbance was measured at 500 nm using a spectrophotometer (Beckman 12v-20, Beckman Coulter, Inc., CA, US). The addition of 0.1% SDS was used to avoid creaming of the emulsion. After 10 min the absorbance was measured at 500 nm again. The t=0 and t=10 absorbances (A_0 and A_{10} respectively) were used in **Equation 2** to determine emulsification stability.

Equation 2

$$Emulsification\ Stability\ (ES)\ in\ minutes = \frac{A_0}{A_0 - A_{10}} \times 10min$$

2.10 Statistical Analysis

Analysis of variance (ANOVA) was carried out using IBM SPSS Statistics software version 23.0 for Windows (SPSS, Inc., Chicago, IL, USA). Significant differences among the means of different treatments were determined when a factor effect was found to be significant ($P \leq 0.05$) using the Tukey-Kramer multiple means comparison test. ANOVA tables can be found in **Appendix E**.

3. Results and Discussion

3.1 Extent of Maillard-induced Glycation of Non-dialyzed WPI Incubated with Maltodextrin

3.1.1 Formation of Amadori Compounds and Browning

Amadori compounds are the first stable Maillard reaction intermediates, so the extent of Maillard-induced glycation of WPI with maltodextrin was estimated by monitoring Amadori compounds formation using differential UV absorbance at 304 nm (Feather et al., 1996). The formation of Amadori compounds at 60°C progressed at a fast rate, and was significant after 48 h of incubation for WPI incubated with maltodextrin at a 1:4 ratio (w/w) and for the WPI control (Fig.1). The Amadori compound formation continued to increase over time up to 144 h for both the WPI incubated with maltodextrin and for the WPI control. The increase in Amadori compound formation seen in the control was not observed in a study on glycation of WPI with dextran done by Wang and Ismail (2012). In that study, ion-exchange produced WPI was used, which has a lower amount of lactose (0.4% lactose) than the membrane produced WPI (2.5% lactose) used in this research. Carbohydrate size plays a role in the rate of the Maillard reaction (Chevalier et al., 2001b). With lactose being smaller than the maltodextrin and thus having higher reducing power, lactose must have contributed to the propagation of Amadori compound formation in both the WPI incubated with maltodextrin and the WPI control (Fig. 1).

In order to monitor the Maillard reaction progression, and ensure it was not advancing to further stages, browning was monitored by measuring absorbance at 420 nm. Browning due to the formation of melanoidins is one of the many advanced stage Maillard reaction end-products (Martins, 2009). After 120 h of incubation the browning in 1:4 non-dialyzed samples barely surpassed an absorbance of 0.025, which is considered to be minimal (Fig. 11, Appendix F). With minimal browning observed in incubated samples, it was evident that advanced stages of the Maillard reaction had not been reached, despite high Amadori compound formation. In order to further evaluate the

extent of Maillard reaction propagation, the following section details measurement of free amine groups in incubated samples.

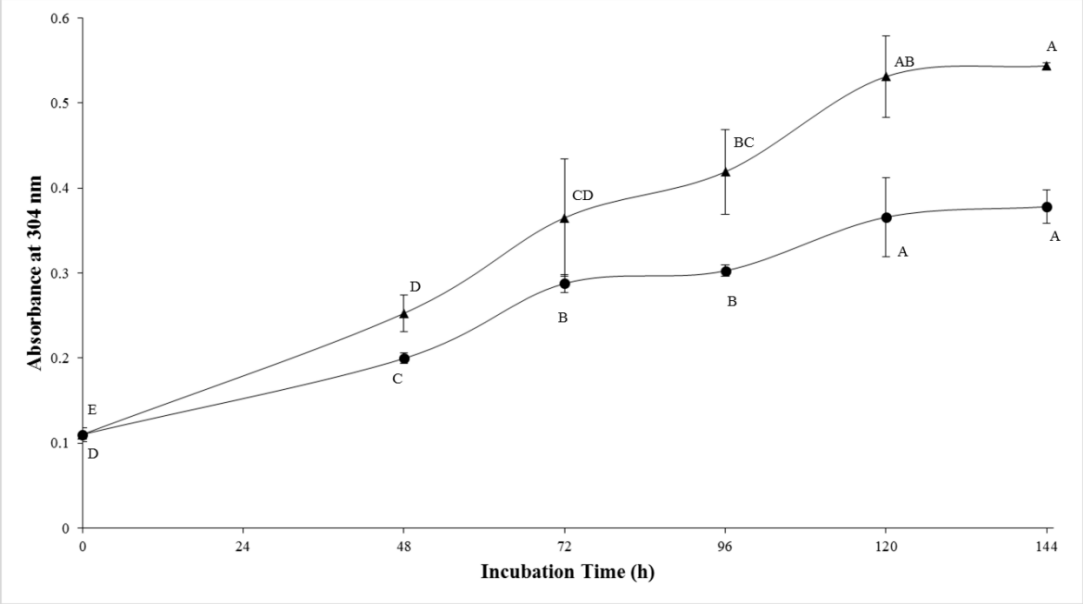

Figure 1. Amadori compound formation in samples of whey protein isolate (WPI) control incubated without maltodextrin (●) and WPI incubated with maltodextrin in a 1:4 ratio (w/w)(▲) at 60°C, 0.45 a_w, for 0-144 h, as determined by UV difference spectroscopy at 304 nm. Error bars represent standard errors (n=3). Different upper case letters above and below the shapes indicate significant differences between different time points within each sample set according to Tukey-Kramer multiple means comparison test ($P \leq 0.05$).

3.1.2 Loss of Free Amino Groups

The extent of glycation can be assessed by determining the extent of amine blockage, since the Maillard reaction involves a reducing sugar and a free amino group. The OPA method was used in order to determine free amino group loss, and is expressed in Fig. 2 as the percent of remaining free amino groups. A significant decrease, 39.5% and 23.1%, in remaining free amino groups was noted for both WPI incubated with maltodextrin and WPI control, respectively, after 48 h of incubation. Maillard-induced glycation continued to occur over time, as the decrease in % remaining free amino groups reached 44.6% and 29.4% for the WPI incubated with maltodextrin and WPI control, respectively, after 144 h of incubation. These results compliment the UV-Vis difference spectroscopy data discussed earlier, and confirm that Maillard-induced glycation was occurring extensively after 48 h in both the samples and controls as noted in the major drop in percent

remaining of free amino groups. The extent of glycation that occurred in the control samples indicated that the lactose was playing a major role in glycation. In previous research on whey protein mixed with lactose (DE = 15) in a 1:1 ratio (w/w) and heated at 60°C for 72 h, the decrease in percent remaining free amino groups was 43% (Chevalier et al., 2001b). However, when whey protein was glycated with dextran (DE = 2.1) at 60°C for 96 h, the decrease in percent remaining free amino groups was only 1.4% (Wang and Ismail, 2012). In this initial incubation, we used non-dialyzed maltodextrin that had a DE of 7.4, with a lower reducing power than lactose, but higher reducing power than the dextran used by Wang and Ismail (2012). The observed decrease in free amino groups indicates that the non-dialyzed maltodextrin and the residual lactose, with its higher reducing power, resulted in a fast propagation of the Maillard reaction. Excessive glycation of WPI incubated with maltodextrin is not desired, as extreme losses of free amino groups (mostly those of the lysine residues) can lead to decreased nutritional value, as well as generation of off-flavors and toxic compounds (Laroque et al., 2008). Blockage of lysine, an essential amino acid, and reduced digestibility of the protein at the site of the glycated amino acid result in decreased nutritional value (Johnson et al., 1977). In order to control the rate and extent of glycation to avoid the potential undesired effects of the advanced stages of the Maillard reaction, there is a need for removal of the residual lactose, and smaller molecular weight maltodextrins.

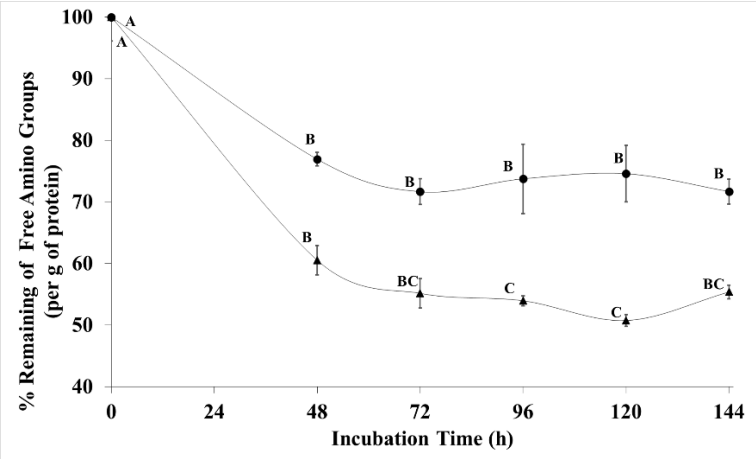

Figure 2. % remaining free amino groups in samples of whey protein isolate (WPI) control incubated without maltodextrin (●) and WPI incubated with maltodextrin in a 1:4 ratio (w/w)(▲) at 60°C, 0.45 a_w, for 0-144 h, as determined by OPA method. Error bars represent standard errors (n=3). Different upper case letters above and below the shapes indicate significant differences between different time points within each sample set according to Tukey-Kramer multiple means comparison test ($P \leq 0.05$).

3.2 Extent Maillard-Induced Glycation of Dialyzed WPI Incubated with Dialyzed Maltodextrin at 1:4 and 2:1 Ratio

3.2.1 Formation of Amadori Compounds and Browning

After WPI and maltodextrin were dialyzed to remove residual lactose and small molecular weight maltodextrin, respectively, there was a noted decrease in the rate of Amadori compound formation at 60°C compared to non-dialyzed samples (Fig. 1 and Fig. 3). The resulting differences in absorbance at 304 nm of WPI incubated in a 1:4 and 2:1 ratio with maltodextrin and the WPI incubated without maltodextrin confirmed that glycation did occur, and that it was reduced in comparison to the non-dialyzed WPI incubated with and without non-dialyzed maltodextrin. Formation of Amadori compounds continued to occur as incubation time increased for the WPI incubated with maltodextrin in a 1:4 ratio. The WPI incubated with maltodextrin in a 2:1 ratio reached a plateau of Amadori compound formation at 96 h, suggesting an equilibrium between new Amadori compound formation and loss of Amadori compounds as a result of conversion to intermediate and advanced glycation end-products.

These results were similar to those observed in a recent study by Wang and Ismail (2012), in which Maillard-induced glycation of WPI was carried out using the same conditions as this study. The WPI incubated with maltodextrin in a 1:4 ratio was the same ratio used in the Wang and Ismail (2012) study, but there was an increase in Amadori compound formation seen past 120 h in this study, that was not observed in the other study. This could be due to the fact that maltodextrin was used in this study had a wider range of molecular weight as opposed to the 9-10 kDa dextran used in the Wang and Ismail (2012) study. Smaller carbohydrates are more readily available to participate in the Maillard reaction. The maltodextrin used in this study had a DE of 4.7 after dialysis, which was still higher than that of the dextran used in the Wang and Ismail (2012) study, which had a DE of 2.1. The higher DE of the maltodextrin means its reducing power was higher than that of the dextran, and was more likely to participate in Amadori compound formation. In the samples of WPI incubated with maltodextrin in a 2:1 ratio, the plateau seen in the Wang and Ismail study was reached at 96 h. This observation can be

attributed to the much lower ratio of carbohydrate to protein when maltodextrin was used instead of dextran. In a previous study by Zhu et al. (2008), when whey protein was glycated with dextran in a 10% WPI, 30% dextran solution ratio at 60°C, Amadori compound formation was greater as denoted by a much higher absorbance reading (~0.7 absorbance units after 24 h incubation) than seen in this study and that of the Wang and Ismail (2012) study. The differing parameters among the studies resulted in difference in the extent of Amadori compound formation. Having moderate Amadori compound formation and plateau reached after 96 h of incubation at 2:1 protein to carbohydrate ratio was desired in order to avoid the advanced stages of the Maillard reaction, as previously discussed.

Amadori compound formation occurred as well in the WPI control, but it was at a lower extent than that observed in the non-dialyzed control. Even though dialysis to remove lactose was performed, analysis of total carbohydrate revealed that a little less than 50% of the original lactose was still in the sample (Table 1), which would account for the slight increase in Amadori compound formation observed in the incubated controls.

