Effect of Maillard-Induced Glycosylation on the Molecular Configuration of Whey Protein and its Solubility, Thermal Stability, and Overall Quality for Beverage Applications

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ABSTRACT

Whey protein has numerous nutritional, physiological and functional benefits. It is therefore a perfect candidate for high protein beverage applications. Whey protein beverages are gaining popularity among athletes, bodybuilders and health conscientious individuals. In spite of the reasonable solubility of whey proteins in acidic beverages (pH 3.4-4.5), thermal processing and prolonged ambient storage can result in protein aggregation via hydrophobic and disulfide molecular interactions between denatured proteins, especially at relatively high protein concentration (>4.2%, w/v). Currently, whey protein acidic beverages available in the market contain at most 4% whey protein. To better deliver the nutritional quality and expand the commercial value, it is desirable to develop shelf stable high whey protein acidic beverages (>4.2% protein). It is also desirable to produce a less acidic beverage, to reduce undesirable sourness and astringency. We hypothesized that glycosylation of whey protein via early stage Maillard reaction results in higher net charge, enhanced thermal stability, increased structural rigidity, and reduced buffering capacity. Partially glycosylated whey protein (PGWP) can thus be used to produce high protein acidic beverages that are shelf stable with acceptable sensory quality.

Therefore, our long term goal was to produce a partially glycosylated whey protein with enhanced solubility and thermal stability over a wide pH range, while maintaining nutritional and sensory quality.

Glycosylation conditions were optimized to promote glycosylation of whey protein, while minimizing browning and maintaining nutritional quality. Unreacted dextran was removed using liquid chromatography, and the protein fractions, both non-reacted protein and glycosylated protein were collected and labeled as PGWP. Extent of glycosylation, browning, % lysine blockage, % loss in free amine, and *in-vitro* digestibility were monitored. Solubility and thermal stability of PGWP were compared to whey protein isolate (WPI), at 5-10% (w/v) protein concentration, pH 3.4, 4.5, 5.5 and/or 7.0, and pre and post thermal treatments. Differential scanning calorimetry (DSC) and SDS-PAGE were employed to monitor onset of denaturation and polymerization, respectively. Exposure of sulfhydryl groups and surface hydrophobicity were monitored during heat

treatment of 5% protein solutions at 75 °C for 60 min. The pI of PGWP was as well. For structural characterization mass spectrometry (MS) analysis and surface enhanced Raman spectroscopy (SERS) were used. The glycosylation site was determined using matrix-assisted laser desorption ionization-time of flight MS (MALDI-TOF MS). Solutions (5% protein, w/v) of WPI and PGWP were analyzed by SERS to determine secondary and tertiary structural changes of the protein as affected by glycosylation, pH and heating. The sourness and astringency of protein acidic solutions/beverages formulated with PGWP and WPI at 5% protein and at different pHs were determined. The storage stability of the formulated solutions/beverages over 6-8 weeks was determined, by measuring solubility, turbidity, and protein aggregation using capillary electrophoresis (EC).

Overall, results proved that the solubility and thermal stability of whey protein, at relative high protein concentrations, can be enhanced over a broad range of pH upon partial and limited Maillard-induced glycosylation. The enhanced solubility and thermal stability of PGWP was attributed to structural rigidity, unique glycosylation sites, resistance to denaturation, and shift to a more acidic pI, all of which contributed to reduced protein/protein interactions. The nutritional quality was maintained and advanced stages of Maillard reaction were not detected. Partial glycosylation of whey protein reduced its buffering capacity, and consequently less amount of acid was required to reach the targeted pH of the protein fortified solution/beverage. The reduced acidity of the PGWP fortified beverages, especially at pH 4, resulted in lower perceived sourness and astringency compared to the WPI fortified beverages. In addition, the solubility and turbidity of PGWP solution prepared at pH 4.5 were maintained fairly well during storage at or below 25 °C.

Our findings demonstrate the possibility of using PGWP in the production of shelf stable high protein acidic beverages (> 4.2% protein) that can be less sour and astringent than what is currently present in the market. Our results also provided information that is essential to understand the structure/function relationship upon Maillard-induced glycosylation of whey proteins. This basic information is useful for directed structural modification research aiming at improving whey protein functionality.

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1. LITERATURE REVIEW

1.1. Introduction and objectives

World-wide an increasing amount of whey protein is available due to increasing cheese production and the development of ultrafiltration, microfiltration and ion-exchange methods to concentrate these proteins. Whey protein is considered a high quality protein since it is rich in all essential amino acids and possesses a number of physiological benefits, including but not limited to antimicrobial activity, growth promotional activity and immunoactivity. Additionally, whey protein has unique physicochemical characteristics, which allow it to have versatile functionality, such as foaming, gelation and emulsification. As compared to other food proteins, whey proteins have great solubility in beverages.

Whey protein beverages are designed to be attractive to a variety of consumers, athletes, bodybuilders and health conscientious individuals. There are two kinds of whey protein beverages available in market, neutral and acidic. Whey protein neutral beverages (pH~6.8) are generally opaque and thus available in chocolate, orange cream, and vanilla flavored beverages. Whey protein acidic beverages (pH<3.5) are relatively clear and available in various fruit flavors. Whey protein acidic beverages are preferred over neutral beverages since beverages with pH < 4.6 require mild heat treatments, thus maintaining better sensory quality and shelf-life stability. Currently, whey protein acidic beverages available in the market contain at most 4 % whey protein. To better deliver the nutritional quality and exploit the commercial value of whey protein beverages, it is desirable to develop high whey protein acidic beverages. FDA requires a protein concentration of at least 4.2 % to make a label claim of a “high protein beverage”. However, maintaining clarity at high protein concentration (>4.2%) in processed beverages (pH < 3.5) over an extended period of time, remains a challenge.

Being globular in structure, whey proteins are relatively heat labile. Denaturation can occur at temperatures as low as 60 ºC. Upon denaturation, hydrophobic and sulfhydryl groups, originally buried inside the protein globular structure, are exposed. These

exposed groups participate in hydrophobic interactions, inter- and intramolecular disulfide linkages, respectively, resulting in protein polymerization, and possibly precipitation when protein percent is relatively high. The newly formed intermolecular disulfide linkages are especially important for heat-induced aggregation, since the formation of disulfide linkages is irreversible. Protein/protein interaction builds up post heat treatment, especially during extended storage, resulting in protein precipitation and loss of clarity, thus reducing the acceptability. Therefore, it is essential to investigate ways to delay onset of whey protein denaturation, reduce disulfide interchange, and decrease intermolecular attractions.

Additionally, a significant sensory challenge is confronted when formulating high whey protein acidic beverages. Since proteins are excellent buffers (especially at pH 3.5-4.5), a considerable amount of acid has to be added to reach the desirable pH (~3.5) as protein % increases. The high acidity will result in excessive sourness and astringency, thus lowering the overall acceptability. Hence, it is important to investigate ways to improve flavor quality of acidic beverages.

Researchers have improved whey protein functionality by using limited enzymatic hydrolysis. Enhanced solubility of whey protein hydrolysate over a wide pH range (pH 3-10) have been reported, and was mostly attributed to the reduced molecular weight and the increased hydrophilicity resulting from the increase in free carboxyl and amine groups. However, complication of thermal stability remains since disulfide linkages can still take place upon denaturation, resulting in protein polymerization and precipitation. Moreover, the freed carboxyl and amine groups cause an increase in the buffering capacity of the proteins.

The Maillard reaction, however, provides a promising alternative targeting the mentioned issues. It is a spontaneous reaction, naturally occurring between amino groups of a protein and the carbonyl groups of a reducing sugar. A variety of functional enhancement upon glycosylation of food proteins under controlled conditions has been reported. Producing Maillard glycoconjugates has to be conducted under controlled conditions to limit propagation to advanced stages with undesirable outcomes, such as browning, development of off-flavor, and production of mutagenic compounds.

Additionally, the digestibility and nutritional quality of the glycosylated protein have to be monitored.

Although there are several attempts made to improve protein functionality via the Maillard reaction, most of them focused on improving protein emulsifying or gelation properties, while few addressed solubility and thermal stability. Moreover, the research on whey protein solubility and thermal stability has shown significant drawbacks limiting the industrial application in high protein acidic beverages. Enhancement of functionality was monitored using isolated conjugated protein fraction, which is not industrially applicable, and the concentration (0.1 %) tested was much lower than what is desired. In addition, and to the best of our knowledge, none of the studies has investigated the shelf-life stability and sensory qualities of the glycosylated whey proteins. Last but not least, structural characterizing of the glycosylated proteins, necessary to understand the improvement of solubility and thermal stability via Maillard reaction, is lacking.

Therefore, our long term goal is to produce partially glycosylated whey proteins with enhanced solubility and thermal stability over a wide pH range, while maintaining nutritional quality. The produced glycosylated whey protein can be used to formulate high protein acidic beverages with improved shelf-life stability and sensory acceptability. To achieve this long term goal, the following hypotheses and supporting objectives were proposed:

It is hypothesized that blockage of protein amine groups by carbonyl groups of reducing sugar and formation of an Amadori compound will result in higher net negative charge, thus cause repulsion between proteins. Additionally, the molecular hydrophilicity of the modified whey proteins will increase due to the attachment of reducing sugars. The increased electrostatic repulsion and hydrophilicity will reduce the chances of protein-protein interactions and formations of intermolecular disulfide linkages, and will increase the protein-water interaction. Additionally, with the increased net charge the protein will resist denaturation, thus enhancing protein solubility and thermal stability. Moreover, the blockage of some amine groups by carbonyl groups of the reducing sugar will lower the protein buffering capacity. At lower buffering capacity, less acid is needed to reach a desirable pH, hence, resulting in a reduction of perceived sourness and astringency.

Objective 1) Determine the effect of controlled and limited Maillard-induced glycosylation on the nutritional quality, solubility, thermal stability and molecular configuration of whey protein.

Objective 2) Characterize the protein structure of PGWP as influenced by pH and heat using surface-enhanced Raman spectroscopy (SERS).

Objective 3) Monitor the sourness and astringency of whey protein (5% protein, w/v) acidic beverages formulated with PGWP at pH 3.4 and 4.0 in comparison to a beverage formulated with whey protein isolate (WPI) at pH 3.4.

Objective 4) Monitor the shelf life stability of whey protein (5% protein, w/v) solutions/beverages prepared using PGWP at acidic pH (3.4 and 4.5) and/or neutral pH (~7.0) compared to those prepared using WPI at pH 3.4 and/or 7.0.

1.2. Significance of whey protein

Whey, which is a by-product of cheese making and casein production, is produced in large amounts in the US, specifically in 2009 over 1.4 billion pounds of whey was produced (Fennem, 1976). This creates waste disposal problem. To solve this problem and to continuously benefit the dairy industry, researchers put intensified efforts to find value-added uses for whey. Whey fluid obtained from cheese or casein production contains approximately 93% water and 0.6-0.9% of protein; the remaining components are mainly lactose (4.5-5.1%), minerals (0.5-0.7%) and lipids (0.1-0.3%) (Foegeding, Davis, Doucet, & McGuffey, 2002). Modern filtration technology has improved dramatically in the past decade, allowing companies to selectively separate and concentrate whey protein to 35-93% purity. Because of its nutritional, physiological, and functional properties, whey protein has been used in many food applications, such as protein bar, protein beverage, meat products, bakery goods as well as ice-cream. Whey protein is usually included as the protein source or food supplement in the diets for athletes, bodybuilders, weight control and immune-compromised populations, such as people with protein allergy, asthma, high cholesterol, and obesity.

1.2.1. Whey protein composition

The protein content of whey represents about 20% of the total milk protein, and is commonly referred to as whey protein or serum protein (Fennem, 1976).Whey protein is composed mainly of β-lactoglobulin (β-lg), α- lactalbumin (α-la), serum albumin, lacto-transferrin and immuno-globulin (Fox & McSweeney, 1998). Among them, β-lactoglobulin represents more than 50% of the total whey protein, and α- lactalbumin represents about 20%. Based on the production process used, acid or rennet whey can be produced, which have different protein fractions. Acid whey contains some γ-casein and proteose-peptones, derived from casein, while rennet whey contains glyco-macropeptides produced upon hydrolysis of κ-casein with rennet enzyme (Fox & McSweeney, 1998).

Since β-lg and α-la are the major constitutes of whey protein and contribute the most to whey protein functionality, their composition and structure will be detailed below.

1.2.1.1. β-lactoglobulin

β-lactoglobulin is a globular protein (Fig. 1), composed of 162 amino acids, with molecular weight of 18,362 Da for genetic variant A and 18,276 Da for genetic variant B. Genetic variants A and B differ in the substitution of a glycine in Variant B for an aspartic acid in Variant A. The secondary structure of β-lg constitutes of 15% α-helix, 50% β-sheet, and 15-20% reverse turn (Wong, Camirand, & Pavlath, 1996). The tertiary structure of β-lg constitutes of a β-barrel structural motif and a single short α-helix. The center of the β-barrel forms a hydrophobic pocket. A hydrophobic pocket also exists on the surface. One β-lg molecule has five cysteine residues; four of which form disulfide bonds (Cys106–Cys119 and Cys66–Cys160), and one is a free thiol group, located at position 121 within the interior hydrophobic pocket (Sava, Van der Plancken, Claeys, & Hendrickx, 2005). This free thiol group is not involved in disulfide linkage, unless the protein is denaturated. The functionality of β-lg is mostly governed by the hydrophobic pockets, the two disulfide bonds and the buried sulfhydryl group (Fennem, 1976).

The intermolecular association of β-lg molecules is governed by the pH of the system. β-lactoglobulin has an isoelectric point at ~ pH 5.4. Under acidic conditions (pH < 3.5), β-lg molecules are very stable as monomers, resisting denaturation due to the excessive net negative charges that cause electrostatic repulsion. Between pH 3.5 and 5.2, close to the isoelectric point, the monomers aggregate into octamers because of the minimized electrostatic repulsion (Fennem, 1976). At neutral pH, β-lg forms dimers. Above pH 7.5, the dimers dissociates to monomers again, as a result of the electrostatic repulsion induced by net positive charges.

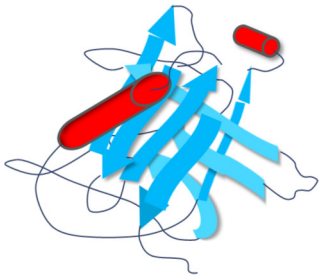

Figure 1. β-lactoglobulin structure.

1.2.1.2. α-lactalbumin

α-lactalbumin is a globular protein (Fig. 2), composed of 123 amino acids, with a molecular weight of 14,147 Da and 14,175 Da for genetic variant A and B, respectively. Genetic Variants A and B differ in the substitution of an Arg in Variant B for a Gln in Variant A. The secondary structure of α-la constitutes of four α-helices, and an antiparallel β-sheet (Fox & McSweeney, 1998). The three-dimensional structure of α-la is nearly spherical. One α-la has four disulfide linkages and no free cycteine.

α-lactalbumin has the lowest denaturation temperature (62 °C) of the whey proteins, but the denaturation is mostly reversible (Chaplin & Lyster, 1986). A unique characteristic of α-la is its high affinity to calcium and some other metal ions, including magnesium, sodium and potassium. This binding of calcium stabilizes the protein against thermal denaturation (Wong, Camirand, & Pavlath, 1996) and may result in bactericidal or antitumor activity. Additionally, the ability to bind calcium enhances the thermally-induced gelation property of the protein (Permyakov, Yarmolenko, Kalinichenko, Morozova, & Burstein, 1981).

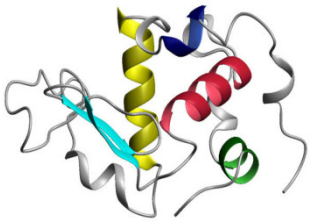

Figure 2. α-lactalbumin structure. http://www.acsu.buffalo.edu/~andersh/research

1.2.2. Nutritional value of whey protein

Whey protein is considered a high quality protein for several reasons. Whey protein is a complete protein rich in all the essential amino acids, and has a biological value that is 15% greater than egg protein. Therefore, it has been used as a protein supplement in several food products, such as baby formula.

Whey protein is also the richest known source of the naturally occurring branched chain amino acids (leucine, isoleucine and valine). Branched chain amino acids are important for active individuals and professional athletes. The body requires higher amounts of branched chain amino acids during and following an exercise. Branched chain amino acids are taken up directly by the skeletal muscles instead of having to be metabolized first through the liver, as in the case of other amino acids. In particular, leucine plays a critical role in initiating the transcription of protein synthesis (Fujita et al., 2007). When leucine is ingested in high amounts, a greater stimulation of protein synthesis occurs, which may speed up recovery and adaption to stress (Ha & Zemel, 2003). Thus, many athletes consume whey protein beverages, both before and immediately after an exercise, to help repair and rebuild lean muscle tissues.

Additionally, whey protein is rich in sulfur-containing amino acids, methionine and cycteine, which are involved in glutathione synthesis. Glutathione serves as an antioxidant that defends the body against free radical damage and neutralizes toxins (Shoveller, Stoll, Ball, & Burrin, 2005).

1.2.3. Physiological properties

The physiological properties of whey protein have been well documented. Whey protein is regarded as a natural food with antimicrobial activity, growth promotional activity and immunoactivity (Smithers, 2008; Kitts & Weiler, 2003). These biological functions are attributed to the naturally present peptides, namely lactoferrin, growth factors and immunoglobulins.

1.2.3.1. Lactoferrin

Lactoferrin, a glycoprotein that binds iron, has a wide variety of uses in biological systems and is considered a first line immune defense in the human body (Nikolaev & Anshakova, 1985). Lactoferrin is generally associated with anti-bacterial and anti-viral activity and immune modulation when in non-denatured form (Berkhout, Van Wamel, Belijaars, Meijer, Visser, & Floris, 2002). Lactoferrin is especially important to the health of the intestinal tract, and has been found to reduce systemic and intestinal inflammation. It enhances the growth of beneficial microflora, such as bifidus, and suppresses the growth of harmful bacteria, such as *E.coli, streptococcus* and *clostridium*. Lactoferrin inhibits viruses by binding to viral receptor sites without cytopathic effects on healthy cells, thus it may potentially combat human immunodeficiency virus.

Another promising property of lactoferrin is its potential as a cancer prevention and treatment agent. Extensive *in-vitro* and *in-vivo* studies have illustrated the anti-cancer effect of lactoferrin against colon, esophagus, lung, and bladder cancer (Tsuda, Sekine, Fujita, & Ligo, 2002).

Additionally, lactoferrin can raise glutathione (GSH) levels in cells. Glutathione is one of the most important antioxidant found in the body essential to the immune system. Lactoferrin itself can also act as an antioxidant by scavenging free iron, thus preventing uncontrolled iron-based free radical reactions, ultimately protecting cells from peroxidation (Togawa et al., 2002; Weinberg, 2001).

1.2.3.2. Growth factors

Whey protein is reported to contain a group of peptides including insulin-like platelet-derived growth factor, fibroblast growth factor, and transforming growth factor. Using animal models, several researchers confirmed the effects of these growth factors on simulating the growth and proliferation of mammalian cell lines (Smithers, 2004).

1.2.3.3. Immunoglobulins

Immunoglobulins (IgG) make up approximately 10-15% of the whey protein. Immunoglobulins provide protection against bacteria, viruses and fungi by agglutination, immobilization and neutralization of their toxins (Berkman, Lee, & Gale, 1988). Immunoglobulins also play an important role in the immune system by taking part in the processes of antibody dependent cell-mediated cytotoxicity and intracellular antibody-mediated proteolysis.

1.3. Whey protein ingredients

The annual production of whey protein is roughly 600,000 metric tons (Fox & McSweeney, 1998). The development of membrane filtration technologies, such as ultrafiltration (UF) and diafiltration (DF), as well as ion-exchange chromatography (IEC), resulted in the development of whey protein ingredients with high protein concentration and various functionalities (Ju & Kilara, 1998).

1.3.1. Whey protein concentrate (WPC) and whey proteins isolate (WPI)

Whey protein concentrate (WPC), which contains 35-80% protein, is usually produced by membrane filtration techniques. The remaining components are mainly lactose (4-47%), fat (< 10%) and minerals (< 8 %). Whey protein isolate (WPI), which contains 90-96% of protein, can be produced using membrane techniques, including cross-flow microfiltration, UF, microfiltration (MF), reverse osmosis (RO), dynamic membrane filtration (DMF), electro-ultrafiltration (EU), and nanofiltration (NF); or by chromatography techniques, such as IEC and radial flow chromatography (RFC) (El-salam, 2009). Compared to WPC, WPI has a cleaner flavor and pronounced functionality, thus is preferred in many food applications.

The components and denaturation state of the whey protein ingredients produced using different technologies are not exactly the same. Compared to WPI produced using membrane filtration techniques, WPI produced using IEC contains less casein glycol-macropeptide, immunoglobulin, lactoferrin, α-lactobumin, but more β-lactoglobulin.

Membrane-produced WPI has variable amounts of glycol-macropeptides with molecular weights from 2 to 10 kDa, and a small amount of peptides with molecular weight less than 2 kDa (Marcelo & Rizvi, 2009). Moreover, the protein components of IEC-produced WPI are more denatured than their counterparts in the membrane-produced WPI (Morr & Ha, 1993). On the other hand, IEC- produced WPI contains a lesser amount of calcium, and extremely low amount of lactose (~0.8%) and fat (~0.5%). This difference in protein content, denaturation state, lactose and mineral content among the whey protein ingredients contributes to different functional behavior in various food applications.

1.3.2. Whey protein hydrolysate (WPH)

To enhance the functionality and to explore more applications of whey protein, whey protein hydrolysate (WPH) was introduced to the market. Whey protein hydrolysate is produced by enzymatic or chemical hydrolysis. However, enzymatic hydrolysis is preferred since chemical hydrolysis may result in loss of essential amino acids, such as tryptophan (Mahmoud, 1994). Additionally, enzymatic hydrolysis is preferred due to the availability of a wide range of enzymes that are considered safe (GRAS) and natural. The enzymes employed to produce WPH include digestive enzymes, such as trypsin, pepsin and chymotrypsin; plant enzymes, such as papain and bromelain; and bacterial proteases, mainly from *Bacillus licheniformis* and *Bacillus subtilis*. The functional and biological properties of the WPH depend to a great extent on the type of enzyme used (specificity and selectivity), hydrolysis conditions employed (enzyme-to-substrate ratio, incubation temperature, pH and time), and the source of the protein, i.e. native vs. denatured, WPI vs. WPC, membrane or IEC product, etc. (Herceg, Lelas, Rezek, & Kresic, 2005).

Compared to WPI and WPC, WPH is considered as a superior alternative, for several reasons, in various food applications, as well as in nutraceutical and therapeutic applications (Potier & Tome, 2008). One reason is that WPH has improved digestibility. Being globular in nature, β-lg and α-la can resist digestion. A portion of the population experiences digestive issues following consumption of whey protein. These symptoms

include gas, bloating, cramps, and irritability (Does 100% whey protein make you have bad gas? Livestrong.com). WPH-based products can be especially beneficial for patients suffering from a variety of gastrointestinal malabsorption syndromes (Brinson et al., 1989), and/or specific metabolic disorders (De Freitas et al., 1993).

Whey protein hydrolysate also has reduced allergenicity. β-lactoglobulin allergy is a hindrance for expanding the use of whey protein as a food ingredient. Enzymatic hydrolysis of whey protein can result in breaking down sequences that trigger an allergic reaction. For instance, WPH can be used in infant formulas intended for infants suffering from cow's milk allergy (Walker-Smith et al., 1989).

Upon enzymatic hydrolysis, a number of bioactive peptides can be released. These peptides, which usually have a molecular weight less than 6 kDa, are inactive within a sequence until they are released by enzymatic hydrolysis. Bioactive peptides contribute to several health benefits, including but not limited to antihypertensive, antioxidant, anticancerous, antibacterial, and mineral binding activity (Fields, Falla, Rodan, & Bush, 2009). The presence of bioactive peptides increases the nutritional and physiological value of WPH.

Additionally, functionality of whey protein can be improved via limited hydrolysis, which imparts changes in the molecular size, conformation, and strength of inter- and intramolecular interactions (Guan, 2007). By reducing the molecular size, solubility is improved compared to WPI over a wide pH range (Perea, Ugalde, Rodriguez, & Serrat, 1993). Thermal stability is enhanced upon partial hydrolysis due to the loss of secondary structure, thus reducing heat-induced structural changes (Foegeding, Davis, Doucet, & McGuffey, 2002). Additionally, upon controlled hydrolysis, gelation, foaming and emulsification properties are improved due to the enhanced balance of the hydrophobic to hydrophilic distribution (Scherze & Muschiolik, 2001; Perea, Ugalde, Rodriguez, & Serrat, 1993).

1.4. Whey Protein Functionality

Whey protein has versatile functionality that is valued by food manufacturers. Some of the special functional aspects of WPI are gelling, water binding, viscosity, emulsification, whipping/foaming, and solubility (Beuschel, Culbertson, Partridge, & Smith, 1992). Whey proteins contain both hydrophobic and hydrophilic regions that facilitate good surface activity after denaturation. Therefore, whey protein can be used as a foaming or emulsifying agent in many food products including dairy products, baked goods, candies, snack foods, and processed meats (Whey Production, Fas.usda.gov). Because whey protein forms heat-induced gels, they can also be used in meat products to improve the water holding capacity (Akoh, 1998). Additionally, whey protein can form cold-set gels, thus is used in salad dressings and mayonnaise (Beuschel, Culbertson, Partridge, & Smith, 1992). Compared to other food proteins, whey protein has high solubility, especially under acidic conditions, therefore, is used in protein fortified acidic beverages (Chobert, Bertrand-Harb, & Nicolas, 1988). Solubility and thermal stability of whey protein are the focus of this research; therefore, they will be discussed in more detail in the following sections.

1.4.1. Solubility

Solubility can be defined as an operational parameter that refers to the retention of protein in the supernatant after centrifugation for a certain period of time under a specific gravitational force (Morrisey, Mulvihill, & O'Neill, 1987). Solubility is mostly governed by the balance between surface hydrophobic interactions (protein-protein) and hydrophilic interactions (protein-water). The surface hydrophilicity of whey protein, including its surface net charge, is critical for its solubility.

Solubility is a function of intrinsic factors, such as the source of the protein and protein composition, and extrinsic factors, such as pH, ionic strength, and temperature. Among the extrinsic factors, pH is the most important factor, as it significantly affects the nature and distribution of charges on the surface of the protein (Pelegrine & Gasparetto, 2005). Whey protein has the lowest solubility around its isoelectric point (pI, pH of 4.5-5.2) and is more soluble as pH decreases or increases (Phillips, Whitehead, & Kinsella,

1994). Being globular in nature, the hydrophobic groups of the protein are mostly buried within the molecule, with one hydrophobic region on the surface (Fox & Mcsweeney, 1998). At the pI of whey protein, the net charge on the molecular surface is zero, thus the repulsion between molecules is at a minimum (Fennem, 1976). Therefore, some hydrophobic interactions between the surface hydrophobic regions of the protein molecules occur, reducing the whey protein solubility. However, because of the dominant hydrophilic interactions between the hydrophilic groups and water, the whey protein still has relatively good solubility at its pI. Jimènez-Castaño et al. (2007) reported that at protein concentration of 1 mg/mL, β-lg has 80-85% solubility at pH 5.0. When pH increases or decreases, whey protein has an excess of net negative or positive charges, respectively, resulting in strong electrostatic repulsion between molecules (Fox & McSweeney, 1998). Additionally, with more charged groups, more water interacts with the protein, thus, the whey protein stays in solution, maintaining high solubility. At extreme pH, whey protein starts to unfold and expose the hydrophobic groups (Pelegrine & Gasparetto, 2005), resulting in aggregation mostly due to hydrophobic interactions.

Ionic strength influences whey protein solubility by having either a salt-in or salt-out effect. Protein solubility increases at low ionic strength, which is a salt-in effect. Whey protein solubility tends to decrease at pI, however, adding a small amount of salt with pairing ions will shield some of the charges around the protein, thus reducing protein-protein interaction and increasing the solubility (Kehoe & Foegeding, 2001). De Wit et al. (1996) found that adjusting the ionic strength to 0.1 M using NaCl, prevented the reduction of whey protein solubility between pH 4.5 and 5.5. On the other hand, addition of a relatively large amount of neutral salt, such as ammonium sulphate, compresses the solvation layer and increases protein-protein interactions (Deak et al., 2006). As the salt concentration of a solution is increased, more of the bulk water becomes associated with the ions. As a result, less water is available to partake in the solvation layer around the whey protein. Proteins may then exhibit hydrophobic interactions, aggregate and precipitate from the solution (De Witt & Kessel, 1996).

The change in solubility as affected by temperature and subsequent denaturation is a function of the protein's thermal stability, which will be discussed in the following section.

1.4.2. Thermal stability

As globular proteins, β-lg and α-la are more heat labile than open structure proteins, such as casein. During thermal treatment, globular proteins will undergo structural unfolding, exposing their hydrophobic regions to the surface, thus initiating intermolecular hydrophobic interactions. The initial denaturation stage starts with reversible conformational unfolding (Wong, Camirand, & Pavlath, 1996) as a result of the breakage of some non-covalent interactions, such as hydrogen bonding, hydrophobic and electrostatic interactions. The buried hydrophobic groups and reactive sulfhydryl groups become exposed. The reactivity of the sulfhydryl groups increases after exposure. Hydrophobic interactions coupled with disulfide interchange start to take place. The newly formed disulfide linkages play an important role in heat-induced aggregation since it is an irreversible process (Sava, Van der Plancken, Claeys, & Hendrickx, 2005). Further unfolding in combination with intermolecular interactions contributes to protein aggregation and precipitation, thus greatly reducing protein solubility (Wong, Camirand, & Pavlath, 1996).

Heating temperature and time affect whey protein denaturation. When heated between 40 °C to 50 °C, the solubility of whey protein increases. Increasing temperature in general promotes protein-water interaction, thus enhancing solubility of the protein in solution. At higher temperatures and longer heating time, whey protein solubility decreases due to the heat-induced denaturation (Sava, Van der Plancken, Claeys, & Hendrickx, 2005). Heating temperature and time also influence the reversibility of whey protein denaturation. When whey protein is heated at low temperature (< 65 °C) for a few seconds, reversible dissociation of oligomers into monomeric species occurs. And a portion of non-covalent chemical bonds stabilizing secondary and tertiary structures get interrupted without drastic conformational change. When the whey protein sample is

cooled down, these broken bonds will re-form, and the denaturation is considered reversible. However, under harsh conditions with high temperature (> 70 °C) for more than 30 min, the secondary and tertiary structure of whey protein are completed disrupted, and intermolecular covalent bonds, such as disulfide linkages, start forming, resulting in irreversible denaturation (Thompson, Boland, & Singh, 2009).

Thermal stability is remarkably affected by protein concentration. Iametti et al. (Imetti, Cairolo, Gregori, & Bonomi, 1995) investigated the relationship of structural changes of β-lg as affected by protein concentration. They showed that the protein association dominated by hydrophobic interactions was highly dependent on concentration. Formation of multimeric species was promoted by increasing protein concentration. More importantly, the formation of intermolecular disulfide linkages, under typical industrial pasteurization processes (70 °C or above), was also promoted at high protein concentration (Iametti, Cairoli, De Gregori, & Bonomi, 1995).

Similar to solubility, thermal stability of β-lg also depends on pH. At neutral pH of ~6.7, β-lg starts to denature at temperatures above 65 °C (Mulvihill & Donovan, 1987). Thermal stability of whey protein increases as pH decreases. At pH 2.5, for instance, denaturation of β-lg occurs at 75 °C. At low pH, whey protein resist denaturation because of the strong stabilizing action of the increased surface net negative charges (Iametti et al., 1995). Whey protein, however, is most heat sensitive near pH 4.0, close to its isoelectric point.

Ionic strength also influences the thermal behavior of β-lg. At high ionic strength, lower β-lg concentration is required to form aggregate under neutral pH (Nicolai, Britten, & Schmitt, 2011). Baussay et al. (Baussay, Bon, Nicolai, Durand, & Busnel, 2004) found that the concentration of β-lg to form thermally-induced aggregates decreased from 100 g/L without added salt to 1 g/L at 0.4 M NaCl concentration.

Due to the relatively good solubility and thermal stability of whey protein at acidic pH (< 3.5), and because of its excellent nutritional and physiological properties, whey protein is an outstanding candidate for protein acidic beverage applications.

1.5. Whey protein acidic beverages

1.5.1. Significance

The United States beverage market is very large and still growing. The major nonalcoholic beverages include carbonated soft drinks ($63 billion/year), juice drinks ($20 billion/year), functional beverages such as energy drinks ($11 billion/year), sports drinks ($4 billion/year), and vitamin water ($8 billion/year). Mostly, the above beverages contain no protein and are of low nutrient density. Soft drinks, such as acidic soda, have a pH of 2.5, and the acidity has to be balanced with sugar. Increasing consumption of these high carbohydrate drinks such as soda and juice can be a reason of the increase in body fat, blood lipids content, insulin resistance (Type 2 diabetes) and appetite (Malik, Popkin, Bray, Després, & Hu, 2010). Additionally, the high degree of acidity of these beverages can cause dental decay.

One alternative is to develop a drink with high protein content and low sugar content with a slightly higher pH than the current soft drinks. Increasing the pH requires a less amount of sugar to balance the sourness. Calories from protein can be compensated by the reduced amount of sugar. Thus, a beverage having a pH between 3.4 and 4.6 and containing 10 g sugar and 10 g protein per 240 mL, equivalent to 4.2 % protein (the FDA reference amount for beverages to have a claim of "high protein beverage") is a suitable target. This beverage could be labeled "high protein", "reduced sugar" and "reduced calorie" (Etzel, 2004). This kind of product can help shift the diet of the consumer from a high carbohydrate-base to high protein-base, thus reducing the risk of obesity and diabetes. Until now, there are no such commercial products available in the market.

Whey protein beverages currently present in the market are regarded as functional beverages that provide physiological benefits beyond high nutritional value. They are currently designed for athletes, bodybuilders and health conscientious individuals. A whey protein beverage is considered a convenient food product that can be consumed as a daily protein intake supplement. In terms of commercial value, whey protein beverages (> $8 billion/year) represent a dominant sector of the functional drinks' market.

There are many forms and flavors of whey beverages available in the market. Whey protein beverages produced at pH close to neural (pH 6.8) are generally opaque and thus available in chocolate, strawberry and vanilla flavor. On the other hand, whey protein acidic beverages (pH < 3.5) are relatively clear and available in various fruit flavors. Acidic beverages are preferred since beverages with pH < 4.6 require mild heating, thus maintain better sensory quality and shelf life stability (Etzel, 2004).