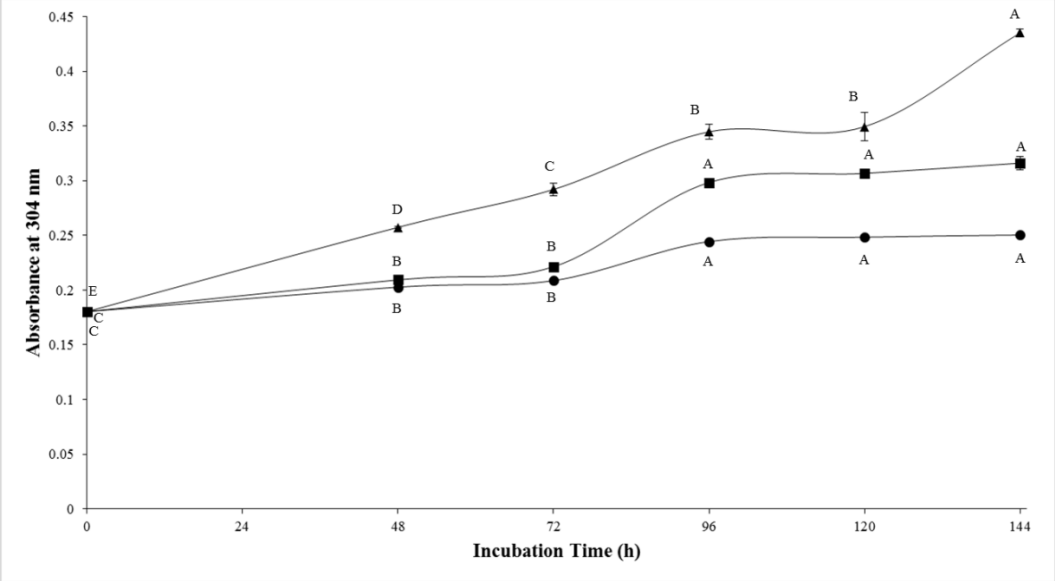

Figure 3. Amadori compound formation in samples of whey protein isolate (WPI) control incubated without maltodextrin (●) WPI incubated with maltodextrin in a 1:4 ratio (w/w) (▲), and WPI incubated with maltodextrin in a 2:1 ratio (w/w) (■) at 60°C, 0.49 a_w, for 0-144 h, as determined by UV-Visible difference spectroscopy at 304 nm. Error bars represent standard errors (n=3). Different upper case letters above and below the shapes indicate significant differences between different time points within each sample set according to Tukey-Kramer multiple means comparison test ($P \leq 0.05$).

Only after 96 h of incubation a significant increase in browning was observed for the samples incubated in a 2:1 ratio, as opposed to the samples incubated in a 1:4 ratio that showed a significant increase in browning after 48 h of incubation. However, for both samples browning never breached an absorbance of 0.025 at 420 nm, which is considered minimal (Fig. 12, Appendix F). This observation confirmed that limited formation of advanced glycation end products occurred. These results are similar to those observed by Wang and Ismail (2012) in which the 420 nm absorbance was below 0.020 for WPI incubated with dextran at 60°C for up to 144 h. β-lactoglobulin incubated with galactose at 50°C and 0.44 a_w up to 60 h of incubation had absorbance readings at 420 nm that remained below 0.020 as well.

3.2.2 Loss of Free Amino Groups

The OPA method was used in order to determine free amino group loss in the incubated samples as discussed earlier. In the WPI controls after dialysis, there was no significant decrease in percent remaining free amino groups over time of incubation (Fig. 4). A significant decrease (~22%), however, in % remaining free amino groups was observed after 48 h of incubation of WPI with maltodextrin in a 1:4 ratio. This decrease in % remaining free amino groups was much less (about half as much) than that (39.5%) of the non-dialyzed WPI incubated with maltodextrin at the same ratio, an observation attributed to the residual lactose, as discussed earlier. On the other hand, no significant decrease was observed for the dialyzed WPI with maltodextrin at a 2:1 ratio until 72 h of incubation (12.6% decrease in % remaining free amino groups). The loss of available amino groups was much greater than that observed in the study by Wang and Ismail (2012), where only a 1.4% loss was observed after 96 h of incubation. This difference is most likely attributed to the smaller molecular weight constituents in the maltodextrin used in this study, even after dialysis, compared to the dextran (molecular weight of ~ 10 kDa) used in the former study. Although there was a greater loss in free amino groups in this study in comparison to that of Wang and Ismail (2012), other research has reported values of up to 14.8% loss (Rufián-Henares et al., 2002; Xu et al., 2011). The results of this study showed lower free amino loss in the 2:1 incubated ratio over time compared to

the reported loss in previous research, which indicates that there was an occurrence of Maillard-induced glycation, but that it was limited. The sample produced after 96 h incubation at 2:1 protein to carbohydrate ratio was selected for further analysis due to the plateau reached in Amadori compound formation observed starting at 96 h of incubation and the limited loss of free amino groups (~11%). Moreover, this sample was chosen because two of the main goals of this study were to eliminate the need for separation of unreacted carbohydrate, and to produce a product that has greater than 90% protein (and be considered an isolate), as opposed to a product with only 60% protein (Wang and Ismail, 2012).

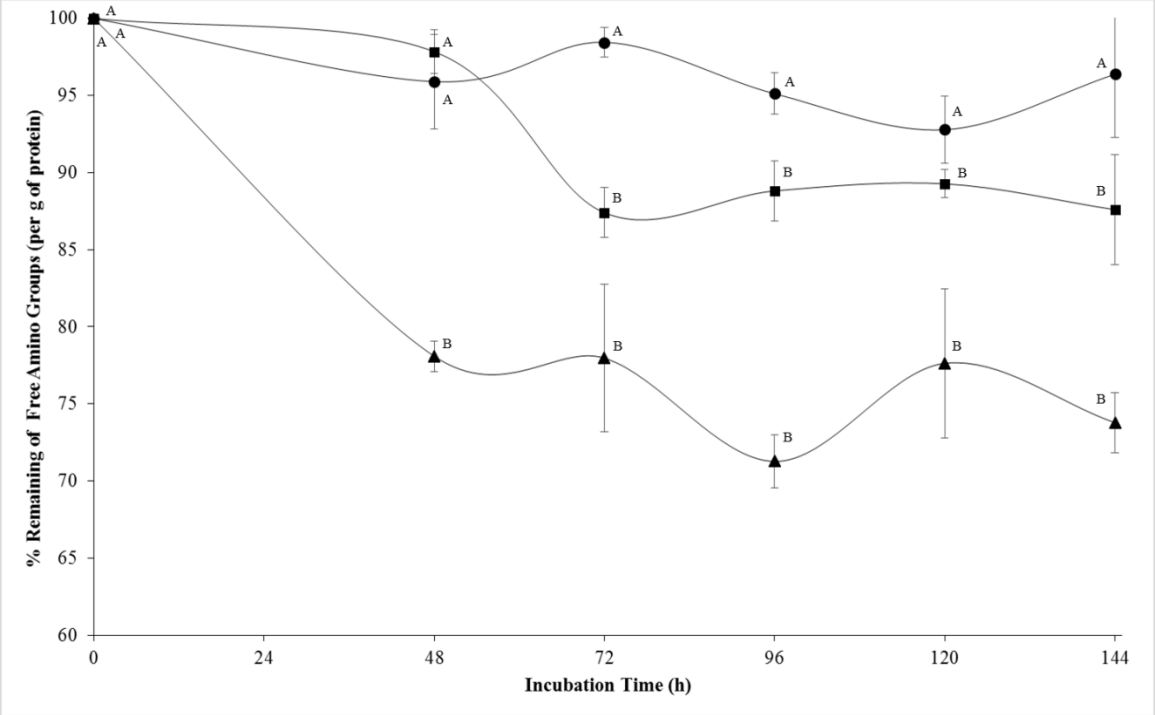

Figure 4. % remaining free amino groups in samples of whey protein isolate (WPI) control without maltodextrin (●), WPI incubated with maltodextrin in a 1:4 ratio (w/w)(▲), WPI incubated with maltodextrin in a 2:1 ratio (w/w) (■) at 60°C, 0.49 a_w, for 0-144 h, as determined by OPA method. Error bars represent standard errors (n=3). Different upper case letters above and below the shapes indicate significant differences between different time points within each sample set according to Tukey-Kramer multiple means comparison test ($P \leq 0.05$).

3.2.3 Determination of Lysine Blockage

The extent of glycation and implications of glycation effect on nutritional quality of WPI incubated with maltodextrin in a 2:1 ratio for 96 h at 60°C and 0.49 a_w, was monitored by measuring furosine, which is formed upon the acid hydrolysis of lysine mediated Amadori compounds. The ε-amino group of lysine is very reactive in the Maillard reaction, and this can result in decreased bioavailability of lysine and a resulting decrease in nutritional quality of whey protein (Finot & Mauron, 1972).

Only 0.26 mg of furosine per gram of protein was detected (equivalent to 3.38 mg furosine per gram of lysine; whey protein has 7.6% lysine). Furosine represents ~43.4% of the total Amadori compounds under the conditions used in this study for acid hydrolysis (Krause et al., 2003). Using that information, lysine blockage was calculated to be 0.45% (on molar basis). The measured lysine blockage represents a very minimal reduction in the bioavailability of lysine, and further confirms that the glycation carried out was minimal. The measured lysine blockage observed by Wang and Ismail (2012) was 0.6% after WPI was incubated with dextran (1:4 protein:carbohydrate ratio) for 96 h at 60°C and 0.49 a_w. The lower percentage of blocked lysine is likely due to the decreased amount of carbohydrate (2:1 protein:carbohydrate ratio) in the current study available to participate in glycation. However, both this study and the Wang and Ismail (2012) study showed limited lysine blockage in comparison to other work. In a study done by Jiménez-Castaño et al. (2007) lysine blockage of β-lg incubated with 10 kDa dextran at 60 °C at a water activity of 0.44, was reported to be 1.65% (on a molar basis) after 72 hours of incubation. Other studies have shown much greater loss of lysine with up to a 13.4% loss shown in commercial whey proteins glycated with maltodextrin at temperatures between 100 to 120°C for 0 to 20 min (Rufián-Henares et al., 2006). The incubation conditions and ingredients used clearly have an impact on the propagation of Maillard-glycation, and lysine blockage, however the WPI incubated with maltodextrin in 2:1 ratio for 96 h at 60°C and 0.49 a_w in this study had limited propagation, and was ideal for further investion of functionality, following removal of unreacted carbohydrate.

3.3 Purification and Compositional Analysis of Whey Protein Incubated with Maltodextrin

3.3.1 Separation of PGWP from Free Maltodextrin

The elution of unreacted maltodextrin from the hydrophobic interaction chromatography (HIC) column was completed after 45 min of 1.25 M ammonium sulfate (10 mL/min) followed by 17 min of DDW (10 mL/min) as confirmed by running a non-incubated 2:1 protein to carbohydrate ratio sample mixture through the column in comparison to the targeted 96 h incubated sample, and quantifying total carbohydrate in the three different elution phases of 1.25 M ammonium sulfate, DDW before protein elution, and DDW elution of protein (Fig. 5). The protein component eluted at 62 min of run time (45 min 1.25 M ammonium sulfate, and 17 min DDW), and was collected between 62-82 min of DDW (10 mL/min). The HIC separation was considered successful in achieving separation of unreacted carbohydrate due to the observed difference in total carbohydrate % of the eluted protein fraction, of non-incubated, and incubated sample. The higher amount of carbohydrate in the protein fraction of the incubated sample is attributed to whey protein glycated with carbohydrate. The protein fraction of non-incubated sample mixture had a much lower amount of carbohydrate, confirming that presence of carbohydrate in the protein fraction of the incubated sample was due to glycation, and unreacted carbohydrates have been successfully separated prior to protein elution.

The need for removal of unreacted carbohydrate is multifaceted. Free carbohydrate remaining in the final sample can be available to take part in the Maillard reaction upon further processing of the PGWP product, leading to initiation and propagation of the reaction past the initial stages during processing and storage of a product containing PGWP. This propagation to advanced stage will result in detrimental effects such as browning, decreased nutritional quality, and change in functionality of the PGWP-containing product. The free carbohydrate could also increase turbidity of the PGWP in solution, which would interfere with spectrophotometric assays to evaluate the product.

The unreacted carbohydrate could also increase the viscosity of PGWP in solution leading to reduced solubility and clarity in a beverage application upon processing.

Although separation of unreacted carbohydrate was achieved, some smaller components of WPI were possibly lost due to HIC separation. Around 20 minutes during the 1.25M ammonium sulfate elution, a small peak was observed (Fig. 5). This peak was attributed to the glycomacropeptide that is found in the membrane separated WPI, and not in ion-exchange separated WPI. Glycomacropeptide is polar and thus was assumed not to interact effectively with the HIC column. Since it is only a minor component of the membrane-separated WPI, and is not found in ion-exchange WPI, it was not attempted to recover the lost glycomacropeptide.

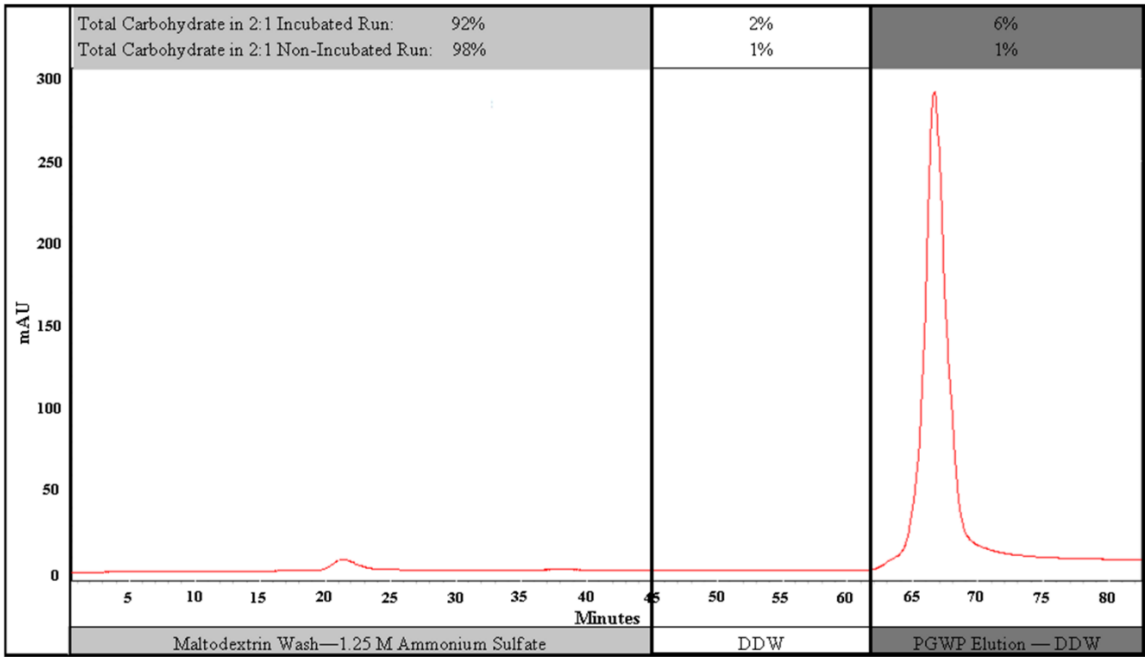

Figure 5. Elution of proteins during separation of free maltodextrin from incubated and non-incubated whey protein and maltodextrin mix (2:1 ratio) using 1.25M ammonium sulfate and DDW (10 mL injection volume, 3.0% protein (w/v)). Chromatogram shows absorbance at 280 nm, scaled to 300 absorbance units (mAU). Total carbohydrate of the three separate washes (1.25M ammonium sulfate (45 min), distilled dionized water (17 min), and distilled dionized water in which protein eluted (20 min), all at 10 mL/min) was measured following the phenol-sulfuric acid total carbohydrate assay. Total carbohydrate percentage is expressed as % total of carbohydrate measured in all 850 mL eluted volume for the incubated (0.19 g carbohydrate) and non-incubated (0.18 g carbohydrate) runs.

3.3.2 Total Protein and Carbohydrate Contents of PGWP and Controls

The PGWP, produced upon incubated of whey protein with maltodextrin at 2:1 ratio for 96 h at 60°C and isolated using HIC separation, was composed of about 94% protein and 4.5% carbohydrate (Table 1). Since the protein content is above 90%, the PGWP would be considered a protein isolate, which is a preferred product claim in the food industry. In previous research done by Wang and Ismail (2012), the protein content was 60% in the produced PGWP, with 30% carbohydrate. The higher protein content in this study would only be beneficial if the improved functionality of PGWP seen in the study by Wang and Ismail (2012) is also observed in the PGWP produced in this study.

The protein and carbohydrate content were also determined for WPI, dialyzed WPI, and WPI (not mixed with maltodextrin) run through the HIC column (HIC WPI). The WPI and dialyzed WPI had differing carbohydrate contents, which indicated that lactose removal was achieved, just not completely as discussed earlier. The HIC WPI had a higher protein content and lower carbohydrate content than the original and the dialyzed WPI, which confirmed that residual lactose was separated out. Lyophilizing, which may have resulted in lower moisture content than in the original WPI sample, contributed to the higher protein content. The difference in carbohydrate content of the PGWP (4.5%) and the HIC WPI (0.51%) confirmed that the carbohydrate content of the PGWP is attributed to the maltodextrin conjugated with the protein. This data indicates that partial glycation did occur under the limited and controlled Maillard reaction conditions used.

Table 1. Protein and carbohydrate content of partially glycated whey protein (PGWP), hydrophobic interaction chromatography (HIC) whey protein isolate (WPI), dialyzed WPI, and membrane produced WPI

	% Protein ± SE[a]	% Carbohydrate ± SE[a]
PGWP	94.0 ± 0.94	4.5 ± 0.01
HIC WPI	93.5 ± 0.79	0.51 ± 0.01
Dialyzed WPI	90.5 ± 0.08	1.2 ± 0.02
WPI	89.1 ± 0.17	2.6 ± 0.10

[a] Standard Error

3.4 Protein Profile Visualization of WPI and PGWP Using SDS-PAGE

Protein profile visualization following SDS-PAGE helped confirm Maillard-induced glycation of whey protein based upon the observed changes in molecular weight of whey proteins by the covalent addition of one or more 3.5-10 kDa maltodextrin molecule per a protein component. Changes in protein profile due to HIC separation was also monitored. HIC separated WPI (Fig. 6, lane 4) had a faint band between 6.5-14.4 kDa, attributed to α-la. Compared to the original and dialyzed WPI samples, this α-la band in HIC separated WPI had much reduced intensity. This observation indicated that during HIC separation some of the α-la in the WPI was lost. Since α-la is one of the two major whey proteins, this reduction in α-la content will have implications on whey protein functionality that will be discussed further in following sections.

Glycation of whey protein is characteristically noted by a longitudinal smear (comparing lanes 1, 2, 3 and 4 to lane 5), indicating formation of glycated proteins. Both β-lg and α-la appeared to have been glycated since the intensity of the bands corresponding to the monomer forms of β-lg and α-la decreased upon glycation. Specifically, in the PGWP sample (Fig. 6, lane 5), the band attributed to α-la has an even lower intensity than that of the HIC separated WPI. The wide spread of molecular weight (~15 to 200 kDa) is attributed to the heterogeneous distribution of conjugated maltodextrin, which means that there was a diverse array of protein molecular weights after glycation. This observation indicated that the α-la and β-lg increased in molecular weight due to attachment of one or more maltodextrin molecules. The smear was visible under both non-reducing and reducing conditions (Fig. 6 a. and b.), which confirmed that the newly formed species were not disulfide-linked polymers. The heterogeneous distribution of glycated proteins was observed in previous research (Lillard et al., 2009; Zhu et al., 2010; Wang & Ismail, 2012). The confirmation of glycation through SDS-PAGE was promising for the 96 h 2:1 incubated samples, but in order to test whether the glycation had produced the desired improvements in functionality for acidic whey protein beverages, solubility and thermal stability testing was employed, as will be discussed in the following sections.

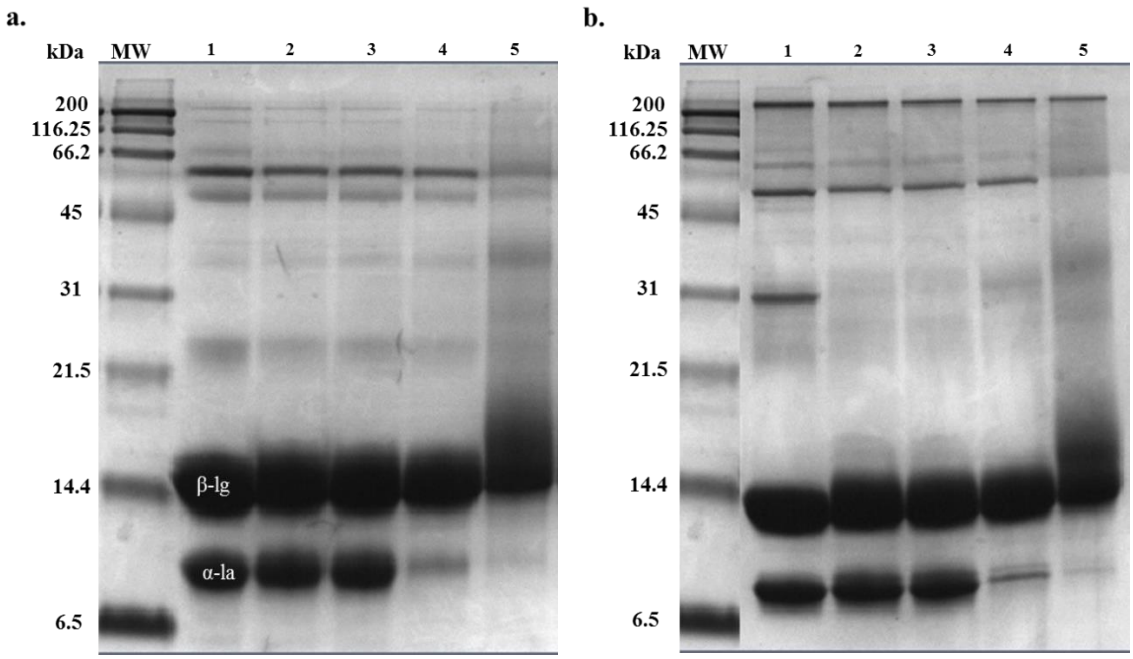

Figure 6. SDS-PAGE gels with Coomasie blue staining under reducing conditions (a) and non-reducing conditions (b). Lane MW- molecular weight standard, lane 1 – ion-exchange produced whey protein isolate (WPI), lane 2 – membrane produced WPI, lane 3 – dialyzed membrane produced WPI, lane 4– hydrophobic interaction chromatography (HIC) separated WPI, lane 5–partially glycated whey protein.

3.5 Changes in Denaturation State of Whey Protein upon Glycation and HIC Separation

Denaturation has a direct bearing on protein functionality. The effect of glycation and HIC separation on denaturation temperature and extent of denaturation has been assessed. No endothermic peak for α-la was seen in the PGWP sample and HIC separated WPI (Fig. 7), even though the SDS-PAGE gel (Fig. 6) indicates there is still α-la present in the samples. In the original WPI sample, α-la content is small to start with, since membrane produced WPI typically contains ~13% α-la, and it must have been slightly denatured in the original protein. Since endothermic peaks are concentration dependent, this loss of α-la, caused a disappearance of the α-la peak in the thermograms for both PGWP and HIC-separated WPI. On the other hand, an endothermic peak for β-lg was observed in all three samples: WPI, HIC-separated WPI, and PGWP. The denaturation temperature of β-lg increased by ~14 °C in both the PGWP and the HIC separated WPI. The lack of α-la in the PGWP and HIC separated WPI compared to the original WPI sample that showed an

α-la endothermic peak at 65.4°C, mean that the 20% protein solution (w/v) of each samples used for DSC were primarily, if not all, β-lg. The higher concentration of β-lg in the samples could have attributed to this shift in denaturation temperature. The increased thermal denaturation temperature of HIC separated WPI was surprising, but could have happened in connection with the 1.25 M ammonium sulfate that both the WPI and PGWP were mixed in to run through the HIC column. It is possible that the ammonium sulfate caused reversible denaturation that resulted in rearrangement of intramolecular interactions within the β-lg. Changes in intramolecular interactions could have led to α-helices being rearranged to form β-sheets, which in turn can lead to better thermal stability (Dàvila et al., 2006). When solutions of proteins that predominantly consist of α-helices are heated and cooled, α-helices are usually converted to β-sheets (Damodaran, 1988). It is assumed that exposure to salt lead to a similar effect on structural rearrangement. In previous work, it has been shown that glycation of whey protein caused complete denaturation (Zhu et al., 2010). However in research done by Wang and Ismail (2012), denaturation temperature was increased. The increase in denaturation temperature was related to partial glycation of whey proteins. However, since there was an increase in denaturation temperature of β-lg in both the PGWP and the HIC-separated WPI, the improved thermal stability could be attributed to multiple factors.