Currently, whey protein acidic beverages available in the market contain at most 4% whey protein, which is below the FDA requirement for a label claim of a "high protein beverage" (21 CFR 101.54 B). To make better use of the nutritional and biological properties whey protein possesses and to raise their market value, it is desirable to develop high whey protein acidic beverages. However, maintaining clarity at high protein concentration (> 4.2%) in thermally processed beverages, at 3.4 < pH < 4.6, over an extended period of time is a challenge.

1.5.2. Manufacturing challenges of whey protein acidic beverages

1.5.2.1. Solubility and thermal stability

Whey protein solubility and thermal stability, as discussed above, are highly affected by protein concentration. High protein content results in shorter distances between protein molecules, more competition for water layers, and higher chances of protein-protein associations, thus lowering the protein solubility. During thermal treatment, the exposed hydrophobic groups and free thiol groups more readily interact with each other to form hydrophobic associations and intermolecular disulfide linkages, leading to protein aggregation and possible precipitation (Pelegrine & Gasparetto, 2005). This process can continue post heat treatment, especially during extended storage, leading to polymerization and precipitation, eventually jeopardizing consumer acceptance.

1.5.2.2. Sensory quality

For acidic beverages, fortified with whey protein, sourness and astringency are often the limitations for customer acceptance. Sourness is refers to a tart and tangy taste, commonly caused by the acids naturally present in or added to foods. Astringency is a sensory term that refers to a tactile sensation felt in the mouth and is commonly described as a drying-out or roughing sensation (Lee & Vickers, 2008). Astringency, however, can be attributed to several reasons. In wine, tea, fruits, and soy-based products, astringency is attributed to endogenous polyphenols (Haslam & Lilley, 1988). Beecher et al. (2006) claimed that the astringency of whey protein beverages is attributed to whey protein binding and precipitating saliva protein under acidic pH. However, Lee and Vickers (2008) found that the astringency in whey protein beverages is caused by the high degree of acidity. Acidic whey protein solutions were not more astringent than acidic water solutions, with matching acidity, i.e. containing same amount of acid without protein.

As an ampholyte, whey protein is an excellent buffer in nature. Increasing protein concentration causes increased buffering capacity, which in turn results in an increased amount of acid needed to reach the target pH of an acidic beverage. This high acidity leads to increased perception of sourness and astringency, thus reducing the overall liking and consumer acceptance. To balance the astringency and sourness, a considerable amount of sugar is added, which is not desirable for health conscientious consumers. Therefore, ways to reduce the amount of acid required to reach a desirable pH in a high protein beverage has to be investigated.

1.5.2.3. Storage stability and shelf life

Shelf life stability for 1.5-6 months at ambient temperature is an economical challenge for whey protein acidic beverages producers (Fox & McSweeney, 1998). Many manufacturers use hot-fill heat treatment (88 °C for 2 min) for acidic beverages to prevent yeast and mold growth and extend shelf life. During ambient storage of thermally processed beverages, turbidity is often observed coupled with sedimentation. After three weeks of storage at room temperature (22 °C), a significant increase in beverage turbidity was observed (Etzel, 2004). Loss of soluble protein was detected after six weeks of

storage of 1.25% whey protein acidic solutions (pH 3.4), especially at storage temperature higher than 25 °C (Etzel, 2004). The gradual aggregation and buildup of protein polymers resulted in cloudiness and loss of acceptability. None of the few studies (Temelli, Bansema, Stobbe, 2004, 1998; Etzel, 2004; LaClair & Etzel, 2009) that investigated the shelf life stability of whey protein acidic beverages was based on high protein beverages (> 4.2% protein). Therefore, shelf life stability of high protein (> 4.2%) beverages remains an open area of investigation.

1.6. Approaches to enhance whey protein solubility

Although whey protein has better solubility compared to other food proteins, solubility in beverages becomes a challenge at high protein concentration. The growing demand of high protein beverages has engaged producers and researchers in a long-term commitment to investigate approaches that can enhance whey protein solubility and thermal stability.

Protein solubility in general can be improved by chemical modification, such as esterification (Taha et al., 2010), and amidation (Zhang, Li, Lu, & Liu, 2009). Chemical modification of the protein, however, may cause an increased risk of impaired *in vitro* metabolism and possible toxicological consequences (Lieske & Konard, 1994). In the last few decades, enzymatic modifications gained preference since the conditions employed are mild and the products have no potential harm. More recently, Maillard-induced glycosylation has been explored as a possible mechanism to improve solubility and thermal stability of protein. The effect of enzymatic hydrolysis and Maillard-induced glycosylation on protein solubility will be discussed in the following sections.

1.6.1. Enzymatic hydrolysis

Enzymatic hydrolysis of whey protein results in the reduction of molecular weight, possible conformational changes, and enhanced hydrophilicity as a result of the freed amine and carboxyl groups (Gauthier, Paquin, Pouliot, & Turgeon, 1993). Enhancement

of solubility is dependent on the degree of hydrolysis (DH), which is the extent of cleaved peptide bonds. Degree of hydrolysis is influenced by the activity of the protease, the physiochemical property of the protein, and the hydrolysis conditions. The solubility of whey protein at 0.1%, hydrolyzed by trypsin to reach a DH of 2.5, 3.9, and 5.3%, was higher than that of intact whey protein over a wide range of pH (pH 1-10) (Chobert, Bertrand-Harb, & Nicolas, 1988). However, enzymatic hydrolysis can also increase surface hydrophobicity of whey proteins due to the exposure of apolar amino acid residues (Gauthier, Paquin, Pouliot, & Turgeon, 1993). These hydrophobic residues may induce aggregate formation via hydrophobic interactions, and thus reduce the solubility. Whey protein hydrolyzed by *Bacillus licheniformis* protease to a DH of 6.8%, aggregated at pH 7.0 (Creusot & Gruppen, 2008). Aggregation and gelation of whey protein at pH 6.0 was also observed upon extensive hydrolysis (DH > 18%) with Alcalase (Doucet, Gauthier, & Foegeding, 2001).

Although the thermal stability of WPH was reported to be slightly improved compared to WPI at pH 5 (Chicon, Belloque, Alonso, & Lopez-Fandiño, 2009), the WPH was relatively unstable under other pH conditions. Additionally, the free thiol group of β-lg can still initiate disulfide interchange between peptides and intact proteins, leading to polymerization. Protein precipitation could occur during manufacturing, shipping and extended storage, thus greatly jeopardizing consumer acceptability. The pH dependency under the thermal conditions applied by the beverage industry, coupled with S-S linkages and hydrophobic interactions that build up over time, limit the use of WPH in beverages. Furthermore, flavor challenges can be encountered when using WPH. With increased hydrolysis (> 10% DH), bitterness can be detected due to the accumulation of hydrophobic peptides, containing phenylalamine, tyrosine, and leucine (Cliffe & Law, 1990). Another prominent flavor challenge is the excessive sourness and astringency when formulating acidic beverages using WPH. Hydrolysis of peptide bonds results in higher percentage of free carboxyl and amine groups, thus increasing the buffering capacity of the proteins. As mentioned earlier, the more the buffering capacity, the more acid is needed to reach the target acidic pH (~3.4), resulting in extreme sourness and astringency. Therefore, it is essential to investigate other ways to improve whey protein

solubility, thermal stability and flavor acceptance in order to incorporate whey protein into high protein beverages.

1.6.2. Maillard-induced glycosylation

1.6.2.1. The Maillard reaction

Maillard reaction is a non-enzymatic browning reaction, named after the French chemist Louis-Camille Maillard. The Maillard reaction occurs between a carbonyl group, usually from a reducing sugar, and an amine group, usually from an amino acid, peptide or a protein. The occurrence of Maillard reaction is exceptionally widespread. It occurs in food products, particularly during processing at elevated temperatures, such as roasting, baking, extruding or during storage for prolonged periods.

The Maillard reaction is very complex in that it involves a number of intermediate pathways. The products of the Maillard reaction between xylose and glycine alone are more than 100 as detected by high performance liquid chromatography (HPLC) (O'Reilly, 1982). Hodge (1953) successfully simplified the Mailard reaction (Fig. 3) as follows:

I. Initial stage: products are colorless, without absorption in the ultraviolet region (280 nm)
   Reaction A: Sugar-amine condensation
   Reaction B: Amadori rearrangement
II. Intermediate stage: products are colorless or yellow, with strong absorption in the ultraviolet region.
III. Reaction C: Sugar dehydration
IV. Reaction D: Sugar fragmentation
V. Reaction E: Amino acid degradation (Strecker degradation)
VI. Final stage: products are highly colored.
VII. Reaction F: Aldol condensation

VIII. Reaction G: Aldehyde-amine condensation and formation of heterocyclic nitrogen compounds

Among the three stages, the early stage is the one that has been well-characterized. The intermediate and final stages are much more complex and involve many poorly characterized compounds (Nursten, 2005). Some color is produced during the intermediate stage while most of the color is produced in the final stage as "melanoidins".

1.6.2.1.1. Characteristics

The primary characteristic of the Maillard reaction is color formation. The pigments are important in the development of flavors and color compounds in coffee, chocolate and other products (Hodge, 1953). The color produced can be measured using absorbance at visible wavelengths of 360 and 420 nm. So far, a wide range of colored molecules (Nursten, 2005), which are relatively small, has been isolated (Fig. 4). However, because of the complexity of the compounds produced, there still is quite a large gap in terms of intensity of color between the compounds identified and melanoidins.

Formation of flavor compounds is another important characteristic of the Maillard reaction. Much has been done on the isolation, identification and separation of aroma compounds. However, the mechanisms of formation of these compounds are very complex and heterogeneous. Additionally, for many of the flavor compounds, the separation and structural information are not clearly outlined (Nursten, 2005).

The Maillard reaction also plays an important role in protein aggregation, especially in whey protein bar systems (a_w 0.5~0.7). During eight weeks of whey protein bar storage at room temperature, significant browning and loss of available lysine were observed due to advanced stages of the Maillard reaction. With the prorogation of the Maillard reaction, disulfide linkages and Maillard-induced polymerization contributed to bar hardening during storage (Liu, Zhou, Tran, & Labuza, 2009).

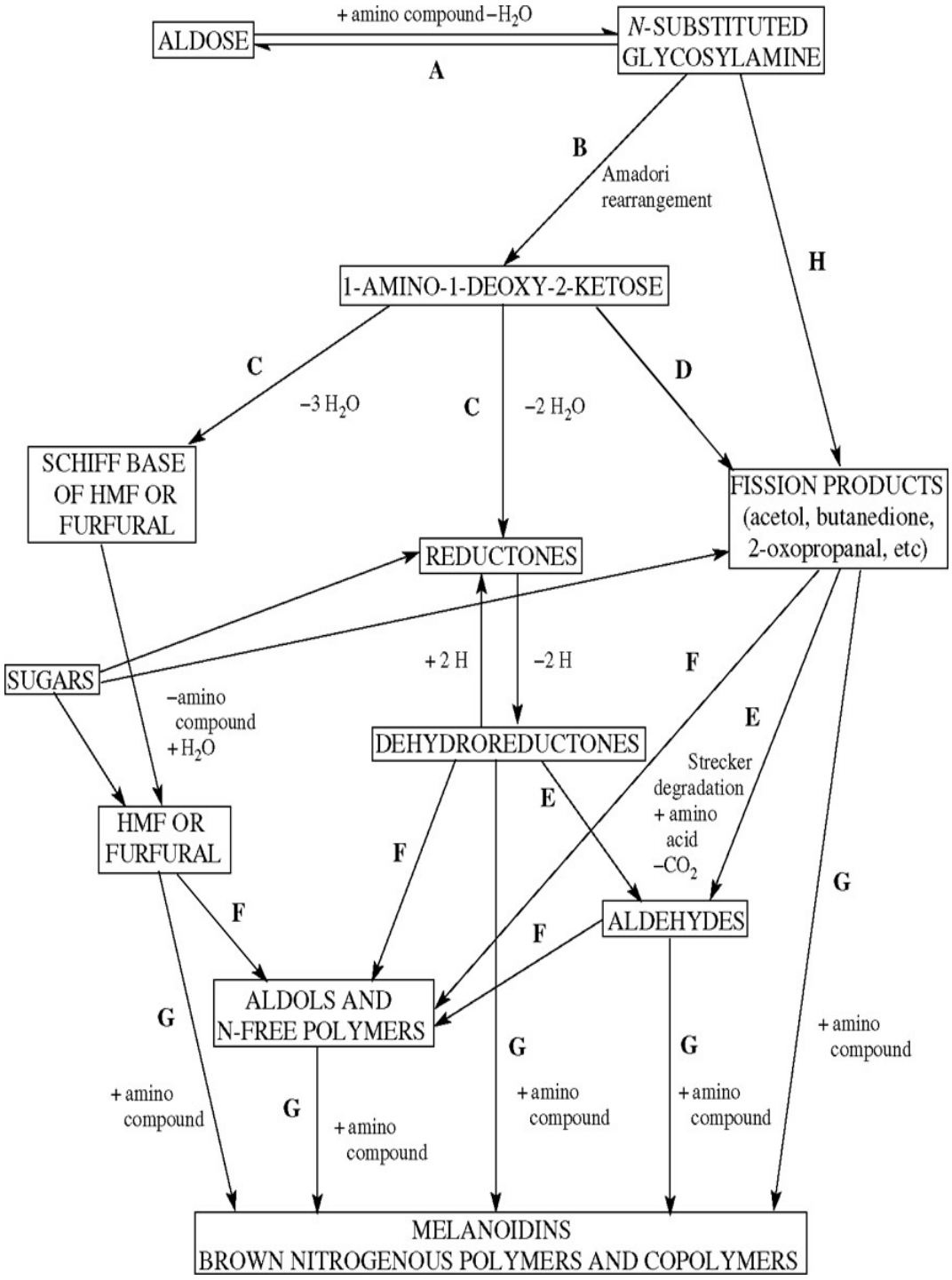

Figure 3. The Maillard reaction, a non-enzymatic browning reaction (Hodge, 1953).

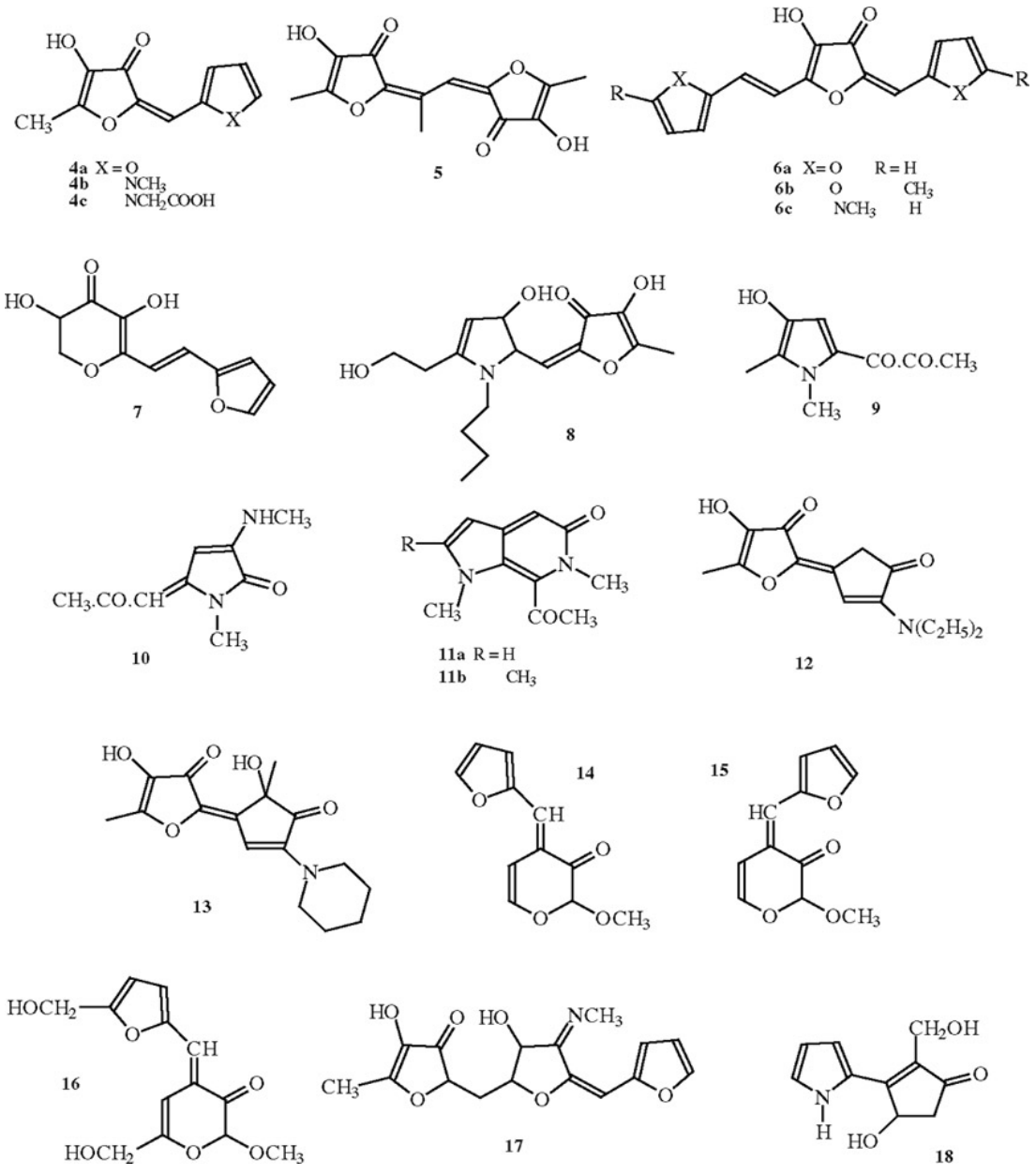

Figure 4. The structure of some low-molecular-mass color compounds formed in model Maillard systems (Nursten, 2005).

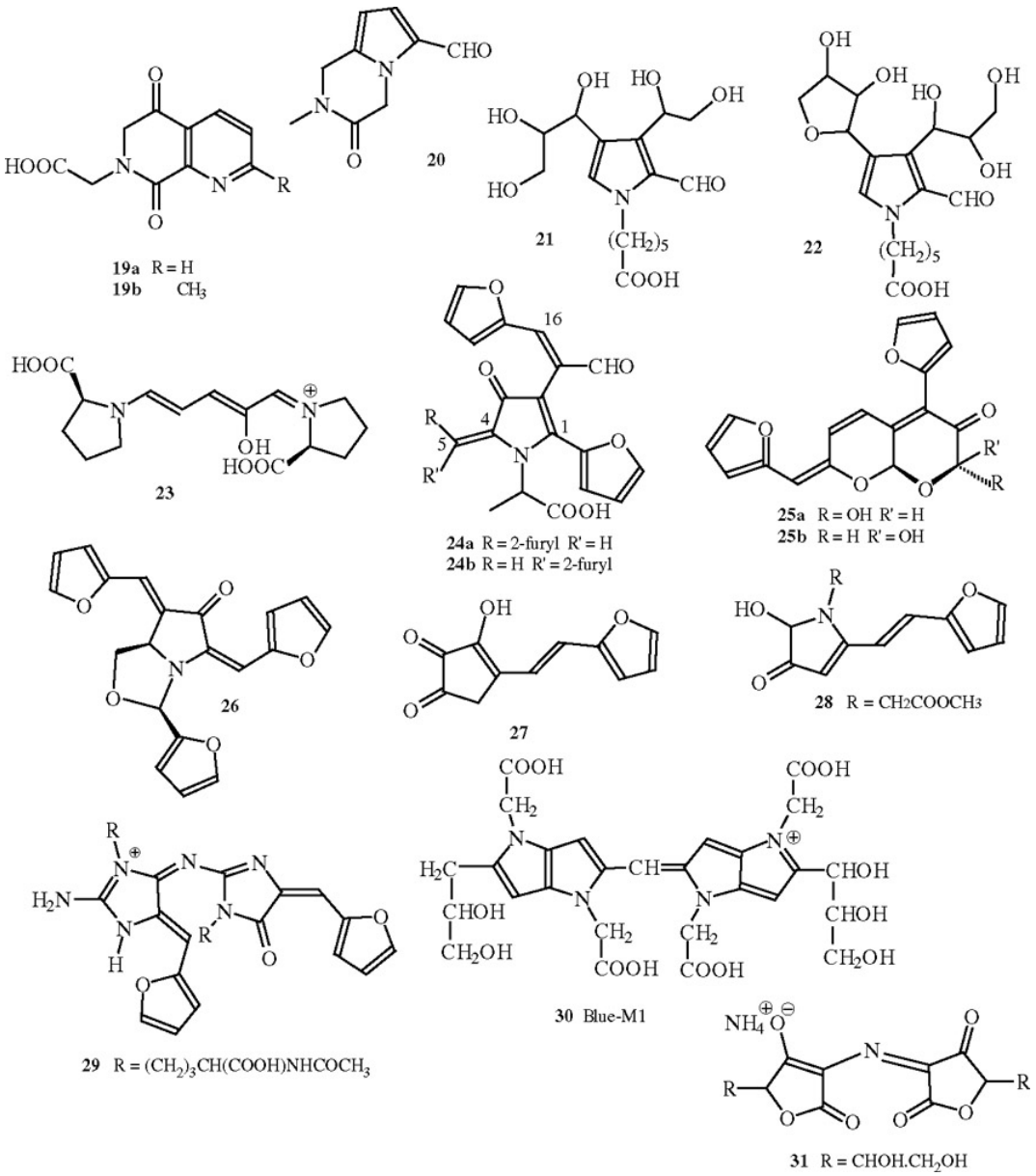

Figure 4. Continued

1.6.2.1.2. Physiological contribution

Some Maillard reaction products produced at intermediate and advanced stages were shown to have physiological properties, such as antioxidant activity and ion chelating activity. Chen and Kitts (2008) reported that glucose and ribose glycosylated lysine had antioxidant activity, while Chawla et al. (2009) reported that glycosylated whey protein were able to scavenge hydroxyl and superoxide anion radicals *in vitro*.

1.6.2.1.3. Toxicology

Toxicological aspects of the Maillard reaction have been a concern for a long time. Many researchers studied the carcinogenic products produced by the Maillard reaction. For instance, Widmark, et al. (1939) observed that an extract of roasted horse muscle had a carcinogenic effect when applied to mouse skin. Currently, acrylamide is the focus of attention with regard to carcinogenicity. Acrylamide could be formed by multiple pathways, and the streecker degradation pathway (E) in the Maillard reaction of asparagines represents the principle one. A significant amount of acrylamide is formed in foods subjected to frying, baking or other processes (Nursten, 2005). However, the formation of acrylamide does not occur below 120 °C (Mottram & Wedzicha, 2002).

Formation of mutagenic products, such as heterocyclic aromatic amines, is another concern. Mutagenic compounds can be formed in many foods subjected to extreme cooking conditions, including high temperature (> 120 °C) or mild heating (< 60 °C) for long time (> 80 days), not expected in western diet (Skog, Solyakov, & Arvidsson, 1998). Mutagens were formed after heating of sugar (glucose, fructose, galactose, tagatose, lactose, and lactulose) and casein mixtures at 120 °C for 60 min (Brands, Alink, Van Boekel, & Jongen, 2000). The authors also reported that mutagenicity of the products strongly varied with the kind of sugar used. Glycation by glucose and galactose showed a higher mutagenic activity, corresponding to a higher Maillard reactivity. Disaccharide glycosylation showed no mutagenic activity (lactose) or a lower mutagenic activity (lactulose) as compared to monosaccharide glycosylation.

1.6.2.2. Mechanism of protein solubility enhancements via Maillard reaction

The review by Olive, Melton, & Stanley (2006), summarized the mechanism of protein functionality enhancement via Maillard-induced glycosylation. Glycosylation of proteins with carbohydrates via controlled Maillard reaction (Fig. 5) has the same effect on protein structure as succinylation, which converts cationic amino groups to anionic residues. By blocking some of the amine groups, the net negative charges surrounding the molecules increases (Fig. 6), thus promoting electrostatic repulsion between molecules. Besides, the attachment of polar polysaccharides increases the surface hydrophilicity of the protein, thus promoting solvation. Due to the bulky polysaccharides attached, the steric hindrance reduces the chances of protein-protein interactions of denaturated protein molecules that have their interior hydrophobic groups exposed. The combined effect of increased electrostatic repulsion, hydrophilibicy and steric hindrance stabilizes the protein in solution, slows down the denaturation rate and reduces the chance of hydrophobic interactions and disulfide interchanges. Therefore, protein solubility and thermal stability are enhanced post early stage Maillard reaction. On the other hand, excessive glycosylation promotes protein cross-linking, especially at elevated temperature and extended heating time (Oliver, Melton, & Stanley, 2006). However, this mechanism remains hypothetical and not fully characterized.

To improve protein functionality, in general, the initial stage including reaction A and reaction B (Fig. 3) is favored, while the propagation of the reaction to advanced stages has to be limited. Beyond the formation of the Amadori compound, the reaction has to be controlled/stopped to avoid undesirable outcomes, such as formation of mutagenic compounds (Brands, Alink, Van Boekel, & Jongen, 2000), development of off-flavors (Moor & Ha, 1991), and excessive browning (Guerra-Hernandez, Gomez, Garcia-Villanova, Sanchez, & Gomez, 2002).

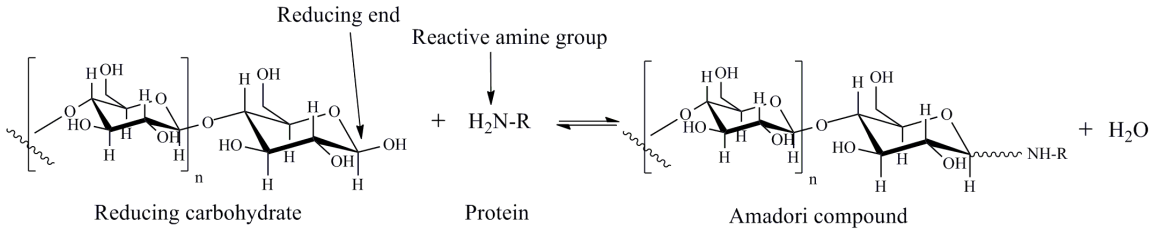

Figure 5. Sugar-amine condensation to form Amadori compounds.

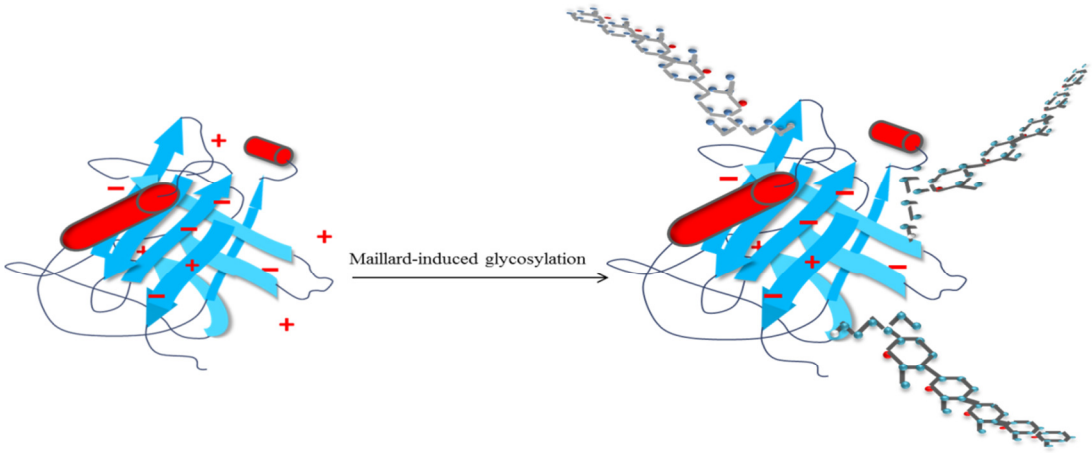

Figure 6. The proposed mechanism of using Maillard-induced glycosylation to improve whey protein solubility and thermal stability.

1.7. Early stage Maillard-induced glycosylation

The Maillard reaction is controlled by many factors, including heating conditions, water activity, pH, type of reducing sugar, and sugar to protein ratio. Maillard reaction occurs spontaneously when protein is mixed with reducing sugar, however, the reaction rate is accelerated by heating at elevated temperatures. Maillard reaction occurs under a_w of 0.2~1.0, with a maximer rate at a_w of 0.6~0.8 (Labuza, 1980). The pH does not affect the reaction speed but affects the ratio of products formed and the occurrence of different pathways. For example, low pH favors the formation of 2-furaldehydes, whereas high pH favors the 2,3-enolization pathway and formation of the furanones (Nursten, 2005). Using early stage Maillard-induced glycosylation to improve whey protein has to occur at a slow rate for better control and avoidance of advanced stages. Thus, the reaction should be conducted below the optimum Maillard reaction conditions. Researchers using early

stage Maillard reaction usually conduct the reaction at neutral pH, a_w of 0.3~0.5 with mild heating (< 65 °C) (Mitchell, 1988). The optimum sugar to protein ratio has to be determined under various conditions since this factor interacts with other factors, such as a_w, temperature and pH.

Different reducing sugars varying in molecular weight and reducing power have different effect on the Maillard reaction rate. The glycosyltaion reaction between polysaccharide and protein is much slower than when mono-, di- or oligosaccharides are used. This is probably due to the steric hindrance of the bulky carbohydrate chain. This can result in better control of the reaction progress and formation of advanced reaction products. Thus, using polysaccharides to modify protein may lead to functionality improvement while limiting undesired effects (Jimenez-Castano, Villamiel, & Martin-Alvarez, 2005). The commonly used polysaccharides include dextran, galactomannan, chitosan, guar gum and carboxymethlcellulos (Shu, Sahara, Nakamura, & Kato, 1996).

Besides their effect on the reaction rate, different reducing sugars may result in different extent of physiochemical changes and functional enhancement (Nursten, 2005). For example, after glycosylation with hexoses and lactose, β-lg exhibited higher solubility than glycosylated β-lg with ribose and glyceraldehyde under the same conditions (49 h, at 60 °C, with excessive sugar) (Doucet, Gauthier, & Foegeding, 2001). Kato et at. (1990) showed that the Maillard reaction between ovalbumin and dextran displayed much higher heat stability than ovalbumin with glucose. Additionally, Shu et al. (Shu, Sahara, Nakamura, & Kato, 1996) reported that the emulsifying properties of whey protein are improved in proportion to the length of the polysaccharide employed in the Maillard reaction.

Since β-lg is the dominant component of whey protein, many studies were conducted on the glycosylation of β-lg with mono-, di- and oligosaccharides, in order to improve its functionality. However, much less is known on the possibility of glycosylation of β-lg or whey protein with polysaccharides. Jimeneze et al. (2005) have investigated the effect of dry heating conditions on the glycosylation of β-lg with dextran (10, 20 and 43 kDa) via Maillard reaction, and reported that the optimum conditions for the first stage Maillard reaction between 43 kDa dextran and β-lg were 60 °C, a_w of 0.44, and 2:1 dextran to β-lg

(w/w). However, since limited work has been done on Maillard glycosylation of polysaccharides and whey proteins, definitive conclusions cannot be drawn about the optimum conditions for Amadori compounds production and subsequent functionality improvements.

The earliest start of using the Maillard reaction to improve protein functionality was by the group of Kato et al. (Kato & Kobayashi, 1991; Matsudomi, Nakano, Soma, & Ochi, 2002; Kato, Mifuru, Matsudomi, & Kobayashi, 1992; Kato, Minaki, & Kobayashi, 1993) who initiated and conducted a series of research on the emulsifying properties of glycosylated proteins. They found that lysozyme with poor emulsifying properties (EP) in the native form showed highly effective EP after glycosylation with dextran. This emulsifier was superior to commercial emulsifiers, even under harsh conditions, such as acidic or high salt environment. Later on, remarkable enhancement of emulsifying properties using a variety of proteins and sugars was reported, for example bovine serum albumin (BSA) glycosylated with galactomannan (GM), caseintate with maltodextrin, ovalbumin with dextran, and protamine with GM (Kim, Choi, Shin, & Moon, 2003; Babiker, Hiroyuki, & Matsudomi, 1998; Kato, Sasak, Furuta, & Kobayashi, 1990; Nakamura & Kato, 2000).

Globular proteins, such as lysozyme, BSA and soy protein, after glycosylation with low molecular weight reducing sugars, such as lactose, ribose, and xylose, form heat-induced gels with higher breaking strength compared with their native forms (Armstrong, Hill, Schrooyen, & Mitchell, 1994; Cabodevila, Hill, & Armstrongm, 1994). The increase in gel strength was attributed to the contribution of sugar-induced covalent cross-linking of proteins. When glycosylated proteins are used, thermally-induced gelation at low protein concentrations can occur. These findings were further confirmed by Easa et al. (1991) who showed that only 0.5-1.2 % of xylose- or ribose-glycosylated BSA was needed to form a heat-induced gel with the same strength of 7% native BSA in a 90 min heating time instead of 160 min at 95 °C. Similar results were reported by Matsudomi et al. (2002) using egg white protein glycosylated with galactomannan.

Although the sugar may protect the protein from denaturation during heating, excessive heating of the glycated protein in solution may lead to gelation. A spontaneous

hydrolytic reaction such as deamidation may occur, facilitating the cross-linking process (Oliver, Melton, & Stanley, 2006).

Compared to emulsifying and gelation, much less research was done on the effect of the Maillard reaction on solubility and thermal stability. This may be due to several early studies showing a marked decrease in protein solubility and thermal stability due to extensive glycation (Chicon, Belloque, Alonso, & Lopez-Fandiño, 2009; Groleau, Morin, Gauthier, & Poliot, 2003).

1.7.1. Effect of Maillard-induced glycosylation on solubility and thermal stability

A few attempts have been made to develop glycosylated β-lg or whey proteins with enhanced solubility and thermal stability under controlled dry conditions (Doucet, Gauthier, & Foegeding, 2001; Jiménez-Castaño et al., 2005; Jiménez-Castaño, et al., 2007) and recently in aqueous solutions (Zhu, Srinivasan, & Lucey, 2008). Dry heating, however, is preferred over heating in aqueous solution because reducing sugars in solution accelerate protein denaturation, leading to gross protein structural modifications (Gauthier, 2001).