The enthalpy (ΔH) is a measure of the extent of denaturation, and is calculated as area under the endothermic peak. A decrease in enthalpy indicates partial unfolding, while absence of a peak indicates complete denaturation. The ΔH (J g^{-1}) of β-lg had a 12% reduction in the PGWP sample in comparison to that of the WPI sample, but was increased by 14% in the HIC separated WPI. The reduction in ΔH of the PGWP indicated that partial unfolding of the tertiary structure was induced by glycation. Since ΔH is concentration dependent, the high relative concentration of β-lg due to the reduction in α-la in the HIC-separated WPI resulted in an increase in the apparent ΔH of β-lg. Compared to the HIC-separated WPI, ΔH of β-lg in PGWP was lowered by 22%. This direct reduction in ΔH of β-lg, taking into account the concentration effect of HIC separation, confirms that glycation resulted in partial unfolding of the protein, similar to the findings of Wang and Ismail (2012). The improvement in thermal stability of the PGWP and HIC-

separated WPI, needed to undergo further investigation in solution for beverage application, which is discussed in the following section.

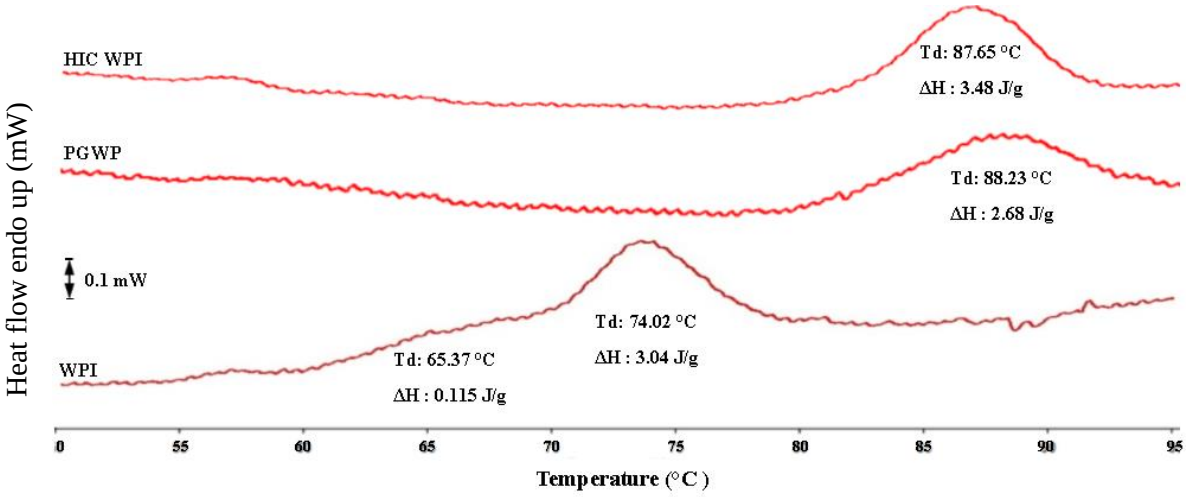

Figure 7. Differential scanning calorimetry of partially glycated whey protein (PGWP), post hydrophobic interaction chromatography (HIC) separation, HIC-separated whey protein isolate (HIC WPI), and original WPI. Td: denaturation temperature and ΔH: enthalpy.

3.6 Changes in Protein Solubility and Thermal Stability of PGWP and Controls

At 5% protein solutions, the membrane-produced WPI exhibited excellent solubility at pH 3.4, 4.5, and 7 when the solutions were not subjected to a heat treatment (Table 2). However, when heated for 30 min at 80°C, membrane-produced WPI had a significant drop in solubility at pH 7, and more so at pH 4.5 (Table 2). The drop in solubility at pH 4.5 can be explained by denatured whey proteins around their pI readily aggregating via hydrophobic interactions, due to no net charge on the proteins and thus a reduction in electrostatic repulsion. The drop in solubility of the membrane-produced WPI after heating at pH 7 indicated that thermal protein denaturation occurred followed by protein aggregation. A similar drop in solubility (down to 68%) was observed when a 1.25% solution of whey protein at pH 6.8 was heated at 60°C for 1 h (Pelegrine & Gasparetto, 2005). However, at pH 3.4 the membrane-produced WPI did not have a significant decrease in solubility in the heated samples compared to the non-heated samples. This observation can be attributed to β-lg having a higher net charge at pH 3.4 than pH 4.5 and

7. The high net charge resulted in electrostatic repulsion even among denatured protein molecules, thus preventing aggregation. Heated PGWP samples, on the other hand, showed enhanced solubility compared to both ion-exchange and membrane-produced WPI at pH 4.5, and compared to membrane-produced WPI at pH 7. When heated at pH 4.5, PGWP had solubility exceeding 90%, whereas ion-exchange WPI almost fell completely out of solution, and membrane WPI had a solubility of as low as 40% (Table 2). Heated PGWP solutions most likely had better solubility at pH 4.5 and 7 due to an increase in net negative charge and overall hydrophilicity upon glycation of whey protein, resulting in resistance/delay of denaturation and polymerization (Wang & Ismail, 2012). The improvement in solubility of the PGWP when heated at pH 4.5 and 7, due to changes in structure are confirmed by the noted increased denaturation temperature of PGWP (88.2°C) in comparison to the membrane WPI (74.0°C) (Fig. 7).

The HIC separated WPI also had improved solubility when heated at pH 4.5 compared to membrane-produced WPI. This observation can be attributed to the noted loss of α-la upon HIC separation, along with the increased denaturation temperature of the HIC separated WPI (87.7°C) in comparison to membrane WPI (74.0°C) (Fig. 7). α-lactalbumin has a lower denaturation temperature compared to β-lg, that may cause increased thermal aggregation resulting in decreased solubility. α-lactalbumin also has a lower isoelectric point (~4.2) compared to β-lg's pI of 5.4. Having a higher pI than α-la, β-lg will have more net negative charge at pH 4.5, and thus will contribute to more electrostatic repulsion in the HIC separated WPI and PGWP at a pH of 4.5. The PGWP heated at pH 4.5 did have a significantly better solubility than the HIC separated WPI, which indicated that glycation contributed as well to the overall improvement of solubility of whey protein, which was not only attributed to the reduced amount of α-la. Glycation blocks some amine groups, which causes net negative charge to increase, and this increase in net negative charge at pH 4.5 helped promote electrostatic repulsion between protein molecules, and thus improved solubility (Oliver et al., 2006). The noted improvement in solubility of PGWP at pH 7 when heated, in comparison to the membrane produced WPI, is attributed to glycation as well, since the HIC separated WPI had even lower solubility than that of the membrane produced WPI. The attachment of bulky carbohydrates through glycation causes an increase in steric hindrance, which in

turn reduces protein-protein interactions of denatured protein molecules that have exposed hydrophobic groups (Oliver et al., 2006; Wang & Ismail, 2012).

The enhanced solubility of PGWP around whey protein's isoelectric point (pH 4.5) under heated and non-heated conditions has been achieved in previous research on whey protein glycation (Akhtar & Dickinson, 2007; Zhu et al. 2010). But, in the previous research, protein solutions were formulated at 0.1% (w/w), which is a very low concentration for protein beverage applications. Additionally, the glycation conditions used by Akhtar and Dickinson, were harsher (a_w = 0.79 and 80°C), and the extent of glycation was not monitored, but was most likely high based on the harsher conditions employed. On the other hand, similar findings to ours were notated at pH 4.5 for 5% protein solutions (Wang & Ismail, 2012), yet ion-exchange produced WPI, along with non-food grade carbohydrate for glycation was used, in addition to the fact that their product only had 60% protein.

Overall, the improvement of solubility of PGWP after 30 min of heat treatment at 80°C at both pH 4.5 and 7 has possible implications for industry usage. The enhanced solubility at pH 4.5 of glycated whey protein with food grade constituents, and optimized glycation conditions, compared to membrane-produced WPI would allow for formulation of acidic whey protein beverages at or near pH 4.5, rather than lower pHs (~ pH 3.4) that are currently used in industry. A pH of 4.5 still allows for milder thermal treatments applied for acidic beverages compared to neutral beverages. Mild thermal treatment preserves color and flavor better. Beverage formulation at a higher pH (4.5 vs. 3.4) would require addition of less acid, which would reduce perceived sourness and astringency, thus improving overall consumer acceptance of acidified whey protein beverages. The enhanced solubility at pH 7 of glycated whey protein compared to membrane-produced WPI would allow for formulation of neutral protein beverages with higher protein content. Since neutral protein beverages require a harsher heat treatment compared to acidic beverages, the improved thermal stability and solubility of the glycated whey proteins would bode well for this type of beverage formulation.

Table 2. Percent solubility of ion-exchange produced whey protein isolate (WPI), membrane-produced WPI, hydrophobic interaction chromatography separated (HIC) WPI, and partially glycated whey protein (PGWP).

		% Solubility at 5% Protein Solution			
pH	Heat Treatment	Ion-Exchange WPI	Membrane WPI	HIC WPI	PGWP
3.4	Non-heated	104.9 A^a*	102.5 Aa	97.1 Aa	98.2 Aa
	80°C	98.6 Aa	96.6 Aa	82.1 Bb	85.9 Bb
4.5	Non-heated	80.2 Ba	95.9 Aa	95.6 Aa	99.4 Aa
	80°C	5.1 Db	39.9 Cb	86.4 Bb	92.2 Ab
7	Non-heated	99.2 Aa	97.9 Aa	96.4 Aa	98.4 Aa
	80°C	99.6 Aa	68.4 Cb	53.5 Db	84.5 Bb

^ Different uppercase letters indicate significant differences in solubility between the different samples under the same pH and heat treatment according to the Tukey-Kramer multiple means comparison test ($P \leq 0.05$).

* Different lowercase letters indicate significant differences in solubility between the heated and non-heated solubility of each sample at each pH according to the Tukey-Kramer multiple means comparison test ($P \leq 0.05$).

3.6.1 Evaluation of pH 4.5 Heat-Treated Original Membrane WPI, HIC-Separated WPI, and PGWP

Upon viewing the solubility data of the HIC-separated WPI and the PGWP samples when in a 5% protein solution at pH 4.5 and following heat treatment of 30 min at 80°C, the pellet formed in the samples after centrifugation seemed too large (Fig. 13, Appendix G) considering the high solubility values (~86-92%) (Table 2).The pellet formed upon heating the original membrane WPI solution at pH 4.5 was larger than that of the HIC-separated WPI and the PGWP, however the size of the pellets did not seem to correlate well with the solubility results. To confirm that the solubility data obtained was accurate, the supernatant was collected, and the pellet was reconstituted in 1% SDS and β-mercaptoethanol as described in section 2.7.1.1. The total carbohydrate content was then determined for both the pellet and supernatant of each of the three samples. The protein content of the reconstituted pellet was also determined, in order to complete a mass balance evaluation. The pellet of the PGWP contained 0.73% carbohydrate as opposed to 3.74% in the supernatant (Table 3). This data confirmed that the majority of the carbohydrate remained in solution as conjugates of the solubilized proteins. The glycated proteins remained soluble due to the improved hydrophilicity of the proteins along with

the bulky glycated constituents inhibiting protein-protein interactions. The protein percentage of the pellet was also less than 1%, confirming the high protein percentage in the supernatant and the high solubility of the PGWP at pH 4.5 after heat treatment for 30 min at 80°C. The visible pellet was determined to be more of a viscous gel with low density rather than a high density and compact pellet. Once the solubility data of the pH 4.5 heat treated samples was confirmed to an extent, further confirmation and visualization of all the heat treated %5 solution samples was desired, which is discussed in the following section through use of SDS-PAGE.