Nacka et al. (1998) produced glycosylated β-lg and α-la with lactose, glucose, galactose and mannose exhibiting higher solubility (1 mg/mL protein solution) as compared to non-glycosylated proteins over a pH range of 3-9. Chevalier et al. (2001) conjugated β-lg with arabinose and ribose and showed enhanced heat stability at 2 mg/mL protein concentration under neutral pH. Glycosylation of β-lg, α-la and BSA with 10 kDa and 20 kDa dextrans had improved solubility (1 mg/mL protein solution) at pH 3-5 . The heat stability of β-lg and BSA was enhanced at pH 5 and 7 (Jiménez-Castaño et al., 2005; Jiménez-Castaño, et al., 2007).

Although improvements in solubility of whey proteins modified via the Maillard-induced glycosylation were observed (Doucet, Gauthier, & Foegeding, 2001; Nacka, & Chobert, 1998; Chevalier et al., 2001; Aoki et al., 1997), none of the proposed protocols are industrially feasible. Investigations focused on isolated whey protein fractions, β-lg, α-la or bovine serum albumin (BSA) and their respective isolated glycosylated proteins.

On an industry scale, it is preferred to use whole whey protein since it is very costly to isolate individual proteins or conjugates. For scaling up purposes, it would be beneficial to investigate the effect of Maillard-induced glycosylation of a whey protein ingredient without further fractionation. Producing modified proteins without the need of fractionation would potentially prevent accrual of processing costs and generation of waste streams. Moreover, the claim of improved solubility and thermal stability is based on very low protein concentrations, for instance 0.1% (Nacka & Chobert, 1998; Jiménez-Castaño et al., 2007), much lower than what is expected for protein fortified acidic beverages. Finally, the processing conditions (heating temperature, heating time, and pH) were not based on industry practices. Therefore, it is critical to investigate whether Maillard-induced glycosylation can be used to improve whey protein solubility and thermal stability at high concentrations, using an industry friendly approach.

1.7.2. Nutritional quality of glycosylated whey protein

Nutritional value is one of the biggest concerns researchers have for products of Maillard reaction. Glycosylation occurs primarily at the ε-amino group of the lysine residues, and to a lesser extent at the imidazole group of histidine, the indole group of tryptophan, and the guanidine group of arginine residues (Ames, 1992). Because lysine is an essential amino acid, its excessive glycosylation leads to nutritional quality loss. Therefore, partial glycosylation is preferred so as to limit excessive loss of lysine.

Lysine blockage can be estimated by monitoring furosine, hydrolyzed from the glycosylated protein (Fig. 7). Furosine (2-furoyl-methyl-Lys) is one of the Maillard reaction products first to be identified. During acid hydrolysis, furosine was produced from Amadori compound N(ε)-fructoselysine and quantified using HPLC (Erbersdobler & Somoza, 2007; Guerra-Hernandez, Gomez, Garcia-Villanova, Sanchez, & Gomez, 2002). Furosine has been widely used for evaluation of heat-damaged dairy products. Furosine also serves as an indicator of the advanced (intermediate) Maillard stage. During the Maillard reaction, formation of advanced Maillard products (AMP) occurs in

parallel with furosine generation. After reaching a steady state, furosine starts to degrade whereas AMP continues to form (Nursten, 2005).

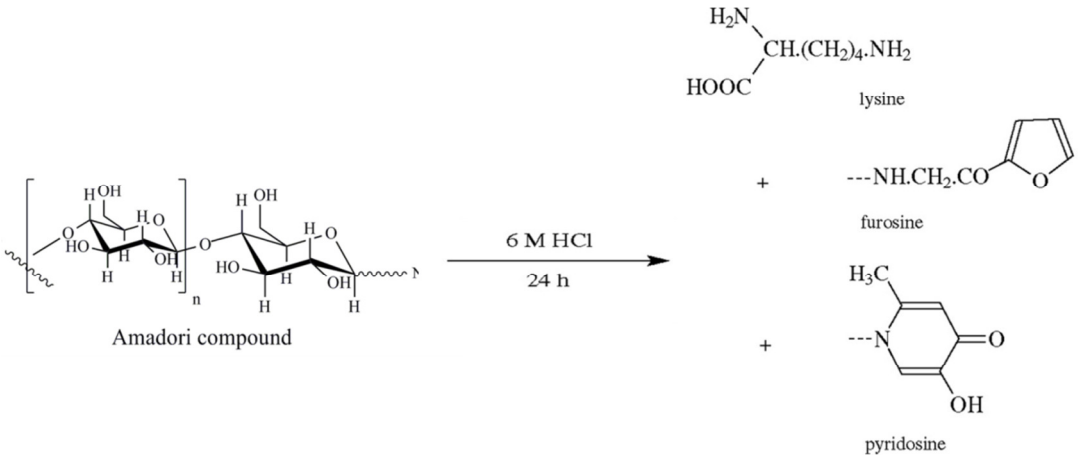

Figure 7. The products of hydrolysis of ε-N-deoxyfructosyllysine under protein-analysis conditions

Decrease of digestibility of the glycosylated protein is another concern. Thus far, the research focused on the *in vitro* digestibility of the glycosylated protein gave controversial results (Erbersdobler, Brandt, Scharrer, & Von Wangenheim, 1981; Hiller & Lorenzen, 2010; Chobert, Mollé, & Haertlé, 2001; Bouhallab, Morgan, Henry, Molle, & Leonil, 1999). Hiller and Lorenzen (2010) examined the digestibility of glycosylated sodium caseinate and glycosylated WPI with glucose, pectin and dextran. The authors reported a significant increase (21%) of *in vitro* digestibility of glycosylated WPI, and a 36-55 % decrease of digestibility for glycosylated sodium caseinate. Chobert, et al. (2001) observed lower tryptic digestibility of glycosylated whey proteins with glucose or lactose, but observed an unaffected peptic digestibility. Bouhallab et al. (Iametti, Cairoli, De Gregori, & Bonomi, 1995) reported an excellent digestibility of lactosylated β-lg upon heat treatment at 90 °C for 10 min.

This discrepancy between results may be attributed to several reasons. The degree of glycosylation among the different studies is different, resulting in different extents of protein structural changes. Additionally, the complex structure of the glycosylated protein with polysaccharides may sterically shield the peptide bonds targeted by the digestive proteases. In other cases, partial unfolding of the protein may occur upon Maillard-

induced glycosylation. This unfolding may facilitate proteolysis by elevating the number of accessible peptide bonds susceptible to the enzymatic cleavage (Oliver, Melton, & Stanley, 2006). Therefore, digestibility is a function of the source of protein, the type of reducing sugar, the glycosylation conditions employed and the extent of glycosylation.

1.7.3. Sensory, storage stability and shelf life of glycosylated whey protein in beverage applications

To the best of our knowledge, no study has investigated the effect of Maillard-induced glycosylation on protein buffering capacity. Whether acidic beverages formulated with glycosylated whey protein will be less sour and astringent than native whey protein, is yet to be determined. Additionally, no work has been done to evaluate the shelf life stability of acidic solutions or beverages formulated with glycosylated whey protein.

1.8. Characterization of protein structural modifications upon glycosylation

Investigation of protein functional/structural relationship based on evaluation of protein functionality and characterization of protein structure is the core focus of researchers' interest. A lot of work has been done to determine the structural characteristics of different types of proteins and their correlation to functionality. To achieve this goal, many rapid chemical assays and novel instrument approaches have been developed. Using these technologies, whey protein structure and physiochemical characteristics, including amino acid sequencing, secondary and tertiary configuration, pI, surface hydrophobicity etc., have been well-illustrated. Besides, the conformational changes induced by various treatments, such as pH adjustment or thermal treatment, have been demonstrated using multiple techniques. More recently, these techniques have been used to investigate the structural changes of chemically and enzymatically modified whey proteins (Nacka & Chobert, 1998). Thus far, no research has been done to characterize the protein structural changes upon Maillard-induced glycosylation.

It is critical for researchers to understand the site and extent of glycosylation of whey protein under different incubation conditions, and analyze the possible effects of the attachment of reducing sugars. The attachment of very polar reducing sugars directly decreases the ratio of surface hydrophobicity to hydrophilicity. The change of surface hydrophobicity and conformation affects the protein/protein and the protein-water interactions. Additionally, the blockage of some amino groups upon glycosylation is hypothesized to cause a shift of the pI to a more acidic pH, resulting in an increase of intermolecular electrostatic repulsion over a wide range of pH. With structural characterization upon glycosylation, researchers can better understand the mechanism of functionality enhancement of the protein via Maillard-induced glycosylation.

1.8.1. Chemical assays

Chemical assays are mostly used to determine the physiochemical characteristics of proteins, and usually employ colorimetric probes binding to functional groups of proteins during or after treatments. The change in surface hydrophobicity, for instance, can be monitored by using spectrofluorometric probes (Cardamone & Puri, 1992), such as 1-aniline 8-naphthalene sulfonate (ANS). The surface thiol availability can be monitored using Ellman's reagent [5,5'-dithio-bis (2-nitrobenzoic acid), DTNB], which binds to free thiol group on the molecular surface and absorb light at 412 nm. The total free thiol group can be measured using Ellman's reagent after the protein is denatured (Manderson, Hardman, & Lawrence, 1999). Determination of surface hydrophobicity as well as total and surface thiol groups of proteins provides structural information and changes incurred upon thermal or other treatments. The pI of the protein can be calculated by determining kinetics of the binding affinity to an ion-exchange material against pH increments (Yang & Langer, 1985) or by isoelectric point gel electrophoresis (Michel et al., 2003). Determination of pI provides information about any changes in charge density and distribution on the protein, incurred upon various chemical, enzymatic or other modifications.

1.8.2. Instrumental approaches

Instrumental approaches, including mass spectrometry (MS), Nuclear magnetic resonance (NMR), circular dichroism (CD), X-ray crystallography, Fourier transform infrared spectroscopy (FTIR), and Raman spectroscopy have been developed and improved over time. These techniques provide a large amount of detailed and accurate structural information, thus have gained popularity.

Protein sequences can be determined by enzymatic digestion followed by tandem MS, or by Edman degradation (Loo, Edmonds, & Smith, 1990; Smith, 2001). For modified protein, MS is a better tool because it provides additional structural information, including the size of the modified protein, the modification site, and the extent of modification. Using electrospray ionization (EI) MS, Leonil et al. (Lillard, Clare, & Daubert, 2009) were able to identify two lysine residues in β-lg as the potential sites for lactose attachment during milk heat processing.

The three-dimensional structure of protein can be determined using NMR, CD, or X-ray crystallography (He, Lin, Li, & Kim, 2010). NMR allows the observation of specific quantum mechanical magnetic properties of the atomic nucleus. When placed in a magnetic field, NMR active nuclei, such as H or C, absorb electromagnetic radiation at a frequency characteristic of the isotope. The resonant frequency, energy of the absorption, as well as the intensity of the signal are proportional to the strength of the applied magnetic field. Based on the distinct characteristics of the chemical shift, the specific functional group can be determined. In addition, the interactions among functional groups result in delicate splits on the spectra, and based on the shape of the splits, the steric structure of the protein can be identified. With the innovation of NMR spectroscopy, high resolution three dimensional structures of protein can be obtained. Recently, multidimensional (> 3D) NMR spectroscopy has been widely applied in pharmaceutical research to determine the interaction between protein and drug candidates (Akitt & Mann, 2000).

Circular dichroism provides information based on the differential absorption of left and right circularly polarized light (Fasman, 1996). It is exhibited in the absorption bands

of optically active chiral molecules, such as protein. UV-CD is used to investigate the secondary structure of proteins, while vibrational CD, which uses light from the infrared energy region, is used to investigate the secondary/tertiary structural of proteins and DNA.

X-ray crystallography is a method that determines the arrangement of atoms within a crystal. It can produce a three-dimensional picture of the density of electrons within the crystal, by which the mean positions of the atoms in the crystal as well as their chemical bonds can be determined (Bragg, 1912).

All of these techniques have been used by various researchers to monitor protein structural changes, the following are a few examples. The structural changes of β-lg under various heat, pH and pressure treatments have been studied using NMR (Edwards, Jameson, Palmano, & Creamer, 2002; Kuwata, Li, Yamada, Batt, Goto, & Akasaka, 2001). More recently, also using NMR, the structural changes of a chemical muted equine β-lg were determined (Kobayashi, Ikeguchi, & Sugai, 2000). Divsalar et al. (2006) investigated the structure of β-lactoglobulin type A and B after they interact with 2,2'-bipyridinn-hexyl dithiocarbamato Pd (II) nitrate, a new heavy metal complex designed for anticancer property, using CD techniques (Divsalar, Saboury, Mansoori-Torshizi, & Hemmatinejad, 2006). Qin, et al (1998) used X-ray crystallography to illustrate the sterical position (open or close) of EF loop over the β-barral of β-lg. This information is very important for understanding the protein aggregation as affected by pH.

More recently, Raman spectroscopy has been employed to analyze protein structural modifications. Raman spectroscopy, a vibrational light scattering technique, allows for the prediction of molecular identity based on unique spectral analysis of stretching and bending of molecular bonds, collectively producing fingerprint spectra for a particular molecule (Sun, Zhao, Zhao, Yang, Cui, & Ren, 2011). It provides complementary information to FTIR, which also measures vibrational and rotational molecular movements. FTIR is a direct light absorption process while Raman spectroscopy is a light scattering process. Raman spectroscopy is sensitive to changes in covalent bonds, such as disulfide linkages, and non-covalent bonds, such as electrostatic and hydrophobic interactions. It is also able to detect transitions from ordered to disordered structure upon

protein denaturation (Sun et al., 2011). Raman spectroscopy can provide information related to protein backbone conformation and the molecular environment of certain side chains. This technique, for instance, has been applied successfully to study structural changes upon chemical modifications of food proteins (Wong, Choi, Phillips, & Ma 2009).

Compared to other techniques, such as X-ray crystallography, NMR, and CD, Raman spectroscopy is a nondestructive and rapid analytical technique that requires minimal sample preparation (He, Lin, Li, & Kim, 2010). Another advantage of Raman spectroscopy is the ability to analyze aqueous protein solutions with no water interferences (He, Haynes, Diez-Gonzales, & Labuza, 2010). However, Raman spectroscopy has low sensitivity, which limits its applications to powders or highly concentrated samples. A more sensitive Raman technique, surface-enhanced Raman spectroscopy (SERS), was developed to characterize various chemical and biological compounds (Han, Zhao, & Ozaki, 2009; Yakes, Lipert, Bannantine, & Porter, 2008). Raman scattering can be amplified tremendously in the near vicinity of nanoscale-roughened noble metal substrates, such as silver (Ag) dendrites (He et al., 2010).). Only trace amount of sample is needed to obtain structural information, improving the limit of detection to parts per billion. The use of SERS for the structural elucidation of glycosylated whey protein is, therefore, a very promising tool.

2. Effect of Maillard-Induced Glycosylation on the Nutritional Quality, Solubility, Thermal Stability and Molecular Configuration of Whey Protein*

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Whey protein isolate (WPI) was subjected to controlled and limited Maillard-induced glycosylation using dextran. Maillard reaction was optimized to promote glycosylation, while minimizing browning and maintaining nutritional quality. Hydrophobic interaction chromatography was used to separate unreacted dextran from unreacted and glycosylated proteins, collectively termed as partially glycosylated whey protein (PGWP). Solubility and thermal stability of PGWP and WPI were compared over a wide range of pH, protein concentrations, and heating temperatures and times. Compared to WPI, PGWP maintained higher solubility and thermal stability at protein concentrations greater than 4.2%, over a wide range of pH, including the pH around the isoelectric point (pI) of whey protein. The enhanced solubility and thermal stability of PGWP was attributed to reduced intermolecular interactions. Several physicochemical/structural changes, including resistance to denaturation, shift to more acidic pI, reduced surface hydrophobicity, reduced exposure of sulfhydryl groups, and unique glycosylation sites, contributed to the reduced intermolecular interactions.

2.1. Introduction

Whey proteins are reasonably soluble in acidic beverages (pH~3.4); however, thermal processing and prolonged storage can result in protein aggregation and subsequent deterioration of quality. Consequently, whey protein acidic beverages available on the market have a short shelf life and contain at most 4% protein, which is below the minimum percentage (4.2%) required by the FDA to claim a "high protein beverage" (21 CFR 101.54 B).

Solubility of whey proteins upon heating is affected by the onset of protein denaturation and the subsequent hydrophobic interactions coupled with disulfide linkages, which lead to protein aggregation (Nicorescu et al., 2009). The onset of denaturation of β-lactoglobulin (β-lg), the major whey protein, is ~ 60 ºC near pH 4.0, ~ 65 °C at neutral pH, and ~ 75 °C at pH 2.5 (Ye, 2009). Therefore, when heated at > 60 ºC and at a pH close to its isoelectric point (pH 4.5-5.5), whey protein solubility is minimized (Pelegrine & Gasparetto, 2005), due to the denaturation of the protein and the suppression of electrostatic repulsions. The delayed onset of denaturation at acidic pH, on the other hand, explains the enhancement in solubility and clarity at pH < 3.5 (LaClair & Etzel, 2009). However, at relatively high protein concentrations (> 4%) solubility and solution clarity are reduced upon heating, even at low pH (Imetti, Cairolo, Gregori, & Bonomi, 1995).

Various protein modification technologies are being investigated to address the solubility and thermal stability challenges of whey protein. Among the researched technologies, Maillard-induced glycosylation has recently gained significant attention.

Maillard cross-linking between the protein and reducing carbohydrates changes protein charge, conformation, and solvation (Oliver, Melton, & Stanley, 2006). It is hypothesized that the blockage of amine groups upon conjugation with the carbohydrate moiety will result in an increase in net negative charge and overall hydrophilicity, thus causing repulsion between proteins and enhanced solvation. However, Maillard-induced glycosylation has to be controlled to limit propagation to undesired advanced stages. Advanced Maillard reactions may result in reduced digestibility (Erbersdobler, Brandt, Scharrer, & Wangenheim, 1981), formation of mutagenic compounds (Brands, Alink,

Boekel, & Jongen, 2000), development of off-flavors (Moor & Ha, 1991), and excessive browning (Guerra-Hernandez, Gomez, Garcia-Villanova, Sanchez, & Gomez, 2002).

Reported results on the solubility and thermal stability of glycoslyated whey protein are quite promising (Chevalier, Chobert, Popineau, Nicolas, & Haertle, 2001; Jiménez-Castaño, Villamiel, & López-Fandiño, 2007). However, the enhancement in solubility and thermal stability was often reported at protein concentrations much lower than those relevant for industrial applications, for instance, 1 mg mL-1 (Nacka & Chobert, 1998 and Jiménez-Castaño et al., 2007). Additionally, previous studies mostly focused on isolated whey protein fractions, β-lg, α-lactalbumin (α-la) or bovine serum albumin (BSA) and their respective isolated conjugates. For scaling up purposes, it would be beneficial to investigate the effect of Maillard-induced glycosylation of a whey protein ingredient without further fractionation. Producing modified proteins without the need of fractionation would potentially prevent accrual of processing costs and generation of waste streams.

Therefore, the objectives of this study were to: 1) determine the effect of controlled and limited Maillard-induced glycosylation on the solubility and thermal stability of whey proteins, at concentrations > 4.2% and over a wide pH range; 2) determine the effect of glycosylation on the nutritional quality of whey proteins; and 3) determine the physico-chemical changes that explain the structure/function relationship upon conjugation.

2.2. Materials and Methods

2.2.1. Materials

Whey protein isolate (WPI, 92.7% protein, as confirmed by Dumas, AOAC 990.03, using a Nitrogen Analyzer, LECO® TruSpec TM), BiPRO, was kindly provided by Davisco Foods International, Inc. (Eden Prairie, MN, USA). Dextran (10 kDa, D9260), porcine pepsin (EC 3.4.23.1 , 3,200-4,500 units mg^{-1}, P6887), porcine trypsin (EC 3.4.21.4, 10,000 BAEE units mg^{-1}, T8003), dextranase (EC 3.2.1.11, 400-800 units mg^{-1}, D8144), 1-aniline 8-naphthalene sulfonate, AQE-Sephadex A-50, β-lg, and 5,5-dithiobis 2-nitrobenzoic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Furosine

standard was purchased from Polypeptide Laboratories (San Diego, CA, USA). SDS-PAGE reagents and molecular weight standards were purchased from Bio-Rad Laboratories, Inc. (Hercules, CA, USA). Other reagent grade chemicals were purchased from Fisher Scientific (Pittsburgh, PA, USA) and Sigma-Aldrich.

2.2.2. Maillard-induced glycosylation

A two-factor experimental design, completely crossed, was performed in triplicate, to determine the optimum incubation conditions for maximum production of Amadori compounds (glycosylated protein) and minimal browning, with incubation temperature (3 levels) and time (5 levels) as factors. WPI was mixed with dextran in 1:4 ratio (w/w), dissolved in phosphate buffer (0.1 M, pH 7) and lyophilized. The lyophilized powder was incubated in a dessicator at 50, 55, & 60 °C for 48, 72, 96, 120, & 144 h. Dextran was chosen because the rate of Maillard reaction is slower using longer chain reducing saccharides vs. small reducing sugars (Jiménez-Castaño et al., 2007), thus allowing for better control of the reaction. Protein-to-dextran ratio and water activity (a_w) were carefully selected based on preliminary trials, and were set as fixed parameters. The a_w was set at 0.49, which is below the optimum for fast propagation of Maillard reaction (Jiménez-Castaño et al., 2005). Incubation temperature was set at or below 60 °C to limit protein denaturation. Dextran and WPI were incubated separately under the same conditions to serve as controls.

2.2.2.1. Purification of the partially glycosylated whey protein (PGWP) from unreacted dextrans

Separation of unreacted dextran from the protein was performed using hydrophobic interaction chromatography (HIC). Pharmacia Biotech fast protein liquid chromatography (FPLC) system, equipped with a UV detector and a fraction collector, was used. An HIC column, 18.5 cm x 16 mm, was packed with phenyl sepharose high performance media (GE healthcare®) and equilibrated with 1M ammonium sulfate. Incubated protein/dextran

mixture was dissolved in 1M ammonium sulfate (4% protein, w/v). An aliquot (3 mL) of the solution was injected into the column and the flow rate was set at 1 mL min^{-1}. Free dextran was washed with 50 mL of 1M ammonium sulfate followed by 20 mL of de-ionized distilled water (DDW). Glycosylated and free proteins, collectively referred to as PGWP, were eluted with 30 mL DDW. Elution volumes were experimentally determined to ensure separation of free dextran and recovery of proteins. Elution of the protein was monitored at 280 nm. Collected PGWP was then dialyzed against DDW, using 3.5 kDa cutoff membrane, and lyophilyzed. Dextran and protein content of the collected fractions were determined by phenol-sulfuric acid assay, AOAC 44.1.30, and Dumas, AOAC 990.03, respectively.

2.2.2.2. Monitoring of Amadori compounds formation, browning, and loss of free amino groups

PGWP samples and controls (incubated and non-incubated WPI), in triplicate, were dissolved in DDW (0.2% protein w/v) and centrifuged (13,800 x g for 10 min at 4 ºC). The absorption of the supernatant was measured at 304 nm (Beckman Coulter DU®, Brea, CA, USA). The difference UV absorption (DUV) at 304 nm of PGWP was recorded against the control as an indication of Amadori compounds formation (Zhu, Damodaran, & Lucey, 2008). Browning was monitored at 420 nm (Jiménez-Castaño et al., 2007). Quantification of free amino groups was determined following the *O*-Phthaladehyde method outlined by Xu et al. (2011), using lysine as a standard.

2.2.3. Nutritional quality

2.2.3.1. Lysine blockage

Quantification of 2-furoyl-methyl-Lys (furosine), the Amadori compound formed upon the reaction of a lysine residue with a reducing sugar (Guerra-Hernández et al., 2002), was carried out following the hydrolysis protocol and the high performance liquid

chromatography (HPLC) method outlined by Jiménez-Castaño et al. (2007) and Krause, Knoll, and Henle (2003), without modifications. 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 RP-18 column (250 mm x 4.6 mm, 5 µm) and a guard column (20 mm x 4 mm) of the same material were used.

2.2.3.2. Protein digestibility

Digestibility of WPI and PGWP was determined, in triplicate, following sequential *in vitro* pepsin and trypsin digestion as outlined by Tang, Li, and Yang (2006), with some modifications. Dispersions (10 mL of 1% protein, w/v) of WPI and PGWP in 0.1 N HCL were prepared at pH 1.5, and warmed up in a water bath at 37 ºC for 10 min, with gentle stirring. Pepsin (100 µL, 3 mg mL^{-1}) was added to the mixture and incubation continued for 2 h. The pH was then adjusted to 7 using 0.1 N NaOH, to terminate pepsin activity. Trypsin (100 µL, 9 mg mL^{-1}) was added, and incubation continued for 2 h. The pH was adjusted as needed during the incubation to maintain a pH of 7. To monitor the peptide profile across the different digests, samples were analyzed using the same Shimadzu HPLC system and column, described in section 2.3.1. A linear binary gradient was employed using HPLC grade water (solvent A) and acetonitrile (solvent B), both containing 0.11% (v/v) trifluoroacetic acid. Following injection of 50 µL of a digest, solvent B was linearly increased from 16% to 40% in 20 min, kept constant for 10 min, increased to 80% in 10 min, then decreased to 16% in 10 min, followed by column equilibration steps. The column temperature was maintained at 35 °C and the flow rate was set at 1.2 mL min^{-1}. Absorbance was monitored at 214 nm, and percent digestibility was determined based on differences in peak areas.

2.2.4. Solubility & thermal stability

2.2.4.1. Solubility and turbidity

Solutions (1 mL in DDW) of WPI and PGWP were prepared in screw-cap microcentrifuge tubes, in triplicate, at 5, 7, and 10% protein concentration (w/v). To simulate the pH used when formulating acidic whey protein beverages, to test the effect of heating at the isoelectric point, and to serve as pH control, solutions were prepared at pH 3.4, pH 4.5, 5.5, and 7, respectively. The solutions were either left with no further treatment, or were heated in a water bath at 70, 75, and 80 ºC for 30 min. The come up time ranged between 10-22 s. Heated and non-heated solutions were centrifuged (13,000 x g for 10 min at 23 ºC), and protein content in the supernatant was measured using a nitrogen analyzer. Solubility was expressed as the percentage of protein content in the supernatant to the total protein content of the initial solution. As an index of turbidity, optical density (OD) of the supernatants was measured at 600 nm as outlined by Sava and Plancken (2005).

2.2.4.2. 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 and PGWP following the method outlined by Haug, Skar, Vegarud, Langsrud, and Draget (2009), with modifications. 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}. Similarly, extent of denaturation was determined by DSC post-thermal treatment (75 and 80 °C for 30 min) of WPI and PGWP solutions (10% protein, w/v) at pH 3.4, 4.5, 5.5 and 7.0.

2.2.4.3. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was carried out to visualize the protein distribution in non-heated and heated WPI and PGWP samples. In triplicate, 1-mL solutions (5% protein, w/v) were prepared in screw-cap microcentrifuge tubes, and were either left with no heating or were heated at 75 °C for up to 1 h, with an aliquot withdrawn every 10 min. Samples were diluted to 1.4 mg mL^{-1} protein concentration, mixed in 1:1 ratio with Laemmli buffer and boiled for 5 min. For reducing conditions, β-mercaptoethanol was added (5%, v/v). Aliquots (10 μL) were then loaded on hand-cast 18% acrylamide and 4% stacking gel. Molecular weight markers and protein standards were loaded as well. Gels were electrophoresed at 200 V for about 50-70 min. Glycoprotein staining was carried out as described by Zhu et al. (2008). Gels were also stained using Coomassie Brilliant Blue (G-250) for 1 h followed by de-staining as outlined by Laemmli (1970).

2.2.5. Physico-chemical/structural characterization of PGWP

2.2.5.1. Isoelectric point (pI) determination

The pI of WPI and PGWP was determined by measuring the electrical charges on the protein surface at pH 1-6, as outlined by Yang and Langer (1985). The pI was calculated by determining kinetics of the binding affinity to an ion-exchange material against pH increments.

2.2.5.2. Determination of surface hydrophobicity and surface sulfhydryl groups

The same heated and non-heated solutions (5% protein, w/v) of WPI and PGWP prepared for SDS-PAGE analysis were used for the determination of surface hydrophobicity, following the method outlined by Sava and Plancken (2005). The relative fluorescence was measured with a Bio-Tek Synergy HT spectrofluorimeter (Winooski, VT, USA). Determination of surface sulfhydryl groups was carried out as outlined by Sava and Plancken (2005), with minor modifications. Samples were diluted to 0.1% protein (w/v) using tris buffer (0.086 M Tris, 0.09 M glycine, and 4mM

Na_2EDTA; pH=8). Ten µL of DTNB (4 mg mL^{-1} in tris buffer) was added to 1-mL sample and absorbance was measured at 412 nm against a reagent blank after 15 min holding time at 23 ºC. A calibration curve was prepared using standard cysteine solutions (10, 20, 40, 60, 80, & 100 µM).

2.2.5.3. Determination of glycosylation sites using matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS)

PGWP was digested with dextranase to reduce the size of the conjugated dextran, facilitating ionization. An aliquot (950 µL) of PGWP solution (5% protein in 0.1 M phosphate buffer, pH 6.0) was mixed with 50 µL of dextranase (33 µg mL^{-1}), incubated at 37 °C for 30 min and lyophilized. Dextranase digested PGWP and WPI were subjected to trypsin digestion following the procedure outlined by Canales et al. (2009). Digests were then analyzed using MALDI-TOF MS. Samples were first desalted using Millipore C18 ZipTips, following manufacturer instructions, then were suspended in 0.1% trifluoroacetic acid, water:acetonitrile (50:50) and an aliquot (1 uL) was spotted on a Bruker target overlaid with 1 µL of α-cyano-4 -hydroxycinnamic acid matrix. A Bruker Daltonics Biflex III mass spectrometer (Fremont, CA, USA), equipped with a N_2-laser (337 nm, 3 nanosecond pulse length) and a microchannel plate (MCP) detector, was used. Mass data was collected in the linear mode, positive polarity, with an accelerating potential of 19 kV. Each spectrum produced was the accumulation of approximately 200 laser shots. External calibration was performed using horse heart cytochrome C (average mass [MH+] 12361) and horse skeletal muscle myoglobin (average mass [MH+] 16953).

2.2.6. Statistical Analysis

Analysis of variance (ANOVA) was carried out utilizing XLSTAT (2010). When a factor effect or an interaction was found significant ($P \leq 0.05$), significant differences among the respective means were determined using Fisher's LSD test.

2.3. Results and Discussion

2.3.1. Maillard-induced glycosylation

2.3.1.1. Formation of Amadori compounds

The formation of Amadori compounds at 50 and 55 °C progressed at a very slow rate, and was hardly significant even after 144 h of incubation. At 60 °C, however, the formation of Amadori compounds was noticeable after 72 h of incubation (Fig. 8a), and continued to increase with incubation time up to 120 h. No significant change was observed when incubation was extended to 144 h. This could be attributed to the propagation of the Maillard reaction beyond the formation of Amadori compounds, which get converted to secondary metabolites, as confirmed by the significant ($P \leq 0.05$) increase in browning (Fig. 8b). While a significant increase in conjugation was noted after 96 h of incubation at 60 °C, little if any increase in browning was observed. Therefore, PGWP samples produced after the incubation at 60 °C for 96 h were selected for the rest of the investigations. Compared to some conjugation conditions used previously, such as 100 °C for 2 h (Lillard, Clare, & Daubert, 2009) and 60 °C for 10 days (Jiménez-Castaño et al., 2005), the conditions chosen for this study were relatively mild.

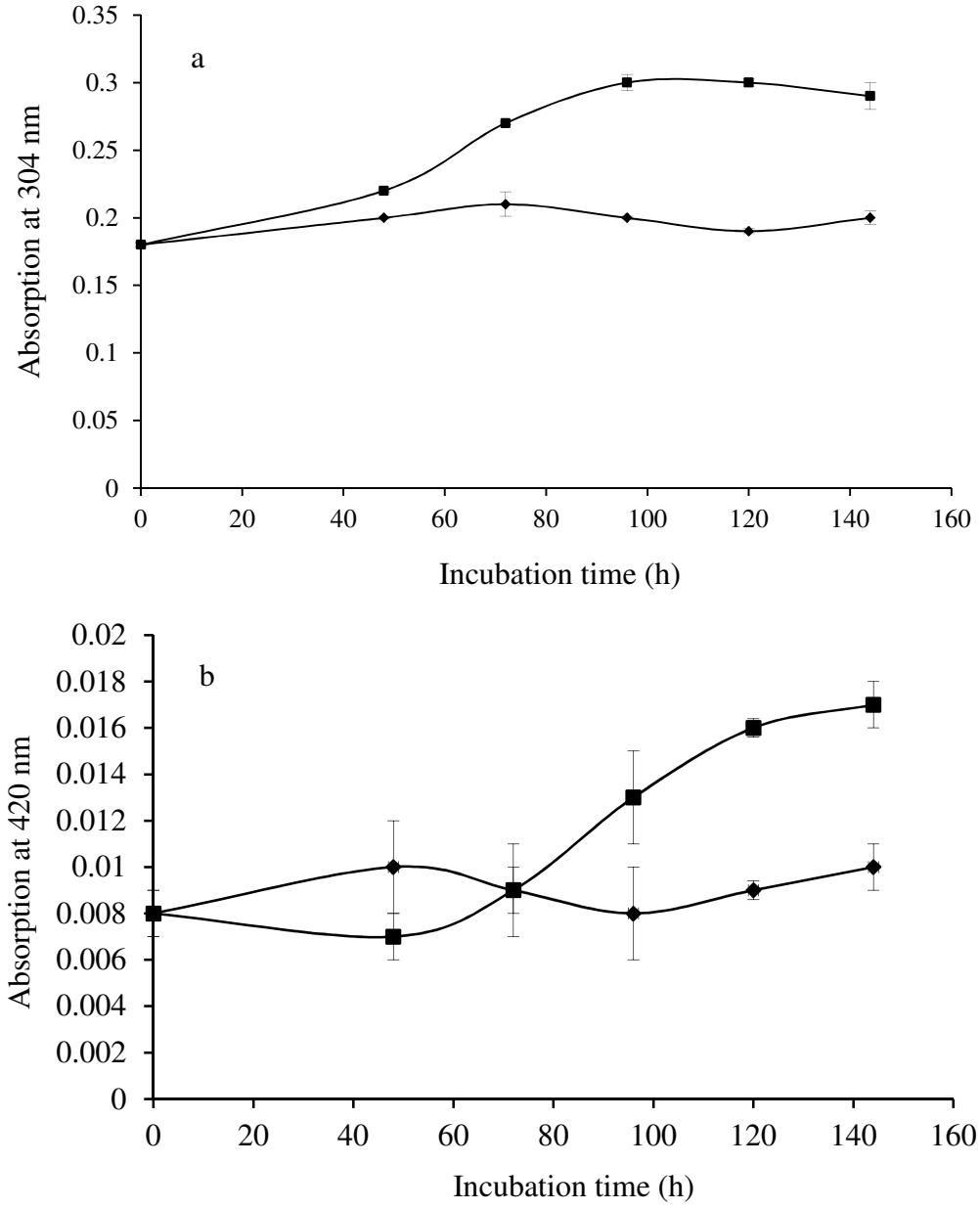

Figure 8. Formation of Amadori compounds monitored at 304 nm (a), and browning monitored at 420 nm (b), in whey protein isolate (WPI) and partially glycosylated whey protein (PGWP) incubated at 60 °C for 0-144 h, at a_w of 0.49. Error bar represents standard deviations; n=3.WPI: ◆ ; PGWP: ■.

2.3.1.2. Purification of PGWP from free dextrans

The elution of unreacted dextran from the HIC column was completed in 70 min as confirmed by running free dextran control through the column (Fig. 9). The protein fraction, constituting both unreacted and glycosylated protein (i.e. PGWP) eluted between 70-100 min. Post dialysis and lyophilization, % protein and carbohydrate in PGWP were ~ 60% and 30% (w/w), respectively. Previously, unreacted carbohydrates and unreacted proteins were separated from glycosylated proteins by dialysis, membrane filtration, ion-exchange chromatography and/or affinity chromatography (Chevalier et al., 2001; Jiménez-Castaño et al., 2007; Zhu, Damodaran, & Lucey, 2010). Functionality enhancement was reported for the isolated protein conjugates after removal of unreacted proteins. From the industry stand point, it is crucial to determine if functionality can be enhanced without the need to remove unreacted proteins. Removal of unreacted proteins will generate new waste streams and economical loss. Furthermore, removal of dextran using HIC vs. other chromatographic separation, involves mild hydrophobic interactions that will not cause significant protein denaturation (Kato, et al. 2002), thus preserving protein functionality.

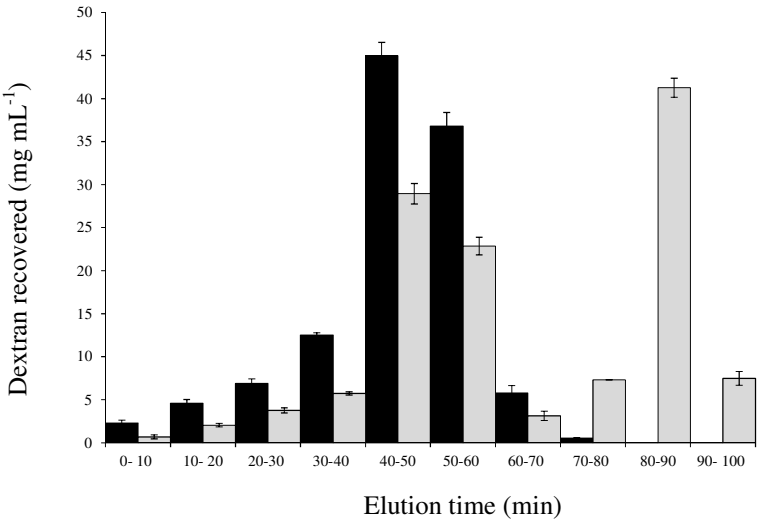

Figure 9. Dextran distribution of fractions collected using hydrophobic interaction chromatography, determined by the phenol-sulfuric acid method for total carbohydrate (AOAC method 44.1.30). Error bar represents standard deviations; n=3. Black bars: Incubated dextran; gray bars: incubated mixture of dextran and whey protein

2.3.1.3. Free amino group loss

Maillard-induced glycosylation occurs primarily at the ε-amino group of the lysine residues, but can also occur to a lesser extent at the imidazole group of histidine, the indole group of tryptophan, the guanidine group of arginine residues, and at the N-terminus of the protein (Ames, 1992). The PGWP lost only 1.4% of the available amino groups, confirming a limited degree of Maillard conjugation as compared to previously reported values of up to 14.8% loss in free amines (Rufian-Henares, Guerra-Hernández, & García-Villanova, 2002; Xu et al., 2011).

2.3.2. Nutritional quality

2.3.2.1. Lysine blockage

Only 0.47 mg furosine g^{-1} of PGWP was detected (equivalent to 4.52 mg furosine g^{-1} of lysine; whey protein used has 10.4% lysine). Given that furosine represents ~43.4% of the total Amadori compounds (Krause et al., 2003), the calculated lysine blockage was 0.6% (on molar basis). This value is significantly lower than some of the previously reported observations (Rufian-Henares, Guerra-Hernández, & García-Villanova, 2006; Jimenez-Castano et al., 2007). The noted difference could be attributed to the mild conjugation conditions, and to the fact that the measurement was on a sample containing both reacted and unreacted proteins compared to previously reported measurements done only on isolated conjugates.

2.3.2.2. Protein Digestibility

Post digestion, the chromatographic peaks representing the hydrolyzed peptides of WPI were quite comparable to those of digested PGWP (Fig. 10), indicating no significant effect of glycosylation on enzymatic activity. The peaks representing the non-digested protein of PGWP (Fig. 10a) were much broader than those of WPI (Fig. 10b). This observation was most likely due to the uneven attachment of dextran residues to the

protein in the PGWP sample, resulting in a heterogeneous increase in molecular hydrophilicity. This heterogeneous hydrophilicity affects the interaction of the protein with the chromatography column, thus causing a change in the shape of the peak that represents a heterogeneous protein fraction.

Based on peak area calculations, percent digestibility of PGWP was greater than that of WPI by 8.9%. Reported *in vitro* digestibility results of glycosylated whey protein are controversial (Erbersdobler, Brandt, Scharrer, & Wangenheim, 1981; Cheveliar et al., 2001; Hiller & Lorenzen, 2010), with some supporting decrease while others showing increase in digestibility. This discrepancy may be due to differences in the extent of glycosylation per protein molecule, size of the carbohydrate moiety, and the conformational change of the protein incurred upon conjugation with the carbohydrate. In some instances, the complex structure of protein-polysaccharides may cause steric hindrance preventing the digestive enzymes from reaching the binding sites. However, partial unfolding of the protein may occur during Maillard reaction that may facilitate proteolysis by elevating the number of accessible enzyme binding sites. Further structural characterization of our PGWP will provide explanation for the observed increase in percent digestibility.

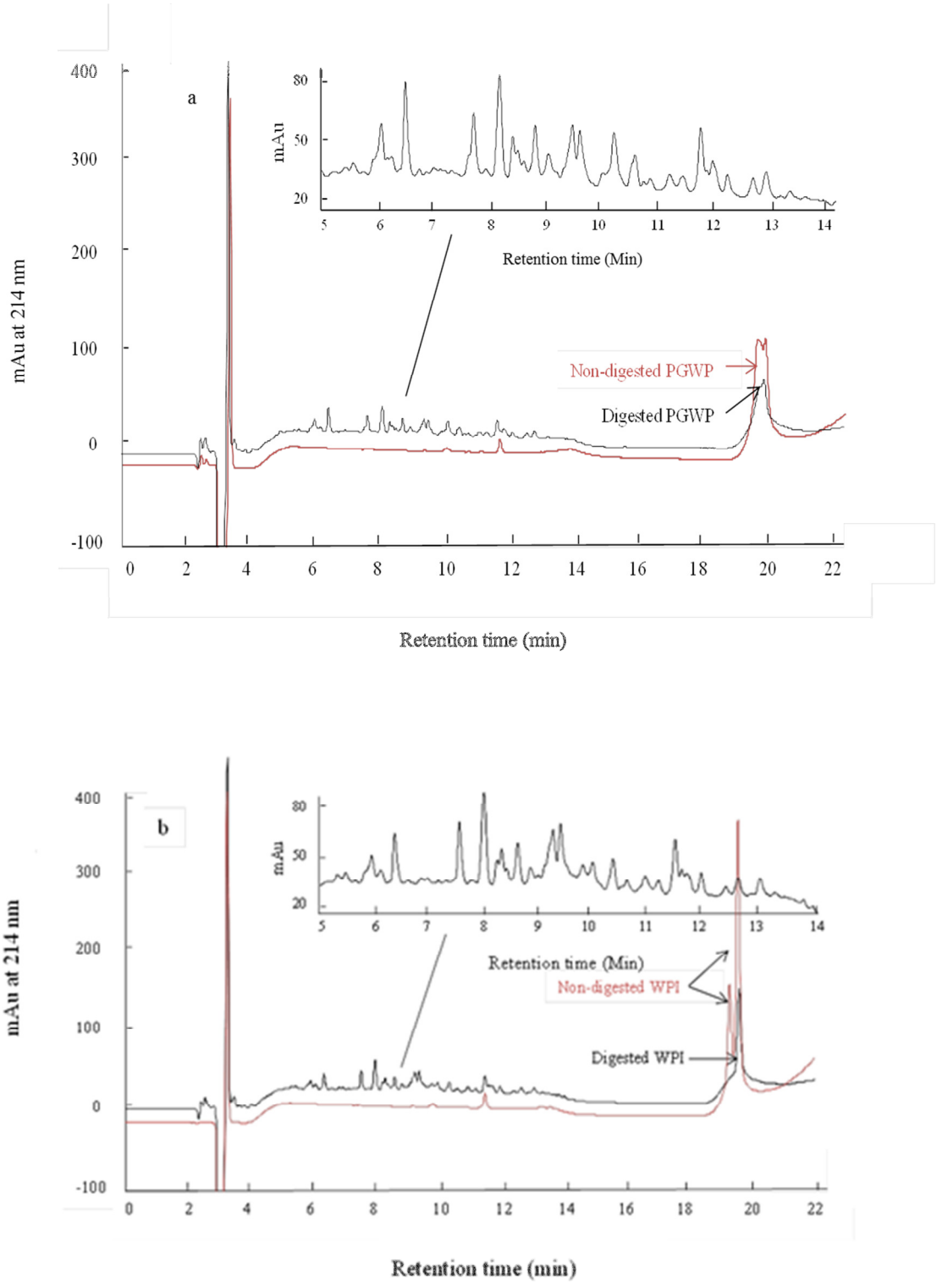

Figure 10. Chromatogram of partially glycosylated whey protein (PGWP) (a) and whey protein isolate (WPI) (b) before and after digestion.

2.3.3. Solubility and thermal stability

2.3.3.1. Solubility

The effect of heating on the solubility of both PGWP and WPI was not significant at pH 3.4 and 7 and protein concentration of 5 and 7% (Table 1). The solubility of WPI solutions at pH 4.5 and 5.5, however, decreased greatly as heating temperature and protein concentration increased. On the other hand, the solubility of PGWP was maintained at ≥ 90% at all pHs and protein concentrations, even after heating at 80 °C, with the exception of 10% protein PGWP solutions. Heating 10% protein solutions of WPI and PGWP at 75 and 80 °C resulted in heat-induced gelation. Heat-induced gelation at 75 and 80 °C and at 80 °C was also observed for 7% protein solutions of PGWP at pH 3.4 and 4.5, respectively. Maillard-induced glycosylation has been shown to enhance heat-induced gelation of whey protein (Handa & Kuroda, 1999; Gan et al., 2009).

The observed solubility trend for WPI was as expected. Whey proteins maintain good solubility even at their isoelectric point mainly because the native globular whey protein, namely β-lg, has low surface hydrophobicity. However, when heated at the isoelectric point, the denatured protein readily aggregates via hydrophobic interactions, due to the suppression of electrostatic repulsion. This phenomenon is enhanced with the increase in protein concentration (Table 1, ANOVA Tables 10-17 in Appendix A). On the other hand, heated PGWP solutions maintained solubility around the isoelectric point, most likely due to an increase in net negative charge and overall hydrophilicity, resulting in resistance/delay of denaturation. However, with harsher heating conditions, partial denaturation will occur, affecting the balance between hydrophilic and exposed hydrophobic groups (Armstrong, Hill, Schrooyen, & Mitchell, 1994). This shift will enhance protein-water-protein interactions, more so at high protein concentration, thus explaining the observed heat-induced gelation.

Table 1. Percent solubility of whey protein isolate (WPI) and partially glycosylated whey protein (PGWP) at various pH and protein %, non-heated and heated at 70, 75 and 80 °C for 30 min.

pH	Heat Treatment	% Protein (w/v)					
		5%		7%		10%	
		WPI	PGWP	WPI	PGWP	WPI	PGWP
3.4	Non heated	102.6aA*	99.2aA	99.1aA	98.5aA	90.1aB	95.1aA
	70 °C	99.4aA	98.1aAB	101.5aA	101.2aA	88.2aA	96.5aA
	75 °C	96.4aA	97.6aA	100aA	--**	--	--
	80 °C	99.7aA	100aAB	98.1aA	--	--	--
4.5	Non heated	86.1aB	97.3aA	81aC	99.4aA	82.4aC	97.2aA
	70 °C	51.2bB	98.9aAB	56.4bC	100.5aA	20.1bC	101.2aA
	75 °C	42.4cC	94.1bA	28.1cC	99.2aA	--	--
	80 °C	3.90dB	89.6cB	4.5dB	--	--	--
5.5	Non heated	90.5aB	99.4aA	91.5aB	101.2aA	84.5aBC	96.5aA
	70 °C	57.9bB	104.9aA	64.6bB	98.7aA	38.4bB	98.1aA
	75 °C	48.7cB	95.4aA	36.9cB	99.5aA	--	--
	80 °C	7.30dB	96.3aAB	7.8dB	100.2aA	--	--
7	Non heated	99.9aA	99.7aA	99.2aA	98.4aA	98.2aA	98aA
	70 °C	93.8aA	94.3aB	98.2aA	100.4aA	96.5aA	97.6aA
	75 °C	96.5aA	95.8aA	97.7aA	102.8aA	99.1a	--
	80 °C	100.2aA	100aA	97.4aA	98.7aA	--	--

* Means in each column, within the same pH treatment, followed by the same small letter are not significantly different across heat treatments, and means within the same heat treatment, followed by the same capital letters are not significantly different across the different pH treatments, according to Fisher least significant difference (LSD) multiple means comparison test ($P \leq 0.05$); n=3. ** The solution gelled.

The observed solubility trend of PGWP around the isoelectric point is in agreement with previous research on isolated protein conjugates (Akhtar & Dickinson, 2007; Zhu et al., 2010). However, our observation confirms this trend at higher protein concentrations (> 5%) than what was tested earlier (1mg mL^{-1}). Additionally, we have demonstrated that solubility enhancement can be achieved for a mixture of proteins (free and glycosylated) and not only for isolated conjugates. Therefore, partial and limited glycosylation, obtained under the mild Maillard conditions employed, is sufficient to cause a remarkable enhancement of whey protein solubility in heated solutions over a wide range of pH and protein concentration.

2.3.3.2. Turbidity

Moderately sized protein aggregates can be soluble yet contribute to turbidity. The turbidity data followed the same trend as the solubility data for both the WPI and PGWP samples. While WPI and PGWP solutions (5%, 7% and 10% protein, w/v) remained clear at pH 3.4 and 7.0 prior and post heating (data not shown), the turbidity of WPI solutions at pH 4.5 and 5.5 were significantly higher than those of PGWP solutions (Fig. 11). These observations confirm that PGWP, unlike WPI, maintains clarity even after heating at pH near the isoelectric point of whey proteins, when the repulsive forces are minimized. Partial glycosylation most likely contributed to rigidity in protein structure, thus resisting complete unfolding and subsequent aggregation due to hydrophobic interactions.

2.3.3.3. Thermal denaturation

The denaturation temperature of β-lg and α-la increased upon partial glycosylation by 15.5 ºC and 18.7 ºC, respectively (Fig. 12). The denaturation enthalpy (ΔH) of β-lg of the PGWP sample (2.04 J g^{-1}) was lower than that of the WPI sample (3.42 J g^{-1}). This reduction in the ΔH indicates partial unfolding of the tertiary structure induced by

glycosylation. This partial unfolding may partially explain the observed increase in digestibility of PGWP as compared to WPI.

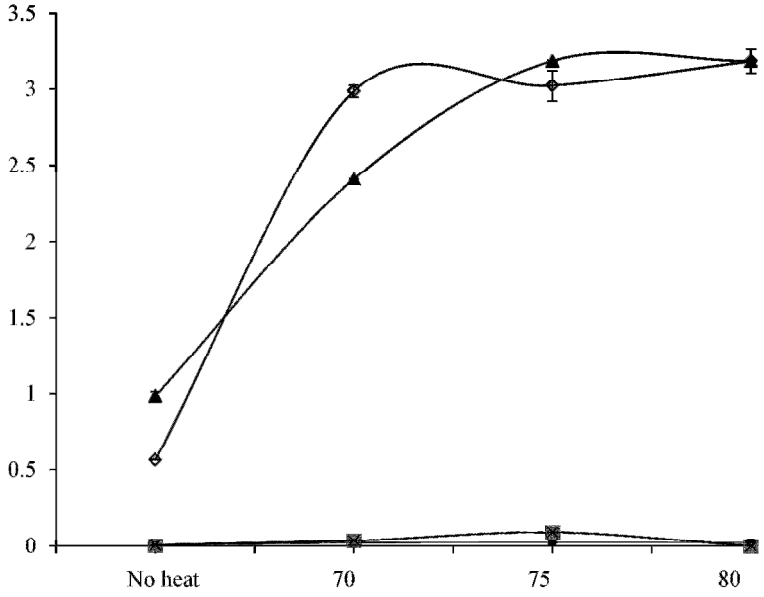

Figure 11. Optical density (OD) of whey protein isolate (WPI) and partially glycosylated whey protein (PGWP) solutions (5% and 7% protein) at pH 4.5, non-heated and heated at 70, 75 and 80 °C for 30 min. Error bar represents standard deviations; n=3. 5% WPI:◇; 5% PGWP:■; 7% WPI:▲; 7% PGWP:☒.

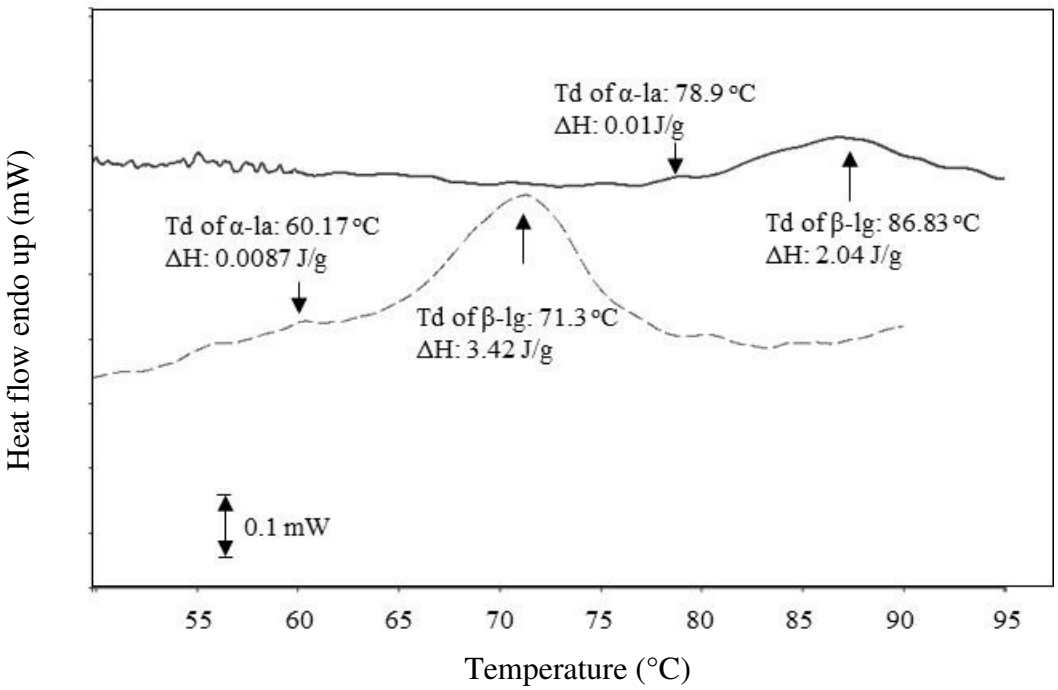

Figure 12. Differential scanning calorimetry of partially glycosylated whey protein (PGWP) and whey protein isolate (WPI) after purification by hydrophobic interaction chromatography. Solid line: PGWP, dotted line: WPI. Td: denaturation temperature and ΔH: enthalpy.

Denaturation pattern was also monitored post thermal treatment of WPI and PGWP at pH 3.4, 4.5, 5.5, and 7.0 (Fig. 13). While β-lg denaturation temperature for PGWP was ~ 84 °C at all four pH levels, the denaturation temperature of β-lg of WPI was ~ 78 °C at pH 3.4, 4.5 and 5.5, and ~ 68 °C at pH 7. Based on β-lg ΔH calculations for both WPI and PGWP, the extent of heat-induced denaturation increased with the increase in pH and heating temperature. However, the heat-induced denaturation of β-lg was more pronounced for WPI than that of PGWP under all pH conditions, especially after heating at 80 °C for 30 min. Upon heating (80 °C for 30 min) WPI at pH 3.4 and 4.5, β-lg ΔH was reduced by 85% and 91%, respectively, and by 100% at pH 5.5 and 7. Upon heating PGWP (80 °C for 30 min), only a 10% and 33% reduction in β-lg ΔH was noted at pH 3.4 and 4.5, respectively. Reduction in β-lg ΔH for PGWP was more pronounced at pH 5.5 and 7.

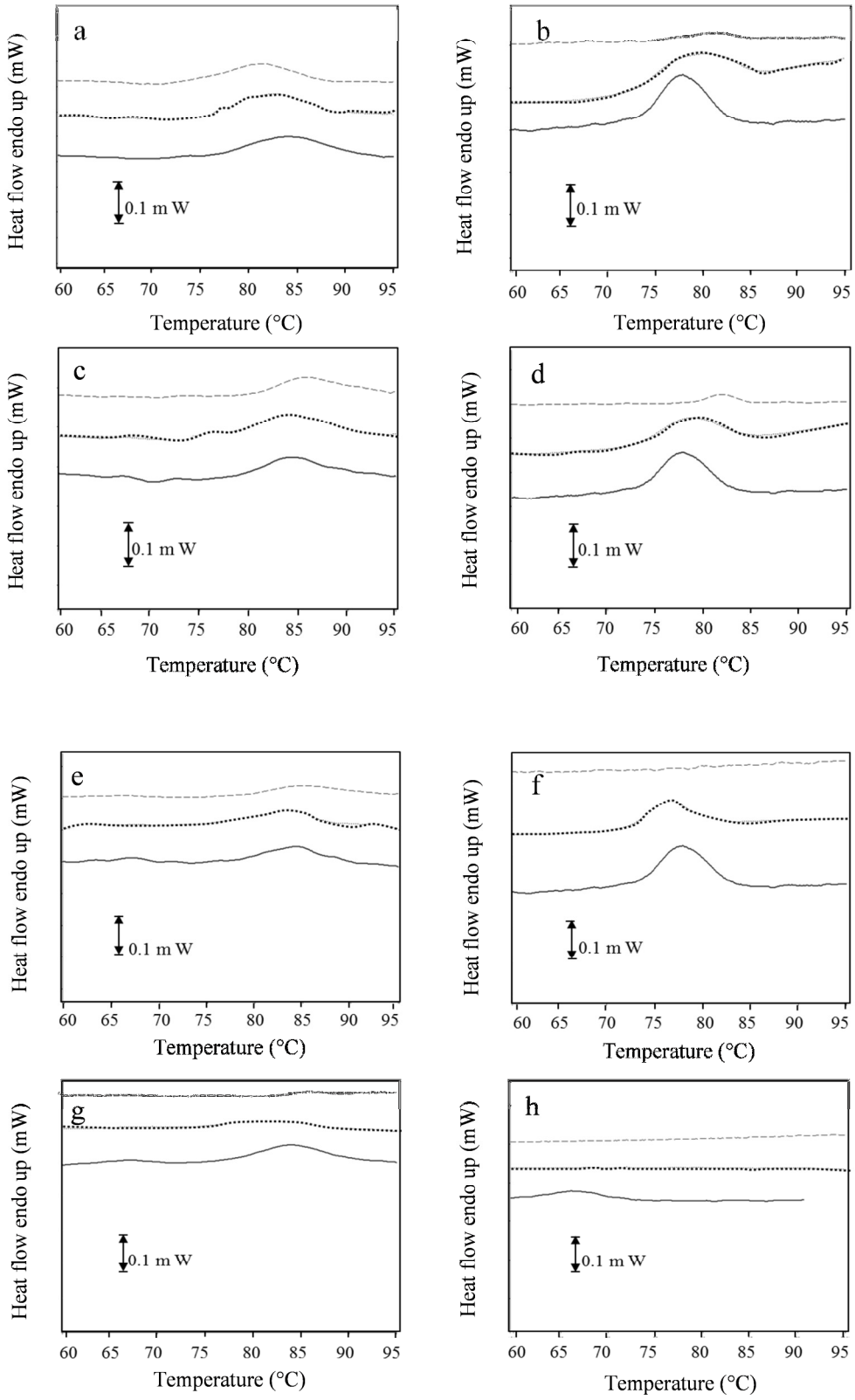
a
b
c
d
e
f
g
h
Heat flow endo up (mW)
0.1 m W
60 65 70 75 80 85 90 95
Temperature (°C)

Figure 13. Differential scanning calorimetry of partially glycosylated whey protein (PGWP) (a) and whey protein isolate (WPI) (b) before and after heating at 75 and 80 °C for 30 min at pH 3.4 PGWP (c) and WPI (d) before and after heating at 75 and 80 °C for 30 min at pH 4.5; PGWP (e) and WPI (f) before and after heating at 75 and 80 °C for 30 min at pH 5.5; PGWP (g) and WPI (h) before and after heating at 75 and 80 °C for 30 min pH 7. Solid line: before heating; dotted line: heated at 75 °C; dashed line: heated at 80 °C.

Previous work has shown that protein isolates were completely denatured post Maillard glycosylation (Zhu et al., 2010). The preservation of the protein structure of our PGWP compared to the glycosylated protein isolates produced by other researchers could be attributed to the fact that our PGWP was a mixture of reacted and unreacted proteins, and to the mild separation conditions we have utilized to isolate the proteins from free dextrans.

2.3.3.4. Protein distribution and heat-induced polymerization

Glycosylation of whey protein was distinctively noted by the appearance of a longitudinal smear (Fig. 14a, comparing lane 2 to lane 9), indicating the formation of new glycosylated proteins, as confirmed by the glycoprotein staining (Fig. 14c). The wide spread of molecular weight (~20 to 200 kDa) is attributed to the heterogeneous distribution of conjugated dextrans. Under reducing conditions (Fig. 14b), the smear remained visible, confirming that the newly formed species were not disulfide-linked polymers. Disulfide polymerization apparently was not promoted during the incubation conditions used to produce PGWP. Similar heterogeneous distribution of glycosylated proteins were are observed by previous researchers (Lillard, et al. 2009; Zhu et al, 2010)

Upon heating of WPI, a noticeable decrease in both β-lg and α-la band intensity was observed with increased heating time, coupled with an increase in disulfide polymerization, as noted by the increase in the intensity of the bands at the top of the resolving gels (Fig. 14a, lanes 3-8). A reduction in β-lg and α-la band intensity and an

appearance of a dark band at the top of the resolving gel was also observed for heated PGWP solutions (Fig. 14a, lanes 10-15). However, no noticeable change in band intensities was noted as heating time increased from 10 to 60 min for the PGWP samples. The dark bands disappeared when both WPI and PGWP samples were run under reducing conditions (Fig. 14b), confirming the formation of disulfide linkages upon heating. Although disulfide polymerization upon heating of PGWP did occur, it was less pronounced than that observed for heated WPI samples, especially with prolonged heating. This observation can be partially explained by the fact that PGWP resisted heat-induced unfolding and complete denaturation. Partial unfolding, as compared to complete denaturation, reduces the chances of hydrophobic interactions and subsequent disulfide linkages. Additionally, PGWP most likely have stronger repulsive forces than WPI due to glycosylation, thus limiting disulfide linkages.

The protein profile on SDS gels of heated WPI solutions at pH 4.5 and 5.5 were comparable to those of samples heated at pH 3.5 and 7 (gels not shown). The SDS buffer, which breaks down non-covalent bonding, solubilized the protein in pH 4.5 and 5.5 solutions, allowing them to move down the gel. This observation indicated that the aggregates/precipitates contributing to turbidity and reduction in solubility of WPI solutions at pH 4.5 and 5.5 were mostly formed by hydrophobic and electrostatic interactions, as well as hydrogen bonding. Since heated PGWP solutions, on the other hand, remained soluble and clear at pH 4.5 and 5.5, it can be presumed that partial glycosylation reduced the non-covalent intermolecular interactions of whey proteins.

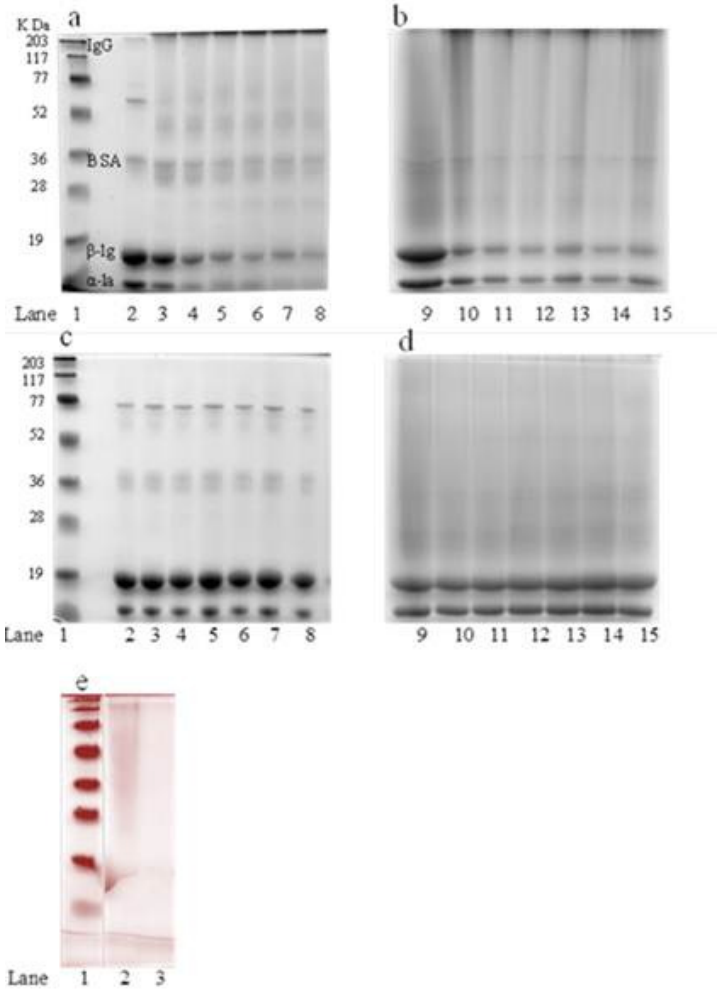

Figure 14. SDS-PAGE gels with Coomassie blue staining under non-reducing conditions (a & b) and under reducing conditions (c & d); lane 1: molecule weight standard, lane 2: non-heated whey protein isolate (WPI), lanes 3-8: WPI heated at 75 °C for 10, 20, 30, 40, 50, and 60 min, lane 9: non-heated partially glycosylated whey protein (PGWP), lanes 10-15: PGWP heated at 75 °C for 10, 20, 30, 40, 50, and 60 min; and SDS-PAGE gel with glycoprotein staining under non-reducing conditions (e); lane 1: molecular weight standard, lane 2: non-heated PGWP, and lane 3: non-heated WPI.

2.3.4. Structural characterization of PGWP

2.3.4.1. Isoelectric point, surface hydrophobicity and surface sulfydryl groups (SH)

As hypothesized, the pI of PGWP shifted to a more acidic pH as compared to WPI, from 4.86 to 4.28. This shift is due to the blockage of some amino groups that are otherwise protonated at a pH lower than their pK_a values, consequently resulting in an increase in net negative charge. The increase in net negative charge partially explains the enhanced solubility at pH 4.5 and 5.5. However, the new pI is close to pH 4.5, suggesting that there are additional mechanisms for the observed enhancement in the solubility of heated PGWP at this pH. One possible mechanism would be a conformational difference between PGWP and WPI related to the exposure of hydrophobic and reactive SH groups.

The initial surface hydrophobicity index of PGWP prior to heating was significantly ($P \leq 0.05$) lower than that of WPI (Fig. 15), indicating a reduction in overall molecular surface hydrophobicity upon conjugation. Upon heating at 75 °C, a marked and gradual increase in surface hydrophobicity of WPI was observed as heating time increased, with a maximum reached at 30 min. The increase in hydrophobicity reflected the exposure of hydrophobic regions originally buried inside the protein globular structure. A gradual decrease in surface hydrophobicity was observed as heating time increased, most likely due to the formation of intermolecular hydrophobic interactions (Sava & Plancken, 2005). The increase in surface hydrophobicity of PGWP upon heating was much lower than that of WPI. While there was a significant increase post heating at 75 °C for 10 min, no significant increase was noticed with increased heating time. This observation correlates with SDS-PAGE results that showed no noticeable change in band intensities post 10 min of heating time (Fig. 14a). Both observations confirmed the reduced potential for intermolecular interactions upon heating PGWP.

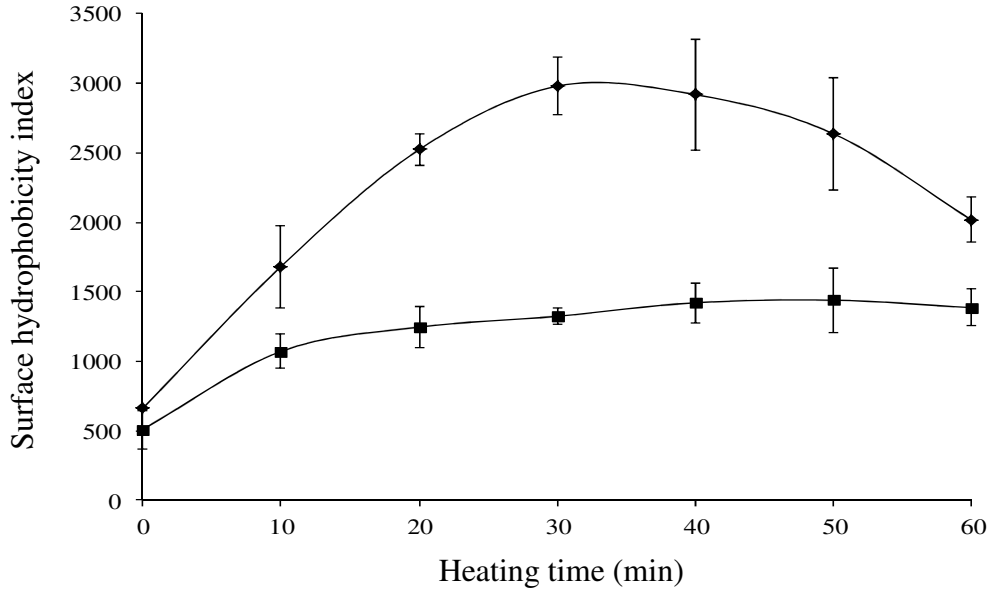

Figure 15. Surface hydrophobicity index of 5% whey protein isolate (WPI) and partially glycosylated whey protein (PGWP) solutions heated at 75 °C for 10-60 min. Error bar represents standard deviations; n=3. WPI: ■; PGWP: ♦.