Table 3. Protein and carbohydrate content of the pellet and supernatant of partially glycated whey protein (PGWP), hydrophobic interaction chromatography (HIC) separated whey protein isolate (HIC WPI), and original membrane WPI, in %5 protein solutions, heated for 30 min at 80°C, at pH of 4.5.

	% Carbohydrate		% Protein	
Sample	Pellet	Supernatant	Pellet	Supernatant
PGWP	0.73	3.74	0.72	4.62
HIC WPI	0.25	0.43	0.77	4.31
WPI	0.73	2.17	2.69	2.02

3.6.2 Protein Profile Visualization of Heat Treated Protein Solutions Using SDS-PAGE

Protein profile and aggregation in heat-treated samples at various pHs were monitored using SDS-PAGE (Fig. 8 a and b). Ion-exchange WPI sample heated at pH 7 showed longitudinal smearing and high intensity bands at the top of the gel ran under non-reducing conditions (Fig 8 a, lane 1). This smearing and the high molecular weight bands were coupled with a reduced intensity of the α-la and β-lg bands indicating that the proteins took part in forming heterogeneous and larger polymers of varying molecular weights. Upon running the same sample under reducing conditions the large polymers and the smearing were no longer visible and the intensity of the monomer's bands was higher (Fig. 8b, lane 1). This observation indicates that polymer formation was due to disulfide interchange (Pelegrine & Gasparetto, 2005). However, this polymerization did not impact solubility of this samples (Table 2). The polymers produced were not large

enough to fall out of solution, they remained suspended. Ion-exchange WPI sample heated at pH 4.5 was not run on the gel, as the extreme loss in solubility due to aggregation (Table 2), made extracting an even suspension of protein difficult due to clogging of pipette tips. Ion-exchange WPI sample heated at pH 3.4 showed no reduction in visualization of β-lg and α-la, and very little longitudinal smearing. Not much of a difference was noted when running the same sample under reducing conditions (Fig 8 a and b, lane 2). The ion-exchange WPI remained soluble after heating at pH 3.4 (Table 2) with the proteins being mostly in monomeric forms compared to the observed polymerization at pH 7 (Fig. 8).

Membrane WPI sample heated at pH 7 showed longitudinal smearing and some bands at the top of the gel ran under non-reducing conditions (Fig 8 a, lane 3). The smearing and the intensity of the bands were less than that of the ion-exchange samples. This is attributed to the fact that solubility was lower for the membrane WPI, and aggregates were larger, and hence an even suspension of the sample was not attained. The smearing and the high molecular weight bands, nevertheless, were coupled with a reduced intensity of the α-la and β-lg bands indicating that the proteins took part in forming heterogeneous and larger polymers of varying molecular weight, just as in the ion-exchange WPI. Upon running the same sample under reducing conditions the large polymers and the smearing was no longer visible and the intensity of the monomers bands was higher. This observation indicates that polymer formation was partially attributed to disulfide interchange. The extent of polymerization had a significant impact on the solubility of this samples, unlike the ion-exchange WPI (Table 2). The polymerization in the membrane WPI can also be attributed to the higher calcium content it has in comparison to ion-exchange WPI (Ju & Kilara, 1998). Increased calcium content in WPI coincides with increased gelation and coagulation of proteins, which means the calcium caused increased polymerization (Ju & Kilara, 1998). Large aggregates may be formed in the presence of calcium, which explains why not all aggregates were adequately pipetted to be loaded on the gel. Membrane WPI sample heated at pH 4.5 was not shown, as the extreme loss in solubility (Table 2) due to extensive aggregation, made extracting an even suspension of protein difficult due to clogging of pipette tips. Membrane WPI sample heated at pH 3.4 showed no reduction in the intensity of β-lg and α-la bands, and very

little longitudinal smearing. Not much difference in band distribution was noted when running the sample under reducing conditions (Fig 8 a and b, lane 4), just as in the ion-exchange WPI. Similar to the ion-exchange WPI, the membrane WPI remained soluble (Table 2) after heating at pH 3.4, with the proteins being mostly in monomeric forms.

For the HIC-separated WPI, the pH 7 heated sample had bands that are faint in both the reducing and non-reducing gels, although more so in the non-reducing gel, where α-la cannot be seen (Fig 8 a and b, lane 5). The sample had a ~50% reduction in solubility upon heating (Table 2), and aggregation caused drawing up an even suspension for visualization to be difficult. Aggregation due to disulfide linkage and other forces was confirmed by the appearance of monomeric a β-lg band when the sample was run under reducing conditions, and the fact that aggregated sample did not remain in solution upon suspension. The heated HIC-separated WPI at pH 4.5 showed very faint longitudinal smearing, that disappeared when the sample was run under reducing conditions. This observation indicated that the sample underwent very little aggregation (Fig 8 a and b, lane 6), which was reflected in the solubility of the sample (Table 2). The heated HIC WPI sample at pH 3.4 showed faint high molecular weight bands under non-reducing conditions (Fig 8 a, lane 7). The high molecular weight bands indicated that the proteins took part in forming heterogeneous and larger polymers of varying molecular weight that caused a 15% reduction in solubility (Table 2).

The heated PGWP at pH 7 underwent some polymerization as noted by the observed smearing (Fig 8 a, lane 8) under non-reducing conditions. Some of the protein in this sample was also difficult to keep well dispersed, even though solubility was high (85%, Table 2), and thus the sample loaded was not a good representative of the mixture. This was confirmed when better suspension was obtained upon adding a reducing agent, and monomer bands were quite visible (Fig 8 b, lane 8). Under reducing conditions, longitudinal smearing of the bands was visible as well, which indicates that glycation was present, since longitudinal smearing due to disulfide linkages would be reduced and would not be present. The heated PGWP at pH 4.5 showed very little difference between non-reducing and reducing conditions (Fig 8 a and b, lane 9). There is noted longitudinal smearing in both, which once again, confirms that glycation occurred, and this glycation maintained the solubility, which was minimally reduced (7%) upon heat treatment (Table

2). The PGWP heat treated at pH 3.4, showed a high molecular weight band around 200 kDa that disappeared under reducing conditions (Fig 8 a and b, lane 10). This would indicate that some aggregation due to disulfide linkages did occur at this pH, and it caused some loss in solubility (~12%) upon heating (Table 2). This decrease in solubility could be attributed to the partial denaturation of the PGWP, as noted by the decreased ΔH, which may have been the result of breakage of intramolecular bonds within individual β-lg proteins, leading to increased potential for protein polymerizations (Nonaka et al., 1993).

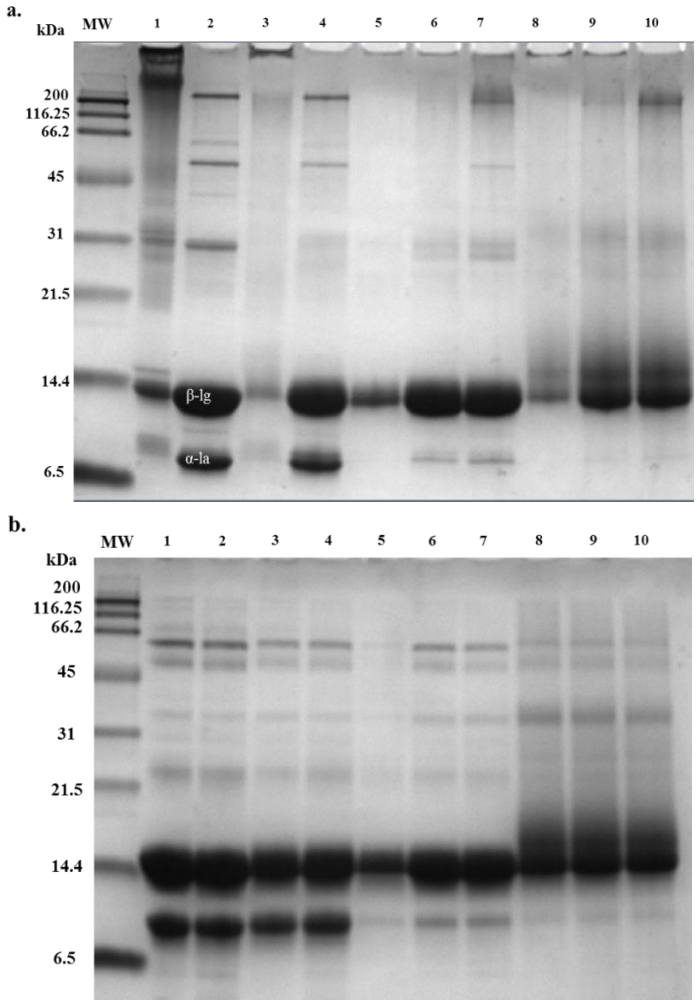

Figure 8. SDS-PAGE gels with Coomasie blue staining under non-reducing conditions (a) and reducing conditions (b). MW- molecular weight standard, lanes 1-2 – ion-exchange produced whey protein isolate (WPI) at pH 7, and 3.4, lanes 3-4- membrane produced WPI at pH 7, and 3.4, lanes 5-7-hydrophobic interaction chromatography (HIC) separated WPI at pH 7, 4.5, and 3.4, lanes 8-10–Partially glycated whey protein at pH 7, 4.5 and 3.4. All samples prepared at 5% were heated for 30 min at 80°C.

3.7 Emulsification Capacity and Stability of Glycated and HIC Separated Whey Protein

The effect of Maillard-induced glycation on emulsion capacity and stability was also investigated. Although partially denatured whey proteins can be efficient emulsifying agents of oil-in-water emulsions, the emulsifying capacity can be significantly reduced when aggregation or precipitation of whey protein occurs. It was, therefore, hypothesized that the observed enhancement of solubility and thermal stability of whey protein may also positively impact the emulsification properties. The emulsification capacity of PGWP was slightly improved in comparison to membrane WPI (Table 4). It has been noted that globular proteins, including whey proteins, show improved emulsifying capacity through moderate heating and the resulting partial unfolding (Zayas, 1997). PGWP did exhibit partial unfolding as determined by the lowered ΔH (2.7 J g^{-1}) in comparison to the membrane WPI (3.0 J g^{-1}) (Fig. 7). The change in structure of the whey proteins could also help explain why HIC separated WPI exhibited a lowered emulsification capacity in comparison to membrane WPI. The increased ΔH of HIC separated WPI (3.5 J g^{-1}) was previously determined to be due to refolding of the structure, and this could have resulted in the burying of hydrophobic groups of the protein. The burying of hydrophobic groups would result in a lowered emulsification capacity, since proteins must have a well-balanced hydrophilic and hydrophobic groups in order to have a high emulsification capacity (Kato et al. 1981; Zayas, 1997). There is not much information on emulsification capacity improvement of whey proteins via Maillard-induced glycation, so these results are promising for further research going forward, such as monitoring enhancement in emulsification capacity at different pH. Unlike emulsion capacity, there has been data compiled on improvements in emulsification stability upon Maillard-induced glycation.

The emulsion stability of PGWP was significantly lower than that of the original membrane WPI (Table 4). The decrease in emulsion stability was also noted in the HIC separated WPI, which was even lower than that of the PGWP (Table 4). The decrease in emulsion stability could be related to the reduced amount of α-la in both of the samples which was discussed earlier, since α-la's molten globule state allows for noted

stabilization in emulsions (Barbana & Perez, 2011). In previous research on emulsification stability improvement, maltodextrin with a DE of 19 was mixed with whey protein at a 1:1 ratio at a water activity of 0.79 for 2 h (Akhtar & Dickinson, 2007). Emulsification stability in that study was increased based on the observed decrease in average droplet size of whey protein-maltodextrin conjugates. The maltodextrin used in this study had a DE of 4.7 after dialysis, which was lower than that of the maltodextrin used in the Akhtar and Dickinson (2007) study, and is indicative of presence of higher molecular weight maltodextrin. Accordingly, the conjugates formed with smaller molecular weight maltodextrins would have potentially contributed to smaller droplets. Also, Akhtar and Dickinson, (2007), did not remove unreacted carbohydrate from their whey protein-maltodextrin mixtures, which means the viscosity of solutions could potentially increase, and viscosity plays a major role in emulsion stability (Zayas, 1997). The loss in stability of PGWP in comparison to WPI could also be due to the larger molecules in PGWP that were formed upon glycation. With larger and slightly unfolded protein molecules in PGWP, fewer proteins can be at the film interface, which results in a thinner film. Film thickness has a major role in emulsion stability, and thinner films are not as stable and lead to coalescence (Zayas, 1997).