Similarly, the amount of surface SH groups of WPI significantly ($P \leq 0.05$) increased upon heating, reaching a maximum after 20 min of heating, and then gradually decreased as heating time increased (Fig. 16). The decrease in SH with extended heating time is most likely attributed to the formation of disulfide linkages. The reactivity of Cys^{121}, buried in the interior moiety of β-lg (Sava, & Plancken, 2005), can be markedly increased upon thermal denaturation, which causes its exposure and participation in SH/S-S interchange reaction, involving both β-lg and α-la. The newly formed disulfide linkages play an important role in protein aggregation and precipitation. The increase in surface SH groups of PGWP, however, did not significantly increase until after 40 min of heating, reaching a max at 50 min. Therefore, moderate heating temperatures and time will not result in enhanced reactivity of Cys^{121} in PGWP.

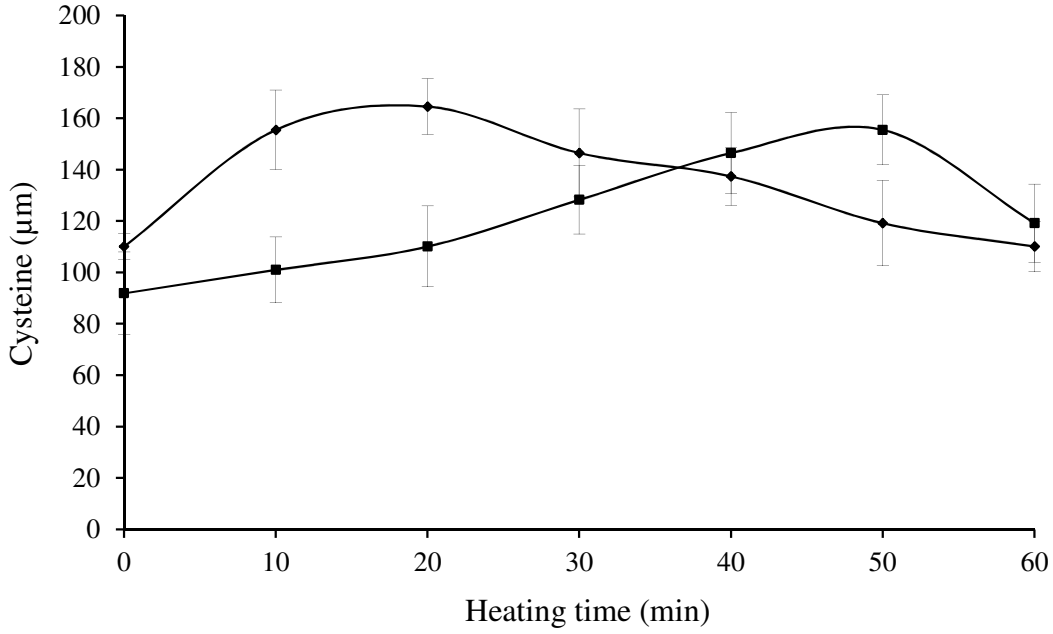

Figure 16. Surface sulfhydryl groups of 5% whey protein isolate (WPI) and partially glycosylated whey protein (PGWP) solutions heated at 75 °C for 10-60 min. Error bar represents standard deviations; n=3. WPI: ■ ; PGWP: ♦.

The observed surface hydrophobicity and SH data clearly demonstrated that PGWP, as compared to WPI, resisted heat-induced unfolding. This resistance to thermal denaturation and unfolding was also demonstrated by the previously discussed DSC measurements. The minimal exposure of the reactive SH of Cys^{121} and the hydrophobic residues upon heating of PGWP, in addition to the reduced pI, will prevent protein aggregation thus greatly contributing to whey protein thermal stability at relatively high concentrations and over a wide range of pH.

2.3.4.2. Glycosylation sites

Compared to the available database for trypsin digested β-lg and α-la peptide profile, the WPI m/z spectra represented a mixture of digested β-lg A, β-lg B and α-la with only a few unknown peaks, most likely attributed to other protein components of WPI, such as BSA and IgG. Similar peaks were found in the spectra of digested PGWP, in addition to

few new peaks (Fig. 17). The peak intensities of these new species were much lower than those of the non-modified peptides, confirming the limited degree of glycosylation. One new peak had m/z of 1727 a.m.u, which was greater than the peak of m/z 917 a.m.u found in both WPI and PGWP. The mass difference of 810 is equivalent to the mass of an oligosaccharide comprised of 5 glucose units. This peptide was determined to be a β-lg peptide (84-91) with the sequence of IDALNENK. Therefore, glycosylation occurred at Lys^{87}, which is located at the EF loop of the β-barrel of β-lg molecule. It is known that the EF loop acts as a gate over the β-barrel to access the hydrophobic core. At low pH, the loop is in the "closed" position, whereas at high pH it is open. The "latch" for this gate is Glu^{89} (Kontopidis, Holt, & Sawyer, 2004). The attachment of a bulky dextran at this position may suppress the function of this "latch", leaving the conformational changes of this loop less sensitive to pH changes. This observation partially explains the observed reduction of pH sensitivity of PGWP, by being soluble over a wide pH range. This also explains the observed reduction in intermolecular hydrophobic interactions discussed earlier.

Another new peak at m/z 5359 a.m.u was determined to have a mass shift equivalent to the mass of an oligosaccharide comprised of 25 glucose units from α-la peptide with m/z 1309 a.m.u (1-10). The amino acid sequence of this α-la peptide is EQLTKC(Carbamidomethyl)EVFR, which has three potential glycosylation sites including the N-terminus of Glu^{1}, the ε-amino of Lys^{3} and the guanidine amino group of Arg^{10}. This glycosylation site is located at the very first loop of the first α-helix structure of α-la, which is easily accessible by the dextran.

There were few other new peaks that could not be distinguished as purely glycosylated peptides. Some of these peaks may correspond to intermediate Maillard reaction products. Several researchers have determined the sites of lactosylation of β-lg and α-la (Leonil et al., 1997; Losito, Stringano, Carulli, & Palmisano, 2010); however, this is the first attempt to determine the glycosylation site when polysaccharides were used. Glycosylation with polysaccharides suppresses ionization, which makes it challenging to determine the glycosylation site without the additional step of hydrolyzing the dextran. Hydrolysis using dextranase, however, was very random and heterogeneous, which added

to the complexity of determining the identity of all the newly observed peaks. Therefore, further investigation is needed to confirm the identity of these peaks.

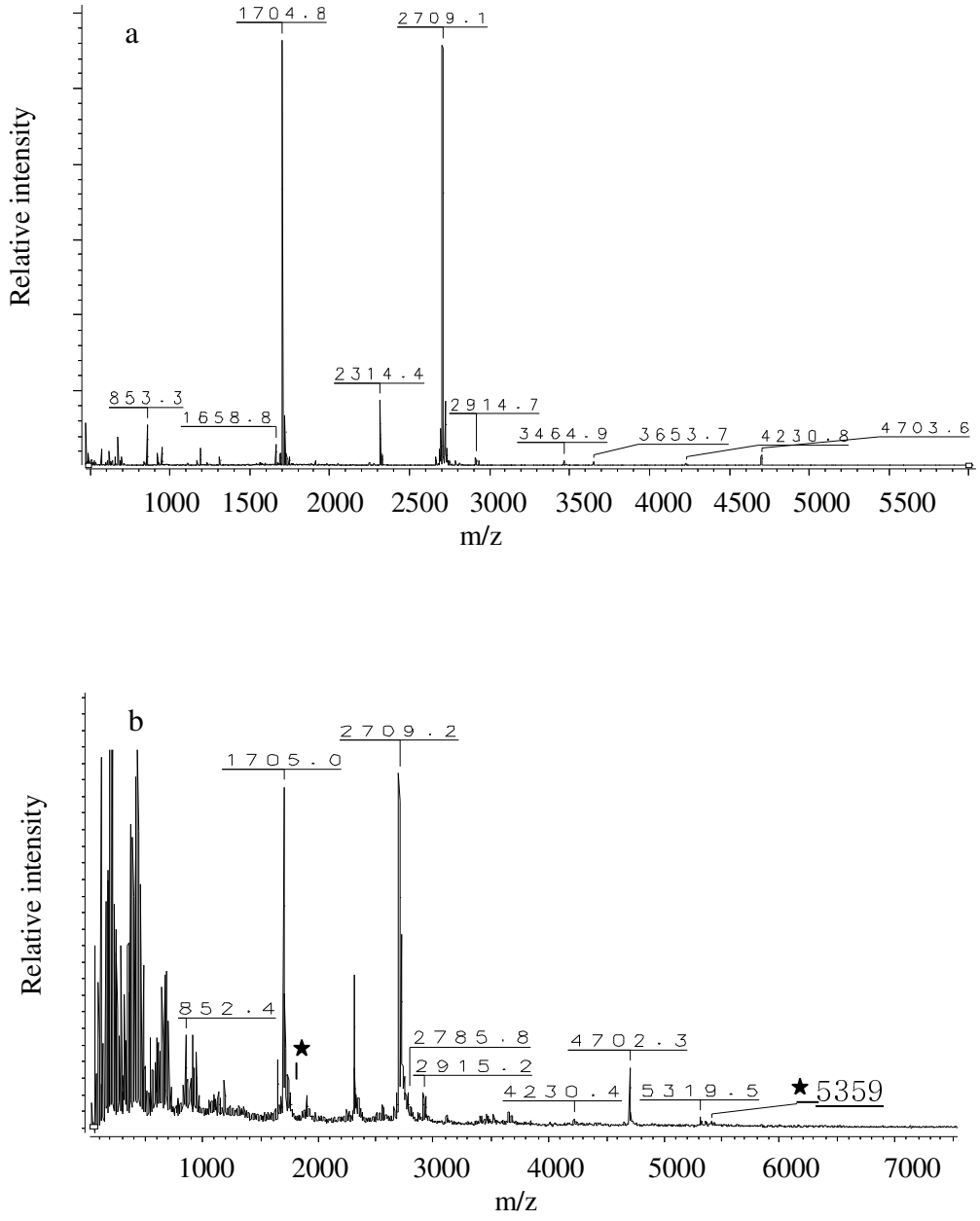

Figure 17. MALDI-TOF MS spectra of trypsin digested whey protein isolate (WPI) (a) and dextranase/trypsin partially glycosylated whey protein (PGWP) (b). ⋆: glycosylated peptide.

2.4. Conclusions

The Maillard-induced glycosylation conditions utilized in this study limited the propagation of Maillard reaction to advanced stages and preserved the nutritional quality of whey protein. Further, partially glycosylated whey protein, i.e., the mixture of free and glycosylated whey protein, remained soluble over a wide range of pH at protein concentrations greater than 4.2%. This observation confirmed that further fractionation to remove unreacted proteins post Maillard-induced glycosylation is not necessary. The enhanced solubility and thermal stability of PGWP, even when the pH was close to the pI of the whey protein, was attributed to the resistance to denaturation and structural modifications. Further structural characterization using Raman spectroscopy is underway. Additionally, thermal stability and solubility of PGWP over an extended period of storage is currently being tested at the pH and protein concentration used in this study. Finally, when using PGWP to produce acidic beverages at protein concentration > 4%, the formulation pH can be 4.5 instead of 3.4. At pH 4.5 sourness perception is reduced and the amount of sugar needed to balance the acidity will be less, thus providing a healthier choice for consumers. The assessment of sensory quality of beverages formulated using PGWP is currently underway.

3. Structural Characterization of Partially Glycosylated Whey Protein as influenced by pH and Heat using Surface-Enhanced Raman Spectroscopy*

*: Content of this chapter was submitted to Food Chemistry

Wang, Q., He, L.[1], Labuza, T.[2], and Ismail, B. *Food Chem.,* submitted, April 3rd of 2012.

[1] Provided help with Raman data analysis and PCA

[2] Provided insights regarding the use and application of SERS

Maillard-induced glycosylation of whey protein improves solubility and thermal stability over a wide pH range. However, the relationship between structural changes and functional enhancement upon glycosylation is not well-characterized. Therefore, our objective was to characterize these structural changes and determine the protein conformation at various pH and thermal treatments, using surface-enhanced Raman-spectroscopy. The spectra of glycosylated protein revealed a new peak at 983 cm^{-1} that can be used as a Raman marker for the early stage glycosylation. Upon glycosylation, structural variations were significant at the disulfide, hydrophobic, Amide III, Amide II, and Amide I zones. Ionization of carboxyl groups at all tested pH values, and increased β-sheet configuration were also observed. The noted structural modifications imparted molecular rigidity and a consequent resistance to denaturation upon thermal treatment over a wide pH range. These findings can be used to explain various functional enhancements of whey protein upon glycosylation.

3.1. Introduction

Thermal processing and prolonged storage of whey protein-based beverages results in protein aggregation and thus deterioration of quality. Protein aggregation is the consequence of thermal denaturation of the protein and the subsequent intermolecular hydrophobic and electrostatic interactions, as well as disulfide linkages. These interactions are governed by the pH of the system, with minimal interactions being at acidic pH (< 3.5). However, even at acidic pH, protein/protein interactions become significant as protein concentration increases (> 4%). Consequently, whey protein acidic beverages available on the market have a short shelf life and contain at most 4% protein.

We have recently demonstrated enhancement of protein solubility and thermal stability upon partial and limited Maillard-induced glycosylation (Wang & Ismail, 2012). As compared to whey protein isolate (WPI), the partially glycosylated whey protein (PGWP) had improved solubility and thermal stability over a broad range of pH, including the isoelectric point (pI, pH 4.5-5.5) of whey protein, and at protein concentrations greater than 4% (5-10%). The enhanced solubility and thermal stability of PGWP was attributed to suppressed intermolecular interactions. Several physicochemical/structural changes, including resistance to denaturation, shift to a more acidic pI, reduced surface hydrophobicity, reduced exposure of sulfhydryl groups, and unique glycosylation sites contributed to the reduced intermolecular interactions.

Although several studies (Xu, Yuan, Wang, Li, Hou, & Gao, 2011; Sinha, Radha, Prakash, & Kaul, 2006; Song, Babiker, & Kato, 2002) have shown functional enhancements upon Maillard-induced glycosysation of food proteins, very few, if any, have characterized the conformational changes responsible for the functional improvements. Raman Spectroscopy, which is gaining popularity in food science applications, is a promising tool to unfold molecular conformation that can explain the structure/function relationship.

Raman spectroscopy, a vibrational light scattering technique, allows for the prediction of molecular identity based on unique spectral analysis of stretching and bending of molecular bonds, collectively producing fingerprint spectra for a particular molecule (Sun, Zhao,

Zhao, Yang, Cui, & Ren, 2011). This technique is sensitive to changes in covalent bonds, such as disulfide linkages, and non-covalent bonds, such as electrostatic and hydrophobic interactions. It is also able to detect transitions from ordered to disordered structure upon protein denaturation (Dàvila, Parés, & Howell, 2006; Ikeda & Li-Chan, 2004; Alizadeh-Pasdar, Nakai, & Li-Chan, 2002). Raman spectroscopy can provide information related to protein backbone conformation and the molecular environment of certain side chains. This technique, for instance, has been applied successfully to study protein unfolding upon heating (Hédoux, Guinet, & Paccou, 2011), and structural changes upon chemical modifications of food proteins (Wong, Choi, Phillips, & Ma, 2009). Reported conformational changes in protein structure included changes in amide I zone, α-helix, β-sheet, CH stretching, and disulfide linkages upon applying various pH, heat, and other treatments (Thawornchinsombut, Park, Meng, & Li-Chan, 2006; Clark, Saunderson, & Suggett, 1981).

Compared to other techniques, such as X-ray crystallography, nuclear magnetic resonance, and circular dichroism, Raman spectroscopy is a nondestructive and rapid analytical technique that requires minimal sample preparation (He, Lin, Li, & Kim, 2010). Another advantage of Raman spectroscopy is the ability to analyze aqueous protein solutions with no water interferences (He, Haynes, Diez-Gonzales, & Labuza, 2010). However, Raman spectroscopy has low sensitivity, which limits its applications to powders or highly concentrated samples. A more sensitive Raman technique, surface-enhanced Raman spectroscopy (SERS), was developed to characterize various chemical and biological compounds (Han, Zhao, & Ozaki, 2009; Yakes, Lipert, Bannantine, & Porter, 2008). Raman scattering can be amplified tremendously in the near vicinity of nanoscale-roughened noble metal substrates, such as silver (Ag) dendrites (He, Haynes, Diez-Gonzales, & Labuza, 2010). Therefore, only trace amount of sample is needed to obtain structural information, improving the limit of detection to parts per billion. Most of the studies on protein structural configuration using Raman spectroscopy analysis were based on the use of the traditional technique. Whereas, most of the studies using SERS analytical approach focused on the ability to detect the presence of trace amount of targets in various

matrices, such as detection of allergens in complex food matrices (He, Haynes, Diez-Gonzales, & Labuza, 2010).

In this study, we took the advantage of SERS coupled with dendritic silver nano-substrates to characterize the protein structure of PGWP as compared to WPI in aqueous solutions, and to determine the conformational changes incurred upon various pH and thermal treatment.

3.2. Materials and Methods

3.2.1 Preparation of partially glycosylated whey protein (PGWP)

Partially glycosylated whey protein (PGWP) was prepared as outlined by Wang and Ismail (2012). Whey protein isolate (WPI, 92.7% protein, as confirmed by Dumas, AOAC 990.03, using a Nitrogen Analyzer, LECO® TruSpec TM) was obtained from a commercial source. WPI was mixed with dextran (10 kDa, D9260 from Sigma-Aldrich, St. Louis, MO, USA) in 1:4 ratio (w/w), dissolved in phosphate buffer (0.1 M, pH 7) and lyophilized. The lyophilized powder was incubated in a dessicator at 60 °C and water activity (a_w) of 0.49 for 96 h. Separation of free dextrans from the reacted and non-reacted proteins, collectively labeled as partially glycosylated whey protein (PGWP), was performed using hydrophobic interaction chromatography (HIC). Pharmacia Biotech fast protein liquid chromatography (FPLC) system, equipped with a UV detector and a fraction collector, was used. An HIC column, 18.5 cm x 16 mm, was packed with phenyl sepharose high performance media (GE healthcare®) and equilibrated with 1M ammonium sulfate. Incubated protein/dextran mixture was dissolved in 1M ammonium sulfate (4% protein, w/v). An aliquot (3 mL) of the solution was loaded on the column and the flow rate was set at 1 mL/min. Free dextran was washed with 50 mL of 1M ammonium sulfate followed by 20 mL of de-ionized distilled water (DDW). Glycosylated and free proteins (PGWP) were eluted with 30 mL DDW. Elution of the protein was monitored at 280 nm. Collected PGWP was then dialyzed against DDW, using 3.5 kDa cut-off membrane (Thermal Scientific, Portsmouth, NH, USA), and lyophilized. Dextran

and protein content of the collected fractions were 30% and 60%, respectively, as determined by phenol-sulfuric acid assay, AOAC 44.1.30, and Dumas, AOAC 990.03.

3.2.2. Preparation of thermally treated WPI and PGWP at different pH

In triplicate, solutions (1 mL) of WPI and PGWP were prepared in screw-cap microcentrifuge tubes at 5% protein concentration (w/v), and the pH was adjusted to 3.4, 4.5, 5.5, and 7. Prepared solutions were then heated in a water bath at 75 ºC for 30 min. After heating, samples were cooled down to room temperature (23 °C) and then subjected directly to SERS analysis. Control solutions with no thermal treatment were also subjected to SERS analysis.

3.2.3. Ag dendrite preparation

Ag dendrite was prepared using a replacement reaction involving both zinc (Zn) and silver nitrate ($AgNO_3$) according to the method described by He, Lin, Li, and Kim (2010). Briefly, a zinc plate (Fisher Scientific, Rochester, NY, USA) was immersed in 200 mM $AgNO_3$ solution for 1 min to form the dendritic Ag. The dendritic Ag was then manually removed from the zinc plate with tweezers, put into a glass vial, and rinsed several times with DDW to remove remaining precursor species such as Zn^{2+} and NO_3^-.

3.2.4. SERS sample preparation

An aliquot (2 µL) of the Ag dendrite suspension was deposited onto a glass microscopic slide and air-dried at room temperature. Then, a sample (2 µL) was deposited onto the spots of Ag dendrites. The aqueous sample, without further drying, was directly analyzed by SERS to avoid conformational changes induced by drying.

3.2.5. Raman instrumentation

A DXR Raman microscope (Thermal Fisher Scientific, Madison, WI, USA) was used. The instrument facilitates 780 nm excitation of SERS spectra through a ×10 confocal microscope objective, resulting in a laser spot of approximately 3 μm in diameter, and a 5 cm^{-1} spectral resolution. Quadruplicate SERS measurements were taken using 4 mW laser power and 25 μm slit aperture for 15 s integration time. Spectra were collected using the Thermo Scientific OMNIC Software with Atlμs. Eight spectra were collected for each sample from randomly picked spots on the Ag dendrite surface.

3.2.6. Spectral data analysis

SERS spectral data were analyzed by the TQ Analyst software (Thermal Fisher Scientific, Madison, WI, USA). Standard normal variant, second derivative transformation, and smoothing were applied when necessary to reduce spectral noise, normalize the spectra, separate overlapping bands, and remove baseline shifts. Principal component analysis (PCA) was applied to analyze the variance of spectral data, indicating patterns of significance. The principal component (PC) score reveals the percentage of data variance. A higher percentage indicates more data variance within the PCA model. Eigenvalue, which is the amount of variance that is explained by a given component, was used to determine the variance of each of the major PCs. The PC correlation coefficients were calculated from the correlation matrix (Massart, Vandeginste, Deming, Michotte, & Kaufman, 2008).

3.3. Results and Discussion

3.3.1. Effect of Maillard-induced glycosylation on whey protein structural conformation

Raman spectra of WPI and PGWP were compared at pH 3.4 since both are stable at this pH. Several conformational changes in protein structure induced by Maillard glycosylation were observed (Fig. 18). PCA of the Raman bands resulted in two PC

scores, PC1 and PC2, with eigenvalues >1, that account for 96.7% and 2.3%, respectively, of the total variations between WPI and PGWP spectra (Fig. 19). The Raman bands that contribute to conformational variations between PGWP and WPI along PC1 are listed in Table 2. The band intensities listed in Table 2 were normalized against phenylalanine (1004-1006 cm^{-1}), whose intensity is unaffected by conformational changes in proteins.

The spectra of PGWP had a unique band at 983 cm^{-1}, which was absent from those of WPI and dextran (Fig.18a). To the best of our knowledge, this band has never been reported by other researchers investigating whey protein structural conformation. A band around this wave number may correspond to C-C, C-O-C, or C-N vibration (Maquelin, Kirschner, Choo, van den Braak, Endtz, & Naumann et al., 2001). Therefore, the newly observed band at 983 cm^{-1} likely corresponds to the C-N bond formed upon the Maillard reaction between the carbonyl carbon of the sugar and the amine group of the protein (Fig. 5, Chapter 1, p30). Alternatively, this band may be attributed to a conformational change in the protein backbone that occurred at the glycosylation site. In any case, this unique band can be considered a useful Raman marker for glycosylated whey protein formed by early stage Maillard reaction.

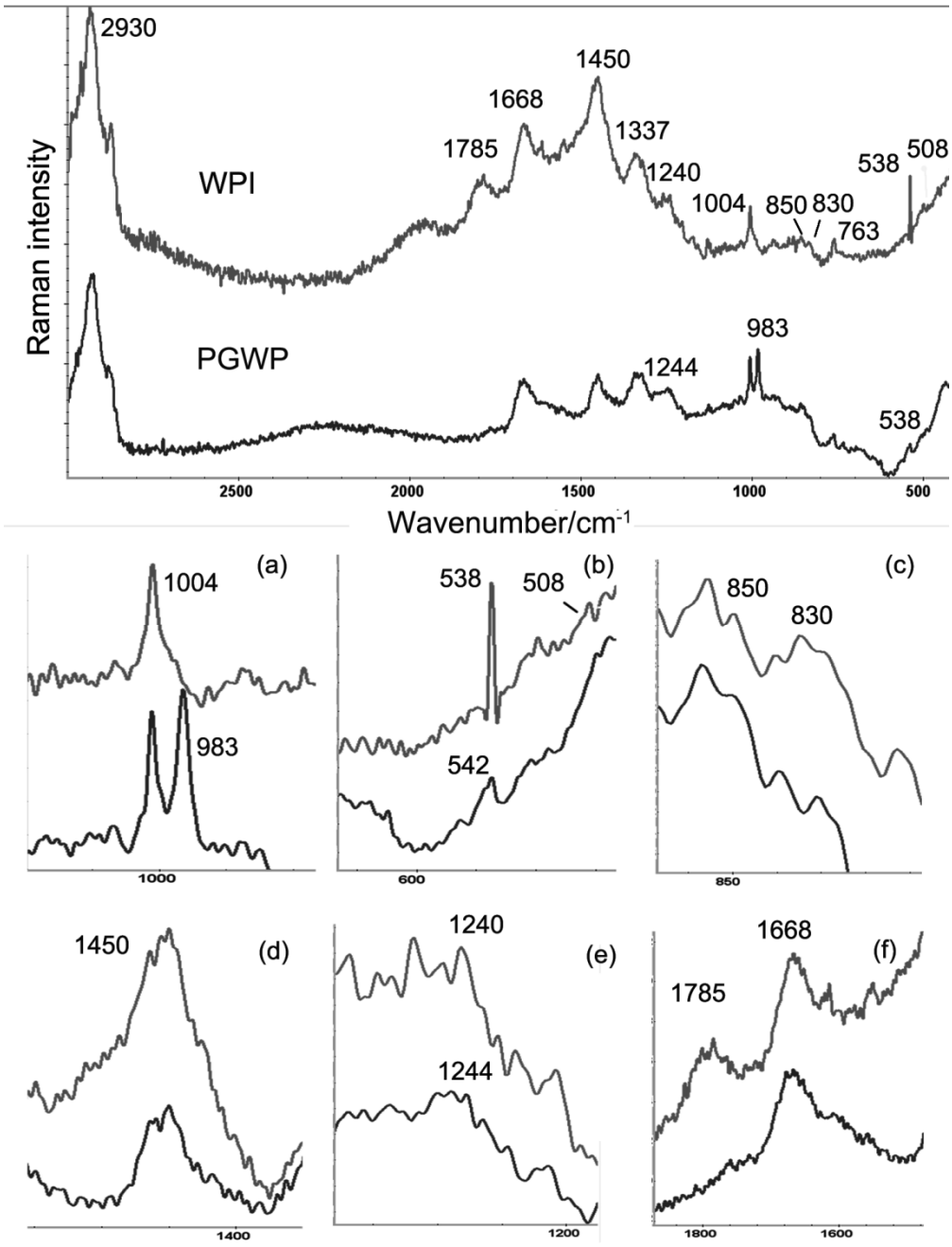

Figure. 18. Raman spectra of WPI and PGWP at 5% protein concentration (w/v) and pH 3.4, and expanded spectral regions near 983 (a), 538 (b), 850 and 830 (c), 1450 (d), 1240 (e), and 1785 (f) cm^{-1}.

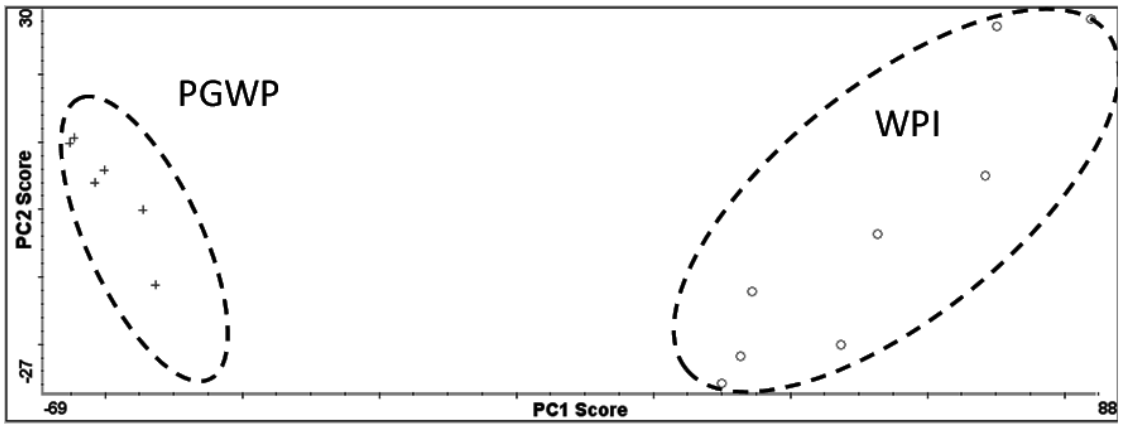

Figure. 19. PCA plot for Raman spectra of WPI and PGWP at pH3.4.

The band at 508 cm^{-1}, which is in the spectral region that corresponds to disulfide bonds (510 to 550 cm^{-1}), was detected in the WPI spectra yet was absent from that of PGWP. Additionally, as compared to WPI spectra, the band intensity at 538 cm^{-1} was less pronounced in the PGWP spectra with a new broader band showing at 542 cm^{-1} (Fig. 18b). According to literature (Maquelin, Kischner, Choo, can der Braak, Endtz, & Naumann et al., 2001), the band at 508 cm^{-1} in the spectra represents disulfide bonds in *gauche-gauche-gauche* conformation, and the one at around 538 cm^{-1} represents disulfide bonds in *trans-gauche-trans* conformation. These changes in the disulfide regions suggest that the native conformation of disulfide linkages in β-lactoglobulin (β-lg) and a-lactalbumin (α-la) were altered upon glycosylation. Similar changes were observed upon heat-induced gelation of β-lg (Dàvila, Parés, & Howell, 2006; Ikeda & Li-Chan, 2004). There are two disulfide bonds in β-lg and one very reactive free cysteine, Cys^{121}. The free Cys^{121} contributes to the flexibility of the protein molecule, which readily unfolds upon heat-induced denaturation facilitating the role of Cys^{121} in intermolecular disulfide exchange reactions leading to protein polymerization (Nonaka, Li-Chan, & Nakai, 1993). Absolute conclusions pertaining to the Cys^{121} reactivity and its role in intermolecular disulfide linkages in heated PGWP solutions cannot be made. However, based on our recent observations in model systems, it is assumed that Cys^{121} induced intramolecular disulfide interchanges with Cys^{106}- Cys^{119} or Cys^{66}- Cys^{160}, causing bands to shift and

change in intensity in the 510 to 550 cm^{-1} region. This possible change in the disulfide linkages may contribute to a more rigid, thermally stable protein structure that resists complete unfolding and subsequent intermolecular disulfide interchange.

Table 2. Relative band intensities and assignments at selected regions of the Raman spectra of WPI and PGWP.

Assignment	PC 1	Wave number (cm^{-1})	Intensity	
			WPI	PGWP
CN	-- [a]	983	- [b]	1.16
SS	++	508	0.07	-
	++	538	1.75	0.64
	--	542	-	0.56
Trp	++	759	0.68	0.23
	++	763	0.59	0.21
Tyr	--	850/830	1.12	1.43
CH	++	2930-2933	3.69	2.56
Amide II, CH_2, CH_3	++	1450	3.53	0.69
	++	1455	3.4	0.62
	++	1463	3.18	0.62
Amide III	--	1240-1244	0.15	0.36
Amide I	--	1668	0.89	1.07
RCOOH	++	1785	1.21	-

[a] Double signs represent a PC coefficient greater than half of the maximum coefficient in absolute values. Positive signs indicate positive correlations while negative signs represent negative correlations. [b] no peak was detected.

The relative intensity of the band at 760 cm^{-1}, which corresponds to the buried tryptophan (Trp) (Ikeda & Li-Chan, 2004), was lower in PGWP than WPI spectra (Table 2). The Trp residues, originally buried within the protein globular structure, became

exposed to the polar environment after glycosylation, indicating partial unfolding of the protein. Observed changes in the tyrosine (Tyr) bands at 850 cm^{-1} and 830 cm^{-1}, also suggested partial unfolding of the protein upon glycosylation. The ratio of the relative intensity at 850 cm^{-1} and 830 cm^{-1} (I_{850}/I_{830}) is commonly used to determine the microenvironment around Tyr (Ikeda & Li-Chan, 2004). A higher I_{850}/I_{830} ratio is an indicator of Tyr exposure to a more polar environment (Dàvila, Parés, & Howell, 2006). The relative intensity of the 830 cm^{-1} band was lower for PGWP than WPI (Fig. 18c), resulting in a higher I_{850}/I_{830} ratio, thus indicating that Tyr became exposed upon glycosylation. Additionally, the reduced intensity at 830 cm^{-1} reflects less ionization of the phenolic hydroxyl oxygen and less hydrogen bonding between Tyr and a negatively charged acceptor, such as a carboxylate ion of aspartic acid (Asp) or glutamic acid (Glu) (Ikeda & Li-Chan, 2004). The partial unfolding of the protein upon glycosylation exposed Tyr to the polar environment and drew it farther away from Asp and Glu residues, thus reducing hydrogen bonding.

A lower band intensity around 2930-2933 cm^{-1} was observed in the spectra of PGWP as compared to that of WPI, reflecting less C-H stretching of aromatic and aliphatic amino acids (Alizadeh-Pasdar, Nakai, & Li-Chan, 2002). Bands from 1440-1460 cm^{-1}, assigned to CH_2 or CH_3 bending in Amid II zone decreased after conjugation, reflecting conformational changes in the protein backbone (Fig. 18d). Differences in intensity of these bands reflect changes in polarity of the environment around the hydrocarbon chains (Zhao, Ma, Yuan, & Phillips, 2004). This observation further confirms partial unfolding of the protein upon glycosylation.