The findings on emulsification properties of PGWP showed a moderate enhancement in emulsion capacity and a reduction in emulsion stability. Improving emulsification capacity of a protein ingredient could be desirable to have more oil emulsified, but if the stability is decreased considerably, the use of this ingredient in products would be ineffective, as most emulsions are expected to be stable over time. However, with improved capacity, the PGWP product could always be blended with a protein that has better emulsification stability for use in emulsified products. In any case, effect of glycation on the emulsification properties of whey protein need further investigation. These preliminary findings indicate that the conditions that result in enhanced solubility may not be ideal for marked enhancement in emulsification properties. It is, therefore, important to optimize glycation conditions for targeted functionality enhancement.

Table 4. Emulsification capacity and stability of membrane produced whey protein isolate (WPI), hydrophobic interaction chromatography (HIC) separated WPI, and partially glycated whey protein (PGWP).

	Emulsification Capacity (g of oil / g of protein)	Emulsification Stability (min)
WPI	954 b*	51.4 a
HIC WPI	850 c	24.5 b
PGWP	1064 a	32.5 b

*Different lowercase letters indicated significant differences in emulsification capacity and stability, across different samples, according to the Tukey-Kramer multiple means comparison test ($P \leq 0.05$).

4. Overall Conclusions, Implications, and Recommendations

Maillard-induced glycation of WPI was successfully carried out in a controlled and limited fashion while incubating WPI with maltodextrin in a 2:1 ratio at 60°C and 0.49 a_w for 96 h. Limited glycation was confirmed by the moderate formation of Amadori compounds, marginal loss in free amino groups, and minimal loss of lysine. Minimal browning confirmed that the Maillard reaction was limited to initial stages with barely any progression to advanced and undesirable stages. The use of HIC for removal of unreacted maltodextrin was successful, however, the process resulted in some loss of α-la. It would be ideal to find optimal incubation conditions in order to eliminate the need for chromatographic separation of maltodextrin, which not only may cause some losses, but may also result in added processing time and waste streams. Nevertheless, a high protein PGWP (94%) product was produced meeting the requirement of a protein "isolate" (>90% protein).

Compared to WPI, PGWP had enhanced solubility and thermal stability at both pH 4.5 and 7 in 5% protein solutions. Enhanced solubility and thermal stability have positive implications for "high protein" (>4.2%) acidic and neutral beverages. Acidic protein beverages formulated around the pI (4.5) of whey protein would be ideal in comparison to the acidic beverages (pH 3.4) currently on the market. There would be less acid required to reach the targeted pH, which would result in less sourness and astringency in the beverages. Reduced sourness and astringency is desirable for improved consumer acceptance of acidic whey protein beverages. The enhanced solubility upon thermal treatment of PGWP at pH 7 is promising for neutral protein beverage formulation, since these beverages require harsher heat treatment in comparison to acidic beverages.

A PGWP product with enhanced solubility and thermal stability is promising, but ideally the product would have multiple uses in the food industry. Preliminary findings on the emulsification properties of the PGWP were promising, warranting further investigations to produce a PGWP product with enhanced emulsification properties. The findings also indicate that conditions that result in enhanced solubility may not be ideal for marked enhancement in emulsification properties. Therefore, glycation conditions may have to be optimized based on targeted functionality enhancement.

Overall, this work was successful in the production of glycated whey protein with enhanced solubility and thermal stability for potential high protein beverage applications. The ingredients used to make this value-added product were food grade, and in abundant availability. But future work on the optimization of glycation for elimination of the HIC separation is needed. Further work is required on the digestibility of this product, in order to ensure that the nutritional quality of whey protein is maintained, since a loss of nutritional quality would devalue the product. Beverage formulation is also required in order to assess consumer acceptance, because without an enjoyable product, the PGWP produced would not have a market use. Further investigation into storage stability would be required as well, in order to evaluate if the noted improvement of solubility and thermal stability would carry over into the speculated improvement in shelf-life of acidic whey protein beverages. With the mentioned future work, production of a glycated whey protein with improved functionality would allow for formulation of beverages at the protein levels needed for a high protein claim, while maintaining longer shelf-life and overall quality. Ability to formulate shelf stable beverages at high protein levels provides economic gain for producers and physiological benefits for consumers.

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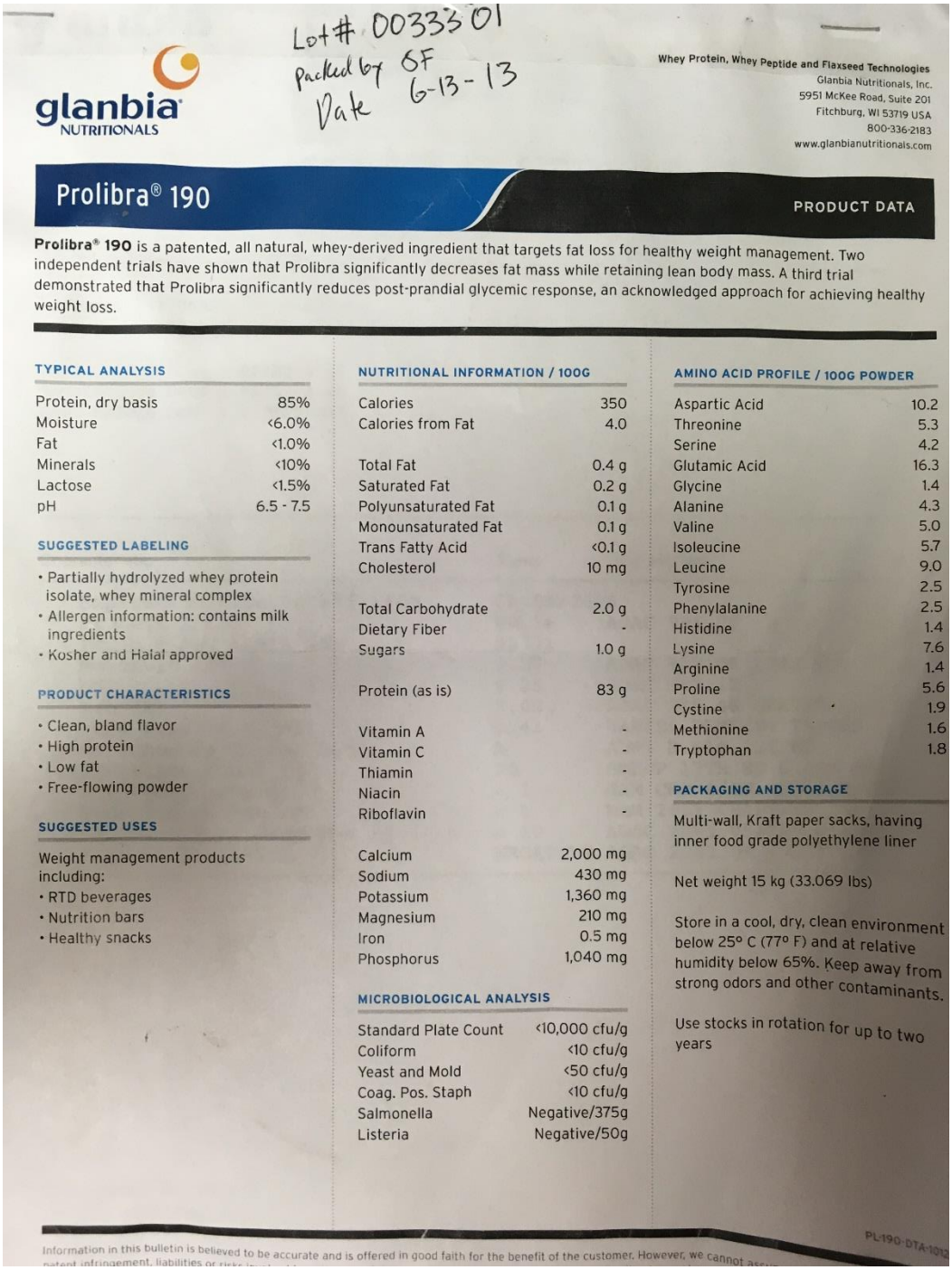
Appendix A: Specification Sheets for Membrane Produced and Ion-exchange Produced WPI



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Date 6-13-13

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Glanbia Nutritionals, Inc.
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Fitchburg, WI 53719 USA
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www.glanbianutritionals.com

Prolibra® 190

PRODUCT DATA

Prolibra® 190 is a patented, all natural, whey-derived ingredient that targets fat loss for healthy weight management. Two independent trials have shown that Prolibra significantly decreases fat mass while retaining lean body mass. A third trial demonstrated that Prolibra significantly reduces post-prandial glycemic response, an acknowledged approach for achieving healthy weight loss.

TYPICAL ANALYSIS

Protein, dry basis	85%
Moisture	<6.0%
Fat	<1.0%
Minerals	<10%
Lactose	<1.5%
pH	6.5 - 7.5

SUGGESTED LABELING

- Partially hydrolyzed whey protein isolate, whey mineral complex
- Allergen information: contains milk ingredients
- Kosher and Halal approved

PRODUCT CHARACTERISTICS

- Clean, bland flavor
- High protein
- Low fat
- Free-flowing powder

SUGGESTED USES

Weight management products including:
- RTD beverages
- Nutrition bars
- Healthy snacks

NUTRITIONAL INFORMATION / 100G

Calories	350
Calories from Fat	4.0
Total Fat	0.4 g
Saturated Fat	0.2 g
Polyunsaturated Fat	0.1 g
Monounsaturated Fat	0.1 g
Trans Fatty Acid	<0.1 g
Cholesterol	10 mg
Total Carbohydrate	2.0 g
Dietary Fiber	-
Sugars	1.0 g
Protein (as is)	83 g
Vitamin A	-
Vitamin C	-
Thiamin	-
Niacin	-
Riboflavin	-
Calcium	2,000 mg
Sodium	430 mg
Potassium	1,360 mg
Magnesium	210 mg
Iron	0.5 mg
Phosphorus	1,040 mg

MICROBIOLOGICAL ANALYSIS

Standard Plate Count	<10,000 cfu/g
Coliform	<10 cfu/g
Yeast and Mold	<50 cfu/g
Coag. Pos. Staph	<10 cfu/g
Salmonella	Negative/375g
Listeria	Negative/50g

AMINO ACID PROFILE / 100G POWDER

Aspartic Acid	10.2
Threonine	5.3
Serine	4.2
Glutamic Acid	16.3
Glycine	1.4
Alanine	4.3
Valine	5.0
Isoleucine	5.7
Leucine	9.0
Tyrosine	2.5
Phenylalanine	2.5
Histidine	1.4
Lysine	7.6
Arginine	1.4
Proline	5.6
Cystine	1.9
Methionine	1.6
Tryptophan	1.8

PACKAGING AND STORAGE

Multi-wall, Kraft paper sacks, having inner food grade polyethylene liner

Net weight 15 kg (33.069 lbs)

Store in a cool, dry, clean environment below 25° C (77° F) and at relative humidity below 65%. Keep away from strong odors and other contaminants.

Use stocks in rotation for up to two years

PL-190-DTA-101

DAVISCO

FOODS INTERNATIONAL, INC.®

*Bi*PRO®
Whey Protein Isolate

Product Description

*Bi*PRO is manufactured from fresh, sweet dairy whey that is concentrated and spray dried. The product is a homogenous, free flowing, semi-hygroscopic powder with a clean, bland flavor.

Product Functionality

*Bi*PRO is a unique, natural and pure dairy protein comprised of beta-lactoglobulin and alpha-lactalbumin. *Bi*PRO's functional protein groups have valuable gelling, water binding, emulsification, and aeration properties. *Bi*PRO replaces larger quantities of other functional ingredients providing improved flavor and mix efficiency. *Bi*PRO is not denatured and is fully soluble over the pH range 2.0 to 9.0. *Bi*PRO is lactose-free†.