Another important conformational change was observed at amide III zone, 1200-1300 cm^{-1}, and amide I zone, 1667-1673 cm^{-1}. The band observed at 1240 cm^{-1}, representing β-sheet in WPI, shifted to 1244 cm^{-1} in PGWP and increased in intensity. The band representing β-sheet configuration in amide III zone is often reported near 1230-1243 ± 2 cm^{-1} (Fig. 18e) (Wong, Choi, Phillips, & Ma, 2009; Ikeda & Li-Chan, 2004; Zhao, Ma, Yuan, & Phillips, 2004). The band intensity at 1668 cm^{-1}, which corresponds to antiparallel β-sheet configuration, also increased significantly in PGWP. It is apparent that β-sheet-like configuration was formed upon glycosylation. β-sheet configuration is more thermally

stable than the α-helix and disordered structure configurations, thus it is assumed that PGWP is more thermally stable than WPI.

A major conformational difference was also noted at 1785 cm^{-1} (Fig. 18f). The band at 1785 cm^{-1} corresponds to the carboxylic groups in the protonated form (Wong, Choi, Phillips, & Ma, 2009). A clear band at 1785 cm^{-1} was observed in the spectra of WPI while it was absent in that of PGWP. This indicates that the carboxylic groups of PGWP were mostly in the ionized form (COO^-), contributing to higher net negative charge, thus facilitating greater interaction with the aqueous environment and providing more structural stability. The band that corresponds to COO^- (centered around 1434 cm^{-1}) (Wong, Choi, Phillips, & Ma, 2009; Dàvila, Parés, & Howell, 2006) was not clearly distinguished due to an overlap with Amid I zone. However, the increase in the intensity of the band centered at 1668 cm^{-1} can partially be attributed to deprotonation of the carboxylic groups (Alizadeh-Pasdar, Nakai, & Li-Chan, 2002).

3.3.2. Effect of pH on the structural conformation of WPI and PGWP

Significant differences were observed in the Raman spectra of WPI at various pHs, while almost none was observed for that of PGWP (Fig. 20). PCA of the Raman spectral data resulted in two PC scores, PC1 and PC2, with eigenvalues >1, that account for 89.3% and 7.1% of the total observed variations, respectively. Spectral data points from WPI at different pH values were separated along PC1, with data points at pH 4.5 and 5.5 clustered together. Data points at pH 3.4 and pH 7 were distinctively separated from each other and from those at pH 4.5 and 5.5 (Fig. 21). Spectral data points from PGWP at different pH values were clustered together but were clearly separated from those of WPI along PC1. The spectral data separation between WPI and PGWP were attributed to variation in bands discussed in the previous section.

For WPI, the band at 1785 cm^{-1}, which corresponds to the protonated form of the carboxylic groups, disappeared completely at pH 7 (Table 3, Fig. 21). This observation was not surprising given the tendency of the carboxylic groups to become ionized as the pH increases. On the other hand, no band was noted at 1785 cm^{-1} for PGWP at all tested pHs,

indicating that the ionization status of the carboxylic groups in PGWP was not affected by changes in pH. This means that the net negative charge of the protein in PGWP remains higher than that of WPI even at acidic pH values. The change in the surrounding environment upon glycosylation may have contributed to the resistance of the carboxyl groups to get protonated at low pH.

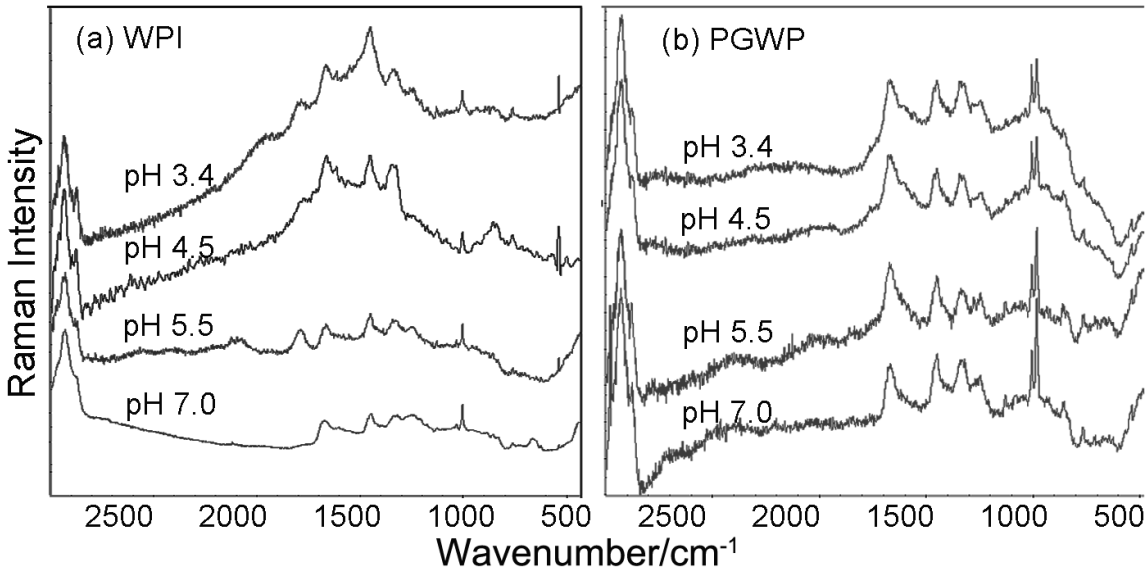

Figure. 20. Raman spectra of WPI (a) and PGWP (b) at pH 3.4, 4.5, 5.5 and 7.

Other structural changes in WPI contributing to variations among the different pH along PC1 were noted at 2930, 1668, 1450 and 538 cm^{-1} (Table 3). This could be attributed to several structural changes including disruption of H-bonding, electrostatic interactions, and disulfide linkages that result in molecular unfolding and reduced stability under stress, such as heat treatment. Whey protein is known to be least thermally stable at its isoelectric point (pH 4.5-4.5) (Sinha, Radha, Prakash, & Kaul, 2006), most likely due to the observed conformational changes around these pH values. Conversely, PGWP across all pH values showed no changes at any of the listed bands, indicating structural stability over a wide pH range.

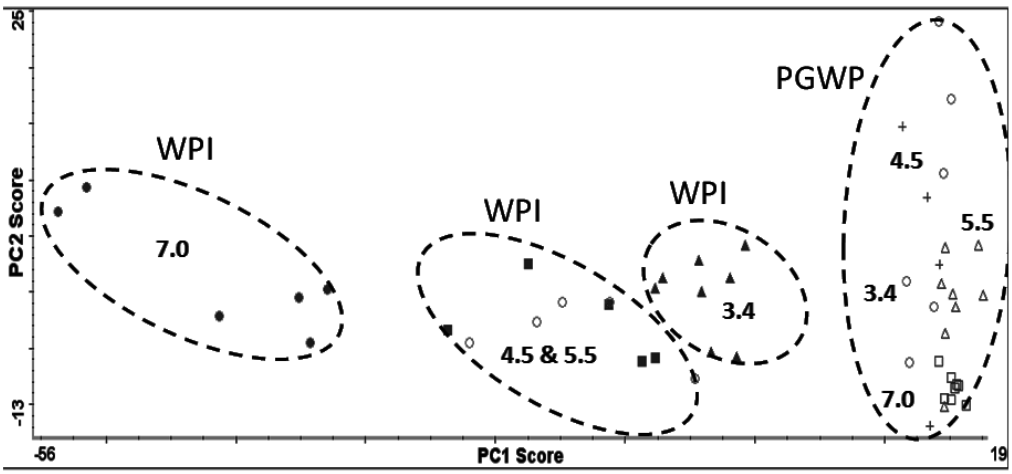

Figure. 21. PCA plot for Raman spectra of WPI and PGWP at pH 3.4, 4.5, 5.5 and 7.

Table 3. Relative band intensities and assignments at selected regions of the Raman spectra of WPI at various pH values.

Assignment	PC1	Wave number (cm^{-1})	Intensity			
			pH 3.4	pH 4.5	pH 5.5	pH 7.0
SS	++ [a]	538	1.75	2.13	0.65	0.17
CH	++	1450	3.53	2.00	1.44	1.07
antiparallel β-sheet	++	1668	0.89	1.13	0.57	0.49
RCOOH	++	1785	1.21	1.05	1.29	0.00
CH	++	2930	3.69	4.55	3.10	3.11

[a] Double signs represent a PC coefficient greater than half of the maximum coefficient in absolute values. Positive signs indicate positive correlations while negative signs represent negative correlations.

3.3.3. Effect of thermal treatment on the structural conformation of WPI and PGWP

Significant differences were observed in the Raman spectra of WPI before and after thermal treatment at pH 3.4, 4.5, 5.5 and 7.0, while minor if any spectral differences were noted for PGWP post thermal treatment regardless of pH (Fig. 22). PCA of WPI spectral

data at pH 3.4, before and after heating, resulted in two PC, PC1 and PC2, with eigenvalues >1, that account for 72% and 21.8%, respectively, of the total observed variations. PCA of WPI spectral data at pH 4.5, before and after heating, resulted in two PC scores, PC1 and PC2, with eigenvalues >1, that account for 88% and 9%, respectively, of the total observed variations. PCA of WPI spectral data at pH 5.5, before and after heating, resulted in one PC score, PC1, with eigenvalues >1, that accounts for 97.8% of the total observed variations. Structural variations upon heating at pH 3.4, 4.5, and 5.5 were mainly attributed to changes in band intensity at 2930, 1785, 1668, 1450, 1337, and 538 cm^{-1} (Table 4), reflecting various conformational changes of the protein backbone, which could be attributed to partial unfolding of the globular protein and exposure of buried groups to polar environment. Additionally, the decrease of band intensities at 1668 cm^{-1}, which was most pronounced at pH 4.5, indicated loss in antiparallel β-sheet structure and thus denaturation of secondary structure.

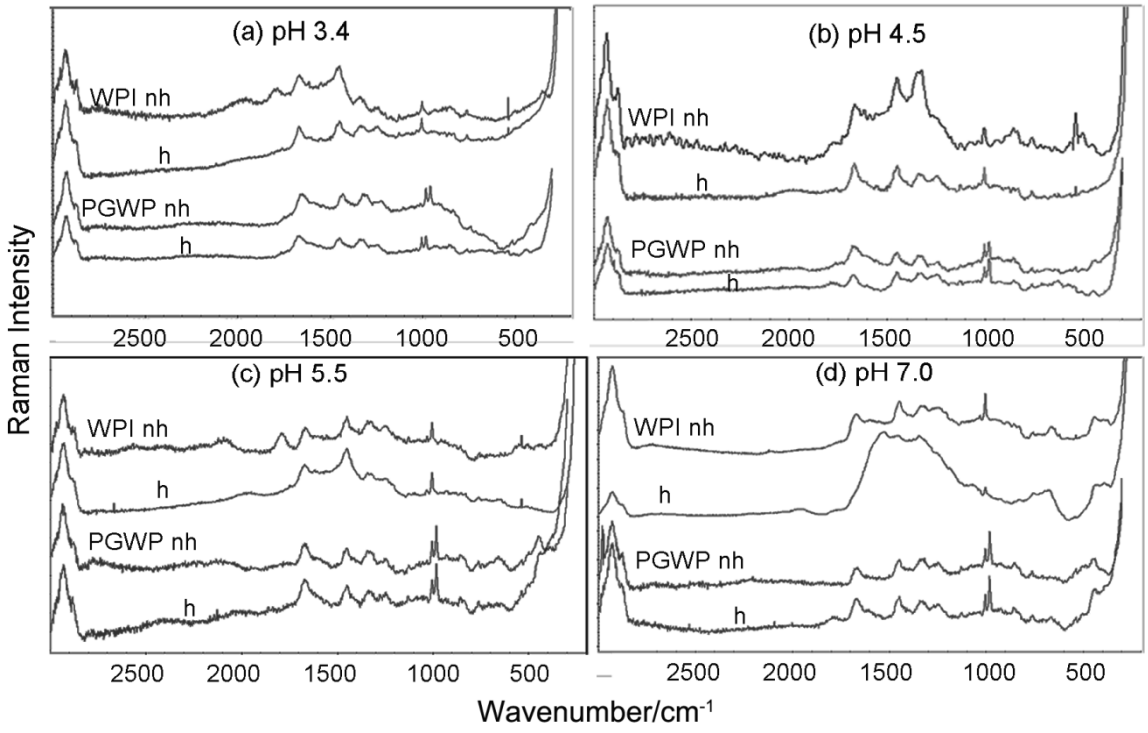

Figure. 22. Raman spectra of WPI and PGWP at pH 3.4 (a), 4.5 (b), 5.5 (c) and 7(d) before (nh) and after heating (h) at 75 °C for 30 min.

PCA of WPI spectral data at pH 7, before and after heating, resulted in two PC scores, PC1 and PC2, with eigenvalues >1, that account for 81.7% and 13.2%, respectively, of the total observed variations. When heated at pH 7, the distinct Amide zones as well as bands representing secondary structures (β-sheet), Try, Tyr, and SS region broadened or disappeared, suggesting a high extent of protein denaturation and loss of tertiary and secondary structure to open random coil.

In contrast to the pH-dependent conformational changes of WPI upon heating, PGWP retained its molecular conformation at all tested pHs. Raman spectral data points of PGWP before and after thermal treatment at each pH were clustered together, without separation along either PC 1 or PC 2 (Fig. 23; PC plot had similar distribution at other pHs, data not shown). This observation confirmed a significant enhancement of structure rigidity upon partial glycosylation. Evidence of denaturation was not seen at any pH, suggesting the resistance to heat-induced molecular unfolding. The enhancement of thermal stability for PGWP was attributed to alterations in secondary and tertiary structures upon partial glycosylation discussed earlier.

Table 4. Relative band intensities and assignments at selected regions of the Raman spectra of WPI at various pH values with (h) and with no heating (nh) at 75 ºC for 30 min.

pH	Assignment	Wavenumber (cm^{-1})	nh	h	PC1	PC2
pH 3.4	SS	538	1.75	0.51	++ [a]	+ [b]
	CH	1450	3.53	0.47	++	-
	CH_2, CH_3	1337	1.17	0.6	++	+
	β-sheet	1240	0.6	0.48	++	+
	antiparallel β-sheet	1668	0.89	0.81	++	-
	RCOOH	1785	1.21	- [c]	++	-
	CH	2930	3.69	4.45	--	+
pH 4.5	SS	538	2.13	0.15	++	
	CH	1450	2.00	1.15	++	
	CH_2, CH_3	1337	1.87	0.3	++	
	β-sheet	1240	0.24	0.48	--	
	antiparallel β-sheet	1668	1.13	0.55	++	
	RCOOH	1785	1.05	-	++	
	CH	2930	4.55	3.85	++	
pH 5.5	SS	538	0.65	0.56	--	
	CH	1450	1.44	2.37	++	
	CH_2, CH_3	1337	0.81	0.42	--	
	β-sheet	1240	0.48	0.42	--	
	antiparallel β-sheet	1668	0.57	0.64	++	
	RCOOH	1785	1.29	-	--	
	CH	2930	3.10	3.65	++	
pH 7.0	SS	538	0.17	-	++	+
	CH	1450	1.07	-	++	+
	CH_2, CH_3	1337	0.86	-	++	-
	β-sheet	1240	0.42	-	++	-
	antiparallel β-sheet	1668	0.49	-	++	+
	RCOOH	1785	-	-	++	+
	CH	2930	3.11	1.02	--	+

[a] Double signs represent a PC coefficient greater than half of the maximum coefficient in absolute values. [b] Single signs represent a PC coefficient less than half of the maximum coefficient in absolute values. Positive signs indicate positive correlations while negative signs represent negative correlations. [c] no peak was detected.

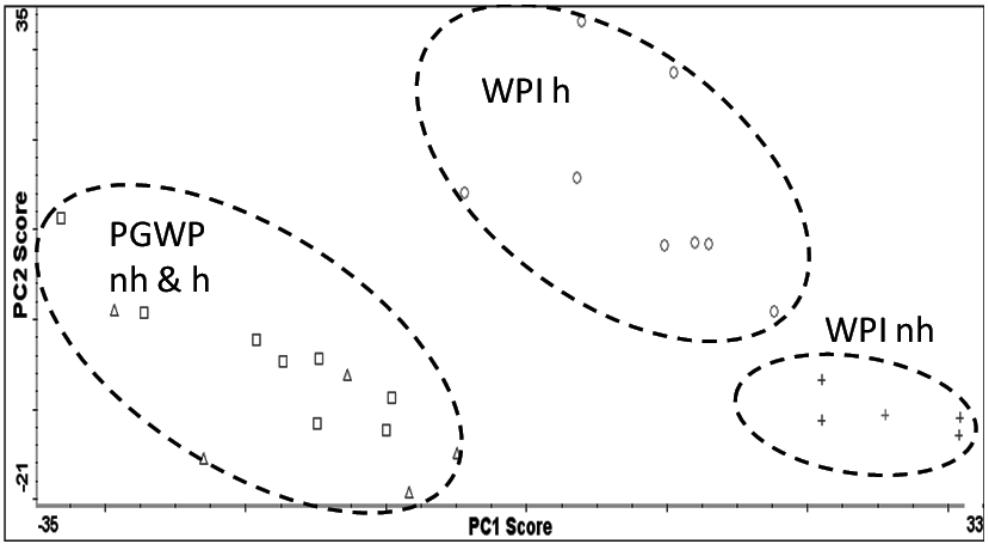

Figure. 23. PCA plot for Raman spectra of WPI and PGWP at pH 5.5 before (nh) and after heating (h) at 75 °C for 30 min.

3.4. Conclusions

SERS was successful in providing data sufficient to determine that significant structural changes of whey protein occurred upon glycosylation and subsequent pH and heat treatments. Distinct structural differences were noted upon glycosylation of whey protein revealing for the first time a Raman marker at 983 cm-1 for glycosylated whey protein formed by early stage Maillard reaction. Other structural changes included ionization of carboxyl groups at all tested pHs, increased β-sheet configuration, reorganization of S-S linkages, and partial unfolding. The noted structural changes imparted structural rigidity to PGWP as compared to WPI, which in turn resulted in resistance to denaturation upon thermal treatment over a wide pH range. These findings can be used to explain various functional enhancement of whey protein upon Maillard-induced glycosylation.

4. Sensory Evaluation of Acidic Beverages Formulated with Glycosylated Whey Protein*

*: Content of this chapter will be submitted to International Dairy Journal

Wang, Q., Vickers, Z.[1], and Ismail, B. *Manuscript in preparation*

[1] Provided insights regarding the sensory evaluation testing and statistical analysis

Partially glycosylated whey protein can be used to formulate a high protein acidic beverage that remains clear post pasteurization at pH greater than 3.4. The sourness and astringency of protein acidic beverages formulated with partially glycosylated whey protein (PGWP) at pH 3.4 and 4.0 were compared to those of a beverage formulated with whey protein isolate (WPI) at pH 3.4. Partial glycosylation of whey protein reduced its buffering capacity, and consequently less acid was required to reach the targeted pH of the fortified beverage. The reduced acidity of PGWP fortified beverages, especially the one formulated at pH 4, resulted in lower (P<0.05) perceived sourness compared to WPI fortified beverage. Results suggested that the perception of astringency of protein beverages can be reduced with lower % acidity. Thus using PGWP in the production of high protein acidic beverages may reduce sourness and astringency compared to products currently present in the market.

4.1. Introduction

Protein fortified beverages, which represent a dominant sector of the functional drinks' market, are consumed mainly by athletes, bodybuilders and health conscientious individuals. Specifically, whey protein beverages are gaining popularity in the market with the increase in consumers' awareness of the physiological benefits of whey protein beyond its high nutritional value. Apart from being a complete protein rich in all the essential amino acids, whey protein is the richest known source of naturally occurring branched chain amino acids (leucine, isoleucine and valine) (Farnfield, Trenerry, Carey, & Cameron-smith, 2009). During and following exercise, the body requires higher amounts of branched chain amino acids, which are taken up directly by the skeletal muscles instead of having to be metabolized first through the liver, as in the case of other amino acids (Morifuji, Koga, Kawanaka, & Higuchi, 2009; Nilsson, Holst, & Björck, 2007). Additionally, whey protein has numerous physiological benefits including but not limited to antimicrobial activity, growth promotional activity and immunoactivity (Sinha, Radha, Prakash, & Kaul, 2007; Manab, Sawitri, Al-Awwaly, & Purnomo, 2011; Petschow & Talbott, 1990; Petschow, Talbott, 1991; Bonomi et al., 2003).

Whey protein beverages produced at pH close to neutral (pH~6.8) are generally opaque and available in chocolate, orange cream, and vanilla flavors. On the other hand, whey protein acidic beverages (pH<3.5) are relatively clear and available in various fruit flavors. Acidic beverages (pH <4.6) are preferred over neutral beverages since they require mild heat treatments, thus maintaining better sensory quality and shelf-life stability (21 CFR 114). Currently, whey protein acidic beverages available in the market contain at most 4% whey protein. To better deliver the nutritional quality and expand the commercial value of whey protein beverages, it is desirable to develop whey protein acidic beverages at concentrations higher than 4.2% protein. FDA requires a protein concentration of at least 4.2% to make a label claim of a "high protein beverage" (21 CFR 101.54 B). It is also desirable to reduce the sugar content and the acidity of these beverages. Producing a beverage at pH 4-4.6, compared to pH 2.5-3.4, will require reduced amount of sugar to

balance the acidity. This beverage could be labeled “high protein”, “reduced sugar” and “reduced calorie” (Etzel, 2004).

The beverage industry, however, is facing major quality challenges in manufacturing shelf-stable whey protein acidic beverages at high protein concentration (>4.2%). Maintaining clarity at such high protein concentration in thermally processed beverages (pH<3.5) over an extended period of time, is a major hurdle. Whey proteins are thermally labile and are thus denatured upon pasteurization of the beverages. After pasteurization and during storage, hydrophobic and disulfide molecular interactions between denatured proteins take place causing undesirable turbidity and precipitation. Another major quality concern is related to flavor. Whey protein acidic beverages are often perceived as astringent and sour. Beecher et al. (2006) claimed that the astringency of whey protein acidic beverages is attributed to the whey protein. Lee and Vickers (2008) clarified that the astringency of whey protein acidic beverages is mostly caused by the high degree of acidity. As an ampholyte, whey protein is an excellent buffer in nature. Increasing protein concentration causes increased buffering capacity, which in turn results in an increased amount of acid needed to reach the target pH of an acidic beverage. This high acidity leads to a high degree of sourness and astringency that greatly reduce the overall liking and consumer acceptance of high protein acidic beverages. Moreover, to offset the astringency and sourness, often a considerable amount of sugar is added, which is not desirable to health conscientious consumers.

We have recently demonstrated enhancement of protein solubility and thermal stability upon partial and limited Maillard-induced glycosylation (Wang & Ismail, 2012). As compared to whey protein isolate (WPI), the partially glycosylated whey protein (PGWP) had improved solubility and thermal stability over a broad range of pH, including the isoelectric point (pI, pH 4.5-5.5) of whey protein, and at protein concentrations greater than 4.2% (5-10%). The enhanced solubility and thermal stability of PGWP was attributed to suppressed intermolecular interactions. Several physicochemical/structural changes, including resistance to denaturation, shift to a more acidic pI, reduced surface hydrophobicity, reduced exposure of sulfhydryl groups, and unique glycosylation sites contributed to the reduced intermolecular interactions. With PGWP, it is possible to

manufacture high protein acidic beverages that remain clear at pH greater than 3.4 (yet less than 4.6 to maintain the acidic conditions). We hypothesized, that at a pH greater than 3.4, an acidic beverage fortified with PGWP would be less sour and less astringent than one formulated with non-modified whey protein at pH 3.4. Therefore, our objective was to monitor the sourness and astringency of whey protein (5% protein, w/v) acidic beverages formulated with PGWP at pH 3.4 and 4.0 in comparison to a beverage formulated with WPI at pH 3.4.

4.2. Materials and Methods

4.2.1. Materials

Food grade whey protein isolate (WPI, 92.7% protein, as confirmed by Dumas, AOAC 990.03, using a Nitrogen Analyzer, LECO® TruSpec™) and food grade maltodextrin (Dry 01956) were kindly provided by Davisco (Eden Prairie, MN, USA) and Cargill (Hammond, IN, USA), respectively. Food grade potassium carbonate (P1472) and sodium sulfate (13464) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Food grade hydrochloride acid (A481-212) and citric acid (77-92-9) were purchased from Fisher Scientific (Pittsburgh, PA, USA). Alum and caramel liquid color (351Q) were purchased from McCormick & Co. (Hunt Valley, MD, USA) and Oringer (Brockton, MA, USA), respectively. Phenyl sepharose high performance media (17108204) was purchased from GE healthcare (Pittsburgh, PA, USA). Ultrafiltration membranes and membrane stand were purchased from Millipore (P3C010C01, Billerica, MA, USA).

4.2.2. Maillard-induced glycosylation

Partial glycosylation of whey protein was produced following the procedure described by Wang and Ismail (2012) with some modifications. Food grade maltodextrin (α-1,4 glycosidic linkages) was used as the reducing polysaccharides instead of dextran (α-1,6 glycosidic linkages) used in the previous study. Maltodextrin has similar reducing power

and molecular distribution as that of dextran. Replacement of the chemical grade dextran with maltodextrin was to meet the food grade requirement for ingredients used in sensory evaluations. WPI was mixed with maltodextin in 1:4 ratio (w/w), dissolved in deionized distilled water (DDW) and lyophilized. The lyophilized mixture was incubated in a sealed dessicator at a_w of 0.50 (using saturated potassium carbonate solution), at 60 ºC for 96 h.

4.2.3. Purification of the partially glycosylated whey protein (PGWP) from unreacted maltodextrin

Separation of unreacted maltodextrin from the protein was performed using hydrophobic interaction chromatography (HIC). Pharmacia Biotech fast protein liquid chromatography (FPLC) system, equipped with a UV detector and a fraction collector was used. A scaled-up HIC column, 20 cm x 50 mm, was packed with phenyl sepharose high performance media and equilibrated with 1 M food grade sodium sulfate. Incubated protein/maltodextrin mixture was dissolved in 1 M sodium sulfate (6% protein, w/v). An aliquot (100 mL) of the solution was injected onto the column and the flow rate was set at 10 mL min^{-1}. Free maltodextrin was washed with 200 mL of 1 M sodium sulfate followed by 50 mL of DDW. Glycosylated and free proteins, collectively referred to as PGWP, were eluted with an additional 280 mL DDW. Elution volumes were experimentally determined to ensure separation of free maltodextrin and recovery of proteins as outlined by Wang & Ismail (2012). Elution of the protein was monitored at 280 nm. Collected sample was then desalted and concentrated (3.5 times) using 10 kDa ultrafiltration membrane set-up against DDW, and lyophilized using a food grade lyophilizer. Approximately 700g of PGWP was produced, sufficient for sensory and other related tests. The protein percentage of PGWP was 41% as determined by Dumas, AOAC 990.03, using a Nitrogen Analyzer (LECO® TruSpec™).

4.2.4. Whey protein acidic beverages formulation

Three beverages were formulated with either WPI or PGWP at 5% (w/v) protein concentration using caramel colored water (0.4% liquid caramel, v/v) (Table 1). The pH of the beverage formulated with WPI was titrated to 3.4 using food grade 6.12 M hydrochloride acid. The pH of beverages formulated with PGWP was titrated to either 3.4 or 4.0 using the same acid. No beverage was formulated using WPI at pH 4.0. This is a pH close to the pI of non-modified whey protein, which will fall out of the solution upon heating (Zayas, 1997). The solubility of PGWP, however, was not altered after thermal treatment at this pH.

Table 5. Whey protein beverages and protein-free controls prepared using caramel colored water (0.4% liquid caramel, v/v) and with added sugar (a final concentration of 2%).

Sample ID	Whey Protein (g/100 mL)	6.12M HCL (mL/100mL)	Total acidity (M)
Beverage formulated with WPI[a] at pH 3.4	5	5	0.29[*]
Beverage formulated with PGWP[b] at pH 3.4	5	4.6	0.27
Beverage formulated with PGWP at pH 4.0	5	3.4	0.20
Negative control for WPI at pH 3.4	0	5	0.29
Negative control for PGWP at pH 3.4	0	4.6	0.27
Negative control for PGWP at pH 4.0	0	3.4	0.20

[*]: Acid calculated based on the product information of HCL provided by the supplier and the dilution factor (2 times): 37.2% (v/v); 1.2 g mL^{-1}; 12.24 M. [a]: whey protein isolate; [b]: partially glycosylated whey protein.

The beverages were all sweetened with sucrose to a final sugar concentration of 2% (w/v), which represents one to two-thirds of the amount of sugar present in a regular protein acidic beverage. A reduced level of sugar was chosen because we hypothesized that with lower acidity, a higher perception of sweetness would be noted. Separate protein-free controls were formulated for each beverage. Each control contained the same amount of acid as the corresponding beverage, however with no added protein. The beverages and their corresponding controls formulated at pH 3.4 and at pH 4 were heated in a water bath at 85 ºC and at 80 ºC, respectively, until the geometric center reached the

desired temperature followed by 3 min holding. A slightly lower temperature (80 ºC instead of 85 ºC) was used for PGWP beverage at pH 4.0 to avoid any thermally-induced gelation. The temperature and time used comply with the pasteurization requirement for acidic food (21 CFR 114.80) following the principles of “hot fill/hold" method.

4.2.5. Test samples

All fortified beverages and protein-free controls were prepared the day before the sensory evaluation, and held at 5 ºC until the morning of the session. Before the session, the beverages were equilibrated for 2 h at room temperature, and then 10 mL were poured into coded 2 oz. plastic cups with random 3-digit numbers. The presentation order of the test samples was based on a Latin Squares design balanced for order and carry-over effects.

4.2.6. Subjects

Ten subjects (8 females, 2 males, ages 18-60 years, questionare in Appendix C) were recruited from the trained panel maintained by the Sensory Center at the University of Minnesota. All subjects were tasters of 6-n propothiouracil (PROP) and free from food allergies or sensitivities. Subjects were paid for participating in a two-day study.

4.2.7. Training session

The subjects were given a training session before they participated in the test session. The training session was conducted as described by Lee & Vickers (2008). During the training, the subjects practiced evaluating astringency and sourness using alum and citric acid standards, respectively. Four citric acid samples at 0.462 g L^{-1}, 0.839 g L^{-1}, 1.366 g L^{-1}, and 2.402 g L^{-1} were used to anchor the sourness intensities of 3, 5, 7, and 10 on a 20-point scale (Appdendix C). The astringency reference was alum at a concentration of 0.25%.

4.2.8. Test sessions

Subjects participated in two sessions. During the first subjects evaluated the sourness, sweetness, and astringency of the whey protein acidic beverages. During the second subjects evaluated the sourness, sweetness, and astringency of the protein-free controls. During testing the subjects were required to use nose plugs to avoid interference of odor with the perception of the attributes of interest. The subjects were asked to put the entire sample in their mouth, hold the sample without moving, and rate sourness and sweetness on 20-point scales with 0 being "no sensation" and 20 "intense sensation". The subjects were then instructed to swish gently for 10 s, expectorate the sample, and perform a standardized mouth movement of three exaggerated "why" (Lee & Vickers, 2008). Subjects then rated the astringency on a 20-point scale. They performed the ratings using SIMS 2000 (Sensory Computer Systems, Morristown, NJ, USA) computerized data collection interface. Between samples, subjects rinsed their mouth at least 5 times with water and waited at least 5 minutes. Subjects were encouraged to wait until no astringency was perceived before they evaluated the next sample.

4.2.9. Statistical Analysis

Analyses of variance (ANOVA) were carried out utilizing XLSTAT® (2010). The dependent variables were sourness, astringency and sweetness. The independent variable was the beverage formulation (see Table 1 for the beverages used). Judges were treated as a random factor. Separate ANOVA were run for the protein-free controls. When the independent variable effect was found significant ($P \leq 0.05$), significant differences among the means were determined using Fisher's LSD test. Differences between a beverage and its protein-free control were analyzed using a student's t-test ($P \leq 0.05$).

4.3. Results and Discussion

4.3.1. Sourness of the formulated whey protein beverages and controls

The beverage formulated with PGWP at pH 4.0 was less sour than the WPI-fortified beverage at pH 3.4 ($P = 0.04$) (Fig. 4). Although the PGWP-fortified beverage at pH 3.4 tended to be less sour than the WPI-fortified beverage, this difference was not significant (ANOVA table in Appendix B).

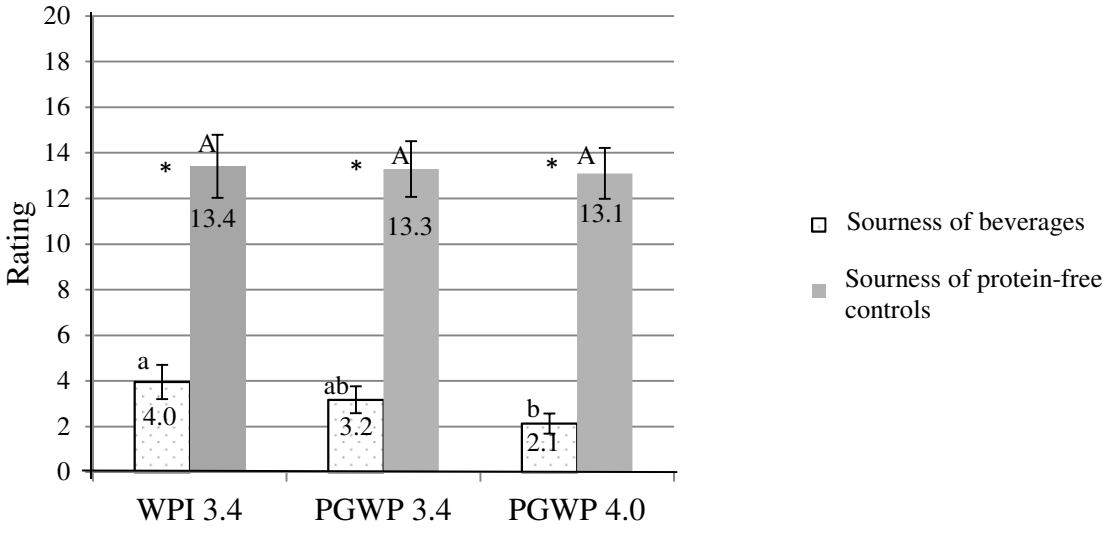

Figure 24. Mean rating of sourness (n=10) of protein beverages (dotted bars) and protein-free controls (gray bars) on a 20-point scale. Error bars represent positive and negative standard error values. Different upper case letters above the gray bars indicate significant differences in sourness among the different protein-free controls, and different lower case letters above the dotted bars indicate significant differences among the different protein fortified beverages, according to Fisher's LSD test ($P \leq 0.05$). The * sign above the bars indicates a significant difference between a protein fortified beverage and its protein-free control, according to student's t-test ($P \leq 0.05$). WPI 3.4: whey protein isolate fortified beverage formulated at pH 3.4; PGWP 3.4: partially glycosylated whey protein fortified beverage formulated at pH 3.4; PGWP 4.0: partially glycosylated whey protein fortified beverage formulated at pH 4.0. Refer to Table 5 for protein beverage and control formulations.

These observations suggested that the sourness can be directly related to the total acidity, i.e. the total amount of acid. When formulating whey protein acidic beverages at a fixed protein concentration of 5% using either WPI or PGWP, different amounts (mL) of 6.12 M HCl were needed to bring the pH to 3.4 and to 4 (Table 1). For the beverage fortified with WPI and that fortified with PGWP at pH 3.4, the total acidities were 0.29 M (1.05%, w/v) and 0.27 M (0.97%, w/v), respectively. This observation indicated that, at a similar protein concentration, PGWP had a lower buffering capacity than WPI, and thus required less acid to reach the same pH. The Maillard condensation between the amine group of the protein and the carbonyl group of the reducing sugar blocks some of the free amine groups, thus reducing the buffering capacity of the protein. Specifically, as free amines become less available, the protein's ability to sequester hydrogen ions is reduced. The % acidity of the beverage formulated with WPI at pH 3.4 (1.05%, w/v) was greater than that of the beverage formulated with PGWP at pH 4.0 (0.73% acidity, w/v), partially explaining the observed significant decrease in sourness.

The judges did not discriminate differences in sourness among the three controls, probably due to the intense sourness (Fig. 1). The protein-free controls received significantly higher sourness ratings than their counterpart protein beverages, although % acidity was maintained constant within a protein beverage and its protein-free control. The protein in the beverage at matching % acidity likely diminished the sourness of the acid by sequestering free hydrogen ions. As observed by Lee and Vickers (2008), protein-free acidic solutions that match the acidity of protein solutions have lower pH and higher perceived sourness.

4.3.2. Astringency of the formulated whey protein beverages and controls

The WPI fortified beverage at pH 3.4 had the highest astringency rating (8.4), while PGWP fortified beverage at pH 4.0 had the lowest (5.8) (Fig. 2), but the difference was not statistically significant (P = 0.18). The observed trend, however, suggested that astringency of the protein beverages can be potentially reduced when using PGWP at

higher pH and lower % acidity. As concluded by Lee and Vickers (2008), total acidity is the main contributor to astringency perception in protein acidic beverages.

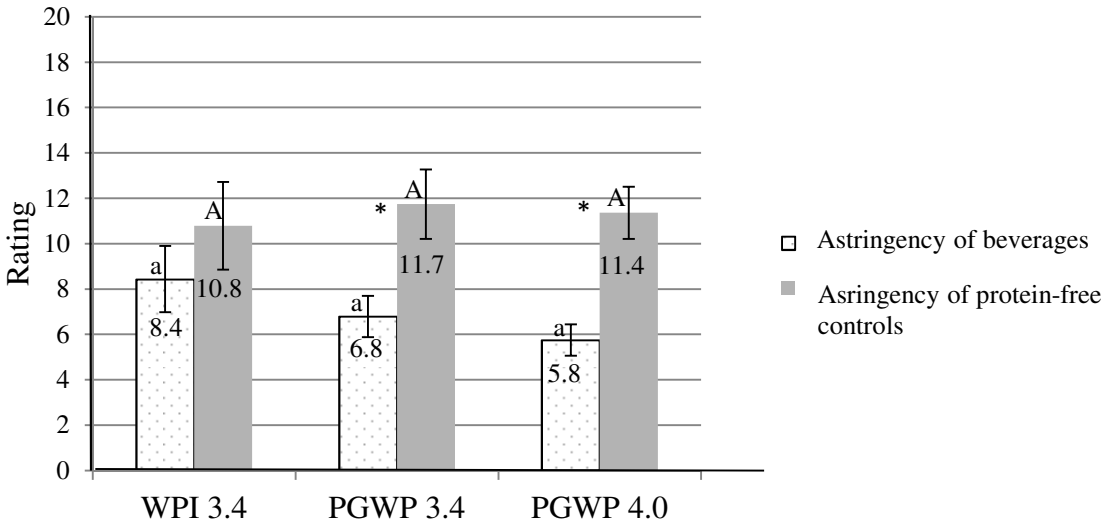

Figure 25. Mean rating of astringency (n=10) of protein beverages (dotted bars) and protein-free controls (gray bars) on a 20-point scale. Error bars represent positive and negative standard error values. Different upper case letters above the gray bars indicate significant differences in astringency among the different protein-free controls, and different lower case letters above the dotted bars indicate significant differences among the different protein fortified beverages, according to Fisher's LSD test ($P \leq 0.05$). The * sign above the bars indicates a significant difference between a protein fortified beverage and its protein-free control, according to student's t-test ($P \leq 0.05$). WPI 3.4: whey protein isolate fortified beverage formulated at pH 3.4; PGWP 3.4: partially glycosylated whey protein fortified beverage formulated at pH 3.4; PGWP 4.0: partially glycosylated whey protein fortified beverage formulated at pH 4.0. Refer to Table 5 for protein beverage and control formulations.

Lack of statistical difference in the astringency ratings among the protein beverages could be partially attributed to the high variation in ratings among the panelists. Astringency is a very hard sensory attribute to evaluate due to unavoidable carry over effects. Carry-over effects on astringency rating have been carefully investigated by

many researchers (Guinard, Pangborn, & Lewis, 1986; Lyman & Green, 1990; Colonna, Adams, & Noble, 2004; Lee & Vickers, 2010). Lee and Vickers (2010) observed that in spite of 5 water rinses and an enforced 5 minute wait between samples, astringency still tended to increase over time. Such carry over effects, could contribute to high variability in astringency ratings.

Comparing control to protein beverages, a significant difference in astringency rating was noted for PGWP beverages at both pH 3.4 and 4 and their respective controls, while no significant difference was observed between WPI fortified beverage and its control. This further suggested that PGWP may have the potential to reduce astringency perception in protein beverages.

4.3.3. Sweetness of the formulated whey protein beverages and controls

No significant differences in sweetness ratings were observed among the three protein beverages (Fig. 3). Sweetness ratings were low compared to sourness and astringency rating, which may explain why the panelists had difficulty discriminating sweetness among the different beverages. Although no statistical difference was noted, the mean sweetness ratings of PGWP fortified beverages at both pH 3.4 and 4 tended to be higher than that of the WPI fortified beverage at pH 3.4. This observation is partially in line with the initial hypothesis that a lower acidity is associated with a higher perception of sweetness (less suppression of sweetness by sourness). However, further confirmation is needed.

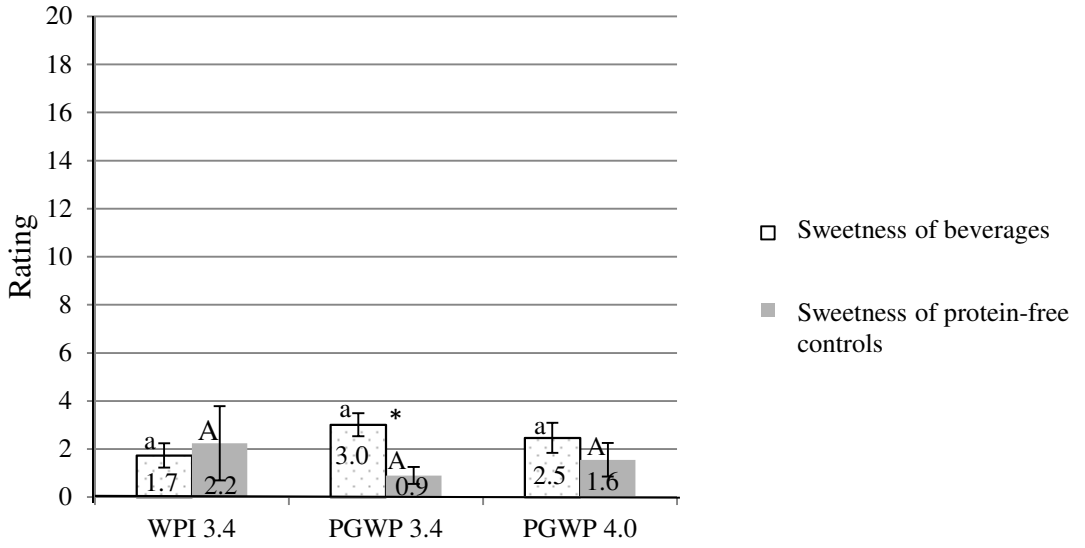

Figure 26. Mean rating of sweetness (n=10) of protein beverages (dotted bars) and protein-free controls (gray bars) on a 20-point scale. Error bars represent positive and negative standard error values. Different upper case letters above the gray bars indicate significant differences in sweetness among the different protein-free controls, and different lower case letters above the dotted bars indicate significant differences among the different protein fortified beverages, according to Fisher's LSD test ($P \leq 0.05$). The * sign above the bars indicates a significant difference between a protein fortified beverage and its protein-free control, according to student's t-test ($P \leq 0.05$). WPI 3.4: whey protein isolate fortified beverage formulated at pH 3.4; PGWP 3.4: partially glycosylated whey protein fortified beverage formulated at pH 3.4; PGWP 4.0: partially glycosylated whey protein fortified beverage formulated at pH 4.0. Refer to Table 5 for protein beverage and control formulations.

4.4. Conclusions

Partial glycosylation of whey protein resulted in lower buffering capacity, and consequently less acid was needed to reach the targeted pH of the fortified beverage. The reduced acidity of the PGWP fortified beverage resulted in lower perceived sourness compared to the WPI fortified beverage. The sourness ratings of the PGWP fortified

beverage at pH 4 were the lowest among the tested beverages. Results also suggested that the perception of astringency of protein beverages can potentially be reduced with lower % acidity. Thus, whey protein acidic beverages produced at pH greater than 3.4 could have better flavor attributes, and ultimately better consumer acceptability. This study has demonstrated for the first time the possibility of using PGWP in the production of high protein acidic beverages that can be less sour and astringent than what is currently present in the market. These findings are relevant to the beverage industry aiming at meeting the growing desire to shift the high carbohydrate-based drinks to high protein-based drinks that are less calorie-dense and more nutritious. Further formula optimization, acceptability evaluations, and storage stability studies are a natural follow up to the present work.

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5. Storage Stability of Glycosylated Whey Protein in Acidic and Neutral Solutions*

*: Content of this chapter will be submitted to International Dairy Journal

Wang, Q., Rao, Q.[1], Labuza, T.[2], and Ismail, B. *Manuscript in preparation*

[1] Provided help with capillary electrophoresis analysis

[2] Provided insights regarding the selection of thermal treatments and storage conditions

Thermal processing and prolonged storage can result in protein aggregation and subsequent quality deterioration of whey protein acidic beverages, especially at protein concentrations >4%. We have recently showed that partial Maillard-induced glycosylation resulted in improved whey protein solubility and thermal stability at concentrations >5% over a wide pH range. Our objective was to compare the storage stability of high whey protein isolate (WPI) and partially glycosylated whey protein (PGWP) solutions prepared at acidic and neutral pH. Solubility and turbidity as well as protein aggregation were monitored over 8 weeks of storage at 25, 35 and 45 °C. The solubility and turbidity of PGWP at pH 4.5 were maintained fairly well during storage at 25 °C. Possible aggregation of PGWP during extended storage was attributed to non-covalent interactions. Our results support the use of PGWP for production of whey protein acidic beverage at pH greater than 3.4, that will have higher protein concentration and be less sour and astringent than whey protein beverages currently present in the market.

5.1. Introduction

Whey protein beverages possess excellent physiological properties and have a pronounced commercial value. They are designed to help individuals recover quickly after a workout, or build muscles in case of bodybuilders. Whey protein beverages also attract health conscientious individuals. Whey protein provides all essential amino acids and an abundant amount of branched chain amino acids (Sinha, Radha, Prakash, & Kaul, 2006) that are taken up directly by the skeletal muscles (Morifuji, Koga, Kawanaka, & Higuchi, 2009; Nilsson, Holst, & Björck, 2007*)*. Beyond the nutritional value, whey protein provides numerous physiological benefits including but not limited to antimicrobial activity, growth promotional activity and immunoactivity (Sinha, Radha, Prakash, & Kaul, 2007; Manab, Sawitri, Al-Awwaly, & Purnomo, 2011; Petschow & Talbott, 1990; Petschow, Talbott, 1991; Bonomi et al., 2003).

Whey protein acidic beverages (pH < 4.6) are preferred over neutral protein beverages because they are relatively clear and require mild heating, thus maintaining better sensory quality and shelf life stability (21 CFR 114). Currently, whey protein acidic beverages available in the market are produced at pH of ~ 3.4, to achieve the best clarity, and contain at most 4% whey protein. FDA requires a protein concentration of at least 4.2 % to make a label claim of a "high protein beverage" (21 CFR 101.54). To make better use of the nutritional and physiological benefits of whey protein and to amplify their market value, it is desirable to develop high whey protein acidic beverages that are shelf stable. Additionally, it is desirable to produce whey protein acidic beverage at pH greater than 3.4 to reduce sourness and astringency.

The beverage industry usually employs a hot-fill heat treatment (88 °C for 2 min or equivalent) to pasteurize acidic beverages. Maintaining clarity, at a relatively high protein concentration, of thermally processed beverages over an extended period of time (6~8 weeks) is a challenge (Koffi, Shewfelt, & Wicker, 2005). Post thermal treatment, denatured whey protein molecules interact via hydrophobic and disulfide interchange, leading to turbidity and sedimentation over time. Laclair and Etzel (2009) observed a significant increase in turbidity of whey protein beverages (pH 4.0, 1.25% protein) after one to three

weeks of storage at room temperature (22 °C). Loss of soluble protein was detected after six weeks of storage, more so at storage temperature > 25 °C.

We have recently demonstrated improvement of protein solubility and thermal stability upon partial and limited Maillard-induced glycosylation (Wang & Ismail, 2012). As compared to whey protein isolate (WPI), the partially glycosylated whey protein (PGWP) had improved solubility and thermal stability over a broad range of pH, including the isoelectric point (pI, pH 4.5~5.5) of whey protein, and at protein concentrations greater than 4% (5~10%). The enhanced solubility and thermal stability of PGWP was attributed to structural rigidity, resistance to denaturation, and shift to more acidic pI, all of which contributed to reduced protein/protein interactions. We hypothesize that using PGWP, whey protein acidic beverages will remain clear at pH 3.4~4.5 and concentrations greater than 4% for an extended period of time.

Studies that have investigated the storage stability of whey protein acidic beverages are limited in number (Temelli, Bansema, & Stobbe, 2004; Laclair & Etzel, 2009; Koffi, Shewfelt, & Wicker, 2005), and none of them was based on protein concentration >4% or on glycosylated whey protein. Therefore, our objective was to monitor the shelf life stability of whey protein (5% protein, w/v) solutions prepared using PGWP at acidic pH (3.4 and 4.5) and/or neutral pH (~7.0) compared to those prepared using WPI at pH 3.4 and/or 7.0.

5.2. Materials and Methods

5.2.1. Materials

WPI (92.7% protein, as confirmed by Dumas, AOAC 990.03, using a Nitrogen Analyzer, LECO® TruSpec™) was kindly provided by Davisco (Eden Prairie, MN, USA). Dextran (10 kDa, D9260) and sodium sulfate (13464) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Phenyl sepharose high performance media (17108204) was purchased from GE healthcare (Pittsburgh, PA, USA). Other reagent grade chemicals were purchased from Fisher Scientific (Pittsburgh, PA, USA) and Sigma-Aldrich.

5.2.2. Maillard-induced glycosylation using dextran

Partial glycosylation of whey protein using dextran was produced following the procedure described by Wang and Ismail (2012) without modifications. Briefly, WPI was mixed with dextran (α-1,6 glycosidic linkages) in 1:4 ratio (w/w), dissolved in phosphate buffer (0.1 M, pH 7) and lyophilized. The lyophilized powder was incubated in a dessicator at 60 °C and water activity (a_w) of 0.49 (using saturated sodium borate solution) for 96 h.

5.2.3. Purification of the PGWP from unreacted dextran

Separation of free dextrans from the reacted and non-reacted proteins was performed using hydrophobic interaction chromatography (HIC) as described by Wang and Ismail (2012), without modification.

5.2.4. Preparation and storage of whey protein solutions

Solutions of WPI and PGWP were prepared, in triplicate, at 5% protein concentration (w/v). The pH of the WPI solutions were adjusted to 3.4 and 7, while the pH of PGWP solutions were adjusted to 3.4, 4.5 and 7, using either 2 M HCl or 2 M NaOH. No solution was prepared using WPI at pH 4.5. At this pH, which is close to the pI of whey protein, non-modified protein will fall out of the solution upon heating (Zayas, 1997). Sodium azide (0.05%, w/v) was added to the solutions to prevent the growth of mold during storage. Prepared solutions were then distributed into screw-cap microcentrifuge tubes, and heated in a water bath at 88 ºC for 2.5 min. The come up time ranged between 10-22 s. After heating, the tubes were inverted, held for 3 min, and then cooled on ice to room temperature (25 °C). Protein solutions were then stored at 25 °C, 35° C, and 45 °C for eight weeks. Samples were withdrawn at the end of each storage week, starting after two weeks of storage, for further analysis.

5.2.5. Solubility and turbidity

Aliquots (1 mL) of protein solutions were centrifuged at 13,000 x g for 10 min, and protein content of the supernatant was measured using a Nitrogen Analyzer (LECO® TruSpec™) following the AOAC Dumas method (AOAC 990.03). Solubility was expressed as the percentage of protein content in the supernatant to the total protein content of the initial solution. As an index of turbidity, optical density (OD) of the solutions was measured at 600 nm as outlined by Sava and Plancken (2005). Solutions with absorption values below 0.2 are clear and have no noticeable sediments.

5.2.6. Capillary electrophoresis (CE)

The protein aggregation was monitored by CE following the method described by Rao et al. (2012), with some modification. A P/ACE™ MDQ CE system (Beckman Coulter, Inc., Brea, CA, USA) equipped with a UV detector (220 nm) was used. Separation was obtained using a 50-µm bare fused-silica capillary, 30.2 cm in length, with a 20.2 cm effective length from the inlet to the detection window. All CE-grade reagents used were provided by the ProteomeLab™ SDS-MW Analysis Kit (Beckman Coulter) that was designed for the separation of protein-SDS complexes using a replaceable gel matrix. The separating gel used provided an effective sieving range of approximately 10 to 225 kDa. The SDS-MW standard (from 10 to 225 kDa, Beckman Coulter) was used to estimate the protein MW distribution of the sample. A capillary conditioning method was run before analyzing each sample. The conditioning method consisted of a basic rinse (0.1 N NaOH, 10 min, 20 psi), followed by an acidic rinse (0.1 N HCl, 5 min, 20 psi), a water rinse (CE-grade H_2O, 2 min, 20 psi) and finally an SDS gel separation buffer rinse (10 min, 70 psi). Voltage equilibration (15 kV for 10 min, 5-min ramping time) was then applied to the filled SDS gel. WPI and PGWP solutions that were stored under various conditions were diluted 1:11.5 (v/v) with 1% SDS (w/v). The mixture was then split into two portions. For reducing conditions, diluted protein samples were mixed (1:3, v/v) with CE sample buffer

containing 5% of 2-mercaptoethanol, and were heated at 100 ºC for 3 min. For non-reducing conditions, diluted protein solutions were mixed with (1:3, v/v) with CE sample buffer containing 5% of 250 mM iodoacetamide, and were heated at 70 ºC for 3 min. All samples were then centrifuged (15,000 x g for 15 min at 23 ºC), and the supernatant collected for CE analysis. The supernatant of each sample was injected into the gel-filled capillary at 5 kV for 20 s. The separation was performed at 15 kV for 30 min with reverse polarity in filled SDS gel. All CE steps were carried out at room temperature. Analysis was performed using a 32 Karat® software (Version 8.0, Beckman Coulter). For measuring the relative percentage of the soluble proteins in the samples, the peak areas of α-La and β-Lg were normalized, respectively. The normalized peak area is the measured peak area divided by its migration time (Harris, 2010).

5.2.7. Statistical Analysis

Analysis of variance (ANOVA) was carried out utilizing XLSTAT (2010). The dependent variables were solubility and turbidity. The independent variables were: 1) formulation (type of protein at a specific pH), with a total of 5 levels (PGWP solutions at pH 3.4, 4.5 and 7, and WPI solutions at pH 3.4 and 7); 2) storage temperature with a total of 3 levels (25, 35 and 45 °C); 3) and storage time with a total of 8 levels, 0, 2, 3, 4, 5, 6, 7 and 8 weeks. When a factor effect or an interaction was found significant ($P \leq 0.05$), significant differences among the respective means were determined using Tukey-Kramer multiple means comparison test.

5.3. Results and Discussion

5.3.1. Solubility of PGWP and WPI solutions during storage

The formulation, storage temperature, storage time, and their two-way interactions had a significant ($P \leq 0.05$) effect on the solubility of the protein solutions (Table 6). Comparing mean square values, it was apparent that storage time had the greatest effect

on solubility, followed by formulation. For all protein solutions at any storage temperature, a steady decrease in solubility (from ~ 100% down to ~ 65% for some protein solutions) was observed with a significant ($P \leq 0.05$) decrease noted after 4 ~ 5 weeks of storage (Table 7).

At 25 °C, no significant difference in solubility among PGWP at pH 3.4, PGWP at pH 4.5 and WPI at pH 3.4 was observed at any specific storage time (Table 7). Interestingly, the solubility of PGWP at pH 4.5 and WPI at pH 3.4 solutions followed a similar trend at both storage temperatures of 25 and 35 °C (see Figure 28 in Appendix D). At this level of protein concentration, WPI solutions at pH 4.5 fell almost completely out of solution immediately after thermal treatment (Wang & Ismail, 2012). In this study, PGWP at pH 4.5 maintained high solubility (≥ ~ 90%) up to 4 weeks of storage at both 25 and 35 °C, and up to 3 weeks of storage at 45 °C (Table 7). At 45 °C, however, the decrease in solubility of PGWP solution at pH 4.5 was more pronounced (down to 84 ~ 64%) than that of WPI solution at pH 3.4, with a significant difference noticeable starting at 4 weeks of storage. At 35 and 45 °C, PGWP solution at pH 3.4 gelled after 3 weeks of storage. This observation indicated that storage at elevated temperatures will result in thermally-induced gelation of PGWP at pH 3.4. For the most part, all formulations at pH 7, under all 3 storage temperatures, maintained high solubility (93 ~ 100%) up to 4 weeks of storage, with a slight decrease (83 ~ 89%) during the remainder of the storage time (Table 7 and ANOVA table 19-24 in Appendix D; Figure 28 in Appendix E).

Under accelerated storage conditions, previous work showed a quicker and more pronounced (as compared to our results) decrease in solubility during storage of whey protein solutions formulated at an acidic pH of 4.0 (Laclair and Etzel, 2009; Temelli et al., 2004). Observations by previous researchers coupled with our observations confirm that non-modified whey protein has relatively good stability even under accelerated storage conditions (below denaturation temperature) only when formulated at lower acidic condition (pH ≤ 3.4). To the best of our knowledge, our study is the first to investigate the storage stability of high whey protein solutions prepared at pH 3.4 instead of 4.0.

Table 6. ANOVA of the solubility and turbidity of protein solutions at 5% (w/v) protein concentration, with formulation (i.e. type of protein and pH of solution), storage temperature, and storage time as independent variables.

Dependent Variable	Source of Variation	DF^	Mean Square	F-Value	Significance ($P \leq 0.05$)
Solubility	Corrected Model	107	202.7	10.71	0.000
	Time	7	1789	94.54	0.000
	Temperature	2	80.75	4.267	0.015
	Formulation	4	745.8	39.41	0.000
	Time*Temperature	14	33.99	1.796	0.041
	Time*Formulation	28	64.95	3.431	0.000
	Temperature*Formulation	8	94.66	5.001	0.000
	Time*Temperature* Formulation	44	18.63	0.984	0.507
	Error	208	18.93		
Turbidity	Corrected Model	107	0.064	119.0	0.000
	Time	7	0.118	221.9	0.000
	Temperature	2	0.250	467.6	0.000
	Formulation	4	0.789	1478	0.000
	Time*Temperature	14	0.015	28.04	0.000
	Time*Formulation	28	0.049	91.66	0.000
	Temperature*Formulation	8	0.098	183.1	0.000
	Time*Temperature* Formulation	44	0.012	23.34	0.000
	Error	208	0.001		

^ DF: degrees of freedom

Table 7. Mean solubility % of WPI and PGWP solutions (5% protein, w/v) at pH 3.4, 4.5, and 7 during storage at 25, 35, and 45 °C for up to 8 weeks.

Protein Solution	Temp (°C)	Storage Time (weeks) 0	2	3	4	5	6	7	8
WPI pH 3.4	25	97.4^{Aa*}	101.5^{Aa}	91.7^{Aab}	91.6^{Bab}	82.5^{Ab}	81.3^{ABb}	80.4^{BCb}	79.2^{BCb}
WPI pH 7.0		95.7^{Aab}	99.1^{Aa}	92.0^{Aabc}	96.9^{Aa}	87.9^{Abc}	86.4^{Ac}	85.7^{Ac}	85.4^{Ac}
PGWP pH 3.4		98.6^{Aa}	96.8^{Aab}	91.0^{Ac}	93.4^{ABbc}	80.8^{Ad}	79.4^{Bd}	77.6^{Cd}	76.5^{BCd}
PGWP pH 4.5		88.9^{Bb}	95.4^{Aa}	94.5^{Aa}	93.8^{ABab}	79.4^{Ac}	78.2^{Bc}	78.8^{BCc}	75.2^{Cc}
PGWP pH 7.0		98.2^{Aa}	104.9^{Aa}	96.8^{Aa}	97.1^{Aa}	84.7^{Ab}	83.2^{ABb}	82.6^{ABb}	81.3^{ABb}
WPI pH 3.4	35	97.4^{Aa}	88.7^{Cab}	90.0^{Cab}	90.0^{Bab}	82.2^{Bb}	84.2^{ABb}	87.8^{Aab}	80.7^{Bb}
WPI pH 7.0		95.7^{Aa}	97.3^{ABa}	101.1^{Aa}	98.0^{Aa}	89.8^{Aa}	86.5^{Aa}	86.8^{Aa}	88.0^{Aa}
PGWP pH 3.4		98.6^{Aa}	94.8^{Ba}	G**	G	G	G	G	G
PGWP pH 4.5		88.9^{Bb}	95.6^{Ba}	96.1^{Ba}	95.5^{Aa}	81.6^{Bc}	80.0^{Bc}	78.0^{Acd}	74.9^{Cd}
PGWP pH 7.0		98.2^{Aa}	100.5^{Aa}	98.9^{ABa}	98.0^{Aa}	84.6^{Bb}	84.8^{Ab}	86.4^{Ab}	83.3^{Bb}
WPI pH 3.4	45	97.4^{Aa}	91.7^{Ba}	91.8^{Ca}	91.2^{Bab}	82.5^{ABc}	84.5^{Abc}	81.8^{Ac}	82.1^{Bc}
WPI pH 7.0		95.7^{Aa}	97.7^{ABa}	96.7^{BCa}	93.8^{Bab}	88.8^{Abc}	88.6^{Abc}	84.8^{Ac}	88.5^{Abc}
PGWP pH 3.4		98.6^{Aa}	94.5^{ABb}	G	G	G	G	G	G
PGWP pH 4.5		88.9^{Bab}	94.2^{ABabc}	94.4^{BCa}	84.4^{Cabc}	75.5^{Babc}	66.8^{Bbc}	67.6^{Bbc}	63.6^{Cc}
PGWP pH 7.0		98.2^{Aa}	99.5^{Aa}	98.1^{Aa}	100.3^{Aa}	85.0^{ABc}	85.1^{Ab}	86.0^{Ab}	84.2^{ABbc}

*: Means in each column, within the same storage temperature and time, followed by the same capital letter are not significantly different across solution formulation, and means in each raw within the same formulation and storage temperature, followed by the same small letters are not significantly different across storage time, according to Tukey-Kramer multiple means comparison test ($P \leq 0.05$); n=3. **: The solution gelled.

5.3.2. Turbidity of PGWP and WPI during storage

The formulation, storage temperature, storage time, all of their two-way interactions, and three-way interaction had a significant ($P \leq 0.05$) effect on the turbidity of the protein solutions (Table 6). Comparing mean square values, it was apparent that storage temperature had the greatest effect on turbidity, followed by time then formulation.

Table 8. Mean absorbance values at 600 nm as a measure of turbidity of WPI and PGWP solutions (5% protein, w/v) at pH 3.4, 4.5, and 7 during storage at 25, 35, and 45 °C for up to 8 weeks.

Protein Solution	Temp (°C)	Storage Time (weeks) 0	2	3	4	5	6	7	8
WPI pH 3.4	25	0.007^{Cd*}	0.029^{Ccd}	0.045^{Cabc}	0.031^{Cbcd}	0.061^{BCa}	0.047^{Babc}	0.051^{Cabc}	0.056^{Cab}
WPI pH 7.0		0.006^{Cb}	0.009^{Cb}	0.021^{Da}	0.014^{Cab}	0.012^{Cab}	0.014^{Bab}	0.021^{Ca}	0.012^{Dab}
PGWP pH 3.4		0.029^{Bb}	0.212^{Ab}	0.259^{Ab}	0.291^{Aab}	0.267^{Ab}	0.272^{Ab}	0.268^{Ab}	0.388^{Aa}
PGWP pH 4.5		0.038^{Ab}	0.097^{Ba}	0.098^{Ba}	0.099^{Ba}	0.092^{Ba}	0.094^{Ba}	0.105^{Ba}	0.096^{Ba}
PGWP pH 7.0		0.031^{Bab}	0.022^{Cb}	0.025^{Db}	0.023^{Cb}	0.022^{Cb}	0.037^{Ba}	0.032^{Cab}	0.024^{CDb}
WPI pH 3.4	35	0.007^{Cab}	0.040^{BCb}	0.057^{Bc}	0.056^{Bab}	0.061^{Bab}	0.059^{Bab}	0.070^{Ba}	0.065^{Ba}
WPI pH 7.0		0.006^{Cd}	0.011^{Ccd}	0.016^{Cbc}	0.028^{Ca}	0.014^{Cbcd}	0.018^{Bab}	0.020^{Bab}	0.019^{Cbc}
PGWP pH 3.4		0.029^{Bb}	0.495^{Aa}	G	G	G	G	G	G
PGWP pH 4.5		0.038^{Ae}	0.084^{Bde}	0.107^{Ade}	0.155^{Acd}	0.145^{Acd}	0.203^{Ac}	0.337^{Ab}	0.539^{Aa}
PGWP pH 7.0		0.031^{Ba}	0.028^{Ca}	0.032^{Ca}	0.034^{Ca}	0.055^{BCa}	0.071^{Ba}	0.078^{Ba}	0.060^{Ba}
WPI pH 3.4	45	0.007^{Cc}	0.039^{Cbc}	0.055^{Bab}	0.071^{Bab}	0.071^{Bab}	0.059^{Bab}	0.059^{Ba}	0.081^{Bab}
WPI pH 7.0		0.006^{Cb}	0.014^{Cab}	0.020^{Cab}	0.030^{Ca}	0.018^{Bab}	0.206^{Bab}	0.024^{Bab}	0.017^{Cab}
PGWP pH 3.4		0.029^{Bb}	0.611^{Aa}	G**	G	G	G	G	G
PGWP pH 4.5		0.038^{Af}	0.137^{Bef}	0.218^{Ade}	0.343^{Acd}	0.496^{Abc}	0.565^{Aab}	0.553^{Aab}	0.698^{Aa}
PGWP pH 7.0		0.031^{Ba}	0.034^{Ca}	0.052^{BCa}	0.039^{BCa}	0.044^{Ba}	0.086^{Ba}	0.048^{Ba}	0.039^{BCa}

*: Means in each column, within the same storage temperature and time, followed by the same capital letter are not significantly different across solution formulation, and means in each raw within the same formulation and storage temperature, followed by the same small letters are not significantly different across storage time, according to Tukey-Kramer multiple means comparison test ($P \leq 0.05$); n=3. **: The solution gelled.

Both PGWP and WPI solutions prepared at pH 7 remained relatively clear (absorbance values were ≤ 0.2) throughout the storage time at all temperatures (Table 8 and ANOVA Tables 19-24 in Appendix D). At 25 and 35 °C, both PGWP at pH 4.5 and WPI at pH 3.4 remained relatively clear throughout storage, with the exception of PGWP at pH 4.5 that had a significant increase in turbidity post 7 weeks of storage at 35 °C (Table 8 and ANOVA table 19-24 in Appendix D). The turbidity of PGWP solution at pH 3.4 was significantly increased post 7 weeks of storage at 25 °C, and post two weeks of storage at both 35 and 45 °C (beyond which gelation was observed) (Table 8 and ANOVA table 19-

24 in Appendix D). At 45 °C, WPI at pH 3.4 remained clear throughout storage, while PGWP at pH 4.5 had a noticeable increase in turbidity after 4 weeks of storage (Table 8 and ANOVA table 19-24 in Appendix D).

The change in turbidity followed a similar trend to that of the solubility of the different formulations. However, under some storage conditions, such as at 45 °C for 5 weeks, the solubility of some protein solutions, for instance WPI at 3.4 and PGWP at 4.5, was not significantly different (Table 7), while their turbidity was (Table 8). The scattering of visible light at 600 nm is related to the size of the particle (Laclair and Etzel, 2009). An increase in optical density during storage is an indication of protein aggregation, although the protein may remain in solution (the size of the aggregate is not large enough to precipitate). The aggregated PGWP remained relatively soluble partially due to the increased surface hydrophilicity upon glycosylation. Turbidity is not detrimental to consumer acceptance of a beverage, but is undesirable. Thus, turbidity in this case is measured as a complementary assessment tool for the evaluation of the overall storage stability of the protein solutions.

Laclair and Etzel (2009) and Temelli et al. (2004) reported that the turbidity of 1.5% whey protein acidic beverage at pH 4 was significantly higher after six to eight weeks of storage at room temperature. The difference in the observations of previous researchers and ours could be attributed to two reasons. Their solutions were formulated at pH 4, close to the pI of non-modified protein, instead of 3.4. Additionally, the WPI used in the previous studies is membrane-produced which ours was IEC-produced WPI. Composition, Ca content, and denaturation state, which affect protein solubility, are different between the two sources of proteins.