Analysis*	Specification	Typical Range	Test Method
Moisture (%)	5.0 max.	4.7 ± 0.3	Vacuum Oven AOAC 927.05
Protein, dry basis (N x 6.38) (%)	95.0 min.	97.7 ± 0.7	Leco Combustion AOAC 990.03
Fat (%)	1.0 max.	<0.5	Mojonnier AOAC 989.05
Ash (%)	3.0 max.	1.9 ± 0.3	Residue on Ignition AOAC 930.30
Lactose (%)	0.5 max.	<0.2	By difference AOAC 986.25
pH	6.7 - 7.5	7.0 ± 0.3	10% Sol. @ 20°C AOAC 945.27
Scorched Particles	15 mg/25g max.	7.5mg	ADPI, AOAC 952.21

Microbiological Profile	Specification	Typical Range	Test Method
Aerobic Plate Count	<10,000/g	<2,500	FDA/BAM, AOAC 966.23
Coliform (MPN)	<10/g	<10	FDA/BAM, AOAC 966.23
E. coli (MPN)	Negative/g	Negative	FDA/BAM, AOAC 966.23
Yeast & Mold	10/g max.	≤10	FDA/BAM
Coag. Pos. Staph(MPN)	<10/g	<10	FDA/BAM, AOAC 966.23
Salmonella sp.	Negative/1500 g	Negative	FDA/BAM, ELISA AOAC 2004.03
Listeria sp.	Negative/25 g	Negative	FDA/BAM, ELISA AOAC 999.06

*All results reported on "AS IS" basis except where noted

Storage and Packaging

Dried dairy products can absorb odors and moisture. Therefore, adequate protection is essential. Shelf life is 3 years from the manufacture date. Shelf life will be enhanced through ideal storage conditions which include temperatures below 25°C., relative humidity below 65%, and an odor free environment. Avoid less than ideal storage conditions.

Packaged in Kraft multiwall bags, incorporating a polyethylene bag liner, individually closed.
Net wt.: 33 lb. Bag
Contact your Sales Representative for additional packaging options.

†Based on US regulatory labeling of sugars and carbohydrates in products that contain less than 0.5g per serving as "0g" or "Sugar Free". Always consult with qualified legal representation for proper labeling and claims on your final product.

Version 15U-1123

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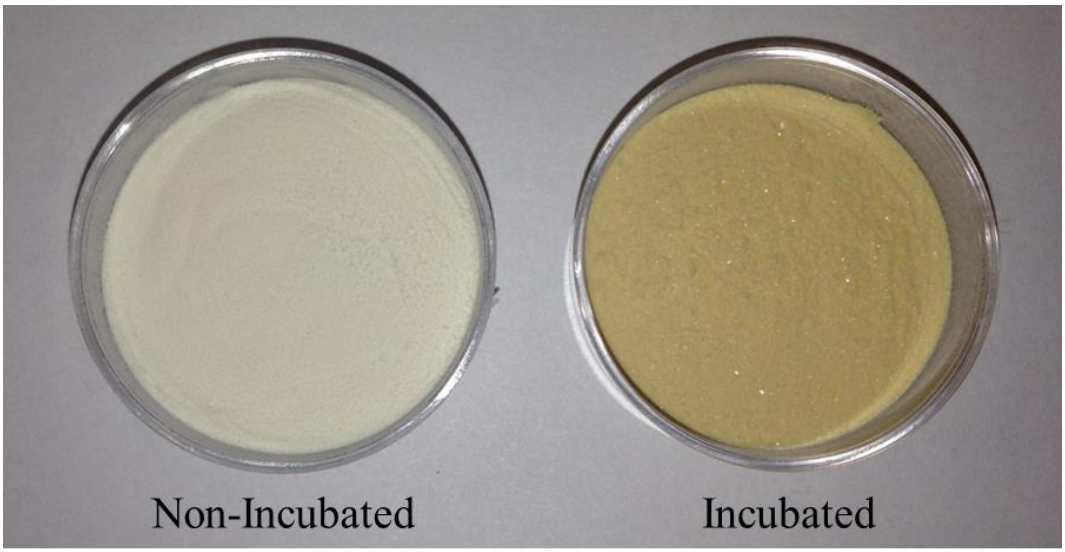
Appendix B: Images of Visible Browning and Caking of a_w= 0.63 and 0.74 Incubated Samples

a.



b.

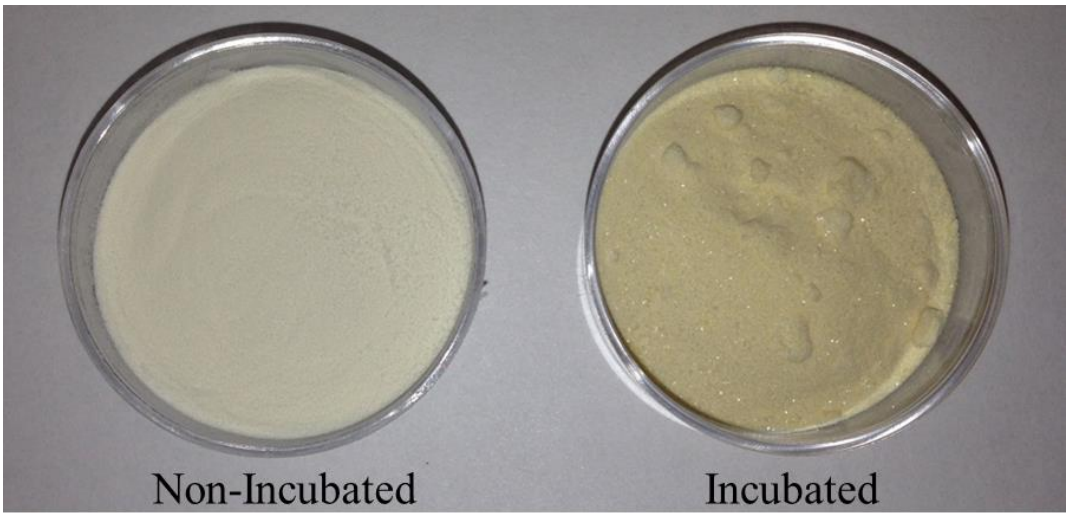


Figure 9. Samples of WPI incubated with maltodextrin in a 2:1 ratio (w/w) incubated at 60°C, and a_w of 0.63 (a) and 0.74 (b) for 48 h.

Appendix C: Calculation for Loss of Free Amino Groups Determined Using OPA Procedure

Equation 1:

$$\%\ \text{Remaining Free Amino Groups} = 100 - \frac{100 * (\%\ \text{free amino groups}_0 - \%\ \text{free amino groups}_{incubated})}{\%\ \text{free amino groups}_0}$$

1:4 (dialyzed incubation) 96 h rep 1

$$\%\ \text{Remaining Free Amino Groups} = 100 - \frac{100 * (11.4 \quad - 8.07)}{11.4}$$

$$= 70.8\%$$

Appendix D: Chromatogram and Sample Calculation for Furosine

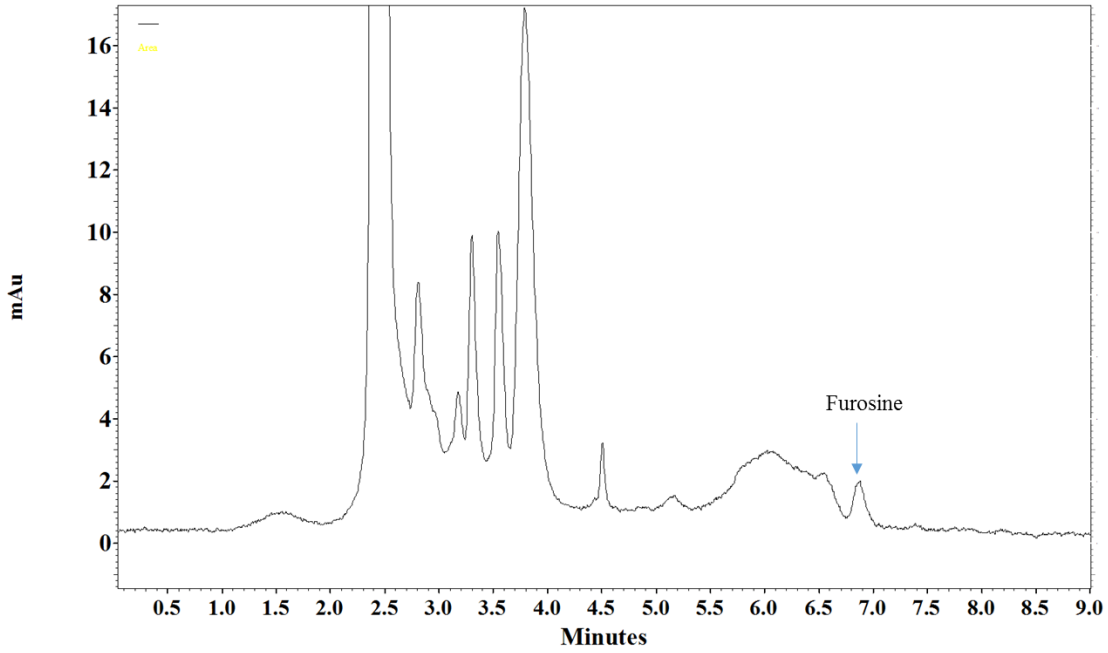


Figure 10. 96 h incubated glycated whey protein sample HPLC chromatogram. Furosine retention time: 6.810 minutes

Furosine peak area from glycated whey protein sample: 19149

Furosine concentration in glycated whey protein sample using standard curve equation:

$$furosine\ concentration\ (ppm) in\ injected\ sample = x = \frac{(y - 6026.8)}{38623}$$

$$= \frac{(19149 - 6026.8)}{38623} = 0.339\ ppm$$

$$0.339\ ppm = 0.339\ \mu g/mL$$

Thus, in the 2.3mL sample solution from which 20 uL was injected onto the column, furosine content was:

$$\frac{0.339\ \mu g}{mL} \times 2.333\ mL = 0.792\ \mu g$$

This 2.333 mL solution was from 0.5 mL of hydrolyzed solution containing 0.02 g sample protein digested in 3.75 mL of HCl. Thus, furosine content in the original 0.02 g protein is:

$$\frac{0.792\ \mu g\ furosine}{1} \times \frac{1}{0.5\ mL\ hydrolyzed\ solution} \times \frac{3.75\ mL\ HCl}{1} = 5.94\ \mu g\ furosine$$

Equation obtained from furosine standard curve:

$$y = 38623x + 6026.8$$

The molecular weight of furosine is 254. The conversion factor of furosine to Amadori compound to furosine is about 0.434 (Krause et al. 2003). Thus, the amount of amadori compound produced per 1 g of glycated protein is:

$$\frac{5.94\ \mu g\ furosine}{0.02\ g\ sample\ protein} \times \frac{M\ furosine}{254\ g\ furosine} \times \frac{1\ amadori\ compound}{0.434\ furosine\ compound} \times$$

$$\frac{1\ g}{10^6 \mu g} = 2.69\ \mu M\ amadori\ compound\ in\ 1\ g\ glycated\ protein$$

According to the manufacturer, whey protein (the sample protein) is comprised of 7.6% of lysine. Molecular weight of lysine is 146.19. Since formation of amadori compound coinsides with lysine blockage, the lysine blockage in the sample protein can be calculated as:

$\%\ Lysine\ Blockage\ (molar\ base)$=

$$\frac{2.69\ \mu M\ amadori\ compound}{1\ g\ sample\ protein} \times \frac{100\ g\ sample\ protein}{7.6\ g\ lysine} \times \frac{146.19\ g\ lysine}{M\ lysine} \times \frac{1\ mM}{1000\ \mu M} \times \frac{1\ M}{1000\ mM} = 0.52\%\ lsyine\ blocked$$

Appendix E: Analysis of Variance (ANOVA) Tables for Determining Significant Effects of Treatments

Table 5. Analysis of variance on the effect of incubation time on 304 nm absorbance of non-dialyzed WPI incubated with (1:4 ratio) or without maltodextrin.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
WPI incubated with maltodextrin (1:4 ratio) at 0.49a_w, 60°C	Incubation Time	5	2.468	49.704	0.000
	Error	12	0.002		
WPI incubated without maltodextrin at 0.49a_w, 60°C	Incubation Time	5	0.032	68.119	0.000
	Error	12	0.000		

Table 6. Analysis of variance on the effect of incubation time on 420 nm absorbance of non-dialyzed WPI incubated with (1:4 ratio) or without maltodextrin.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
WPI incubated with maltodextrin (1:4 ratio) at 0.49a_w, 60°C	Incubation Time	5	0.000	9.592	0.002
	Error	9	1.433E-5		
WPI incubated without maltodextrin at 0.49a_w, 60°C	Incubation Time	5	8.542E-5	2.680	0.087
	Error	10	3.187E-5		

Table 7. Analysis of variance on the effect of incubation time on % free amino groups remaining of non-dialyzed WPI incubated with (1:4 ratio) or without maltodextrin.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
WPI incubated with maltodextrin (1:4 ratio) at 0.49a_w, 60°C	Incubation Time	5	1036.073	262.992	0.000
	Error	12	3.940		
WPI incubated without maltodextrin at 0.49a_w, 60°C	Incubation Time	5	357.299	34.354	0.000
	Error	12	10.401		

Table 8. Analysis of variance on the effect of incubation time on 304 nm absorbance of dialyzed WPI incubated with (1:4 and 2:1 ratio) or without maltodextrin.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
WPI incubated without matltodextrin at 0.49a_w, 60°C	Incubation Time	5	0.002	228.781	0.000
	Error	6	7.167E-6		
WPH incubated with matldoextrin (1:4 ratio) at 0.49a_w, 60°C	Incubation Time	5	0.023	282.768	0.000
	Error	12	8.078E-5		
WPH incubated with matldoextrin (2:1) at 0.49a_w, 60°C	Incubation Time	5	0.010	128.774	0.000
	Error	12	8.111E-5		

Table 9. Analysis of variance on the effect of incubation time on 420 nm absorbance of dialyzed WPI incubated with (1:4 and 2:1 ratio) or without maltodextrin.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
WPI incubated without matltodextrin at 0.49a_w, 60°C	Incubation Time	5	2.385E-5	1.472	0.298
	Error	8	1.621E-5		
WPH incubated with matldoextrin (1:4 ratio) at 0.49a_w, 60°C	Incubation Time	5	2.302E-5	9.098	0.001
	Error	11	2.53E-6		
WPH incubated with matldoextrin (2:1 ratio) at 0.49a_w, 60°C	Incubation Time	5	5.089E-5	45.800	0.000
	Error	12	1.111E-6		

Table 10. Analysis of variance on the effect of incubation time on % free amino groups remaining of dialyzed WPI incubated with (1:4 and 2:1 ratio) or without maltodextrin.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
WPI incubated without matltodextrin at 0.49a_w, 60°C	Incubation Time	5	12.848	2.284	0.172
	Error	6	5.625		
WPH incubated with matldoextrin (1:4) at 0.49a_w, 60°C	Incubation Time	5	316.841	35.205	0.000
	Error	12	9.000		
WPH incubated with matldoextrin (2:1) at 0.49a_w, 60°C	Incubation Time	5	91.948	104.937	0.000
	Error	12	0.876		

Table 11. Analysis of variance on the effect of pH on % solubility of different non-heated WPI samples (ion-exchange produced WPI, membrane-produced WPI, HIC-separated WPI, and PGWP)

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
pH 3.4	Sample Type	3	4.0554	0.866	0.497
	Error	8	46.817		
pH 4.5	Sample Type	3	220.316	30.381	0.000
	Error	8	7.252		
pH 7	Sample Type	3	4.084	0.568	0.651
	Error	8	7.188		

Table 12. Analysis of variance on the effect of pH on % solubility of different heated (80°C, 30 min) WPI samples (ion-exchange produced WPI, membrane-produced WPI, HIC-separated WPI, and PGWP)

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
pH 3.4	Sample Type	3	193.180	15.850	0.001
	Error	8	12.188		
pH 4.5	Sample Type	3	5072.894	1343.111	0.000
	Error	8	3.777		
pH 7	Sample Type	3	1193.123	170.667	0.000
	Error	8	6.991		

Table 13. Analysis of variance on the effect of heat treatment (none or 80°C 30 min) on % solubility of ion-exchange WPI at different pHs.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
pH 3.4	Heating Time	1	60.968	0.743	0.437
	Error	4	82.071		
pH 4.5	Heating Time	1	8447.058	2596.707	0.000
	Error	4	3.253		
pH 7	Heating Time	1	0.245	0.068	0.807
	Error	4	3.589		

Table 14. Analysis of variance on the effect of heat treatment (80°C, 30 min) on % solubility of membrane-produced WPI at different pHs.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
pH 3.4	Heating Time	1	52.003	2.247	0.208
	Error	4	23.145		
pH 4.5	Heating Time	1	4701.717	1257.586	0.000
	Error	4	3.739		
pH 7	Heating Time	1	1300.706	1172.075	0.000
	Error	4	1.110		

Table 15. Analysis of variance on the effect of heat treatment (80°C, 30 min) on % solubility of HIC-separated WPI at different pHs.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
pH 3.4	Heating Time	1	335.092	35.874	0.004
	Error	4	9.341		
pH 4.5	Heating Time	1	132.284	16.221	0.016
	Error	4	8.155		
pH 7	Heating Time	1	2770.282	384.622	0.000
	Error	4	7.946		

Table 16. Analysis of variance on the effect of heat treatment (80°C, 30 min) on % solubility of PGWP at different pHs.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
pH 3.4	Heating Time	1	226.972	65.736	0.001
	Error	4	3.453		
pH 4.5	Incubation Time	1	77.325	11.189	0.029
	Error	4	6.911		
pH 7	Incubation Time	1	292.925	18.641	0.012
	Error	4	15.714		

Table 17. Analysis of variance of sample type (membrane-produced WPI, HIC-separated WPI, and PGWP) on emulsification stability and capacity.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
Emulsification Stability	Sample Type	2	569.606	40.114	0.000
	Error	6	14.200		
Emulsification Capacity	Sample Type	2	34389.620	79.000	0.000
	Error	6	435.312		

Appendix F: Visible Browning in WPI Samples Incubated with Maltodextrin and WPI Controls as Shown by Absorbance at 420 nm

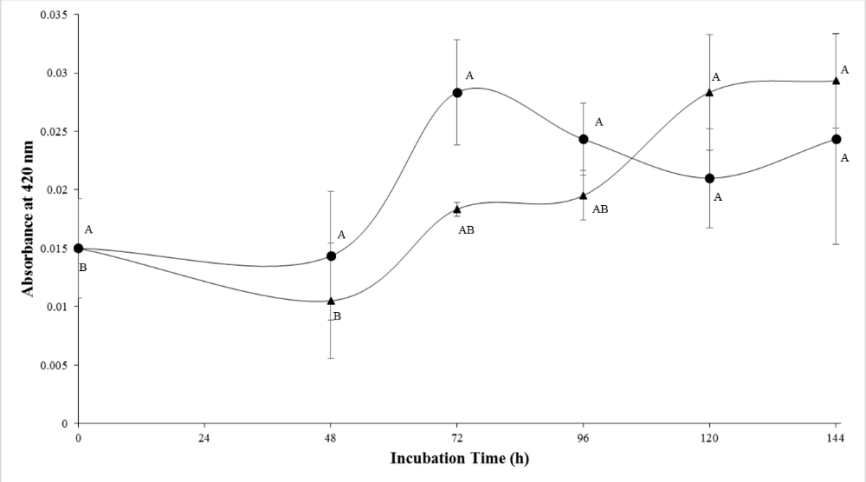


Figure 11. Visible browning in samples of whey protein isolate (WPI) control incubated without maltodextrin (●) and WPI incubated with maltodextrin in a 1:4 ratio (w/w)(▲) at 60°C, 0.49 a_w, for 0-144 h, as determined by UV difference spectroscopy at 420 nm. Error bars represent standard errors (n=3). Different upper case letters above and below the shapes indicate significant differences between different time points within each sample set according to Tukey-Kramer multiple means comparison test ($P \leq 0.05$).

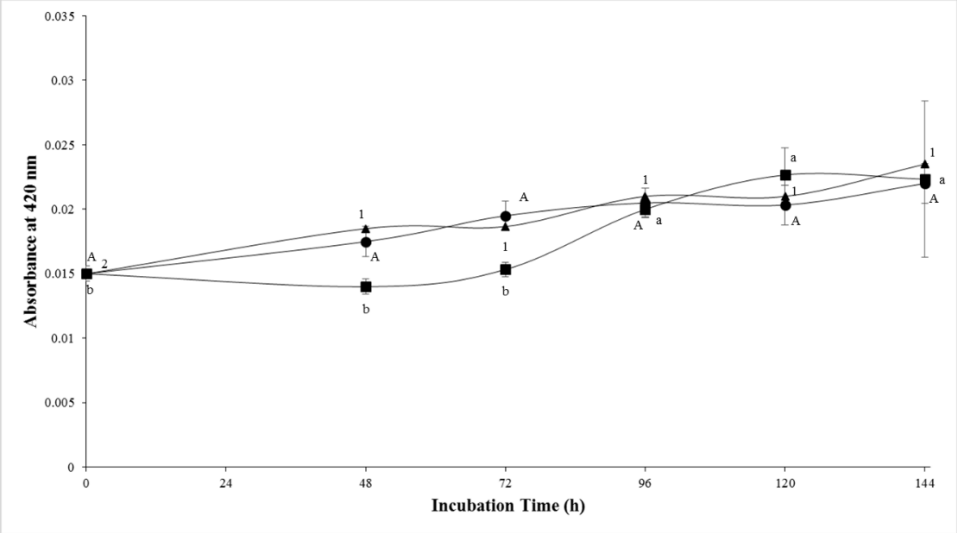


Figure 12. Visible browning in samples of whey protein isolate (WPI) control incubated without maltodextrin (●) WPI incubated with maltodextrin in a 1:4 ratio (w/w) (▲), and WPI incubated with maltodextrin in a 2:1 ratio (w/w) (■) at 60°C, 0.49 a_w, for 0-144 h, as determined by UV-Visible difference spectroscopy at 304 nm. Error bars represent standard errors (n=3). Different upper and lower case letters, and numbers above and below the shapes indicate significant differences between different time points within each sample set according to Tukey-Kramer multiple means comparison test ($P \leq 0.05$).

Appendix G: Image of Pellets Formed in Heat Treated WPI Samples

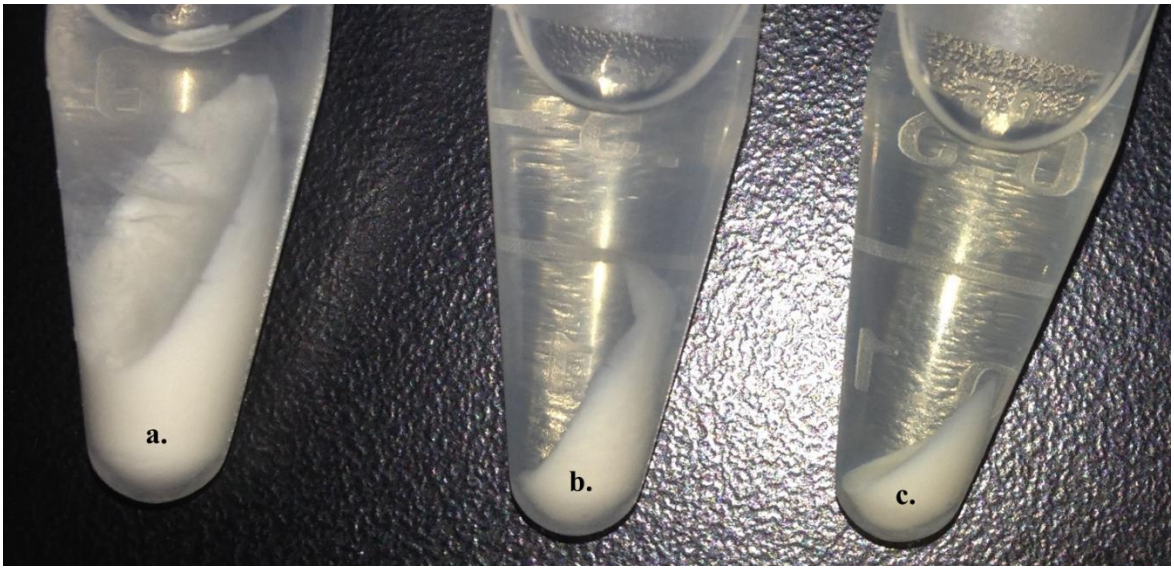


Figure 13. Pellets formed in samples when in a 5% protein solution at pH 4.5 and following heat treatment of 30 min at 80°C. a. membrane-produced WPI b. HIC-separated WPI c. PGWP.