5.3.3. Protein aggregation

Two prominent CE peaks, corresponding to β-lg and α-la monomers, were observed in a typical electropherograms of WPI or PGWP sample (Fig. 27). No other peaks corresponding to polymers were observed. This indicates that any polymer formation was

either bigger in size than 225 kD, or there was a distribution of polymers of different sizes and at lower concentrations than the detection limit of the CE system used.

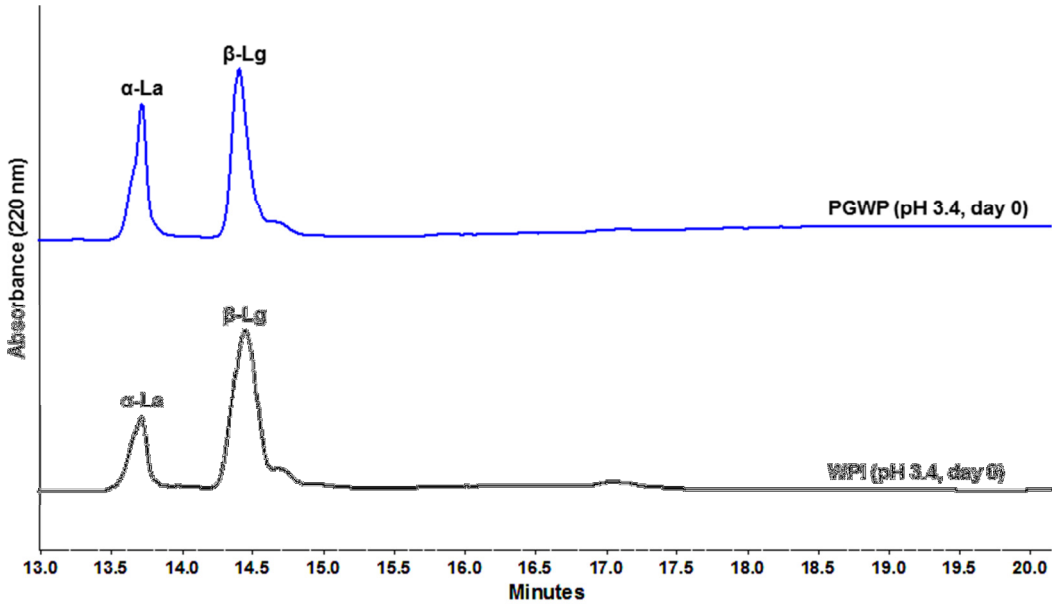

Figure 27. Typical CE electropherograms of WPI (black) and PGWP (blue) systems during storage at three different temperatures.

Under non-reducing conditions, relative percentage of soluble proteins (α-la and β-lg) in WPI decreased during storage, more so at pH 7 than 3.4. The decrease was more pronounced at elevated storage temperature (Table 9). The relative percentage of (α-la and β-lg) in PGWP decreased to a lesser extent compared to that of WPI at both pH 7 and 3.4 under the storage conditions tested.

All possible non-covalent protein interaction/aggregation was disrupted when the samples were dissolved in 1% SDS solution in preparation for CE analysis. Therefore, any protein aggregation, which corresponds to a reduced relative percentage of α-la and β-lg monomers, is mostly attributed to disulfide linkages. Apparently, protein aggregation that resulted in increased turbidity and or reduced solubility under specific storage conditions, are mostly attributed to non-covalent interactions. Upon glycosylation disulfide linkages between proteins partially inhibited compared to non-modified proteins. This information can be useful when formulating PGWP-based beverages. For instance, addition of an ingredient, such as salt at a specific concentration, can result in

reduced non-covalent interactions between the glycosylated protein molecules that might take place during storage.

Table 9. Relative percentage* of the soluble proteins (α-La and β-Lg) under non-reducing conditions, in WPI and PGWP solutions (5% protein w/v) during storage.

Sample system	pH	Temp. (°C)	Storage time (day)					
			0		21		42	
			α-La	β-Lg	α-La	β-Lg	α-La	β-Lg
WPI	3.4	25	100	100	84	76	73	69
		35			67	69	55	63
		45			59	71	22	36
	7.0	25	100	100	95	98	59	61
		35			30	26	31	28
		45			16	17	12	16
PGWP	3.4	25	100	100	110	109	105	102
		35			103	117	83	112
		45			64	91		
	7.0	25	100	100	92	89	79	80
		35			57	66	60	79
		45			31	40	35	53

* Relative percentage = 100 × (normalized peak area at day 21 or day 42/ normalized peak area at day 0)

5.4. Conclusions

The storage stability for PGWP at pH 4.5 was comparable to that of WPI at pH 3.4 when the storage temperature was kept at 25 °C. Unlike WPI, PGWP remained soluble post thermal treatment at pH 4.5 and at protein concentration greater than 4.2%, and was stable for an extended period of time. The results further confirms the finding of our previous studies that support the use of PGWP for production of whey protein acidic beverage pH greater than 3.4, the pH that is currently used. Formulating stable beverages using PGWP at pH 4.5, is desirable due the anticipated reduction in sourness and astringency, and ultimately better consumer acceptability. This study has demonstrated for the first time the possibility of using PGWP in the production of high protein acidic beverages that can be kept eight weeks stable under room. Additionally, our results provided some suggested

mechanisms of possible aggregation during storage. Further optimization studies using food grade dextrin to formulate shelf stable beverages using PGWP are a natural follow up to the present work.

6. OVERALL CONCLUSIONS, IMPLICATIONS, AND RECOMMENDATIONS

Controlled and limited Maillard-induced glycosylation resulted in improved solubility and thermal stability of whey protein over a broad range of pH, including whey protein pI, and for extended period of time. Partially glycosylated whey protein (PGWP), i.e., the mixture of free and glycosylated whey protein, remained soluble at protein concentrations greater than 4.2% (5-10%). This study showed, for the first time, that further removal of un-reacted whey protein or isolation of the glycosylated whey protein is not required to achieve improved solubility and thermal stability. This is important for industry since removal of unreacted proteins will generate new waste streams and economical loss. Unlike other studies, we have utilized a successful and feasible industrial approach to enhance the solubility and thermal stability of whey protein via Maillard-induced glycosylation. A relatively small % loss in amine groups was observed, indicating that limited glycosylation of whey protein in a mixture of free and reacted proteins can elicit a substantial enhancement in solubility over a wide pH range and at relatively high concentrations. The Maillard-induced glycosylation conditions utilized in this study limited the propagation of Maillard reaction into advanced stages and preserved the nutritional quality of the whey protein.

This study is innovative, providing for the first time link between structural/physicochemical changes and enhanced solubility and thermal stability of whey protein upon Maillard-induced glycosylation. We have confirmed, using novel techniques, that the observed enhancement in solubility and thermal stability of PGWP was attributed to structural rigidity, unique glycosylation sites, resistance to denaturation, and shift to more acidic pI. All of these structural and physicochemical changes contributed to reduced protein/protein interactions. Such structural information is crucial for understanding the mechanism of functionality enhancement, which in turn is necessary for achieving a directed enhancement in functionality in an industry setting.

Another important aspect of this study was providing substantial evidence that PGWP can be utilized to manufacture a shelf stable high protein beverage formulated at pH 4.0-

4.5. Beverages produced at pH 4.0-4.5 are still considered acidic beverages, requiring mild heating, yet are less acidic than the protein beverages available in the market (at pH < 3.4). The reduced acidity resulted in a significant drop in perceived sourness and astringency. This will improve consumer acceptability of the high protein acidic beverages, resulting in increased market demand and consequently increased health benefits and economical revinue to the dairy farmer and industry.

The promising enhancements were achieved using an analytical grade dextran for the most part. To apply the results to industrial production, more work has to be done on optimizing glycosylation conditions using food grade maltodextrin. Further beverage formula optimization, acceptability evaluations, and storage stability studies using maltodextrin are a natural follow up to the present work.

Although we have provided ample structural information on the produced PGWP, further investigations need to be conducted to confirm the functional enhancement mechanisms we have proposed. For instance, tandem MS can be done to further confirm the glycosylation sites and determine the sequence of the glycosylated peptides. Our proposed Raman marker of the glycosylated whey protein has to be further investigated using different analytical conditions and other whey protein sources.

We have observed enhancement in the thermally-induced gelation of whey protein upon Maillard-induced glycosylation, especially at concentrations greater than 5%. The thermally-induced gelation of our PGWP needs to be further characterized under various heat treatments, pH, and protein concentration.

Lastly, the solubility and thermal stability of our starting material (ion exchange WPI) was much better than what we expected based on literature information. We noted that some researchers do not indicate the source of their starting protein powder, making it hard to deaive conclusive and accurate information. Due to the extent of controversial results reported in the literature regarding whey protein solubility, thermal stability and shelf life, further Maillard-induced glycosylation studies need to be conducted utilizing different whey protein sources, for example ion-exchange- vs. membrane- produced WPI, and different polysaccharides.

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Appendix A: Analysis of Variance Tables for Solubility Evaluation

Table 10. ANOVA of the solubility of protein solutions without heat treatment with pH as the independent variable.

Sample	Dependent Variable	Source of Variation	DF^	Means Square	F-Value	Significance ($P \leq 0.05$)
WPI 5%	Solubility	pH	3	173.2	12.06	0.004
		Error	7	14.37		
WPI 7%	Solubility	pH	3	220.8	24.45	0.000
		Error	7	9.029		
WPI 10%	Solubility	pH	3	149.8	17.13	0.001
		Error	7	8.740		
PGWP 5%	Solubility	pH	3	3.650	0.163	0.918
		Error	7	22.39		
PGWP 7%	Solubility	pH	3	4.034	0.500	0.694
		Error	7	8.061		
PGWP 10%	Solubility	pH	3	1.709	0.297	0.826
		Error	7	5.746		

Table 11. ANOVA of the solubility of protein solutions heated at 70 °C for 30 min with pH as the independent variable.

Sample	Dependent Variable	Source of Variation	DF^	Means Square	F-Value	Significance ($P \leq 0.05$)
WPI 5%	Solubility	pH	3	1539	92.62	< 0.0001
		Error	7	16.62		
WPI 7%	Solubility	pH	3	1472	116.2	< 0.0001
		Error	7	12.67		
WPI 10%	Solubility	pH	3	3888	156.3	< 0.0001
		Error	7	24.88		
PGWP 5%	Solubility	pH	3	56.66	6.260	0.022
		Error	7	9.051		
PGWP 7%	Solubility	pH	3	2.340	0.260	0.852
		Error	7	9.000		
PGWP 10%	Solubility	pH	3	17.53	1.574	0.279
		Error	7	11.14		

Table 12. ANOVA of the solubility of protein solutions heated at 75 °C for 30 min with pH as the independent variable.

Sample	Dependent Variable	Source of Variation	DF^	Means Square	F-Value	Significance ($P \leq 0.05$)
WPI 5%	Solubility	pH	3	2336	336.5	< 0.0001
		Error	7	6.943		
WPI 7%	Solubility	pH	3	4099	468.4	< 0.0001
		Error	7	8.752		
PGWP 5%	Solubility	pH	3	3.872	0.507	0.690
		Error	7	7.635		
PGWP 7%	Solubility	pH	2	13.43	1.095	0.403
		Error	5	12.27		

Table 13. ANOVA of the solubility of protein solutions heated at 80 °C for 30 min with pH as the independent variable.

Sample	Dependent Variable	Source of Variation	DF^	Means Square	F-Value	Significance ($P \leq 0.05$)
WPI 5%	Solubility	pH	3	8083.3	822.1	< 0.0001
		Error	7	9.832		
WPI 7%	Solubility	pH	3	7552	993.2	< 0.0001
		Error	7	7.604		
PGWP 5%	Solubility	pH	3	60.33	4.246	0.053
		Error	7	14.21		
PGWP 7%	Solubility	pH	2	13.43	1.095	0.403
		Error	5	12.27		

Table 14. ANOVA of the solubility of protein solutions at pH 3.4 with thermal treatment as the independent variable.

Sample	Dependent Variable	Source of Variation	DF^	Means Square	F-Value	Significance ($P \leq 0.05$)
WPI 5%	Solubility	Thermal	3	44.52	0.683	0.622
		Error	7	61.86		
WPI 7%	Solubility	Thermal	3	6.025	0.648	0.609
		Error	7	9.297		
WPI 10%	Solubility	Thermal	1	6.348	0.734	0.455
		Error	3	8.647		
PGWP 5%	Solubility	Thermal	3	3.632	0.252	0.858
		Error	7	14.42		
PGWP 7%	Solubility	Thermal	1	3.267	0.959	0.400
		Error	3	3.408		
PGWP 10%	Solubility	Thermal	1	0.108	0.012	0.919
		Error	3	8.887		

Table 15. ANOVA of the solubility of protein solutions at pH 4.5 with thermal treatment as the independent variable.

Sample	Dependent Variable	Source of Variation	DF^	Means Square	F-Value	Significance ($P \leq 0.05$)
WPI 5%	Solubility	Thermal	3	2723	223.9	< 0.0001
		Error	7	12.16		
WPI 7%	Solubility	Thermal	3	2833	252.8	< 0.0001
		Error	7	11.21		
WPI 10%	Solubility	Thermal	1	4658	158.5	0.001
		Error	3	29.39		
PGWP 5%	Solubility	Thermal	3	63.56	14.65	0.002
		Error	7	4.337		
PGWP 7%	Solubility	Thermal	2	5.064	0.739	0.524
		Error	5	6.856		
PGWP 10%	Solubility	Thermal	1	11.16	3.082	0.177
		Error	3	3.622		

Table 16. ANOVA of the solubility of protein solutions at pH 5.5 with thermal treatment with pH as the independent variable.

Sample	Dependent Variable	Source of Variation	DF^	Means Square	F-Value	Significance ($P \leq 0.05$)
WPI 5%	Solubility	Thermal	3	2996	215.2	< 0.0001
		Error	7	13.91		
WPI 7%	Solubility	Thermal	3	3211	244.4	< 0.0001
		Error	7	13.14		
WPI 10%	Solubility	Thermal	1	2725	144.1	0.001
		Error	3	18.91		
PGWP 5%	Solubility	Thermal	3	62.21	4.467	0.047
		Error	7	13.93		
PGWP 7%	Solubility	Thermal	3	5.054	0.581	0.646
		Error	7	8.695		
PGWP 10%	Solubility	Thermal	1	7.500	0.372	0.585
		Error	3	20.17		

Table 17. ANOVA of the solubility of protein solutions at pH 7.0 with thermal treatment with pH as the independent variable.

Sample	Dependent Variable	Source of Variation	DF^	Means Square	F-Value	Significance ($P \leq 0.05$)
WPI 5%	Solubility	Thermal	3	27.98	2.304	0.164
		Error	7	12.14		
WPI 7%	Solubility	Thermal	3	4.552	0.663	0.601
		Error	7	6.864		
WPI 10%	Solubility	Thermal	2	5.100	0.300	0.753
		Error	5	17.00		
PGWP 5%	Solubility	Thermal	3	25.97	2.747	0.122
		Error	7	9.455		
PGWP 7%	Solubility	Thermal	3	13.84	1.028	0.436
		Error	7	13.46		
PGWP 10%	Solubility	Thermal	1	0.075	0.008	0.936
		Error	3	9.728		

Appendix B: Analysis of Variance Table for Sensory Evaluation

Table 18. ANOVA of the sourness, astringency, and sweetness of beverage and protein-free controls.

Sample	Dependent Variable	Source of Variation	DF^	Means Square	F-Value	Significance ($P \leq 0.05$)
Beverage	Sourness	Formulation	10	5.948	2.636	0.041
		Error	16	2.257		
	Astringency	Formulation	10	14.249	1.656	0.178
		Error	16	8.605		
	Sweetness	Formulation	10	3.310	1.363	0.280
		Error	16	2.428		
Protein-free control	Sourness	Formulation	11	33.065	11.559	< 0.0001
		Error	18	2.861		
	Astringency	Formulation	11	46.110	5.135	0.001
		Error	18	8.980		
	Sweetness	Formulation	11	15.988	2.762	0.027
		Error	18	5.788		

Appendix C: Questionare and Scale for Sensory Evaluation

List of questions

1) What is your gender? ___ Female ___ Male

2) What is your age?

3) How frequently do you drink protein fortified beverages?

___ More than once a day

___ Less than once a day bout one or more times a week

___ Less than once a week, but one or more times a month

___ Less than once a month but more than once a year

___ Less than once a year

4) How frequently do you drink whey protein formulated beverages?

___ More than once a day

___ Less than once a day bout one or more times a week

___ Less than once a week, but one or more times a month

___ Less than once a month but more than once a year

___ Less than once a year

5) Which type of sports beverage you drink? (check all that apply)

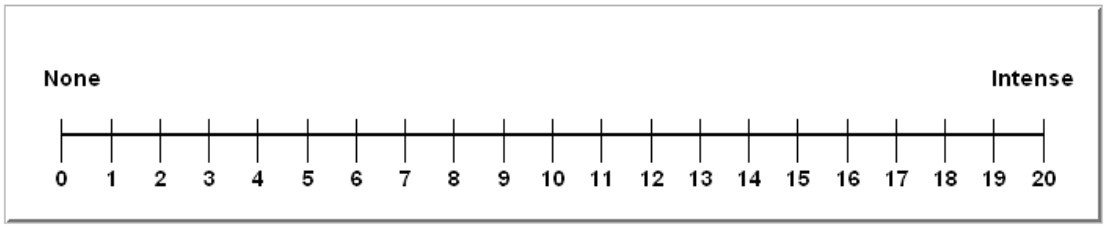
___ Gatorade® Energy Drinks

___ Red Bull®

___ Ensure® Protein Shake

___ Others

20-point scale



Appendix D: Analysis of Variance Tables for Storage Study

Table 19. ANOVA of the solubility and turbidity of protein solutions at 25 °C with storage time as the independent variable.

Sample	Dependent Variable	Source of Variation	DF^	Means Square	F-Value	Significance ($P \leq 0.05$)
WPI pH 3.4	Solubility	Time	7	212.2	8.779	0.000
		Error	15	24.17		
	Turbidity	Time	7	0.001	11.68	0.000
		Error	15	0.00007		
WPI pH 7.0	Solubility	Time	7	91.92	9.556	0.000
		Error	16	9.619		
	Turbidity	Time	7	0.00007	5.783	0.002
		Error	16	0.00001		
PGWP pH 3.4	Solubility	Time	7	249.6	84.29	0.000
		Error	16	2.961		
	Turbidity	Time	7	0.031	19.18	0.000
		Error	16	0.002		
PGWP pH 4.5	Solubility	Time	7	215.2	62.99	0.000
		Error	16	3.417		
	Turbidity	Time	7	0.001	21.22	0.000
		Error	16	0.00006		
PGWP pH 7.0	Solubility	Time	7	249.2	25.09	0.000
		Error	16	9.931		
	Turbidity	Time	7	0.00008	5.408	0.003
		Error	16	0.00002		

^ DF: degrees of freedom

Table 20. ANOVA of the solubility and turbidity of protein solutions at 35 °C with storage time as the independent variable.

Sample	Dependent Variable	Source of Variation	DF^	Means Square	F-Value	Significance ($P \leq 0.05$)
WPI pH 3.4	Solubility	Time	7	84.13	4.189	0.008
		Error	16	20.09		
	Turbidity	Time	7	0.001	18.27	0.000
		Error	16	0.00006		
WPI pH 7.0	Solubility	Time	7	75.73	0.820	0.585
		Error	15	92.31		
	Turbidity	Time	7	0.000	15.97	0.000
		Error	15	0.000008		
PGWP pH 3.4	Solubility	Time	1	20.76	6.274	0.066
		Error	4	3.309		
	Turbidity	Time	1	0.326	341.7	0.000
		Error	4	0.001		
PGWP pH 4.5	Solubility	Time	7	213.5	103.7	0.000
		Error	14	2.060		
	Turbidity	Time	7	0.080	106.7	0.000
		Error	14	0.001		
PGWP pH 7.0	Solubility	Time	7	175.2	25.64	0.000
		Error	16	6.832		
	Turbidity	Time	7	0.001	3.113	0.028
		Error	16	0.000		

^ DF: degrees of freedom

Table 21. ANOVA of the solubility and turbidity of protein solutions at 45 °C with storage time as the independent variable.

Sample	Dependent Variable	Source of Variation	DF^	Means Square	F-Value	Significance ($P \leq 0.05$)
WPI pH 3.4	Solubility	Time	7	96.77	17.83	0.000
		Error	14	5.427		
	Turbidity	Time	7	0.002	10.93	0.000
		Error	14	0.000		
WPI pH 7.0	Solubility	Time	7	65.42	13.23	0.000
		Error	15	4.945		
	Turbidity	Time	7	0.000	3.506	0.020
		Error	15	0.00004		
PGWP pH 3.4	Solubility	Time	1	25.05	10.837	0.030
		Error	4	2.312		
	Turbidity	Time	1	0.509	915.0	0.000
		Error	4	0.001		
PGWP pH 4.5	Solubility	Time	7	378.5	5.845	0.002
		Error	15	64.76		
	Turbidity	Time	7	0.163	57.68	0.000
		Error	15	0.003		
PGWP pH 7.0	Solubility	Time	7	260.2	24.31	0.000
		Error	16	10.71		
	Turbidity	Time	7	0.001	1.203	0.356
		Error	16	0.001		

^ DF: degrees of freedom

Table 22. ANOVA of the solubility and turbidity of protein solutions at 25 °C with formulation as the independent variable.

Time	Dependent Variable	Source of Variation	DF^	Means Square	F-Value	Significance ($P \leq 0.05$)
0	Solubility	Formulation	4	47.95	21.85	0.000
		Error	10	2.195		
	Turbidity	Formulation	4	0.001	154.7	0.000
		Error	10	0.000004		
2	Solubility	Formulation	4	43.24	1.105	0.406
		Error	10	39.12		
	Turbidity	Formulation	4	0.021	187.7	0.000
		Error	10	0.000		
3	Solubility	Formulation	4	17.44	1.189	0.373
		Error	10	14.67		
	Turbidity	Formulation	4	0.030	811.2	0.000
		Error	10	0.00004		
4	Solubility	Formulation	4	16.76	8.586	0.003
		Error	10	1.952		
	Turbidity	Formulation	4	0.041	426.4	0.000
		Error	10	0.00009		
5	Solubility	Formulation	4	33.73	3.487	0.055
		Error	9	9.672		
	Turbidity	Formulation	4	0.032	70.99	0.000
		Error	9	0.000		
6	Solubility	Formulation	4	31.79	6.805	0.007
		Error	10	4.671		
	Turbidity	Formulation	4	0.033	20.50	0.000
		Error	10	0.002		
7	Solubility	Formulation	4	31.02	11.29	0.001
		Error	10	2.749		
	Turbidity	Formulation	4	0.031	80.88	0.000
		Error	10	0.000		
8	Solubility	Formulation	4	49.32	13.40	0.001
		Error	10	3.680		
	Turbidity	Formulation	4	0.073	334.2	0.000
		Error	10	0.000		

Table 23. ANOVA of the solubility and turbidity of protein solutions at 35 °C with formulation as the independent variable.

Time	Dependent Variable	Source of Variation	DF^	Means Square	F-Value	Significance ($P \leq 0.05$)
0	Solubility	Formulation	4	47.95	21.85	0.000
		Error	10	2.195		
	Turbidity	Formulation	4	0.001	154.7	0.000
		Error	10	0.000004		
2	Solubility	Formulation	4	56.46	25.94	0.000
		Error	10	2.176		
	Turbidity	Formulation	4	0.126	314.8	0.000
		Error	10	0.000		
3	Solubility	Formulation	3	60.84	55.57	0.000
		Error	7	1.095		
	Turbidity	Formulation	3	0.005	104.7	0.000
		Error	7	0.00005		
4	Solubility	Formulation	3	42.20	23.04	0.001
		Error	7	1.832		
	Turbidity	Formulation	3	0.007	597.6	0.000
		Error	7	0.00001		
5	Solubility	Formulation	3	39.09	13.28	0.003
		Error	7	2.943		
	Turbidity	Formulation	3	0.007	33.19	0.000
		Error	7	0.000		
6	Solubility	Formulation	3	20.84	7.546	0.013
		Error	7	2.761		
	Turbidity	Formulation	3	0.017	32.55	0.000
		Error	7	0.001		
7	Solubility	Formulation	3	62.16	1.302	0.339
		Error	8	47.73		
	Turbidity	Formulation	3	0.061	42.44	0.000
		Error	8	0.001		
8	Solubility	Formulation	3	89.11	45.22	0.000
		Error	8	1.971		
	Turbidity	Formulation	3	0.182	2079	0.000
		Error	8	0.00008		

Table 24. ANOVA of the solubility and turbidity of protein solutions at 45 °C with formulation as the independent variable.

Time	Dependent Variable	Source of Variation	DF^	Means Square	F-Value	Significance ($P \leq 0.05$)
0	Solubility	Formulation	4	47.95	21.85	0.000
		Error	10	2.195		
	Turbidity	Formulation	4	0.001	154.7	0.000
		Error	10	0.000004		
2	Solubility	Formulation	4	27.88	5.610	0.015
		Error	9	4.969		
	Turbidity	Formulation	4	0.192	744.9	0.000
		Error	9	0.000		
3	Solubility	Formulation	3	22.89	5.814	0.021
		Error	8	3.937		
	Turbidity	Formulation	3	0.024	156.4	0.000
		Error	8	0.000		
4	Solubility	Formulation	3	104.7	29.07	0.001
		Error	6	3.603		
	Turbidity	Formulation	3	0.049	315.6	0.000
		Error	6	0.000		
5	Solubility	Formulation	3	94.94	5.168	0.028
		Error	8	18.37		
	Turbidity	Formulation	3	0.154	43.87	0.000
		Error	8	0.004		
6	Solubility	Formulation	3	267.6	80.57	0.000
		Error	7	3.321		
	Turbidity	Formulation	3	0.188	71.05	0.000
		Error	7	0.003		
7	Solubility	Formulation	3	216.4	19.63	0.001
		Error	7	11.03		
	Turbidity	Formulation	3	0.183	151.4	0.000
		Error	7	0.001		
8	Solubility	Formulation	3	361.1	64.22	0.000
		Error	8	5.623		
	Turbidity	Formulation	3	0.325	2627	0.000
		Error	8	0.000		

Appendix E: Solubility and Turbidiy Figures for Storage Study

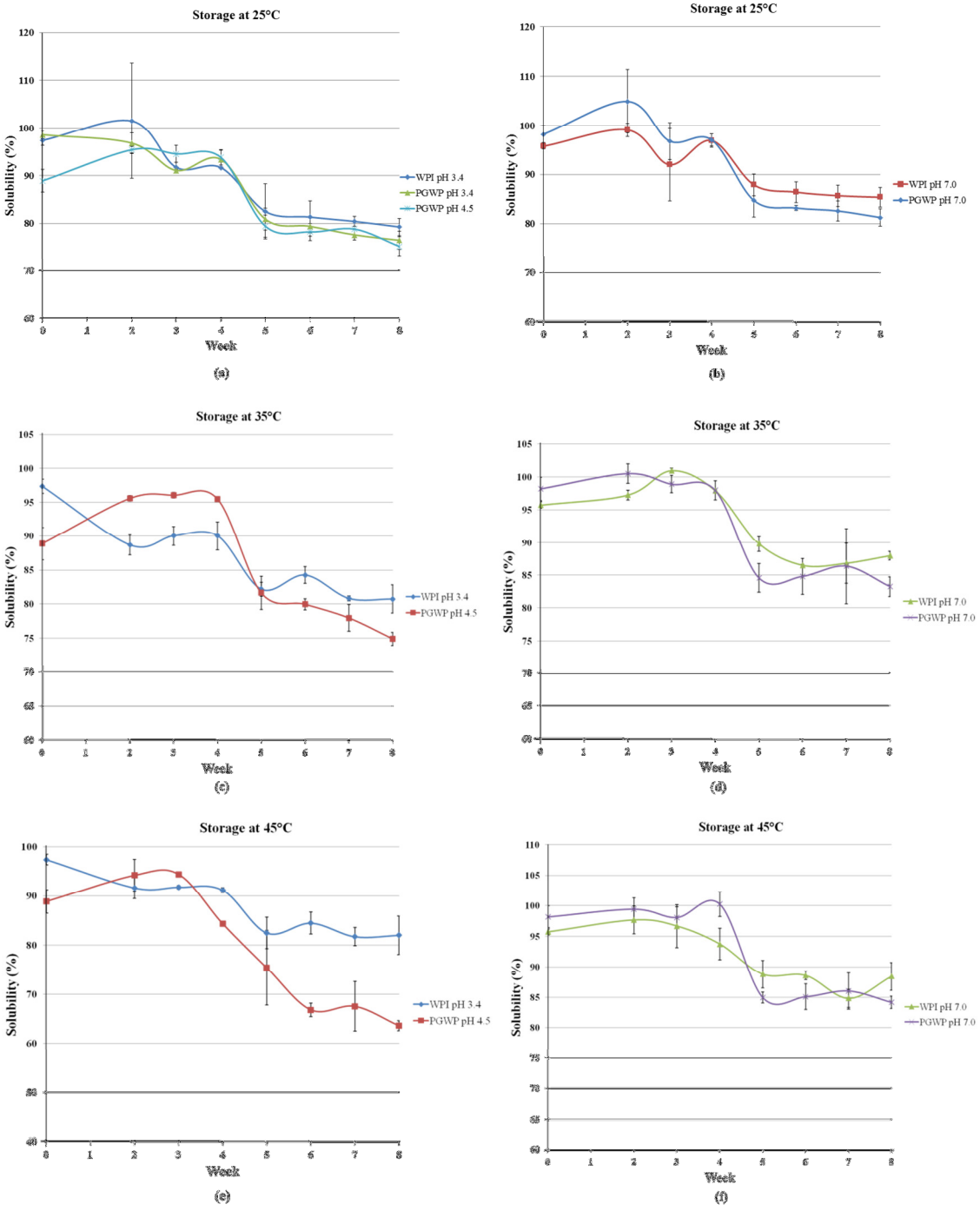


Figure 28. Solubility (%) of WPI and PGWP solutions, prepared at 5% protein (w/v) at pH 3.4 or 4.5 and stored at 25 (a), 35 (c), and 45 °C (e) for eight weeks; and at 7.0 stored

at 25 (b), 35 (d), and 45 °C (f) for eight weeks. Error bar represents standard deviation (n=3).

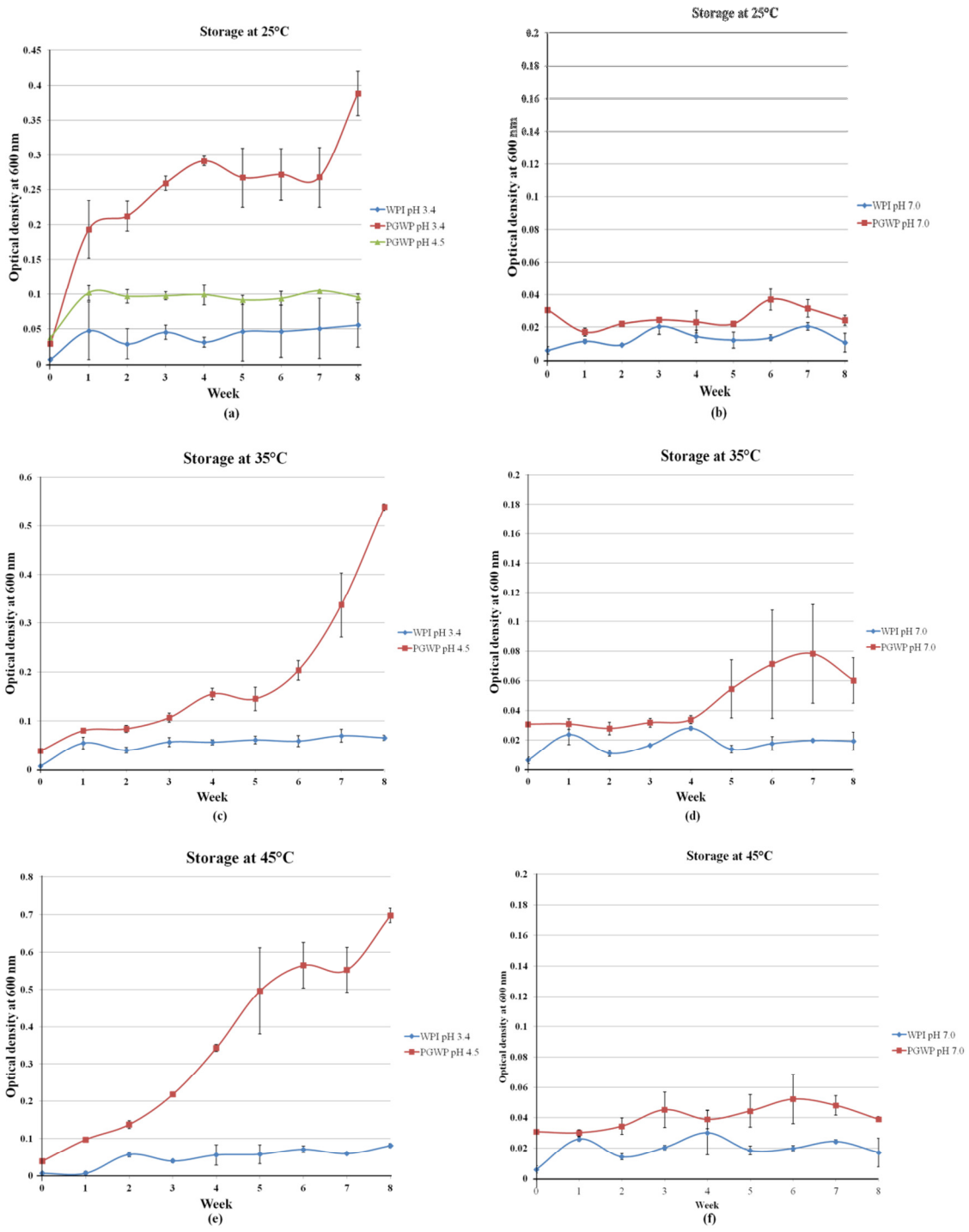


Figure 29. Optical density (turbidity) of WPI and PGWP solutions, prepared at 5% protein (w/v) at pH 3.4 or 4.5 and stored at 25 (a), 35 (c), and 45 °C (e) for eight weeks;

and at 7.0 stored at 25 (b), 35 (d), and 45 °C (f) for eight weeks. Error bar represents standard deviation (n=3).